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Lyophilization as a tool for enhance the stability of microemulsion systems containing Amphotericin B for leishmaniasis treatment

Andreza Do Vale Morais

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Intérêt de la lyophilisation pour améliorer la stabilité des microémulsions chargées en Amphotéricine B destinées au traitement de la leishmaniose

Thèse de doctorat de Universidade Federal do Rio Grande do Norte
et de l'Université Paris-Saclay
préparée à Université Paris Sud

École doctorale n°569 Innovation Thérapeutique, du fondamental à
l'appliqué.

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Thèse présentée et soutenue à Châtenay-Malabry, le 20 Octobre 2017, par

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Eryvaldo Sócrates Tabosa do Egito Professeur, Universidade Federal do Rio Grande do Norte	Co-Directeur de thèse

« J'ai appris que l'on mesure le succès non pas par la situation que l'on a atteinte dans la vie,
mais par les obstacles que l'on a surmontés pour essayer de réussir. »

Booker T. Washington

ABSTRACT

Visceral leishmaniasis is a neglected tropical disease that can be fatal if left untreated. Amphotericin B (AmB) is effective in the treatment of this disease, but the conventional formulation, Fungizone[®] has dose-limiting toxicity while the less toxic lipid-based forms such as Ambisome[®] are expensive. Therefore, the need for new therapeutic systems remains. In this respect, the heating of the Fungizone[®] formulation (H-AmB), and the development of a microemulsion (ME) containing AmB (MEAmB) are possible solutions. In addition, it is desirable to remove water from microemulsion systems in order to reduce instability due to microbiological contamination and hydrolysis. Therefore, the objective of this work was to develop and to evaluate the activity and toxicity *in vitro* and *in vivo* of H-AmB and MEAmB against *Leishmania donovani* (strain LV9) and, furthermore, to optimize a lyophilized microemulsion system containing AmB. Rheological, size and morphology studies showed that MEAmB presented average droplet sizes of 35 nm, a Newtonian behavior and spherical morphology. Spectroscopic characterization of H-AmB showed the formation of superaggregates, which are less toxic than the other states of aggregation. *In-vitro* evaluation on both the axenic and intramacrophagic amastigote forms showed that H-AmB and MEAmB showed similar activity to Ambisome[®]. A high selectivity index of H-AmB and MEAmB was observed compared with unheated Fungizone[®]. Furthermore, both new formulations showed high activity against AmB-resistant strains compared with Ambisome[®]. *In-vivo* experiments designed to evaluate their activity and toxicity did not reveal significant differences in activity between the new and reference formulations. There were no significant differences either in indicators of renal and hepatic toxicity. Therefore, both H-AmB and MEAmB can be used as an alternative for the treatment of LV9, presenting an advantage over AmBisome[®] in their lower costs of production. Therefore, a complete experimental design was performed in order to optimize the lyophilisation of the microemulsion system. It was observed that microemulsions with smaller droplet sizes were obtained using maltose as a cryoprotectant at a concentration of 5%, with freezing at -80 ° C, and a lyophilization process period of 24 h. Furthermore, it was observed that ME containing AmB showed no significant changes in drug content before and after the lyophilization process. Therefore, in its lyophilized form, the ME can remain stable and avoid degradation due to the presence of water.

Keywords: Amphotericin B, microemulsion, visceral leishmaniasis, lyophilization

RESUMO

A leishmaniose visceral é uma doença tropical negligenciada que pode ser fatal se não tratada. A Anfotericina B (AmB) é eficiente no tratamento desta doença, porém o seu elevado custo ou sua alta toxicidade torna necessário o desenvolvimento de novos sistemas terapêuticos para solucionar tais inconvenientes. Nesse contexto, pode-se utilizar o aquecimento da formulação micelar de AmB aquecida (A-AmB), e o desenvolvimento de uma microemulsão (ME) contendo AmB (MEAmB). Adicionalmente, é desejada a remoção da água desses sistemas microemulsionados a fim de diminuir instabilidades relacionadas à contaminação microbiológica e a hidrólise. Desta forma, o objetivo deste trabalho foi desenvolver e avaliar a atividade e toxicidade *in vitro* e *in vivo* da A-AmB e da MEAmB contra *Leishmania donovani* (LV9), além de otimizar um sistema microemulsionado liofilizado contendo AmB. Caracterizações da reologia, do tamanho e da morfologia da gotícula mostraram que a MEAmB apresentou tamanhos médios de 35 nm, um comportamento Newtoniano e uma morfologia esférica. A caracterização da A-AmB mostrou a formação de superagregados, que, são menos tóxicos que os outros estados de agregação. Análises *in vitro*, tanto para a forma amastigota axênica como para a intramacrofágica mostraram que as atividades da A-AmB e da MEAmB foram semelhantes ao do Ambisome®. Além disso, foi observado um alto índice de seletividade da A-AmB e da MEAmB comparada a formulação não aquecida. Adicionalmente, essas duas formulações, quando comparadas ao Ambisome®, mostraram elevada atividade contra cepas AmB resistentes. Essas formulações foram testadas *in vivo* a fim de avaliar sua atividade e toxicidade. Os resultados não apresentaram diferenças significativas entre as atividades das amostras contendo AmB, e, com relação a toxicidade, não mostraram diferenças expressivas capazes de causar uma disfunção renal ou hepática. Portanto, tanto a A-AmB como a MEAmB podem ser usados como alternativa no tratamento contra LV9, apresentando a vantagem sobre o Ambisome® devido aos menores custos de suas produções. Por conseguinte, a fim de liofilizar o sistema microemulsionado, foi realizado um planejamento experimental completo, no qual observou-se que MEs com menores tamanhos de gotículas foram obtidos quando utilizado maltose como crioprotetor na concentração de 5 %, com congelamento a - 80 °C e por um período de liofilização de 24 h. Além disso, foi observado que a ME contendo AmB não teve mudanças significativas quanto ao conteúdo do fármaco, quando comparado o produto antes e após o processo de liofilização. Desta forma, a ME, em sua forma liofilizada, pode manter a estabilidade do sistema perante os danos que poderiam ser causados pela quantidade de água.

Palavras-chave: Anfotericina B, microemulsão, leishmaniose visceral, liofilização

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General Introduction

Leishmaniasis

Leishmaniasis is a vector-borne zoonotic disease caused by a protozoa parasite from one of over 20 *Leishmania* species that have dogs as a common reservoir and humans as an incidental hosts (1). Despite being present worldwide, this disease is neglected because it is associated with poverty due to factors such as malnutrition, poor housing, migration, deforestation, irrigation schemes, urbanization and the building of dams. Furthermore, the existing programs to control leishmaniasis are not sufficient and there is no important investment in the development of new drugs (2).

This parasite is transmitted by the bite of an infected female sandfly (over 90 species) belonging to the genus *Phlebotomus* and *Lutzomyia*. These sandflies are more often found in tropical and temperate regions, which, together with the risk factors mentioned above, explains why Brazil, Ethiopia, India, Somalia South Sudan and Sudan account for more than 90% of the 50 000 to 90 000 new cases of visceral leishmaniasis, that are reported worldwide each year (3).

The *Leishmania* parasite is an obligate intracellular protozoon with two different morphological stages: a promastigote flagellar form and an amastigote form. The infection occurs when an infected sandfly regurgitates promastigotes into the mammalian host during blood feeding. Thereafter, the promastigotes are phagocytized by macrophages and other types of mononuclear phagocytic cells in which they differentiate into amastigotes form that multiply until the host cell bursts and then proceed to infect other macrophages. Many factors, including ones concerning the parasites and the host, influence the clinical manifestations and form of the disease. Sandflies become infected by ingesting the amastigotes while feeding on an infected person. The transformation of amastigotes to promastigotes occurs in the sandfly's gut, from which the promastigotes migrate to the proboscis, ready to infect another mammalian host (4).

The parasite may survive for decades in asymptomatic infected people or can induce symptoms ranging from localized ulcers to fatal infections depending on the parasite cell and organ tropism, which characterize the clinical form of the disease. There are three main forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL). CL, the most common form of leishmaniasis, is caused by two *Leishmania* species:

Leishmania major and *L. tropica*. The parasites remain at the site of insect vector bite, causing localized symptoms such as ulcers on the skin. In MCL, usually considered as a subset of CL, the parasite (*L. guyanensis*, *L. panamensis* and *L. braziliensis*) can spread to mucous regions such as the nose, throat, and mouth, where can lead to partial or complete destruction of the mucous membranes. On the other hand, VL occurs when the parasite causes a systemic infection, spreading through various organs, such as liver and spleen, in which it may destroy the immune cells and can be fatal if untreated. Several *Leishmania* species can cause VL, in the Old World examples are *L. donovani* and *L. infantum* and in the New World *L. chagasi* (5).

VL has a large spectrum of clinical manifestations in reticuloendothelial tissues, involving the spleen, liver, lymph nodes, and bone marrow. Its symptoms include abdominal pain, hepatomegaly, splenomegaly, diarrhea, epistaxis, pancytopenia, cachexia, fever and cough.

Its pathogenesis is characterized by Interleukin (IL)-12 and Interferon (IFN)- γ production by T cells, as well as the presence of IL-1, IL-6, IL-8, IL-15, and tumor necrosis factor (TNF)- α in the spleen and bone marrow. Furthermore, the death of the parasite also involves the complement system. The main innate response to *Leishmania* is the natural killer (NK)-cell-activated macrophage system. However, the *Leishmania* parasites have evolved strategies of immune evasion, the principal mechanisms of which being resistance to complement-mediated lysis, impaired macrophage microbicidal function, impaired antigen presentation to T cells, synthesis of immunosuppressive mediators, generalized depression of T-cell responses and expansion of regulatory T-cell population (1).

Current treatment for leishmaniasis is based mainly on pentavalent antimonials, Miltefosine, micellar Amphotericin-B (M-AmB) and Liposomal Amphotericin-B (L-AmB). The advantages and disadvantages of these different therapies are listed in Table 1.

Pentavalent antimonials were first choice for leishmaniasis treatment for many years due to their low price, despite their variable efficacy; however due to its dangerous side effects the patients needed be monitored, and sometimes the treatment had to be suspended. Furthermore, the resistance of *Leishmania* against pentavalent antimonials has increased. Miltefosine

(hexadecylphosphocholine) is the first effective oral treatment against VL and the most frequently used antileishmanial drug on the market, however its teratogenicity poses a problem, as does the rapid emergence of resistance. Amphotericin B (AmB) is highly active against *Leishmania* and is the drug of choice to treat immunosuppressed patients. There are different formulations of AmB on the market, the main ones being M-AmB (Fungizone®) and L-AmB (Ambisome®). M-AmB, which contains deoxycholate to solubilize the drug, has good activity, but is highly toxic, requiring slow IV administration, whereas L-AmB has the advantage of lower side effects, however its high cost is a limiting factor for its use in poor countries (4, 6).

Table 1: Current VL treatments and their main properties

Drugs	Administration	Regimen	Efficacy (*)	Resistance	Toxicity	Price of treatment
Pentavalent antimonials	IV, IM and IL	30 days 20 mg/kg/day	35–95% (depending on area)	Common (>60% in Bihar, India)	+++ Cardiotoxicity, pancreatitis, nephrotoxicity, hepatotoxicity	\$50–70
Micellar amphotericin-B	IV	30 days 1 mg/kg (15mg/kg total dose)	>90%	Laboratory strains	+++ Nephrotoxicity	\$100
Liposomal amphotericin B	IV	5–20 mg/kg total dose 4–10 doses over 10 – 20 days	>97%	Not documented	+/- Rigors and chills during infusion	\$280
Miltefosine	PO	28 days 1.5–2.5 mg/day	94–97%	Laboratory strains	+ Gastrointestinal, nephrotoxicity, hepatotoxicity, teratogenicity	\$70
Paromomycin sulfate	IM	21 days 15 mg/kg/day	94% (India) 46–85% (Africa, depending on dose)	Laboratory strains	+ Nephrotoxicity, ototoxicity, hepatotoxicity	\$10

IV = intravenous administration; IM = intramuscular administration; IL = intralymphatic administration; PO = oral administration. * Definitive cure at 6 months. Based on (4).

AmB is a potent drug which has a good antileishmanial activity without showing many cases of resistance in the field. However its toxicity and the price of some AmB-based formulations render it unfeasible for use in many situations. Strategies to overcome these drawbacks would be of a great value to improve leishmaniasis treatment.

Amphotericin B

AmB has been used for a long time as the major antifungal drug to treat systemic infections. It possesses a wide spectrum of activity, so that it remains almost 100 % effective as a first-line therapy. This drug is also been used for treatment of VL (7).

AmB was extracted from a strain of *Streptomyces nodosus* in the 1950s (8). Its amphoteric structure is composed of a hydrophilic polyhydroxyl chain and a mycosamine sugar with a lipophilic polyene hydrocarbon chain (Figure 1). AmB has low solubility in water, which leads low bioavailability by oral route, so that its use is limited to the intravenous route and local application. However, this makes the drug an ideal candidate for incorporation into lipid-based preparations (9).

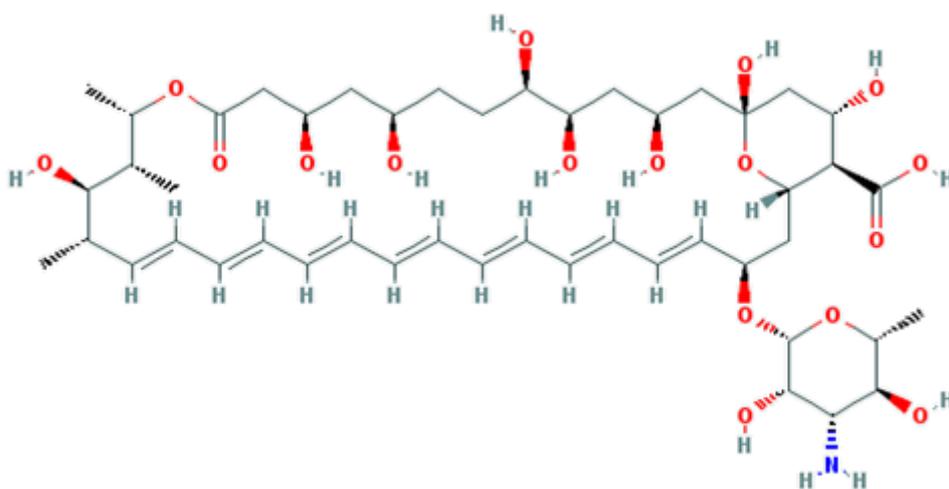


Figure 1: Chemical structure of amphotericin B (10)

Different aggregation states of AmB can be observed, depending on the concentration. In water AmB exists as a mixture of water-soluble monomers and oligomers (the most toxic form) with

insoluble aggregates. The toxicity of the micellar formulation is related to the different aggregate forms of AmB. Monomeric AmB interacts with ergosterol in membranes, forming ion channels, whereas soluble self-associated oligomers cause damage to sterol-free and cholesterol-containing membranes and are susceptible to oxidation, thereby enhancing their toxicity. As well as these two forms, super-aggregates has been shown to be less toxic because they are less susceptible to oxidative degradation, and in mammalian cells, it is less selective for the cholesterol membrane. This form is obtained by heating M-AmB (11).

Both the therapeutic and the toxic effects of AmB are related to its interactions with sterols in biological membranes, such as ergosterol from fungal and parasite cells and cholesterol from mammalian cells. A complex of eight AmB molecules hydrophobically interacting via the polyene chain with eight sterol molecules forms a pore which leads the leakage of electrolytes and other cell components, causing acidification of the interior with precipitation of the cytoplasm, leading the cell death (12).

Different approaches have been applied to decrease the toxicity of AmB. Since the *Leishmania* parasite is an obligate intracellular found within macrophages of the reticuloendothelial system, this presents a major challenge in the treatment of leishmaniasis, since traditional antileishmanial drugs are not readily able to penetrate inside the macrophages to kill the parasite. Therefore this disease is suited for drug delivery therapy, in which the delivery systems are able to accumulate in specific locations (spleen, liver, bone marrow, dermis) after administration. Lipid-based nanocarriers are able to penetrate into macrophages and delivery the drug within the cell in order to reach the therapeutic concentration necessary to kill the protozoa. Therefore the main strategy is to target the drugs directly to mononuclear phagocyte system system (hepatic macrophages), where *Leishmania* parasites accumulate (4, 13). Therefore, new AmB lipid-based formulations have been proposed for the VL treatment.

These lipid formulations have some advantages over native AmB, such as improving the solubility of the drug, protection of the drug from enzymatic degradation and/or host immune factor inactivation; improving its bioavailability and pharmacokinetic properties; preventing uncontrolled

drug leakage by the association of AmB with the carrier; and facilitating the uptake of the complex by the cells of the mononuclear phagocyte system in order to targeted the drug to desired sites of infection (12).

Developments in nanotechnology have provided new approaches to the treatment of VL treatment using lipid formulation in order to decrease the toxicity while maintaining the efficacy and decreasing the product cost. In addition to the formulations which are already on the market such as AmB deoxycholate, Abelcet[®] and Amphocil[®] or Amphotec[®] and Ambisome[®], AmB-loaded emulsified systems have been developed.

Microemulsion

A microemulsion (ME) is a emulsified system which was first studied by Hoar and Schulman in 1943 (14). The ME is a thermodynamically stable system comprised of two immiscible liquids (e.g water and oil) stabilized by a mixture of surfactants. This system is clear, stable, isotropic and possesses low surface tension and small droplet size. It has been used as a drug delivery system improving both drug absorption and permeation (15).

MEs are generally formed spontaneously, without any significant energy input. They can be classified as water-in-oil, oil-in-water and bicontinuous. Their advantages over conventional emulsions are the thermodynamically stability and the minimum energy requirement for formation, ease of manufacturing, enhanced drug solubilization and improved bioavailability, and as a result they have widespread applications as colloidal drug delivery systems for drug targeting and controlled release (16).

Drug release from ME can be described by two models. The first one takes drug diffusion through the droplet as the rate-limiting step of drug release. The other one considers the rate-determining step of drug release to be passage through the interfacial barrier between the droplet and the surrounding medium (17).

However the presence of free water in MEs can destabilize the system by hydrolysis, drug leakage or microbiological contamination. To overcome these drawbacks, a drying technique, such

as freeze-drying, could be used to increase the shelf-life of the preparations.

Freeze-drying

Freeze-drying (FD), also known as lyophilization, is a dehydration technique that transforms the water in the sample from the ice state directly to the gas state at low temperatures and pressures. There are three main steps: freezing, primary drying and secondary drying. These steps ensure the water removal, however specific conditions that includes temperature, time and pressure are necessary for the success of the process (18).

However, during these drying steps as the sample may undergo physical or chemical damage, making it necessary to use cryoprotectants. These compounds can prevent the stresses that occur in lipid bilayers during freeze-drying (19).

Therefore, it is important to evaluate the various parameters which can influence the process and lead to an acceptable freeze-dried product, such as the nature of the surfactants, the solubility and partitioning characteristics of the drug, the type and concentration of cryoprotectants and the time and temperature of each step (20).

Previous study by our research team

AmB has been one of the themes of the research group run by Prof. Sócrates Egito at Laboratório de Sistemas Dispersos (LASID)-UFRN since his PhD studies (11, 21-33). The strategies used by this group to improve AmB therapy are loading into emulsified systems, such as microemulsions, and changing the aggregate state of micellar Amphotericin B (M-AmB) by heating (H-AmB).

The formulation (ME-AmB) used in this work was developed, characterized and evaluated for its toxicity and antifungal activity by Silveira et. al. (2016) and Damasceno et. al. (2012) (29, 33). This work focused on the development of ME-AmB for ocular application and systemic fungal infections, in order to increase its efficacy and decrease its toxicity. Its physicochemical properties, polarized light microscopy, droplet size, drug-loading efficiency, minimum inhibitory concentration

against fungal strains and microemulsion toxicity and in-vitro toxicity against red blood cells were all evaluated. The results showed that the presence of AmB within the system did not cause significant changes in the physicochemical properties of the microemulsion compared with the unloaded system. Furthermore, AmB molecules were strongly and individually bound to the microemulsion droplets. The *in-vitro* assay showed that ME-AmB had high activity against *Candida* strains and showed lower toxicity against human red blood cells compared with the commercial product (M-AmB). Therefore, the microemulsion is an eligible drug carrier for Amphotericin B and has potential applications against fungal infections.

Another formulation of ME-AmB was developed and characterized by Silva et al (2013) (31) for the oral route. This AmB-loaded ME based on pseudoternary phase diagrams was able to improve the apparent solubility of the drug. Moreover, it was observed that part of the AmB in the MEs was aggregated and could act as an AmB reservoir carrier, explaining its low toxicity to macrophage-like cells. Therefore, this formulation may be considered as a promising carrier for AmB for oral delivery.

Our research group has recent results concerning the other alternative formulation proposed in this thesis: H-AmB. Silva-Filho et al. (2012) (11) evaluated how an aqueous M-AmB could be restructured after heating treatment. Physicochemical studies were performed, as well as an *in-vitro* pharmacotoxicity assay using red blood cells from human donors and *Candida parapsilosis*. The results of a spectrophotometric study revealed a blue shift of the aggregate peak after the heating procedure. Furthermore, they showed that the heated micellar AmB was less toxic to mammalian cells while keeping its activity against fungal cells. Therefore, it is a simple, inexpensive, and safe way for the treatment of systemic fungal infections. Siqueira et al. (2014) (32) continued these studies by evaluating the behavior of H-AmB after a freeze-drying process, examining their physicochemical and biological properties by spectrophotometry, antifungal activity and red blood cell toxicity assay. It was observed that H-AmB could be freeze-dried and could retain its physicochemical properties, activity and low toxicity, and was therefore a suitable method to increase the long-term stability of H-AmB systems.

Presentation of the Thesis Work

In the light of these observations, the aim of the work described in this thesis was to develop lipid formulations containing Amphotericin B as microemulsions (MEAmB) and as its heated micellar form (AmB-H). Furthermore, freeze-drying of MEAmB in order to diminish the water content and thereby increase drug stability was also optimized.

In view of the importance of these subjects, an overview highlighting leishmaniasis, microemulsion, AmB and lyophilization was written and published. It is presented here as the first chapter of this manuscript.

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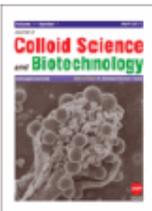
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CHAPTER I

Freeze-dried microemulsion containing amphotericin B for leishmaniasis treatment:

An overview



Freeze-Dried Microemulsion Containing Amphotericin B for Leishmaniasis Treatment: An Overview

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Abstract:

Visceral leishmaniasis is a neglected tropical disease that can be fatal if left untreated. Amphotericin B (AmB) has been quite effective against visceral leishmaniasis, but its conventional micellar form, Fungizon[®], is nephrotoxic. On the other hand, the less toxic AmB liposomal formulation, AmBisome[®], is very expensive. An alternative to solve these drawbacks is to develop a new lipid formulation such as microemulsions (MEs) containing AmB. Additionally, it is desired to remove the water from such systems in order to reduce instabilities related to microbiological contamination and hydrolysis. The aim of this study was to provide a comprehensive overview on the current knowledge regarding AmB-loaded MEs for the treatment of leishmaniasis. Furthermore, the influence of the freeze-drying process to this system was evaluated. This review also discusses the advances regarding leishmaniasis such as general features, drug-resistance parasites, current treatment and new approaches from colloidal nanocarriers for treating leishmaniasis. Likewise, it was emphasized the treatment with AmB addressing its molecular characteristics, mechanism of action, resistance mechanism and overviewing the clinical use of current available formulations. An approach on the ME as nanocarriers for AmB was carried out discussing some aspects on the ME structure, physicochemical characteristics, formation theories, advantages, and studies from the literature and from our preliminary results involving AmB-loaded ME for leishmaniasis. Moreover, it was also provided a theoretical overview on the freeze-drying process taking into account aspects such as its steps, cryoprotectant, parameters that would interfere on the process and design of experiment. Furthermore, studies on the freeze-dried AmB-load ME from our group and from the literature were approached.

Keywords: AMPHOTERICIN B; COLLOIDAL NANOCARRIES; DRUG DELIVERY SYSTEMS; LEISHMANIA; LYOPHILIZATION

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Freeze-dried microemulsion containing Amphotericin B for leishmaniasis treatment: An overview

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Abstract

Visceral leishmaniasis is a neglected tropical disease that can be fatal if left untreated. Amphotericin B (AmB) has been quite effective against visceral leishmaniasis, but its conventional micellar form, Fungizone[®], is nephrotoxic. On the other hand, the less toxic AmB liposomal formulation, AmBisome[®], is very expensive. An alternative to solve these drawbacks is to develop a new lipid formulation such as microemulsions (MEs) containing AmB. Additionally, it is desired to remove the water from such systems in order to reduce instabilities related to microbiological contamination and hydrolysis. The aim of this study was to provide a comprehensive overview on the current knowledge regarding AmB-loaded MEs for the treatment of leishmaniasis. Furthermore, the influence of the freeze-drying process on this system was evaluated. This review also discusses advances regarding leishmaniasis such as general features, drug-resistance parasites, current treatment and new approaches from colloidal nanocarriers for treating leishmaniasis. Similarly, it emphasizes treatment with AmB, addressing its molecular characteristics, mechanism of action, resistance mechanism and reviewing the clinical use of currently available formulations. ME as nanocarriers for AmB are also considered, discussing some aspects of the ME structure, physicochemical characteristics, formation theories, advantages, and studies from the literature and from our preliminary results involving AmB-loaded ME for leishmaniasis. Moreover, a theoretical overview on the freeze-drying process is also provided, taking into account aspects such as its steps, cryoprotectors, parameters that influence the process and experimental design. Furthermore, studies on the freeze-dried AmB-loaded ME from our group and from the literature are mentioned.

Keywords: drug delivery systems, *Leishmania*, lyophilization, amphotericin B, colloidal nanocarriers.

Introduction

Leishmaniasis is a neglected tropical disease caused by 20 different species belonging to the genus *Leishmania*, which is transmitted by the bite of a female mosquito of the genus *Phlebotomus* and *Lutzomyia*, commonly known as sandflies^{1,2}. In vertebrates, this parasite multiplies inside the cells of the mononuclear phagocyte system after acquiring intracellular growth ability. Moreover, it is capable of escaping the host immune response due to complement activation. The clinical manifestations of leishmaniasis recognized by the World Health Organization are divided into three main forms: cutaneous, mucocutaneous and visceral (VL), the latter being the most severe form^{1,3}.

Conventional treatment of VL is based on parenteral administration of salts of pentavalent antimony. However, parasite resistance to these drugs and their high toxicity have restricted their use to some areas and conditions. When this treatment is not effective and/or in patients seropositive to the human immunodeficiency virus, second-line agents, such as Amphotericin B (AmB), have been used. AmB interacts with ergosterol and cholesterol, which are presented as a cell membrane component in *Leishmania* and mammalian species, respectively. For this reason the toxic effects of AmB are exerted not only in the parasites, but also in humans⁴.

AmB, a natural antibiotic obtained from *Streptomyces nodosus* cultures^{5,6}, can present different aggregation states in aqueous media: a monomeric form, water-soluble oligomers and non-water-soluble aggregates, the first being the least toxic form against mammalian cells⁷. Besides these, a recent study revealed that the AmB within the micelle system, Fungizone[®], can exist as a super-aggregate form by the condensation of monomeric AmB with its aggregates, showing larger size, increased stability and efficacy, and low toxicity against mammalian cells⁸. Currently, as well as the traditional AmB-sodium deoxycholate micelle system (Fungizone[®]), there are several pharmaceutical dosage forms of AmB available in the market: i.e. liposomal AmB (AmBisome), AmB lipid complex (ABLCA BELCET[®]) and AmB colloidal dispersion (Amphocil[®]). However, the high cost of such products makes their use prohibitive⁹. Therefore, the development of novel and inexpensive AmB dosage forms has become mandatory to spread its use to the treatment of Leishmaniasis and other neglected diseases¹⁰.

In this context, an alternative to reduce the AmB adverse effects could be the development of an oil-in-water microemulsion (ME) system. MEs are thermodynamically stable, transparent and isotropic systems comprised by hydrophilic and lipophilic phases and stabilized by surfactants^{11,12}. Furthermore, these systems are able to solubilize drugs in their internal phase or at their interface¹³.¹⁴ In this way, therapeutic efficacy can be enhanced while minimizing adverse effects¹⁵.

However, the presence of water in the ME system may decrease its stability due to the hydrolysis¹⁶ and to microbial contamination¹⁷, which can lead to loss of biological activity of the drug. Thus, drying procedures may be useful in order to decrease the moisture and thereby, increase the stability of the system¹⁸.

Freeze-drying, also called lyophilization, is a dehydration method capable of removing water by vacuum sublimation¹⁹. Thereby, it is not necessary to use high temperatures for drying, which could destabilize the system or degrade thermosensitive drugs such as AmB²⁰.

The purpose of this manuscript is to overview the use of AmB containing ME for the treatment of Leishmaniasis. Additionally, the ability of this system be freeze-dried was evaluated. Therefore, aspects such as Leishmaniasis, AmB, ME, freeze-drying and studies from the literature and from our group addressing these issues are discussed.

Leishmaniasis

Leishmania is an intracellular protozoan that causes a complex of diseases called Leishmaniasis, which is classified into visceral (VL), cutaneous and mucocutaneous forms^{2,3}. Among them, the VL is the most severe one, because if left untreated it can be fatal. According to the World Health Organization (WHO), approximately 1.3 million of new cases are annually detected, provoking 20,000 to 30,000 deaths. The number of new patients worldwide suffering from VL each year is estimated in 300,000, and over 20,000 deaths are reported annually. Leishmaniasis is widespread on almost all continents, but highly prevalent (90 % of cases) in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan²¹.

Leishmaniasis is a public health problem associated with poor housing, population

displacement, malnutrition and HIV–*Leishmania* co-infection. Concerning VL, the poor access to diagnosis and treatment is one of the most important drawbacks to its control²². Furthermore, there is no satisfactory drug treatment so far, due to the inherent high drug toxicity, the drug resistance and the high cost of drugs that present low-toxicity.

The bite of infected female *Phlebotomine* spp. (“Old world” – Africa, Asia, Europe) and *Lutzomyia* spp.(New world – Americas) sandflies transmits leishmaniasis to humans and to animals²³. The parasite in the promastigote form enters the vertebrate host when the sandfly is feeding; then it is phagocytosed by macrophages in which it transforms into an amastigote form and reproduces. When the host cell becomes overwhelmed by parasites, the amastigotes are released and infect other macrophages and finally reach the circulation and infect the organs. The amastigote-infected blood is sucked by uninfected sandflies. Moreover, the amastigote forms are metamorphosed into promastigote ones in the insect gut to subsequently infect a new host²⁴.

The usual immune response to VL is to kill the intracellular parasites by increasing the level of reactive oxygen metabolites or nitric oxide by activation of the infected macrophages through the antigen presentation, with consequent activation of Th1 and production of cytokines (IL-2, ITF- γ). However, in VL infections remarkable interference with the cell signaling is observed. The complex immune response pathways were well described by Gupta et al.²⁵.

However, sometimes the intracellular amastigote forms escape from the immune system, obtains nutrients from the host cell and proliferates in it. Additionally, some strategies are used by the amastigote forms to survive and adapt their life in the inhospitable environment of the phagolysosome of the macrophage in which the pH range from 4.5 to 5.0. For instance, they can interact with surface molecules such as lipophosphoglycan and proteases (H⁺-ATPase, transporters²⁶, GP63 metalloproteases, which disable the key microbicide pathways, disturbing the metabolism and the signaling pathways to provide the parasite growth, and induction of DNA methylation in order to turn off genes controlling microbicidal pathways²⁷.

Anti-leishmanial chemotherapy is related to the parasites' biology. Drugs with a suitable molecular weight and pK_a can be designed to be accumulated into the phagosome and to be taken

up by the amastigote form in the low pH environment²⁶. The current Leishmaniasis treatment is based on five group of drugs²⁸, pentavalent antimonials, miltefosine, paromomycin, pentamidine and AmB. The first introduced treatment against Leishmaniasis was based on the antimonials in the early 1900s²⁹. The pentavalent antimonials, commercially available as Glucantime[®] and Pentostam[®], were first used between 1935 and 1945 and have been extensively used as the first-line treatment³⁰. Nevertheless, an increase in clinical resistance has been reported³¹. On the other hand, Miltefosine (hexadecylphosphocholine [HePC]) was initially used for treatment of cutaneous metastases of breast cancers. Nowadays, it is the first oral drug on the market effective against VL. However, this drug has a long half-life in the body and is very expensive³². Paromomycin, an intramuscular drug used to treat intestinal infections, has been recently approved for Leishmaniasis treatment because it has bactericidal activities. However, it has shown serious adverse reactions, such as renal and ototoxicity. Furthermore, its administration by the parenteral route proved to be less effective than AmB for cutaneous leishmaniasis. Pentamidine, on the other hand, is less active than other drugs. For this reason, it has been used as an alternative treatment for both visceral and cutaneous leishmaniasis, although this engenders an increase in the treatment failure against Leishmaniasis^{33,34}.

AmB has been quite effective against fungal infections caused by *Candida albicans* and *Aspergillus fumigatus*. Its use as leishmanicidal dates from 1959³⁵. Despite being very effective, Fungizone[®] (the micellar delivery system for AmB containing deoxycholate), has severe side effects³⁶. Consensus recommendations from the WHO recognized in 2010 AmBisome[®], the liposomal form of AmB, as the first choice for VL. However, the treatment remains quite expensive, even with the reduced price negotiated by the WHO (\$18 per 50-mg ampoule)³⁷.

The onset of resistance to the current chemotherapeutics is a major concern for the current Leishmaniasis treatment. Although the major resistance mechanisms are not elucidated, there are some studies about how the *Leishmania* develop resistance to these chemotherapies. For example, the antimonials, which are the most affordable drug treatment, have been revealed to be the most vulnerable to resistance. The mechanisms of drug resistance are related to a decrease of drug uptake

and an increase in the efflux mechanism, which reduce the drug concentration inside the parasite by the inhibition of the drug activation, the inactivation of the active drug and the gene amplification^{34, 38}. The Pentamidine-resistant promastigote is also able to reduce the drug uptake and increase the drug efflux³⁹. The resistance for Miltefosine is associated to the suppression of oxidative stress-induced programmed cell death and, probably, to the increase of the gene expression (FeSODA and SIR2)⁴⁰. Studies regarding Paromycin-resistant *Leishmania* have shown that the binding of the drug to the parasite cell surface is reduced⁴¹. Some AmB-unresponsive cases encountered in India were explained by the absence of ergosterol in the membranes of resistant *Leishmania* and the upregulated AmB efflux and reactive oxygen species scavenging machinery⁴².

Therefore, all these issues concerning drug resistance revealed that the development of new therapies is an urgent need. The major challenge in the treatment of Leishmaniasis is the ability of the drug to penetrate inside the macrophages. Since nanocarriers have the ability to penetrate into these cells, their combination with antileishmanial drugs would be a promising approach in Leishmaniasis treatment. As well as the ability to overcome biological barriers, nanocarriers can release the drug inside the macrophage, leading to a local high drug concentration that kills the parasite⁴³.

Nanotechnology has brought promising results for the treatment of Leishmaniasis. There are numerous studies involving drug delivery systems for this disease. Among them, colloidal carriers are the most experimentally investigated⁴⁴. Examples of these nanocarriers include liposomes⁴⁵, emulsified systems (emulsions, nanoemulsions and MEs)⁴⁶⁻⁴⁸, solid lipid nanoparticles⁴⁹, polymeric nanoparticles⁵⁰ and polymeric drug conjugates⁵¹. Such systems are able to improve drug bioavailability and to reduce drug toxicity, improving the selectivity, enhancing the treatment efficacy, increasing the drug solubilization, modulating the drug pharmacokinetics and protecting the drug from degradation⁴³.

The development of nanostructured carriers applied to the drugs currently used in Leishmaniasis has gained notoriety. AmB has been the main drug incorporated in these colloidal carries, since it has an excellent cure rate⁵².

In our work, ME was chosen as the nanostructured carrier due to the aforementioned advantages, besides their relatively easy handling compared with other formulations such as liposomes. Indeed, these system usually do not use organic solvent in their production, has an impact for the environment, and for ease of manufacturing and scale-up⁵³. Moreover, ME systems would allow targeting of AmB to the cells and organs affected with VL (liver, spleen and bone marrow).

Amphotericin B

AmB is an antifungal and antiprotozoal drug that was originally isolated from *Streptomyces nodosus*^{5, 6}. Chemically, this molecule is considered a macrolide due to its large macrocyclic lactone ring composed of 37 carbons in the main chain. This molecule contains a heptaene from the carbon 20 to the carbon 33, providing a non-polar characteristic to AmB⁵⁴. The opposite side of the molecule provides polarity due to the presence of seven hydroxyl groups and one carboxyl group directly attached to the main cycle. As a result, this molecule has amphiphilic properties⁵⁵. As well as the carboxyl group (pK_a 5.5), AmB has a primary amine group (pK_a 10) attached to the mycosamine ring (3-Amino-3,6-dideoxy- β -D-mannopyranose). This amino sugar group is connected to the main macrocyclic chain by an o-glycoside linkage at the carbon 19. The anionic carboxylic acid and the cationic protonated amino group result in a zwitterion, providing the molecule with its amphoteric properties⁵⁶. Figure 1 shows the chemical structure of AmB.

Figure 1

AmB is known for interacting with sterol molecules, such as cholesterol and ergosterol, the latter being present in fungal and *Leishmania* cell membranes³⁸. AmB interaction with ergosterol in fungal cells was the primary suggested information that led to studies of AmB's mechanism of action. It is suggested that the AmB may act on several different targets in fungal cells leading to their disruption. The first and most accepted mechanism of action is that the AmB binds to the

ergosterol molecules on the fungal cell membrane and form pores, which increase the permeability of the membranes to small cations, promoting depletion of intracellular ions and cell death^{57, 58}. Another possible mechanism is the induction of ergosterol sequestration resulting in membrane disruption⁵⁹.

Although pore formation is a well established mechanism, Sokol-Anderson *et al.* showed that without oxidative damage the pre-lethal ionic leakage would not be sufficient to kill fungal cells⁶⁰. It is now known that the AmB acts in the intracellular level inducing an oxidative burst. Possible mechanisms are its action as a pro-oxidant, inducing the formation of reactive oxygen species (ROS) that cause protein, DNA and membrane damage. In order to this to happen, it is necessary for the cell to take up AmB by interaction with ergosterol. Another possible mechanism is the interference of the ROS generated with the cells' mitochondrial activity since these reactive species are part of the respiratory chain⁵⁹.

Although more extensive studies on the AmB mechanism of action have been conducted in fungal cells, the mechanism of action involving the interaction with ergosterol and the formation of pores that result in osmotic changes and cell lysis has been confirmed to happen in *Leishmania*, as well as oxidative damage⁶¹. Additionally, it has been reported that the interaction of AmB with human cholesterol in macrophages inhibits the binding of *Leishmania donovani* promastigotes to human macrophage⁶².

Various studies have shown that *L. donovani* promastigotes can develop resistance to AmB in order to reduce the drugs' lethal effect. Purkait *et al.*⁶³ and Mbongo *et al.*⁶⁴ studied different strains of *L. donovani* in order to investigate the mechanisms of resistance. Purkait and his co-workers investigated the intracellular ROS levels for AmB-resistant and AmB-sensitive promastigotes. Their study showed that the ROS level of the AmB-sensitive strain was approximately 3-fold greater than that of the AmB-resistant strain. The authors suggested that resistant strains have upregulated ROS scavenging machinery and also enhanced AmB efflux.

Mbongo and co-workers⁶⁴ observed that the AmB-sensitive strains had ergosterol as the major sterol in their membrane, whereas the AmB-resistant strain membrane sterol composition

showed no ergosterol, containing cholesta-5, 7, 24-trien-3 β -ol as the major sterol. Changes in the sterol chains, such as methylation, would decrease the AmB affinity to the membrane. Since the interaction of AmB with ergosterol leads to the formation of pores and cell disruption, the reduction of ergosterol would decrease the AmB effectiveness on *Leishmania* membranes^{64, 65}. It was also observed that the membrane fluidity is fairly well correlated to the parasite resistance⁴². Indeed, AmB-resistant cells presented higher membrane fluidity, mainly due to the presence of the cholesta-5, 7, 24-trien-3 β -ol instead of the ergosterol⁶⁴. However, it is noteworthy that both AmB-resistant and AmB-sensitive promastigotes strains were infective for macrophages *in vitro*, whereas the AmB-sensitive strain was infective only *in vivo*⁶⁴. Therefore, it is still unclear whether the AmB-resistant strains represent a health issue, because the study showed reduced infectivity due to the change of the sterol composition and the decreased ability to infect macrophages.

AmB has a broad antifungal spectrum of action, including *Candida* spp., *Aspergillus* spp., and other filamentous fungi³⁶. However, the high nephrotoxicity induced by the AmB micelle system, Fungizone[®], has limited its use as second-line treatment when first course with azoles or echinocandins (regarding patients' conditions and specific infection) fails to eliminate the infections⁶⁶⁻⁶⁸. AmB has been used to treat VL in cases of *Leishmania* resistance to antimonial drugs. However, its toxicity can limit the treatment due to the severe side-effects and poor patient compliance⁶⁹. The infusion of Fungizone[®] can be accompanied by shaking, fever, vomiting, tachypnea and may require co-administration of antipyretics, antihistamines, antiemetics and hydrocortisone to reduce the side effects³⁶.

Severe toxicity is usually attributed to the interaction of AmB aggregates with cholesterol molecules in human cells. AmB is able to self-associate and generate different aggregated forms. In its monomeric form this molecule is able to interact only with ergosterol and thus exhibit its pharmacological effect. Aggregated structures such as dimers are suggested to also be able to interact with cholesterol-rich membranes present in human cells and cause side-effects. Lambing et al.⁷⁰ and Huang et al.⁷¹ suggested that dimerization is a necessary event for AmB interaction with cholesterol. On the other hand, studies have shown that heat-induced AmB superaggregates are able

to reduce toxicity against human cells, possibly by reducing the affinity to cholesterol-rich membranes and acting as a reservoir by releasing monomers to the media ^{8, 72}.

Regarding the stability of AmB, few studies have reported on its chemical stability in formulations. In particular, the identity and toxicological safety of AmB degradation products and the kinetics of their formation in various environmental conditions and drug delivery systems have not been described. Studies have demonstrated that the AmB toxicity is reduced in lipid-based delivery systems ^{73, 74}. However, it is unclear whether reduced toxicity is only due to the reduced dimer and AmB-aggregate concentrations or if the changes in the pattern and rate of degradation product formation can play a role in the toxicity.

Because Fungizone[®] was the first approved formulation containing AmB for intravenous use, it is considered the traditional formulation of AmB. This micellar system was developed in order to improve drug solubility in water. Due to the acute and long-term side effects of this formulation, other formulations containing AmB have been studied. Nowadays, it is also known that the reduced side-effects of lipid formulations of AmB enables the use of this drug for prophylaxis ⁷⁵. Currently, several lipid-base formulations are available and approved to treat leishmaniasis in different countries, such as the liposomal formulation (AmBisome[®]) and the lipid other complexes (Amphotec[®] and Abelcet[®]). However, their high cost of such formulations limits their use, especially in emerging and underdeveloped countries.

AmBisome[®] was the first drug approved by the U.S. Food and Drug Administration (FDA) for VL in the United States ⁷⁶. This approval was a consequence of increasing cases of resistance to antimonials in parts of Europe, India, East Africa and South America. In Brazil, a study compared different treatment regimens using AmBisome[®]. Patients were children younger than 12 years old. The study showed 100% success in a regimen consisting of 2mg/kg on days 1-10 (total dose, 20 mg/kg) ⁷⁷. The Brazilian government recommends a regimen with AmBisome[®] consisting of 3mg/kg on days 1-7 (total dose, 21 mg/kg) or 4mg/kg on days 1-5 (total dose, 20 mg/kg) ⁷⁸. However, this treatment is suggested only in patients with renal insufficiency due to its high cost. The WHO suggested a single dose of liposomal AmB (AmBisome) (10mg/kg) as first line

treatment of VL in the South-East Asia region. The treatment was suggested on the Regional Technical Advisory Group (WHO) meeting in 2009 and later endorsed by the expert committee meeting in Geneva, 2010 ⁷⁹.

A clinical study performed by Mondal and co-workers ⁸⁰ evaluated the feasibility of the suggested treatment from WHO in patients from rural areas in Bangladesh. The study was conducted with 398 patients diagnosed with VL. After the patient's consent and some exclusions due to other criteria, 298 patients received complete treatment consisting of 10mg/kg of liposomal AmB as a single dose. Patients were discharged and returned for follow-up visits on day 30 and day 180 after treatment. After 30 days, 36 patients had not attained the necessary increase in the hemoglobin concentrations (part of initial cure protocol). However, they did not show any sign of active disease. On the additional follow-up visits (day 60 and day 90), they were considered initially cured. On the other hand, between days 30 and day 180, 7 patients relapsed and were retreated. The overall cure rate was 97%. The study provided an important assessment on the feasibility of single-dose treatment with AmBisome for rapid elimination of Leishmaniasis as recommended by the WHO. It is noteworthy that the most common adverse events during and 2 hours after treatment were fever with rigor, fever, vomiting and hypotension. In addition, the incidence of adverse events was 47% during treatment and 63% after 2 hours of treatment.

Although the commercially available liposomal formulation of AmB have shown positive results in the treatment of Leishmaniasis, the occurrence of toxicity and the high cost of the product still limits its use. Therefore, further research regarding the development of new drug delivery systems containing AmB, such as nanoparticles, nanoemulsions and MEs would be a suitable alternative to obtain safe formulations to deliver this drug in a controlled way and to protect the human cells against the AmB toxicity, since it has been proved that some of these systems are capable of reducing AmB toxicity when compared to AmBisome ⁸¹⁻⁸³.

