UNIVERSIDADE D COIMBRA FACULDADE DE MEDICINA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

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Sucupira oil-loaded nanostructured lipid carriers: a new approach in diabetes mellitus management

PROJETO DE INVESTIGAÇÃO

ÁREA CIENTÍFICA DE FARMACOLOGIA

Trabalho realizado sob a orientação de:

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NOVEMBRO/2019

Sucupira oil-loaded nanostructured lipid carriers: a new approach in diabetes mellitus management

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Publications | Related to this master thesis there were meanwhile published the following two journal articles and a book chapter.

Vieira R, Souto SB, Sánchez-López E, Machado AL, Severino P, Jose S. *et al*. Sugar-Lowering Drugs for Type 2 Diabetes Mellitus and Metabolic Syndrome – Review of Classical and New Compounds: Part-I. Pharmaceuticals 2019;12(4):152

DOI: 10.3390/ph12040152; Part of ISSN: 1424-8247

Available at: https://doi.org/10.3390/ph12040152

Vieira R, Souto SB, Sánchez-López E, Machado AL, Severino P, Jose S. *et al*. Sugar-Lowering Drugs for Type 2 Diabetes Mellitus and Metabolic Syndrome – Strategies for In Vivo Administration: Part-II. Journal of Clinical Medicine 2019;8(9):1332

DOI: 10.3390/jcm8091332; Part of ISSN: 2077-0383

Available at: https://doi.org/10.3390/jcm8091332

Vieira R, Fortuna AC, Silva AM, Souto SB, Souto EB. Mechanism of Action and Toxicological Profile of Essential Oils in Foodstuff. In: Ramos OL, Pereira RN, Cerqueira MA, Teixeira JA, Vicente AA, (ed). Advances in Processing Technologies for Bio-based Nanosystems in Food. 1st ed. Boca Raton, FL: CRC Press, Taylor & Francis Group; 2019, 20.

DOI: 10.1201/9781315177328-10; Part of ISBN: 9781315177328

ABBREVIATIONS LIST

AIC – Akaike information criterion

BSA – Bovine serum albumin

CLSM – Confocal laser scanning microscopy

DDS – Drug delivery system

DLS – Dynamic light scattering

DM – Diabetes mellitus

DSC – Differential scanning calorimetry

EE – Encapsulation efficiency

ELS – Electrophoretic light scattering

EO – Essential oils

FT-IR – Fourier-transform infrared spectrometer

GC-MS – Gas-chromatography associated with mass spectrometry

GK – Goto Kakizaki

HbA1c – Hemoglobin A1c

HOMA-IR – Insulin resistance index

HPH – High-pressure homogenization

HPLC – High performance liquid chromatography

ITLC-SG – Instant thin-layer chromatography with silica-gel strips

LC – Loading capacity

NLC – Nanostructured lipid carriers

PBE – Phosphate-buffered saline

PdI – Polydispersity index

SEM – Scanning electron microscopy

SLN – Solid lipid nanoparticles

STZ – Streptozotocin

t1DM – Type 1 diabetes mellitus

t2DM – Type 2 diabetes mellitus

^{99m}Tc – Metastable technetium

TEM – Transmission electron microscopy

TPGS – D-α-Tocopherol polyethylene glycol succinate

XRD – X-ray diffraction

YP – Yield of production

z-AVE – Mean particle size

ZP – zeta potential

1. Project description

Scientific domain | Life and Health Sciences

Main scientific area | Diagnostic, Therapies and Public Health

Secondary scientific area | Pharmacology and Toxicology

Acronym | DTP-FTO

Project title (in Portuguese) | Incorporação de óleo de sucupira em transportadores lipídicos nanoestruturados: uma nova abordagem no tratamento da diabetes mellitus

Project title (in English) | Sucupira oil-loaded nanostructured lipid carriers: a new approach in diabetes mellitus management

Project start date | 1st December 2019

Duration | 36 months

Requested funding | 97 617.37 €

Keywords | essential oil, sucupira oil, diabetes mellitus, drug delivery systems, nanoparticles, nanostructured lipid carriers, cutaneous administration, transdermal patch, animal experimentation

2. Institutions involved and their roles

2.1. Proposing organization/ Principal contractor

University of Coimbra

Address | Pólo das Ciências da Saúde – Azinhaga de Santa Comba, 3000-548 Celas, Coimbra, Portugal

Institution description | In 1290 D. Dinis signed "*Scientiae thesaurus mirabilis*" thus founding the University of Coimbra (UC), the oldest public institution for superior education in the country. Having over 22000 students, UC is organized across 3 Campuses and comprises 8 Faculties (Literature, Law, Medicine, Science and Technology, Pharmacy, Economics, Psychology and Educational Sciences, Sports Sciences and Physical Education) and several teaching and research units. It is particularly notable in technology and science research fields. During 2018, UC had 40 active research centers, was involved in 474 research projects and published 2997 articles on the Web of Science. Both the Department of Pharmaceutical Technology (Faculty of Pharmacy) and Physiology Institute (Faculty of Medicine) have demonstrated great results among the Scientific Community over the years. Along with high

quality standards on scientific research, UC also plays an active role in the community and is considered UNESCO Mundial Heritage since 2013.

2.2. Participating institutions

Institute of Technology and Research

Address | Av. Murilo Dantas, 300, 49010-390 Aracaju, Brazil

Institution description | The Institute of Technology and Research (ITR) was founded in 1998 by the Tiradentes Group in order to provide adequate facilities and equipment to a proper science, technology and innovation development in Sergipe and consequently to give some visibility to Northeast Region of Brazil. Tiradentes Educational Society has initially brought together some researchers and since then ITR is related to several academic fields, having approved more than 500 projects with several institutions, thus establishing strategic research networks and strengthening both national and international partnerships with companies, public and private institutions and universities, which allow researchers internationalization and students exchange programs. In 2016, ITR opened a new facility in Maceió given its exponential growth. It now operates in 8 main areas (Energy, Environment, Biotechnology, Engineering, Education, Law, Health and Social Technologies) and is comprised of 19 research laboratories and 3 exclusive branches for provisional services, counting on 55 researchers. In particular, the Laboratory of Nanotechnology and Nanomedicine (LNMed) concerns about lipid, polymeric and metallic nanoparticles applied to drug encapsulation strategies, proteins and cosmetics, but also develops research regarding polymer membranes, hydrogels and grafts. Finally, ITR counts for 82 patents, deposited in the National Institute of Intellectual Property and via Patent Cooperation Treaty, and is co-holder in four patents.

University of Tiradentes

Address | Av. Murilo Dantas 300, 49032-490 Aracaju, Brazil

Institution description | Founded in 1962 and considered the second largest private higher education institution in Northeast Region of Brazil, University of Tiradentes (UNIT) is a coeducational for-profit institution with 5 learning poles situated in Aracaju, Serpige (Aracaju, Farolândia, Itabaiana, Propriá and Estância) and 25 more at distance. UNIT has modern infrastructures and laboratories equipped with the latest technology just to ensure a training of excellence for professionals in several areas of interest, namely human, health, exact and technology sciences. In 2014, UNIT consisted of the "Cambridge University Excellence Guide" as reference of great quality in education and, in 2015, was awarded by the Program "100.000 Strong in the Americas Innovation Fund" for student exchange, sponsored by ExxonMobil. The

initiative aiming the internationalization of the higher education contributed to accelerate the establishment of Tiradentes Institute in Dorchester (Boston), in 2017. Finally, Grupo Tiradentes was founded as a corporative entity of institutional management from UNIT (Serpige), Tiradentes University Center (Maceió), Integrated Faculty of Pernambuco (Recife) and Tiradentes Faculty (Jaboatão dos Guararapes). It is now considered one of the major educational groups in Northeast Brazil.

Tiradentes Institute

Address | 150 Mt Vernon St, Dorchester, MA 02125, USA

Institution description | Tiradentes Institute (TI) is a study center established in 2015 as a result from the partnership between Tiradentes Group and Massachusetts Boston University (UMass Boston). It aims the development of scientific research and to encourage knowledge sharing, student exchange and professional experiences abroad, in order to better motivate and qualify human resources in a globalized world. The TI was inaugurated in 2017 at the UMass Boston Campus and provides orientation programs regarding academic mobility and free courses, particularly in Law, Engineering and Health Sciences, to prepare students or even teachers and researches that mean to spend some time abroad. Unprecedentedly, there has been a great UMass Boston effort in promoting other partnerships between the TI and several American institutions maintaining the main objective: to develop scientific research of quality and to allow students and professionals to have some experiences abroad.

São João Hospital

Address | Alameda Prof. Hernâni Monteiro, 4200–319 Porto, Portugal

Institution description | The Centro Hospitalar Universitário de São João (CHUSJ), officially inaugurated in 1959, is located in Oporto and provides direct assistance to patients from Oporto, Bonfim, Paranhos, Campanhã, Aldoar, Maia and Valongo. CHUSJ is also part of the Referencing Hospital Networks thus providing healthcare to populations in distant geographic areas. The hospital is organized in multiple services and organic units and has room for 1105 beds and 45 cradles. The institution has a close relationship with the Faculty of Medicine from University of Oporto thus contributing to research developments in medical-related issues. CHUSJ aims to give the best healthcare with elevated levels of competence, excellence and rigor, promoting pre- and pos-graduated training and investigation, always respecting the humanization principle.

University of Trás-os-Montes e Alto Douro

Address | Quinta de Prados, P-5001-801 Vila Real, Portugal

Institution description | The University of Trás-os-Montes e Alto Douro (UTAD), located in Vila Real, was founded in 1973 as Instituto Politécnico de Vila Real but was only raised to University of Trás-os-Montes e Alto Douro in 1986. UTAD is a high-level institution focused on the creation, transmission and diffusion of culture, knowledge and science. It promotes entrepreneurship in a close relationship with the community (citizens, institutions and business-related subjects), deepens scientific knowledge, develops technology and tries to respond to several global and national issues. Settled in an eco-campus integrating one of the largest Botanical Gardens in Europe, UTAD offers modern facilities, libraries, laboratories, online services and sports infrastructures and its Social Services are known for their excellence. UTAD comprises 5 schools (agrarian and veterinary sciences, human and social sciences, science and technology, life and environmental sciences and health school) and 17 research centers. Among these it may be highlighted the Centre for Research and Technology of Agro-Environmental and Biological Sciences (CITAB), which is focused on the agro-food and forestry systems using the production-chain approach as a whole. CITAB researchers defend the use of natural resources to produce multipurpose biofuels and biomaterials thus solving human-related issues and preserving ecosystems sustainability in parallel.

University of Naples Federico II

Address | Via D. Montesano 49, 80131, Naples, Italy

Institution description | The University of Naples Federico II (UniNa) dates to 1224, when the Swabian emperor and the king of Sicily issued the institutive edict, thus being the oldest public non-secretarian university in the world. It comprises a great infrastructure, consisting of 13 faculties, 4 schools, 26 departments, 49 inter-departmental centers and also libraries, museums, a botanic garden, agricultural company, among others. UniNa is considered one of the best universities in Italy, being notably recognized for research. In 2015 it was ranked among the top 100 universities in the world by citations per paper and, since 2016, it is considered the only generalist Italian university in the Times higher education reputation (compared to 200 other universities worldwide). Also, in 2016, UniNa hosted the first ever Apple IOS Developer Academy and then, in 2018, the Cisco Digital Transformation Lab. Several professors from various disciplines are among the top Italian Scientists by H-index and, in 2018, expertscape recognized UniNa as the world's 10th expertise in Celiac Disease.

