



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA



PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

**Carreadores lipídicos nanoestruturados à base de compostos naturais com atividade
antineoplásica**

Aluno: Ma. Letícia Santos Pimentel

Orientador: Dra. Lígia Nunes de Moraes Ribeiro

Coorientadora: Dra. Luciana Machado Bastos

UBERLÂNDIA - MG
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Aos trinta e um dias do mês de janeiro de dois mil e vinte e três, às 09:00 horas, reuniu-se via web conferência pela Plataforma Google Meet, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Dr^a. Viviane Aparecida Guilherme, Dr^a. Simone Ramos de Castro, Dr^a. Thaise Gonçalves de Araújo, Dr^a. Belchiolina Beatriz Fonseca e Dr^a. Lígia Nunes de Moraes Ribeiro, orientadora da candidata. Iniciando os trabalhos a presidente da mesa, Dr^a. Lígia Nunes de Moraes Ribeiro apresentou a Comissão Examinadora e a candidata e concedeu à Discente a palavra para a exposição do seu trabalho. A duração da apresentação da Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir a senhora presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir a candidata. Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou a candidata:

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antineoplásica**

Ma. Letícia Santos Pimentel

COMISSÃO EXAMINADORA

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As sugestões da Comissão Examinadora e as Normas do PPGGB para o formato da Tese foram contempladas.

UBERLÂNDIA - MG
2023

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“If I have seen further, it is by standing on the shoulders of giants.”

Isaac Newton (1643 – 1727)

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Apresentação

O câncer é um termo usado para abranger mais de 200 doenças multifatoriais caracterizadas por crescimento e invasão descontrolados de células anormais levando à formação de tumor. O câncer de próstata é o segundo câncer mais prevalente em homens em todo o mundo e o quarto em geral. O tratamento para o câncer de próstata permanece limitado à cirurgia, radioterapia e quimioterápicos que atuam não apenas nas células cancerígenas, mas também nos tecidos normais. Produtos naturais possuem grande potencial no tratamento do câncer, podendo apresentar menos ou nenhum efeito colateral.

Óleos essenciais (OEs) são misturas complexas de compostos aromáticos voláteis de ocorrência natural presentes nas folhas, flores, frutos, sementes, brotos, rizomas, raízes e cascas. Por sua grande diversidade química, os OEs possuem diversas propriedades medicinais e têm sido utilizados desde a antiguidade no tratamento de diversas doenças. Dentre inúmeras atividades biológicas, podemos citar a atividade anticancerígena. Eles podem combater o câncer por meio de mecanismos antioxidantes, antimutagênicos, antiangiogênicos, antiproliferativos, por indução enzimática e modulação de mecanismos de resistência a múltiplas drogas e do sistema imunológico.

O capítulo I apresenta uma revisão bibliográfica integrativa sobre os efeitos terapêuticos dos OEs de várias espécies vegetais e seus principais componentes ativos em células de câncer de próstata.

O capítulo II contempla o artigo científico no qual consta estudos experimentais baseados no desenvolvimento pioneiro de formulações nanoestruturadas compostas por excipientes naturais que encapsulam diferentes OEs como tratamentos promissores contra o câncer de próstata. Para tanto, uma triagem de dez OEs foi realizada para avaliar os efeitos citotóxicos em células saudáveis de próstata e de câncer de próstata com o intuito de selecionar os lipídios líquidos estruturais e bioativos mais promissores para a preparação subsequente de carreadores lipídicos nanoestruturados (Nanostructured lipid carriers -NLC). Com o intuito de desenvolver NLCs robustos, três formulações foram otimizadas empregando planejamentos fatoriais do tipo 2³ e avaliadas quanto a estabilidade físico-química de longo prazo e térmica, morfologia, estrutura e atividade biológica, garantindo sua qualidade, segurança e eficácia.

Chapter I – Therapeutic effects of essential oils and their bioactive compounds on prostate cancer treatment

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Abstract

Since prostate cancer relies on limited therapies, more effective alternatives are required. Essential oils (EOs) and its bioactive compounds are based on natural products that play an important role in cancer treatment. Several action mechanisms of EOs have been described against prostate cancer, such as antioxidant, antimutagenic, antiangiogenic, antiproliferation, enhancement of immune system, enzyme induction and modulation of multidrug resistance. This review covers the most recent works to summarize anti prostate cancer mechanisms of EOs from several plant species and their main active compounds including the perspectives and the remained challenges.

Key words: Essential oil, prostate cancer, natural treatment

1. Introduction

1.1 Overview of prostate cancer

Cancer is considered one of the most alarming diseases in the last few decades worldwide. It is a term used to cover more than 200 multifactorial diseases characterized by uncontrolled growth and invasion of the abnormal cells leading to the formation of tumor in healthy tissues (Vecchi et al., 2021). Prostate cancer (PCa) is the second most prevalent cancer in men throughout the world and the fourth overall (World Health Organization, 2022). Risks factors include age, ethnicity, diet, genetic, enviromental and others. The incidence rate for men under the age of 50 years is 1 in 350 men but it increases to nearly 60 % in men over the age of 65 years (Rawla, 2019). PCa is a highly heterogeneous disease whose subtypes remains poorly understood (Kaffenberger & Barbieri, 2016).