Microemulsion

Different carriers have been used as drug delivery systems to treat infectious diseases; e.g. MEs^{48, 84, 85}, nanoemulsions⁸⁶, nanoparticles⁸⁷⁻⁹¹, solid lipid systems⁹²⁻⁹⁷, carbon nanotubes^{98, 99}, liposomes¹⁰⁰⁻¹⁰⁵, micelles^{102, 106-111} and dendrimers¹¹². Among them, MEs show interesting properties for delivering drugs to *Leishmania* parasites. MEs are colloidal optically transparent systems, with thermodynamic stability, low viscosity and surface tension, small droplet size, composed of two immiscible liquids (water and oil), stabilized by an interfacial film of a surfactant, usually in combination with a co-surfactant¹¹³⁻¹¹⁸. These systems, first described by Hoar and Schulman¹¹, can also be classified as three major microstructures: swollen micellar (oil-in-water, O/W), reverse micelles (water-in-oil, W/O) and bicontinuous^{119, 120}. Other types of microstructures formed in MEs are cubic, cylinder-like (such as rod-micelles or reverse micelles), plane-like (e.g., lamellar structures) and hexagonal structures^{121, 122}, depending on the concentration, nature and arrangement of the molecules in the formulation¹²³.

MEs differ from emulsions by their smaller droplet size as a result of the low interfacial tension^{124, 125}. The dispersed phase occurs as very small droplets (10 – 100 nm) that hardly scatter light in the visible wavelength domain, explaining their tendency to appear transparent. Moreover, these systems present a spontaneous formation and thermodynamic stability due to a relatively large amount of surfactant stabilizing their interfacial area¹²⁶. The low interfacial tension compensates the dispersion entropy; hence the system is thermodynamically stable¹²⁷ and forms spontaneously under a specific set of composition and environmental conditions¹²⁴.

Surfactants play an important role in ME development due to their self-association into a variety of equilibrium phases when dispersed in water or in non-aqueous solvents^{127, 128}. During film formation, the type of system formed is dependent on the surfactants' affinity for water or oil^{127, 128}. Hydrophilic lipophilic balance (HLB), the critical packing parameter (CPP) and the solubility parameters approaches have been proposed to rationalize surfactant selection for ME production. HLB takes into account the relative contribution of the hydrophilic and the hydrophobic fragments of the surfactant molecule^{127, 129, 130}. CPP relates the preferred geometry adopted by the

surfactant to form particular aggregates¹¹⁵. The size and shape of the ME are mainly governed by the curvature free energy of the interface between water and oil, the bending elastic constant and the curvature of the surfactant film. In the solubility parameter theory, two chemical compounds are infinitely soluble and capable of formation of ME when the chemical intermolecular forces are equivalents between them¹³¹⁻¹³⁵.

ME formation has been explained by the following three theories: interfacial or mixed film theory, solubilization theory and thermodynamic theory^{115, 136, 137}. The interfacial or mixed film theory considers that the interfacial film can be considered duplex in nature (region bounded by water by one side and oil by the other) with an inner and an outer interfacial tension acting independently to form the ME spontaneously due to the low interfacial tension, which tends to zero¹³⁸. The solubilization theory is based in the principle that MEs are swollen micelles in which either the water or the oil are solubilized in reverse and normal micelles, respectively^{139, 140}. The thermodynamic theory considers that the free energy must be negative to form thermodynamically stable MEs, which depend on the reduction of the surface tension of the oil – water interface by the surfactants and the change in the entropy of the system^{11, 137, 141}.

Although MEs may form spontaneously, external factors can be used to overcome kinetic barriers, thereby reducing the time of formation of these systems. Some factors that can accelerate and facilitate the formation of the ME system are the order of component addition, the application of mechanical agitation and the use of ultrasounds or heat. Moreover, ME can be formed by phase inversion and phase titration methods¹²⁶.

The ME system has many advantages as a drug delivery system, including enhancement of drug solubility, high stability and small droplet size, which provides a large surface area improving the absorption or the permeation ability of the drugs through membranes compared to the conventional formulations^{115, 119, 127, 142}. Due to the ability to enhance the bioavailability of poorly soluble drugs by maintaining them as a molecular dispersion, MEs can promote an effective control and sustained release of their active agent^{127, 143}. Moreover, MEs can store drugs in their internal phase, protecting them against digestive enzymes and from light (photosensitive drugs)^{144, 145}.

Additionally, they are easy to manufacture (not process-dependent) and scale-up^{120, 146, 147}.

MEs have been used by different routes of administration, such as percutaneous, oral, topical, transdermal, ocular and parenteral^{116, 148}. In particular, in parenteral administration, ME improves the drug residence in the blood circulation and may reduce the drug adverse effects¹⁴⁹. Additionally, this system causes minimum immune reactions or fat embolism in contrast to emulsions¹⁵⁰.

ME has been an efficient carrier for AmB with potential fungicide and fungistatic applications against histoplasmosis, aspergillosis and candidiasis^{151, 152}, as well as, for protozoan infections such as visceral leishmaniasis¹⁵³. In this context, a recent study carried out by our research team aimed to use an oil-in-water (o/w) ME system to improve the solubility of AmB for the oral route⁸⁴. Different nonionic surfactants were tested to evaluate their solubilization ability in combination with several oils. AmB-loaded MEs with mean droplet sizes of about 120 nm were successfully produced. These systems were able to improve the drug solubility up to 1000-fold. Cytotoxicity studies revealed limited toxicity against macrophages, suggesting that this formulation could be a suitable carrier for the AmB that would also reduce its *in-vivo* toxicity. Some years ago Gupta et al. developed an o/w ME containing quercetin based in a pseudoternary diagram study for Leishmaniasis treatment in hamster models⁴⁸. They showed that clove oil/Tween 20[®]/water can form a very stable (temperature and salt resistant) o/w ME system with reasonably low droplet dimension. A significant improvement in the pharmacological efficacy of the drug was observed. Although the vehicle showed some hepatotoxic and nephrotoxic effects, the drug-loaded ME seemed to function better against experimental leishmaniasis in hamster models than the free drug.

In another study, Brime et al demonstrated the *in vivo* efficacy of a new AmB o/w lecithin-based ME compared to the Fungizone[®]. The study was conducted in immunocompetent and neutropenic murine models of systemic candidiasis¹⁵⁴. The doses administered (1, 2 and 3 mg/kg) were well tolerated due to their reduced toxicity. The AmB-loaded ME mice treated group had a longer survival time than the infected mice without treatment. According to the authors, the ME presented better results than the Fungizone[®] in an established infection in both immunocompetent

and neutropenic mice.

Recently, a topical ME containing AmB was investigated for the treatment of invasive fungal infections¹⁵⁵. The influence of the surfactant and co-surfactant mass ratio on the ME formation was evaluated. Also, the permeation of the ME through excised rat skin was studied. The authors produced an optimized formulation consisting of 0.1% (w/w) AmB, 5% (w/w) Isopropyl Myristate and 35% (w/w) surfactant (Tween 80: Propylene glycol (3:1)). The ME showed an average droplet size of 84.2 nm, a polydispersity index of 0.164, a pH of 7.36 and a conductivity of 229 μ S. The formulation was found to be stable at 2-8 °C and at room temperature, for the period of three months. Also, it exhibited a 2-fold higher drug permeation when compared to the plain drug solution. The *in-vitro* anti-fungal activity against *Trichophyton rubrum* fungal species have shown that the developed ME showed a higher zone of inhibition of fungal growth (5.1mm after 72h) due to its high fluidity compared to other ME systems.

AmB in a lipid-based preparation as a ME was developed by Darole et al. (2008). Its toxicity was also evaluated in comparison to the commercial formulation Fungizone[®]¹⁵⁶. Pseudoternary phase diagrams were constructed to identify areas of existence of ME composed of glyceryl monooleate as oil phase and polyethylene glycol (40)-stearate and polyethylene glycol (15)-hydroxystearate as surfactants. The average droplet size of the ME was 84 nm. The ME showed a 92% decrease in the hemolysis of human red blood cells (RBCs) *in vitro* when compared with the commercially available Fungizone[®]. The LD₅₀ in mice was estimated to be 3.4 mg/kg. The authors concluded that the formulation would be a safe and effective alternative to classical AmB therapy. In a similar study, an AmB-loaded ME was developed in order to increase its efficacy and decrease its toxicity compared to Fungizone[®]¹⁵⁷. On this case, the AmB-loaded ME showed an average size of 300 nm. The selectivity for fungal cells was maintained after the incorporation of the drug in the ME and this system had the same efficacy of Fungizone[®] against *C. albicans*. Additionally, the ME showed lower leakage of hemoglobin and K⁺ from the RBCs compared to Fungizon[®].

Patents involving the formation of AmB-loaded ME have also been developed. A stable ME

composition including of AmB with additives suitable for intravenous administration was disclosed. The formulation was designed to provide safety, to improve stability and to be produced by an efficient, simple and rapid process^{158, 159}.

Considering the high cost of Ambisome[®] and the high toxicity of Fungizone[®], in our work¹⁶⁰ (data not shown) the strategy to improve the therapeutic index of AmB with inexpensive ingredients was to produce a biocompatible ME, which was prepared with phosphate buffer pH 7.4, Tween 80[®], Lipoid S100[®] (Soybean phosphatidylcholine) and Mygliol 810[®]. The AmB load content was 5 mg/mL. The ME droplet size measured by Dynamic Light Scattering was about 40 nm. The spherical shape of the system was confirmed by the Transmission Electron Microscopy. Additionally, the rheological analysis showed low viscosity and Newtonian behavior of the ME system.

Our group has also evaluated the biological activity of this AmB-ME formulation against *Leishmania donovani* LV9¹⁶⁰ (TO BE PUBLISHED SOON 1). The overall result allowed us to conclude that the IC₅₀ on axenic amastigotes for the AmB-ME was 0.2 μM, and the IC₅₀ on intramacrophagic amastigotes growing in J774 cells was 0.81 μM. The AmB-ME selectivity Index was higher than Anforicin B[®] (a Brazilian product similar to Fungizone[®]). Furthermore, the AmB-ME showed a high activity against AmB-resistant amastigotes in comparison with Ambisome[®]. The *in-vivo* efficacy was evaluated in Balb/C mice infected with *Leishmania donovani* LV9 after IV administration of the different formulations (three doses of 1 mg/kg AmB). The AmB-ME showed a reduction of the parasite burden of 78% compared to 72% for the Anforicin B[®]. The *in-vivo* toxicity was determined by the changes in total serum cholesterol, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatinine and urea concentrations. The results did not show important differences among the samples analyzed. Furthermore, they were not able to cause kidney or liver dysfunction. Therefore, the ME containing AmB developed in our work seems to be an inexpensive alternative formulation for this molecule, showing good efficacy and selectivity for the *Leishmania donovani* and merits further investigation.

Freeze-drying

Many pharmaceutical industries have used this technique as a method to obtain more stable products, since the aqueous media, present in large amount in colloidal systems, may cause some chemical and microbiological reactions that can destabilize them ¹⁶¹. Freeze-drying is a dehydration technique capable of bringing the product to an anhydrous or almost anhydrous state, using low temperatures and pressures ¹⁶².

This process consists of three consecutive stages: (1°) freezing (solidification of the water), (2°) primary drying (ice sublimation) and (3°) secondary drying (desorption of water) ¹⁹. The first step is of utmost importance since the ice crystal formation can induce physicochemical changes, as well as droplets aggregation ¹⁶³. The temperatures to which the samples are exposed must be below the glass transition temperature (T_g). If this is not the case, during the subsequent step, the sample will collapse, losing its integrity and stability ¹⁶⁴. Furthermore, the freezing rate should be carefully chosen, since different water crystals can be formed, influencing the primary and the secondary drying in different ways. Large crystals are produced in small quantities when a slow freezing is used. On the other hand, fast freezing is able to form small and numerous ice crystals ¹⁶⁵.

After freezing, the next step, the primary drying, is the most time- and energy-consuming, because the sublimated water vapor has to be continuously removed from the sample, maintaining the freeze-dryer chamber pressure below the vapor pressure of the ice, with subsequent condensation on a cooling coil ²⁰. Therefore, the temperature during this step should be optimized in order to reduce the cost of the process. The freezing rate influences the length of this step, because if the process was conducted at the fast rate, the small ice crystals formed will hinder the movement of the water vapor ¹⁶³. Furthermore, the higher the sample temperature, the lower the time required for the primary drying. However, the structure of the product could be lost if its temperature reaches values above the T_g, leading to a collapse that affects the quality of the freeze-dried product ¹⁶⁴.

The unfrozen water that was not removed by sublimation will be removed by desorption from the solute phase during the secondary drying, once the shelf temperature has been increased and the chamber pressure reduced. As for the primary drying, the temperature of the sample during

this step must be below the Tg in order to prevent the product from collapsing. This step is also influenced by the freezing rate. Since the adsorption-desorption equilibrium is the most important factor during the secondary drying, the large surface area of samples submitted to a fast freezing procedure will favor water desorption and reduce the time for this step ¹⁶⁶.

At the end of the freeze-drying process the moisture content can be reduced to levels below 1% ¹⁹. However, the system may undergo some damage during these steps, resulting in the loss of its properties, such as the increase in the interaction between the system components, which can lead to aggregation of the droplets and change in the ME pH¹⁸.

The system integrity can be protected against these drawbacks and destabilization by proper selection of cryoprotectant molecules that are capable of reducing the stresses caused by the temperature reduction and the dehydration ¹⁶⁷.

Cryoprotectants are able to bind to the surfactants, avoiding droplet aggregation when the water separates from the other components during the freezing process ¹⁶⁸. These compounds are also able to decrease the interaction between the hydrocarbon chains of the phospholipids during the drying steps ¹⁶⁹, as well as to replace the hydrogen bonds with water ¹⁶⁵. Although the mechanism of action of cryoprotectants is not yet well understood, the theory currently used to explain their action is the ability of these molecules to form hydrogen bonds that replace water during the sublimation ¹⁶⁷.

The main cryoprotectant molecules used are carbohydrates, such as trehalose, sorbitol, glucose, mannitol, sucrose, fructose and lactose ¹⁷⁰. The concentrations used are dependent on factors such as temperature and rate of cooling and freezing ¹⁷¹. The concentration of the molecule reaches a limit of stabilization, whereas above this limit the cryoprotectant destabilizes the system ¹⁸.

An acceptable freeze-dried product has to maintain the same physical and chemical properties of the system before and after reconstitution. Therefore, it is important to analyze all the parameters that affect the final freeze-dried product ¹⁷². Several important factors must be considered during the lyophilization; for example, the thickness of the frozen layer, the formulation, the type of container, the equipment and the process variables such as the shelf temperature, the

chamber pressure, the time (freezing, primary and secondary drying), and the nature and concentration of the cryoprotectant ¹⁹.

Freeze-drying is an excellent drying method for dehydrating biological products and drug delivery systems with high water content ¹⁹. Examples of such systems are MEs; however the lyophilization process has been rarely studied for these systems. Moreno et al. freeze-dried a ME system using 5% of mannitol as cryoprotectant, freezing at -40°C for 2 h, and drying for 24 h (primary drying) and 48 h (secondary drying). The process did not interfere on the parameters studied for these formulations (macroscopic aspects, droplet size distribution, residual moisture, viscosity and rheological behavior). Furthermore, the freeze-dried MEs showed better stability than the non-lyophilized ones ¹⁷³. This work also evaluated the efficacy of the AmB-ME against *Candida* strains, its safety ¹⁷⁴ and its pharmacokinetics parameters ¹⁵⁴. The freeze-dried ME maintained their antifungal activity and reduced toxicity. The system also demonstrated good efficacy in animals and showed biphasic elimination from the blood.

Experiments on the freeze-drying process were also performed for MEs developed by our group ¹⁷⁵ (data not shown). Since this system has a large volume of aqueous phase, the surfactants Tween 80[®] and Lipoid S100[®] or the medium chain triglycerides may undergo hydrolysis, as well as being susceptible to drug leakage and microbial growth. Therefore, water removal might improve the stability of the ME, enhancing its shelf life. However, a large number of freeze-drying factors may influence the quality of the final product. For this reason, we decided to use a 2³ full factorial design with three replicated points to optimize the parameters: freezing temperature, freeze-drying time, type and concentration of cryoprotectants.

Design of experiment (DOE) is a tool used to improve the quality of products and processes, that is able to evaluate a large number of variables using a reduced number of samples through matrix combinations between the levels of the variables investigated. Moreover, it is possible to identify the variables and their interactions that influence on the parameters of interest, and optimize multiple variables ^{176, 177}.

The results showed that maltose presented the best protective effect. It was possible to

observe in the DOE using this cryoprotectant that the freezing was the only studied parameter that had a significant effect on the droplet size of the freeze-dried ME. This is probably because of the separation of ice from the concentrated solution, which can cause interaction among the droplets, and, consequently, their fusion ¹⁹.

The response surface analysis showed that the concentration of 5% of maltose led to the lowest droplet size ¹⁷⁵ (TO BE PUBLISHED SOON 2). In addition, the best quality product was obtained by freezing the sample at around -88°C and freeze-drying for 24 hours. The optimized conditions were then evaluated by comparing the physicochemical characteristics of the system before and after the freeze-drying process. The changes observed in the freeze-dried ME did not impact on the overall properties of this system, which could be administered by the parenteral route.

The same optimized parameters were used to freeze-dry the AmB-loaded ME. The results observed were similar to the system without the drug. Moreover, the drug content remained the same as that before freeze-drying. Therefore, this process did not induce drug degradation.

The ME morphology (with and without AmB), examined by transmission electron microscopy and scanning electron microscopy, was found to be similar before and after the freeze-drying process. However, the droplet size was slightly increased after reconstitution of the system to the original volume with vortex stirring. During the lyophilization, the phosphatidylcholine present in the ME returned to the dry state. Therefore, in order to reform the ME droplets to the same size, the reconstituted ME must be heated above its transition temperature of 52°C.

The influence of the heating on the phase behavior of the ME system reconstituted from a lyophilized state was also studied ¹⁷⁸ (TO BE PUBLISHED SOON). The changes in the droplet size and the transparency were evaluated. The use of vortex stirring followed by heating was able to reconstitute the ME after the lyophilization process, since at 60°C a phase transition from emulsion to ME occurred and at approximately 65°C the system showed a great transparency and a small droplet size. Indeed, by heating, the nonionic surfactant becomes more hydrophobic; therefore, less lipophilic surfactant is needed to reach the optimum HLB ¹⁷⁹. Furthermore, with increasing temperature the spontaneous curvature of the membrane becomes negative, which favors the

formation of O/W structures¹⁸⁰.

Conclusion

VL is a parasitic disease that can be fatal. It affects numerous countries, especially in emerging and undeveloped areas. The resistance to traditional antimonials, the toxicity of the micellar AmB formulation and the high cost of the liposomal AmB formulation have hindered the eradication of VL. Thus, the development of a less toxic and more affordable system containing AmB has been pursued. This review shows that promising studies have been carried out in order to obtain an affordable and effective ME system containing AmB to treat VL. *In-vitro* studies from our group have shown that the ME containing AmB was more effective against AmB-resistant strain of *Leishmania donovani* than the commercially available liposomal AmB formulation. *In-vivo* studies also showed a good profile of efficacy for the ME system. The literature suggests that the freeze-drying is as an effective technique to improve the stability of drugs and pharmaceutical products. Preliminary studies from our group aiming to improve the AmB-containing MEs' stability have shown that the freeze-drying MEs require optimum conditions and parameters to guarantee the success of the entire process. Our studies revealed that the freeze-drying technique was an excellent method to obtain a dried AmB-containing ME that was easy to redisperse. Despite the recent developments aiming to improve the treatment of VL, further studies with the freeze-dried ME containing AmB are still necessary in order to obtain all necessary information to establish this system as a new pharmaceutical product to treat VL.

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Andreza Rochelle do Vale Morais, Freeze-dried microemulsion containing amphotericin B for leishmaniasis treatment: An overview, **Figure 1**

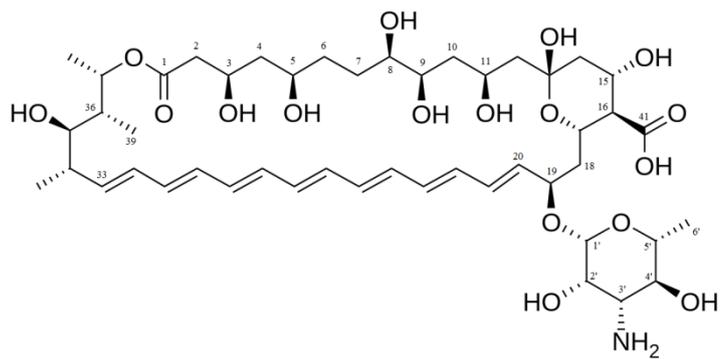


Figure 1 – Structure of Amphotericin B showing the numbering of carbon atoms in the molecule

(Molecular Mass 924.08 g/mol)

SECTION I

MICROEMULSION SYSTEM CONTAINING AMPHOTERICIN B FOR
LEISHMANIASIS TREATMENT

BRIEFING

Amphotericin B (AmB) has known pharmacological activity against the genus *Leishmania*. However, the formulations available on the market have a high cost, high toxicity and/or are subject to resistance from the parasite. Therefore, the work described in Chapter II was designed to find new ways to deliver AmB with low cost of production and low toxicity, which would make it possible for the populations at risk to have access to an affordable, efficient and safe formulation.

Therefore, two systems were produced: a microemulsion containing this drug and a system derived by heating the commercially available AmB micellar form. These systems were characterized for their physico-chemical properties and their toxicity and activity were evaluated *in vitro* and *in vivo*.

This work was carried out in Paris with the collaboration of Professor Philippe Loiseau (UMR 8076 CNRS BioCIS), responsible for the team “Chimiothérapie Antiparasitaire”.

CHAPTER II

In-vitro and in-vivo antileishmanial activity of inexpensive Amphotericin B formulations: heated Amphotericin B and Amphotericin B-loaded microemulsion

***In-vitro* and *in-vivo* antileishmanial activity of inexpensive Amphotericin B formulations:
heated Amphotericin B and Amphotericin B-loaded microemulsion**

Running title: Antileishmanial activity of Amphotericin B formulations

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Abstract:

Amphotericin B (AmB) is effective against visceral leishmaniasis (VL), but the renal toxicity of the conventional form, mixed micelles with deoxycholate (M-AmB), is often dose-limiting, while the less toxic lipid-based formulations such as AmBisome[®] are very expensive. Two different strategies to improve the therapeutic index of AmB with inexpensive ingredients were evaluated on this work: (i) the heat treatment of the commercial formulation (H-AmB) and (ii) the preparation of an AmB-loaded microemulsion (ME-AmB). M-AmB was heated to 70°C for 20 min. The resulting product was characterized by UV spectrophotometry and circular dichroism, showing super-aggregates formation. ME-AmB was prepared from phosphate buffer pH 7.4, Tween 80[®], Lipoid S100[®] and Mygliol 812[®] with AmB at 5 mg/mL. The droplet size, measured by dynamic light scattering, was about 40 nm and transmission electron microscopy confirmed a spherical shape. Rheological analysis showed low viscosity and Newtonian behavior. All the formulations were active *in vitro* and *in vivo* against *Leishmania donovani* (LV9). A selectivity index (CC₅₀ on RAW/IC₅₀ on LV9) higher than 10 was observed for ME-AmB, H-AmB and AmBisome[®]. Furthermore, no important *in vivo* toxicity was observed for all the samples. The *in-vivo* efficacy of the formulations after IV administration was evaluated in Balb/C mice infected with LV9 (three doses of 1 mg/kg AmB) and no significant difference was observed between H-AmB, M-AmB, ME-AmB and AmBisome[®]. In conclusion, these two inexpensive alternative formulations for AmB showing good efficacy and selectivity for *Leishmania donovani* merit further investigation.

INTRODUCTION

Leishmaniasis is a parasitic infectious disease caused by obligate intra-macrophage protozoa of the *Leishmania* species, transmitted to humans via the bite of female sand flies of the genera *Phlebotomus* and *Lutzomyia*. This disease is widespread in tropical and subtropical areas as three different clinical manifestations: mucocutaneous, cutaneous, and visceral leishmaniasis (VL), also known as kala-azar, which is fatal if untreated (1). Pentavalent antimonials are the most commonly used drugs to treat VL; however, they are no longer recommended due to their toxicity and the emergence of drug resistance (2). Amphotericin B (AmB) and miltefosine are the two alternative drugs able to replace antimonials, although neither drug is completely safe (3). This work is focused on the exploitation of AmB as low-cost and low-toxic formulations.

AmB has been used as a second-line drug for leishmaniasis treatment (4). Despite its high efficacy, toxic effects, such as cardiotoxicity and nephrotoxicity, limit its successful therapeutic use. The AmB selectivity and toxicity level depend on its aggregation state, which can exist as monomeric, aggregated and super-aggregated states. In order to reduce the side effects credited to AmB, several lipid formulations have been developed. These systems prevent the AmB self-aggregation and slowly release AmB monomers to the surrounding medium (5). An example of success of the lipid-based approach is the liposomal AmB formulation (AmBisome[®]), which is less toxic than Fungizone[®], the commercial available micellar AmB (M-AmB). Nevertheless, AmBisome[®] is not widely available due to its cost.

To summarize, the current visceral leishmaniasis treatment remains limited by the issues of drug resistance, toxicity and high cost (6). The therapeutic properties and pharmacological profile of AmB can be optimized by modifying the aggregation state of the drug (7) and by developing new drug delivery systems.

M-AmB has good availability and relatively low cost; hence it is the most widely used AmB-based product. However, the presence of AmB in the aggregated state allows

the formation of ion channels in membranes containing cholesterol, which causes toxicity (8). Studies have shown that the heating of M-AmB is capable of inducing a new type of aggregate, called super-aggregates, which is less selective for the cholesterol in the mammalian cell membranes. Therefore, the toxicity can be significantly reduced without loss of activity (5, 7, 9, 10). The molecular rearrangement that yields super-aggregate structures can be achieved by appropriate heating of M-AmB (5, 11).

An alternative formulation without the drawbacks of cost and toxicity could be a microemulsion (ME). Microemulsions are anisotropic, clear, small droplet sized and thermodynamically stable drug delivery systems, comprised of an oil and aqueous phase, stabilized by surfactants (12). The ME would be able to carry AmB (ME-AmB) in its dispersed phase, increasing its solubilization and bioavailability, and decreasing its toxicity (13, 14).

The aim of this work was to investigate Heated M-AmB (H-AmB) and ME-AmB in comparison with M-AmB and AmBisome[®] with regard to their *in-vitro* and *in-vivo* safety and antileishmanial activity against *Leishmania donovani*, in order to improve the therapeutic index of AmB while using inexpensive ingredients.

MATERIALS AND METHODS

Chemicals

Micellar AmB (Anforicin B[®]) was a gift from Cristália (Itapira, Brazil). Liposomal AmB (AmBisome[®]) was purchased from Gilead (Foster City, USA). Miglyol[®] 812, used as the oil phase of the ME, was obtained from CONDEA Chemie GMBH (Hamburg, Germany). Lipoid[®] S100, used as surfactant, was purchased from LIPOID GMBH (Ludwigshafen, Germany). The AmB, used to load the ME, Tween[®] 80, used as surfactant, Sodium Phosphate Monobasic and Sodium Phosphate dibasic, used to prepare the phosphate buffer pH 7.4, were all acquired from Sigma Aldrich Inc (St. Louis, USA).

Sample preparation

AmBisome[®] was prepared according to the manufacturer's instructions. The commercial M-AmB was prepared by adding 10 mL of water for injection into the vial containing 50 mg of AmB (final concentration of 5×10^{-3} M), followed by vortex shaking until dissolution. In order to obtain the H-AmB, the M-AmB was heated at 70°C for 20 min.

In order to prepare the ME, 68 %_(w/w) of phosphate buffer pH 7.4, 14.7 %_(w/w) of Tween[®] 80, 6.3 %_(w/w) of Lipoid[®] S100 and 11 %_(w/w) of Miglyol[®] 812 were weighed. They were mixed under magnetic stirring followed by 3 cycles of probe sonication (80 watts power output) in a Branson Digital Sonifier S-250 (Branson Ultrasonic Corporation – Danbury, USA) for 1.5 min and followed by 3 min in a 1210E-MTH Bransonic Ultrasonic bath (Branson Ultrasonic Corporation – Danbury, USA). To incorporate AmB into the ME, solid AmB was added to a final concentration of 5×10^{-3} M. Then, the system was alkalinized with NaOH 1M until complete AmB solubilization. After that the loaded ME was neutralized by addition of HCl 1M to a final pH of 7.4. Afterwards, the MEAmB was filtered in 22µm membrane.

M-AmB and H-AmB characterization

AmB molecules exhibit absorbance bands correlated to their aggregation state in the UV/Vis range of 300 to 450 nm. A PerkinElmer Lambda 25 UV/VIS spectrometer (Waltham, MA, USA) was used to analyze AmB-containing samples in 1cm path length cuvettes. Samples were diluted in pure water and analyzed immediately after dilution. M-AmB and H-AmB were diluted to 5×10^{-6} M of AmB (5, 13).

Circular dichroism (CD) spectra were recorded with a Jasco J-180 dichrograph (Easton, MD, USA). Samples were diluted in water to $5 \cdot 10^{-5}$ M AmB 24h before analysis. Measurements were conducted at room temperature using a 0.1 cm path length cuvette (15). Results are expressed as $\Delta\epsilon$ (differential molar absorption dichroic coefficient).

Microemulsion characterization

Droplet size distribution analyses were carried out by Dynamic Light Scattering (DLS) using a Malvern-Zetasizer Nano ZS (Malvern, UK). Transmission electron

microscopy (TEM) was used to investigate the ME morphology. The MEs were observed after staining with 2% phosphotungstic acid, using a JEOL 1400 apparatus (SamXPlus, France), operated at 80kV as the acceleration voltage, equipped with a high resolution CCD Gatan digital camera (SC1000 Orius, France). Before performing both types of analysis, the MEs were diluted 1:20 with water.

The rheological properties of the MEs were evaluated by a Haake Rheo Stress 600 rheometer equipped with 35mm cone-plate geometry (Thermo Scientific, USA). The flow curve and the viscosity curve of the samples were performed by controlled shear rate rotation tests (from 0.1 s⁻¹ to 1000 s⁻¹).

The aggregation state of the AmB in the ME-AmB was also evaluated by spectrophotometry and CD as described above.

***In vitro* antileishmanial activity**

The promastigote stages of *Leishmania donovani* (MHOM/IN/80/DD8) wild-type and of the AmB-resistant (AmB-R) strain obtained from the wild-type parasites by *in-vitro* drug pressure were grown in M-199 medium supplemented with 40 mM HEPES, 100 μM adenosine, 0.5 mg/L haemin, and 10% heat-inactivated fetal bovine serum (FBS) at 26 °C in a dark environment under an atmosphere of 5 % CO₂. Parasites in their logarithmic phase of growth were used for all experiments. Promastigotes were grown at 37 °C in 5% CO₂ atmosphere for 24 hours before treatment for differentiation. The axenic amastigotes were generated by differentiation of promastigotes. To achieve this, a 1 × 10⁶ promastigote suspension was diluted in 5mL of axenic amastigote media (1 X M-199, 40 mM HEPES, 100 μM adenosine, 0.5 mg/L haemin, 2 mM CaCl₂, 2 mM MgCl₂ and 20% FBS) and the pH was adjusted to pH 6.

Evaluation of the in vitro antileishmanial activity on axenic amastigote stage

Axenic amastigotes were suspended at 5 × 10⁶ cells/mL in a final volume of 200 μL. Serial dilutions from 100 to 0.39 μg AmB/mL was performed by distributing AmBisome[®],

M-Amb, H-Amb, ME-Amb, and ME in 96-well plates. Triplicates were used for each concentration. The viability of the axenic amastigote stage was assessed using the trypan blue exclusion method after a 3-day incubation period at 37 °C, in the dark, under a 5% CO₂ atmosphere. Parasite growth was determined by using SYBR Green I (Invitrogen, France), a dye whose fluorescence is greatly enhanced upon contact with the parasite DNA. The plates were frozen 3 times and the DNA was extracted from the plates according to the Direct PCR (cell) protocol (Viagen Biotech Inc, Eurogentec, France). Into the PCR plate 10µL of Lysate and 40 µL of direct PCR (cell) supplemented with SYBR green I (5 µL of SYBR Green I / 10 mL of lysis buffer) were added. Untreated parasites were used as control. The fluorescence was directly evaluated on a Mastercycler® ep realplex real-time PCR system (Eppendorf, France). The program was as follows: 90 °C, 1 min and ramp time of 5 min to obtain 10 °C, during this period the fluorescence was obtained in continue and in a hold step at 10 °C. The curves of fluorescence were analyzed and the fluorescence at 10°C was used to determine the concentrations inhibiting parasite growth by 50 % (IC₅₀). Indeed, the IC₅₀, the concentration inhibiting the parasite growth by 50%, was determined using the software in line at <http://www.antimalarial-icestimator.net/MethodIntro.htm>.

Evaluation of the in vitro antileishmanial activity on intramacrophagic amastigotes

RAW 264.7 cells (mouse leukemic monocyte macrophage cells) cultured in DMEM with L-glutamine (Life technologies) and 10% FBS (Life technologies) were suspended to yield 1.5×10^5 cells/mL in 96-well plate, in a final volume of 100 µL. After a 24 h incubation period, cells were infected by a suspension of 1×10^6 *Leishmania donovani* axenic amastigotes/mL in DMEM, and incubated for 24 h at 37 °C in the dark and under a 5 % CO₂ atmosphere. The free parasites were, then, eliminated by washing and intramacrophagic amastigotes were treated with the same AmB samples and concentrations as those in the assay on axenic amastigotes. After 48 h of incubation under

the same conditions, the experiment was stopped and the plate was treated as for the axenic amastigote stage assay. Each experiment was performed in triplicate (16). Fluorescence was compared to that obtained with untreated infected and uninfected macrophages and untreated axenic parasites used as controls.

***In-vivo* evaluation**

All procedures involving animals were conducted in compliance with the standards for animal experiments and were approved by the local committee for animal care (0858.01/2014, Versailles, France).

***In-vivo* antileishmanial activity**

A suspension of *Leishmania donovani* (LV9) amastigotes (1×10^7 /100 μ L M199 medium supplemented with 40 mM HEPES, 100 μ M adenosine and 0.5 mg/L haemin, 10% FBS), obtained from infected hamster spleen, was injected into female BALB/c mice (weighing 20 g) by intravenous route. The mice were randomly sorted into six groups seven days after the infection. Each group was treated with 100 μ L of one formulation (AmBisome[®], M-AmB, H-AmB, ME-AmB and ME), administered by intravenous route for 3 alternate days (1mg/kg/day). Samples were diluted prior to use with 5% glucose to give a final AmB concentration of 2×10^{-4} M. Animals were sacrificed three days after the end of treatment. Livers and spleens were weighed and drug activity was estimated microscopically by counting the number of amastigotes/500 liver cells in Giemsa stained impression smears to calculate the *Leishmania donovani* units (LDU) for liver parasite burdens, using the Stauber's formula (17). The mean number of parasites per gram of liver among treatment groups and controls was compared. Three independent counting were performed and the results were expressed as the mean values \pm SD. The parasite burden of treatment groups and controls were compared using the Kruskal–Wallis nonparametric analysis of variance test for comparing two groups. Significance was established for a P value < 0.05.

***In vitro* cytotoxicity assay**

RAW 264.7 cells maintained in DMEM supplemented with 10% of heat-inactivated FBS (Life technologies) were seeded to early confluence in a 96-well plate at density of 7500 cell/well and incubated in 5 % CO₂ at 37 °C for 24 h in a final volume of 100 µL. Thereafter, M-AmB, H-AmB, ME-AmB, AmBisome[®] and ME were added to achieve final AmB concentrations from 0.1 to 108 µM and incubated for a further 24 h under the same conditions. In order to estimate background absorbance due to light scattering, wells without cells, but containing the same concentration of the samples, were analyzed. The cytotoxicity of the formulations was evaluated using the MTS [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay, whereupon 20 µL of MTS solution were added into the wells and incubated for further 2 h. Finally, the absorbance was measured using a 492-nm high-pass filter in a Multiskan MS microwell plate reader (Labsystem, Ramat-Gan, Israel) and the IC₅₀ for each formulation was calculated. For this assay 4 replicates were performed.

***In vivo* toxicity assay**

Blood samples taken from the mice from the antileishmanial activity experiment were kept at room temperature for about 30 min and, then, centrifuged at 3,500 g for 10 min. In order to evaluate the renal and liver toxicity, serum was harvested from each blood sample and total serum cholesterol (CHO), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanineaminotransferase (ALT), creatinine (CREA) and urea (URE) concentrations were determined by commercially available kits according to the manufacturer's instruction.

Statistical analysis

Statistical analyses were performed by using analysis of variance or Kruskal-Wallis tests (nonparametric), followed by the Tukey's or the Dunn's multiple comparison test. GraphPad Prism5 version (GraphPad Software Inc., La Jolla, CA) was used to perform the analysis. Significance was established for a P value < 0.05.

RESULTS

M-AmB and H-AmB characterization

The UV-Vis spectrum for M-AmB (Figure 1a) showed four main peaks at 329, 367, 388 and 408 nm. The first peak is credited to the aggregated form of AmB whereas the one at 408 is ascribed to the monomeric form (15). After heat treatment it was observed that H-AmB (Figure 1a) presented similar peaks to M-AmB regarding the monomeric form, but the aggregate-related peak underwent a blue shift and became centered on 323 nm in this preparation. The UV-Vis results were in accordance with those observed by circular dichroism (Figure 1c), in which the doublet related to the AmB aggregation was also blue-shifted for H-AmB.

Microemulsion characterization

The produced ME was optically clear with a mean droplet size of 22 nm for the unloaded ME and 36 nm for the ME containing AmB, with polydispersity indexes of 0.170 and 0.594, respectively, indicating that the addition of AmB led to slightly larger particles. TEM revealed that the droplets were spherical in shape even after the addition of AmB (Figure 2). Furthermore, the average size was similar to the DLS results. The rheological analysis revealed that both the unloaded ME and the ME containing AmB (Figure 3) showed a linear relationship between shear stress and shear rate, which is characteristic of a Newtonian material. The apparent viscosity of the unloaded ME and ME containing AmB were $0.0623 \text{ Pas} \pm 0.0101$ and $0.1141 \text{ Pas} \pm 0.0049$, respectively, which is low because the ME was of the oil-in-water type. The low viscosity would ensure good syringeability as well as ease of mixing with intravenous fluids using minimum mechanical agitation. The AmB molecule showed to be in both aggregated and monomeric forms (Figure 1b and 1d), since peaks at 323 nm (aggregated form), 367, 388 and 408 nm (monomeric form) were observed. All these results: the small spherical droplet size, the transparency and the low viscosity, allow these samples to be characterized as a true ME system. The incorporation of AmB did not affect these parameters.

Toxicity of the formulations

The CC_{50} was evaluated *in vitro* for each formulation (Table 1). M-AmB showed high toxicity towards RAW cells (CC_{50} $4.5 \pm 0.4 \mu\text{M}$). However, when this formulation was heated this value increased approximately 8-folds to $39.8 \pm 3.2 \mu\text{M}$, indicated a decrease in toxicity. In fact, in this study, the toxicity of H-AmB was similar to that of AmBisome[®]. On the other hand, although the incorporation of the AmB into the ME decreased the CC_{50} of the formulation compared to the unloaded ME, the cytotoxic effect of ME-AmB (CC_{50} 9.0 ± 0.3) was slightly lower than that of M-AmB.

The results of the evaluation of the toxicity *in vivo* are given in Table 2. There were no significant changes ($p > 0.05$) among the samples for the two transaminase activities levels (AST and ALT) and the creatinine levels. However, all treatments caused a significant increase ($P < 0.05$) in the urea level except for mice treated with unloaded ME. A significant decrease in this parameter was also observed in the mice treated with the ME and the ME-AmB compared to the AmBisome[®]. Furthermore, it is noteworthy that heating of M-AmB slightly decreased the blood urea levels. The ALP value increased significantly with all treatments compared to the untreated group. Nevertheless, the ALP level for the mice treated with the AmBisome[®] was significantly lower than that of the mice treated with the ME-AmB. The CHO results showed no difference between the ME, the AmBisome[®] and the untreated group. However, M-AmB, H-AmB and ME-AmB caused a significant decrease in this parameter, while the lowest values were observed in the mice treated with the ME-AmB.

Antileishmanial activity

The activity of the formulations against *Leishmania donovani* was evaluated *in vitro* and *in vivo*. The *in-vitro* assays were performed on both Wild-type (WT) and AmB-R intramacrophagic and axenic amastigotes (Table 1). All formulations, except ME (unloaded microemulsion), showed high activity against WT parasites. ME-AmB and H-AmB yielded

lower IC₅₀ values than the AmBisome[®] for both amastigote stages. As far as the activity on axenic amastigotes was concerned, all the formulations exhibited lower IC₅₀ compared to the intramacrophage amastigote. However, the IC₅₀ of the AmBisome[®] did not change significantly (1.73 μM and 1.76 μM).

The H-AmB showed a higher IC₅₀ than the M-AmB, when the *Leishmania* was within macrophage, and similar antileishmanial activity (IC₅₀ = 0.05 μM) against the axenic stage. However, the selectivity index for the H-AmB was approximately 3-fold higher than that of the M-AmB. Moreover, the ME-AmB showed a better selectivity index than the M-AmB, although it remained lower than the AmBisome[®].

Some clinical isolates of *Leishmania donovani* have shown resistance against the AmB (18). Therefore, the issue of AmB resistance should be considered early in the development of any new AmB delivery system. With this in mind, the formulations were evaluated against both AmB-resistant axenic and intramacrophagic amastigotes. The AmBisome[®] was active against AmB-resistant intramacrophage amastigotes, but it did not show any activity on AmB-resistant axenic amastigote. On the other hand, the other formulations were active in a range from 0.8 to 4 μM on both axenic and intramacrophagic amastigotes. The drug pressure to get the AmB-R line was previously carried out using M-AmB. Indeed, the IC₅₀ value of M-AmB on the AmB-R line was about 30 times higher than those of the WT, both on axenic and intramacrophage amastigotes. Interestingly, the IC₅₀ values of H-AmB, AmBisome[®] and ME-AmB on the AmB-R intramacrophage amastigotes were only about twice as high as those on the WT. These results suggest that these formulations could be useful for the treatment of AmB-R leishmaniasis.

The *in-vivo* antileishmanial evaluation was performed on the *Leishmania donovani*/Balb/C mice model. The percentage of parasite burden reduction *in vivo* is shown in the Table 2. Whereas the unloaded ME alone did not show good activity *in vivo*, M-AmB, H-AmB, ME-AmB and AmBisome[®] all reduced the parasite burden greatly, with no significant difference between the formulations. No sign of acute toxicity was observed in

the mice and the biological parameters did not show significant toxicity, as shown in Table 2.

DISCUSSION

The MEs prepared in this study showed a spherical shape and a small droplet size, which is in agreement with other studies that describe these systems as having diameters less than 150 nm (12, 14). Although the addition of AmB to the ME increased its droplet size and polydispersity index, probably due to the large molecule size and its deposition on the ME interface, to the heterogeneous distribution of AmB in ME or to the formation of other nanostructures as a result of interaction between AmB and components of the ME's. Nevertheless, the ME-AmB droplet size remained within the acceptable range. Such small droplets within MEs produce only weak scattering of visible light, and as a result the system is transparent, as observed macroscopically in this work. Moreover, the probability of embolus formation after injection of MEs is insignificant due to their small droplet size (12).

Since the nanosized droplets do not interact with each other, the resulting MEs have low viscosity (19). The viscosity analysis is of utmost importance since the parenteral administration of very viscous samples could be painful for the patient. Furthermore, it is well known that the syringeability is affected by the viscosity of the parenteral formulations (20). The ME and ME-AmB showed a constant low viscosity and proportionality between shear stress and shear rate, for which they were deemed to behave as Newtonian fluids. As a consequence, they can be considered suitable for the intravenous route. This low viscosity ensures ease of mixing with intravenous fluids with minimum mechanical agitation as well as good syringeability and, therefore, can ensure good patient compliance.

Spectrophotometric studies revealed that after the heating process, super-aggregates are formed from M-AmB, manifested as a blue-shift in the electronic spectra as well as a decrease on the dichroic doublet, which is also blue-shifted in the spectra of H-AmB (15). When AmB was loaded into the ME, a pattern similar to H-AmB was observed.

Since the system was not heated in order to form AmB super-aggregates, the peak at 323 nm probably represents the association of the AmB with the ME-oil phase. Pham et al, (2014) (21) have shown blue-shifts in the AmB dichroic doublet when the molecule is loaded into liposomes. Our results are in agreement with Silva et al (2013) (22), who have developed ME systems to carry AmB and have demonstrated peaks of AmB aggregates and monomers at the same time. However, the aggregate peak is not the same as one observed in our work, supporting the theory of that such a peak is a result of a complex formed between the molecule and the system, which for instance depends on the used raw material, mainly the surfactants. Larabi et al (2004) (23) reported studies in which absorption spectrum and circular dichroism varied according to the proportion of lipid in the formulation. Knowing the aggregation state of the molecule inside the carrier is very important since it influences the drug efficacy and toxicity (5, 9, 13, 15, 24).

The treatment of visceral leishmaniasis was dominated by pentavalent antimonials for a long time. However due to their toxicity and adverse side effects, these drugs have progressively been replaced as the first-line treatment by the AmB, which is considered as the best existing drug against this disease. Among the different commercial formulations, Fungizone[®] (a mixture of AmB with deoxycholate in a phosphate buffer) is the most frequently used. However, the liposomal formulation is the most effective and safe, despite its high cost (4).