2.3. Principal research unit

Department of Pharmaceutical Technology, Laboratory of Development and Drug Technologies, Faculty of Pharmacy, University of Coimbra (FFUC) | Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Celas, Coimbra, Portugal

2.4. Additional research units

Institute of Physiology, Faculty of Medicine, University of Coimbra (FMUC) | Subunidade 1, Pólo III, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Celas, Coimbra, Portugal

Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra | Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Celas, Coimbra, Portugal

Institute of Technology and Research (ITR), Laboratory of Nanotechnology and Nanomedicine (LNMED) | Av. Murilo Dantas, 300, 49010-390 Aracaju, Brazil

Industrial Biotechnology Program, University of Tiradentes (UNIT) | Av. Murilo Dantas 300, 49032-490 Aracaju, Brazil

Tiradentes Institute (TI) | 150 Mt Vernon St, Dorchester, MA 02125, USA

Department of Endocrinology, Hospital de São João (HSJ) | Alameda Prof. Hernâni Monteiro, 4200–319 Porto, Portugal

Department of Biology and Environment, University of Trás-os-Montes e Alto Douro (UTAD) | Quinta de Prados, P-5001-801 Vila Real, Portugal

Centre for Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro (UTAD) | Quinta de Prados, P-5001-801 Vila Real, Portugal

Department of Pharmacy, University of Napoli Federico II (UniNa) | Via D. Montesano 49, 80131, Naples, Italy

2.5. Host institution

University of Coimbra

Pólo das Ciências da Saúde, Azinhaga de Santa Comba

3000-548 Celas, Coimbra, Portugal

3. Scientific component

3.1. Abstract

3.1.1. In Portuguese

Os óleos essenciais são produtos líquidos aromáticos que consistem numa complexa mistura de compostos voláteis obtidos a partir de matéria-prima vegetal e que têm cada vez mais provado ser potenciais agentes naturais no tratamento de várias patologias humanas.[1,2] Sendo considerada um grande problema de saúde pública, deve ser prestada uma especial atenção à diabetes mellitus (DM), uma doença metabólica caracterizada por hiperglicemia crónica e que se pode apresentar sobretudo de duas formas: DM tipo 1 (DMt1), que resulta da destruição de células β-pancreáticas secretoras de insulina sobretudo por um processo autoimune, e DM tipo 2 (DMt2) que, por sua vez, se deve a insulinorresistência periférica e hepática e deficiência relativa de insulina.[3,4] Nestes doentes, a hiperglicemia correlaciona-se com um risco aumentado para doenças micro- e macrovasculares, tais como doença cardíaca coronária, doença vascular periférica e doença cerebrovascular.[4] Assim, terapêuticas que tenham um efeito positivo na redução dos níveis de glicose são a estratégia privilegiada no tratamento da DM. Infelizmente, os fármacos atualmente disponíveis têm evidenciado cada vez mais frequentemente efeitos adversos, perda de eficácia ao longo do tempo, elevado custo e adesão subótima do doente à terapêutica.[3] Neste sentido, novas abordagens para o tratamento da DM devem ser exploradas. O nosso grupo pretende encapsular óleo essencial de sucupira, obtido a partir dos frutos da planta brasileira *P. emarginatus* em transportadores lipídicos nanoestruturados (NLC), nanopartículas lipídicas de segunda geração que consistem num novo sistema de transporte controlado de fármacos. Este sistema assegura a preservação das propriedades físico-químicas do óleo até que seja alcançado o seu alvo biológico, evitando desta forma alguns inconvenientes inerentes aos óleos essenciais, nomeadamente a sua lipofilicidade, hidrofobicidade, volatilidade e elevada suscetibilidade a fatores externos.[1,5-7] São reconhecidas várias atividades biológicas do óleo de sucupira,[8] embora ainda sem evidência científica do seu efeito anti-hiperglicémico até à data. NLC com óleo de sucupira incorporado serão produzidas por homogeneização a alta pressão a quente e minuciosamente caracterizadas quanto às propriedades físico-químicas e morfologia, usando as técnicas difusão e eletroforese dinâmicas de luz, microscopia eletrónica de varrimento e transmissão e difração de raios-X. De seguida, ensaios de estabilidade serão realizados armazenando as NLC a diferentes temperaturas e analisando-as por calorimetria de varrimento diferencial. Adicionalmente, um ensaio de citotoxicidade será realizado usando células Caco-2 como modelo e será ainda avaliado o perfil de libertação de fármaco *in vitro*. Já que as NLC serão administradas por via transdérmica através de um adesivo, ensaios de

permeabilidade *ex vivo* serão igualmente realizados. A propósito, células de difusão de Franz e uma membrana com a espessura total da pele humana (a separar os compartimentos dador e recetor) serão usadas e a quantidade de óleo de sucupira que ficar retida quer no compartimento recetor quer na membrana de pele será analisada por cromatografia líquida de alto desempenho. De seguida, serão desenhados e testados adesivos transdérmicos para libertação *in vitro* e estudos de permeabilidade serão igualmente realizados pelo método acima descrito. A distribuição dos NLC através da pele será avaliada por microscopia de varrimento a laser confocal. Finalmente, estudos farmacodinâmicos *in vivo* serão realizados em dois modelos animais: modelo de DMt1 induzida por streptozotocina (STZ) e modelo de DMt2 – ratos Goto Kakizaki (GK). Um adesivo transdérmico contendo NLC com óleo de sucupira será aplicado na pele de cada rato e a biodistribuição das NLC será avaliada, já que as nanopartículas são previamente revestidas com albumina de soro bovino e radiomarcadas com tecnécio (^{99m}Tc). O efeito farmacológico do óleo de sucupira será avaliado pela determinação dos níveis plasmáticos de glicose e insulina em jejum, índice de insulinorresistência (HOMA-IR), teste de tolerância à glicose intraperitoneal e a HbA1c. O nosso grupo de investigação pretende assim desenvolver NLC com óleo de sucupira devidamente otimizadas e estáveis para que sejam usadas como sistema controlado de libertação de fármaco por via transdérmica (adesivo); desta forma, o óleo de sucupira poderá alcançar os seus alvos biológicos, atuar no metabolismo dos hidratos de carbono (redução dos níveis plasmáticos de glicose nos modelos animais de DM) e ser reconhecido como agente promissor na terapêutica da DM.

3.1.2. In English

Essential oils are odorant liquid oily products consisting of a complex mixture of volatile compounds obtained from a plant raw material which are increasingly proven to be potential natural agents in the treatment of several human conditions.[1,2] Consisting of a major public health problem, special attention should be given to diabetes mellitus (DM), a metabolic disorder characterized by chronic hyperglycemia and which has mainly two presentations: type 1 DM (t1DM), which results from the destruction of insulin-secreting pancreatic β-cells mainly by an autoimmune-mediated process, and type 2 DM (t2DM) which is, in turn, due to peripheral and hepatic insulin resistance and relative insulin deficiency.[3,4] In these patients, hyperglycemia correlates with an increased risk for micro- and macrovascular diseases, such as coronary heart disease, peripheral vascular disease and cerebrovascular disease.[4] Thus, therapies having a blood glucose lowering effect are the main approach on DM treatment strategy. Unfortunately, the available drugs have increasingly reported side effects, efficacy loss over time, high cost and suboptimal patient compliance.[3] In this way, novel approaches

for DM management must be explored. Our group aim to encapsulate sucupira essential oil, obtained from the fruits of the Brazilian plant *P. emarginatus* in nanostructured lipid carriers (NLC), a second-generation lipid nanoparticles which act as a new controlled drug delivery system (DDS). This system assures the preservation of the oil physicochemical properties until it reaches the biological target, thus overcoming essential oil-related issues, namely their lipophilicity, hydrophobicity, volatility and high susceptibility to external factors.[1,5-7] Several biological activities of sucupira oil have been reported,[8] although there is no scientific evidence of its anti-hyperglycemic effect until now. Sucupira-oil loaded NLC will be produced by hot high-pressure homogenization (HPH) technique and carefully characterized in terms of their physicochemical properties and morphology, using dynamic (DLS) and electrophoretic (ELS) light scattering, scanning (SEM) and transmission (TEM) electron microscopy, and X-ray diffraction (XRD) techniques. Then, stability assays will be performed by NLC storage at different temperatures and differential scanning calorimetry (DSC) analysis. Additionally, a cytotoxicity assay will be performed using Caco-2 cells as cell model and an *in vitro* drug release profile must be also assessed. Furthermore, since the sucupira-oil loaded NLC will be administrated by transdermal route using an adhesive patch, *ex vivo* skin permeation assays will be also performed. For the purpose, Franz glass diffusion cells and a full-thickness human skin membrane (to separate the donor and receptor compartments) will be used and the amount of sucupira oil retained in both the receptor compartment and the skin membrane will be analyzed by high performance liquid chromatography (HPLC). Then, transdermal adhesive patches will be designed and tested for *in vitro* release and permeation studies by the methods above described. NLC distribution over the skin will be also determined using the fluorescence image analysis by confocal laser scanning microscopy (CLSM) and eventual skin irritation must be investigated. Finally, *in vivo* pharmacodynamic studies will be performed in two animal models: streptozotocin (STZ)-induced t1DM model and Goto Kakizaki (GK) rat t2DM model. The adhesive patch containing the sucupira oil-loaded NLC will be applied onto the rat skin and the NLC dispersions, previously coated with bovine serum albumin (BSA) and radiolabeled with technetium (^{99m}Tc) will be tracked for the biodistribution assessment. The pharmacological effect of sucupira oil will be assessed by the determination of both fasting plasma glucose and insulin levels, insulin resistance index (HOMA-IR), intraperitoneal glucose tolerance test (PTGIP) and HbA1c. Thus, our research group aim to develop optimized and stable sucupira-oil loaded NLC, which will be applied to a transdermal controlled DDS (adhesive patch); this way, sucupira oil will reach its biological targets, play an active role on carbohydrate metabolism (lowering the plasma glucose levels in diabetic animal models) and will be recognized as a promising new approach in diabetes mellitus treatment.

3.2. Technical description

3.2.1. Literature review

Essential oils (EO) are odorant and oily natural liquids consisting of a complex mixture of volatile compounds which are synthesized as secondary metabolites in the organs of aromatic plants, namely seeds, fruits, leaves and flowers.[1,2] EO have generally three major compounds that may be responsible for their biological properties: isoprenes, terpenoids and aromatic compounds.[1,2,5] Isoprenes are hydrocarbon terpenes consisting of head-to-tail linked isoprene units (2-methyl-1,3-butadiene) with the general structure $(C_5H_8)_n$, where n represents the number of isoprene units, and they comprise two main groups: monoterpenes, with two coupled isoprene units ($C_{10}H_{16}$) and sesquiterpenes, with three isoprene units ($C_{15}H_{24}$).[1,2,7] In turn, terpenoids or isoprenoids derive from C5-units of isopentenyl diphosphate and dimethylallyl diphosphate.[7] Finally, aromatic compounds occur as phenylpropane derivatives.[2,7] EO have been increasingly implicated in food, cosmetic and pharmaceutical industries and several medicinal properties have been reported, due to their antiseptic, analgesic, sedative, anesthetic and anti-inflammatory activities.[2] Furthermore, EO are able to permeate biological membranes, enter the systemic circulation and act simultaneously in several targets, with no resistance mechanisms or genotoxicity documented to date.[2,7]

DM is considered one of the major global public health concerns, accounting for 422 million cases in 2014 and a prevalence of 8.5%, according to the World Health Organization data.[3] DM is a metabolic disease characterized by a status of chronic hyperglycemia and has mainly two presentations: type 1 DM (t1DM), which represents 10% of the cases and results from the destruction of insulin-secreting pancreatic β-cells mainly by an autoimmune-mediated process, and type 2 DM (t2DM), which in turn occurs on the remaining 90% of the cases and is due to peripheral insulin and hepatic resistance and relative insulin deficiency, associated with an impaired β-cell function and insulin secretion.[3,4] Current DM therapeutic approaches include insulin analogues, for both t1DM and t2DM, and, for t2DM, several classes of antidiabetic drugs: biguanides (in which metformin is the main choice), thiazolidinediones, sulfonylureas, α-glucosidase inhibitors, meglitinides, sodium-glucose transporter protein 2 inhibitors, incretin mimetics such as glucagon-like peptide 1 analogues and glucose-dependent insulinotropic peptide analogues, and dipeptidil peptidase 4 inhibitors.[3] Unfortunately, the above-mentioned drugs report several side effects, mainly gastrointestinal complications, efficacy loss over time, high cost and suboptimal patient compliance.[3] In this way, it is urgent to develop novel antidiabetic drugs, preferentially more effective, safer and “patient- and environmental-friendly”.

Essential oil of sucupira, obtained from the fruits of the Brazilian plants of the genus *Pterodon* and commonly known as “faveira”, “sucupira”, “sucupira-branca”, “fava-de-sucupira”

or “sucupira-lisa”, seems to be a promising agent for DM management.[8] Traditional medicine had long ago recognized its biological activity, namely its antinociceptive, anti-inflammatory, antioxidant, antimicrobial, anticancer, hypoglycemic and lipolytic properties, apparently due to its main constituents: diterpenes (especially 6α,7β-dihydroxyvouacapan-17β-oic acid and 14,15-epoxygeranylgeraniol), sesquiterpenes and isoflavones.[8] However, there remain some difficulties in delivering EO such as sucupira oil to target tissues mainly due to their characteristic lipophilicity, hydrophobicity and volatility.[1,5,6] Furthermore, EO are very sensitive to external factors, especially light, elevated temperature and high oxygen availability, and thus highly susceptible to conversion and degradation reactions (mainly oxidation reactions) that produce free oxygen radical species and other oxidation products, such as lipid hydroperoxides, aldehydes, hydrocarbons, ketones and epoxides, which contribute to lose their main properties.[1,5-7]

To overcome the above-mentioned issues, several encapsulation strategies and controlled drug delivery system (DDS) have been developed.[6,9] Lipid nanoparticles are considered a DDS since 1991 and comprise two categories: solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).[6] Both SLN and NLC (**Figure 1**) have a structure of pure lipids or a lipid compound mixture (including triacylglycerols, fatty acids, steroids, waxes and oils) and a surrounding single surfactant or an association of this with a co-surfactant.[9-11] SLN, the first generation lipid nanoparticles, comprise solid lipids, either highly purified triglycerides, complex glyceride mixtures or waxes, stabilized by a surfactant agent and report high encapsulation efficiency, great physical stability, flexible profile of controlled drug release and good tolerability.[9,10,12,13] In turn, NLC result from the mixture of solid and liquid lipids covered by a surfactant solution and were designed to overcome some issues related to SLN, such as reduced loading capacity, relatively high water content in the dispersion and drug release after crystallization.[9-11] Their nanostructure allows an increased loading capacity and prevents oil leakages and abrupt drug expulsion.[9-11]

Our research group aim to develop optimized and stable sucupira-oil loaded NLC to apply in a transdermal controlled DDS in order to deliver sucupira oil to its biological targets, thus lowering plasma glucose levels in diabetic animal models and being recognized as a promising antidiabetic agent.

3.2.2. Research plan and methods

Based on the literature review and own experience, the following unsolved/unconsidered issues related to the development of sucupira-loaded NLC for diabetes mellitus management can be stated:

(1) The current DM drug therapies have been associated with some side effects, loss of efficacy over time, high cost and suboptimal patient compliance and there is a great need to develop new treatment approaches.

(2) Traditional medicine has recognized several biological properties of the sucupira oil but there is no previous study of scientific value respecting its likely effects on carbohydrate metabolism and application as an antidiabetic agent.

(3) Since EO chemical profile varies according to climate, soil, age and vegetative cycle stage of the plant and extraction method, it is important to characterize the chemical composition of the sucupira oil and establish the characteristics that must be present for the NLC loaded with sucupira oil be considered as reproducible.

(4) Essential oil of sucupira is highly lipophilic and very sensitive to environmental factors, easily suffering conversion and degradation reactions that compromise its properties; encapsulation in NLC may be the solution to a controlled delivery of sucupira oil to its cellular and organ targets.

(5) NLC have been increasingly studied for food industry and cosmetic applications but no investigation related to its use in metabolic diseases treatment was ever runned out.

(6) The NLC production by hot HPH technique allows the scale-up and industrial production of sucupira oil-loaded NLC by the pharmaceutical industry.

In this project, main attention will be given to the biological effect of sucupira oil on glucose profile of diabetic animal models after a controlled release of the NLC from a transdermal delivery system. Oil extract from the fruits of *P. emarginatus* will be firstly chemically characterized by gas-chromatography associated with mass spectrometry (GC-MS) technique and further incorporated in NLC using hot HPH technique, after performing the proper assays to select both the adequate solid lipid and surfactant agent to incorporate in the formulation. Then, after a careful evaluation of sucupira oil-loaded NLC physicochemical properties, morphology and physical stability upon storage, *in vitro* assays regarding cytotoxicity and drug release profile will be performed. Finally, adhesive patches will be designed in order to incorporate the stable sucupira oil-loaded NLC for transdermal delivery into tissues of diabetic t1DM and t2DM animal models used for *in vivo* experiments. It will be demonstrated if sucupira oil-loaded NLC are able to enter systemic circulation and reach their biological targets thus delivering sucupira oil to cells and tissues in a controlled manner.

According to the above-mentioned statements, the following ideas and their implementation strategies are proposed:

(1) Characterize the chemical composition of the sucupira oil obtained from *P. emarginatus* fruits using GC-MS method, thus recognizing its main constituents which are also the ones responsible for the oil biological properties.

(2) Produce sucupira oil-loaded NLC using the hot HPH technique, assess their loading capacity (LC), encapsulation efficiency (EE) and yield of production (YP), and then identify their physicochemical properties by both DLS and ELS techniques and evaluate their morphology by SEM, TEM and XRD techniques.

(3) Optimize the sucupira oil-loaded NLC by 2^2 factorial design using Statistica 7.0 software, thus understanding the most appropriate concentrations of the NLC constituents.

(4) Perform *in vitro* stability assays firstly by storage of sucupira oil-loaded NLC at different temperatures (4ºC, 25ºC and 40ºC) and then by both LumiSizer® and DSC analysis.

(5) Perform an *in vitro* cytotoxicity assay using Caco-2 cell lines as cell model in order to evaluate if any relevant cytotoxic events occur when treating Caco-2 cells with sucupira oil-loaded NLC at different concentrations (5, 10, 15 and 20 µg/mL), using Alamar Blue at 10% (v/v) to estimate cell survival rate by means of absorbance monitoring at both 570 and 620 nm wavelengths after 4, 24 and 48 hours of culture.

(6) Evaluate the *in vitro* drug release profile of the NLC formulations, framing them into one of the following kinetic models: zero order kinetics, first order kinetics, Higuchi model or Korsmeyer-Peppas model.

(7) Perform *ex vivo* skin permeation assays using Franz glass diffusion cells and a full-thickness human skin membrane, and then measure the amount of sucupira oil retained in both the receptor compartment and skin membrane by the HPLC technique.

(8) Design adhesive patches and perform *in vitro* release and permeation studies following the same protocol as for NLC formulations alone and also evaluate skin NLC distribution using fluorescence image analysis.

(9) Perform *in vivo* pharmacodynamic studies in STZ-induced t1DM model and GK-rat t2DM model and assess sucupira oil antidiabetic activity by evaluating blood glucose profile. This will require the determination of fasting plasma glucose and insulin levels, insulin resistance index (HOMA-IR) and HbA1c, as well as the intraperitoneal glucose tolerance test.

(10) Assess formulations biodistribution by tracking the sucupira oil-loaded NLC, previously coated with BSA and radiolabeled with technetium (^{99m}Tc), using a radiotracer and acquiring all the images by camera-gamma.

The main expected results at the end of the project are:

1. To chemically characterize sucupira oil of *P. emarginatus* by GC-MS method.
2. To produce optimized sucupira-loaded NLC by hot HPH technique, allowing large scale production and assuring either the preservation of sucupira oil biological properties and the nanoparticle physicochemical stability upon storage.
3. To ensure that sucupira-loaded NLC are not associated with any cytotoxic features, thus considering these systems as a novel and safe treatment approach.
4. To prove that NLC drug release profile *in vitro* may be appropriate to deliver sucupira oil to target tissues via transdermal application in a controlled manner.
5. To demonstrate *in vivo* sucupira-loaded NLC activities on glucose homeostasis, thus recognizing both NLC as a proper drug-controlled delivery system and sucupira oil as a promising antidiabetic agent.

3.2.3. Tasks

Task 1 | **Characterization of the sucupira essential oil**

Start date 01-12-2019 | **End date** 31-12-2019 | **Duration** 1 month | **Person*month** 6.25

Description and expected results | This first task aims to identify the constituents of sucupira oil. For that, sucupira essential oil extracted from the *P. emarginatus* fruits collected as endemic source from Serpige (Brazil), in September 2014, will be analyzed by a combined GC-MS technique following the experimental protocol used by Valentim *et al.*: injector temperature set on 200°C; detector temperature set on 250°C; helium chosen as carrier gas, at a flow rate of 1 $mL.min^{-1}$ and 1:40 ratio split injection; oven temperature firstly at 50°C (for 10 min), then increasing 2°C per minute until it reaches 200°C and finally increasing 10°C per minute to 290°C (for 10 min).[14] Additionally, the C18 Agilent column parameters will be defined as follows: internal diameter of 0.25 mm, length of 30 m and film thickness of 0.25 mm.[14] Kovats retention indices will be also obtained by the co-injection with a mixture of liner hydrocarbons (C8-C32).[15] Finally, mass spectrometry conditions will comprise an ionization voltage of 70 eV, a scan rate of 1 scan per second and a mass range (m/z) of 50-400.[14] At the end of the task, we expect to have identified at least the three major constituents of the sucupira essential oil.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra) and Patrícia Severino, PhD (from University of Tiradentes, Aracaju, Brazil and Tiradentes Institute, Dorchester, USA).

Task 2 | **Production of sucupira oil-loaded nanostructured lipid carriers**

Start date 01-01-2020 | **End date** 30-06-2020 | **Duration** 6 months | **Person*month** 37.49

Description and expected results | In this task we aim to select the best solid lipid and surfactant agent to integrate the NLC structure and to produce NLC formulations loaded with sucupira oil using the hot HPH technique. Thus, this is one of the most important and labor-consuming tasks and the experimental protocol will mainly consider the group experience in the field.