In this work, we have proposed two new formulations of AmB which retain its activity while reducing its toxicity, and are furthermore inexpensive, with costs accessible for patients from the tropical endemic countries and easy to manufacture. Both H-AmB and ME-AmB were thoroughly characterized and shown to be appropriate for parenteral use against visceral leishmaniasis. In order to investigate the antileishmanial activity of these formulations, their IC₅₀ in axenic and intramacrophage amastigote were evaluated *in vitro*, as well as the percentage of reduction of parasite burden *in vivo*. In addition, the cytotoxicity of the formulations was examined *in vitro*, allowing calculation of the Selectivity

Index, while the toxic effects *in vivo* were analyzed by assessment of renal and liver function, because the most serious side effect of AmB is nephrotoxicity (25).

Both *in-vitro* and *in-vivo* experiments with the WT line demonstrated that, as expected, all formulations containing AmB were effective against *Leishmania donovani*. On the other hand, despite a moderate effect *in-vitro*, unloaded ME did not show significant *in-vivo* antileishmanial activity, proving that it was the addition of AmB that produced this effect.

Some differences in the *in-vitro* effectiveness were observed when comparing the axenic and intramacrophagic amastigotes. The axenic amastigote assay allowed us to evaluate an intrinsic activity on parasites, whereas the intramacrophage amastigote assay demonstrates the ability of the active drug to be delivered through the macrophage membrane, the parasitophorous vacuole membrane and finally into the parasite (26, 27). It is noteworthy that AmBisome[®] was not active against AmB-R axenic amastigotes while the other formulations did have some activity. This result cannot be a result of the ME diameter being less than that of AmBisome[®] (35 vs 80 nm, respectively) since H-AmB, AmBisome[®] and ME-AmB had similar activities on the WT intramacrophage amastigotes. This observation could be related to the composition of the AmB-R membranes, poor in sterols, more fluid than the WT membranes and, therefore, more stable and less sensitive to a liposomal effect. With the intramacrophagic amastigotes, this difference was not observed since all the formulations after macrophage phagocytosis released AmB within the parasitophorous vacuole, probably after fusing with lysosomes. Indeed, AmBisome[®] is a very stable formulation with cholesterol and a high-transition-temperature phosphatidylcholine making a very impermeable membrane and phosphatidylglycerol forming an electrostatic complex with AmB. It is possible that parasite-derived enzymes are necessary to release the AmB from the liposomes and that the AmB-R parasites have a different enzyme profile and are less efficient at this. On the other hand, AmB may be less strongly associated in M-AmB, H-AmB and ME-AmB. Further studies could be

focused on the ME 78 behavior within the infected macrophages through confocal studies.

M-AmB showed the lowest selectivity index, while H-AmB presented the highest index. Therefore, the heating process was capable of considerably reducing the cytotoxicity of AmB *in vitro* as demonstrated by the CC₅₀ values. This result is supported by Gaboriau et al. (1997)(28) and Petit et al. (1998) (10) whose have found the mild heating as a simple way to decrease the toxicity of micellar AmB systems. In addition, the therapeutic window between the IC₅₀ (1.53 µM) and CC₅₀ (39.8 µM) of H-AmB is wider than that of M-AmB, thus improving its safety. It is interesting to mention that H-AmB have shown to favor drug uptake by macrophage-like cell line (9). The authors suggest that this fact could be interesting since the macrophage could act as a reservoir, releasing monomeric AmB on the infection site and, thus, improving antiparasitic effect (9). ME-AmB (selectivity index = 10.23) also showed a better selectivity compared to M-AmB. When the selectivity index is greater than 10, it is generally, considered that the pharmacological efficacy is not due to the *in-vitro* cytotoxicity (29), therefore, the incorporation of AmB in ME systems was able to improve the effectiveness of the drug.

The potential of the new formulations (H-AmB and ME-AmB) was confirmed by the *in-vivo* studies, which showed no significant differences in their ability to reduce the parasite burden compared to the AmBisome[®]. In addition, both formulations demonstrated minimal cytotoxicity *in vivo*.

The biochemical evaluation of kidney and liver parameters showed that the untreated infected mice had lower values than those with no parasite burden (reference values were found in (30)). Some studies have described a reduction of the serum cholesterol concentrations as a function of the splenic parasite burden, since this organ is responsible for cholesterol biosynthesis (31). An overview of all the biochemical results showed that despite some values, which were statistically different, there was a minimal variation among the groups, showing that none of the formulations induced toxicity at the doses used in this study. Therefore, ME-AmB and H-AmB were considered safe regarding

the limited damage to macrophage and the *in vivo* evaluation.

Furthermore, ME-AmB, M-AmB and H-AmB showed efficiency against AmB-R intramacrophagic amastigotes. AmB-unresponsive cases have been reported at the Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Bihar, India. These drug-resistant cases are to be expected due to the very high frequency of AmB use (18). Since visceral leishmaniasis is fatal, the development of new systems, which do not demonstrate drug resistance is very important to ensure that suitable treatment would be available. Therefore, these two inexpensive alternative formulations for AmB showing good efficacy and selectivity for *Leishmania donovani* merit further investigation. Unfortunately, it was not possible to verify the *in-vivo* efficacy of the formulations on an AmB-R/ mice model since the AmB-R parasites are poorly infectious for Balb/c mice (32).

CONCLUSION

These experiments show a novel approach to AmB therapy, addressing both the cost of formulations and the AmB-resistance. The formulations H-AmB and ME-AmB proved to be very successful in the treatment of a *Leishmania donovani* Balb/c mouse model. Both *in-vivo* and *in-vitro* evaluations showed good efficacy and low toxicity for these formulations. Since the method of preparing the H-AmB is just the heating of the reconstituted M-AmB for a few minutes, it appears to be a low cost alternative to the commercially available products. The cost of producing MEs is also lower than that of producing the liposomal AmB, since the components are commonly used pharmaceutical excipients rather than expensive phospholipids. Therefore, the formulation developed in this work is a promising way to reduce toxicity while maintaining the efficacy of AmB.

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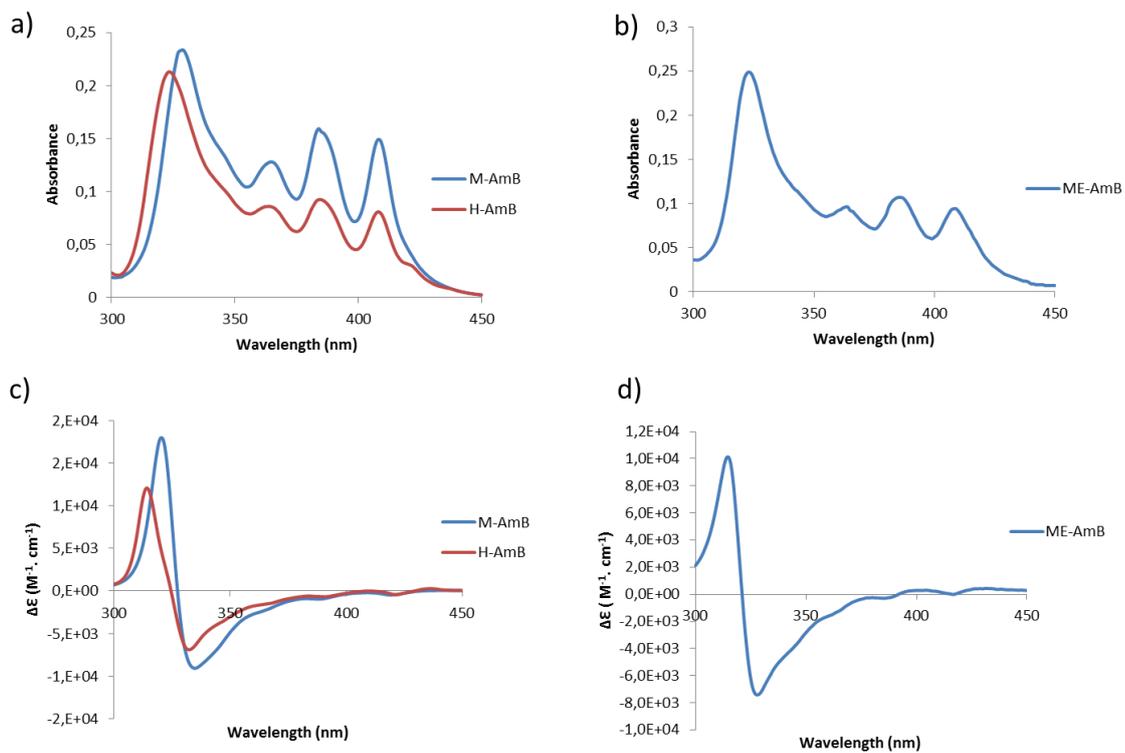


Figure 1: Spectroscopy of M-AmB and H-AmB (a) and of ME-AmB (b) and circular dichroism of M-AmB and H-AmB (c) and of ME-AmB (d).

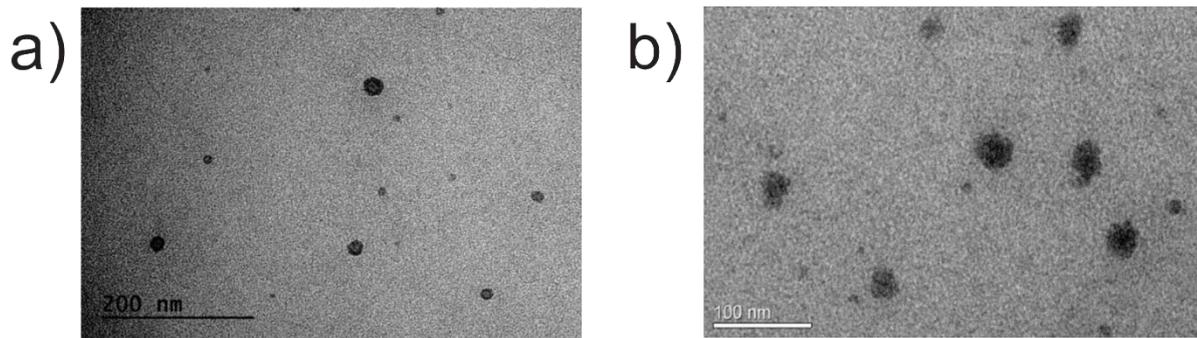


Figure 2: Transmission electronic microscopy of ME (a) and ME containing AmB (b).

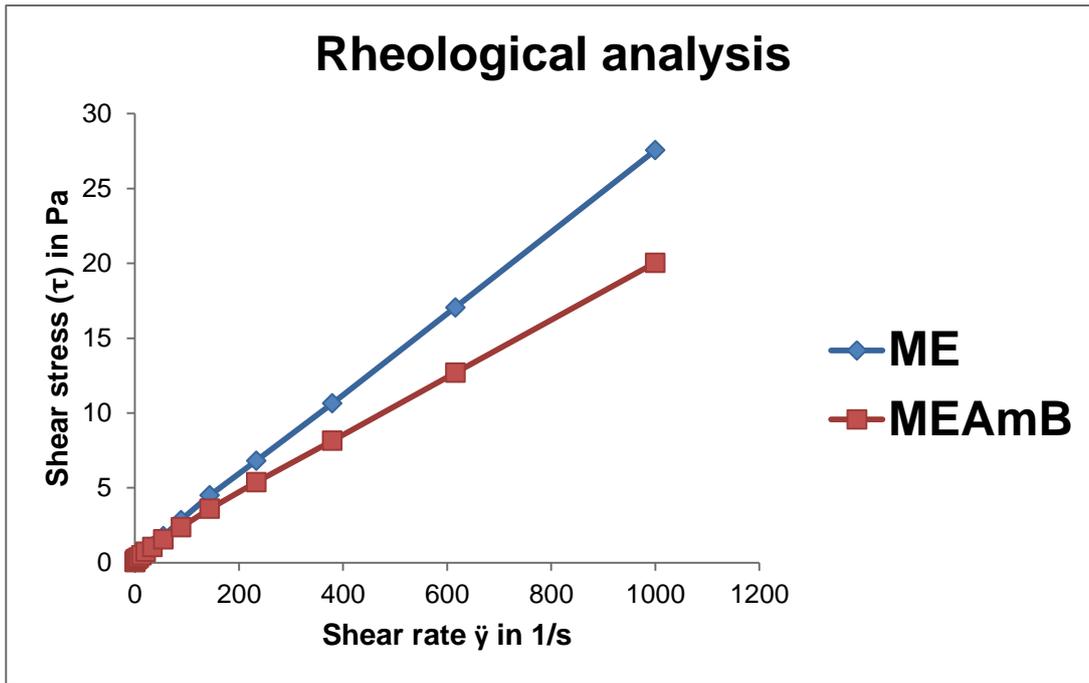


Figure 3: Rheological behavior of ME and ME containing AmB.

Table 1: *In vitro* evaluation of the cytotoxicity and the anti-leishmanial activity on *L. donovani* WT and AmB-R.

Formulations	Intramacrophage amastigotes IC ₅₀ (μM) ± SD		Axenic amastigotes IC ₅₀ (μM) ± SD		Cytotoxicity RAW 264.7 macrophages CC ₅₀ (μM) ±SD	Selectivity index SI= CC ₅₀ /IC ₅₀
	WT	AmB-R	WT	AmB-R		WT
M-AmB	0.67 ± 0.25	2.15 ± 0.18	0.05 ± 0.03	1.69 ± 0.04	4.5 ± 0.4	6.7
H-AmB	1.54 ± 0.34	2.31 ± 0.32	0.05 ± 0.03	0.82 ± 0.03	39.8 ± 3.2	25.8
AmBisome®	1.76 ± 0.69	3.54 ± 0.10	1.73 ± 0.87	>100	36.5 ± 2.3	20.7
ME-AmB	0.88 ± 0.26	1.56 ± 0.01	0.22 ± 0.11	3.38 ± 0.08	9.0 ± 0.3	10.2
ME (equivalent AmB)	27.98 ± 2.71	27.34 ± 0.24	6.82 ± 0.54	27.06 ± 0.52	22.8 ± 2.9	0.8

Table 2: *In-vivo* antileishmanial activity and acute toxicity of H-AmB and ME-AmB compared to M-AmB and AmBisome®. Mice were treated intravenously at 1 mg/kg equivalent AmB at Day 8, Day 10 and Day 12 post-infection.

	<i>In vivo</i> antileishmanial activity	<i>In vivo</i> toxicity					
		^a AST U/L	^a ALT U/L	^b ALP	^b CHO mM	^a CREA μM	^b URE
M-AmB	72	233.0	134.0	225.5	2.515**	19.5	10.70
H-AmB	78	189.5	171.5	241.0	2.575**	18.0	9.65
AmBisome®	83	242.5	167.0	229.0	*2.755	20.0	10.15
ME-AmB	78	224.5	169.5	246.0**	2.415**	18.5	8.35**
ME	*33	293.0	181.0	225.0	*2.710	16.0	*7.60**
Untreated	-	313.0	239.0	199.0**	2.810	18.0	7.40**

AST : aspartate aminotransferase. Normal value : 456 (U/L) (30).

ALT: alanine aminotransferase. Normal value : 304 (U/L) (30).

CHO : Cholesterol. Normal value: 3.5 mM (30).

CREA : Creatinine. Normal value: <18 μM (30).

URE : Urea. Normal value 7.4 mM.

ALP: Alkaline Phosphatase

* There was no significant difference in comparison to untreated (P>0.05)

** There was significant difference in comparison to AmBisome® (P<0.05)

^a P > 0.05

^b P < 0.05

SECTION II

Freeze-drying as strategy for improve emulsified systems stability

BRIEFING

Since the results shown in the previous chapter demonstrated that a microemulsion containing Amphotericin B (MEAmB) has a good activity against *Leishmania donovani* and low toxicity, it is important to think about the fate of the formulation during storage. The high water content (63%) in the MEAmB makes the formulation susceptible to the drawbacks of high humidity, such as microbial contamination and hydrolysis. A way of solving this problem could be to remove the water through a drying process that does not use high temperatures, since AmB is a thermolabile drug.

Lyophilization, also known as freeze-drying, is a process that removes the frozen water from a material by sublimation using low temperatures, which has already been used to improve the stability of emulsified systems. However, an in-depth study on lyophilization of emulsified systems is required since these systems are not easily freeze-dried due to the components present in their formulation and to the process, which can cause damage such as drug loss, precipitation, loss of system structure, coalescence and phase separation.

Several factors may influence the outcome of freeze-drying, including the thickness of the frozen layer, composition and concentration of the product, type and concentration of cryoprotectant, the equipment and parameters of the lyophilization process, such as time and temperature (freezing, primary and secondary drying). For this reason, it is important to use appropriate techniques to identify the variables that influence the process and to choose the best parameters to obtain the optimal response. Design of experiment (DOE) is a tool used to improve the quality of products and processes, able to evaluate a high number of variables using a reduced number of samples. Therefore, DOE was employed in this study to determine the best parameters for the lyophilization of AmB-loaded microemulsions in order to increase their stability.

A theoretical review on this topic it was an essential prerequisite to facilitate experimental decision-making based on studies already carried out and to avoid unnecessary experiments, thereby increasing the probability of success. Therefore, a review about freeze-drying of emulsified systems was carried out to assist the development of a freeze-dried microemulsion containing AmB. This review helped to define the types of cryoprotectant to be used in the experimental study and their concentration range, as well as the time and temperature ranges to be tested.

CHAPTER III

Freeze-drying of emulsified systems: A review



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Review

Freeze-drying of emulsified systems: A review



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ABSTRACT

Colloidal systems such as emulsions, microemulsions and nanoemulsions are able to transport active molecules, enhance their solubility and stability and minimize their side effects. However, since they are dispersions with an aqueous continuous phase they have some disadvantages such as the risk of microbiological contamination, degradation by hydrolysis, physico-chemical instability and loss of pharmacological activity of the drug. Freeze drying, in which the water is removed from the preparation by sublimation under vacuum, has been suggested as a means to resolve these problems. Lyophilized products are very stable and are easy to transport and store. However, there is very little information in the literature about the application of this technique to emulsified systems. The aim of this review is to evaluate the lyophilization process as a tool for increasing the shelf life of emulsified systems such as emulsions, microemulsions and nanoemulsions. In addition, the mechanism of cryoprotection and the techniques that can be used to characterize the freeze-dried systems are discussed.

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Abbreviation: FD, freeze-drying.

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1. Introduction

Colloidal dispersions have been studied because of their ability to protect, encapsulate and deliver bioactive components such as vitamins, antimicrobials, drugs, antioxidants and nutraceuticals. The results have attracted the attention of the food, pharmaceutical, agrochemical and other industries. These dispersions have advantages over systems containing larger particles because, due to the small particle size, low sedimentation and good colloidal stability can be incorporated into the optically clear products, and may increase the bioavailability of insoluble drugs (lipophilic or hydrophilic) encapsulated within them (McClements, 2012). Furthermore, these systems may be able to provide prolonged blood circulation, drug delivery, specific targeting, and by-passing endosome-lysosome processing (Mishra et al., 2010). All colloidal dispersions are two-phase based systems consisting of a dispersed phase and a continuous phase, which may be solid, liquid or gaseous (Bolintineanu et al., 2014; Bonacucina et al., 2009). However, this review will focus only on those emulsified systems which are liquid-liquid dispersions.

Emulsified systems can be classified as emulsions, nano-emulsions or microemulsions. There are many similarities between these systems. However, they differ in their physico-chemical properties and thermodynamic stability, making each one more suitable for a particular application compared to others (Rao and McClements, 2011).

All three systems have a similar basic composition: an aqueous phase, an oily phase and a mixture of surfactants. Moreover, the structure formed by these components is also similar. When the oil is dispersed in the aqueous phase the system is the oil-in-water (O/W) type, whereas when the oil is the continuous phase and the

water is the internal phase the system is the water-in-oil (W/O) type. It is also possible to obtain multiple emulsions in which O/W and W/O systems co-exist, forming oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) structures, in which smaller droplets are dispersed in larger ones (Fig. 1) (Date and Nagarsenker, 2008b; Tamilvanan, 2004).

However, there are also important differences between emulsions, nanoemulsions and microemulsions. In particular, microemulsions are thermodynamically stable, isotropic, clear and usually contain droplets of less than 100 nm in diameter (Lawrence and Rees, 2012), and generally close to the corresponding micelles, whereas nanoemulsions and emulsions are thermodynamically unstable. Emulsions have a milky aspect and droplet sizes between 100 nm and 1 μ m (Ranade and Cannon, 2011). Nanoemulsions (well known as miniemulsion) have smaller droplet sizes, around 300 nm, which modifies their physicochemical and biological properties and leads to better kinetic stability, avoiding processes such as flocculation and coalescence that cause instability in classical emulsified systems (Anton and Vandamme, 2011).

Emulsified systems have some interesting pharmaceutical applications, such as acting as a reservoir for active substances, enhancing the therapeutic effect, reducing drug doses and thereby side effects and toxicity. In addition, they can increase the absorption of the active substance due to the presence of surfactants that can modify membrane fluidity (Gupta et al., 2013). Furthermore, they show advantages over systems containing larger droplet size because they can be designed with original rheological properties, such as good colloidal stability, low phase separation velocity (i.e. sedimentation) and the improvement of

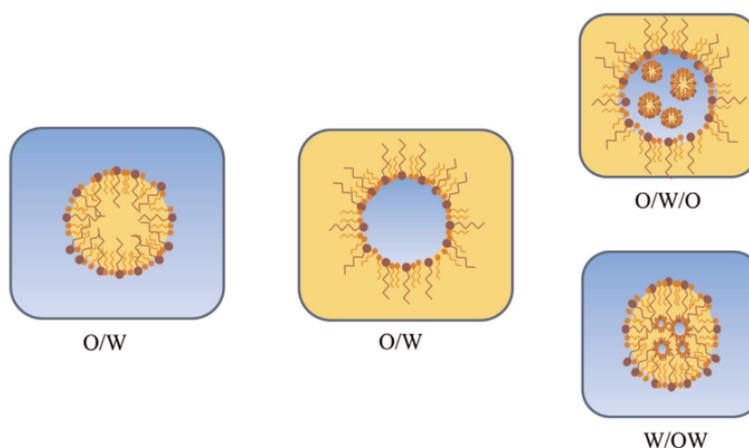


Fig. 1. Different types of emulsified systems.

the bioactivity of encapsulated active molecule (McClements, 2012).

However, a large volume of water is required to obtain these systems and this reduces the stability of the final product. The presence of free water can promote microbial growth, drug leakage, and hydrolysis of phospholipids and triglycerides to free fatty acids, all leading to a decrease in therapeutic efficiency. As a result, the final phase composition is an important factor that determines the shelf life of the product (Abdelwahed et al., 2006b).

As a means of overcoming the drawbacks mentioned above, freeze-drying (FD) as a strategy of increasing long-term stability has been studied. FD, also known as lyophilization, is a dehydration technique based on the removal of water from the system by sublimation under vacuum, at low pressure, leading to an anhydrous or almost anhydrous product (Franks, 1998). This technique has often been used for food products, biological materials and drug delivery systems. However, during this process several types of stress that could potentially destabilize the colloidal structure of emulsified systems may be generated. To prevent this damage, cryoprotectant agents have been used (Abdelwahed et al., 2006b).

The purpose of this paper is to review the freeze-drying process as a means of improving the stability of emulsified systems. Therefore, all the stages of the process are considered, as well as the use and mechanism of action of cryoprotectants. Characterization methods that can be applied to lyophilized systems and results that have already been obtained concerning the freeze-drying of emulsions, microemulsions, and nanoemulsions are also discussed.

2. Freeze-drying

The process of drying a frozen product using a vacuum was first described in 1906 (Bordas and d'Arsonval, 1906); however it remained as a laboratory technique until the second world war, when it was used as an industrial process to preserve plasma when the demand reached major proportions (Franks, 1998).

Nowadays, many pharmaceutical and food companies use the freeze-drying process to obtain dry products (Najjar and Stubenrauch, 2009). In the pharmaceutical industry, a number of products such as viruses (Chen et al., 2012), vaccines (Anamur et al., 2015),

peptides (Lim et al., 2008), proteins (Michael, 1994) and colloidal carriers (Abdelwahed et al., 2006b) are subject to chemical reactions such as oxidation, disulfide rearrangements, cross-linking, hydrolysis and aggregation in aqueous solution, leading to their destabilization in the short or long term. Freeze-drying process would appear to be a good method to improve their stability, since in the dry state these undesired reactions are slowed down (Franks, 1998).

The majority of emulsified systems used in the pharmaceutical industry are freeze-dried from aqueous solution, since water is the main solvent used to produce pharmaceutical products. However, some systems may contain organic solvents, which are able to sublimate under reduced temperature and pressure. However, in this case, lower temperatures are necessary in order to freeze and condense such the organic solvents. Thereby, well adapted freeze-drying equipment is required for most organic solvents. Furthermore, the type and the concentration of the organic solvent should be studied, since it impacts on the freezing and drying steps (Rey and May, 2004).

On the other hand, there may be some advantages in using organic solvent in emulsified freeze-dried systems. These are increased drug wetting, drug solubility, and chemical stability that could decrease the drying time, improve the reconstitution process of the product and make it easier to obtain a sterile product. However, there are also some drawbacks in manufacturing a system containing organic solvent, such as the high toxicity of these solvents. Therefore, it is necessary to ensure that the residual solvent in the final product is at an acceptable level. It is also very important to consider the high flammability of the organic solvent, its difficult handling properties, the special manufacturing facilities/equipment that must be used and the high cost. Nevertheless, a safe emulsified system can be produced by freeze-drying from a preparation containing organic solvents with correct safe handling, the use of suitable equipment for the particular solvent, analysis of the residual solvent levels, as well as the evaluation of the acute and other types of toxicity (Rey and May, 2004).

Freeze-drying is able to improve stability and facilitate handling of the final product. Since the processes are performed at low temperature, they present a lower risk for labile products than at the high temperatures used in spray-drying. Therefore,

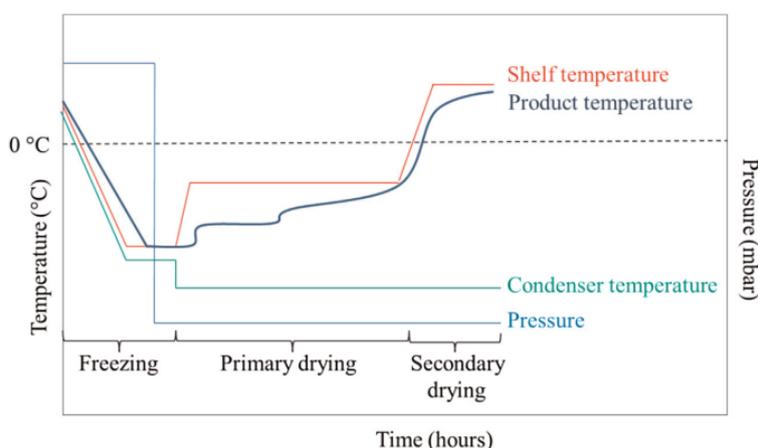


Fig. 2. Freeze-drying cycle, showing shelf, product and condenser temperatures, and chamber pressure. Figure adapted from (Franks, 1998).

freeze-drying is used for samples sensitive to heat that cannot be treated using other processes involving high temperatures. However, freeze-drying is a slow and expensive method, since the cycle may be long, necessitating considerable energy consumption.

2.1. Freeze-drying cycles

A typical freeze-drying cycle consists of three stages: freezing, primary drying and secondary drying (Tang and Pikal, 2004). Fig. 2 gives an example of a recorder output trace from a freeze-drying cycle showing all these steps. In the first step the liquid is cooled, and as it freezes the solids become more concentrated in the remaining liquid. When this step is performed in a freeze-dryer, the shelf temperature is reduced. Next, the primary drying begins when the pressure is lowered to values below the vapor pressure of ice and the shelf temperature increases in order to supply the latent heat removed by ice sublimation (ΔH). This step ends when the product temperature approaches the shelf temperature. Thereafter, an open network of pores is formed due to the sublimation of the ice crystals, which provide a pathway for desorption of water from the sample during the secondary drying, when the shelf temperature is increased and the chamber pressure is reduced in order to remove liquid water. In contrast to the primary drying step that is long and has a large impact on the cost of the process, the secondary drying period is short and does not have a significant impact on the cost (Wang et al., 2012).

2.1.1. Freezing

The first step of the freeze-drying process is freezing, in which the samples are exposed to a temperature sufficient low for them to become solid. This can be achieved using liquid nitrogen, in a separate freezer or in the freeze-dryer itself. Changes in the physical properties caused by ice crystal formation that may cause aggregation of the droplets in the emulsified systems (Tang and Pikal, 2004; Wang, 2000) and pH changes due to crystallization of buffer salts (Wang, 2000) makes this step the most critical for product integrity.

It is important to understand the critical parameters in the formulation in order to optimize the process design. These include the eutectic temperature (T_{eu}) for samples that form a eutectic (a mixture of crystalline solids, which presents the same physical properties as if they were alone) and the glass transition temperature (T_g), which represents the temperature at which the transformation of amorphous materials from a viscous to a glassy state occurs. This glassy state is accompanied by an increase of the viscosity as the temperature decreases. It differs from the crystalline solid, which has an ordered molecular structure (Levi and Karel, 1995). Different values of T_g can exist in the same sample, since the T_g is dependent on the moisture content. During the drying process, the T_g usually increases as the water is removed. Therefore, at the lowest moisture content the highest T_g value is reached (Fissore et al., 2011; Meister and Gieseler, 2009; Tang and Pikal, 2004). The frozen amorphous material takes up the (glassy) solid form. However, its molecular structure remains disordered as in a liquid. Physical parameters such as T_g and T_{eu} have great importance because of the structural disorganization that occurs when the temperature of the process rises above these temperatures (Meister and Gieseler, 2009). The freezing rate is a parameter that should be optimized, since different rates result in the formation of different types of ice crystals. Fast freezing leads to small and numerous ice crystals whereas slow freezing forms larger and less numerous crystals. The surface area of these crystals will influence the further freeze-drying steps (Ingvarsson et al., 2011).

2.1.2. Annealing

Annealing is a process during which the product is maintained, for a defined time, at a subfreezing temperature above the T_g and below the T_{eu} of the bulking agent in order to allow efficient crystallization of the crystalline bulking agent (e.g. mannitol). It has been observed that this process can lead to the growth of ice crystals, and is able to accelerate the primary drying (the longest step), optimizing the freeze-drying cycle. Furthermore, this procedure can eliminate the layer formed on the top surface of the freeze-dried cake that hinders the transfer of water vapor during the primary drying step (Tang and Pikal, 2004).

2.1.3. Primary drying

After samples have been frozen, the freeze-drying cycle proceeds to the next step, the primary drying. This step allows ice sublimation, driven by low pressures and temperatures (Tang and Pikal, 2004). The sublimation of the ice starts from the top surface of the sample and continues to the bottom. In this way, the sublimated vapor is removed by diffusion or convection through the porous layers. Since the diffusivity is related to the pore size, for samples that have been frozen fast and have formed small ice crystals that hinder the mass transfer of vapor through the dry layer, the primary drying step will be long. On the other hand, slow freezing forms large ice crystals that facilitate the movement of water vapor (the mass transfer rate is high) and as a result the primary drying time is reduced. In addition, the pressure plays an important role during this stage, since it is the driving force for the transport of the water vapor. Therefore, lower chamber pressure leads to faster ice sublimation (Ingvarsson et al., 2011).

Another factor which should be controlled during this step is the collapse temperature (T_c), which marks the change from a glassy solid matrix state into a rubbery amorphous one. The structure of the product is lost or damaged when collapse occurs during the primary drying and this could affect the quality of the freeze-dried product. This temperature is related to the T_g , although a decrease in viscosity is not able to induce collapse; furthermore, some studies have shown that the T_c is few degrees higher. Therefore, the temperature of the samples must remain below their T_c during this step in order to avoid these drawbacks (Levi and Karel, 1995).

2.1.4. Secondary drying

During secondary drying the residual water is removed by desorption from the solute phase (Wang, 2000). The aim of this step is to reduce the moisture content to an optimal level for stability, since even after the sublimation during the primary drying the product still contains 10–35% of bound water. During the secondary drying step the most important factor is the adsorption-desorption equilibrium of the moisture and the porous medium. Thus both temperature and moisture content need to be controlled in order to obtain an acceptable product quality (Wang et al., 2012).

The surface area affects the secondary drying rate (Rey and May, 2004). Samples submitted to fast freezing produce numerous small crystals with a large surface area, which favors water desorption during the secondary drying, as well as reducing the disruption of the bilayer structure. However, with a slow freezing rate the surface area of the dry powder is small, which impairs water desorption, resulting in a slow drying rate during the secondary drying (Wang et al., 2012).

Rehydration of the dried product has to be done slowly because of its anhydrous state. Rapid addition of the water could disrupt the porous structure, leading to collapse of the system matrix that could compromise the product quality (Schaffazick et al., 2003; Wang, 2000).

2.2. The effect of various parameters on freeze-drying

An acceptable freeze-dried product should possess the same physical and chemical properties as the system before being submitted to the process, acceptable humidity, long-term stability and an elegant cake appearance. In order to achieve this, it is important to analyze all the parameters that could influence the final freeze-dried product carefully (Rey and May, 2004).

The freeze-drying process is affected by many parameters related to the product, the container, the equipment and the process. Due to the presence of so many variables, the production of freeze-dried emulsified systems needs be optimized in order to provide the most stable product possible (Tang and Pikal, 2004).

The formulation influences how stable the system will be in the face of the stress imposed by the freeze-drying process. The most important parameters are (1) the nature of the surfactants, due to the different interactions they may have with the drugs and the cryoprotectants, as well as the different rearrangements that occur during the freeze-drying and rehydration; (2) the solubility and partitioning characteristics of the drug that will determine whether it can easily permeate the bilayer and leak out from the emulsified systems during the freeze-drying and reconstitution processes; and (3) the type and concentration of cryoprotectants, since they have different protective mechanisms and exert their best protective activity at specific concentrations; some cryoprotectants do not have any activity in certain formulations (Chen et al., 2010).

The nature and shape of the container in which the samples are placed also influence the process conditions. Containers with a large contact surface area and low thickness are desired and preferred in order to obtain the maximum sublimation (Franks, 1998). Finally, different models of freeze-dryer yield different qualities of dried product, because some equipment cannot control the parameters for each step and some cannot freeze-dry samples containing organic solvents (Abdelwahed et al., 2006a).

3. Cryoprotectants

Cryoprotectants are compounds used to prevent the stresses that occur in the lipid bilayers during the freezing and drying steps that can lead to physical and chemical damage of the used

emulsion. The ideal cryoprotectant would cause minimal stress to the system and provide full protection during the process (Abdelwahed et al., 2006a).

Water becomes ice during the freezing step, separating from the other components of the formulation. Thereafter, the highly concentrated droplets can start to aggregate and fuse irreversibly, leading to the destabilization of the system. However, in the presence of cryoprotectants this damage during freezing can be avoided. At a slow rate of freezing, the cryoprotectant can migrate to the concentrated phase liquid and prevent droplet aggregation. This mechanism cannot occur during fast freezing, when the cryoprotectants do not have enough time to diffuse completely, resulting in poor redispersion. However, the addition of a suitable amount of cryoprotectant at a high freezing rate can improve such problems (Lee et al., 2009).

Water removal during the drying steps causes the phase transition of the fully hydrated phospholipid from the liquid to the gel phase. The head group spacing between the phospholipids is decreased, which results in an increase in the phase transition temperature due to stronger van der Waals interactions (Koster et al., 2000). However, during rehydration, there is another phospholipid phase transition from the gel to liquid phase, leading to a transitory rearrangement of the phospholipids, which can cause aggregation; this is the most important reason for drug leakage. These changes are illustrated in Fig. 3 (Cacela and Hinch, 2006).

The addition of cryoprotectant can decrease the interaction between the hydrocarbon chains, avoiding destabilization, because it can increase the distance between these chains due to its intercalation between the lipids. Hence, the van der Waals attraction forces are decreased, reducing the temperature of phase transition (Ingvarsson et al., 2011). In addition, during the drying process the hydrogen bonding of water with the phospholipid is replaced by the hydrogen bonding of the cryoprotectants, which reduces the phase transition, avoiding phospholipid rearrangement and the resultant drug loss.

There are several explanations for the protective mechanism (Fig. 4), which are mainly related to the structure of these substances that contain multiple hydroxyl groups and are able to form a eutectic mixture with water, leading to the formation of an amorphous solid or imperfect ice crystals, inhibiting the extrusion

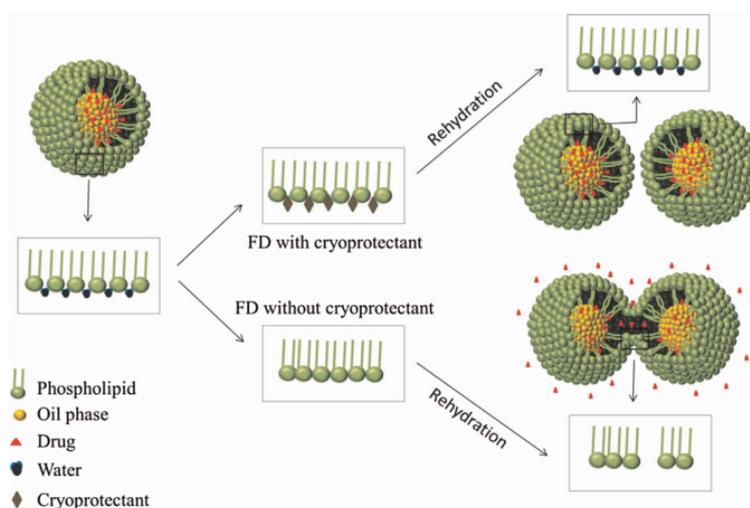


Fig. 3. Effect of freeze-drying on the phospholipid rearrangement, droplet size and drug leakage.

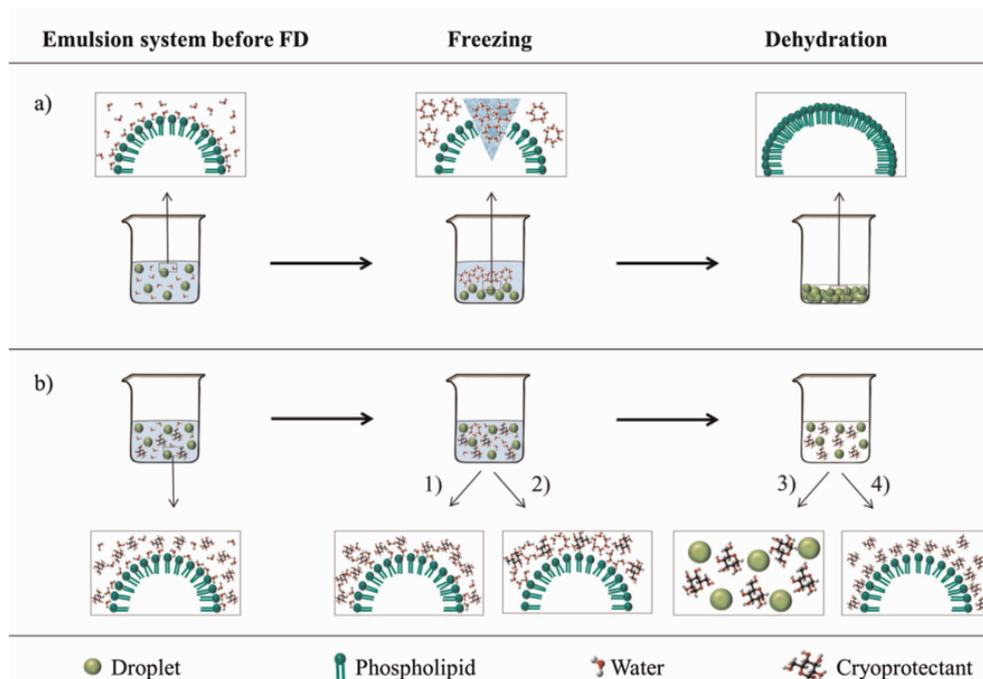


Fig. 4. Effect of freezing and dehydration on emulsified systems without (a) and with (b) cryoprotectant. The mechanisms of protection are explained by (1) formation of a eutectic mixture (2) the vitrification theory (3) maintaining the special organization and (4) replacement of the water molecules.

of ice crystals and mechanical damage (Zhang et al., 2008). Another protective mechanism described by some authors is the vitrification theory, in which the interaction between the hydroxyl groups of the cryoprotectant with water molecules and the free hydroxyl groups on the surface of the system increase the viscosity of the solution, reducing ice crystallization and limiting mechanical stress (Sussich et al., 2001).

In addition to these mechanisms, it has also been suggested that these compounds can maintain the spatial organization and the distance between the droplets of the emulsified systems during the drying steps (Abdelwahed et al., 2006b). Furthermore, the hydroxyl groups of the cryoprotectant can replace water molecules during drying, avoiding the aggregation of the surfactant layer (which would increase the droplet size), since the water molecule binds to the polar head group of the lecithin forming a hydrated layer which provides a repulsive force to prevent fusion of the lecithin layers when they are close enough (Zhang et al., 2008).

The concentration of the cryoprotectant is very important for the stabilization of the dispersions. High concentrations of cryoprotectant show better protective activity. However, once the limit of stabilization is reached, a higher concentration can induce destabilization of the dispersion (Abdelwahed et al., 2006b). The ideal concentration of cryoprotectant depends on other factors besides the composition of the system, such as the cooling rate and the freezing temperature (Sussich et al., 2001).

Many sugars have been used as cryoprotectants, including monosaccharides, disaccharides and polysaccharides. The cryoprotectants most often described in the literature for emulsified systems are trehalose, sucrose, maltose, glucose and mannitol, which have similar chemical properties (Table 1). However, they

yield different results, since the protection is also related to the chemical and physical structure of the system (Zhang et al., 2008).

4. Characterization of a freeze-drying product

Once the systems have been freeze-dried, it is important to characterize them in order to verify whether the procedure has been successful and has produced a suitable freeze-dried matrix. Also, it is necessary to evaluate whether the properties of the sample have not been changed by the process. Furthermore, these characterization methods can be used to validate the optimal process conditions, as well as the optimized formulation.

4.1. Macroscopic aspect

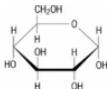
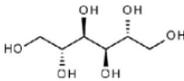
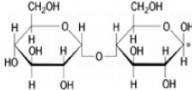
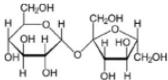
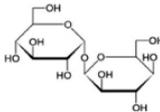
The macroscopic integrity of the lyophilizate is very important. The ideal freeze-dried product should not present any shrinkage or collapse and retain its initial volume with a high surface area. A product showing loss of the structure often contains a higher level of water and a lower surface area (Rey and May, 2004).

4.2. Differential scanning calorimetry

Calorimetry studies the thermal behavior of a sample by analyzing the specific physical properties as a function of the temperature. In particular, differential scanning calorimetry (DSC) assesses the heat uptake of a sample as a function of changing temperature. This technique is often used to track phase transitions changes, such as freezing, melting, glass transition, boiling, crystallization and degradation, and to evaluate the temperature at which these events occur (Gill et al., 2010).

Table 1

Cryoprotectants used for freeze-drying of emulsified systems with their molecular formulae, molar mass and collapse temperature (Bolton et al., 2008; Wang et al., 2012).

Cryoprotectants	Molecular formula	Structural formula	Molar mass (g/mol)	T _c (°C)
Glucose	C ₆ H ₁₂ O ₆		180.16	-40
Mannitol	C ₆ H ₁₄ O ₆		182.17	-30
Maltose	C ₁₂ H ₂₂ O ₁₁		342.30	-32
Sucrose	C ₁₂ H ₂₂ O ₁₁		342.30	-32
Trehalose	C ₁₂ H ₂₂ O ₁₁		342.30	-28

Therefore, DSC is very useful because it is possible to observe the phase transition which occurs as a result of the interactions between the cryoprotectant and the phospholipids, as well as measuring the T_g, which is a very important parameter of the freeze-drying process that ensures the quality of the final product (Abdelwahed et al., 2006b).

Furthermore, this technique is an important tool for investigating the recrystallization process that can occur with some cryoprotectants such as mannitol, which may destabilize the system (Zhang et al., 2008).

4.3. Residual moisture

Other important parameter that should be evaluated in freeze-dried samples is the residual moisture. Products with a higher percentage of residual water have a reduced T_g. Therefore, it is essential to control the moisture content to ensure that the T_g is high enough to provide stability of the product during storage (Duddu and Dal-Monte, 1997).

One of the most commonly used methods to measure the water content is the Karl Fischer titration. This method is based on the oxidation of sulfur dioxide by iodine in a methanolic hydroxide solution (Hua et al., 2010).

4.4. Size distribution and zeta potential

In dispersed systems, the droplet size and the size distribution before freeze-drying and after reconstitution (redispersion in appropriate medium) can be measured by dynamic light scattering (DLS). An increase in the size and a wide size distribution after the process can be explained by the fusion or aggregation of the droplets (Moretton et al., 2012).

Zeta potential deduced from electrophoretic mobility measurements can be used to evaluate the surface charge density on the

surface of the droplet. The electrokinetic study can be useful to verify the influence of the drying process on the colloidal stability of the dispersion, since higher zeta potential in absolute values lead to high colloidal stability induced by repulsive electrostatic interaction. Furthermore, this technique can be used to detect possible interactions between the cryoprotectant and the droplet surface or any particles surface modification before and after freeze-drying process (Abdelwahed et al., 2006b).

4.5. Electron microscopy

Microscopy is an essential technique for observing the morphology of emulsified systems in a liquid or freeze-dried form, providing information about the shape, size, stability and internal structure of the droplets. Moreover, any changes in the morphology of the system which occur during the freeze drying process (e.g. collapse) can be observed in this way. The most common types of electron microscopy used for these systems are transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Klang et al., 2013). With SEM is possible to obtain information about the topography of the sample surface, whereas TEM allows the internal structure of the emulsified systems to as well as coexisting structures and microstructure transitions to be observed (Klang et al., 2012).

4.6. Rheological behavior

Analysis of the rheological behavior of a sample consists of application of a specific force to the material and measurement of the resulting flow and/or deformation. Knowledge of the rheological properties gives much information about emulsified system, since changes in the internal viscosity, the droplet concentration, the type of droplets, the size distribution, deformability and the nature of the droplet interaction can all affect these

properties (McClements et al., 2007). Furthermore, an analysis of viscosity can reveal vitrification, since the highly viscous glassy matrix that can be formed with the addition of cryoprotectants reduces the molecular mobility (Ingvarsson et al., 2011).