First, a lipid screening assay must be performed by mixing sucupira oil in concentrations of 5%, 10% and 15% (w/v) with a solid lipid at a time, each one varying in the concentration of 95%, 90% and 85% (w/v), respectively: Imwitor® 900 K (glycerol monostearate, type II), Dynasan® 116 (tripalmitin), Kollivax® GMS II (glycerol monostearate), Compritol® 888 ATO (glyceryl dibehenate) and Cetostearyl alcohol. The obtained fifteen samples of 1 mL each will be heated on a drier at 95ºC for 30 minutes (above the selected solid lipids melting point) and then observed at naked eye for macroscopic appearance at every 15 minutes within an hour and then after 24 hours to evaluate if phase separation occurred, that is, to evaluate if lipids are miscible. The same procedure must be repeated if samples demonstrate no or slightly phase separation to confirm the obtained results.

Simultaneously, a surfactant screening assay may be done by ultrasonication of twelve samples, 50 mL each, with different concentrations of TPGS (also known as D-α-Tocopherol polyethylene glycol succinate, vitamin E polyethylene glycol succinate or vitamin E-TPGS), Poloxamer 188 (also known as Kolliphor® P188), Tween® 80 (also known as polysorbatum 80, polysorbate 80 or polyoxyethylenesorbitan monooleate), soy lecithin (Phospholipon® 80H, phosphatidylcholine hydrogenated) and ultra-purified water (from a MilliQ Plus system and filtered through a 0.22 µm nylon filter before use). The proposed concentrations (% w/v) for each component are illustrated in **Table 1**. They will be mixed on a stirring plate at 500 rpm, without heating, for 10 minutes and then submitted to a Vibra-Cell™ ultrasonic processor at maximum amplitude, with no pulse, for 2 minutes.

The solid lipid and surfactant agent showing the best results in the previous assays will be chosen to produce the sucupira oil-loaded NLC by the hot high-pressure homogenization technique. The lipid phase consisting of sucupira oil [0.5% and 0.75% (w/v)] and the solid lipid [4.5% and 4.25% (w/v)] will be dispersed in an aqueous phase consisting of the selected surfactant [0.475%, 0.950% and 1.425% (w/v)] and ultra-purified water, at 68ºC (approximately 5ºC above the melting point of the solid lipid), with a sample final volume of 50 mL. Each of the obtained heated coarse pre-emulsions will be then processed by high-shear mixing Ultra-Turrax T25 at 16000 rpm for 15 minutes, followed by hot HPH, operating continuously for 20

minutes with a set pressure of 600 bar. The best formulations obtained in Task 2 will be then characterized and optimized.

The expected results are (i) selection of the best solid lipid to integrate in the NLC structure, (ii) selection of the appropriate surfactant agent to stabilize NLC structure and (iii) obtainment of NLC formulations loaded with sucupira oil by hot high-pressure homogenization technique.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra) and Amélia M. Silva, PhD (from Department of Biology and Environment, UTAD, Vila Real and Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real).

Task 3 | Characterization and optimization of the sucupira oil-loaded NLC

Start date 01-02-2020 | **End date** 31-07-2020 | **Duration** 6 months | **Person*month** 37.49

Description and expected results | In this third task we aim to optimize sucupira oil-loaded NLC in order to have a mean particle size (z-AVE) below 200 nm, polydispersity index (PdI) below 0.3 and a surface zeta potential (ZP) above the absolute value 30, and to assess NLC morphology and phase separation.

Firstly and according to group experience in the field, the selected samples will be analyzed by DLS for z-AVE and PdI, either immediately after production and also after 24 hours, using a Zetasizer Nano ZS, which has a 0.3 nm to 10 μm particle size range and is equipped with a laser beam (λ = 633 nm and 4 mW) and a scattered light detector positioned at an angle of 173° (non-invasive backscatter) in order to unmask scattered light signals of low intensity originated by the smaller particles.[16,17] All the samples will undergo 100 times dilution in ultra-purified water, placed in disposable cells at 25°C and analyzed in triplicate measurements (n = 3), performing 33 runs per measurement. In turn, ZP will be determined by ELS at 25°C by using the same device. The samples will be also diluted 100 times in ultra-purified water and analyzed in triplicate measurements (n = 3), performing 30 runs per measurement. The Henry's equation with the Smoluchowsi approximation is used to determine ZP. Data is expressed as arithmetic mean ± standard deviation.[17] The best formulation, i.e., with z-AVE below 200 nm, PdI below 0.3 and a surface ZP above the absolute value 30, will be subject to a full factorial design approach gathering all the possible combinations between the factors and their levels.

A 2^2 factorial design consisting of two factors, each one set at 2-levels, will be used to evaluate surfactant and solid lipid concentrations influence on the NLC formulations in order to maximize the efficiency of the experiment with a minimum number of attempts and thus

optimizing the sucupira-loaded NLC. As dependent variables we consider z-AVE, PdI and ZP. For each factor, the lower and higher values of the lower and upper levels will be represented by (−1) and (+1), respectively. The central point, represented by (0), will be replicated three times to estimate the experimental error, as showed in **Table 2**. Data will be analyzed by the Statistica 7.0 software. NLC dispersions randomly produced will suffer a variance analysis by the ANOVA statistical test, performed for each response parameter to identify effects significance and their interactions. A *p* value < 0.05 is considered statistically significant.

The optimized sucupira-loaded NLC will be then evaluated in terms of LC, EE and YP, determined by the equations below [11]:

$$\boldsymbol{LC} = \frac{\boldsymbol{Wa - Ws}}{\boldsymbol{Wa - Ws + W_L}} \times \mathbf{100} \qquad (1)$$

$$\boldsymbol{EE} = \frac{\boldsymbol{Wa - Ws}}{\boldsymbol{Wa}} \times \mathbf{100} \qquad (2)$$

$$\boldsymbol{YP} = \frac{\boldsymbol{W_L}}{\boldsymbol{V_D}} \times \mathbf{100} \qquad (3)$$

, where W_L is the weight of lipid added; V_D is the volume of NLC aqueous phase (surfactant and water); W_a is the weight of liquid lipid (sucupira oil) added in the formulation; and W_s is the weight of liquid lipid analyzed after NLC centrifugation, in the supernatant.

Then, the polymorphic forms of the lipids in the nanoparticle matrix and NLC phase separation profile will be determined using XRD techniques and the morphology, surface characteristics and particle size will be measured by electron microscopy (SEM and TEM).[11]

The expected results are: (i) obtention of optimized sucupira-loaded NLC with z-AVE below 200 nm, PdI below 0.3 and a surface ZP above the absolute value 30, by DLS and ELS analysis, (ii) assessment of an amorphous morphology and well-defined rounded shape of the sucupira-loaded NLC by SEM and TEM measurements, and (iii) characterization of the NLC phase separation profile by XRD techniques.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra) and Amélia M. Silva, PhD (from Department of Biology and Environment, UTAD, Vila Real and Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real).

Task 4 | Perform a stability analysis of the sucupira-loaded NLC

Start date 01-08-2020 | **End date** 31-03-2021 | **Duration** 8 months | **Person*month** 49.98

Description and expected results | In this task we aim to evaluate sucupira-oil loaded NLC physicochemical stability upon storage. For that and after a full sucupira-oil loaded NLC characterization in Task 3, stability assays must be performed.

According to group experience in this field, the formulations will be stored at three different temperatures (4ºC, 25ºC and 40ºC) and z-AVE, PdI and surface PZ will be monitored for 24 hours and after 3, 7, 15, 30, 60, 90, 180 days to assess the real-time sucupira-oil loaded NLC stability. Additionally, accelerated stability, demixing behavior and dispersion profile will be assessed using a LUMiSizer® analyzer: NLC dispersions (1 mL) will be slowly added to the bottom of a disposable polyamide cell, which will be capped and horizontally placed into the instrument and a centrifugal force under 2300 g, at 4000 rpm and 25°C will be applied; 750 profiles are recorded in 20 second-intervals and the instability indexes will be also calculated by a SEPView® software.[17]

Lastly, physicochemical stability of the NLC formulations will be assessed using DSC analysis, by the evaluation of the fusion and crystallization events, polymorphic forms, transition temperatures and enthalpies, resumed on phase diagrams.[18]

It is expected that the (i) sucupira-oil loaded NLC are stable upon several months of storage at the temperatures of 4ºC 25ºC and 40ºC, (ii) dispersion pattern of the NLC formulations remains favorable over time and (iii) physicochemical stability is maintained over time.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra) and Antonello Santini, PhD (from Department of Pharmacy, University of Naples Federico II, Naples).

Task 5 | Perform *in vitro* cytotoxicity assays

Start date 01-08-2020 | **End date** 30-11-2020 | **Duration** 4 month | **Person*month** 24.99

Description and expected results | With this task we aim to assess if there is any evidence of relevant cytotoxic events when treating the Caco-2 cells with sucupira oil-loaded NLC at different concentrations, thus deciding whether they may be suitable for *in vivo* administration.

For that, *in vitro* cytotoxicity assays of sucupira oil-loaded NLC will be performed using Caco-2 cell lines. The Dulbecco's Modified Eagle Medium, which contains glucose (25 mM) and is supplemented with 10% (v/v) fetal bovine serum , L-glutamine (2 mM) and the antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL), will be used as culture medium and kept at 37ºC in air with a CO_2 concentration of 5%, thus providing a controlled humidity atmosphere.[19]

Cells will be concentration-dependently treated over time with both non-loaded NLC and sucupira oil-loaded NLC formulations at four different concentrations: 5, 10, 15 and 20 µg/mL. The latest formulations will be produced in a laminar flow chamber ensuring all the aseptic conditions. First, surfactant will be removed from confluent cells, which were then exposed to trypsin, for 10 minutes and at 37ºC, until their detachment and disaggregation is reached. Culture medium will then finish this trypsin reaction, cells will be re-suspended, meticulously counted, seeded into microplates of 96 wells having 100 µL/well (which equals to a density of 5×10^4 cells/mL) and cultured for 24 hours. Subsequently, after culture medium removal and replace for FBS-free culture media containing the NLC formulations, cells will be incubated 24h more. Finally, Alamar Blue at 10% (v/v) will be then added to the medium in order to estimate cell survival rate by means of absorbance monitoring using an ELISA microplate reader at the wavelengths of 570 and 620 nm, at three different moments: after 4, 24 and 48 hours of culture (as the manufacturer protocol describes). All the obtained results will be then analyzed and the percentual reduction of absorbance will be measured and expressed as control percentage, which corresponds to the untreated cells. Further statistical analysis will be performed using Student's t-test ($p < 0.05$).[20]

The expected result is the absence of relevant cytotoxic events when treating the Caco-2 cells with sucupira oil-loaded NLC at different concentrations, proven by a cellular viability greater than 90%, thus confirming their suitability for *in vivo* administration.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra), Amélia M. Silva, PhD (from Department of Biology and Environment, UTAD, Vila Real and Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real) and Selma B. Souto, MD (from Department of Endocrinology, Hospital de São João, Porto).

Task 6 | Perform *in vitro* release studies for sucupira oil-loaded NLC

Start date 01-12-2020 | **End date** 31-03-2021 | **Duration** 4 months | **Person*month** 24.99

Description and expected results | This sixth task aims to establish the release profile of the best sucupira-oil loaded NLC formulations.