4.7. Drug content

The drug content should be measured before and after the freeze-drying process in order to detect any leakage during the process. The encapsulation efficiency can be measured by removing the free (non encapsulated) drug from the system using column chromatography, ultracentrifugation, size exclusion chromatography, equilibrium dialysis or ultrafiltration, before any quantitative aspect of the loaded material by analytical techniques such as UV/VIS Spectrometry, Electron Spin Resonance Spectroscopy, Spectrofluorimetry and High Performance Liquid Chromatography (Maherani et al., 2011).

It is known that the leakage of lipophilic drugs during rehydration occurs less readily than that of hydrophilic and amphiphilic drugs, since molecules that have poor solubility in water have difficulty in permeating the bilayer and leaving the droplet. Therefore, it is also important to know the partitioning and solubility characteristics of the drug to predict the drug content after the drying process (Chen et al., 2010).

5. Emulsions

Emulsions are defined as a mixture of two immiscible liquids, one dispersed within the other in droplet form with the aid of surfactants, which are amphiphilic compounds responsible for decreasing the interfacial tension between two liquids. The first study of the use of surfactants as stabilizers of mixture of liquids was published by Griffin (Griffin, 1949).

Emulsions are considered to be metastable and thermodynamically unstable systems because the contact between oil and water molecules is energetically unfavorable. However, emulsions are kinetically stable, which may be explained by the fact that their energy of formation is greater than zero. Essentially, the adsorbed surfactant layer at the water/oil interface provides the emulsion's kinetic stability (Capek, 2004).

Emulsion droplets behave in a way typical of metastable colloids, showing Brownian motion and reversible or irreversible transitions due to droplet interactions, which usually lead to their destruction (Bibette et al., 1999), and as a result they are physically unstable. These phenomena occur in order to reduce the total free energy through the increase of droplet size, which may lead to phase separation. The main phenomena causing instability on emulsions are creaming, flocculation, coalescence and Ostwald ripening. In addition, many other processes may contribute to their instability, including microbiological and chemical effects that can also lead to phase separation (Jafari et al., 2008).

Emulsions are well established systems for the delivery of water-insoluble drugs. Thus, many formulations have been developed in order to improve the biopharmaceutical properties and reduce toxicity of widely used drugs, such as amphotericin B (Egito et al., 1996) and penicillin G (Lee, 1999). Anticancer agents as chlorambucil have also been incorporated into emulsions in order to produce a dosage form for intravenous administration. These systems have also proved to be suitable for targeting specific sites after the attachment of suitable ligands. Furthermore, they can be concentrated in the lymphatic system and because their distribution in vivo involves uptake by the reticuloendothelial system, which gives them the potential for lymphatic, liver and brain targeting (Khan et al., 2006). From the pharmacokinetic point of view, emulsions can improve the drugs' concentration-time

profiles, reducing the plasma clearance and increasing the elimination half-life when compared with aqueous solutions (Ganta et al., 2008).

Emulsions are also extensively used for parenteral nutrition, because commercial emulsion formulations have proven to be safe and efficient for intravenous use and have made it possible to administer the necessary calories to patients in critical situations, such as sick neonates and low birth-weight premature babies (Bryan et al., 1976).

Emulsions containing natural products have been studied as a possible alternative to treatment with synthetic drugs. Delivery systems are necessary to improve the natural products' bioavailability and to achieve desirable pharmacokinetics and permeability profiles (Alencar et al., 2015; Xavier-Júnior et al., 2012).

Emulsions, microemulsions and nanoemulsions often have the advantage over aqueous solutions with respect to the chemical stability of drugs, since in these systems the drug will be preferably distributed in the oil core or in the surfactant layer. It is known that hydrolysis is one of the most common reactions leading to instability in liquid dosage forms with high water content. Furthermore, hydrolysis is often the main degradation pathway for drugs that have amide or ester functional groups in their structure. In this context, several studies have investigated the ability of emulsions to decrease drug instability compared with aqueous solutions. Tian et al. (2007) developed a forced degradation study of etoposide incorporated within an O/W emulsion, while Shah et al. (Shah et al., 1989) studied this drug in aqueous solution under the same conditions. After comparison of the k_{obs} values for the loss of etoposide at 25 °C in the two studies, it was possible to conclude that the chemical stability of the drug was better in the emulsion than in the aqueous solution. This phenomenon was explained by the high entrapment efficiency of the drug in the oily phase of the emulsion.

Despite all these advantages, emulsions, nanoemulsions and microemulsions still have significant amounts of water that might catalyze hydrolysis of the other components of the system. Thus, the use of drying processes is an essential factor for increasing product stability and shelf life, improving their conditions of use and facilitating transport (Choi et al., 2007; Kaushik and Roos, 2007; Marefati et al., 2013; Wang et al., 2010).

5.1. Freeze-drying of emulsions

Among the emulsified systems, ordinary emulsions are the most frequently used in the pharmaceutical and cosmetic industries. For this reason, the freeze-drying of these systems has been studied in detail, from its usefulness for protecting the ingredients of the systems to its application to the production of tablets. In this section, some examples of freeze-drying studies of different types of emulsions, from regular O/W emulsions to multiple and Pickering emulsions, will be presented.

Many O/W emulsions are produced in order to allow the administration of essential oils, such as fish oil. This type of compound is sensitive to thermal and oxygen-induced oxidation. Heinzelmenn and co-workers (Heinzelmenn and Franke, 1999) studied the ability of dried emulsions to retain the oil content and stability over time as a function of different freeze-drying conditions. The variables were the freezing rate, the addition of anti-oxidants, the addition of different cryoprotectants, the rate of homogenization and the starting temperature for freezing drying. As a result of their analysis, the authors suggested that the addition of anti-oxidants to the formulation was essential due to the porosity of the powders and pellets obtained, which still allowed contact of oxygen with the unsaturated compounds. As far as the cryoprotectants were concerned, it was observed that different ones produced different dried products, as will be explained below

in the section on freeze-drying of nanoemulsions. Furthermore, it was observed that different conditions of freezing improved or decreased the stability of the dried emulsion. However, the authors explained this change as being due to the shear rate to which the emulsions were submitted in one of the freezing methods. Finally, no optimal formulation was obtained, but it was possible to distinguish suitable and unsuitable techniques for future applications.

Multiple emulsions have also been used for the administration of fragile water-soluble molecules. However, these are delicate systems to dry because after re-hydration of the dried emulsion the double water phase structure needs to be preserved. During a study of the freeze-drying of this sort of system by Choi and co-workers (Choi et al., 2007), a decrease in the mean droplet size was observed, which was dependent on the cryoprotectant and the conditions used for freeze-drying. The authors found that low molecular weight cryoprotectant (e.g. glucose and trehalose) could maintain the droplet size, while high molecular weight ones (e.g. κ -carrageenan) induced aggregation. It was also possible to observe variations in the properties of the rehydrated emulsions at different concentrations of cryoprotectant.

In addition, when Potier et al. (1992) studied the freezing of multiple emulsions they observed a transfer of water from the inner phase to external phase at the beginning of the freezing process. The authors explained this movement of water from one phase to another by the difference of vapor pressure created between ice and undercooled water at the same temperature in different compartments. This interpretation was corroborated by the results of Choi et al. (2007), who observed that at high freezing rates the droplet size was reduced. They also suggested that due to this transfer of water, W/O/W multiple emulsions could become O/W after drying and reconstitution. On the other hand, when the leakage of active molecule from the inner phase to the external phase was evaluated, the emulsions containing κ -carrageenan showed better results. However, this cryoprotectant was not considered for further studies, and trehalose was chosen as the best cryoprotectant for these systems, corroborating other reports in which trehalose also showed higher protective power (Choi et al., 2007).

For most emulsions, the use of a cryoprotectant has been described as essential for the production of an acceptable lyophilisate. However, Pickering emulsions, which are emulsions stabilized by solid particles, might be an exception, according to Marefati et al. (2013). When these authors freeze-dried heated or non-heated starch-stabilized emulsions they observed large or small variations of droplet size distribution depending on the formulation and the freeze-drying parameters. In particular, they found that low temperatures and a high freezing rate led to the aggregation of the oil droplets and consequently to a bigger droplet

size. They also observed the appearance of a smaller peak on droplet size analysis, which was attributed to the free starch that had left the water/oil interface.

In addition to the importance of the freezing step, Marefati et al. (2013) also observed that the nature of the oil used to produce the emulsions had influenced the result. Liquid oils shrink more than solid oils (at room temperature), which decreases the displacement of starch particles from the interface and maintains the structure of the emulsion after re-hydration. The author's explanation for this phenomenon was that the higher amount of shrinkage leads to oil crystals from liquid oil protruding more into the aqueous phase than the crystals from solid oils. However, coalescence may not be completely avoided. Furthermore, the effect of the swelling of the starch at the interface may also be a factor. On the other hand, larger differences were only observed with liquid oil droplets, which were more resistant to freeze-drying in the emulsion with non-swollen starch.

In summary, it is evident that regardless of the type of the emulsion all the parameters of freezing and drying must be carefully chosen, as well as the ingredients of the formulation, which will strongly interact with each other when the formulation starts to freeze and their water volume begins to decrease while water crystals expand in volume. The process conditions that have led to successful freeze-drying of emulsified systems are summarized in Table 2.

6. Microemulsions

Microemulsions are colloidal systems defined as transparent and isotropic mixtures of an aqueous phase, an oily phase and surfactants. These systems were first described by Hoar and Schulman (1943) and can also be classified as O/W, W/O and bicontinuous systems; the last having approximately equal volumes of water and oil (Date and Nagarsenker, 2008a).

Although a system may be macroscopically and physico-chemically characterized as a microemulsion, it can adopt different microstructures depending on the proportions of the components. Indeed, not only bicontinuous systems and spherical droplets, but also a wide variety of microstructures, such as cubic, hexagonal, cylindrical and lamellar patterns can be formed in microemulsions (Vinod et al., 2013). Due to their small droplets, microemulsions shows higher positive entropy compared with emulsions. Therefore, microemulsions are considered to be thermodynamically stable because the entropy of formation exceeds the interfacial free energy (Ruckenstein, 1978).

These systems have been developed for transdermal delivery of established drugs. Fouad et al. (2013) prepared a microemulsion containing diclofenac epolamine. Their studies demonstrated the ability of the microemulsion to provide an *in vivo*

Table 2
Freeze-drying conditions for different emulsified systems.

Systems	Freezing	Primary drying	Secondary drying	Cryoprotectants	Cryoprotectant concentration	Ref
Emulsion	-20°C -40°C -45°C -80°C	-10°C and -37°C 24 h, 72 h	20°C and 25°C 6 h	Glucose, Trehalose, Maltodextrin and Sucrose	5%–20%	Choi et al. (2007); Corveleyn and Remon (1998); Kaushik and Roos (2007); Zhao et al. (2008)
Microemulsion	-40°C	24 h	40 h	Mannitol	5%	Moreno et al. (2001)
Nanoemulsion	-70°C	-35°C/ 4 h → -20°C/ 13 h 24 h	20°C/5 h	Maltose, Trehalose and Mannitol	15%–20%	Gupta et al. (2011); Li et al. (2008)

anti-inflammatory activity for 12 h. Furthermore, the presence of drug deposits in the skin was observed.

Biocompatible propofol-loaded microemulsions have been developed for intravenous administration. After characterization and evaluation of the anesthetic effect it was observed that the propofol-loaded microemulsions had the same efficacy as a classical emulsion loaded with the same amount of the drug. However, the physico-chemical properties of the microemulsion, such as viscosity, globule size and stability, were different, and would give to these systems some advantages over classical emulsions (Li et al., 2012).

Recently, microemulsions have attracted attention from the pharmaceutical industry for their possible application as safe, effective and comfortable ocular delivery systems. Fungal infections of the eye are very difficult to treat because of the location of the micro-organisms in the ocular tissue and the poor permeation of drugs through the corneal membrane. As an example, microemulsions containing voriconazole developed by Kumar and Sinha (2014) can be cited. *Ex-vivo* studies showed that the microemulsions enhanced permeation through the cornea by about 3-fold compared with a drug suspension. Furthermore, recent work has shown that microemulsions are less irritating than some eye drop solutions available on the market (Habib et al., 2012).

In general, microemulsions possess several advantages over other delivery systems, such as drug solubility enhancement and good stability (Mehta et al., 2008). As well as being successfully evaluated as drug carriers, microemulsions are being studied for other applications, such as vehicles for food and cosmetic ingredients. Furthermore, these systems could be used for bioseparation and act as microreactors for optimization of chemical and biochemical reactions, (Date and Nagarsenker, 2008a; Fanun, 2007).

6.1. Freeze-drying of microemulsions

Moreno and co-workers prepared phospholipid-based microemulsions, to which 5%_(w/v) mannitol was added. The system was cooled at -40°C for 2 h. The primary and secondary drying steps were carried for 24 h and 40 h respectively. The authors reported that the final freeze-dried microemulsions exhibited the same properties as the original ones (Brime et al., 2004; Moreno et al., 2001).

Moreno et al. (2001) used most of the characterization techniques described above in this review to evaluate the microemulsions: macroscopic aspects, droplet size distribution, residual moisture, viscosity and rheological behavior. In this way, they demonstrated that the reconstituted microemulsions had similar properties to the initial formulations.

Indeed, droplet size distribution and viscosity were shown to be reduced in the freeze-dried systems, although these changes were not considered to be significant. Lyophilized microemulsions were also found to be more stable than their non-lyophilized counterparts. At the end of the freeze-drying procedure, all the parameters evaluated remained within the limits set for this sort of formulation. (Brime et al., 2004; Moreno et al., 2001).

Brime et al. (2004) later evaluated the efficacy and pharmacokinetics of microemulsions loaded with amphotericin B (AmB). Efficacy was evaluated against *Candida* strains and it was observed that the antifungal activity was maintained, and the toxicity was reduced compared with traditional formulations of amphotericin B. Furthermore, the administered dose could be increased using the microemulsion without increasing lethal toxicity, leading to an improvement in the therapeutic index of amphotericin B and to a smaller risk of developing resistance among fungi (Brime et al., 2004; Moreno et al., 2001).

A multiple-dose toxicity study was performed in albino CR1 mice as well as a pharmacokinetic study after a single dose given to New Zealand white rabbits. It was observed that the elimination of the amphotericin B given as the microemulsion from the blood was biphasic, as for the traditional formulation. Furthermore, a single dose of freeze-dried microemulsion led to lower values of C_{max} and $\text{AUC}_{0-\infty}$ than the traditional formulation. In addition, the microemulsion produced no histologically demonstrable renal lesions or changes in blood chemistry (Brime et al., 2003). Therefore, the authors concluded that the freeze-dried microemulsion was a useful system for the delivery of amphotericin B, since it presented low toxicity and good efficacy in animals.

7. Nanoemulsions

Nanoemulsions are dispersions in the nanometric size range containing oil droplets in an external aqueous phase stabilized by an appropriate surfactant mixture. These systems show good physical stability due to their small droplet size and low viscosity (Chen et al., 2011; Delmas et al., 2011). They differ from microemulsions in that nanoemulsions have a spontaneous tendency to phase separation. However, they are considered to be kinetically stable, similarly to emulsions (Chen et al., 2011; Mason et al., 2006; Rao and McClements, 2011). In contrast to classical emulsions, nanoemulsions have small-sized droplets so that gravitation effects are slower in these systems, which, together with Brownian movement, prevents creaming and sedimentation. In addition, steric stabilization also protects the system from flocculation and coalescence. Thus, nanoemulsions (well known as miniemulsion in polymer in disperse media science) show shelf-lives comparable to microemulsions, remaining stable for longer periods of times than traditional emulsions (Tadros et al., 2004).

Nanoemulsions are susceptible to one main mechanism of instability: Ostwald ripening. This phenomenon is responsible for enlargement of the droplet size of nanoemulsions, thereby causing instability; this is usually associated to the aging of the system. The process occurs the same way as in traditional emulsions and the Ostwald ripening rate may be determined from the change in the droplet size (Capek, 2004).

The use of nanoemulsions is frequently preferred to that of microemulsions because they have good stability, while the concentrations of surfactants are usually lower (Capek, 2004). This is particularly relevant for the cosmetic industry. A recent study (Ribeiro et al., 2015) has shown that nanoemulsions are suitable formulations for incorporating a hydroglycolic mill extract of *Opuntia ficus-indica* (L.) for anti-aging and moisturizing purposes. The nanoemulsion developed in this work proved to be a better moisturizer than a traditional emulsion containing the same extract.

Intravenous administration of water-insoluble substances has always posed a problem in pharmaceutical technology. In this context, nanoemulsions have proved to be a good solution for the administration of many drugs. As well as academic research, there are already several commercially available drug-loaded nanoemulsions. In addition, drug-free nanoemulsions are on the market for use in hospitals for parenteral nutrition and extemporaneous preparations in cases when there is no other treatment available. At the moment, nanoemulsions are still the most frequently used type of emulsion for intravenous administration of drugs, based on the number of commercially available products (Bruxel et al., 2012).

Nanoemulsions have also been investigated as non-viral vectors for genetic material; given that the use of viral vectors may cause serious side effects such as immune and inflammatory responses in the host as well as posing limitations on the size of

nucleic acid that can be packed (Verissimo et al., 2010). Silva et al. (2012) developed a nanoemulsion for plasmid DNA incorporation. An electrophoresis assay showed that the genetic material was incorporated within the nanoemulsions as a result of the inclusion of a positive charged component, stearylamine, in the formulation.

7.1. Freeze-drying of nanoemulsions

Like microemulsions, the freeze-drying of nanoemulsions has not been extensively studied. However, the studies reported in literature are more detailed. Drying techniques have been applied to nanoemulsions mainly to increase their shelf life and for the production of Self Nanoemulsifying Drug Delivery Systems (SNeDDS). In fact, Gupta and co-workers (Gupta et al., 2011) recently published a study in which they observed that the droplet size of SNeDDSs was increased after the freeze-drying process. However, the observed size increase was acceptable since the range of droplet size remained below 200 nm, and all other physico-chemical properties, such as polydispersity index, conductivity and zeta potential, were unchanged.

In most published work, the addition of the cryoprotectant to the nanoemulsions is done at the end of the process. However, (Li et al., 2008) have shown that this step should be considered to be as important as the freezing and drying steps. From the results of their study that adopted an experimental design, they observed that the addition of cryoprotectant at different steps led to variations in nanoemulsion properties such as droplet size and drug loading efficiency. When the cryoprotectants were added during the preparation of the nanoemulsions, an increase in droplet size and polydispersity was observed. On the other hand, when the cryoprotectants were dissolved in the final formulation, it was difficult to solubilize some of the sugars, such as mannitol and lactose, and if ultrasound was used, it led to droplet size increase and the leakage of the drug. However, if the cryoprotectant was added as a concentrated solution at the end of the production process, this step seemed to disrupt the system less. This procedure could be easily performed in a laboratory-scale production.

Li et al. (2008) also studied another parameter that was neglected in many studies which is the way of reconstituting the lyophilizate. Two methods were evaluated: (1) hand shaking and (2) sonication in a water bath. When sonication was used, a significant increase in the droplet size was observed, making this method unacceptable for clinical use. However, with hand shaking a significant difference in the time necessary for reconstitution was observed, mainly depending on the solubility of the cryoprotectants used. For example, dried nanoemulsions containing trehalose were easily re-dispersed than the ones dried with mannitol as cryoprotectant.

Nanoemulsions are also used in the biotechnology field, mainly as carriers for genes or as adjuvants for vaccine formulations. In the latter area, a study reported by Orr et al. (2014) monitored the stability of a nanoemulsion formulation destined to be used as an adjuvant for a vaccine formulation, after submitting these freeze-dried formulations to heat stress for 30 days at 50 °C. The freeze-drying was carried out over a period of 37 h from freezing until the end of the secondary drying. After drying, some samples were reconstituted in order to evaluate the direct influence of the process on the formulations. The only difference observed was in the Z-average diameter, which was 10 nm higher for the formulations after freeze-drying, while all the other parameters examined were unaffected by the process. When the formulations were compared before and after heating stress, no macroscopic differences were observed by the authors. However, no further characterization was performed. On the basis of these results, the

authors concluded that the freeze-drying would be a suitable technique to increase the stability of the systems.

8. Summary of the freeze-drying process for emulsified systems

Some critical steps should be carefully studied before the freeze-drying process of emulsified systems.

- Firstly, all the components of the emulsified system must be carefully selected. Several aspects of the formulation are crucial, such as its T_g and the presence of organic solvents, in order to choose the correct freeze-dryer able to reach temperatures below the T_g and safe to be used with these solvents. In addition, the nature and concentration of the surfactants will also affect the process. The lower the degree of unsaturation or the longer the length of the acyl chains in the surfactant, the higher the phase transition temperature of the phospholipid, at which rearrangement may occur and cause further drug loss (Komatsu et al., 2001). Since the emulsion, nanoemulsion and microemulsion could be prepared from exactly the same ingredients, the main difference with respect to freeze-drying is the concentration of the components, given that the microemulsion requires a higher surfactant-to oil ratio (McClements, 2012).
- The next step is the choice of the type and the concentration of the cryoprotectant. The polar P=O and/or C=O groups, as well as the CH₃ groups, of the surfactants may interact with the cryoprotectant. Hydroxyl, phosphate, methyl choline and/or carbonyl groups in the cryoprotectant are usually responsible for this bonding (Sum et al., 2003). Therefore, the interaction between the surfactant and the cryoprotectant should be assessed in order to ensure maximum stabilization. Furthermore, the cryoprotectant concentration should be sufficient to interact with the droplet surface, protecting it from damage during lyophilization. As shown in Table 2, the type of cryoprotectant that can be used successfully for freeze-drying emulsified systems is saccharides and disaccharides. Among these, trehalose has been shown to be one of the most effective because of its reduced hygroscopy, which allows it to form hydrogen bonds easily, its low chemical reactivity and its high T_g (Hedoux et al., 2013). The ideal cryoprotectant concentration of emulsified systems ranges from 5% to 20%. It has also been observed that a microemulsion system could be freeze-dried using a small quantity of mannitol; probably because of the high affinity of this cryoprotectant for the surface.
- In order to proceed with the freeze drying process, the freezing conditions should be carefully determined. To ensure the total solidification of the emulsified system, the temperature of the samples during the freezing process should be below the T_g . Furthermore, the product should be maintained at the set temperature long enough to freeze all the liquid (Meister and Gieseler, 2009). The formulation of the systems also exerts an influence. Nanoemulsions that contain a high quantity of water and a low surfactant concentration will more quickly than emulsions and microemulsions. During slow freezing, since the water can diffuse slowly, the osmotic pressure is reduced, preventing leakage of the droplets (Wang et al., 2012). However, the use of moderate freezing rates could be advantageous, since preventing the system from freezing extremely would protect it from suffering much damage while also maintaining the freezing rate high enough to facilitate secondary-drying. The emulsified systems discussed by Wang et al. used a moderate freezing temperature and rate.
- The next step is to optimize the primary drying. The temperature should remain constant during this entire step and it should be high enough to sublimate the water, but not to melt the sample. The choice of the pressure for this step should be based on the

frozen temperature of the sample, since the sublimation can only occur at pressures lower than the vapor pressure of the ice. It has been recommended that the chamber pressure does not exceed one-half of the vapor pressure of ice at the desired sample temperature, but that should not be less than one-quarter (Wang et al., 2012). The product temperature should be close to the T_g , because the higher the temperature the faster the process: a 1 °C increase in the product temperature decreases the primary drying time by 13%. A small safety margin between the product temperature and the T_c between 2 °C and 5 °C depending on the freeze-drying time has been suggested (Tang and Pikal, 2004). Among the systems discussed in this work, nanoemulsions are probably the ones with the shortest primary-drying time because they contain the largest proportion of water, with a faster mass transfer. On the other hand, microemulsions, which have a higher concentration of surfactant, undergo a more difficult vapor mass transfer. The different primary drying times for different freeze-dried systems can be observed in Table 2.

- Next, the parameters of the secondary-drying should be defined. This stage begins by slowly increasing the shelf temperature (0.1 or 0.15 °C/min), because a fast temperature ramp might cause the collapse of the amorphous products, which contain high residual moisture and hence have a low T_g (Tang and Pikal, 2004). The pressure values used for the secondary-drying can be the same as those used in the previous step, because the pressure does not affect water desorption (Wang et al., 2012). Furthermore, samples containing higher solute concentrations have a smaller surface area in the dry form. Hence, they need higher temperatures and/or a longer time to remove the water (Wang et al., 2012). As shown in Table 2, among the formulations studied in this work, microemulsions are the type that require most time for this step.
- Finally, the dried product obtained from this process has to be sealed correctly to avoid absorption of humidity from the environment and thereby ensure its quality during the storage.

9. Conclusion

The use of colloidal systems as pharmaceutical carriers has been able to improve the efficiency of many drugs. This is a result of their ability to increase drug bioavailability while decreasing the systemic toxicity. However, the presence of an aqueous phase can decrease the stability of these systems. The freeze-drying process is an effective way to overcome this drawback by removing the water content of pharmaceutical formulations. However, during this process, some mechanical stress is produced, especially during the freezing stage in which the contact of the crystallized water with the droplet surface can destabilize the system and provoke drug release from the carrier, interfering in its pharmacological response. Therefore, the addition of cryoprotectants to the formulations is necessary to prevent such damage. The success of the freeze-drying process is influenced by several factors related to the formulation, the container, the equipment and the freeze-drying process. For this reason, the freeze-drying of emulsified systems has been a challenge, since it demands a thorough investigation of the formulation and the process conditions in order to ensure the long-term stability of these systems. As a result, there are no freeze-dried emulsions, microemulsions or nanoemulsions on the market as yet. Freeze-dried emulsified systems promise to be good drug delivery systems, since the drug will not be present in liquid medium that presents a risk of microbial contamination and the stability will be enhanced. However, to ensure proper delivery of the drug, the freeze-drying process must be validated and performed correctly, taking the necessary precautions discussed in this review.

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CHAPTER IV

*Application of design of experiment to optimize the freeze drying process of
microemulsion systems*

Application of design of experiment to optimize the freeze drying process of microemulsion systems

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Application of design of experiment to optimize the freeze drying process of microemulsion systems

ABSTRACT

The high aqueous content of oil-in-water microemulsion systems may be responsible for instability of the encapsulated molecule due to chemical reaction or microbiological attack. This drawback may be overcome by the use of the freeze-drying process; however this is not easy to optimize for colloidal systems like microemulsions. Design of Experiment can be used to find the optimal working conditions to achieve this goal. The aim of this work was to develop a freeze-dried microemulsion using Design of Experiment, using Amphotericin B, which is quite unstable in the aqueous media, as a drug model. Different types of cryoprotectants were added to the microemulsion and a 2^3 full factorial design with three replicated points was performed. Microemulsion, unloaded or loaded with Amphotericin B, were fully characterized before and after the freeze-drying process. Microemulsion containing Maltose at 5% as a cryoprotectant frozen at a temperature of $-80\text{ }^\circ\text{C}$ using a 24 h of freeze-drying time yielded the best results. It was observed that after the freeze-drying process, the droplet size became smaller and no significant changes in the Amphotericin B content were observed. The microemulsion containing Maltose at 5% are suitable for drug incorporation and the freeze-drying was able to enhance drug stability in the system.

KEYWORDS: Amphotericin B, Statistical experimental design, Lyophilization, Drug delivery, Dispersed system.

INTRODUCTION

Colloidal drug delivery systems are become more attractive in pharmaceutical technology because they are able to control drug release and to improve bioavailability.(1) Microemulsions (MEs), optically transparent systems with low viscosity, are thermodynamically stable dispersions of two immiscible liquids, stabilized by an interfacial film of a surfactant, usually in combination with a co-surfactant.(2) They have shown several advantages over conventional formulations, including enhanced drug solubility, good thermodynamic stability, an increased surface area improving absorption and enhancement of transdermal passage.(3) These properties give MEs potential applications in the food, cosmetic and pharmaceutical industries when solubilization of lipophilic or hydrophilic ingredients is necessary.(4) In pharmaceuticals, MEs are used as vehicles to deliver a number of drugs due to their thermodynamic stability, simple preparation and good appearance.(2)

Amphotericin B (AmB) is the drug of choice for the treatment in immunodeficient patients affected by systemic fungal infections, as well as local treatment of fungal infections in the eye.(5, 6) However, due to the high incidence of adverse drug reactions, i.e. cardiotoxicity and hepatotoxicity of conventional formulations,(7) AmB has been incorporated into a number of colloidal drug delivery systems, including MEs in order to improve its therapeutic index.(8, 9)

Generally, pharmaceutical MEs are produced with water as the continuous phase, which carries some risks such as microbiological contamination, degradation by hydrolysis and loss of pharmacological activity of the drug. A possible solution to these problems consists of the use of freeze-drying process, also known as lyophilization. Freeze-dried products have good stability and are easy to transport and store.(10) However, the freeze-drying process for MEs has rarely studied in been studied in pharmaceutical technology due to the difficulties that arise during freezing of colloidal systems. Lyophilization consists in removing water by sublimation

through three steps: freezing, primary drying and secondary drying.(11) During this process stress may be generated that could destabilize the colloidal structure of the MEs, in particular the freezing stage, during which the crystallization of ice may produce mechanical stress. However, cryoprotectants (CP) can be used in order to protect these systems against damage.(12) Among the compounds that can exert cryoprotective effects, carbohydrates are interesting because are chemically innocuous and can be easily vitrified during freezing, supporting their use as CPs in the freeze-drying process.(9)

The freeze-drying process has been used for several years in the pharmaceutical and biotechnology industries, leading to products destined for various administration routes such as parenteral, oral, nasal or pulmonary. However, the freeze-drying of colloidal systems is delicate due to the tendency of the droplets to interact and damage caused by the freezing process, which can increase the droplet size and destabilize the system.(10) In fact, there are very few reports in the literature of the successful freeze-drying of colloidal systems such as ME.

The quality of the final freeze-dried product can be influenced by several factors relating to the formulation, to the container, to the equipment and to the freeze-drying process.(10) Therefore, the process efficiency must be optimized through adjustment of these factors and process monitoring is crucial in order to obtain a product with the desired quality. It is necessary to select suitable freeze-drying parameters, evaluate the effects of these parameters and their possible interactions with the entire process. Therefore, Design of Experiment (DOE) has been used for the optimization, modeling and characterization of the freeze-drying process of some pharmaceutical products that are not colloidal systems.(13)

DOE is a statistical approach used to determine the influence of several independents variables on the dependent variable of the process. The optimal design allows the time and cost of the experimentation to be reduced, as well as improving the

process yield.(14) Therefore, this method is used much more often than the One Factor at a Time Method, which is time-consuming and expensive because it requires a large number of experiments and does not examine interactions between the variables.(15) The response surface methodology is a technique of DOE that combines mathematics and statistics to analyze the relative significance of different parameters, finding the optimal working conditions, by combining a small number of variables, resulting in fewer experiments.(16)

The aim of this work was to develop a freeze-dried ME product as a model to design freeze-dried ME systems containing drugs and thereby improve their stability. Therefore, AmB was used as a model drug incorporated within the ME. A DOE approach was used in order to establish the ideal CP content and the technical procedure to produce freeze-dried colloidal systems. Important parameters, which can influence the droplet size of this system, such as concentration and type of CP, freezing temperature and freeze-drying time were also evaluated.

MATERIALS AND METHODS

Materials

Miglyol 812[®] was obtained from CONDEA Chemie GmbH (Hamburg, Germany), Lipoid S100[®] was purchased from LIPOID GMBH (Ludwigshafen, Germany), Tween 80[®], Mannitol (MN), Glucose (GC), Lactose (LT) Sorbitol (ST) and Maltose (MT) were obtained from Sigma Aldrich Inc (St. Louis, USA), and the Na₂HPO₄ and the NaHPO₄, used to produced the phosphate buffer pH 7.4, were purchased from Vetec Química Fina Ltda (Rio de Janeiro, Brazil). Ultra-pure water obtained by a Milli Q water purification system (Merck Millipore, Massachusetts, U.S.A.) was used along the experiments.

ME preparation

The MEs were prepared by mixing 68% of phosphate buffer pH 7.4, 14.7% of Tween 80[®], 6.3% of Lipoid S100[®] and 11% of Mygliol 812[®] using magnetic stirring, following by three cycles of sonication (40 watts power output for 1.5 min) using a Vibra Cell 75041 (Bioblock scientific) and ultrasound bath (120 watts power for 3 min) using a USC-1800A (unique). The MN, MT, GC and LT were added to the MEs prior to the freeze-drying process for the DOE study.

Sample characterization

Macroscopic aspect, pH evaluation, isotropy and conductivity analysis

The color and homogeneity of the freeze-dried products (cake) were evaluated with the naked eye. The isotropy of the samples was evaluated by polarized light microscopy using an Olympus BX4 (Olympus Corporation, Tokyo, Japan) apparatus. The electrical conductivity was measured using a DM-32 conductivity meter (Digicrom Analytical, SP, Brazil), with a cell constant of 0.11 cm⁻¹. The pH values were measured by a PG-2000 pHmeter (GEHAKA, SP, Brazil). All analyses were performed in triplicate at 25 ± 2 °C.

Dynamic Light Scattering (DLS)

In order to evaluate the influence of the different CP types and concentration on the ME droplet size before the DOE study, MT, MN, GC, LT and ST were added to the MEs at 5%, 10%, 15% and 20% and the droplet size of all preparations was analyzed.

The samples were previously diluted with water in a ratio of 1:20 and their droplet size distribution was evaluated using a DLS, ZetaPlus (Brookhaven Instruments

Corporation, NY, USA). Previous experiments revealed that this dilution process has no influence on the DLS measurements.

Differential Scanning Calorimetry (DSC)

In order to characterize the type of system (water-in-oil, bicontinuous or oil-in-water),(17) the thermal behavior of the ME and its components were analyzed by DSC using a DSC-60 Shimadzu (Shimadzu Scientific Instruments, Kyoto, Japan). The samples (5-15mg) were weighed into aluminum pans. A temperature ratio of 5 °C/min, a nitrogen atmosphere at the flow of 50 ml/min and cooling at a pre-determined rate from 25 °C to -40 °C (for 10 min) followed by heating at a constant scanning rate up to 110 °C were used. An auto-cooling system TAC-60i (Shimadzu Scientific Instruments, Kyoto, Japan) was used to decrease the temperature during the cooling curve.

DOE methodology for freeze-drying conditions

In order to determine the optimum parameters for the freeze-drying process, the DOE methodology was performed for ME systems for which no changes in droplet size were observed after CP incorporation. The CP type and concentrations, the freezing temperature and the freeze-drying time were chosen as independent variables. The ME droplet size after freeze-drying process, on the other hand, was chosen as the dependent output response variable. The experimental levels of independent variables for ME droplet size after freeze-drying are given in Table 1.

Table 1

The levels of the variables CP concentration and freeze-drying time were chosen in accordance with the values commonly found on the literature. On the other hand, the freezing temperature used was selected according to the temperature attained by the use of liquid nitrogen (-196 °C – the minimum temperature), the temperature produced by a regular freezer (Frost Free 260 (Brastemp, São Paulo, Brazil) (-20 °C – the maximum temperature) and the temperature produced by a ultra-freezer Glacier NU-9438 ULT Freezer (NuAire, Inc., Minnesota, U.S.A.) (-80 °C – which is the DOE replicated point). A two-level three-factor full-factorial design with three replicated points leading to 8 experimental randomizations runs was performed for each CP (Table 2).

Table 2

Freeze-drying was performed using a Christ Alpha 1-2 LD freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). After the freeze-drying process, the MEs were reconstituted by adding the same amount of water as that lost during the process, followed by vortex mixing and one cycle of sonication (40 watts power output for 1.5 min), after which the droplet size was measured by DLS.

The effect of the studied variables was graphically and statistically interpreted using the Statistic software version 7.0 (StatSoft Inc., Oklahoma, USA). Based on the statistical results, the parameters that would produce a dry powder ME after freeze-drying and that would present the smallest droplet size values after reconstitution were determined. Thereafter, the ME systems that showed the best aspect and concentration of CP, and the best freezing temperature and the freeze-drying time, was produced and thermodynamically characterized for their pH, conductivity and droplet size before and after the freeze-drying process. The optimized formulation was then used for further studies, including drug loading.

Incorporation of AmB to the ME system

AmB was incorporated, as a model drug, into the optimal ME system determined by the DOE methodology. The aim was to observe the efficiency of the freeze-drying process on the production of dried ME systems containing drugs. The production of a dried powder for this system was verified and pH, conductivity and droplet size before and after freeze-drying were measured. Furthermore, the influence of drug loading on the thermodynamic stability of the ME was evaluated.

The incorporation of AmB was performed by adding the drug to the ME to a final concentration of 2×10^{-3} M, under continuous stirring. In order to improve the AmB dissolution, the pH of the ME was increased ($\text{pH} \geq 10.0$) by the addition of sodium hydroxide solution (NaOH 1 N). Afterwards, the pH was reduced using hydrochloric acid solution (HCl 1 N) to physiological pH (pH 7.4).(8)

Spectrophotometric analysis

A spectrophotometric assay was performed to analyze and compare the amount of AmB before and after the freeze-drying process, and to evaluate its stability. The experiment was carried out using a Biochrom Libra S32 UV-VIS spectrophotometer (Biochrom US, Massachusetts, USA), at the wavelength of 405 nm, by measuring the absorbances of the reference solution (methanol) and of the ME with and without AmB. The assay was performed in triplicate. The ME samples were first diluted with DMSO: methanol (1:9) at the ratio of 1:10 and, then, 50 μL of this solution was diluted with 20 ml of methanol. The drug loading efficiency was calculated by comparing the AmB concentration before and after centrifugation (Centrifuge 5410, Eppendorf, Hamburg, Germany), which was performed in triplicate by centrifuging 1 ml of ME with and

without AmB at 14.000 g for 20 min to precipitate any insoluble content. Moreover, an aliquot of 100 μL of the supernatant of each sample was used to evaluate the AmB content as previously described.

Statistical Analysis

The statistical tests used in the experiments were the Analysis of Variance (ANOVA) performed to analyze the statistical significance between 3 groups, followed by Dunnet post-test, to compare with the control or Bonferroni to compare all samples. Finally, a paired t-test was used in case of 2 unpaired groups. P values less than 0.05 ($p < 0.05$) were considered to indicate significance.

RESULTS AND DISCUSSION

Characterization of the ME system before adding the CP

Macroscopic aspect, pH evaluation, isotropy and conductivity analysis

The ME, a homogeneous and clear yellowish product that appeared dark under cross-polarized light microscopy (no birefringence), was classified as an isotropic system. Its pH value was 7.1 ± 0.09 , which is considered physiologically acceptable.

In general, MEs can be divided into 3 types: water-in-oil (W/O), bicontinuous, and oil-in-water (O/W).(18) A correlation between ME structure and electrical conductivity has been demonstrated and can be used as a tool to assess its properties.(19) The electrical conductivity of the ME studied was $832 \pm 22 \mu\text{S cm}^{-1}$. It is well known that O/W MEs have conductivity values similar to the aqueous phase.(20, 21) This high value is probably due to the large volume percentage of water in the system, from which can be inferred that the ME is of the O/W type.(22, 23)

DSC analysis of ME components

The thermal behavior of the ME and its individual components (phosphate buffer solution alone, oil phase and surfactants), was evaluated by DSC analysis. Figure 1 shows thermal events associated with the ME: a large exothermic peak at $-13.54\text{ }^{\circ}\text{C}$ during the cooling curve, which was due to water freezing, following by an endothermic peak at $4.26\text{ }^{\circ}\text{C}$ during the heating curve explained by the melting of water. A second, broad endothermic peak for ME at a temperature between $50\text{ }^{\circ}\text{C}$ and $100\text{ }^{\circ}\text{C}$ with an enthalpy value of -1.57 kJ/g probably represents water loss. It was also possible to observe small exothermic peaks for Tween 80[®] and Miglyol 812[®], following by only one endothermic peak for Miglyol 812[®].

Figure 1

Since it represents the freezing of water during the cooling curve, the position of the exothermic peak may indicate the state of water in the ME. In fact, the strong interaction among water and surfactant molecules compared to the weaker interactions among the water molecules themselves or water molecules and phosphate buffer may induce a large change in the temperature at which the water freezes.(24) The exothermic peak on Figure 1 indicates the freezing of water with less molecular interaction with surfactant molecules, since the onset freezing temperature of ME is approximately, at $-18\text{ }^{\circ}\text{C}$, which is near to that of phosphate buffer solution ($-19.09\text{ }^{\circ}\text{C}$). Moreover, no peaks characteristic of Lipoid or Tween 80[®] were observed in the ME thermal profile, which suggests not only a strong interaction between the surfactants and other components of the system, but also that they could be located at the internal phase or at the interface of the ME droplet and not in contact with the continuous phase.

Therefore, the results of DSC measurements lead to the conclusion that water is the continuous phase,(3) thus the ME is of the O/W type, confirming the results of the electrical conductivity.

Droplet size evaluation after addition of CP

Based on the hypothesis that ME stability could be modified by the type and concentration of the CP used, while ,on the other hand, the CP could affect both the droplet size distribution and the freeze-drying process, the influence of different CP types and concentrations in the ME systems were evaluated.

Macroscopically, all the ME systems, with and without CP, were clear, isotropic and homogeneous. On the other hand, those containing LT and the MN presented a turbid aspect at 15% _(w/w) and at 20% _(w/w), respectively, showing that these additives are not very soluble in the ME system.

The influence of CP addition on the droplet size can be observed in Figure 2. MEs showed an average diameter of 22 nm that do not change with the addition of MT, MN, GC and LT ($p > 0.05$). However, in the case of ST, the addition of even a small amount caused an increase in the ME droplet size (28 nm) and with the highest concentration it reached a 62 nm. Those variations, compared with the ME without CP, were statistically significant ($p < 0.05$).

Figure 2

Therefore ST was excluded from the DOE, while the other CPs: MN, MT, GC and LT, at concentrations from 5% _(w/w) to 20% _(w/w) may be used to protect MEs during the freezing and drying steps required for the freeze-drying process, since they did not

cause significant changes in the ME droplet size.

DOE for freeze-drying parameters

Among the samples submitted to the freeze-drying process, those containing MT and LT produced dry powders. Disaccharides, the class to which these CPs belong, are more effective freeze-drying protectants than other types of molecule because they are able to form an amorphous sugar glass.(10)

Although the ME-LT at the lower level (5% _(w/w)) produced a powder, it did not appear to be completely dry. This is probably because the concentration of CP was not high enough to completely stabilize the ME.(25) On the other hand, all MEs containing MT produced dry powders, showing the high capacity of this CP to stabilize freeze-dried MEs.

The use of the monosaccharide GC resulted in freeze-dried cakes, which might have been generated due to the primary drying temperature during the freeze-drying process being above the glass transition temperature (transition temperature range in which an amorphous sample in the glassy state changes to a rubbery state or the reverse(26) of the MEs containing GC, causing the collapse of the product.(27)

Only the ME containing MN at 20% _(w/w), frozen at the highest level (-196 °C), yielded dry powders after freeze-drying, probably because only this MN concentration was sufficient to stabilize the system while the rapid cooling inhibited the MN crystallization.(28) However, at this concentration, this CP was not soluble in the system and after a period of time at room temperature the dry powder changed to a rubbery state, revealing that MN was not the best choice as CP. The MEs containing MN at the other DOE levels (5% _(w/w) and 12.5% _(w/w)) did not produce powders, probably due to the tendency of MN to partly crystallize into MN hemi-hydrate. This

could release water on conversion to the anhydrous crystal form and thereby cause phase separation in the cryo-concentrated portion of the frozen samples, resulting in the loss of CP activity and destabilization of the system.(15, 29)

Formulations in which a dry powder were generated, ME containing MN 20% (w/w) and all samples of MEs containing MT and LT, were reconstituted with the same amount of water as that lost during the freeze-drying process and presented a Winsor IV ME system. Moreover, the droplet sizes of these samples were evaluated and a Pareto's Chart to investigate the standardized effect of the independent variables and their interaction with the ME droplet size was constructed. The results for MEs containing MT showed that the "square freezing" is the most important factor influencing the droplet size. The negative coefficients for the square freezing variables indicated an unfavorable or antagonistic effect on the droplet size. Therefore, the closer to the lower limit (-1 level) of the square freezing temperature (-20 °C), larger the droplet sizes. In other words, the higher the freezing temperatures, the larger the ME droplet sizes (Figure 3a).(30)

Freezing is the stage at which most of the water is separated from the solutes to form ice and concentrated solution.(11) In the specific case of MEs, the "concentrated solution" is composed of oil, surfactants and buffer salts. Increasing the concentration may enhance the interaction between the droplets, leading to their aggregation or fusion and increasing the droplet sizes.(25)

The Pareto's Chart analysis of the MEs containing LT (Figure 3b) showed positive coefficients for model components (x_2 , x_3 , x_2x_3 , x_1x_3) showed a synergistic or favorable effect on the droplet size. Additionally, the CP concentration was the most significant variable for droplet size variation.(30)

Figure 3

Date *et al*(31) also observed that the droplet size of MEs increased with CP concentration. Additionally, Abdelwahed *et al.* (2006) demonstrated that certain amount of CP was necessary to effectively preserve the droplet size and ensure a maximum stabilization of nanoparticulate systems.(10)

An empirical second order polynomial equation was developed for the response variable, droplet size, in terms of the three independent variables. This equation for MEs containing MT is expressed by $y = 23.4 + 1.31x_1 + 6.11x_1^2 + 1.41x_2 + 0.96x_3 + 1.61x_1x_2 + 0.41x_1x_3 - 0.54x_2x_3$, and for MEs containing LT by $y = 30.93 + 2.04x_1 + 3.00x_1^2 + 9.86x_2 + 4.19x_3 + 2.26x_1x_2 + 3.19x_1x_3 + 4.16x_2x_3$, where y represents the response variable (droplet size), x_1 , x_2 and x_3 are the coded values of the test variables, which are freezing (x_1), CP concentration (x_2) and freeze-drying time (x_3), respectively.