Studies *in vitro* on sucupira oil release from NLC will be performed using Franz glass diffusion cells (three cells per NLC formulation) and a synthetic cellulose membrane Millipore® separating the donor and receptor compartments. This membrane has a mean diameter per well of 0.45 µm, 0.64 cm^2 of surface area and is pre-hydrated in methanol-water [7:3 (v/v)] for 24 hours before being incorporated in the Franz diffusion cell. Sucupira oil-loaded NLC (1 mL)

will be placed in the donor compartment, in contact with the cellulose membrane surface, and phosphate-buffered saline (PBS) will be used as receptor medium at 37ºC and pH 7.40, under continuous stirring at 700 rpm to assure sink conditions. Finally, at strictly defined time intervals (0.5, 1, 2, 3, 4 and 6 h), 200 µL of each sample are collected from the receptor compartment (of 5 mL), with a syringe, and the equivalent volume restored with fresh PBS at the above-mentioned conditions.

The amount of sucupira oil contained in the collected samples will be then analyzed by spectrophotometry and GraphPad® prism 6, adjusted to four kinetic models represented by the equations below [21,22]:

Zero order kinetics	$F = K_0 \times t$	(5)
First order kinetics	$F = 100 \times (1 - e^{-K_1 \times t})$	(6)
Higuchi model	$F = K_H \times t^{1/2}$	(7)
Korsmeyer-Peppas model	$F = K_{KP} \times t^n$	(8)

, where K_0 is the zero order release constant, K_1 is the first order release constant, K_H is the Higuchi release constant, K_{KP} is the release constant considering both structural and geometric characteristics of the drug-dosage form, n is the diffusional exponent that refers to the drug-release mechanism and F is the fraction of released drug (%) in time, t.

In general, zero order kinetics model is applied to modified release systems with less soluble substances, ideal to a prolonged drug release; first order kinetics model is related to matrix systems in which the drug release profile directly varies with the amount of remaining drug inside the dosage form; and both Higuchi and Korsmeyer-Peppas models are related to modified release matrix systems that have diffusion as drug release mechanism.[22]

For each model, the regression coefficient (R_2) and the Akaike information criterion (AIC), calculated by the equation below,[23] will be measures of fit to compare all these models and the model with the highest R_2 and lowest AIC will be chosen.

$$AIC = n \times \ln WSSR + 2 \times p \quad (9)$$

, where n is the number of dissolution data points, p is the number of the parameters of the model and WSSR is the weighed sum of square of residues.

Finally, the dissolution efficiency (DE) will be also determined from the dissolution area under the curve at time t, accordingly to the following equation [21,23]:

$$DE\ (\%) = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100 \qquad (10)$$

, where y is the percentage of drug dissolved at the measured time, t.

The group is expecting to identify a zero order kinetics release profile for the sucupira-oil loaded NLC formulations, indicating their suitability for prolonged drug release.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra), Amélia M. Silva, PhD (from Department of Biology and Environment, UTAD, Vila Real and Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real) and Antonello Santini, PhD (from Department of Pharmacy, University of Naples Federico II, Naples).

Task 7 | Perform *ex vivo* human skin permeation assay for sucupira oil-loaded NLC

Start date 01-04-2021 | **End date** 31-07-2021 | **Duration** 4 months | **Person*month** 24.99

Description and expected results | In this task we aim to define the most appropriate diffusional membrane area and the permeation parameters for sucupira oil-loaded NLC formulations to be delivered to target organs *in vivo*, from an adhesive patch.

Skin permeation studies were performed using Franz glass diffusion cells (three cells per NLC formulation) and a Strat-M® artificial membrane for transdermal diffusion testing, with 0.64 cm^2 of surface area, separating the donor and receptor compartments, having the stratum corneum facing the donor chamber and the subcutaneous side in contact with the receptor medium. Sucupira oil-loaded NLC (1 mL) will be placed in the donor compartment, onto the membrane surface and PBS will be used as receptor medium at 37ºC, pH 7.40 and stirred at 700 rpm. After 24 hours the cells will be stopped and the amount of sucupira oil present in the receptor compartment analyzed by HPLC: first, tissue membranes will be dismantled and cleaned, using gauze soaked in a 0.05% sodium dodecyl sulfate solution and washed in distilled water, and permeation areas will be then excised and weighed; second, sucupira oil retained in skin will be extracted using the extraction medium ethanol water transcutol® P (50:40:10) under sonication, in an ultrasound bath for 20 minutes; and third, the resulting solutions will be measured by HPLC to measure the amount of sucupira oil retained in the skin.

Then, permeation parameters will be determined with GraphPad® prism 6 software and according to the equations below [24-26]:

Cumulative amount permeated through the membrane (Q_t)

$$Q_t = \frac{V_r \times C_t + \sum_{t-0}^{t-1} V_s \times C_i}{S} \quad (11)$$

Permeability coefficient (K_p), partition (P_1) and diffusion (P_2) parameters	$K_p = \frac{J}{C_0}$	(12)
	$K_p = P_1 \times P_2$	(13)
	$T_L = {}^1/_6 \times P_2$	(14)
Predicted steady-state plasma concentration (C_{ss})	$C_{ss} = \frac{J \times A}{Cl_p}$	(15)

, where V_r and V_s are the volumes of the receptor solution and the sample, respectively; C_t and C_i are the drug concentrations of the receptor solution and the sample at each time, respectively; S is the skin surface area; J is the flux of sucupira oil through the skin membrane, calculated from the slope of linear portion of the cumulative amounts permeated through the membrane per surface area unit versus time plot; T_L is the lag time, calculated from this line when intercepting the X-axis; A is the hypothetical area of application and Cl_p is the plasmatic clearance.

The group is expecting to establish the most appropriate diffusional area for the adhesive patch and to assess the ideal permeation parameters for sucupira oil-loaded NLC delivery *in vivo* by transdermal route.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra), Selma B. Souto, MD (from Department of Endocrinology, Hospital de São João, Porto) and Patrícia Severino, PhD (from University of Tiradentes, Aracaju, Brazil and Tiradentes Institute, Dorchester, USA).

Task 8 | Preparation of the transdermal adhesive patches

Start date 01-08-2021 | **End date** 31-10-2021 | **Duration** 3 months | **Person*month** 18.74

Description and expected results | In this task we aim to develop an adhesive transdermal drug delivery system for sucupira oil-loaded NLC incorporation.

First, adhesives will be produced by the solvent evaporation technique, using ethanol as cosolvent and propylene glycol, at a concentration of 3.77% (w/w), as enhancer. Compounds will be homogenized by magnetic stirring, added to NLC dispersions and dissolved in the adhesive. The formulations will be placed in a backing layer, with 25 cm^2 of area, followed by solvent evaporation for 48 h, in an oven setted at 35°C. Then, a part of the adhesive (an area of 1.44 cm^2) will be cutted and diluted under sonication in the mobile phase until a complete redispersion is achieved. Finally, samples will be filtered and the sucupira oil incorporated into transdermal patches will be quantified by HPLC analysis.[27,28]

Second, the interaction skin-adhesive must be investigated, essentially based on initial and long-term adhesion, lift and residue. Adhesive properties – adhesiveness, adhesion energy and separation distance of adhesives – will be assessed by the transdermal adhesive tape test, using a texture analyzer with an analytical probe P/1S to assure a strict contact of the adhesive with a double-sided tape. It will be measured the adhesion and separation probe-adhesive mechanisms, separation distance and the adhesion energy. Data will be managed by the Texture Exponent 3.0.5.0 software. Additionally, interactions between the patch components will be also evaluated with a Fourier-transform infrared (FT-IR) spectrometer.[27,28]

Third, *in vitro* release and permeation studies will be performed following the above-mentioned protocols but using a piece of the transdermal patch attached onto the membrane surface, in the donor compartment, instead of sucupira-oil loaded NLC alone.

Fourth, NLC distribution over the skin will be assessed by fluorescence image analysis using CLSM. After fulfill the permeation study protocol, each piece of skin will be embedded in Tissue-Tek O.C.T. compound and frozen at -20°C; then, using a cryostat, there will be obtained transversal sections of 50 μm thickness, from dermis to stratum corneum, and which are integrated in a Dako fluorescent medium to preserve the fluorescent signal during the CLSM measurements. Fluorescence images will be acquired by a confocal microscope and the digital images will be obtained with an EM charge-coupled device camera by using ZEN software (CarlZeiss).[27,28]

A skin irritation test must be also performed. Histological changes will be assessed after treatment with sucupira oil-loaded NLC to verify if there is any evidence of skin irritation. A limited area of skin (2 x 2 cm^2) will be cutted, fixed, dehydrated and embedded in paraffin. Lastly, sections of 5 μm, produced using a cryostat, will be stained with hematoxylin and eosin, observed by optical microscope and compared with untreated skin (used as control).[29]

The group is expecting to develop an adhesive transdermal drug delivery system with sucupira oil-loaded NLC incorporated, having efficient loading, proper stickiness to skin and the ability to preserve sucupira oil physicochemical properties with no or minimal skin irritation.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra), Selma B. Souto, MD (from Department of Endocrinology, Hospital de São João, Porto), Patrícia Severino, PhD (from University of Tiradentes, Aracaju, Brazil and Tiradentes Institute, Dorchester, USA), Amélia M. Silva, PhD (from Department of Biology and Environment, UTAD, Vila Real and Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real) and Antonello Santini, PhD (from Department of Pharmacy, University of Naples Federico II, Naples).

Task 9 | **Perform *in vivo* glycemic profile of sucupira oil-loaded NLC delivered by transdermal route**

Start date 01-11-2021 | **End date** 31-10-2022 | **Duration** 12 months | **Person*month** 74.97

Description and expected results | In this last task group aims to transdermally deliver sucupira oil-loaded NLC in DM animal models and establish the effect of sucupira oil on glucose profile, respecting the standards from the Federation of European Laboratory Animal Science Association and the European Union Council Directive 86/609/EEC and the ethical principles and guidelines of the Direcção Geral de Alimentação e Veterinária related to housing and experiments on animals.

To be traced on body tissues immediately after administration, sucupira oil-loaded NLC will be firstly coated with bovine serum albumin (BSA) and radiolabeled with technetium (^{99m}Tc) as follows: first, BSA (outer layer) will be labelled with a mixture of sodium thiosulfate (10 mg/mL), HCl (1 N) and 1110-2220 MBq of $Na^{99m}TcO^{4-}$ (1.0, 1.0 and 0.5% (v/v), respectively) and heated at 100ºC for 3.5 min within a dry bath to be then cooled to room temperature; more BSA (2.5 mL) will be added, the mixture will be stirred and PBS (2 mL at pH 7.4) added. Once coated, the suspension will be washed by centrifugation and re-dispersed in ultrapurified water.

Using instant thin-layer chromatography with silica-gel strips (ITLC-SG) radiolabeling efficiency of ^{99m}Tc will be measured with two systems: (1) quantifying sample $^{99m}TcO^{4-}$ using sodium chloride at 0.9% (w/v), and (2) isolating sample ^{99m}Tc-BSA and ^{99m}Tc-BSA-NLC, with a retention value (Rf) of 0, using acid citrate-dextrose, since they differ from $^{99m}TcO^{4-}$ and reduced or hydrolyzed ^{99m}Tc, which have a Rf of 1.[30] NLC biodistribution will be assessed by an administered radiotracer, images will be acquired by camera-gamma and the activity (%) per mass unit (g) will be calculated.[30]

Animal models will be arranged as follows: STZ-induced t1DM model will use male Wistar rats having 250 to 350 g of weight. An intraperitoneal injection of 60 mg/kg STZ prepared in citrate buffer at pH 4.5 will be given to destroy the pancreatic β-cells and within the first 24 h it will be given a 5% (w/v) glucose physiologic solution to prevent hypoglycemia. In 15 to 21 days, twenty rats showing weight loss, frequent urination and fasting plasma glucose levels higher than 14 mM will be randomizedly distributed in two groups (**I** – control and **II** – treated with sucupira oil-loaded NLC), with N = 10 per group, to perform *in vivo* studies on glycemic profile.[30] In turn, t2DM model will use genetic non-obese and insulin-resistant GK rats, weighing 250 to 350 g, characterized by islet glucose-dependent insulin secretion deficiency and peripheral insulin resistance. Twenty rats will be randomizedly selected and distributed in two groups (**III** – control group and **IV** – treated with sucupira oil-loaded NLC), with N = 10 per group.[30] As such, NLC-free adhesive patches will be applied to rats from groups I and III and the previously radiolabeled sucupira oil-loaded NLC will be incorporated in the adhesive

patches applied to groups II and IV, in a dose of 100 IU/Kg. All patches will be applied onto rats' tricotomized neck skin for a four-hour period, at the beginning of the fourth week of the experiment.[30] All rats will be accommodated under controlled temperature, humidity and a 12-12 h light–dark cycle, with standard laboratory feed and free access to tap water.[30]

The pharmacological effect of sucupira oil will be assessed by (1) measuring fasting plasma glucose before and after NLC-administration to both STZ-induced and GK rat models and (2) measuring fasting insulin and HbA1c levels using an ELISA kit and determining insulin resistance index (HOMA-IR) in both STZ-induced and GK rat models after 8 weeks of experiment, at the time of rat sacrifice; and (3) performing intraperitoneal glucose tolerance test (PTGIP) in GK rats, after an intraperitoneal injection of 1.75 g/Kg glucose solution.

The group expects to achieve a transdermal delivery of sucupira oil-loaded NLC in DM animal models (with minimal skin irritation), trace NLC and recognize the effect of sucupira oil on the glycemic profile.

Members of the research team who will participate in this task | Raquel Vieira, MS (from Faculty of Medicine, University of Coimbra), Eliana B. Souto, PhD (from Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra), Selma B. Souto, MD (from Department of Endocrinology, Hospital de São João, Porto), Amélia M. Silva, PhD (from Department of Biology and Environment, UTAD, Vila Real and Centre for Research and Technology of Agro-Environmental and Biological Sciences, CITAB, UTAD, Vila Real), Raquel Seiça, MD, PhD (from Institute of Physiology and Coimbra Institute for Clinical and Biomedical Research, Faculty of Medicine, University of Coimbra) and a PhD researcher (from Institute of Physiology and Coimbra Institute for Clinical and Biomedical Research, Faculty of Medicine, University of Coimbra).

3.2.4. Project schedule and management

3.2.4.1. Description of the management structure

The project management involves:

1. Administrative and financial execution of the project by elaboration of administrative and financial reports every 12 months.
2. Coordination of the technical execution of the project through semiannual meetings and elaboration of technical reports every 12 months. There must be considered eventual work plan adjustments as a result of the experimental analysis.
3. Coordination of the results disclosure by participating in both national and international conferences and scientific publications.

The administrative and financial management of the resources allocated to the project will be performed by PI under supervision of the Project Support Office and the Financial Services Office of UC. These offices will assure that all management, acquisitions and reporting will be provided accordingly to rules established by Fundação para a Ciência e a Tecnologia (FCT).

The communication strategy of the project aims to keep all partners fully informed on the project status, achievements and related issues to encourage the synergy of the cooperation. Since participants come from distinct backgrounds and have different skills, the cooperation and knowledge sharing among partners is especially important in this project and contributes to enrich each one and give some versatile experience.

The project includes nine tasks and the responsibility for their execution is distributed among the partners according to their expertise and participation in a particular task. Five milestones are established in this project, as well as five meetings which are planned at that time, when the results should be reported and discussed together with the elaboration of future work plans. Moreover, unplanned meetings can be arranged by any participant if it is necessary for the implementation of the project.

3.2.4.2. List of milestones

Milestone M1

Date: Month 1 | **Denomination:** Characterization of sucupira essential oil

Description: At least three major components of sucupira essential oil, which determine its biological activity, will be identified.

Milestone M2

Date: Month 8 | **Denomination:** Sucupira-NLC production, characterization and optimization

Description: Sucupira oil-loaded NLC formulations will be first produced using different solid lipid, surfactant and oil concentrations, then characterized for z-Ave, PDI, ZP, surface and phase separation profile, and finally optimized by 2^2 factorial design.

Milestone M3

Date: Month 16 | **Denomination:** *In vitro* stability, cytotoxicity and release assays

Description: Sucupira oil-loaded NLC formulations will be tested *in vitro* for physicochemical stability over time, cytotoxicity potential in Caco-2 cells and their release profile will be identified using Franz diffusion cells.

Milestone M4

Date: Month 23 | **Denomination:** *Ex vivo* permeation assay and adhesive patches design

Description: Skin permeation studies will be performed thus assessing the diffusional area and permeation parameters for further sucupira oil-loaded NLC incorporation on the originally designed adhesive patches for transdermal drug delivery.

Milestone M5

Date: Month 35 | **Denomination:** *In vivo* pharmacodynamic studies for NLC transdermal delivery

Description: Sucupira oil will be evaluated for its effect on glucose profile after sucupira oil-loaded NLC controlled transdermal delivery to body tissues in t1DM and t2DM animal models.

3.2.4.3. Timeline

The timeline designed for this project is presented in section 9. Appendices.

3.3. References

Reference	Year	Publication
1	2015	El Asbahani A, Miladi K, Badri W, Sala M, Ait Addi EH, Casabianca H, El Mousadik A, Hartmann D, Jilale A, Renaud FN, Elaissari A. Essential oils: from extraction to encapsulation. Int J Pharm 2015;**483**(1-2):220-43.
2	2008	Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils-a review. Food Chem Toxicol 2008;**46**(2):446-75.
3	2017	Habtemariam S. Antidiabetic Potential of Monoterpenes: A Case of Small Molecules Punching above Their Weight. Int J Mol Sci 2017;**19**(1).
4	2015	Ali MA, Wahed MI, Khatune NA, Rahman BM, Barman RK, Islam MR. Antidiabetic and antioxidant activities of ethanolic extract of Semecarpus anacardium (Linn.) bark. BMC Complement Altern Med 2015;**15**:138.
5	2013	Turek C, Stintzing FC. Stability of Essential Oils: A Review. Comprehensive Reviews in Food Science and Food Safety 2013;**12**(1)
6	2016	Rodríguez J, Martín MJ, Ruiz MA, Clares B. Current encapsulation strategies for bioactive oils: from alimentary to pharmaceutical perspectives. Food Research International 2016; **83**:41-59.
7	2014	Bilia AR, Guccione C, Isacchi B, Righeschi C, Firenzuoli F, Bergonzi MC. Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach. Evid Based Complement Alternat Med; **2014**:651593.

8	2015	Hoscheid J, Cardoso ML. Sucupira as a Potential Plant for Arthritis Treatment and Other Diseases. Arthritis; **2015**:379459.
9	2012	Wilczewska AZ, Niemirowicz K, Markiewicz KH, Car H. Nanoparticles as drug delivery systems. Pharmacol Rep 2012;**64**(5):1020-37.
10	2007	Souto EB, Muller RH. Lipid nanoparticles (solid-lipid-nanoparticles and nanostructured lipid carriers) for cosmetic, dermal and transdermal applications. In. Nanoparticulate Drug Delivery Systems. New York: Informa Healthcare USA, Inc; 2007, 22.
11	2010	Souto EB, Muller RH. Lipid nanoparticles: effect on bioavailability and pharmacokinetic changes. Handb Exp Pharmacol 2010(197):115-41.
12	2009	Souto EB, Doktorovova S. Solid lipid nanoparticle formulations: pharmacokinetic and biopharmaceutical aspects in drug delivery. In. Methods in Enzymology. 2009/11/12 ed: Academic Press; 2009, 105-129.
13	2016	Geszke-Moritz M, Moritz M. Solid lipid nanoparticles as attractive drug vehicles: Composition, properties and therapeutic strategies. Mater Sci Eng C Mater Biol Appl 2016; **68**:982-994.
14	2018	Valentim DSS, Duarte JL, Oliveira A, Cruz RAS, Carvalho JCT, Conceicao EC, Fernandes CP, Tavares-Dias M. Nanoemulsion from essential oil of Pterodon emarginatus (Fabaceae) shows in vitro efficacy against monogeneans of Colossoma macropomum (Pisces: Serrasalmidae). J Fish Dis 2018;**41**(3):443-449.
15	2014	Alves SF, Borges LL, Santos TO, Paula JR, Conceição EC, Bara MT. Microencapsulation of essential oil from fruits of Pterodon emarginatus using gum arabic and maltodextrin as wall materials: composition and stability. Drying Technology 2014;**32**:96-105.
16	2016	Bhattacharjee S. DLS and zeta potential - What they are and what they are not? J Control Release 2016; **235**:337-351.
17	2018	Pereira I, Zielinska A, Ferreira NR, Silva AM, Souto EB. Optimization of linalool-loaded solid lipid nanoparticles using experimental factorial design and long-term stability studies with a new centrifugal sedimentation method. Int J Pharm 2018;**549**(1-2):261-270.
18	2019	Ramesh N, Mandal AKA. Encapsulation of epigallocatechin-3-gallate into albumin nanoparticles improves pharmacokinetic and bioavailability in rat model. 3 Biotech 2019;**9**(6):238.
19	2014	Severino P, Andreani T, Jager A, Chaud MV, Santana MH, Silva AM, Souto EB. Solid lipid nanoparticles for hydrophilic biotech drugs: optimization and cell viability studies (Caco-2 & HEPG-2 cell lines). Eur J Med Chem 2014; **81**:28-34.
20	2014	Fangueiro JF, Andreani T, Fernandes L, Garcia ML, Egea MA, Silva AM, Souto EB. Physicochemical characterization of epigallocatechin gallate lipid nanoparticles (EGCG-LNs) for ocular instillation. Colloids Surf B Biointerfaces 2014; **123**:452-60.