The statistical significance, evaluated using the Fisher's F-test and ANOVA, showed, based on probability values inferior than 0.05, that it was indicated for the quadratic term of freezing in MEs containing MT. On the other hand, MEs containing LT presented statistical significance for concentration, freeze-drying, interaction between them, and interaction between freezing and freeze-drying. Furthermore, the ANOVA results (Table 3) for both MEs showed that the calculated F value was found to be greater than the tabulated F value at the 5% level, indicating that the treatment combinations are significant. Moreover, concerning the lack of fit and pure error, the calculated $F < \text{tabulated } F$ demonstrated that the model was predictive.(16) The value of the coefficient of determination for MEs containing MT and LT were, respectively, $R^2 = 0.9236$ and $R^2 = 0.9120$, indicating that 92.36% and 91.2% of the response variability

could be explained by the previously discussed models.

Table 3

A marginal means chart was plotted to observe the average responses for each level of each factor (Figure 4). It was observed that for ME containing MT (Figure 4a) when observing the effect of CP concentration at the lowest level of freeze-drying time, the lowest droplet size was observed using the lowest CP level (5% _(w/w) of MT), regardless of the freezing temperature. However, at 48 h of freeze-drying time, the droplet size varies depending on the freezing temperature. In fact, freezing at -196 °C generates smaller ME droplet size when 5% _(w/w) of MT was used, while for high freezing temperatures (-20 °C) a 20% _(w/w) of MT was necessary to achieve the same result. It was also observed that the droplet size increased with the CP concentration when the rapid freezing process (-196 °C) was used.

The analysis of the marginal means of ME containing LT (Figure 4b) showed that for both the high and the low level of freeze-drying times (48 h and 24 h) the ME droplet size increased with increasing CP concentration, regardless of the freezing temperature. Nevertheless, at the minimum freeze-drying time level (20 h) the freezing temperature of -20 °C produced a larger droplet size than the one at -196 °C. Otherwise, the freeze-drying time of 48 h led to a lower droplet size at this longer freezing time. Both marginal levels of freezing temperature showed decreased droplet size with decreasing CP concentration.

Figure 4

The relationship between the dependent and independent variables was further

elucidated by the analysis of response surface diagrams, which was able to evaluate the relative significance of all factors involved in the process (Figure 5). Throughout this analysis it was possible to determine the optimum operational conditions for the system and the process.(32) It was observed that the ME droplet size decreased with the reduction on the freeze-drying time and MT concentration in a linear way. However, when freezing time and other independent variables were compared, no direct linear relationship among them was observed. Nevertheless, it was possible to identify the smaller droplet size values of ME-MT at - 80 °C of freezing temperature (Figure 5a and Figure 5b).

The response surface analysis revealed that the droplet size increased with increasing LT concentration and freeze-drying time. In fact, the smallest droplet size was found at the central point of freezing temperature (Figure 5c and Figure 5d).

Figure 5

Therefore, the best parameters for producing small droplets for freeze-dried MEs using either MT or LT as CP were a concentration of 5%, a freezing temperature of -80 °C and a freeze-drying time of 24 h. Since at this concentration ME-LT become rubbery few minutes after the freeze-drying process and at a higher concentration the CP could not be dissolved in the system, MT was chosen as the best CP for this ME.

Characterization of the ME before and after freeze-drying

The general physicochemical characteristics of the ME-MT at 5% showed some changes before and after the freeze drying process. Before freeze drying, the ME-MT presented a droplet size of $52 \text{ nm} \pm 0.7$, a pH value of 7.1 ± 0.09 and a conductivity

value of $832.9 \mu\text{S cm}^{-1} \pm 22.1$. After the freeze drying process and subsequent dispersion with water, its macroscopic appearance remained homogeneous and a clear yellowish product was obtained. However its pH, conductivity values and droplet size were slightly different (Table 4).

Table 4

The pH value decreased, probably due crystallization of the buffer salts during the freezing drying process.(11) However, even at this pH (6.86), the ME-MT is physiologically acceptable.

The reduction on the droplet size of the ME-MT might be due to the co-surfactant role that CP could play. The short poly-hydroxyl chain-alcohol could be adsorbed and intercalated into the interfacial film of the ME after the freeze-drying process and reconstitution, thereby decreasing its surface tension.(33, 34) However, at this droplet size (20.13 nm) the sample remains within the ME droplet size range of 10 – 100 nm.(35) The decrease of conductivity just after CP addition and before the freeze drying process was probably due to its protective mechanism that enhances the viscosity of the system due to the interaction between the hydroxyl from the CP and the water molecules. In fact, it is well known that the crystallization of ice can be suppressed by the increase on the viscosity of the medium, which limits the mechanical damage.(25) Therefore, a decrease in the amount of ions can induce a reduction in the conductivity value of the ME-MT.

On the other hand, the increase of this conductivity value in the reconstituted ME-MT can be explained because when the water is removed during the drying process, the ions from the buffer remain in the sample. Thus, when the water was added to

reconstitute the freeze-dried ME, these ions dissolve in the water and cause the electrical conductivity to increase.

The protective mechanism of the CPs may be also explained by (i) the formation of an eutectic in the presence of water, due the multi-hydroxyl compounds, that leads to the formation of amorphous or imperfect ice crystalloids; (ii) the maintenance of the spatial orientation and distance between the droplets when the ice sublimates during the freeze-drying process, preventing the formation of aggregates;(36) and (iii) the amorphous glass formation into which the cryo-concentrated solution may vitrify at its T_g. The immobilization of the droplets within a glassy matrix of CP can prevent their aggregation and protect them from the mechanical stress of ice crystals.(27)

Incorporation and freeze-drying of ME containing AmB

The incorporation of AmB into the ME generated a product with a homogeneous and clear yellowish aspect. The addition of MT at 5% did not cause any changes in the ME appearance. However, the freeze-drying process (24 h of freezing at - 80 °C and freeze-drying for 24 h) altered the AmB-ME formulation, although a dry powder is produced. In fact, the reconstituted AmB-ME, which was made by adding the same amount of water as that lost during the freeze-drying process and using a probe sonicator with an iced bath for fast reconstitution, showed changes in its electrical conductivity, pH and droplet size (Table 5). The aforementioned reasons for changes in unloaded ME-MT formulations can also explain such changes in the AmB-ME product.

Table 5

The addition of AmB increased the ME droplet size and the conductivity value

(Table 4 and Table 5). In fact, the ionization of functional groups on AmB (carboxyl and/or amino groups) may increase the amount of ions in the system, thereby increasing the electrical conductivity.(37) The increase in the droplet size may be explained by the partitioning of AmB molecules into the oil phase since it is insoluble in water, increasing the oily phase volume. Furthermore, due its amphiphilic properties, the molecule might be located in the system interface, also increasing the droplet size.(38)

A standard curve (data not shown) with increasing concentrations of AmB was constructed in order to determine the amount of AmB in the ME. The method showed to be robust, efficient and sensitive. Thus, the linear equation ($y = 1.2565x - 0.0257$ and $R^2 = 0.9992$) could fit more than 99% of the experimental data. Therefore, the methodology may be confidently used for quantitative analysis of AmB. The AmB content of the ME remained unchanged after the freeze-drying process ($p > 0.05$). Therefore, MT at 5% was effective in preventing ME diameter changes and loss of AmB.

Although the AmB loading efficiency (encapsulation of the drug) was quite low (24.4%), the freeze-drying process did not change the concentration of entrapped drug ($p > 0.05$). Therefore, no loss of drug occurred during the process.

MT was an efficient CP avoiding drug loss, probably as a result of its ability to form hydrogen bonds to phospholipid head groups and replacing the water by reducing the gel to liquid crystalline phase transition that occurs when the dried phospholipids are rehydrated. In this way, the MT did not lead to inhomogeneous rearrangement of phospholipids that could result in particle aggregation and loss of incorporated drug into the aqueous medium.(10)

CONCLUSION

Research and development of freeze-dried microemulsion systems has been a challenger for several research teams. In fact, several parameters may affect the system stability during the freeze-drying process. However, this work revealed that DOE can be a successful tool allowing determining the optimal freeze-drying conditions to achieve this task. By taking in account the type and concentration of CP, the freeze-drying time and freezing time temperature on the DOE, and using the droplet size as variable of response, a ME dried product can be produced using a low number of experiments. MT at 5% as CP, - 80 °C of freezing time temperature and 24 h of freeze-drying time were the best parameters to produce a good aspect brick of freeze-drying ME. The incorporation of AmB did not dramatically change the ME physicochemical properties. Moreover, AmB-ME can become a new antifungal product to treat not only systemic candidiasis, but also leishmaniasis infection.

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Table 1: Experimental levels of independent variables of the DOE for droplet size evaluation of ME after freeze-drying process.

Independent variable	Level		
	-1	Replicated (0)	+1
Freezing temperature (°C)	- 20	- 80	-196
CP concentration (% _{w/w})	5	12.5	20
Freeze- drying time (h)	24	36	48

Table 2: DOE for the freeze-drying process of MEs for each CP type.

Samples	Freeze-drying temperature	CP concentration	Freeze – drying time
1	- 1	- 1	- 1
2	+ 1	- 1	- 1
3	- 1	+ 1	- 1
4	+ 1	+ 1	- 1
5	- 1	- 1	+ 1
6	+ 1	- 1	+ 1
7	- 1	+ 1	+ 1
8	+ 1	+ 1	+ 1
9	0	0	0
10	0	0	0
11	0	0	0

Table 3: Statistical Analysis (ANOVA) of results of DOE for ME-MT and ME-LT samples.

Source	ME – MT				ME –LT			
	Mean square	Calculate d F	Tabulate d F	R^2	Mean square	Calculated F	Tabulated F	R^2
Regression	81.518	9.9861	5.117	0.9236	25.,3153	12.57359	5.05	0.9120
Residue	8.163				19.9080			
Lack of fit	9.755	3.7666	19.35		31.2845	11.00275	19.16	
Pure Error	2.590				2.8433			
Total	15.498				135.1116			

Table 4: Physicochemical characterization of the ME-MT before and after the freeze-drying process (FDP).

Samples	Appearance	pH	Electrical conductivity ($\mu\text{S cm}^{-1}$)	Droplet size (nm)
ME + MT at 5% before FDP	Homogeneous and clear	7.23 ± 0.04	749.1 ± 25.65	48.13 ± 1.57
	yellowish			
ME + MT at 5% after FDP	Homogeneous and clear	$6.86 \pm 0.01^*$	$876.5 \pm 14.80^*$	$20.13 \pm 3.30^*$
	yellowish			

Average values \pm SD

* $p < 0.05$ compared with the same sample before freeze-drying.

Table 5: Physicochemical characterization of ME with AmB containing 5% of MT before and after freeze-drying process.

Samples	Appearance	pH	Electrical conductivity ($\mu\text{S cm}^{-1}$)	Droplet size (nm)	AmB concentration (M)
Before	Homogeneous				
freeze-drying	and clear yellowish	7.59 \pm 0.10	1583 \pm 39.55	54.07 \pm 2.35	1.97 x 10 ⁻³ \pm 0.03
After	Homogeneous				
freeze-drying	and clear yellowish	7.4 \pm 0.11*	1639 \pm 53.75*	32.30 \pm 4.20*	1.94 x 10 ⁻³ \pm 0.02**

Average values \pm SD reported

*p < 0.05 compared with the same sample before freeze-drying process.

**p > 0.05 compared with the same sample before freeze-drying process.

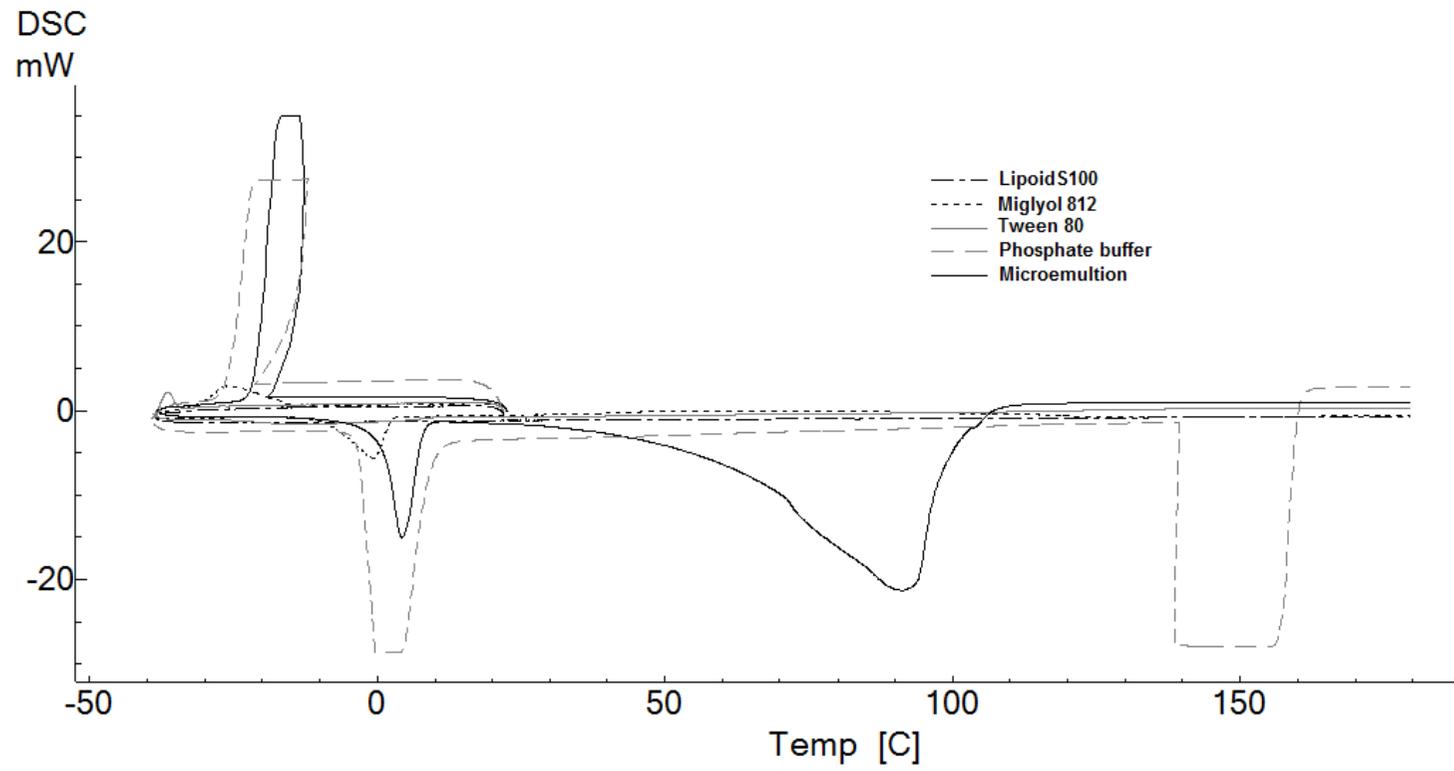


Figure 1

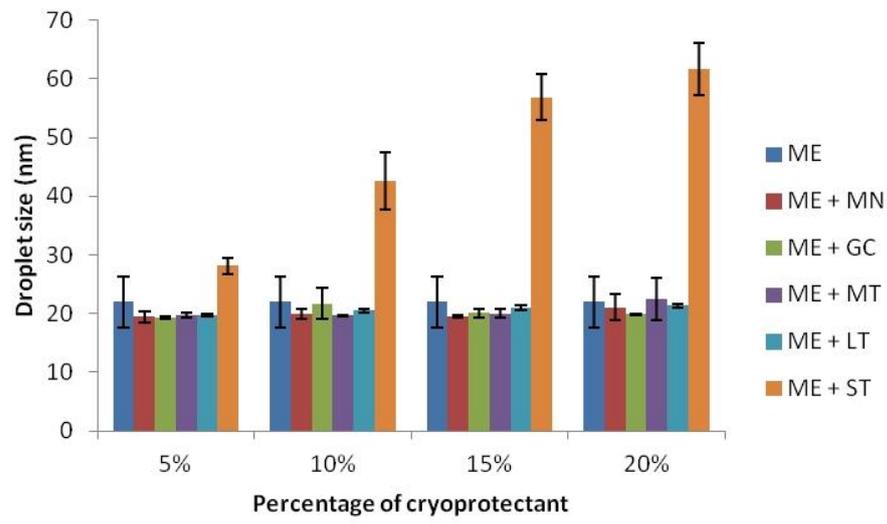


Figure 2

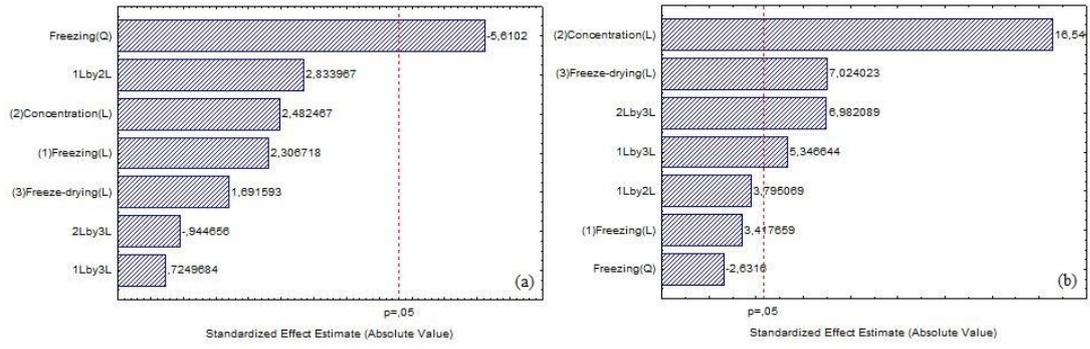


Figure 3

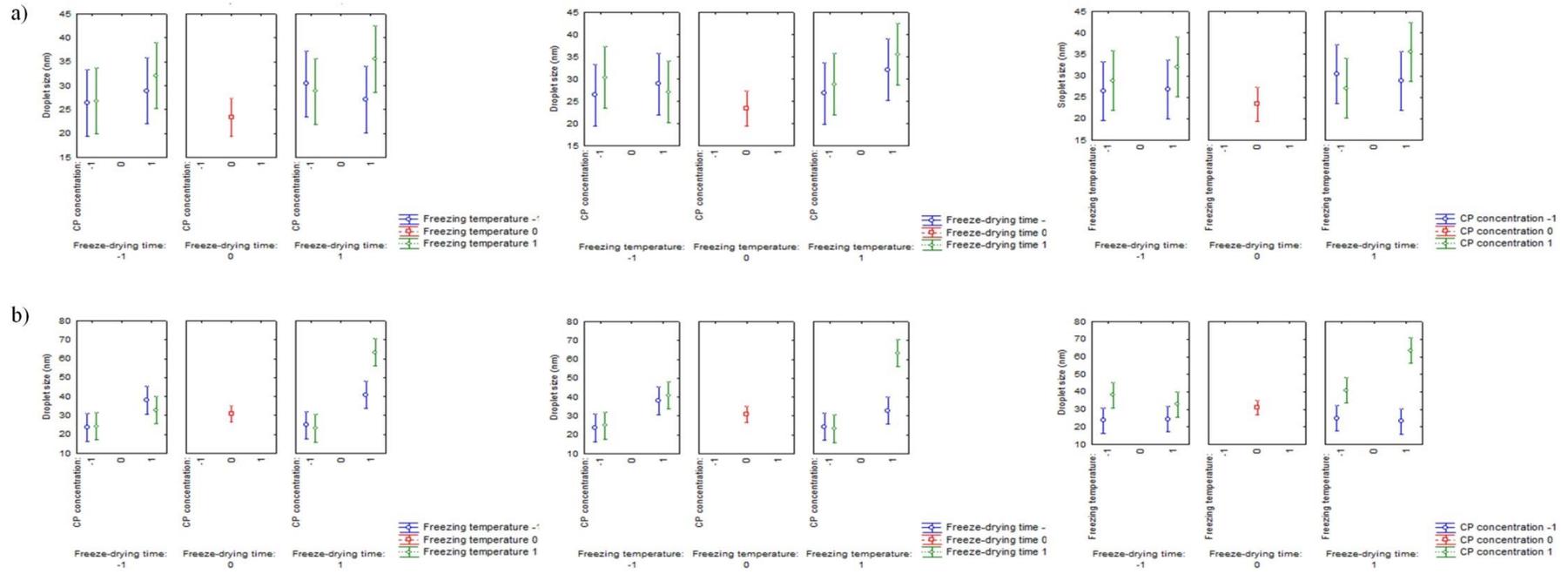


Figure 4

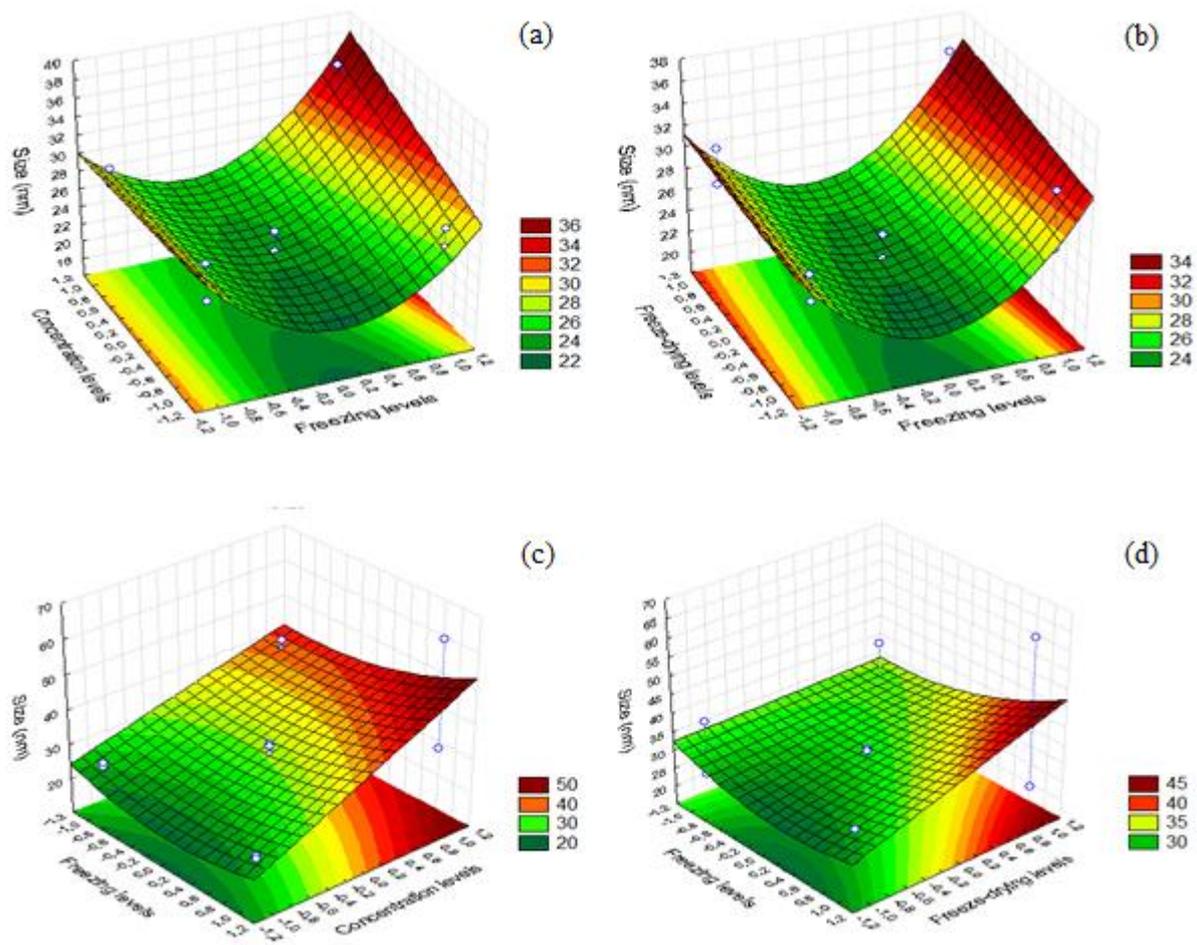


Figure 5

Figure captions

Figure 1: DSC thermograms of ME and its components.

Figure 2: Influence of the type and concentration of CP on the droplet size of the ME formulations before the freeze-drying process.

Figure 3: Pareto chart of the effects of independent variables and their interaction on the (A) ME containing MT droplet size (B) ME containing LT droplet size. (L) is the linear and (Q) is the quadratic interaction of variables

Figure 4: Plots of marginal means for CP concentration, freezing temperature and freeze-drying time to verify the effects of each variable on the droplet size of ME containing MT (a) and ME containing LT (b).

Figure 5: Response surface plot showing the effects of the mutual interactions between two independent variables on the droplet size. The effect of the freezing temperature and CP concentration on the MEs containing MT (a) and MEs containing LT (c). The effect of the freezing temperature and freeze-drying time on the MEs containing MT (b) and MEs containing LT (d).

General discussion

The aim of this thesis was to evaluate the activity and toxicity of two formulations: Amphotericin B-loaded microemulsion (ME-AmB) and Heated Micellar Amphotericin B (H-AmB), developed in order to improve antileishmanial therapy while reducing costs and toxicity. A further objective was to improve the stability of ME-AmB by reducing the amount of free water, and thereby the possibility of microbial contamination and hydrolysis reactions. Hence, the manuscript is divided in two sections.

The first section concerns the *in-vitro* and the *in-vivo* evaluation of the lipid systems: microemulsion and micelles containing AmB, against *Leishmania*.

Amphotericin B (AmB) is a potent drug for antifungal and antileishmanial therapy which was discovered more than 60 years ago. Its efficacy has not changed very much over the years and not many cases of resistant strains have been reported. However, its toxicity makes it difficult to use and it is necessary to develop strategies to solve this problem.

Some research teams, as well as our group, have also been investigating AmB incorporation into microemulsions. The ME-AmB developed by Darole et. al (2007) (1) showed the same efficacy as Fungizone[®] formulation; however with lesser hemolytic potential and a higher LD₅₀ value. Butani et al (2014) (2) designed a ME-AmB for the treatment of invasive fungal infections based on pseudo-ternary phase diagrams. Among all this work we would like to highlight the studies on permeation of AmB as ME through excised rat skin which exhibited 2-fold higher drug permeation than a plain drug solution. Pestana et al (2008) (3) and Franzini et. al. (2012) (4) analyzed the diameter distribution of the oil phase in as a function of the surfactant used and the oil phase content in the presence and absence of AmB incorporated into the ME system. The results showed that AmB incorporation into the ME was dependent on both the oil phase and the surfactant proportions, reaching a plateau at high contents, and that it influences the droplet size of the microemulsion. Furthermore, it was observed that increasing the surfactant concentration decreased the droplet size, demonstrating the stabilizing effect of the surfactant, which is a normal observation for MEs.

The other alternative formulation proposed in this thesis, H-AmB, has been investigated by other

research teams, who have looked at features of M-AmB and H-AmB such as the AmB aggregation state, demonstrating that H-AmB contained superaggregated and monomeric forms (5). The morphology examined by cryo-transmission electron microscopy showed pleiomorphic cobweb structures and laser diffraction revealed a mean particle size of approximately 300 nm (6). Fluorescence studies showed similar stability to disaggregation by addition of the surfactant sodium dodecyl sulfate, providing the groundwork for future fluorescence characterization of the interactions of M-AmB and H-AmB with cell membranes (7). The interaction between AmB formulations and lipid monolayers has been studied, showing a large disruptive effect for both M-AmB and H-AmB (8). The effect of the aggregation state of AmB on the toxicity of the formulation was examined in detail, indicating that the smaller the aggregation size fraction the higher the toxicity determined by hemolysis (9). Finally, it was observed *in vitro* and *in vivo* that heat treatment preserved the activity of M-AmB against fungal cells (5, 10, 11) and parasites (12, 13), while reducing toxicity probably as result of its increased uptake by macrophages (14).

Furthermore, the serum pharmacokinetics, tissue distribution, and renal toxicity of AmB were determined after administration to rabbits. M-AmB caused serum creatinine concentrations to rise compared with baseline while H-AmB showed no difference. The AmB area under the concentration-time curve after H-AmB administration was significantly lower than that of M-AmB. On the other hand, total body clearance of AmB was significantly greater for H-AmB. Kidney, lung and spleen AmB concentrations were higher after H-AmB administration than M-AmB. However, liver AmB concentrations were significantly higher after H-AmB administration (15).

As mentioned above, one of the main aims of this work was to test the activity of these two systems, MEAmB and H-AmB. The experimental studies were carried out at the Institut Galien Paris Sud at the Université Paris Sud XI, in Châtenay Malabry, France, under the supervision of Dr. Gillian Barratt and Prof. Philippe Loiseau.

There is not much information in the literature about the use of these two systems for leishmaniasis treatment. In this current work, it was observed that both strategies were very successful *in vitro* and

in vivo for the treatment of this disease since both of them revealed an efficacy that was not significantly different from Fungizone[®], whereas they showed much lower toxicity. These results are in agreement with the literature mentioned previously. In addition, it should be emphasized once the materials used to prepare the formulations are inexpensive and, as far as H-AmB is concerned it is not necessary to buy ingredients to prepare the formulation since its preparation involves only the heating of a formulation that is already available in the market.

The second section of this thesis addresses the freeze-drying of emulsified systems. The experimental study was carried out partly in the LASID guided by Prof. Sócrates, and partly at the Laboratoire d'Automatique et de Génie des Procédés (LAGEP), Lyon, under the guidance of Prof. Hatem Fessi and Dr. Abdelhamid Elaissari.

Firstly, a comprehensive literature survey was made of the most interesting findings about freeze-drying of emulsified systems. This survey was focused on the databases compiled by ISI Web of Knowledge and PUBMED. Publications were selected for having one of these following keywords in the abstract: “emulsion”, “nanoemulsion”, “microemulsion”, “cryoprotectant”, “freeze-drying” and “lyophilization”. The aim of this survey was to summarize and provide an update of knowledge on this subject.

Emulsified systems are very important since they are able to transport active molecules, increase the drug solubility and stability as well as improve the pharmacokinetics and decrease the adverse effects of some drugs. These systems are comprised of oil, surfactants and water. The presence of this aqueous phase may lead to microbial contamination of the system, as well as some physical-chemical instability: degradation by hydrolysis and drug loss. Freeze-drying is a good removal water technique; however it should be thoroughly studied with respect to the many parameters that influence the quality of the dry powder and the reconstituted product. These parameters are related to the product, the container, the equipment and the process, including the time and temperature of each freeze-drying step (freezing, primary-drying and secondary drying), and type and concentration of cryoprotectant.

As a result of the literature survey, the experimental work plan was developed. The literature search revealed that for emulsified systems the main cryoprotectants used are sugars at concentrations from 5% to 20%, the freezing temperatures range from - 20 °C to - 196 °C (liquid nitrogen) and the freeze-drying time ranges from 24h to 72h. Faced with the problem of choosing suitable parameters on many variables, the second chapter of the second section is about the development of a freeze-dried microemulsion containing AmB based on optimization process by design of experiment. The results showed that the best conditions were 5 % of maltose as cryoprotectant, freezing at - 80 °C and freezing drying for 24 h. The amount of AmB did not change after freeze-drying and the other parameters such size, pH and conductivity remained acceptable. Therefore, the process did not influence ME properties greatly and would be a good strategy to increase the stability of such products.

Work described the freeze-drying of microemulsions is not frequent in the literature. Moreno et al (2001) (16) and Brime, et al (2002, 2003 and 2004) (17-19) have reported that the microemulsion conserved its original physical-chemical properties after the lyophilization process. The freeze-dried systems were found to be more stable than their nonlyophilized counterparts. In addition, the efficacy against *Candida* strains and pharmacokinetics were evaluated. The activity was maintained and the toxicity was reduced compared with traditional formulations on the market; moreover no histologically demonstrable renal lesions or changes in blood chemistry were observed.

Future perspectives

This work was designed to advance the development of new strategies to combat visceral leishmaniasis using an old drug, AmB, while overcoming some drawbacks such as adverse effect and cost. Two alternative systems have been studied and discussed: the heating of AmB deoxycholate and incorporation of AmB into a microemulsion . Furthermore, during this work we developed a method to dry the microemulsion without losing its properties, which represents a major challenge as far as dispersed systems are concerned. However, further studies are needed in

order to deepen our knowledge of these strategies and to answer some questions that have appeared during the development of this work.

It is necessary to understand some aspects better; for example how AmB is incorporated into the ME. What happens to the ME structure after AmB addition? Are new nanostructures formed? Where the drug is located? What is its aggregation state? Is the ME-AmB stable? How is the drug released?

Furthermore, it is important to know how these systems act inside the body; that is, their pharmacokinetics and pharmacodynamics. The good results in AmB-resistant strains emphasize the importance of studying these formulations in greater depth, to understand why AmB loaded in ME is able to destroy resistant strains.

Moreover, more experiments are required to understand some issues related to freeze-dried ME-AmB, such as its stability compared with non-freeze-dried systems, the drug release, its antileishmanial efficacy *in vitro* and *in vivo*, the AmB aggregation state and its relationship with biological activity, and the influence of temperature in reconstituting freeze-dried systems.

In the appendices of this manuscript it is possible to find articles describing the use of natural oils to treat fungal and cancer diseases. An alternative approach using natural oils could be interesting for leishmaniasis treatment. There are few studies using natural oils to treat this disease, especially Copaiba oil. Therefore, the development of dispersed systems containing Copaiba oil could be another possibility for AmB-resistant strains.

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Conclusions

The use of AmB for leishmaniasis treatment has been studied, showing Ambisome[®] and Fungizone[®] as the most frequently used products. However, both have drawbacks: high cost and toxicity respectively. This work presents two alternative products containing AmB that are effective but do not show a high toxicity and whose production cost are lower than those of Ambisome[®].

Fungizone[®] is cheap and has a good efficacy; however, it has high toxicity. The heating of this formulation is one alternative to reduce its toxicity. The results showed that the superaggregates present in heated Fungizone are probably the reason for the decrease in the *in-vitro* and *in-vivo* toxicity of the commercial product, but the heated product remained effective, as observed by the high selectivity index against *Leishmania donovani*.

Other promising results were obtained regarding the AmB-loaded microemulsion. Its small droplet size and observed Newtonian behaviour would sanction its use by injection. The AmB toxicity in ME also decreased compared with Fungizone[®], and the efficacy was not significantly different. Therefore, this system is another alternative to treat visceral leishmaniasis.

Furthermore, this work demonstrated the possibility of freeze-drying the microemulsion with or without AmB. Thereby, the storage stability of the system increased, avoiding drug loss due to reactions with water and decreasing the possibility of microbial contamination. The use of design of experiment as a tool was important to find the optimum conditions of cryoprotectant concentration, freezing temperature and freeze-drying time. Therefore, the freeze-dried ME-AmB could be another pharmaceutical dosage form to treat leishmaniasis. However, more studies are necessary in order to evaluate the efficacy of this system after lyophilization.

Appendix

SYSTEMS BASED ON NATURAL OILS

INTRODUCTION

Since the beginning of my undergraduate studies, I have been working on the topic of nanostructured systems and the use of natural products to produce them. During the PhD, I entered into collaboration with other researchers, working on Copaiba oil and Bullfrog oil.

The review article “Microemulsion systems containing bioactive natural oils: an overview on the state of the art” was published in collaboration and it concerns the same subject as this thesis, microemulsions. In addition to being produced with synthetic oils, these systems can be produced with natural and vegetable oils, in which the therapeutic activity is due to the presence of these natural products.

In addition to microemulsions, other emulsified systems, such as emulsions and nanoemulsions, can also be produced using natural oils, in order to treat a particular disease according to the therapeutic characteristic of the oil chosen to be part of the formulation. The article “Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions” emphasizes the use of the Copaiba oil as an antimicrobial agent in emulsion systems. On the other hand, the article “New Trends on Antineoplastic Therapy Research: Bullfrog (*Rana catesbeiana* Shaw) Oil Nanostructured Systems” discusses the development of a nanoemulsion containing bullfrog oil as anti-tumoral treatment.

The design of experiment used as a tool in this thesis to optimize the freeze-drying process can also be used to develop nanostructured systems containing natural oils. The article “Experimental design approach applied to the development of chitosan coated poly(isobutylcyanoacrylate) nanocapsules encapsulating copaiba oil” illustrates nanocapsule production from an experimental design evaluating the pH of the polymerization media, temperature of production and concentration of chitosan.

Document I

*Microemulsion systems containing bioactive natural oils: an overview on the state of
the art*

REVIEW ARTICLE

Microemulsion systems containing bioactive natural oils: an overview on the state of the art

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ABSTRACT

Natural oils are extremely complex mixtures containing compounds of different chemical nature. Some of them have physiological or therapeutic activities that may act either alone or in synergy. Therefore, they are used in the pharmaceutical, agronomic, food, sanitary and cosmetic industries. Today, the interest in bioactive natural oils is growing due to their immense potential to prevent and treat numerous human diseases. Formulation in microemulsions (MEs) containing natural oils appeared suitable to improve pharmaceutical and biopharmaceutical properties of bioactive compound derivatives from these oils. Microemulsion systems are thermodynamically stable, transparent, and are isotropic dispersions consisting of oil and water stabilized by an interfacial film of surfactants, typically in combination with a cosurfactant. They can protect labile compounds from premature degradation, control release, increase solubility and hence enhance the bioavailability of poorly bioavailable compounds. The aim of this work was to review the various advantages of bioactive compounds presented in natural oil loaded ME systems to be used as delivery systems. First, the state of the art of the parameters involved in the ME formation, including the basic concepts of the physicochemical formulation of the ME systems, and the main aspects of production and the energy responsible for their formation were reported. The second section describes the use of ME systems and reviews the recent applications of natural oil-loaded in the ME systems as the bioactive compound in the formulation.

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Introduction

Microemulsion (ME) has attracted much interest for several years in terms of delivery and target potential¹⁻³. Microemulsions are transparent, optically isotropic, and thermodynamically stable-phase transition systems, which possess low surface tension and small droplet size^{4,5}. These systems are formed by two immiscible liquids (water and oil) mixed to form a single phase stabilized by an interfacial film of alternating surfactant and cosurfactant molecules^{6,7}. MEs can appear in at least three major microstructures: swollen micellar (oil-in-water, O/W), reverse micelles (water-in-oil, W/O) and bicontinuous structures^{8,9}.

Microemulsions have many advantages as drug delivery systems, including improved appearance, high stability, easiness of preparation and small droplet size, resulting in a large surface area from which the active substances can partition and be absorbed or permeate through membranes^{1,6,8,10}. Also, such systems possess the ability to enhance the bioavailability of poorly soluble drugs by maintaining them in a molecular dispersion, consequently allowing for controlled or sustained release of their active agent^{1,11}. Microemulsions form spontaneously (zero energy input). Therefore, they are easy to manufacture (not process dependent) and scale-up^{9,12,13}. These special properties of the ME offer a high potential for numerous practical applications, including enhanced oil recovery, pharmaceutical and cosmetic formulations, edible coatings for food, and other industrial applications^{7,14}.

Recently, MEs containing natural oils have been of increasing interest to researchers and have shown great potential in

industrial applications. MEs' utility lies in their ability to incorporate a large amount of active natural oil products in the continuous or dispersed phase, which are otherwise difficult to formulate^{6,15-17}. Natural oil products are extremely complex mixtures containing compounds of various chemical natures, which act either alone or in synergy with other compounds, giving a global therapeutic activity when incorporated in formulations¹⁸⁻²². These oils are used widely to the prevention and treatment of very severe diseases including cancer, Alzheimer's, and cardiovascular diseases, as well as their bioactivity as spasmolytic, revulsive, anti-inflammatory, analgesic and acaricide, antibacterial, antiviral, antipsoriatic, antioxidant, and antidiabetic agents^{17,23-28}.

Due to the therapeutic advantages and the complex composition of the natural oil, various formulation approaches including carrier technology such as MEs offer an intelligent approach for the delivery of bioactive compounds from the natural oil products. Therefore, the aim of this work was to review the various advantages of natural oils loaded ME systems to be used as delivery systems for these bioactive compounds. The present review is divided into two sections. First, the state of the art of parameters involved in the ME formation, including the basic concepts of the physicochemical formulation of the ME systems, and the main aspects of production and the energy responsible for their formation were reported. The second section describes the use of ME systems and reviews the recent applications of natural oil-loaded in the ME systems as the bioactive compound in the formulation.

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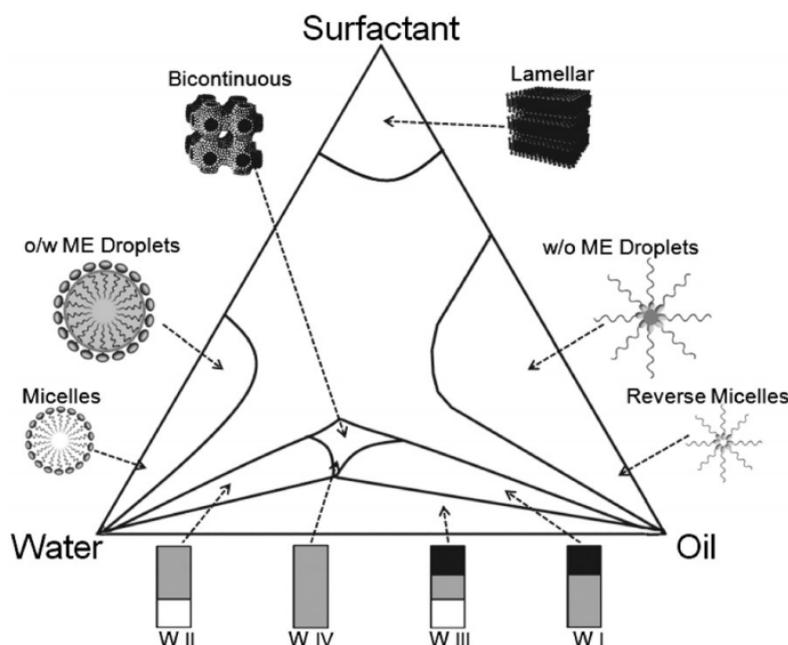


Figure 1. A predictive pseudo-ternary phase diagram with the existing regions of different systems: conventional micelles, reverse micelles, W/O ME, O/W ME and bicontinuous ME, and also the Winsor systems (white is the water-excess region, black is the oil-excess region, and gray is the ME) (adapted from Lawrence and Rees 2012⁶).

Microemulsions: background

The term “microemulsion” was first introduced in 1943 by Hoar and Schulman²⁹. It is characterized as a self-assembled homogeneous isotropic system, with thermodynamic stability that contains extremely high oil/water interfacial areas, offering ultra-low interfacial tension (less than 0.1 mN/m) and viscosity. ME systems present small droplets (10–100 nm) that hardly scatter light in the visible wavelength domain, explaining their tendency to appear as transparent.

Microemulsion comprised of two immiscible liquids, such as oil and water, which are stabilized by surfactants and co-surfactants²⁹. Surfactants are amphiphilic molecules that possess within their structure a part that has an affinity for oil and a part that has affinity for water and during the mixing, they migrate to the oil–water interface to form a film. The surfactants decrease the surface tension of the oil–water interface and change the entropy of the system. However, in ME systems a relatively large amount of surfactant is required in order to stabilize their large interfacial area³⁰. The low interfacial tension compensates the dispersion entropy; hence, the system is thermodynamically stable¹ and forms spontaneously under a specific set of composition and environmental conditions³¹.

When dispersed in water or in non-aqueous solvents, the surfactants self-associate into a variety of equilibrium phases. The monolayer of surfactant in the interface can exert a two-dimensional surface pressure due to the expansion of the film until the pressure at both sides of the interface becomes constant. After the surfactants occupy the entire interface, the addition of more surfactant will result in micelle formation^{1,32}. Depending on the nature of the surfactants presented (greater affinity for water or for oil), it will be determined which type of system will be formed. A surfactant more soluble in water than in oil will influence the direction of the ME to the oil in water type (O/W ME). Similarly, a

more oil soluble surfactant favors W/O ME formation, while bicontinuous type formation occurs when similar contents of water and oil are used. Various structures may be formed when the surfactants are combined with water, oil or both, such as spherical micelle, reverse micelle, rod-shaped micelle, hexagonal phase, lamellar phase and reverse hexagonal phase (Figure 1)⁶. In addition, the required concentration of surfactant in the systems will depend on its structure, since a lower concentration of surfactant is required for the one that strongly favors orientation to the oil/water interface compared to a surfactant that partitions strongly into either the oil or the water phase³³. Therefore, concerning the concentration and the geometry of the surfactant structures, it is understandable that the surfactant films in MEs may have different shapes³⁴. Additionally, the concentration of water also plays an important role in the ME structure. Indeed, it is possible to verify the formation of elongated, rod-like micelles and W/O spherical droplets at low water content, whereas at high water concentration the most frequent form observed is O/W droplets. Additionally, as previously mentioned, bicontinuous structures may be formed in MEs with similar contents of water and oil³⁵.

At low surfactant concentration, four types of ME phases, commonly referred to as Winsor phases (Figure 1)³⁶, exist in equilibrium: Winsor I (with two phases, a lower O/W ME phase in equilibrium with the upper excess oil); Winsor II (with two phases, an upper (W/O) ME phase in equilibrium with the water excess); Winsor III (with three phases, a middle ME phase O/W plus W/O, called bi-continuous) in equilibrium with the upper oil excess and lower excess water; and Winsor IV (in single phase, in which both oil and water are completely dispersed in the surfactant ME phase). Inter-conversion among the above-mentioned phases can be achieved by adjusting proportions of the constituents.

ME droplets have a larger effective interaction volume for the O/W type than the W/O type, which is due to a strong repulsive

term introduced by the presence of an electrical double layer on the surface of the O/W droplet when the ionic surfactants are used. However, when a nonionic surfactant is used to stabilize the O/W ME, the predominant repulsive factor might be attributed to steric interactions, although the polar head groups produce a hydration shell. Additionally, the preparation process of the W/O ME is generally easier than that of the O/W ME, since its interfacial tension tends to be lower due to the easier surfactant arrangement at an interface with high curvature, given that the surfactant tails extend outwards into a continuous oil phase, which is entropically more favorable as the hydrocarbon tails have more directional freedom⁶.

Attempts have been made to rationalize surfactant behavior in ME formation. These approaches are fairly empirical, but can be a useful guide to surfactant selection. In this context, the hydrophilic-lipophilic balance (HLB), the critical packing parameter, and the solubility parameter approach are proposed in order to support surfactant selection for ME application. The HLB takes into account the relative contribution of the hydrophilic and the hydrophobic fragments of the surfactant molecule. The W/O ME is formed through the surfactants' high dispersion rates in oil, beyond the limit to form reverse micelles^{33,37}. Surfactants used to produce this type of ME have an HLB ranging from 3 to 8. On the other hand, to produce W/O ME, the oil is dispersed using surfactants with an HLB ranging from 8 to 18. The formation of bicontinuous ME (HLB \approx 10) is explained by various models, such as the Scriven model, the Random-lattice model, the Cubic random-cell model and the disordered open-connected model (subject outside the scope of this review)¹.

In contrast, the critical packing parameter relates the ability of surfactants to form particular aggregates to the geometry of the molecule itself. This parameter measures the preferred geometry adopted by the surfactant and, consequently, is predictive of the type of aggregate that is likely to form⁶. The geometric position of the surfactant at the interface can be another factor influencing the ME structure³⁸. The size and shape of the ME are mainly governed by the curvature free energy of the interface between water and oil and are determined by the bending elastic constant and curvature of the surfactant film. The elasticity of the film depends not only on the type of surfactant and on the thermodynamic conditions, but also on the presence of additives such as alcohols, electrolytes, block copolymers and polyelectrolytes. Co-surfactants such as short chain alcohols can improve the film's flexibility³⁹.

The solubility parameter theory is based on the premise that when the solubility parameters of two chemical compounds are equal, the compounds are infinitely soluble⁴⁰⁻⁴². The intermolecular forces that cause chemical species to dissolve are the same forces that prevent those materials from boiling away until a specific temperature is reached⁴³. Hansen et al. included molecules interacting by dipolar and hydrogen bonding forces (as well as dispersion forces) of this theory, by making the assumption that the solubility parameter could be represented by an additive function of three components⁴⁴. In this theory, for complete miscibility, two liquids need each of these parameters to be similar. The solubility parameter offers a far more comprehensive system than the HLB system concept. The HLB does not take into account the chemical match between the surfactant and the phase components of the ME. In other words, it does not take into account the miscibility properties of the surfactants with solvents composing each phase of the ME. However, the solubility parameter has the disadvantage of being very complex with several alternative available expressions⁴⁵.

Theory of ME formation

The formation and stability of MEs can be affected by various factors such as the nature and molecular weight of surfactant, alcohol chain length of the co-surfactant, temperature of the formulation, etc. The reduction of the interfacial free energy to a very low value is of prime importance in the ME formation. Accordingly, the ME formation has been explained by the following three approaches: interfacial or mixed film theory, solubilization theory and thermodynamic theory^{6,46,47}.

Interfacial or mixed film theory

Postulated by Bowcott and Schulman in 1955, this theory describes that the interfacial film is considered to be duplex in nature (region bounded by water on one side and oil on the other), with an inner and an outer interfacial tension acting independently⁴⁸. Such specialized liquid has been based on the assumption that interactions in the interface and reducing the original O/W interfacial tension to zero are capable of, spontaneously, forming an ME. Nevertheless, the ME formation is not ensured by zero interfacial tension, although the interfacial tension is generally extremely low, but it depends on the kind of molecular interactions in the liquid interface.

Based on this, Robbins et al. developed the theory of ME phase behavior, which discusses that the changes in the direction and extent of curvature are due to the interactions in a mixed film, which, on the other hand, can estimate the type and size of the ME droplets⁴⁹. Furthermore, the differential tendency of water to swell the heads and oil to swell the tails of the surfactants imposes the ideal kind and degree of curvature of the surfactant film molecules included in the interface to ME formation.

Solubilization theory

Since the 70s it has been possible to explain that MEs are swollen micelles in which either the water is solubilized in reverse micelles, or the oil is solubilized in normal micelles⁵⁰. A model of this theory was presented by Adamson et al. reporting that the W/O ME is formed because of the balance achieved in the Laplace and osmotic pressure and that the electrical double layer system with internal aqueous phase is partially responsible for the interfacial energy, which presents positive free energy, contradicting the concept of negative interfacial tension⁵¹.

Thermodynamic theory

Concerning the thermodynamic theory, it is important to consider that the free energy must be negative to form thermodynamically stable MEs. The ME formation depends on the reduction of the surface tension of the oil-water interface by the surfactants and the change in entropy of the system⁵². Schulman explained that the formation of a thermodynamically stable ME occurs with a very low interfacial tension on the order of 10^{-7} to 10^{-5} dynes/cm. Moreover, the interfacial charge is responsible for controlling the phase continuity, as the thermodynamic approach accounts for the free energy of the electric double layer along with the van der Waals and the electrical double layer interaction potentials among the droplets^{29,47}. However, a significant favorable entropic change should be accompanied by large reductions in the surface tension in order to achieve a negative free energy of formation, resulting in spontaneous microemulsification and a thermodynamically stable system. This entropic change arises from the monomer-micelle surfactant exchange, the surfactant diffusion in the interfacial layer

and the mixing of one phase in the other in the form of large numbers of small droplets⁶.

Methods of preparation of MEs

Although MEs may form spontaneously, external factors can be used to overcome kinetic barriers, thus reducing the time to obtain the formation of these systems. Some factors that can accelerate and facilitate the formation of the ME system can be the order of component addition, the application of mechanical agitation, and the use of ultrasound or heat, for instance. Therefore, in order to accelerate the ME formation from a kinetic standpoint, two different methods have been proposed: the phase inversion and the phase titration methods. The change in the spontaneous curvature of the surfactant is used by the phase inversion method. The phase inversion may occur in response to the temperature or upon dilution of the excess of the dispersed system inducing drastic physical changes, such as changes in the particle size that can affect drug release both *in vivo* and *in vitro*³⁰. The concept of phase inversion temperature (PIT), introduced by Shinoda and Arai, shows the importance of the temperature on the surfactant properties (particularly nonionic ones)^{53,54}. In the PIT method, the interfacial properties of the system are balanced and very small droplet sizes are produced. The nature of the emulsified oils as well as the HLB and concentration of surfactants are important parameters for the PIT⁵⁵. Additionally, changing the water volume fraction can induce a transition in the spontaneous radius of curvature³⁰.

The phase titration method uses the spontaneous diffusion of surfactant or solvent molecules into the continuous phase due to ultra-low interfacial tension. Diagrams are a useful tool to understand the complex series of interactions that can occur when different components are mixed together. A pseudoternary phase diagram is often constructed when there are four components in the formulation, wherein one corner is the mix of surfactants and the others are the oil and the water. To construct the phase diagram, all the components of the formulation are mixed in proportions varying from 0 to 100%. Subsequently, each system is characterized and the phase boundaries formed are demarcated^{2,4,55,56}.

Uses and applications of ME systems

Microemulsions have been used in a variety of chemical and industrial processes, such as to enhance oil recovery; as fuels, lubricants, detergents, cutting oils, and corrosion inhibitors; in coatings and textile finishing; and in cosmetics, agrochemicals, food, among others. Additionally, MEs have been used in biotechnology, such as in environmental remediation and detoxification, in analytical applications, in microporous media synthesis, in pharmaceuticals, and as liquid membranes as well^{57,58}.

Pharmaceutical preparations such as liquid crystals, micelles and emulsion forming systems have been studied by several authors as a method to solubilize drugs, since solubilization using co-solvents was the conventional approach. However, the use of co-solvents cannot be employed for parenteral administration for several drugs. Furthermore, other disadvantages such as precipitation of the drug on dilution, severe pain at the injection site, and hemolysis are related to the use of co-solvents⁵⁹. Nevertheless, the instability of emulsions and low solubilization capacity of micelles are disadvantageous. MEs are a better proposition over other compartmentalized systems due to their thermodynamic stability, minimum energy necessary for formation, easiness of preparation,

long-term shelf life, low viscosity, surfactant-provoked permeability and reduction of various diffusion barriers by acting as penetration enhancer, protecting against enzymatic degradation, improving drug stability and solubilization capacity, allowing a large amount of drug to be incorporated.

These structures have been investigated as drug delivery systems for the purpose of bioactive targeting and controlled release. They improve the bioavailability of poorly soluble drugs due to the capacity of solubilizing both lipophilic or hydrophilic drugs, and partitioning them between the dispersed and the continuous phases, or even administering them together in the same preparation⁶⁰. Another important factor is their small droplet size, which results in a large surface area from which the drug can partition. Hence, they solve problems regarding the dissolution of drug that can be better absorbed or permeated through biological membranes³. Accordingly, the enhancement of the bioavailability of the drug can reduce the dose required to provide the same pharmacological action and, hence, reduce associated side-effects⁵⁷. In addition to these advantages, concerning the parenteral delivery systems, MEs improve the drug residence in the blood circulation and reduce the irritation⁶¹. Furthermore, MEs cause minimum immune reactions or fat embolism in contrast to emulsions. However, MEs show the disadvantage of having a high concentration of surfactants, which can be toxic to cells depending on their nature^{62,63}.

Natural products

Mother Nature has been a source of medicinal agents for thousands of years. Humans started to use plants as medicine approximately 60,000 years ago, and today 65% of the world's population relies on plants for their primary health care²⁰. Various medicinal plants have been used for years in daily life to treat disease all over the world^{64,65}. The oldest forms of health care include the use of leaves, flowers, stems, berries and roots of herbs because of their therapeutic or medicinal value⁶⁶. Today, it is estimated that 250,000 to 500,000 plant species have been identified so far, and about 35,000 are used worldwide for medicinal purposes^{67,68}.

Natural medicine is based on the premise that plants contain bioactive substances that can promote health and alleviate illness, usually with minimal toxic side effects^{19,69-71}. The focus on plant research, especially medicinal plants used in traditional systems, has increased all over the world^{72,73}. Plant extracts represent excellent renewable resources for human applications. Ethnobotanical information has contributed to health care worldwide through the isolation of bioactive compounds for direct use in medicines⁷⁴⁻⁷⁶.

The plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives⁷⁷. The most important of these biologically active constituents are alkaloids, flavonoids, tannins and phenolic compounds⁷⁸. These secondary metabolites in the plants have a prominent function of protection such as antibacterial, antiviral, anti-fungal, insecticides and also against herbivores by reducing their appetite for such plants²⁰. It is believed that most of the 100,000 known secondary metabolites are involved in plant chemical defense systems. However, only 12,000 have been isolated, a number estimated to be less than 10% of the total^{79,80}.

Some metabolites are involved in defense mechanisms against abiotic stress (e.g. UV-B exposure) and are important in the interaction of plants with other organisms (e.g. attraction of pollinators)^{26,81}. Some, such as terpenoids, give plants their odors and flavor (e.g. the capsaicin from chili peppers); others (quinones and tannins) are responsible for plant pigment, and several herbs

and spices have been used by humans to season food²⁰. In humans, these natural compounds are predominantly used for their antioxidant, antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic effects. They even have vasodilatory and neuroprotective properties^{82–84}.

Bioactive natural oils

Phytochemical composition of natural oils

Regarding natural oil studies, the composition of most seed oils are made up of a wide range of fatty acids with six dominating fatty acids: palmitic, stearic, oleic, linoleic, linolenic and lauric acids. Such fatty acids include those with chain lengths between 8 and 24 carbon atoms, containing a different number of double bonds, conjugated systems, or functional groups such as acetylenic bond, epoxy group and hydroxyl group⁸⁵. The advantage of using plant oil is that it is an excellent renewable source for industrial usage and, further, they are structurally similar to the long-chained hydrocarbons derived from petroleum⁸⁶.

Essential oil is one of the main classes of secondary metabolites. Among the secondary metabolites, it is estimated that over 3000 essential oils are known, of which about 300 are commercially important, especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries^{18,87}. These oils are volatile, natural, highly enriched in isoprene structure, and characterized by a strong odor^{18,20}. Essential oils are liquid at room temperature, though a few of them are solid or resinous, limpid, rarely colored and hydrophobic. Generally, they have a lower density than that of water and are soluble in lipids and in organic solvents^{18,88}. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes. Essential oils are extracted from various aromatic plants generally localized in temperate to warm countries such as Mediterranean and tropical countries, where they represent an important part of the traditional Pharmacopeia¹⁸. It is known that the percentage of the components of essential oils varies among species, plant parts, age and stage in the vegetative cycle, as well as according to environmental factors such as light, temperature, soil composition, season, geographical origin of the plants and period of harvest^{89,90}.

Complex mixtures of volatile compounds come from two groups of distinct biosynthetic pathways. The main group is composed of terpenes and terpenoids and the other one of aromatic and aliphatic constituents, all characterized by low molecular weight^{91–94}. Terpenes are made from combinations of several isoprene precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The mevalonate route operates in the cytoplasm and mitochondria, and the deoxyxylulose pathway in the plastids⁹⁵ (Figure 2). After isoprene precursor's formation, the terpenes biosynthesis consists of the repetitive addition of IPP and DMAPP molecules and modification by terpene specific synthetases to form the terpene skeleton (geranyl pyrophosphate). Finally, secondary enzymatic modification of the skeleton occurs to attribute functional properties to the different terpenes^{18,94}. A monoterpene has a general chemical structure of C₁₀H₁₆ and it occurs as diterpenes, triterpenes and tetraterpenes (C 20, C 30, and C 40), as well as hemiterpenes (C 5) and sesquiterpenes (C 15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids^{20,96}. These compounds are largely synthesized from acetate units, and despite sharing their origins with fatty acids, they differ from these in that they contain

extensive branching and are cyclized. Aromatic compounds, which occur less frequently than terpenes, are generated from phenylpropane (C₆–C₃) derivatives^{20,22}.

Extraction of bioactive natural oil

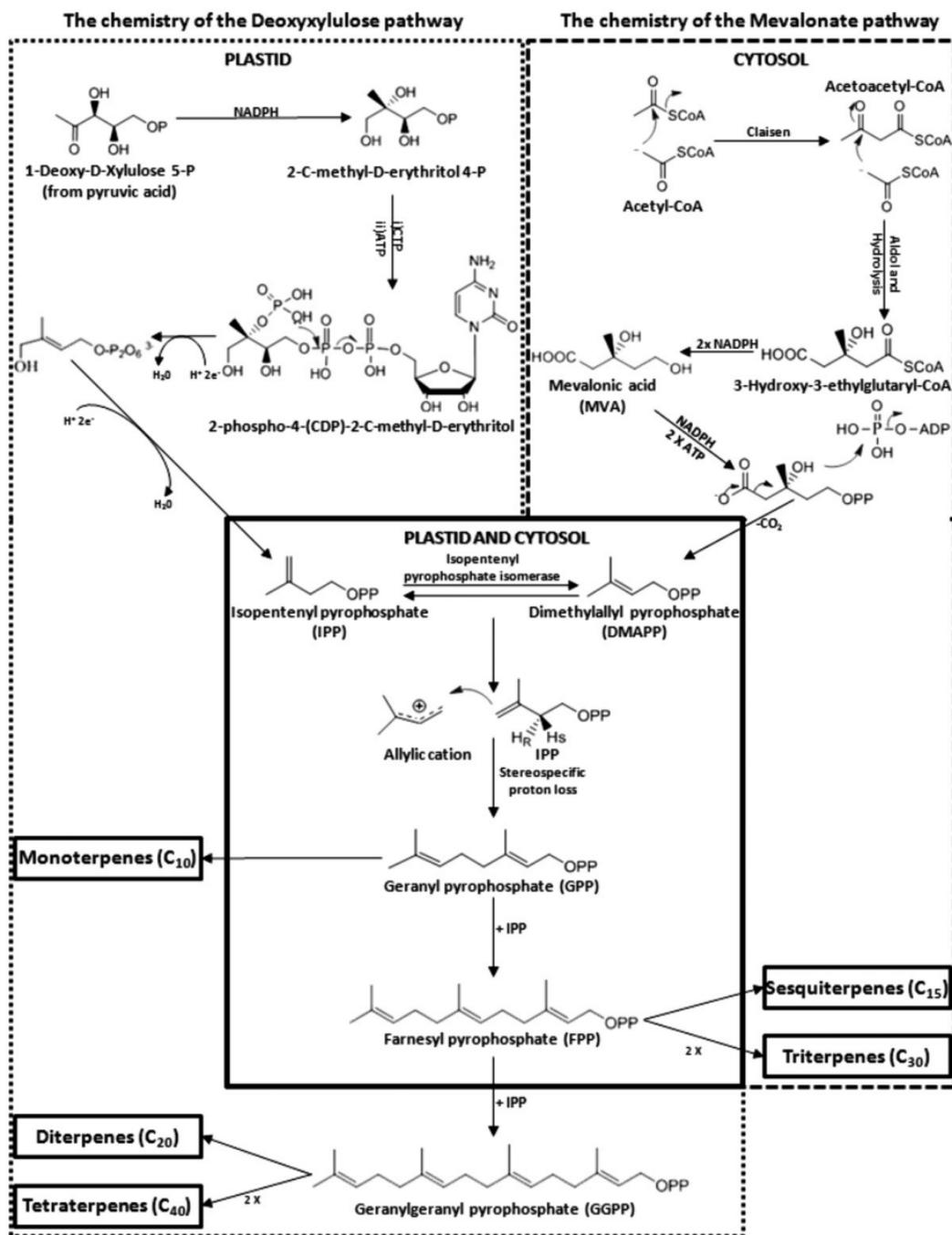
Several techniques can be used to extract essential oils from different parts of the aromatic plant, including solvent extraction, expression under pressure, effluage and distillation extractions. However, hydro, or steam, distillation is the most commonly used method^{26,97,98} (Figure 3). The steam distillation is a separation process based on the difference in composition between a liquid mixture and the vapor formed from it. The mechanical process is used exclusively for citrus fruit because their essential oils are contained in microvesicles located in the peel and may be extracted by pressure or friction. Dry distillation, without addition of water vapor, is used for wood, bark and roots²². For perfume uses, extractions with lipophilic solvents and sometimes with supercritical carbon dioxide are desired.

The chemical profile of the essential oil products differs not only in the number of molecules, but also in the stereochemical types of extracted molecules, according to the type of extraction. Therefore, the type of extraction is chosen according to the purpose of the use¹⁸. The essential oil composition can also be changed after extraction. Depending on the storage conditions, they can quickly become oxidized, and this oxidation is responsible in some cases for variation on the pharmacological activities⁹⁹. To monitor these phenomena, most of the commercialized plant extracts are chemotyped by gas chromatography and mass spectrometry analysis⁹⁷.

Pharmacological aspects of bioactive natural oils

Some essential oils appear to exhibit particular medicinal properties that have been claimed to cure certain organ dysfunctions or systemic disorders^{100,101}. The new attraction for natural products as essential oils in ME delivery systems is due not only to their effectiveness, but also to their use as an alternative to synthetic compounds from the chemical industry, which may show same secondary effects and do not belong from renewable resources, which may affect the ecological equilibrium^{18,102}. Regarding their biological properties, it has to be kept in mind that essential oils are complex mixtures of numerous molecules that have different mechanisms of action^{21,103}. It is likely that several components of the essential oils play a role in cell penetration and distribution due to the lipophilic or hydrophilic attraction and fixation on the cell walls and membranes^{18,104}. In general, the terpene compounds including limonene^{105,106}, menthol, terpineol, menthone, pulegone, carvone¹⁰⁷, thymol, carvacrol, trans-anethole, linalool¹⁰⁸, 1,8-cineole¹⁰⁹ and geraniol^{110,111} show a low systemic toxicity and skin irritancy in addition to having good penetration-enhancing abilities¹¹². This feature is very important because the distribution of the oil in the cell determines the different types of produced radical reactions, depending on their compartmentalization in the cell¹¹³.

Studies also showed that antioxidant activity may be attributed primarily to the high content of phenolic components of the essential oils^{114,115}. In general, these compounds can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential, affecting ionic Ca²⁺ cycling and other ionic channels, reducing the pH gradient, collapsing the proton pump, and depleting the ATP pool^{116–120}. They may change the fluidity of membranes, which might become abnormally permeable,



Q10 Figure 2. The terpene biosynthesis by deoxyxylulose and mevalonate pathways. Modified from Dubey et al.⁹⁵.

resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetics failure, which may explain their pharmacological and possible toxic effects¹²¹⁻¹²³. Other studies indicated that the anti-mutagenic properties of plant extracts may be due to the inhibition of penetration of the mutagens into the cells, inactivation of the mutagens by direct scavenging, inhibition of metabolic conversion by P450 of promutagens into mutagens, or activation of enzymatic detoxification¹⁸. The major related anti-mutagenic compounds are

tannic acid, apigenine¹²⁴, α -bisabolol¹²⁵, thuyone, 1,8-cineole, camphor, limonene¹²⁶, (-)-menthol, (-)- α -pinene, (+)- α -pinene, β -ionone, α -terpinene, α -terpineol, citronellal and others^{127,128}. Essential oils possess antibacterial, anti-fungal^{17,26} and antiviral²⁴ properties. They may be used for prevention and treatment of cancer^{129,130}, and cardiovascular diseases including atherosclerosis and thrombosis^{131,132}. They might also be used for their analgesic¹³³, sedative²⁵, anti-inflammatory^{100,101}, spasmolytic¹³⁴, local anesthetic, and antipyretic activities¹³⁵ and as a

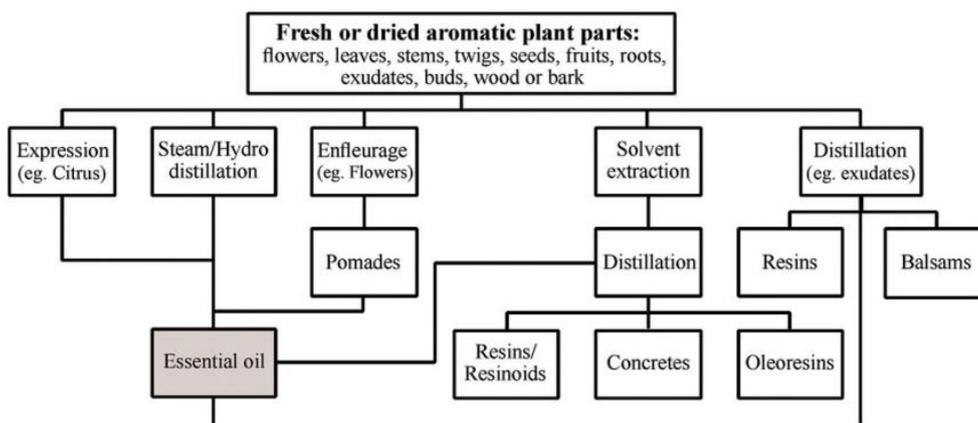


Figure 3. Main extraction process for essential oils from fresh or dried aromatic plant parts (adapted from Douglas et al.⁹⁸).

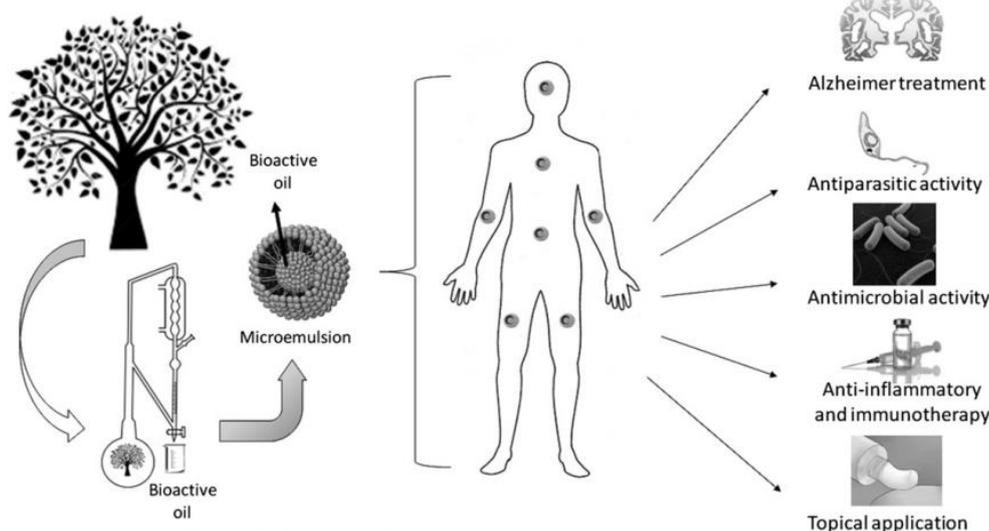


Figure 4. Development of microemulsion containing natural-oils as bioactive compounds to human applications.

food preservative¹³⁶. Moreover, their fragrance can be used in cosmetic applications^{23,25}.

In some cases, the bioactivities of essential oils are closely related to the activity of the main components of the oils¹³⁷. However, some studies have demonstrated that whole essential oils usually have higher activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity. Additionally, antagonistic and additive effects have also been observed^{114,138–141}. In that sense, for ME application, it is more relevant to study the whole oil rather than some of its components because the concept of synergism appears to be more meaningful, and the addition effect of the disperse system and the bioactive compounds' derivatives from the natural oil product can be expected.

Microemulsion based on bioactive natural oils

Development of MEs using bioactive natural oil products has been of increasing interest to researchers and has shown great potential

in industrial applications^{18–22}. The association of the intrinsic advantages of the ME may be able to act in synergy with the natural compounds^{6,15,16}. The flowchart of the development of a ME containing natural oils as bioactive compounds to human application is presented in Figure 4. For the purposes of this review, the main focus will be an evaluation of the recent developments of MEs with natural oil products as bioactive compound. Therefore, the isolated compounds derived from plants or loaded-ME systems containing synthetic oils will not be emphasized on this review. This section will be focused on ME systems containing natural oils and their respective therapeutic application (Table 1). Moreover, the main studies using bioactive natural oil loaded MEs systems will be reported in the next section.

Alzheimer treatment

Bioactive natural oil loaded MEs systems have been studied to treat Alzheimer. The strategy has been developed using essential

Table 1. Microemulsion systems containing natural oils.

Oil	Natural source	Data about therapeutic applications	Reference
Babassu oil	<i>Orbignya phalerata</i>	Immunotherapy strategies	149
Babchi oil	<i>Psoralea corylifolia</i>	Psoriasis	27
Cassia oil	<i>Cinnamomum cassia</i>	Antifungal activity against <i>Geotrichum citri-aurantii</i>	144
Cinnamon bark oil	<i>Cinnamomum zeylanicum</i>	Antimicrobial activity against <i>L. monocytogenes</i> and <i>E. coli</i>	160
Cinnamon oil	<i>Cinnamomum</i>	Fungicide	145
Citrus oil	<i>Citrus</i>	ND	151
Clove oil	<i>Syzygium</i>	ND	16
Clove oil	<i>Syzygium aromaticum</i>	Leishmaniasis	146
Coconut oil	<i>Cocos</i>	ND	157
Copaiba oil	<i>Copaifera Langsdorffii</i>	ND	147
Corn oil	<i>Zea</i>	ND	16
Cottonseed oil	<i>Gossypium</i>	ND	16
Davana oil	<i>Artemisia pallens</i>	Topical delivery	152
Eucalyptus oil	<i>Eucalyptus Spp</i>	Absorption promoter to transdermal drug	153,154
Lemon grass	<i>Cymbopogon citratus</i>	Alzheimer's	28
Lemon oil	<i>Citrus</i>	ND	31
Linseed oil	<i>Linum</i>	ND	155
Lizard tail	<i>Houttuynia Cordra</i>	ND	158
Makrut lime	<i>Citrus hystrix</i>	Alzheimer's	28
Neem oil	<i>Azadirachta indica</i>	Acaricidal activity against <i>Sarcoptes scabiei</i> var. <i>cuniculi</i> larvae	167
Olive oil	<i>Olea europaea</i>	Ocular irritation	161
Orange oil	<i>Citrus</i>	Absorption promoter to transdermal drug	150
Orange oil	<i>Citrus</i>	ND	16
Palm oil	<i>Elaeis</i>	ND	155
Peppermint oil	<i>Mentha piperita L.</i>	ND	156
Peppermint oil	<i>Mentha piperita</i>	ND	16
Plai oil	<i>Zingiber cassumunar</i>	Alzheimer's	142
Ricebran oil	<i>Oryza sativa</i>	ND	155
Sesame oil	<i>Sesamum</i>	ND	155
Soybean oil	<i>Glycine max</i>	ND	155
Soybean oil	<i>Glycine max</i>	Antimicrobial activity against <i>L. monocytogenes</i> and <i>E. coli</i>	160
Tea tree oil	<i>Melaleuca Alternifolia</i>	Psoriasis	148

ND: not demonstrated.

oils of three edible Thai plants, *Cymbopogon citratus* (Gramineae), *Citrus hystrix* (Rutaceae) and *Zingiber cassumunar* (Zingiberaceae). The loaded-MEs have been produced in order to enhance the acetylcholine levels in Alzheimer's patients²⁸. Among the three oils, the *C. citratus* oil exhibited the highest cholinesterase inhibitory activity. Brij[®] 97, Triton X[®]-114, Tween[®] 20 and Tween[®] 85 were used as surfactants whereas ethanol and hexanol were used as cosurfactants in the formulation of the ME. The pre-formulation studies of the *C. citratus* oil ME, revealed that the type and concentration of surfactant and co-surfactant exhibited different characteristics. Therefore, such parameters influenced the formation of a large ME region in the pseudoternary phase diagram. Tween 20[®] was used in combination with ethanol rather than hexanol, to be more suitable for the ME formation. The mixture Tween[®] 20/ethanol was hardly influenced by both the pH and the ionic strength of the aqueous phase regarding the formation of the ME. The inhibitory activities of the ME based on water/*C. citratus* oil/Tween[®] 20/ethanol was significantly greater when compared to the native oil.

In other study, the acetylcholinesterase and butyrylcholinesterase inhibitory activities of *Zingiber cassumunar* oil were enhanced by 20–25 times respectively while formulating in an ME compared to the activity reported for the non-formulated native oil¹⁴². Chaiyana et al. interestingly developed a system with the alkaloidal extract from *Tabernaemontana divaricata* loaded in the *Zingiber cassumunar* oil (Plai oil) ME¹⁴³. This system was composed of Plai oil, Triton X[®]-114, ethanol and water with the oil:surfactant ratios of 1:5 and 2:5. Formulations with the oil:surfactant ratio of 1:5 containing 0.1 µg/mL of an extract from *T. divaricata* showed significantly higher acetylcholinesterase inhibition than those with the oil:surfactant ratio of 2:5.

Antimicrobial activity

Microemulsions have been developed as antimicrobial formulations. Xu et al. developed a food-grade water-dilutable ME containing cassia oil (*Cinnamomum cassia*) as oil, ethanol as cosurfactant, Tween[®] 20 as surfactant, and water¹⁴⁴. Antifungal activity *in vitro* and *in vivo* against *Geotrichum citri-aurantii* was assessed. According to the authors, the phase diagram confirmed the feasibility of formulating such an ME including cassia oil. The ME was composed of cassia oil/ethanol/Tween 20 at a weight ratio of 1:3:6 (w/w/w) for each of these ingredients, respectively. The average droplet size of the ME was 6.3 nm. The *in vitro* antifungal experiments showed that the ME inhibited fungal growth on solid medium and prevented arthroconidium germination in liquid medium. Cassia oil had a stronger activity when encapsulated in the ME. Yifei et al. also developed the ME as a potential alternative to chemical fungicides, but their system was composed of cinnamon essential oil. The ME significantly reduced the decay incidence by 18.7% of postharvest gray mold of pears (*Pyrus pyrifolia*) in comparison to that non-formulated natural oil ME after four days storage at 20°C. In the vapor phase, the cinnamon ME with the lowest concentration had the best result for decay incidence and lesion diameter. The authors concluded that the ME may be an alternative way to control the gray mold of pears without a negative influence on its qualities¹⁴⁵.

Antiparasitic activity

Several studies have considered the development of MEs based on natural oil products as antiparasitic agents. Gupta et al. studied the phase diagram of a new pseudoternary system consisting of

clove oil (*Syzygium aromaticum*)/Tween[®] 20/water at ratio of 5/30/65. Several compositions from the single-phase region were selected and their stability toward time, temperature and electrolytes was examined¹⁴⁶. The authors performed an *in vivo* study in golden hamsters (*Mesocricetus auratus*) against *Leishmania donovani* strain AG83 from Indian Kala-azar patient. Among the treatment groups, the authors tested the free drug, the quercetin containing ME and a placebo ME, i.e. a blank ME without quercetin. The placebo ME suppressed 11% of the parasite spleen load, while the ME-incorporated drug suppressed 50% and the free-drug 30% of the parasite load. The results were important to prove that the placebo ME containing clove oil without quercetin was able to suppress the parasite load by itself and that the ME containing quercetin was able to suppress parasite load more effectively than the free drug in synergistic action between the clove oil based ME system and the drug.

In another study, the preparation of neem (*Azadirachta indica*) oil ME was investigated as well as its acaricidal activity *in vitro*. In this system, the mixture of Tween[®] 80 and the sodium dodecyl benzene sulfonate (4:1) w/w was used as surfactants; the mixture of surfactants and hexyl alcohol (cosurfactant) (4:1) w/w was used as emulsifiers. The ME was composed of a mixture of neem oil, emulsifiers and water (1:3.5:5.5) w/w. The ME was formed after stirring at 800 rpm for 15 min at 40 °C. It showed globular and uniform droplets, and had a viscosity of 9.96 mPa s at 25 °C. The ME was still clear after six months at room temperature. The lethal time was used to evaluate the acaricidal activity *in vitro* using *Sarcoptes scabiei* var. cuniculi larvae. The ME containing 10% neem oil showed a median lethal time value (LC50) of 81.75 min against *Sarcoptes scabiei* var. cuniculi larvae. These results acknowledged an effective anti-parasite activity for the neem oil ME¹⁴⁵.

Anti-inflammatory and immunotherapy application

MEs based on natural oil products can be applied as anti-inflammatory and immunotherapy treatment. Recently, an O/W ME including a high volume fraction of a natural oil (copaiba oil) (19.6%) has been developed¹⁴⁷. The formulation approach was based on the chemical match between components of the oil and the lipophilic part of the surfactants according to the Hansen approach. This ME showed a reduced concentration of surfactant and high values of oil/surfactant ratio (1.43). The ME for the oral route was obtained using copaiba essential oil at a final composition of 19.6%, Pluronic F-68[®] 0.15%, Brij O10[®] 13.55% and milli-Q[®] water 66.7% (w/w). This system showed an incorporation rate of 3.8 mg mL⁻¹ of β -caryophyllene from copaiba oil and can be used as a potent anti-inflammatory formulation.

In another study, an ME formulated with tea tree oil, a natural oil containing active molecules against psoriasis including terpinen-4-ol, was proposed by Khokhra et al. The ME was formulated with 5% of tea tree oil, different concentrations of polysorbate 80, as surfactant, and isopropyl myristate and isopropyl alcohol, as cosurfactants. The tea tree oil ME showed droplets of spherical shape with a size ranging from 84 to 115 nm. The maximum terpinen-4-ol compound content observed was 1.68 μ g/mg of ME. The release profile of terpinen-4-ol from the ME depicted that there was a total of 14.5% release through the excised skin from Wistar rats using Franz-type diffusion cells after 24 h. Also, no signs of erythema and skin irritation were found during the experiment¹⁴⁸.

Ali et al. have investigated and evaluated an ME gel-based system of babchi oil (*Psoralea corylifolia*) for the treatment of psoriasis. Babchi oil was used because it contains psoralen, an active compound that acts by inhibiting the DNA synthesis and causing

a decrease in cell proliferation. The authors prepared an ME by titration of the aqueous phase into the mixture of oil and surfactant. It consisted of 1.67% (v/v) babchi oil, 8.33% (v/v) oleic acid, 55% (v/v) Tween[®] 80/Transcutol-P (S/Co ratio 1:1) and 35% (v/v) distilled water. The authors suggested that the ME gel could be a potential vehicle for improved topical delivery of psoralen; hence, it could be a potential vehicle for improved topical delivery of babchi oil in psoriasis lesions²⁷.

Pessoa et al.¹⁴⁹ developed a babassu oil ME aiming to improve the functional activity of phagocytes. Indeed, they show that the babassu oil ME could modulate the blood phagocyte functional activity. The blood from 20 healthy volunteers was processed in order to obtain samples containing 95% of pure mononuclear cells. *Ex vivo* studies showed that the babassu oil ME increased the human phagocyte viability index. In addition, the babassu oil increased the microbicidal activity of the mononuclear blood phagocytes compared to the untreated phagocytes. Therefore, the authors claim that this system can be applied for future immunotherapy strategies, in particular for infectious diseases.

Topical application

MEs containing natural oils have been largely applied as a promoter of absorption through the skin. Yotsawimonwat et al. (2006) investigated the formation of an ME containing orange oil. Pseudoternary phase diagrams of orange oil, ethyloleate or a 1:1 mixture (w/w) of orange oil and ethyloleate as oil components, and a 6:4 (w/w) mixture of polyoxyethylene 20 sorbitan monooleate and sorbitan monolaurate as surfactant components and water or propylene glycol as hydrophilic components were investigated. The authors observed a smaller ME region on the phase diagram when orange oil was used as a substitute for ethyloleate. In addition, the dimension of the solution-type ME areas in the phase diagrams was likely to depend on the miscibility of the components. Larger ME areas were found when ethyloleate was used instead of orange oil and when propylene glycol was used instead of water. Moreover, orange oil incorporation as a penetration enhancer into a topical ME affects its physical characteristics which may lead to instability of the ME and/or can influence the release patterns of the drugs¹⁵⁰. Other system containing water, propylene glycol, sucrose laurate, ethoxylated mono-di-glyceride and citrus oil was formulated. In this ME, the free energy of solubilization decreased with water content in the water-in-oil ME region and increased in the oil-in-water region. Furthermore, the free energy of solubilization decreased with increasing ethoxylated mono-di-glyceride content in the mixed surfactants. The authors also observed that the hydrodynamic diameter of the diluted ME decreases with the increase in temperature¹⁵¹.

Topical davana oil (*Artemisia pallens*) ME formulation was also developed. The authors found that the increase in Tween[®] 80 concentration, enhanced the solubilization capacity of the davana oil into the ME system, leading to enhancement in the ME region. However, transcutol P decreased the interfacial free energy and reduced the surface tension generating the homogenized droplet formation. Optimized formulation was prepared using *Artemisia pallens* based oil (15% w/v), tween 80 (15% w/v), transcutol P (5% w/v) and water (65% w/v)¹⁵².

A number of reports detail natural oil loaded-ME formulations designed to potentiate the topical or transdermal permeability of synthetic drugs^{150,153,154}. Maghraby analyzed the effects of cosurfactants on the transdermal delivery of hydrocortisone from the eucalyptus oil ME. Pseudoternary phase diagrams were constructed in the presence and absence of cosurfactants.

ME formulations containing 20% eucalyptus oil, 20% water and 60% of either Tween[®] 80 or 1:1 surfactant/cosurfactant mixture were compared. In most cases during this study, the incorporation of cosurfactants expanded the ME zone. The cosurfactant-free ME was viscous and showed pseudo-plastic flow. In contrast, the ME prepared with cosurfactants was less viscous and showed a Newtonian flow. In this study, the hydrocortisone was used as the model drug. The drug loading and release rates were increased in the presence of cosurfactants (ethanol being the most efficient among the tested cosurfactants) with the release depending on the viscosity, affecting the phase behavior and the transdermal delivery potential of the ME¹⁵³. The author also used a rabbit skin model to monitor the skin delivery of the drug. The skin was obtained from the inner side of freshly excised ears of 10 male rabbits. For the release studies, the rabbit skin was placed with the stratum corneal side uppermost on the vertical diffusion cell. The study showed that the presence of cosurfactants on the formulation increased the transdermal drug delivery of drug-loaded ME. Among different co-surfactants, ethanol was the most efficient in increasing the permeability.

Other studies

Numerous other systems with potential therapeutic applications have been developed based on bioactive natural oils dispersed in MEs. Microemulsification of vegetable oils (ricebran, saffola, soybean, sesame, palm and linseed) with water using aerosol-OT and cinnamic alcohol as mixed amphiphiles has been studied¹⁵⁵. The developed MEs covered on average approximately 27% of the single-phase area in the triangular phase diagram. The saffola oil ME at a reasonable water/aerosol mole ratio presented a moderate increase in conductance with temperature. Among the studied systems (sesame, saffola and ricebran), the viscosity of the first two decreased with the rate of the shear, whereas the ricebran's viscosity increased. When cinnamic alcohol was used as the oil, the trend of viscosity was similar to that of the sesame and saffola oils. Other authors have observed in detail the development in the phase behavior of Eucalyptus oil/Tween 20/Butanol/Water and Eucalyptus oil/Tween[®] 20/Cinnamic Alcohol/Water systems¹⁵⁴. Triangular and tetrahedral representations have been considered to understand the topological nature of the multicomponent mixtures between the compound mixtures.

Gupta et al. studied microemulsification of various combinations of water with corn oil, cottonseed oil, clove oil, orange oil and peppermint oil using several nonionic surfactants (Tween[®] 20, Brij[®] 30 and Brij[®] 92) and cosurfactants (ethanol and isopropanol). Both ternary (oil/surfactant/water) and pseudoternary (oil/surfactant + co-surfactant/water) phase diagrams were constructed. The ternary systems produced larger ME-forming zones than the pseudoternary systems. Interestingly, the peppermint oil/isopropyl alcohol/water and 1:1 (v/v) peppermint oil + isopropyl myristate/isopropyl alcohol/water combinations were used to form the proportion of the single-phase of the majority of the MEs. All systems showed excellent stability for one year and they withstood temperature variations¹⁶. Fanum et al. developed a peppermint oil ME. The author observed O/W ME formation with droplets of up to 12 nm diameter. The solubilization capacity of the water in the oil was dependent on the surfactants and ethanol/oil mixing ratios (w/w). In addition, a progressive transformation of the W/O to bicontinuous and inversion system (O/W ME) occurred upon dilution with water (Figure 5). The diffusion coefficients of the

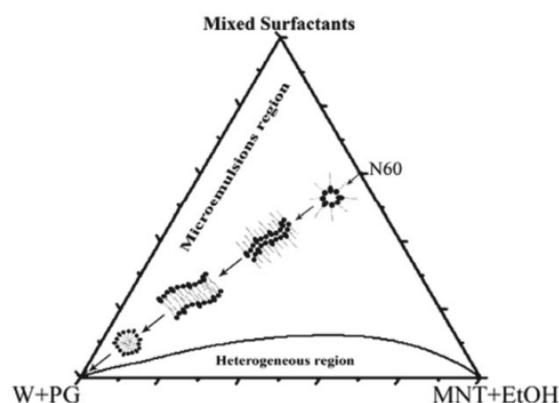


Figure 5. Schematic presentation (not for scale) of the structural transitions along the N60 dilution line in the pseudo-ternary phase diagram. Pseudoternary phase behavior of water (W)/propylene glycol (PG)/sucrose laurate (L1695)/ethoxylated mono-di-glyceride (EMDG)/peppermint oil (MNT)/ethanol (EtOH) system at 25 °C. The mixing ratios (w/w) were mixed surfactants (L1695/EMDG) = 1/1, W/PG = 2/1 and MNT/EtOH = 1/1¹⁵⁶.

surfactants at the interface increased while increasing the water volume fraction¹⁵⁶.

A stable coconut oil ME was prepared based on the HLB concept by using a ternary mixture of nonionic surfactants. The W/O ME was successfully formulated with a surfactant blend composed of 16.6% of Tween[®] 20, 15.0% of Span[®] 20 and 68.4% of Span[®] 80. Transparent MEs could only be formed when the ratio of water and surfactants was at least 1:4.5 and the ratio of water/surfactants and coconut oil was kept below 1:3.5. These MEs remained stable during storage for up to two months, even after centrifugation, but they were not stable when subjected to heating at 70 °C or higher¹⁵⁷. Rao et al. established conditions to produce a stable ME from a nonionic surfactant (Tween[®] 80) and flavor oil (lemon oil). The stable lemon oil ME was produced only by heating the colloidal dispersion containing high surfactant-to-oil ratio. The authors suggested that there was a kinetic barrier at ambient temperature that prevented the system from reaching its most kinetically or thermodynamically stable state. In addition, the application of heating appeared to be much more effective than the application of mechanical energy at overcoming this kinetic barrier. In this study, when higher temperatures (from 62 to 90 °C) were applied at high surfactant concentration (20% Tween[®] 80), the system was not transparent by turbidity analyses, while upon cooling back to the ambient temperature, the turbidity of the system decreased and remained low³¹.

Some authors have also studied ME formulations for solubilization of volatile oils. Yi et al. studied the solubilization of volatile oil from *Houttuynia Cordra* in an O/W ME. The ME system was developed by the titration method, but the varieties and amount of surfactant and co-surfactants had effects on the solubilization of the volatile oil. The ME was composed of medium-chain triglycerides as oil phase, polyoxyethylene castor oil EL-35 as surfactant, and propylene glycol as cosurfactant (at a ratio of 2). This system was able to solubilize the volatile oil from *Houttuynia Cordra* and presented a broad range of therapeutic activities¹⁵⁸. Zhong and Ma developed a Soybean oil-based ME containing cinnamon bark essential oils¹⁵⁹. The system produced was capable of expanding the regime region of MEs and reduced the droplet dimensions, which were stable over 90 days. In another study, biopolymer film-based MEs containing cinnamon bark oil and soybean oil were developed to improve microbiological safety and film transparency¹⁶⁰.