21	2019	Mateus D, Marto J, Trindade P, Goncalves H, Salgado A, Machado P, Melo-Gouveia A, Ribeiro HM, Almeida AJ. Improved Morphine-Loaded Hydrogels for Wound-Related Pain Relief. Pharmaceutics 2019;**11**(2).
22	2016	Ochiuz L, Grigoras C, Popa M, Stoleriu I, Munteanu C, Timofte D, Profire L, Grigoras AG. Alendronate-Loaded Modified Drug Delivery Lipid Particles Intended for Improved Oral and Topical Administration. Molecules 2016;**21**(7).
23	2018	Rincon M, Calpena AC, Fabrega MJ, Garduno-Ramirez ML, Espina M, Rodriguez-Lagunas MJ, Garcia ML, Abrego G. Development of Pranoprofen Loaded Nanostructured Lipid Carriers to Improve Its Release and Therapeutic Efficacy in Skin Inflammatory Disorders. Nanomaterials (Basel) 2018;**8**(12).
24	2011	Gonzalez-Mira E, Nikolic S, Garcia ML, Egea MA, Souto EB, Calpena AC. Potential use of nanostructured lipid carriers for topical delivery of flurbiprofen. J Pharm Sci 2011;**100**(1):242-51.
25	2019	Soriano-Ruiz JL, Suner-Carbo J, Calpena-Campmany AC, Bozal-de Febrer N, Halbaut-Bellowa L, Boix-Montanes A, Souto EB, Clares-Naveros B. Clotrimazole multiple W/O/W emulsion as anticandidal agent: Characterization and evaluation on skin and mucosae. Colloids Surf B Biointerfaces 2019; **175**:166-174.
26	2014	Clares B, Calpena AC, Parra A, Abrego G, Alvarado H, Fangueiro JF, Souto EB. Nanoemulsions (NEs), liposomes (LPs) and solid lipid nanoparticles (SLNs) for retinyl palmitate: effect on skin permeation. Int J Pharm 2014;**473**(1-2):591-8.
27	2018	Regenthal R, Voskanian M, Baumann F, Teichert J, Bratter C, Aigner A, Abraham G. Pharmacokinetic evaluation of a transdermal anastrozole-in-adhesive formulation. Drug Des Devel Ther 2018;**12**:3653-3664.
28	2017	Mendes M, Nunes SCC, Sousa JJ, Pais A, Vitorino C. Expanding Transdermal Delivery with Lipid Nanoparticles: A New Drug-in-NLC-in-Adhesive Design. Mol Pharm 2017;**14**(6):2099-2115.
29	2015	Peng X, Zhou Y, Han K, Qin L, Dian L, Li G, Pan X, Wu C. Characterization of cubosomes as a targeted and sustained transdermal delivery system for capsaicin. Drug Des Devel Ther 2015;**9**:4209-18.
30	2017	Lopes M, Aniceto D, Abrantes M, Simoes S, Branco F, Vitoria I, Botelho MF, Seica R, Veiga F, Ribeiro A. In vivo biodistribution of antihyperglycemic biopolymer-based nanoparticles for the treatment of type 1 and type 2 diabetes. Eur J Pharm Biopharm 2017; **113**:88-96.

3.4. Previous publications

There are no previous applications related to this project.

3.5. Re-submissions of applications

There are no re-submissions of applications.

4. Research team

4.1. List of members

Name	Role	Degree	% Time	Core CV	CV
Eliana M. B. Souto	Principal investigator	PhD	60	✓	FCTSIG/cv
Raquel M. F. Seiça	Investigator	MD, PhD	35	✓	FCTSIG/cv
Raquel M. Vieira	Master student	MS	60	✗	FCTSIG/cv
Patrícia Severino	Investigator	PhD	25	✗	FCTSIG/cv
Amélia M. Silva	Investigator	PhD	60	✓	FCTSIG/cv
Selma B. Souto	Investigator	MD	35	✗	FCTSIG/cv
Antonello Santini	Investigator	PhD	30	✗	FCTSIG/cv
PhD researcher	Investigator	PhD	35	✗	FCTSIG/cv

Total: 8

4.2. List of members to be hired during execution of the project

There are no members to be hired during the execution of this project.

5. Other projects

5.1. Funded projects

Reference	Status
M-ERA-NET/0004/2015-PAIRED	In progress
UID/AGR/04033/2019 (CITAB)	In progress

Total: 2

5.2. Similar applications

There are no similar applications.

5.3. Other proposals

There are no other proposals.

5.4. SR&TD Projects in research lines of excellence

There are no SR&TD Projects in research lines of excellence.

6. Outcome indicators

6.1. Outcome indicators for the project

Description	2019	2020	2021	2022	Total
A. Publications					
Books	1	0	0	0	1
Papers in international journals	1	0	3	2	6
Papers in national journals	0	0	0	1	1
B. Communications					
In international meetings	0	1	1	2	4
In national meetings	0	0	1	1	2
C. Reports	0	0	0	1	1
D. Organization of seminars and conferences	0	0	0	0	0
E. Advanced training					
PhD theses	0	0	0	0	0
Master theses	1	0	0	0	1
Other	0	0	0	0	0
F. Models	0	0	0	0	0
G. Software	0	0	0	0	0
H. Pilot plants	0	0	0	0	0
I. Prototypes	0	0	0	0	0
J. Patents	0	0	0	0	0
K. Other	0	0	0	0	0

6.2. Dissemination of scientific activity

Diabetes has become a concern for most individuals worldwide, either for elderly or youth, and they want to get informed of the disease pathophysiology, currently available treatment options and preventive care. Also, scientific community needs to understand the disease establishment and progression and to develop novel therapies. In this way, the results of this

project will be broadly available through publications in scientific journals and presentations at national and international conferences and seminars. Additionally, the most interesting and promising ideas and results of the project will be divulgated at the open days for school students at Pharmacy and Medicine Faculties of the several universities involved and will also be presented to university students, young scientists and school teachers as a part of lectures. Finally, the highlights of the project will appear at the website of all the participating institutions.

7. Budget

Principal Contractor | University of Coimbra

Description	2019	2020	2021	2022	Total
Human resources	19 677.60 €	00 000.00 €	00 000.00 €	00 000.00 €	19 677.60 €
Travel	00 000.00 €	1 213.63 €	1 917.71 €	1 684.71 €	4 816.05 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	500.00 €	14.550.00 €	27 679.16 €	00 000.00 €	42 729.16 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	4 035.52 €	3 152.73 €	5 919.37 €	336.94 €	13 444.56 €
Total	**24 213.12 €**	**18 916.36 €**	**35 516.24 €**	**2 021.65 €**	**80 667.37 €**

Participating Institution 1 | Institute of Technology and Research

Description	2019	2020	2021	2022	Total
Human resources	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Travel	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	1 550.00 €	1 700.00 €	550.00 €	00 000.00 €	3 800.00 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	310.00 €	340.00 €	110.00 €	00 000.00 €	760.00 €
Total	**1 860.00 €**	**2 040.00 €**	**660.00 €**	**00 000.00 €**	**4 560.00 €**

Participating Institution 2 | University of Tiradentes

Description	2019	2020	2021	2022	Total
Human resources	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Travel	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	950.00 €	250.00 €	325.00 €	00 000.00 €	1 525.00 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	190.00 €	50.00 €	65.00 €	00 000.00 €	305.00 €
Total	**1 140.00 €**	**300.00 €**	**390.00 €**	**00 000.00 €**	**1 830.00 €**

Participating Institution 3 | Tiradentes Institute

Description	2019	2020	2021	2022	Total
Human resources	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Travel	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Total	**00 000.00 €**	**00 000.00 €**	**00 000.00 €**	**00 000.00 €**	**00 000.00 €**

Participating Institution 4 | São João Hospital

Description	2019	2020	2021	2022	Total
Human resources	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Travel	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €

Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Total	**00 000.00 €**	**00 000.00 €**	**00 000.00 €**	**00 000.00 €**	**00 000.00 €**

Participating Institution 5 | University of Trás-os-Montes e Alto Douro

Description	2019	2020	2021	2022	Total
Human resources	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Travel	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	00 000.00 €	4 950.00 €	1 025.00 €	00 000.00 €	5 975.00 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	00 000.00 €	990.00 €	205.00 €	00 000.00 €	1 195.00 €
Total	**00 000.00 €**	**5 940.00 €**	**1 230.00 €**	**00 000.00 €**	**7 170.00 €**

Participating Institution 6 | University of Naples Federico II

Description	2019	2020	2021	2022	Total
Human resources	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Travel	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	00 000.00 €	2 050.00 €	775.00 €	00 000.00 €	2 825.00 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	00 000.00 €	410.00 €	155.00 €	00 000.00 €	565.00 €
Total	**00 000.00 €**	**2 460.00 €**	**930.00 €**	**00 000.00 €**	**3 390.00 €**

Overall Budget |

Description	2019	2020	2021	2022	Total
Human resources	19 677.60 €	00 000.00 €	00 000.00 €	00 000.00 €	19 677.60 €
Travel	00 000.00 €	1 213.63 €	1 917.71 €	1 684.71 €	4 816.05 €
Consultants	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Acquisition of goods and services	3 000.00 €	23 500.00 €	30 354.16 €	00 000.00 €	56 854.16 €
Patent registration	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Adaptation of buildings and facilities	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Equipment	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Overheads	4 535.52 €	4 942.73 €	6 454.37 €	336.94 €	16 269.56 €
Total	**27 213.12 €**	**29 656.36 €**	**38 726.24 €**	**2 021.65 €**	**97 617.37 €**

Funding plan |

Description	2019	2020	2021	2022	Total
Requested FCT funding	27 213.12 €	29 656.36 €	38 726.24 €	2 021.65 €	97 617.37 €
Own funding	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Other public-sector funding	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Other private funding	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €	00 000.00 €
Total of the project	**27 213.12 €**	**29 656.36 €**	**38 726.24 €**	**2 021.65 €**	**97 617.37 €**

8. Justification of the budget

8.1. Human resources rationale

Type	Cost
PhD studentship	**19 677.60€**

Rationale for requested funding | Task 9 is very demanding and prolonged in time and thus the team decided to include a PhD researcher for a 12-month period.

8.2. Travel rationale

Type	No. of participations	Venue	Cost
Congress participation	1	Rome	**830.72€**

Rationale for requested funding | The International conference "*International Conference on Advancements in Obesity Therapies and Diabetes Research*" (ICAOTDR) will be held in October 2020. The requested amount will cover the participation expenses (conference fee, travel and accommodation) for 1 team member.

Congress participation	1	Lisbon	**382.91€**

Rationale for requested funding | The international conference *"ISER - International Conference on Nanoscience, Nanotechnology & Advanced Materials"* (IC2NAM) will be held in January 2021. The requested amount will cover the participation expenses (conference fee, travel and accommodation) for 1 team member.

Congress participation	1	London	**855.00€**

Rationale for requested funding | The International conference *"International Conference on Biomechatronics and Nanotechnology For Biomedical Applications"* (ICBNBA) will be held in May 2021. The requested amount will cover the participation expenses (conference fee, travel and accommodation) for 1 team member.

Congress participation	1	Athens	**932.71€**

Rationale for requested funding | The International conference "1*0th Annual International Conference on Health & Medical Sciences*" (AICHMS) will be held in May 2022. The requested amount will cover the participation expenses (conference fee, travel and accommodation) for 1 team member.

Congress participation	1	Copenhagen	**1 062.71€**

Rationale for requested funding | The International conference *"International Conference on Nanotechnology in Drug Delivery"* (ICNDD) will be held in July 2022. The requested amount will cover the participation expenses (conference fee, travel and accommodation) for 1 team member.

Congress participation	1	Lisbon	**752.00 €**

Rationale for requested funding | The international conference "*Nanomedicine International Conference*" (NIC) will be held in October 2022. The requested amount will cover the participation expenses (conference fee, travel and accommodation) for 1 team member.

8.3. Consultants rationale

Not applicable to this project.