Agami and Maghraby¹⁶¹ developed ME systems based on olive oil and castor oil for ocular delivery. Indeed, the MEs were composed of water, Tween® 80 and each of the two natural oils for ocular delivery of tropicamide. The authors evaluated the drug release using *in vitro* protocols and evaluated the mydriatic response and the ocular irritation using *in vivo* protocols using albino New Zealand rabbits as a model. The mydriatic response was evaluated by the pupil dilatation after the application of each formulation containing the drug. On the other hand, ocular irritation was assessed by the tear volume measurement using the phenol red thread test. Results showed similar mydriatic response for both systems containing the drug. In addition, lower irritation to the eye was found for both formulations containing the natural oils compared to a ME developed with synthetic oil.

Patented systems based on natural oil-loaded ME

In addition to published papers focused on ME formulations containing natural oils, an increasing number of patents have been developed around the world under known patent depositories such as the European Patent Office and the United States Patent and Trademark Office. These formulations have been patented for the past decades since their development¹⁶². A number of these formulations have not yet been tested *in vivo*; however, due to their potential applicability and viability, researchers and companies have taken precautions in protecting their intellectual property products. ME formulations containing natural essential oils have been patented for use as food preservatives. The invention is based on the antimicrobial activity of the oil and the improvement of microbiological stability provided by the ME¹⁶³. Another system was recently patented to prevent hair loss and to increase hair density. This formulation is a complex mixture of natural oils, essential oils and plant extracts developed with the aim to impact the lives of men and women with alopecia and baldness¹⁶⁴. A non-alcohol bioactive essential oil mouth rinse was patented as an O/W ME containing antimicrobial essential oils as active components¹⁶⁵. Another patent of MEs in the cosmetic industry presents a system developed to hold fragrances, being described as an aqueous, perfumed ME. This formulation claims that different natural oils, such as rose oil, tree oil, lemon oil, among others can be incorporated¹⁶⁶.

Future perspectives

A wide variety of research studies regarding the development of formulations with natural oils as bioactive compound or as lipophilic carrier for incorporation of lipophilic drug molecules have been conducted nowadays. Indeed, MEs containing natural oils present many advantages, as previously mentioned in this review. Moreover, studies continue to be conducted in order to demonstrate adequate pharmaceutical properties of such systems to become viable delivery systems able to incorporate drugs that present drawbacks in traditional formulations. In addition, the discovery of new bioactive compounds, which are mostly found in natural products and need a suitable delivery system, have been directed as a consequence of new diseases that appeared every day.

In the following years, bioavailability studies with ME formulations are expected to be carried out because the absorption behavior of mostly MEs containing natural oils are still very erratic and the reproducibility in humans became a challenger for pharmacokinetic researchers. In fact, natural oils often present different chemical composition according to the extraction variations, the plant location, the soil changes and the seasonal changes, and

this induces a great variation of the bio-absorption of the oil itself or its carriers. Additionally, more reliable information on the pharmacokinetics and pharmacodynamics of bioactive compounds present in the natural oils and how they influence the properties of the incorporated drug in the human body became mandatory. Another important point is the expectation of an increase on the *in vivo* studies using humans for the next few years in order to better indicate the viability of new lipid formulations. Such studies would be able to clearly reveal the synergy among the active compounds provided by the natural oil resource and their carrier such as ME systems.

Conclusion

Natural oil products have promising potential in maintaining and promoting health, as well as preventing and potentially treating some diseases. The natural oil products are extremely complex mixtures of different functional-group classes. Delivery systems of complex bioactive compounds from natural oil represent a promising strategy for overcoming the limitations of these oils, such as low solubility, bioavailability, and efficacy, and for preventing degradation of their active components in the presence of air, light, moisture and temperature. Several studies have been conducted over the last few decades concerning the formulation of new ME systems containing bioactive compounds from natural oil products. Such studies reveal that these systems are promising and are also an innovative approach with potential applications in medicinal and health research, which can result in decreasing the dose, enhancing the absorption and the bioavailability as well as reducing the systemic side effects and the patient variability. Therefore, bioactive oil loaded-ME represents potential strategies to increase the therapeutic proprieties of natural oils.

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Document II

*Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil
Nanostructured Emulsions*

Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions

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The aim of this work was to investigate the antimicrobial activity of nanostructured emulsions based on copaiba (*Copaifera langsdorffii*) resin-oil, copaiba essential oil, and bullfrog (*Rana catesbeiana* Shaw) oil against fungi and bacteria related to skin diseases. Firstly, the essential oil was extracted from copaiba resin-oil and these oils, along with bullfrog oil, were characterized by gas chromatography combined with mass spectrometry (GC-MS). Secondly, nanostructured emulsion systems were produced and characterized. The antimicrobial susceptibility assay was performed, followed by the Minimum Inhibitory Concentration (MIC) determination, the bioautography assay, and the antibiofilm determination. Strains of the genera *Staphylococcus*, *Pseudomonas*, and *Candida* were used. The GC-MS analysis was able to identify the components of copaiba resin-oil, copaiba essential oil, and bullfrog oil. The MIC assay in association with the bioautography revealed that some esters of palmitic and oleic acids, α -curcumene, α -himachalene, isothujol, and α -fenchene—probably inhibited some strains. The nanostructured emulsions based on copaiba resin-oil and essential oil improved the antimicrobial activity of the pure oils, especially against *Staphylococcus* and *Candida*, resistant to azoles. The bullfrog oil nanostructured emulsion showed a lower antimicrobial effect when compared to the copaiba samples. However, bullfrog oil-based nanostructured emulsion showed a significant antibiofilm activity ($p < 0.05$). Given the significant antimicrobial and antibiofilm activities of the evaluated oils, it may be concluded that nanostructured emulsions based on copaiba and bullfrog oils are promising candidates for the treatment of infections and also may be used to incorporate other antimicrobial drugs.

Keywords: Nanostructured Emulsion, Copaiba (*Copaifera langsdorffii*) Oil, Bullfrog (*Rana catesbeiana*) Oil, Antimicrobial Activity.

1. INTRODUCTION

Natural oils have been used in popular medicine as antimicrobial agents for treatment of various infections.¹ Nowadays, due to the substantial number of drugs resistant to microorganisms, these oils and other natural products

have become scientifically recognized, encouraging the introduction of new products originated from animal and vegetable sources in the market.² Among these products, bullfrog (*Rana catesbeiana* Shaw) and copaiba (*Copaifera langsdorffii*) oils are widely used in popular medicine.

Copaiba oil is extracted from trees known as Copaiibeiras (*Copaifera* spp.), which are distributed in South America

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and Africa.³ The copaiba resin-oil is traditionally used in popular medicine due to its anti-inflammatory and antimicrobial activities, among other pharmacological and cosmetic properties already elucidated in the literature.^{3–6} Phytochemical studies of resin-oil reveal that it contains about 28 diterpenes and 72 sesquiterpenes hydrocarbons.⁷ The essential oil extracted from the copaiba resin-oil concentrates the majority of the sesquiterpenes, which are associated with the pharmacological activity of this compound. Therefore, the copaiba essential oil has become a highly demanded product in the pharmaceutical field.⁸

The bullfrog oil is biotechnologically extracted from bullfrog adipose tissues, which are usually discarded in the frog farms in the food industry. In this way, it is possible to reuse an animal residue to provide an active oil to treat diseases.⁹ Because it belongs to the muscular tissue and skin, the bullfrog oil is rich in polyunsaturated fatty acids such as the omega group, which are pharmacologically relevant for their medicinal use.¹⁰ Additionally, other chemical components from bullfrogs, such as peptides from their stomach, have been studied due to their antimicrobial activity.¹¹

Because the skin acts as a physical barrier of the human organism, it is constantly exposed to several pathogenic microorganisms through the contact with the external environment. Therefore, this organ may be susceptible to infections. Among the microorganisms that are involved with such infections, gram-positive bacteria of the genus *Staphylococcus*, gram-negative bacteria such as *Pseudomonas aeruginosa*, and yeasts belonging to the genus *Candida* are prevalently found.^{12,13} It is well known that many antimicrobial agents are no longer effective against several microorganisms that may have become resistant to antimicrobial agents. Nevertheless, these microorganisms are still sensitive to natural products. On the other hand, the use of oils *in natura* leads to low adherence to treatment due to its unpleasant organoleptic characteristics. Natural oils may also not provide desirable pharmacokinetic properties and permeability. Therefore, the delivery systems, such as nanostructured emulsions, are viable systems to improve these characteristics. Additionally, such systems can help the understanding of the mechanism of action of the substances present in these natural oils, a subject barely discussed on the literature.

Nanostructured emulsion systems containing natural oils are suitable alternatives against infectious pathogens because of the synergic effect induced by the antimicrobial activity of the renewable source of oils and the advantages conferred to the nanostructured emulsions.¹⁴ The development of these systems is economically viable and aims to improve the bioavailability, to enhance or modify the release of the oil's active components, and to improve their organoleptic characteristics.¹⁵ Those emulsified systems may be defined as homogeneous milky systems formed by two phases that are immiscible, in which one phase is

dispersed within the other in the form of droplets stabilized by surfactant molecules.¹⁶

The aim of this work was to determine the antimicrobial activity of nanostructured emulsions based on bullfrog and copaiba (resin and essential) oils. Therefore, first, a chemical characterization of the oils used to develop the nanostructured emulsions was performed to identify their antimicrobial compounds. Moreover, the antimicrobial activity of the nanostructured emulsion systems produced through different methods was evaluated.

2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Chemicals

The copaiba (*Copaifera langsdorffii*) resin-oil was purchased from Flores and Ervas (Piracicaba, SP, Brazil). Bullfrog (*Rana catesbeiana* Shaw) oil was a gift from Asmarana Natural Products (Natal, Brazil). Span 80[®], methylene blue dye, glucose, and tetrazolium chloride were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Tween 20[®], DMSO, hexane, ethyl acetate, ethanol, and violet crystal were purchased from Vetec Quimica Fina LTDA (Rio de Janeiro, RJ, Brazil). The copaiba essential oil was obtained by hydrodistillation of copaiba resin-oil using a Clevenger apparatus for 4 h at 100 °C. Brain Heart Infusion (BHI) broth, Sabouraud dextrose agar, Mueller-Hinton agar, and Mueller-Hinton broth came from Himedia (Mumbai, MU, India). Yeast Extract-Peptide-Dextrose (YPD) broth was a gift from MMLL (Medical and Molecular Mycology Laboratory), UFRN (Natal, Brazil). All other chemicals were of at least analytical grade.

2.1.2. Microorganisms

The bacteria strains used in this study were *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC 27853, and two clinical strains of each species. The yeasts used during the experiments were *Candida albicans* ATCC 90027, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 2001, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 13803, and also a clinical strain of each aforementioned tested species. Yeasts clinical strains isolated from patients were collected according to two different protocols approved by the local Research Ethics Committee from the Onofre Lopes University Hospital, Natal, Rio Grande do Norte, Brazil under the numbers: 595/11p (vaginal isolates) and 494/10 (skin and mucosal isolates). All the fungal isolates belong to The Laboratório de Micologia Médica e Molecular culture collection, UFRN, Rio Grande do Norte, Brazil. The bacteria were maintained in Nutrient broth (Mumbai, MU, India) medium, and the yeasts were maintained in Sabouraud Dextrose broth medium (Mumbai, MU, India) containing 20% glycerol, frozen at –80 °C until the moment of the experiment. All stored bacteria and yeasts were cultured in

BHI agar for 24 h and Sabouraud dextrose agar for 48 h, both at 37 °C respectively, before the tests.

2.2. Methods

2.2.1. Chemical Characterization

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the oils was performed on a Hewlett-Packard 6890 gas chromatograph interfaced to a HP-5975 mass selective detector. The column used was a HP-5MS cross-linked fused silica capillary column (30 m × 0.25 mm × 0.25 μm). Before the experiments, the bullfrog oil and copaiba resin-oil were methylated using diazomethane aiming to perform the methyl ester's identification of acid compounds. The work temperatures for the copaiba essential oil were as follows: oven temperature started at 60 °C, isothermal, then heating 3 °C/min to 240 °C, and isothermally for 7 minutes at 250 °C. The injector temperature was 220 °C. The following are the conditions for both copaiba resin-oil and bullfrog oil: oven temperature started at 110 °C, isothermal, then, heating 5 °C/min to 280 °C, and isothermally for 26 minutes at 300 °C. The injector temperature was 250 °C. The volume injected for all samples was 1 μL. The split ratio was 1:25 and the ionization voltage 70 eV. Helium was the carrier gas at a flow rate of 1 mL/min. The separated components were identified by matching them with the National Institute of Standards and Technology (NIST) mass spectral library data and by comparing their Kovat's indices with those of authentic components and with published data. The quantitative determination was carried out by peak area integration.

2.2.2. Nanostructured Emulsion Preparation and Characterization

The three different nanostructured emulsions were prepared containing copaiba resin-oil, copaiba essential oil, and bullfrog oil according to previous studies.¹⁵ The system was produced using the following composition: oil at 5% (w/w), water at 93% (w/w), Tween 20[®] at 1.56% (w/w), and Span 80[®] at 0.44% (w/w). Phase inversion technique (PIT) was used to produce the nanostructured emulsion.¹⁷ The amount of Span[®] 80 was dispersed in the oil (phase 1) while Tween[®] 20 was dispersed in the water (phase 2). The phases were heated separately to 70° C and then phase 1 was emulsified into phase 2 by an Ultra-Turrax[®] T25 (IKA, Staufen, Germany) homogenization for 10 minutes at 13,000 rpm.

Additionally, physicochemical analyses were carried out. pH was measured using a PG-2000 pHmeter (GEHAKA, Morumbi, SP, Brazil) and electrical conductivity measurement was performed using a DM-32 conductivity (Digicrom Analytical, Campo Grande, SP, Brazil). Droplet size, polydispersity, and zeta potential analysis were evaluated by Dynamic Light Scattering (DLS) using a ZetaPlus (Holtville, NY, USA). All analyses were performed at 25 ± 2 °C.

2.2.3. Antimicrobial Susceptibility Assay

Bacterial and fungal sensitivity of all strains described above was initially performed using a susceptibility test with Muller-Hinton agar for bacteria and Mueller-Hinton agar + 2% glucose and 0.5 μg/mL methylene blue dye for yeasts.^{18,19} The inocula were prepared in NaCl 0.9% (w/v) solution and adjusted to the 0.5 McFarland standard. Subsequently, 10 μL of the copaiba resin-oil, copaiba essential oil, bullfrog oil, and the three nanostructured emulsions containing each of these oils were added to the wells. The oils were dispersed in DMSO (1% (w/w)) for all tests. Chloramphenicol (1.5 mg/mL) and ketoconazole (2.5 mg/mL) were used as synthetic antimicrobial controls for bacteria and yeasts, respectively. Tween 20[®] at 1.56% (w/w), Span 80[®] at 0.44% (w/w), and DMSO 1% (w/w) were also tested individually. Assays were carried out in triplicate, three times ($n = 9$).

2.2.4. Broth Microdilution Assay

The inocula of microorganisms in NaCl 0.9% (w/v) solution were adjusted to the 0.5 McFarland standard. They were then used to prepare further dilutions in Mueller-Hinton Broth, containing 0.5 to 2.5 × 10³ CFU/mL. A broth microdilution assay with serial dilutions of the samples was performed with sterile Muller-Hinton Broth in a 96-well microplate from 0.0001 to 248.7 mg/mL.²⁰ Subsequently, 100 μL of each microorganism cell suspension was then added to each well. The plates containing bacteria were incubated for 24 hours at 35 °C while the fungi were incubated for 48 hours at 37 °C in an orbital shaker (TE-420 Tecnal, Piracicaba, SP, Brazil) at 150 rpm for Minimal Inhibitory Concentration (MIC) determination. Assays were carried out in triplicate.

2.2.5. Bioautography Assay

The Thin Layer Chromatography (TLC) was developed with copaiba-oil/methanol and bullfrog-oil/methanol (2:8) and the hexane/ethyl acetate (9:1) solution as the eluent system. The TLC plates were revealed with vanillin-sulphuric acid to observe the chromatographic profile. Subsequently, the TLC plates were reproduced and loaded into culture plates prepared with Mueller-Hinton agar and the bacterial inoculum adjusted to the McFarland 0.5 standard. For the fungi strains, Mueller-Hinton agar plus 2% (w/w) glucose and 0.5 μg/mL methylene blue dye were used. The inhibition halos were observed using tetrazolium chloride (TTC) and the Retention Factors (R_f s) were calculated after an incubation period of 24 and 48 hours for bacteria and fungi, respectively, at 35 °C. The silica was removed at the R_f for extraction of the chemical retained compounds. The extraction was performed with ethyl acetate in an ultrasound bath for 20 minutes and filtration. Moreover, the samples were analyzed in GC-MS. Assays were carried out in triplicate.

2.2.6. Antibiofilm Assay

Cell suspensions were prepared according to the microdilution assay. The nanostructured emulsions and the respective oils (99:1 (w/w) in DMSO) were added (12.5% (v/v)) separately in sterile 96-well microliter plates containing sterile Mueller-Hinton Broth and microorganism suspensions, incubated at 35 °C for 24 h and 48 h for bacteria and yeasts, respectively, in an orbital shaker at 150 rpm. Then, the supernatants were removed and the wells were washed with sterile water to remove non-adherent cells. The biofilms were stained with crystal violet for 20 minutes. Subsequently, 200 μ L of absolute ethanol was added and the optical density (OD) was measured with an ELISA microplate reader (BioTek, μ Quant) at 570 nm.²¹ Ketoconazole and chloramphenicol were used as control antimicrobial agents according to the antimicrobial screening. Assays were carried out in triplicate.

2.2.7. Statistical Analysis

The results are presented as the mean \pm S.D. Statistical significance between 3 groups was performed by Analysis of Variance (ANOVA) followed by Tukey's post-test. Student's *t*-test was used between 2 unpaired groups. *p* values less than 0.05 (*p* < 0.05) were considered significant.

3. RESULTS AND DISCUSSION

3.1. Chemical Characterization

Concerning the samples' characterization, the GC-MS was a useful tool to identify the chemical compounds of the studied oils. The technique showed a required resolution and peak separation, allowing analyzing the compounds individually in comparison with the spectra library and the retention times found in the literature. Prior to the CG-MS analysis, the extraction of copaiba essential oil showed a 10% (v/v) yield, which was superior to that reported by Gramosa and Silveira, 2005.²² The CG-MS analyses showed that terpenes have lower retention time in copaiba resin-oil analysis when compared to the same compounds in the copaiba essential oil (Figs. 1(A) and (B)). These characteristic terpene compound peaks can be observed in two other studies that analyzed the chemical composition of copaiba oils.^{3,23}

The analysis of copaiba resin-oil chromatograms suggests that β -bisabolene was the major identified compound (Table I). Other components, such as β -caryophyllene and α -bergamotene, were also present at high amounts. The residue fraction obtained after extraction of the essential oil showed a lower amount of sesquiterpenes, indicating that these compounds were extracted by hydrodistillation and were concentrated in the essential oil (data not shown).

The bullfrog oil GC-MS analyses showed separated peaks in which methyl oleate was the ester presented as the most abundant compound. The oleic acid esters were the predominant compounds, followed by esters of palmitic and linoleic acid (Fig. 1(C)). Thus, these three

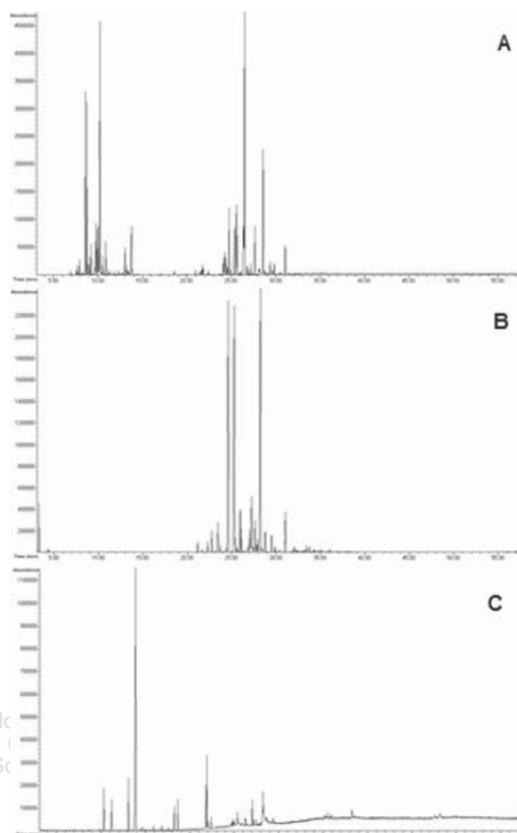


Figure 1. Chromatogram of natural oils. (A) Copaiba resin-oil; (B) copaiba essential oil; (C) bullfrog oil.

compounds were the predominant monounsaturated, saturated, and polyunsaturated fatty acids, respectively.

These results are also in accordance with those observed in the literature concerning the composition of bullfrog oil,^{9,24} which reported different methodologies for bullfrog oil extraction and also indicated the predominance of oleic, linoleic, and palmitic fatty acids (Table II).

3.2. Nanostructured Emulsion Characterization

The systems were macroscopically presented as milky and fluid dispersions. Concerning their organoleptic characteristics, a change in their natural oils inherent odor was observed after the nanostructured emulsion process of these oils. Furthermore, the formulations were not creamy on top. The conductivity (from 187.51 μ S to 226.20 μ S) confirmed the external aqueous phase of the systems. The DLS analysis showed a nanosized droplet size for all nanostructured emulsions of about 200 nm, as well as a low polydispersity. Additionally, pH values were low, ranging from 3.22 to 3.48, probably due to the presence of

Table I. Percentage of terpenes from copaiba (*Copaifera langsdorffii*) oils.

Chemical composition	RT (min)	CO (%)	RT (min)	CEO (%)
δ -elemene	6.90	0.37	21.17	0.63
(+)-cyclosativene	7.52	0.45	22.31	0.67
α -copaene	7.66	0.93	22.76	1.39
β -elemene	7.92	1.44	23.46	2.41
β -caryophyllene	8.57	18.30	24.62	21.68
α -bergamotene	8.74	15.61	25.29	20.53
α -guaiene	8.83	1.17	25.38	0.88
β -farnesene	9.03	1.61	26.11	1.56
α -caryophyllene	9.22	2.80	25.95	2.85
τ -muurolene	9.60	0.82	26.88	0.53
β -cubebene	9.75	4.83	27.06	1.72
β -selinene	9.88	4.34	27.27	6.16
α -selinene	–	–	27.62	2.31
τ -gurjenene	–	–	27.78	0.89
β -chamigrene	10.04	5.63	–	–
β -bisabolene	10.21	24.76	28.24	23.67
β -sesquiphellandrene	10.55	2.44	–	–
α -curcumene	12.29	0.35	–	–
α -cedrene	12.85	0.53	–	–
β -cedrene	–	–	28.76	1.40
α -himachalene	13.01	1.74	33.39	0.46
Kaurene	20.98	0.39	–	–

Notes: RT (min): Retention time; CO: Copaiba resin-oil; CEO: Copaiba essential oil; (–) Not detected.

fatty acids from both oils. The zeta potential was distinct for the three systems, probably because this property is influenced by the chemical composition of the nanostructured emulsion components, especially the oils (Table III). The bullfrog oil nanostructured emulsion showed the lowest zeta potential (-11.86 ± 1.99), which could predict low stability. However, all the nanostructured samples remained quite stable over three months at 25 °C.

3.3. Antimicrobial Susceptibility Assay

The antimicrobial screening performed by the agar diffusion method was performed to investigate whether the tested strains were sensitive to the oils used to produce the nanostructured emulsion systems. The inhibition halos showed that some strains were sensitive to copaiba oils. The resin-oil and the essential oil exhibited an inhibition halo of 6.6 mm and close to 9.0 mm, respectively, against *S. aureus* (Table IV). This result corroborates the findings of other studies with copaiba oil from other species

Table II. Percentage of fatty acid esters from bullfrog (*Rana catesbeiana* Shaw) oil.

Chemical composition	RT (min)	BO (%)
Methyl palmitoleate	18.25	2.23
Methyl palmitate	18.65	5.87
Methyl linoleate	21.84	4.79
Methyl oleate	21.94	9.26
Glyceryl monooleate	28.22	7.59

Notes: RT (min): Retention time; BO: Bullfrog oil.

Table III. Physicochemical characterization of the nanostructured emulsions.

	pH	Conductivity (μ S)	Droplet size (nm)	Polydispersity	Zeta potential
COE	3.40	187.51	200 \pm 0	0.24 \pm 0.01	-34.37 ± 2.50
CEOE	3.48	226.20	280 \pm 1	0.14 \pm 0.02	-27.08 ± 0.89
BOE	3.22	220.30	260 \pm 0	0.27 \pm 0.01	-11.86 ± 1.99

Notes: COE: Copaiba resin-oil nanostructured emulsion; CEOE: Copaiba essential oil nanostructured emulsion; BOE: Bullfrog oil nanostructured emulsion.

against *S. aureus*, including the copaiba oil extracted from *Copaifera multijuga*, which showed inhibition halos of 7.0 mm against *S. aureus*.²⁵

On the other hand, the bullfrog oil showed no significant antibacterial or antifungal activity against all tested strains. However, it is important to emphasize that studies based solely on susceptibility tests using agar diffusion are not conclusive. In some occasions the antimicrobial compounds are not able to migrate through the agar or are present at low concentrations. Therefore, further microbiological assays such as broth microdilution and bioautography would be required to confirm these results.²⁰

The microbiological behavior of the resin-oil and that of the essential oil revealed in this work were significantly different, probably because of their different chemical compositions. In fact, the essential oil showed an activity of about 50% more efficient than the resin-oil against *S. epidermidis* ATCC 12228 and was more effective than the resin-oil for most of the tested strains. This indicates that the highest concentration of sesquiterpenes, such as β -caryophyllene and α -himachalene, provides greater antibacterial and antifungal activity to the essential oil (Table IV).²⁶

Several works found in the literature concerning the antimicrobial activity of *C. langsdorffii* oil by agar diffusion technique show a different profile of response. In fact, the copaiba oil used in this work presented an inhibition halo that was about 50% lower for some reference strains. This could be explained by the variability of the methodology or by the variability of the chemical composition of the oil in plants of the same species. Therefore, the

Table IV. Microbial sensitivity (inhibition halos (mm)) of copaiba resin-oil and copaiba essential oil against susceptible strains.

Microorganisms	CO	CEO	Ref.
<i>S. aureus</i> ATCC 29213	6.66 \pm 1.32	9.88 \pm 2.14	*27.78 \pm 2.04
<i>S. epidermidis</i> ATCC 12228	5.44 \pm 0.72	14.00 \pm 2.39	*35.11 \pm 3.68
<i>S. epidermidis</i> CS1	–	11.89 \pm 1.96	*27.89 \pm 3.14
<i>S. epidermidis</i> CS2	–	13.11 \pm 3.14	*24.44 \pm 6.34
<i>C. glabrata</i> ATCC 2001	6.33 \pm 2.06	12.89 \pm 6.03	**28.33 \pm 2.50
<i>C. glabrata</i> 15V3C (CS)	–	9.88 \pm 0.78	**28.11 \pm 2.08
<i>C. krusei</i> ATCC 6258	–	13.67 \pm 3.42	**24.67 \pm 2.87
<i>C. krusei</i> LMM54 (CS)	–	13.78 \pm 3.23	**24.22 \pm 4.35

Notes: (–) Exhibited no inhibition halo; CO: Copaiba resin-oil; CEO: Copaiba essential oil; Ref: Antimicrobial synthetic reference; *Chloramphenicol; **Ketoconazole.

evaluation of the chemical composition of the oils is a crucial step in the development of nanostructured emulsions using natural oil products.²⁷

Concerning the antifungal activity, it must be emphasized that copaiba essential oil has potential activity against reference strains of *C. krusei* (12.89 ± 6.03) and *C. glabrata* (13.67 ± 3.42), which are known for possible resistance to azole antifungals widely used for the treatment of superficial infections. On the other hand, strains of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* had full growth and were not sensitive to the essential oil. Therefore, each strain should be tested with the essential oil independently of the genus or species. However, these *Candida* species are considered less able to develop resistance to synthetic antifungal drugs currently in use.²⁸

It is important to note that both the DMSO (used to solubilize the oils) and the surfactants (Tween 20® and Span 80®) present no activity when separately used. This finding is relevant to prove that the surfactants would not directly influence the antimicrobial activity of the nanostructured emulsion systems.

3.4. Broth Microdilution Assay

Following the evaluation of the sensitivity of the strains to the oils, a broth microdilution assay was performed with the nanostructured emulsion systems containing copaiba resin-oil, copaiba essential oil, and bullfrog oil. The copaiba oil-based nanostructured emulsions presented lower MIC values when compared to the pure oil samples, specifically for *C. glabrata* and *C. krusei* (Table V). It is important to highlight that, although with only 5% of oil, the nanostructured emulsion systems containing the copaiba resin-oil and the copaiba essential oil showed activity equivalent to or better than that of the pure oil alone (Table V). Such improvement of activity could be due to the nanostructured emulsion system, in which the oil was dispersed as droplets and which may improve the activity of the compounds, resulting in a better activity of natural oils.^{15,29} This was again demonstrated by the significant decrease ($p < 0.05$) in the MIC for *C. glabrata* ATCC 2001 using the emulsion based on the copaiba resin-oil rather than the oil itself.

However, a likely paradoxical effect phenomenon for the clinical strain of *C. glabrata*, which presented inhibition only at low concentrations, but cells restarted to grow with higher concentrations of the oil, might have occurred (results not shown). Despite the fact that Chamilos et al. (2007), testing the effect of a new group of antifungal agents, the echinocandins,³⁰ have described that only *Candida* species other than *C. glabrata* presented paradoxical growth effect, we cannot rule out the possibility that this phenomenon has happened for this species in the presence of the oil. Nevertheless, additional studies are mandatory to confirm this finding.

Since the nanostructured emulsion based on bullfrog oil and the pure oil showed no activity in the susceptibility assay, the MIC was determined only against the ATCC strains in order to confirm the preliminary results. As expected, both the bullfrog oil nanostructured emulsion and the pure oil exhibited MIC values $> 228.5 \pm 0.0$ mg/mL for all tested strains. However, because the oils presented different composition, the ineffective activity found for the bullfrog oil compared to the copaiba oils could be due to the choice of the strains used in this study. Concerning its active compounds, other strains not discussed in this study might show sensitivity to the bullfrog oil chemical compounds. It is important to note that the action of natural products may be either species specific or even strain specific. It is well described in the literature that susceptibility to antifungal drugs may vary within different strains belonging to the same species.²⁷ Therefore, a microbiological activity assay with additional strains would reveal the efficacy of such product and the fatty acids found in its composition, as claimed by the literature.³¹

3.5. Bioautography

The bioautography of copaiba resin-oil revealed two different chromatographic zones that caused inhibition on the microorganisms' growth. The first chromatographic band inhibited *P. aeruginosa* ATCC 27853 (*Rf* 0.2) and the second one inhibited the growth of *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 (*Rf* 0.15). The GC-MS analysis showed traces of α -curcumene, α -himachalene,

Table V. Microdilution assay results (MIC) of copaiba oils and nanostructured emulsions (mg/mL).

Microorganisms	CO	COE	CEO	CEOE
<i>S. aureus</i> ATCC 29213	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	$55.4 \pm 0.0^*$	$> 249.3 \pm 0.0^*$
<i>S. epidermidis</i> ATCC 12228	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	221.7 ± 0.0	$> 249.3 \pm 0.0$
<i>S. epidermidis</i> CS1	$0.0009 \pm 0.0^{**}$	$0.0455 \pm 0.022^{**}$	221.7 ± 0.0	$> 249.3 \pm 0.0$
<i>S. epidermidis</i> CS2	$> 234.0 \pm 0.0$	$> 249.750 \pm 0.0$	221.7 ± 0.0	$> 249.3 \pm 0.0$
<i>C. glabrata</i> ATCC 2001	$> 234.0 \pm 0.0^*$	$0.9717 \pm 0.00^{***}$	$0.1083 \pm 0.076^{***}$	$15.6 \pm 0.0^{***}$
<i>C. glabrata</i> 15V3C (CS)	0.0009 ± 0.0	0.0001 ± 0.0	$0.1083 \pm 0.038^{***}$	$0.9736 \pm 0.0^{***}$
<i>C. krusei</i> ATCC 6258	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	$34.7 \pm 0.0^*$	$15.6 \pm 0.0^*$
<i>C. krusei</i> LMM54 (CS)	$> 234.0 \pm 0.0$	$> 249.7 \pm 0.0$	$34.7 \pm 0.0^*$	$3.9 \pm 0.0^*$

Notes: CO: Copaiba resin-oil; COE: Copaiba resin-oil nanostructured emulsion; CEO: Copaiba essential oil; CEOE: Copaiba essential oil nanostructured emulsion; MIC = Minimum Inhibitory Concentration. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, when comparing the emulsions with the oils themselves.

Isothujol, and α -fenchene in the first band, while the second fraction showed α -himachalene (1.46%) and traces of α -curcumene in its composition (results not shown). Pharmacological and toxicological data involving these isolated terpenes are not well described yet in the literature. Therefore, their mechanism of action of such substances was not elucidated yet. However, there are some registers about derivatives and isomers of himachalene, especially β -himachalene, as being a potential antifungal and insecticidal agent in the food poisoning.^{32,33}

Additionally, other terpenes from the copaiba oil have been reported as active compounds by several studies in which the antimicrobial activity of β -caryophyllene, caryophyllene oxide, and copalic acid, usually present in large quantities in the copaiba resin-oils, was investigated.³⁴ Thus, it may be suggested that the terpenes, identified by the GC-MS after the bioautography method, were probably responsible for the antimicrobial activity of the copaiba oil. However, further studies are required in order to investigate their activity individually.

The bioautography of bullfrog oil followed by CG-MS analysis revealed the presence of esters of oleic (48.6%) and palmitic (0.9%) acids in the zone related to the inhibition growth of *P. aeruginosa* ATCC 27853 (*Rf* 0.6). As it can be seen, the oleic acid was predominant in this oil (results not shown). Issacs et al. (1995) demonstrated that oleic acid and monoglycerides are responsible for the antimicrobial activity in breast milk.³¹ Therefore, these fatty acids present in large amounts in the bullfrog oil may be the compound responsible for its antimicrobial activity. It is important to note that although the bioautography results did not corroborate with the antimicrobial screening, it provides the possibility of testing concentrated compounds through chromatographic bands, which provides better sensitivity to the method and highlights the effective antimicrobial activity of the bullfrog oil.

3.6. Antibiofilm Activity

The reduction in biofilm formation is an important tool to corroborate the antimicrobial activity because phenotypic changes may occur with microorganisms, making them more invasive than planktonic cells. In most cases, biofilm formation confers greater resistance to antimicrobial molecules and the immune defense of the host.³⁵

Both copaiba resin-oil and copaiba essential oil were able to provide effective inhibition on the biofilm formation (Fig. 2). The nanostructured emulsions were able to inhibit the biofilm formation of all ATCC strains tested. Additionally, considering most of the strains, the oils themselves and their nanostructured emulsions presented the same profile of inhibition with no significant difference ($p > 0.05$). However, for *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 strains, a significant decrease ($p < 0.05$) in the biofilm formation was observed for the copaiba essential oil nanostructured emulsion when compared to the pure oil. In contrast, *C. krusei* CS improved

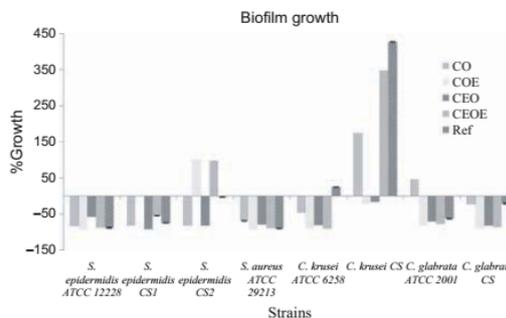


Figure 2. Percentage of biofilm growth in the presence of copaiba samples. CO: Copaiba resin-oil; COE: Copaiba resin-oil nanostructured emulsion; CEO: Copaiba essential oil; CEOE: Copaiba essential oil nanostructured emulsion; Ref.: Chloramphenicol (bacteria) and Ketoconazole (fungi).

the biofilm formation when cells were grown in the presence of this system and ketoconazole (Fig. 2). However, the copaiba essential oil nanostructured emulsion showed a better antibiofilm activity than ketoconazole for *C. krusei* ATCC 6258, clearly demonstrating that the improvement of biofilm formation may vary within different *C. krusei* strains. Concerning *C. glabrata* ATCC 2001 and CS, the nanostructured emulsions were more effective by inhibiting biofilm formation than the oils themselves (Fig. 2).

The biofilm inhibition induced by natural oils has been already studied for different bacteria and fungi. As discussed by Carvalho and Fonseca (2007),³⁶ terpene compounds may be useful to inhibit the biofilm formation by acting in both the cellular membrane structure and the cell surface hydrophobicity. Interestingly, in spite of the parenteral lipid emulsion induced *Candida* biofilm formation on medical catheters,³⁷ the nanostructured emulsions based on copaiba oil containing the terpene compounds were able to inhibit it (Fig. 2).

The analysis of the biofilm formation for bullfrog oil nanostructured emulsion showed a significant inhibitory activity against most of the yeast strains when compared to the pure oil (Fig. 3). It is important to highlight that although bullfrog oil nanostructured emulsion did not present positive results for *Pseudomonas aeruginosa* ATCC 90027 in the microbial sensitivity assay and in the broth microdilution assay, a significant inhibition of the biofilm formation was observed, suggesting that the bullfrog oil may act on the cells that form the biofilm, impairing either adhesion initial steps or exopolymeric matrix secretion, but not in planktonic cells of *P. aeruginosa*.

Additionally, bullfrog oil nanostructured emulsion inhibited the biofilm formation of most of the tested *Candida* species. Thus, this system could be used to treat fungal infections triggered by the biofilm formation caused by yeasts of the genus *Candida*, which are responsible for about 80% of fungal infections in the hospital environment.³⁸

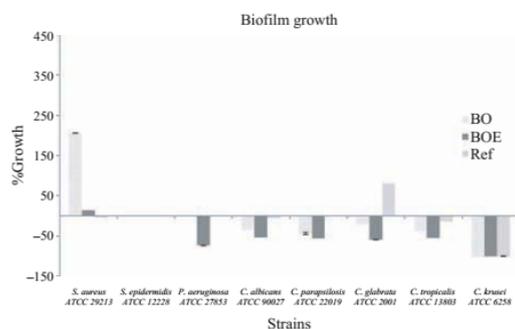


Figure 3. Percentage of biofilm growth in the presence of bullfrog samples. BO: Bullfrog oil; BOE: Bullfrog oil nanostructured emulsion; Ref: Chloramphenicol (bacteria) and Ketoconazole (fungi).

It is important to highlight that the biofilm formation is related to the severity of the infection. Therefore, its inhibition is a mandatory step to evaluate whether a product has a good antimicrobial activity, even if the concentration used to inhibit biofilm formation is below the MIC. Moreover, the nanostructured emulsion system here evaluated could be a therapeutic choice to treat patients without the use of synthetic antibacterial or antifungal agents.

4. CONCLUSION

Copaiba essential oil assembles the majority of sesquiterpenes, identified as main antimicrobial compounds, presented in the copaiba resin-oil while bullfrog oil contains a pool of omega fatty acids. Moreover, copaiba essential oil exhibited better antimicrobial activity than the similar resin-oil, especially against *Staphylococcus* and *Candida* species. Bullfrog oil and its nanostructured emulsion, on the other hand, showed no significant antimicrobial sensitivity against the tested strains in some experiments. However, the oleic acid, which was identified as the main antimicrobial compound in this product, exhibited antimicrobial activity against *P. aeruginosa*.

The nanostructured emulsions, even containing only 5% (w/w) of oil in its formulation, showed their importance in preserving and enhancing the antimicrobial activity of copaiba oils. Moreover, the nanostructured emulsion systems were able to improve the antimicrobial activities of the original oils, especially the bullfrog oil nanostructured emulsion, which demonstrated a poor antimicrobial activity. Additionally, this system showed a significant result concerning the biofilm inhibition against *Pseudomonas aeruginosa*, an important multidrug resistant pathogen responsible for hospital infections in immunocompromised patients.

Therefore, given the relevant antimicrobial and antibiofilm activities found for the copaiba essential oil and the bullfrog oil nanostructured emulsions against pathogenic species of bacteria and fungi involved with

cutaneous infections, it may be concluded that these formulations are suitable alternatives to develop new medicines for future use in the treatment of infections triggered by several microorganisms.

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Document III

*New Trends on Antineoplastic Therapy Research: Bullfrog (Rana catesbeiana Shaw)
Oil Nanostructured Systems*

1. Introduction

Ethnopharmacological exploration concerning the bio-prospection of drug discovery based on natural sources has become quite common since the isolation of new pharmacologically active compounds, increasing the therapeutic arsenal against various diseases [1–5]. In this context, natural oils have been widely used in the folk medicine for the treatment of several diseases due to their rich content of secondary metabolites and fatty acids [6]. These oils have a renewable character [7], low toxicity, high biodegradability, and presence of substances with pharmacological activity [8].

Bullfrog oil is a natural oil extracted through biotechnological reuse of adipose tissue, from the amphibious *Rana catesbeiana* Shaw, which is usually discarded by food industries after the bullfrog meat processing [9,10]. Previous chemical characterization of the bullfrog oil suggests a pharmacological potential due to the presence of fatty acids such as oleic, linolenic, stearic, palmitic, and myristic acids [10,11]. The oil *in-nature* has been used in folk medicine due to its inflammatory, antioxidant, antiedematogenic, and antimicrobial activities [9]. However, although there are studies showing that the presence of fatty acids can promote the viability reduction of tumor cells due to damage to the cellular membrane [12], there are no studies that evaluate this activity on bullfrog oil. Additionally, its unpleasant organoleptic characteristics, its low spread ability, its poor skin absorption, and its low viscosity can contribute to low patient adherence to the treatment [9].

Based on these advantages, the development of an emulsified system based on bullfrog oil would become a simple and effective alternative to its use. Emulsified systems are thermodynamically unstable dispersions, composed of a mixture of two or more immiscible liquids stabilized by surfactants. According to the physicochemical properties of a specific oil, a suitable surfactant mixture can efficiently stabilize the droplets into an aqueous solution. The composition of this mixture is assessed by the evaluation of its hydrophile-lipophile balance (HLB) [13,14], which is an empirical number proposed by Griffin [15] that affects the stability and the type of emulsion systems [15–17].

The aim of this study was to develop a stable nanoemulsion containing bullfrog oil for topical application. Initially, the oil was extracted and characterized, followed by HLB study. A pseudo-ternary phase diagram was plotted according to the visual aspect of different samples prepared by mixing the oil, water, and surfactants, and a point was chosen to produce the bullfrog oil nanoemulsion system. In addition, the *in vitro* biocompatibility and cytotoxicity were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for normal fibroblasts (3T3) and melanoma (B16F10) cell lines, respectively.

2. Results and Discussion

2.1. Extraction and Physicochemical Characterization of the Bullfrog Oil

The extraction of bullfrog oil was performed following two different methods in order to produce the BOH (bullfrog oil extracted by hot method) and BOHx (bullfrog oil extracted using n-hexane as organic solvent). The yields of bullfrog oil extractions were 60.6% and 74.8% concerning the fatty tissue used in the extraction processes for hot and solvent extractions, respectively. Differences in the yield of oil extraction can be obtained using various techniques in association with changes in temperature, solvents, and pressure [18]. Lopes and colleagues [10] evaluated bullfrog oil extraction yield under different temperatures and found that the income is directly proportional to the extraction temperature, which corroborates our findings.

The quality of the oils extracted was evaluated by following their physicochemical characterization. These parameters were important because they can be used to evaluate the quality of the bullfrog oil extracted, since the standards of the bullfrog oil were not available in the literature [19]. The acid index (AI) was used to determine the hydrolytic degradation of the bullfrog oil, as high AI suggests the presence of water [19]. BOH and BOHx showed the same AI (2.9 mg of KOH/g of oil) (Figure 1), demonstrating good preservation of bullfrog oil after the extraction process. The iodine index (II) was performed to evaluate the degree of unsaturation of the fatty acids in the oil, allowing

may be related to both the feeding of these amphibians and the climatic conditions of the frog farms. The results obtained are relevant, considering that the arachidonic acid acts as a proinflammatory agent stimulating the synthesis of leukotrienes and prostaglandins, promoting leukocyte migration and accelerating the onset of the first phase of the skin healing process [26–29]. EPA and DHA play important roles in the human body, such as preventing cardiovascular disease and improving mental capacity [30], besides providing greater permeability of drugs in topical treatments [31].

Table 1. Chemical characterization by GC-MS of the bullfrog oil obtained by hot extraction (BOH).

Substance	Retention Time (min)	Concentration (%)
Myristic acid	10.3	1.4
Arachidonic acid	12.0	8.4
Palmitic acid	12.2	10.3
EPA, Timnodonic acid	13.7	17.6
Oleic acid	13.7	29.9
Stearic acid	14	2.5
DHA, Cervonic acid	16.5	0.8
Cholesterol	20.6	9.5
Ethyl iso-allocholate	27.7	3.5
Total		83.9
Not identified		16.1

GC-MS (gas chromatography mass spectrometry); EPA (eicosapentaenoic acid); DHA (docosahexaenoic acid).

Ethyl iso-allocholate was also identified in the BOH sample, even though it was not described in previous studies. This bioactive compound presents antioxidant, antibacterial, anti-tumor, cancer preventive, diuretic, anti-inflammatory, and anti-asthma properties [32,33].

The presence of all these compounds with therapeutic activities in the bullfrog oil makes it especially suitable for topical use, mainly by its compound interaction with ceramides, reducing skin dehydration [11,25,34]. Therefore, the bullfrog oil could present several therapeutic applications in the context of different diseases, with great pharmacological potential, mainly considering its low cost production through biotechnological processes.