8.4. Acquisition of goods and services

Type	Cost
Chemical reagents	**3 000.00€**
Rationale for requested funding | Several chemical reagents, namely solid lipids, surfactants, soy lecithin and ultra-purified water, necessary for to produce sucupira oil-loaded NLC will be bought.	
Equipment consumables	**6 000.00€**
Rationale for requested funding | High-purity solvents and compounds for preparation of standard solutions for HPLC will be bought. Also, some consumables necessary to MS and FT-IR spectrometers, TEM, SEM, AFM and CLSM analysis will be also purchased.	
Equipment maintenance and running costs	**13 000.00€**
Rationale for requested funding | The requested amount will be used to cover maintenance and running costs of the MS and FT-IR spectrometers, TEM, SEM, AFM, and CLSM which includes services acquisition, technical assistance and periodic replacement of the spectrometer components due to their limited time of life or degradation (namely laser, beam-splitters, radiation sources, detectors, mirrors and electronic components).	
Measurement costs	**2 000.00€**
Rationale for requested funding | The majority of measurements are planned to be done using centralized equipment in UC and its partner institutions, thus free of charge. Meanwhile, the consumables necessary for TEM should be paid.	
***In vivo* experiments-related costs**	**15 535.84€**
Rationale for requested funding | The budget required for *in vivo* experiments takes into account the purchase and transportation of 40 rats (20 Wistar and 20 GK adult male rats), permanence in bioterium (food, drink, hygiene, vigilance, sacrifice-related actions), substances to be administered according to the experimental protocol (e.g. glucose, STZ) and required material for measurements (Kits ELISA for HbA1c and insulin measurement, glucometer and blood glucose test strips).	
Regular laboratory and office consumables	**4 500.00€**
Rationale for requested funding | The requested amount includes expenses related to regular laboratory consumables and office accessories, printing costs for posters, publishing costs of scientific refereed articles and postages.	

8.5. Equipment rationale

8.5.1. Already available equipment

Equipment Type	Manufacturer	Model
Gas-chromatography and Mass spectrometry system	ThermoFisher	ISQ 7000 Single Quadrupole
High-performance liquid Chromatographer	Agilent Technologies	1250 infinity

HPLC Column	Agilent Technologies	C18
High-pressure homogenizator	Avestin Inc	Emulsiflex®-C3
Ultra-Turrax	Ystral GmbH	T25 D-7801
Zetasizer®	Malvern Instruments Ltd	Nano ZS
LUMiSizer®	LUM, GmbH	610 model
Differential scanning calorimetry	Schimadzu	DSC-60 Plus Series
X-ray powder diffractometer	Bruker	D8 Advance
Scanning Electron Microscope	TESCAN	Veja 3 SBH
Transmission Electron Microscope	JEOL	2200 PS
FT-IR Spectrometer	Matson	700
Atomic Force Microscope	NT-MDT	NTEGRA Prima
Confocal laser scanning microscope	Carl-Zeiss	LSM 510 Meta
Optical microscope	Olympus	CX23
UV-Visible Spectrophotometer	Shimadzu	UV-2600
Instant thin-layer chromatography with silica-gel strips	FisherScientific	SGI0001
Horizontal laminar flow chamber	Foster	Flow Fast H
Texture analyzer	Stable Micro Systems Ltd	TA.XT Plus
ELISA microplate reader (absorbance measurer)	Labsystems	Multiskan EX
Franz glass diffusion cells	PermeGear Inc.	U9-CA-02 30401
Microtome	Carl-Zeiss	HM 550
Ultrasonic Probe	SONICS	Vibra-Cell™ 435-A
Agitation plate	J.P.Selecta, s.a.	Agitmatic-E
Water and Oil Bath	J.P.Selecta, s.a.	Precisterm

8.5.2. Equipment to be acquired

Not applicable to this project.

8.6. Patent registration

Not applicable to this project.

8.7. Adaptation of buildings and facilities

Not applicable to this project.

9. Appendices

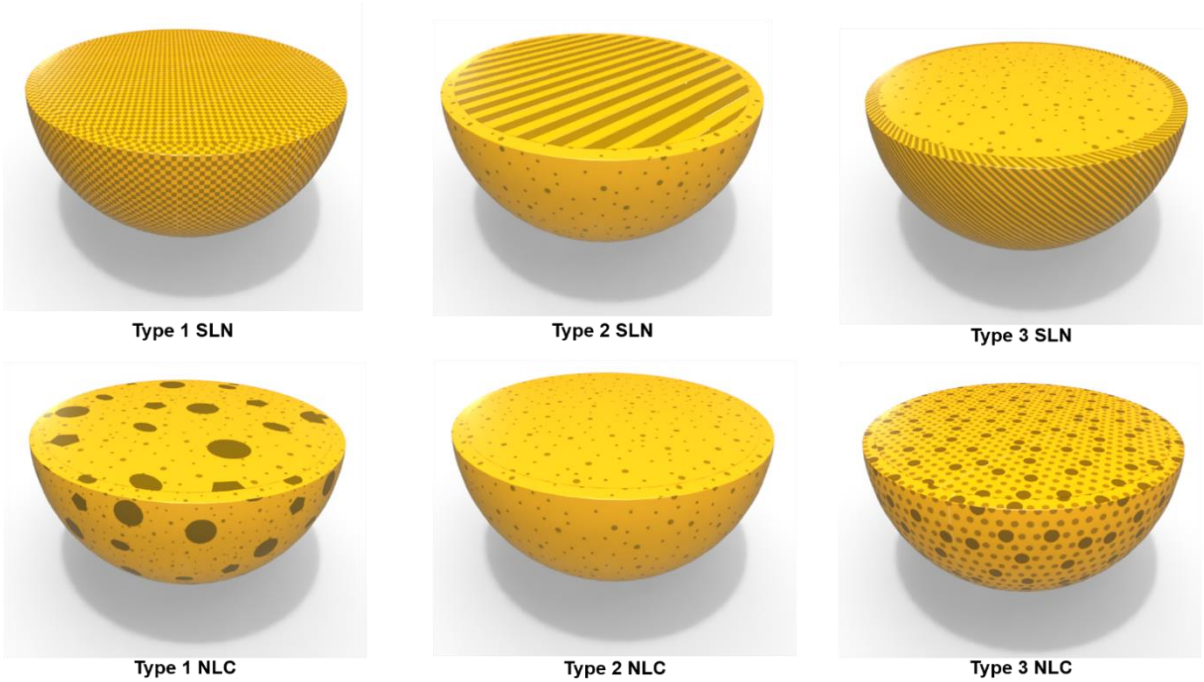


Figure 1. Tridimensional structure of solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).

Table 1. Composition of the surfactant screening formulations.

Samples (V_T 50 mL)	**TPGS** % (w/v)	**Tween 80** % (w/v)	**Poloxamer® 188** % (w/v)	**Lecithin** % (w/v)	**Sucupira** % (w/v)	**Kollivax® GMS II** % (w/v)
1	4,5	1	1	0,5	-	-
2	4,5	1	1	-	-	-
3	4,5	2	-	0,5	-	-
4	4,5	2	-	-	-	-
5	4,5	-	2	0,5	-	-
6	4,5	-	2	-	-	-
7	-	0,5	-	-	0,5	4,5
8	-	1	-	-	0,5	4,5
9	-	1,5	-	-	0,5	4,5
10	4,5	1	1	-	0,5	-
11	-	1,5	-	-	0,5	-
12	-	1,5	-	0,5	0,5	-

Table 2. Initial 2-level full factorial design providing the lower (−1), upper (+1) and central point (0) level values for each variable.

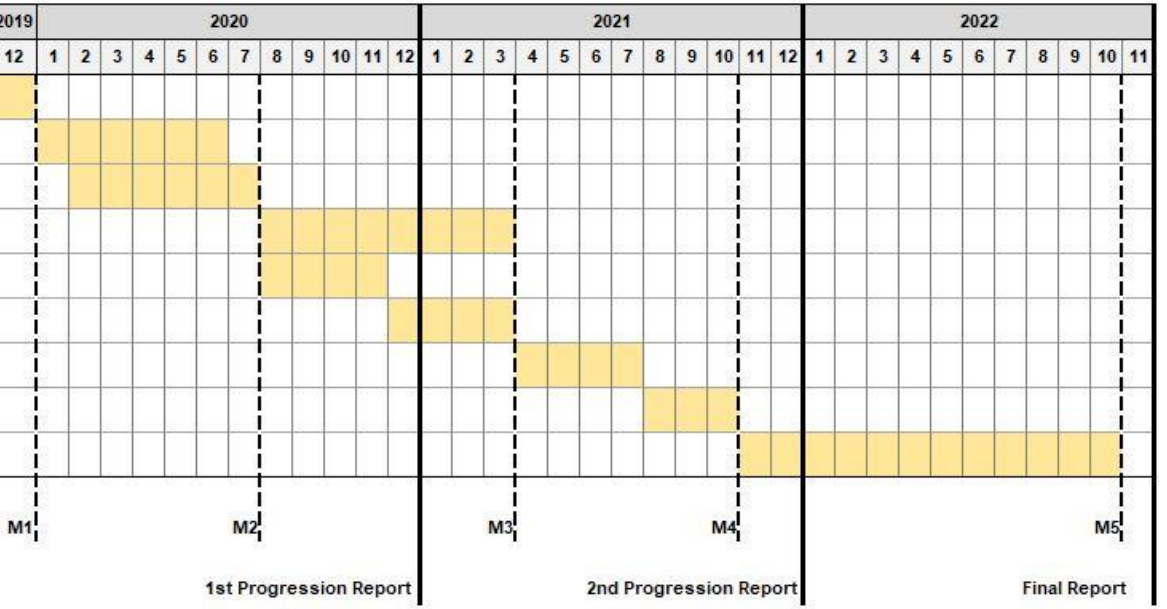
Factors	**Levels**		
	– 1	**0**	**+ 1**
Solid lipid	2,25% (w/v)	4,5% (w/v)	9% (w/v)
Surfactant agent	0,7125% (w/v)	1,425% (w/v)	2,85% (w/v)

Project timeline

Project Reference | xxxx/xxx/xxxxx/2019

Project Title | Sucupira oil-loaded nanostructured lipid carriers: a new approach in diabetes mellitus management

Nr	Task Denomination	Person*month	Partner responsible for the task	Partners Involved in the task
1	Characterization of sucupira essential oil	6,25	UNIT	UNIT, FFUC, FMUC
2	Production of sucupira oil-loaded NLC	37,49	FFUC	FFUC, FMUC, UTAD
3	Characterization and optimization of sucupira oil-loaded NLC	37,49	FFUC	FFUC, FMUC, UTAD
4	Perform a stability analysis of the sucupira oil-loaded NLC	49,98	NU	FFUC, FMUC, NU
5	Perform *in vitro* cytotoxicity assays	24,99	FFUC	FFUC, FMUC, HSJ, UTAD
6	Perform *in vitro* release studies for sucupira oil-loaded NLC	24,99	FFUC	FFUC, FMUC, UTAD, NU
7	Perform *ex vivo* human skin permeation assay for sucupira oil-loaded NLC	24,99	FFUC	FFUC, FMUC, HSJ, UNIT
8	Preparation of transdermal adhesive patches	18,74	UTAD	FFUC, FMUC, NU UTAD, HSJ, UNIT
9	Perfom *in vivo* glycemic profile of sucupira oil-loaded NLC delivered by transdermal route	74,97	FMUC	FFUC, FMUC, NU UTAD, HSJ, UNIT



10. Conflicts of interest

The authors report no conflict of interests.

11. Acknowledgements

I would like to express my gratitude to my advisor, Eliana Maria Barbosa Souto, for all the support, availability, knowledge sharing and opportunities given along this journey.

A special acknowledgement must also be given to my co-advisor, Raquel Maria Fino Seiça, for all the availability, support and encouragement given.

I am also thankful to Ana Cláudia Santos, Irina Pereira and Regina Vieira for all the companionship, support and experience sharing in laboratorial tasks.

And finally, a huge acknowledgement to my family who has always supported me and given all the strength to concretize the present work.