2.2. Pseudo-Ternary Phase Diagram (PTPD) for Bullfrog Oil Compositions

Table 2 shows the physicochemical properties of different bullfrog oil nanoemulsions prepared by using a surfactant blend composed of Tween® 20 and Span® 80 at distinct weight:weight (*w/w*) ratios (HLB_r from 4.5 to 15.5). HLB is an important parameter to produce stable emulsified systems, since the mixtures must interact better with a specific oil phase [35]. In the first batch, it was possible to observe that nanoemulsions with HLB between 12.5 and 13.5 showed the smallest droplet size variations. No significant differences between the systems at HLB 12.5 and 13.5 were established, due to the small droplet size variation (259.9 nm up to 275.6 nm) and the lowest polydispersity (PDI) (0.190 up to 0.218). This interval was considered the most capable of producing stable nanoemulsion systems. In addition, the lowest creaming rates were also observed for the same interval (2.8% at 3.7%). All samples showed an acid pH, which is the result of the large number of long chain fatty acids presented in the chemical composition of the bullfrog oil.

In a second step, this HLB range was investigated by using smaller variations (about 0.1) with an interval of confidence corresponding to half of the variation of the HLB range used in the first batch. Table 3 shows the results obtained for the characterization of the nanoemulsion systems for the second batch (HLB_r ranged from 12.0 to 14.0) after 60 days at 25 °C. The higher creaming rate observed in the bullfrog nanoemulsion was 3.2% (±0.8) for the formulation with the HLB 13.6 (Table S1). The pH remained weakly acid during all the studied time, ranging from 5.0 (±1.6) to 5.6 (±1.4). The pH value is an important factor in the stability of the emulsified systems, particularly when the emulsifier agents are obtained by the saponification process, since the acid compounds may promote destabilization

The pseudo-ternary diagram is a useful tool in the development of dispersed systems since it is possible to obtain the optimal compositions able to produce the required system [46–51]. Different emulsified systems such as emulsion, microemulsion and nanoemulsion, can be characterized by this method. These systems differ in their thermodynamic stability and physicochemical properties [45,52]. Indeed, microemulsion is defined as a thermodynamically stable system, whereas emulsion and nanoemulsion are considered unstable. However, nanoemulsions, which present a smaller droplet size (around 300 nm) than emulsions, presented a better kinetic stability [53]. A large region of emulsified systems was obtained during the pseudo-ternary construction (68%) (Figure 2). Different emulsion systems with water ratio ranging from 20% to 80% or with oil ratio up to 60% were observed. Similar results were observed for emulsions prepared with mono-, di- and triglyceride of medium chain fatty acids as oil phase (30% to 50%) [51] and Espinosa and colleagues [47] showed the formation of the emulsion with lipid concentrations ranging from 5% to 70%. The presence of clear flow-resistant dispersions (gels, 2%) and clear and transparent liquid dispersions (microemulsions, 4%) were also observed. The pseudo-ternary phase diagram was successfully used in similar studies as a tool to determine the best concentration of components for emulsified systems [48–50]. Therefore, in order to guarantee a suitable topical nanoemulsion, the oil-in-water (O/W) basic nanoemulsion formulation containing 12% of bullfrog oil, 8% of surfactant blend (Tween® 20:Span® 80 6.29:3.71 ratio), and 80% of distilled water was chosen for further studies.

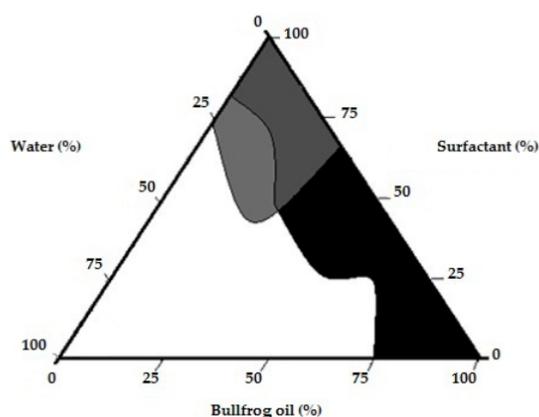


Figure 2. Pseudo-ternary phase diagram of the bullfrog oil produced with HLBr 12.1. Black (phase separation), dark gray (microemulsion), medium gray (gel), white (emulsion).

2.3. Development of a Topical Bullfrog Oil Nanoemulsion

Some additives were added to the chosen nanoemulsion point on the PTPD studies in order to improve the organoleptic characteristics of the bullfrog oil. The effect of these substances on the stability of the bullfrog oil nanoemulsion was evaluated for 90 days. The basic nanoemulsion sample (without additives) was used as a control.

Macroscopic aspects such as white color and absence of the characteristic odor of bullfrog oil remained in the samples throughout the studied time. The basic nanoemulsion presented himself as a fluid system; however, this characteristic was changed in the topical nanoemulsion due to the addition of xanthan gum at 1%, which generated a high viscosity system. The creaming rate value of about 2.4% was observed for the basic nanoemulsion. A similar result (about 2.5%) was observed in the HLBr study. Nevertheless, the topical nanoemulsion did not show creaming at all, which probably occurred due to the high viscosity found in these systems. This feature reduces the motility of the droplets, improving the emulsion stability [54]. The creaming rate is an important parameter to evaluate the stability of the emulsion systems, since it precedes the phase separation phenomena.

2.4. Biocompatibility and Cytotoxicity Study

The biocompatibility of the 3T3 and the cytotoxicity of B16F10 cells treated with bullfrog oil or topical nanoemulsion at three different concentrations for 24 h, 48 h, and 72 h, respectively, was provided by the colorimetric MTT assay (Figure 4).

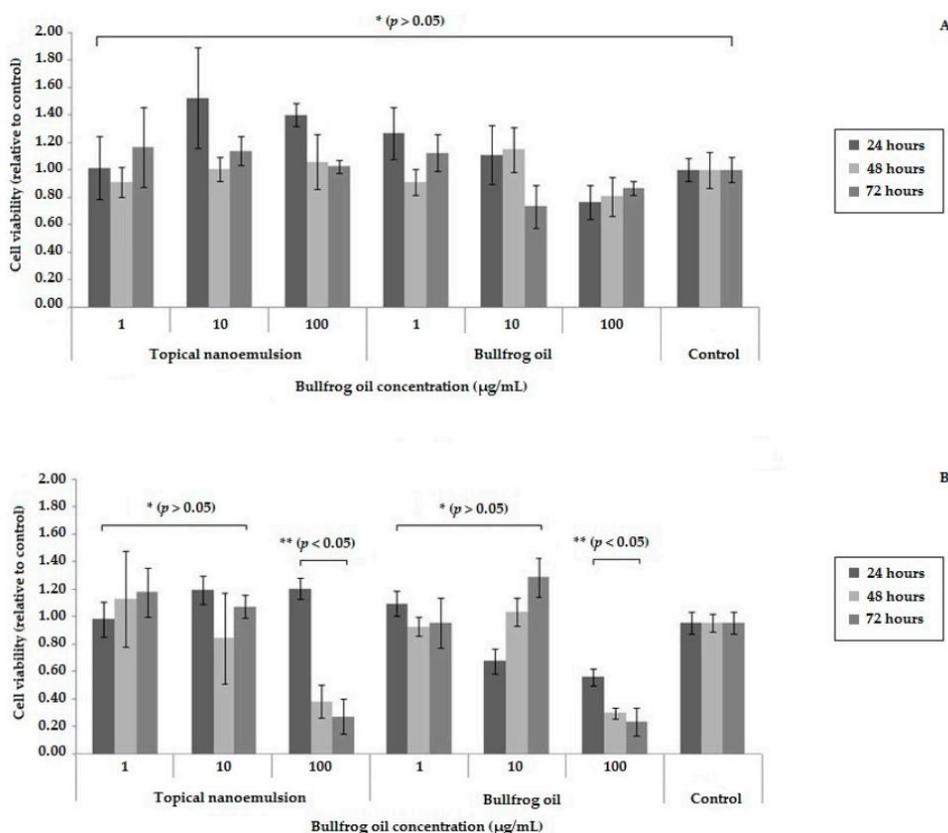


Figure 4. Cell viability of the topical nanoemulsion and the bullfrog oil at three concentrations. (A) 3T3 cell viability; (B) B16F10 cell viability. Dark gray: 24 h; Light gray: 48 h; Medium gray: 72 h. * No statistical difference compared to the control. ** Statistical difference compared to the control.

Compared to the positive control, 3T3 cell growth showed no statistical difference when treated with bullfrog oil or topical nanoemulsion samples ($p > 0.05$). These results were observed for all samples over 72 h (Figure 4A). Therefore, both samples presented biocompatibility at these concentrations for 3T3 cells, suggesting that the bullfrog oil may be suitable to be applied into a formulation. This is an important result because the safety knowledge of the natural products is very important for food and pharmaceutical industry application [3,4,9].

Similarly, results obtained for the melanoma cell line B16F10 showed that the lower concentration of both bullfrog oil and topical nanoemulsion (1 µg/mL and 10 µg/mL) did not interfere with the cell viability. However, at higher concentrations (100 µg/mL), the viability of B16F10 cells was reduced over the time. In the first 24 h, it was observed that the bullfrog oil promoted a greater inhibition of cell growth compared to topical nanoemulsion, and this occurs because the bullfrog oil is internalized in the droplet, requiring more time to be delivered to the cellular membrane. However, cell growth

inhibition was not significantly different after 48 h of cell culture. Both the topical nanoemulsion and the bullfrog oil showed concentration and time-dependent cytotoxicity against B16F10 cell lineage. Similar results were obtained by our team with other tumor cell lines (cervical tumor cells—(HeLa) and liver tumor cells—(HepG2)) [60].

This potential antineoplastic activity of the topical nanoemulsion may be related to the presence of ethyl iso-allocholate and fatty acids, which were identified in the bullfrog oil. Indeed, some studies have reported the anti-tumor activity of these compounds [12,32].

Based on these results, it is possible to suggest that the bullfrog oil is a safe natural oil compound. Therefore, using it *in natura* form or adding it to a nanoemulsion system for topical use would be suitable, as it shows no cytotoxicity. However, when incorporated in a nanoemulsion system, an improvement in the organoleptic characteristics of the bullfrog oil was observed. Additionally, the bullfrog oil and the topical nanoemulsion showed inhibitory activity on the cellular growth of B16F10 cells, suggesting that this system would be a potential anti-melanoma agent. However, further *in vitro* studies with other normal and melanoma cell lines need to be conducted to confirm this hypothesis.

3. Materials and Methods

3.1. Materials

Bullfrog adipose tissue was kindly provided by the Asmarana Produtos Naturais (Natal, RN, Brazil). Span[®] 80 (sorbitan monooleate 80), penicillin-streptomycin, and MTT reagent ((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Ethanol, dimethylsulfoxide (DMSO), n-hexane, propylene glycol, and Tween[®] 20 (polyoxyethylene 20 sorbitan monolaurate) were acquired from VETEC (Rio de Janeiro, RJ, Brazil). Cetostearyl alcohol ethoxylate and isopropyl palmitate were purchased from ViaFarma (São Paulo, SP, Brazil). Xanthan Gum and Germall[®] were purchased from Mapric (São Paulo, SP, Brazil). Butylhydroxytoluene was acquired from Galena (Campinas, SP, Brazil). Floral green fragrance was obtained from Bio Inter (São Paulo, SP, Brazil).

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Cultilab (Campinas, SP, Brazil). The 3T3 fibroblasts and B16F10 cell lines from the American Type Culture Collection (ATCC) used in this study were provided by the Laboratory of Biotechnology of Natural Polymers at Federal University of Rio Grande do Norte (Natal, RN, Brazil).

3.2. Methods

3.2.1. Bullfrog Oil Extraction

Two bullfrog oil samples were extracted from the amphibian's adipose tissue by two different methods. The first bullfrog oil sample (BOH) was produced following the method adapted from Lopes and colleagues [10]. The adipose tissue was heated under controlled magnetic stirring (IKA[®], RH basic model KT/C, Staufen, Germany) at 80 °C for 40 min. The second bullfrog oil sample (BOHx) was obtained by the solvent-extraction technique by adding n-hexane in the adipose tissue followed by Ultra Turrax[®] T-18 stirring at 5000 r.p.m (IKA[®], Staufen, Germany). Then, the solvent was evaporated in a rotary evaporator (Quimis Diadema, SP, Brazil) for 30 min at 40 °C. After the extraction process, both bullfrog oil samples were filtrated in membranes of 0.45 µm (Merck Millipore, Hessen, Germany) and stored in amber glass bottles at room temperature.

3.2.2. Physicochemical Characterization of Bullfrog Oils

Physicochemical analyses of bullfrog oil samples (BOH and BOHx) were carried out by titration according to the methodologies described in the United States Pharmacopeia (USP 35) [61] for the iodine index (II) and in the American Oil Chemists Society guidelines [19] for the saponification index (SI), acid index (AI), and peroxide index (PI). The II was evaluated by titration of the samples (200 mg),

3.2.6. Development of Topical Nanoemulsion Based on Bullfrog Oil

A basic nanoemulsion was defined as an O/W-emulsified system containing high oil concentration and low surfactant concentration without additives obtained from the pseudo-ternary phase diagram. In order to improve the organoleptic properties and the stability of the systems containing bullfrog oil, a topical nanoemulsion with additives was produced (Table 4). The basic and topical nanoemulsions were prepared by the phase inversion technique, as previously described in Section 3.2.4.

Table 4. Composition of the topical nanoemulsion.

	Excipients	% (w/w)	Function
Aqueous phase	Tween® 20	5.03	Surfactant
	Propylene glycol	4.00	Humectant
	Germall	0.30	Antimicrobial preservative
	Xanthan gum	1.00	Stabilizing agent
	Distillated Water	62.55	Dispenser agent
Oily phase	Span® 80	2.97	Surfactant
	Butylhydroxytoluene	0.10	Antioxidant
	Cetostearyl alcohol ethoxylate	8.00	Viscosity-increasing agent
	Isopropyl palmitate	4.00	Emollient and skin penetrant
	Bullfrog oil	12.00	Oil
After preparation	Fragrance	0.05	Essence

3.3. Characterization of the Emulsions

Characterization studies were performed for basic and topical nanoemulsions containing bullfrog oil.

3.3.1. Macroscopic Aspect

Organoleptic changes (color and odor), as well as the presence of emulsion instability phenomena (creaming or phase separation) were determined by visual observation of the samples stored in scintillation vials at 25 and 45 ± 2 °C. The storage time for the HLB and stability studies was 60 and 90 days, respectively.

3.3.2. pH and Conductivity Evaluation

The pH and the electrical conductivity of the nanoemulsions were evaluated in triplicate by pH-meter (Tecnal, TEC-2, Piracicaba, SP, Brazil) and conductometer (Digimed, DM-32, São Paulo, SP, Brazil) pre-calibrated at 25 ± 2 °C, respectively.

3.3.3. Droplet Size Distribution and Zeta Potential Analysis

The hydrodynamic diameter and the size distribution of the nanoemulsions were determined in triplicate by dynamic light scattering using a ZetaPlus (Brookhaven instruments, Holtsville, NY, USA) apparatus at 25 °C. The scattered angle was fixed at 90°. Samples were diluted at 1:100 with distilled water before analysis. Results were expressed as the mean hydrodynamic diameter, the standard deviation of the size distribution, and the polydispersity index (PDI). The zeta potential of the nanoemulsions was measured by Laser Doppler Electrophoresis using a ZetaPlus (Brookhaven instruments, Holtsville, NY, USA) apparatus. To maintain a constant ionic strength, samples were diluted (1:100) in saline solution (NaCl) at 1 mM. All results corresponded to the average of three determinations.

3.4. Stability Study of the Emulsion

A stability study for over 90 days for the basic and the topical nanoemulsions containing bullfrog oil was performed by using three different approaches.

First, the micro-emultocrit technique (short-term stability) [17] was assessed to evaluate the creaming rate presented by the emulsions in a micro-centrifuge (Microspin, SPIN 1000 model, Equipar, PR, Brazil) at 11,500 r.p.m for 10 min. Second, the freeze/thaw cycle stability was performed by methodology adapted from Roland and Xavier [42,57] when the emulsified systems were added in hermetically sealed tubes, vertically stored for 24 h in a freezer at $-5\text{ }^{\circ}\text{C}$, followed by another period of 24 h at $45 \pm 2\text{ }^{\circ}\text{C}$. Cycles were repeated six times and the changes were recorded. Finally, the stability under centrifugation was determined by using 10 mL of the emulsion, placed in the centrifuge at 3000 g for 30 min at $25\text{ }^{\circ}\text{C}$.

3.5. Biocompatibility and Cytotoxicity Study

Two representative cell lines of skin tissue, fibroblasts (3T3) and melanoma (B16F10) lineages, were used for the biocompatibility and cytotoxicity evaluation, respectively. The assay was performed in four replicates for each cellular line and at three different concentrations of bullfrog oil or nanoemulsion (1 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$). To attain such concentrations, the bullfrog oil was firstly diluted to 1% (*v/v*) with DMSO, and, then, both the bullfrog oil DMSO solution and the topical nanoemulsion (amount corresponding to the same bullfrog oil concentration) were diluted with DMEM medium. DMEM medium was also used as the positive control. Then, 100 μL of cells in DMEM medium supplemented with 10% of fetal bovine serum were placed into 96-well plates (5×10^4 cells/well) and incubated overnight at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 for a period of 24, 48, and 72 h in the presence of the aforementioned concentrations of topical nanoemulsion and bullfrog oil. Posteriorly, 100 μL of MTT reagent at 1 mg/mL was added to each well to analyze the cell viability. After 4 h, formazan crystals were dissolved in 100 μL of ethanol and the absorbance was measured in a Multiskan Ascent Microplate Reader (Thermo LabSystems, Franklin, MA, USA) at 570 nm. The biocompatibility and cytotoxicity was evaluated by the relative absorbance value between the control, the bullfrog oil, and the nanoemulsion system.

3.6. Statistical Analyses

All results were performed in triplicate and expressed as mean \pm standard deviation. Statistical significance among three or more groups was evaluated by the variance analysis (ANOVA) followed by the Tukey test for multiple means comparisons. Analyses between the two groups were performed by the Student's *t*-test. $p < 0.05$ was considered statistically significant.

4. Conclusions

The bullfrog oil, which showed a rich composition of unsaturated fat acids such as eicosapentaenoic acid (17.6%) and arachidonic acid (8.4%), was obtained by hot extraction from the adipose tissue of *Rana catesbeiana* Shaw. The HLB 12.1 produced a stable bullfrog oil emulsion. The basic nanoemulsion developed in this work was composed of Span[®] 80 (2.97%), Tween[®] 20 (5.03%), bullfrog oil (12%), and water (80%) (*w/w*). The selected additives improved the organoleptic characteristics of the bullfrog oil nanoemulsion for topical application, allowing the development of a stable nanoemulsion. The bullfrog oil and topical nanoemulsion showed biocompatibility in the normal fibroblast lineage. In addition, the bullfrog oil and the topical nanoemulsion showed a cytotoxicity activity over the melanoma cell lines (B16F10). Thus, this discovery may contribute to the design of new drugs for cancer therapy through the use of a natural product at low cost and with no toxicity.

Supplementary Materials: Supplementary materials can be accessed at: www.mdpi.com/1420-3049/21/5/585/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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Document IV

Experimental design approach applied to the development of chitosan coated poly(isobutylcyanoacrylate) nanocapsules encapsulating copaiba oil.

Keywords:
Nanocapsules
Copaiba oil
Chitosan
Poly(isobutylcyanoacrylate)
Experimental design approach

spherical objects when analyzed by transmission electron microscopy. The copaiba oil encapsulated in the nanocapsules showed all compounds of the parent oil. Nanocapsules with positive zeta potential were obtained consistently with the expected distribution of chitosan on the nanocapsule surface. Optimal nanocapsules showed a diameter of 473 nm, a zeta potential of +34 mV and an encapsulation efficiency of the oil of 74% including 55.5 µg of β-caryophyllene/mg of nanocapsules. The obtained nanocapsules can be suggested as oral delivery system for anticancer molecules including paclitaxel assuming a synergistic effect with anticancer active components of the oil.

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1. Introduction

Nanoparticles composed of mucoadhesive polymers are promising systems for oral drug delivery applications [1,2]. Generally, nanoparticles are defined as solid colloidal particles that include both nanospheres and nanocapsules [3]. The latter are vesicular systems in which the drug is confined in a liquid/solid cavity surrounded by a polymer envelope.

Proposed to improve drug delivery, nanoparticle systems can modulate drug biodistribution and release in a controlled manner, increase intracellular uptake and improve the stability of active substances [4–6]. Many types of nanoparticles made of biodegradable polymers, including poly(isobutylcyanoacrylate), were considered to improve drug delivery by oral route. To this purpose, their surface property may be tuned to increase mucoadhesion [7,8]. Chitosan has been a widely used polysaccharide to formulate mucoadhesive systems [9,10]. In addition, this polysaccharide is biocompatible for oral administration. Its inherent mucoadhesive properties come from the amino groups included in the chemical structure that can interact with sialic acid groups of mucins composing the mucus via electrostatic interactions [11]. Besides, the positive charges of chitosan are also believed to play an essential role increasing the permeability of the intestinal epithelium thanks to its capacity to disturb the calcium concentration balance near the tight junction [12]. Although widely used to improve mucoadhesion of nanospheres, this polysaccharide was not yet used to improve mucoadhesion of polymeric nanocapsules, which are interesting as drug delivery systems for lipophilic drugs.

The copaiba oil-resin (*Copaifera langsdorffii*) is an oily plant extract used in folk medicine in its *in-natura* form [13]. Phytochemical studies on oil-resin reveal that it contains a complex mixture of diterpene and sesquiterpene hydrocarbons [14], giving this oil many interesting therapeutic activities. For instance, these include anti-inflammatory, antitumor, anti-tetanus, antimicrobial, antileishmania activities, among others [15–17]. Although used for years in folk medicine, it is believed that pharmacological activities of this oil may be increased by developing appropriate formulations.

Therefore, the aim of the present work was to develop an original formulation of nanocapsules coated with chitosan as mucoadhesive compound and including copaiba oil in the reservoir of the vesicle. The polymer composing the nanocapsule envelope was a critical choice. Poly(isobutylcyanoacrylate) was selected because of its capacity to formulate nanocapsules that resist well to the gastric medium and promote release in the intestinal medium [18]. The development of mucoadhesive copaiba oil-containing nanocapsules of poly(isobutylcyanoacrylate) have not been described before. The formulation process was based on the use of an experimental design approach that was never applied so far while developing new formulations of oil-containing nanocapsules prepared by interfacial polymerization of isobutylcyanoacrylate. It is noteworthy that experimental design approach was not so much applied for the development of nanocapsules while such an approach could be helpful to optimize formulations,

limiting the number of experiments to perform hence the amount of reagent and time [19].

2. Materials and methods

2.1. Materials

Copaiba resin oil was purchased from Flores & Ervas (Piracicaba, SP, Brazil). Isobutylcyanoacrylate was provided by ORAPI engineered solutions worldwide (Vaulx-en-Velin, France). Water soluble chitosan (Mw 20,000 g/mol) was purchased from Amicogen (Jinju, South Gyeongsang, South Korea). Ethanol, acetone, 2-propanol, sodium hydroxide, ethyl acetate, nitric acid were provided by Fisher Scientific (Pittsburgh, PA, EUA). Diazomethane and β-caryophyllene were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was obtained from a Millipore purification system (Milli-Q® plus, Millipore, St Quentin en Yvelines, France). All chemicals were reagent grade and used as received.

2.2. Copaiba essential oil extraction

Copaiba essential oil was obtained from the hydrodistillation of 400 mL of copaiba resin oil using a Clevenger-type apparatus for 3 h. The extracted essential oil was dried with sodium sulphate, filtered, stored in the refrigerator and protected from light until use.

2.3. Preparation of the nanocapsules

Copaiba oil-loaded chitosan-decorated poly(isobutylcyanoacrylate) nanocapsules were elaborated by the method of interfacial polymerization [20,21] that was adapted because of the use of chitosan. A preliminary study investigated the influence of the molecular weight of chitosan (20, 70 and 250 kDa), the type of copaiba oil (resin and essential oil) and the nature of the solvent phase (ethanol, 2-propanol and acetone) in order to identify the best substances to produce nanocapsules. Thus, 0.25 mL of copaiba oil and 0.032 mL of isobutylcyanoacrylate were solubilized in 6.25 mL of solvent to produce the organic phase. This phase was slowly injected dropwise in 12.5 mL of the polymerization medium prepared with 0.6% of chitosan at pH 6 and homogenized for 10 min at 250 rpm at 25 °C (Fisher- Bioblock Scientific AM 3001 K, Illkirch, France). The obtained colloidal dispersion was concentrated by rotary evaporator for 20 min at 35 °C/43 mBar (BÜCHI Rotavapor R-125, Heating Bath B-491, Vacuum pump V-700, recirculating Chiller F-108, Flawil, Switzerland) to eliminate the solvent. Then, the dispersion was filtered through a 5 µm minisart NML membrane (Sartorius GmbH, Goettingen, Germany). The obtained nanocapsule dispersions were purified by dialysis (Spectra/Por Biotech membranes, cellulose ester, 100,000 g/mol molecular weight, Rancho Dominguez, CA, USA) against ultrapure water three times for 60 min and once overnight to remove non-associated chitosan.

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model, *t*-tests and *F*-test with a 95% confidence level ($p < 0.05$) were performed. The softwares Graph Pad Prism (Version 5.0, La Jolla, CA, USA) and Statistic software (Version 7.0, StatSoft Inc., USA) were used to perform the statistical analysis of the data.

3. Results

Copaiba oil nanocapsules were obtained by designing new conditions of interfacial polymerization of poly(isobutylcyanoacrylate), performed with a bioactive natural oil, chitosan and in the absence of surface active compounds. As a first step of the work that aimed to identify the best conditions to be used to produce copaiba oil containing nanocapsules by interfacial polymerization, several experiments were performed to select suitable materials. To this aim, preparations of nanocapsules were performed with chitosan (0.6%) of different molecular weight (20, 70 and 250 kDa), with copaiba resin and essential oils and with different solvents including ethanol, 2-propanol and acetone at pH=6 and temperature of 25 °C. The solvents were chosen based on their miscibility with water, a condition to achieve the synthesis of nanocapsules by interfacial polymerization of alkylcyanoacrylates. Particle size and zeta potential of the obtained nanocapsules are summarized in Fig. 1.

The mean diameters of the nanocapsules ranged from 440 ± 8 nm to 932 ± 28 nm when they were prepared with copaiba resin oil and copaiba essential oil, respectively. Nanocapsules obtained with chitosan 20 and 70 kDa were spherical as observed by transmission electron microscopy. The morphology of nanocapsules suggests that they were formed by an oily core surrounded by a polymer envelope. The size of copaiba resin oil-loaded nanocapsules varied with the different solvents. The average particle size were 440 ± 8 , 669 ± 8 and 536 ± 7 nm, respectively ($p < 0.05$) for nanocapsules prepared with ethanol, 2-propanol and acetone (Fig. 1A).

All nanocapsules were characterized by positive values of zeta potential indicating the presence of chitosan on the nanocapsule surface. The charges of the nanocapsule envelope were statistically different whether they were prepared with copaiba resin oil or copaiba essential oil. Nanocapsules prepared with copaiba essential oil displayed the lowest zeta potential (less than +12 mV).

Based on results obtained from this first series of preparations, optimization of conditions for the preparation of chitosan-coated nanocapsules containing copaiba oil was pursued using chitosan 20 kDa, ethanol and copaiba resin oil. The experimental design approach used in this work represented an easy methodology to provide important data about the natural oil-containing nanocapsules limiting the number of experiments to be performed hence

the amount of reagent and time. Thus, the optimization was carried out following a 2^3 full-factorial experimental design approach with center points. Three independent variables were considered including pH of the polymerization media (x_1), the temperature of production (x_2) and the concentration of chitosan 20 kDa (x_3) (Table 1). The optimal parameters chosen were those which produced small size nanocapsules with positive surface charges.

The mean hydrodynamic diameter of the produced nanocapsules was strongly influenced by the selected variables, emphasizing their relevancy as critical parameters influencing the preparation process. The size ranged from 200 to 1200 nm. The Pareto chart (Fig. 2) gives the relative importance of the individual and interaction effects [22]. Initially, Pareto chart was created to analyse the individual standardized effects of studied variables, and later their interaction effects on the size and zeta potential, as well as it permitted the identification of the statistically significant variables for the process, with a significance level (ρ -value of 0.05). The length of each bar in the chart indicated the standardized effect of the corresponding variable on the response. Negative values indicated unfavorable or antagonistic effect for the development of nanocapsules with the desired characteristics, i.e. small size and positive zeta potential, while positive coefficients showed a favorable or synergistic effect.

As shown by the Pareto's chart and considering each variable separately, a decrease of the polymerization medium pH had a statistically positive effect in the decrease of the nanocapsule size. Furthermore, it was observed that the nanocapsule size decreased with an increase of the temperature while the concentration of chitosan had no significant effect on this parameter. Interactions between the different variables can be highlighted by investigating the combined influence of several variables. The Pareto's chart highlighted a clear significant interaction between the pH of the polymerization medium and the other studied variables, i.e. pH \times temperature and pH \times concentration. In the acid solution at high concentration of chitosan, the increase of the temperature from 5 to 45 °C induced an increase of the nanocapsule size from 250 to 1070 nm. In contrast, in basic polymerization medium, the temperature variation did not show significant size changes (approximately 930 nm). Opposite effect on the nanocapsule size was observed between the concentration of chitosan and the pH of the medium.

The analysis of variance was used to test the significance of independent variables and their interactions. The model's goodness of fit was checked by the coefficient of determination (R^2). The R^2 values provide a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions (fluctuation) [23]. The matrix of

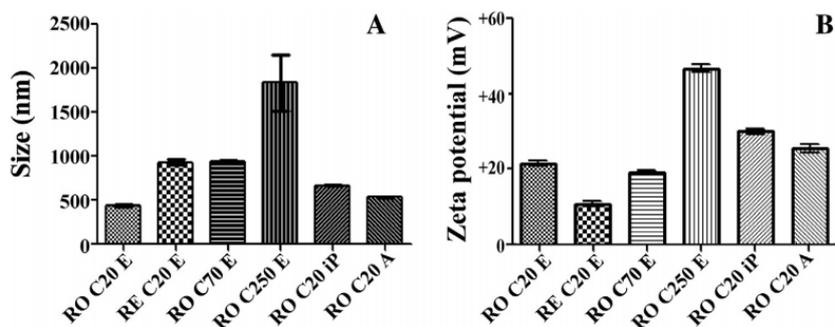


Fig. 1. Particle size (A) and zeta potential (B) of copaiba oil nanocapsules with chitosan coated in the surface at pH = 6 and temperature of 25 °C. Wherein RO and RE corresponds to nanocapsules produced with copaiba resin and essential oils, respectively; C20, C70 and C250 referred to the three different chitosan (0.6%) molecular weights of 20, 70 and 250 kDa; and E, IP and A indicated the type of organic solvent used, ethanol, 2-propanol and acetone respectively, used to nanocapsule production.

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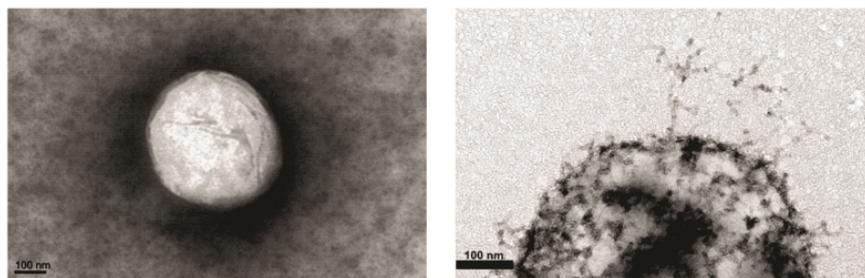


Fig. 4. Transmission electron microscopy analysis of the optimal formulation of copaiba oil-loaded chitosan-poly(isobutylcyanoacrylate) nanocapsules stained with phosphotungstic acid (2%) for 30 s and observed at 60 kV (Electron Microscopy facilities of I2BC, CNRS, Gif-sur-Yvette, France (<http://www.cgm.cnrs-gif.fr/spip.php?article282&lang=fr>)). Scale bar of 100 nm.

actions between each of the independent variables and the size of the nanocapsules obtained from the different tested conditions.

Regarding the zeta potential, the copaiba oil nanocapsules showed values ranging from +15 to +55 mV. The Pareto's chart showed a higher increase of the zeta potential of the nanocapsules increasing the temperature and decreasing the pH of the polymerization medium comparatively to the smaller influence revealed by varying the concentration in chitosan (Fig. 2). The first-order polynomial equation was established to zeta potential analyzing the responses given in the experiments (Eq. (3)):

$$Y_2 = 31.8 - 3.8x_1 + 6.4x_2 - 3.4x_1x_2 + 4.2x_1x_3 - 5.9x_2x_3 + 1.9x_1x_2x_3 \quad (3)$$

Where Y_2 was the predicted zeta potential (mV), x_1 , x_2 and x_3 were the coded terms for three independent test variables: the pH of the polymerization medium, the temperature at which the interfacial polymerization was carried out and the concentration of chitosan added in the polymerization medium, respectively.

These experiments presented the linearity regression of $R^2 = 0.94$, therefore this model explains 94% of the variability in the response. The value of the adjusted determination coefficient (Adj $R^2 = 0.92$) was accepted as high significance of the model. The F test applied in the mathematical model shows the significant ($F_{model} > F_{tab}$) and predictive results ($F_{residues}/F_{Tabresidues} < 1$) of these experiments. In addition, the full first-order response surface was plotted for analysis of the optimal zeta potential on the nanocapsules (Fig. 3B). Nanocapsules with high positive values of the zeta potential can be obtained at low pH of the polymerization medium and performing the polymerization at high temperature. However, increasing the temperature of the polymerization increased the size of the nanocapsules that was not a desired result. To define optimal conditions it was taken into account that the size decreased more than the zeta potential increased in the same range of variation of the temperature at which the polymerization was performed.

The result of the optimization study following a 2^3 full-factorial experimental design approach allowed to identify optimal conditions for nanocapsule preparation that have both a small particle size and positive zeta potential. The optimal characteristics of the nanocapsule preparation were pH 3, temperature 4°C and a concentration of chitosan of 0.9% (w/v). The samples prepared under these conditions showed a size of 473 ± 1 nm, the size distribution was unimodal with a polydispersion index of 0.20 ± 0.02 and a zeta potential of $+34.8 \pm 0.2$ mV. Observation of the sample by transmission electron microscopy showed that the optimal nanocapsules prepared by interfacial polymerization were spherical and well individualized (Fig. 4). They showed typical images of nanocapsules composed of an oily core surrounded by a polymer envelope.

The composition of copaiba oil recovered from the nanocapsules was determined by gas chromatography and compared with the oil

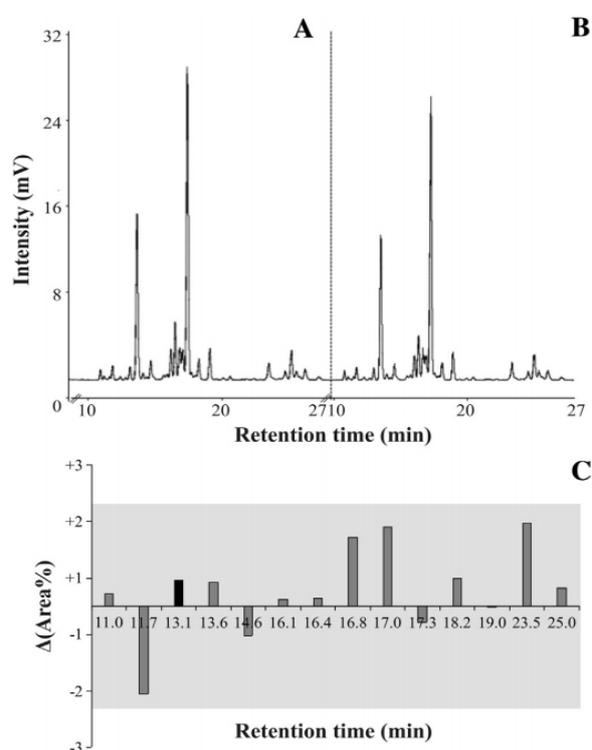


Fig. 5. Major compounds encapsulated in copaiba resin oil-nanocapsules coated with chitosan. Figures A and B represent the copaiba resin oil chromatograms before and after recovery from the nanocapsule. Figure C gives the difference in the area of the peaks between the copaiba resin oil in native form and the oil encapsulated in the nanocapsules. The gray color represents the precision of the method to determination of copaiba oil compounds.

prior to its nanoencapsulation (Fig. 5). All components found in the original oil were also found in the nanocapsules. Differences in concentrations of components present in copaiba oil versus the copaiba oil extracted from the nanocapsules were in the range of precision (below 2%) of the gas chromatography method used to perform the analysis (Fig. 5C). Results indicated that the encapsulated oil was identical in term of its composition than the initial oil. The nanoencapsulation process fully preserved the composition complexity of the oil. Therefore, the encapsulation efficiency of copaiba oil was high at $75.8 \pm 3\%$. It could be determined that the nanocapsule dispersion contained 2.5 mg of β -caryophyllene encapsulated in the

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RÉSUMÉ

La leishmaniose est une zoonose provoquée par un parasite protozoaire de l'espèce *Leishmania* transmise par la piqure d'un phlébotome femelle infecté souvent présent dans les zones tropicales et tempérées. Il en existe trois formes principales : la leishmaniose cutanée, la leishmaniose mucocutanée et la leishmaniose viscérale, cette dernière étant la plus sévère, car fatale si elle n'est pas traitée, avec des manifestations cliniques dans les tissus du système réticulo-endothélial dont la rate, le foie, les ganglions lymphatiques et la moelle osseuse.

Les traitements principaux sont les sels d'antimoine pentavalents, la miltéfosine, l'Amphotéricine B sous forme de micelles mixtes et l'Amphotéricine B sous forme de liposomes. Malgré leur prix abordable pour les pays pauvres où la maladie est endémique, les sels d'antimoine ainsi que la Miltéfosine ont des inconvénients tels que la toxicité et l'émergence rapide de résistances. Les formulations à base d'Amphotéricine B (AmB) sont efficaces contre la maladie mais la formulation conventionnelle en micelles mixtes, Fungizone[®] présente une toxicité limitant les doses qui peuvent être administrées tandis que les formulations à base de lipides telles qu'Ambisome[®] sont moins toxiques mais chères. Ainsi, le besoin de nouveaux systèmes thérapeutiques demeure. Dans cette optique, le chauffage de la Fungizone[®] (H-AmB) ainsi que la mise au point de microémulsions (ME) chargées en AmB (ME-AmB) représentent des solutions potentielles.

Les microémulsions (ME) sont des systèmes thermodynamiquement stables composés d'huile, d'eau et de surfactants. Ils peuvent transporter une molécule active dans la phase huileuse et ainsi la protéger contre les enzymes digestives et la lumière, augmenter sa solubilité apparente et améliorer son absorption et, de cette façon, accroître la biodisponibilité des molécules faiblement solubles dans l'eau. Cependant, la quantité importante d'eau dans les ME peut favoriser la croissance microbienne, la fuite du principe actif ainsi que l'hydrolyse des composants du système, conduisant à une perte d'activité thérapeutique. La lyophilisation – l'élimination d'eau par sublimation sous vide - pourrait pallier cet inconvénient. Cependant ce procédé peut déstabiliser le système, aussi est-il nécessaire d'évaluer l'impact de plusieurs paramètres de formulation sur l'échantillon. Un

plan d'expérience a donc été mis en œuvre afin d'optimiser la lyophilisation de nos formulations. Ainsi, les objectifs de ce travail ont été de développer des formulations H-AmB et ME-AmB et d'évaluer leur activité sur *Leishmania donovani* (souche LV9) ainsi que leur toxicité *in-vitro* et *in-vivo* et, de plus, de mettre au point une formulation lyophilisée de Me-AmB.

La formulation H-AmB a été préparée en chauffant la forme conventionnelle micellaire à 70°C pendant 20 minutes. Les MEs ont été préparées en mélangeant un tampon phosphate à pH 7,4, du Tween 80[®], du Lipoid S100[®] et du Mygliol 812[®] sous agitation magnétique suivie par trois cycles d'ultrasons. Afin d'incorporer l'AmB, la molécule a été ajoutée à la ME sous agitation continue, avec alcalinisation du milieu suivie de neutralisation.

H-AmB a été caractérisé par spectrophotométrie UV/vis et dichroïsme circulaire, tandis que pour la ME-AmB la taille de gouttelettes, la morphologie par microscopie électronique de transmission, le comportement rhéologique ainsi les spectres en UV/vis et dichroïsme circulaire ont été déterminés. Des expériences *in-vitro* et *in-vivo* ont été menées afin d'évaluer l'efficacité et la toxicité des formulations en comparaison avec les formes commerciales Fungizone[®] et AmBisome[®].

Avant la lyophilisation des systèmes, des cryoprotectants - mannitol, maltose, glucose, sorbitol ou lactose – ont été ajoutés. Ensuite un plan factoriel complet en 2³ avec trois répétitions au centre a été effectué. ME et ME-AmB ont été caractérisés en termes de taille de gouttelettes, pH, conductivité et contenu en AmB avant et après lyophilisation selon un protocole optimisé. Les déterminations de taille, morphologie et comportement rhéologique ont montré un diamètre moyen de gouttelette de 35 nm, une morphologie sphérique et un comportement newtonien. L'analyse spectrophotométrique d'H-AmB a révélé la formation de superagrégats, moins toxiques que d'autres types d'agrégats. L'activité anti-leishmanienne *in-vitro* de H-AmB et ME-AmB sur les parasites axéniques ainsi que sur les amastigotes à l'intérieur des macrophages est similaire à celle de l'AmBisome[®]. L'indice de sélectivité observé pour H-AmB et ME-AmB est plus grand que celui

de la Fungizone[®] non chauffée. De plus, les deux nouvelles formulations s'avèrent avoir une activité importante sur les souches résistantes à l'AmB, à la différence de l'AmBisome[®].

Des expériences *in-vivo* visant à évaluer leur activité et leur toxicité n'ont démontré de différence ni en activité ni en toxicité entre les nouvelles formulations et les formulations de référence. Il n'y avait pas de différence significative dans les indicateurs plasmatiques de toxicité rénale et hépatique. Par conséquent, H-AmB et ME-AmB pourraient tous les deux être considérés comme des traitements alternatifs pour la leishmaniose viscérale, tout en ayant des coûts de production inférieurs à ceux de l'AmBisome[®].

Concernant l'étude de la lyophilisation de la ME, il s'est avéré que l'addition de sorbitol modifiait la taille des gouttelettes, ainsi seuls le mannitol, le maltose, le glucose et le lactose ont été utilisés dans le plan d'expériences. Les résultats ont indiqué que les microémulsions avec les gouttelettes les plus petites étaient obtenues avec le maltose comme cryoprotectant à une concentration de 5%, une température de congélation de -80°C et une durée de lyophilisation de 24h. Les échantillons contenant du glucose ont collapsé, probablement à cause d'une température au-dessus de la température de transition vitreuse au cours du premier séchage. Certains échantillons contenant du mannitol ou du lactose présentaient un bon aspect de la poudre mais après reconstitution la taille des gouttelettes a augmenté ou le cryoprotectant est devenu insoluble. Quand la ME a été lyophilisée selon les paramètres optimisés, il n'y avait pas de différences de pH, conductivité ou taille des gouttelettes, susceptibles de déstabiliser le système. De plus, la quantité d'AmB de la ME-AmB n'était pas modifiée après lyophilisation.

En conclusion, la ME demeure stable et résiste à l'hydrolyse sous sa forme lyophilisée. De plus, la ME-AmB lyophilisée a le potentiel de devenir un nouveau traitement pour la leishmaniose viscérale.

Titre : Intérêt de la lyophilisation pour améliorer la stabilité des microémulsions chargées en Amphotéricine B destinées au traitement de la leishmaniose

Mots clés : amphotéricine B, microémulsions, leishmaniose viscérale, lyophilisation

Résumé : La leishmaniose viscérale est une maladie tropicale grave qui est létale sans traitement. Le médicament Amphotéricine B (AmB) est efficace mais la forme conventionnelle Fungizone® est dotée d'une toxicité considérable et la forme à base de lipides AmBisome®, moins toxique, est chère. Le but de cette thèse était de préparer et d'évaluer deux formulations alternatives d'AmB : la Fungizone® chauffée (H-AmB) et une microémulsion formulée à partir d'excipients peu coûteux (MEAmB). L'activité de ces formulations sur les parasites est comparable à celle des produits commercialisés et leur toxicité vis-à-vis des cellules humaines est moindre que celle de la Fungizone®.

De plus, elles ont montré une activité contre les parasites résistants à l'AmB. Dans un modèle animal de la maladie, les nouvelles formulations ont démontré leur activité sans engendrer de toxicité. Elles pourraient donc se substituer à l'AmBisome®.

Afin d'augmenter sa stabilité pendant le stockage et le transport, une forme lyophilisée de MEAmB a été mise au point. Ces résultats ouvrent la voie à de nouveaux traitements pour la leishmaniose plus accessibles pour les pays en voie de développement.

Title : Freeze-drying to increase stability of Amphotericin B-loaded microemulsion for leishmaniasis treatment

Keywords : Amphotericin B, microemulsion, visceral leishmaniasis, lyophilization

Abstract : Visceral leishmaniasis is a serious parasitic tropical disease that is fatal without treatment. Amphotericin B is effective against the parasites but has dose-limiting side-effects in its conventional form (Fungizone®) while a less toxic lipid-based form (AmBisome®) is expensive. The aim of this thesis was to prepare and evaluate two alternative formulations: heat-treated Fungizone® (H-AmB) and a microemulsion from low-cost ingredients (MEAmB). These formulations showed activity comparable to that of the commercial products against the parasites, and lower toxicity than Fungizone® to human cells.

Furthermore, they showed activity against parasites that were normally resistant to AmB. In an animal model of leishmaniasis, these new formulations were found to be effective and not to cause toxicity. Therefore, they could be used as a cheaper alternative to AmBisome®.

In order to improve its stability during transport and storage, a freeze-dried form of MEAmB was optimized. These results are promising for the development of new treatments for leishmaniasis that would be accessible in the countries where the disease is endemic.