In early stages, primary treatment is based on radiation and surgery. However, up to 53% of these progress to recurrence. Recurrence or metastatic tumors are usually treated with androgen deprivation therapy (ADT) in the form of gonadotropin-releasing hormone (GnRH) and androgen receptor (AR) antagonists, so progression can be prevented and patient survival can be improved. However, in most cases, despite initial response, cells become therapeutic resistant, progressing to castration resistant PCa (CRPC) and metastatic CRPC (mCRPC) wich leds to lethal disease (Y. A. Wang et al., 2020). CRPCs are characterized by constitutive activation of AR signaling through expression of AR variants, AR gene/enhancer amplification, AR mutations, overexpression of AR coactivators, AR-independent pathways and other mechanisms (Feng & He, 2019; Jernberg et al., 2017).

In PCa cells, defective phosphatase and tensin homolog (PTEN) and uncontrolled activation of phosphatidylinositol-3 kinase (PI3K)/AKT signaling frequently promotes cancer progression (Figure 1). The tumor supressor PTEN is frequently mutated and shows loss of function in PCa allowing strong activation of PI3K/AKT signaling pathway. Downstream of PI3K and AKT, the protein kinase mechanistic target of rapamycin (mTOR) plays an important role in cell growth regulation, being often associated to tumorigenesis (Wise et al., 2017). Other target molecules of downstream PI3K/AKT signaling, such as cyclin-dependent kinases (CDK) and forkhead box subgroup O (FoxO), contribute to suppress the cell cycle control and apoptotic mechanisms leading to resistance to chemotherapeutic drugs (West et al., 2002).

The Ubiquitin-Proteasome (UPS) pathway plays a key role in degradation of intracellular proteins involved in regulation of several cellular processes, such as cell cycle, DNA damage

response, cell growth, apoptosis, angiogenesis and others (Figure 1). In PCa cells, degradation of PTEN, cyclin-dependent kinase inhibitor p27 and Bcl2-associated x (Bax) by UPS pathway are increased (Chen & Zhao, 2013).

DNA damage repair (DDR) is another pathway altered in PCa cells. DDR deficiency induces cell dependence on poly (ADP-ribose) polymerase (PARP)-1 protein for DNA repair. Use of a PARP-1 inhibitor leads to PCa cell death (Risdon et al., 2021; Teyssonneau et al., 2021). Treatment options for PCa remain limited, once chemotherapeutics presents severe side effects, acting not only on cancer cells, but also on normal tissues. Therefore, searching for new safe and efficient active compounds to PCa treatment is mandatory (Machado et al., 2022). In this sense, natural products could be great potential therapeutics in cancer treatment, once that several pharmaceutical drugs have been developed through compounds derived from plants.

1.2 PCa culture cell lines

Before proceeding with the review, it is important to understand the main PCa cell line models. DU145, PC3 and LNCaP were the first three PCa cell lines established from metastasis tumor and are the most used until nowadays, even with several others cell lines and sublines being developed (Namekawa et al., 2019). The DU145 is an androgen-resistant cell line isolated in 1975 from a brain metastatic PCa of a 69-year-old white man (D D Mickey et al., 1977). The androgen-resistant PC3 cell line was established in 1979 and derived from a lumbar vertebral metastatic PCa of a 62-year-old white man (M E Kaighn et al., 1979). A subline was developed in 1984 from a PC-3 xenograft tumor in athymic mouse, called PC-3M, and revealed to be more aggressive than the parental PC3 cell line (J M Kozlowski et al., 1984). The androgen-responsive LNCaP cell line was established in 1980 and obtained from a needle aspiration biopsy of a lymph node metastatic lesion from a 50-year-old white man (Horoszewicz et al., 1980). A subline from a vertebral metastasis of LNCaP xenografts in castrated mouse was isolated in 1994 and called C4-2 cell line. These cells became androgen-resistant upon interaction with bone fibroblasts (H C Wu et al., 1994). Another subline (C4-2B) was developed from bone metastasis of C4-2 xenografts in the same year (G N Thalmann et al., 1994). The 22Rv1 is an androgen-responsive cell line was isolated in 1999 from a CWR22R xenograft tumor in mouse isolated from a patient with bone metastasis (R M Sramkoski et al., 1999).

1.3 Essential oils: nature and biological activities

Essential oils (EOs) are natural lipid products present in various aromatic plants derived from their secondary metabolites. They are usually obtained from leaves, flowers, fruits, seeds, buds, rhizomes, roots and/or bark (Cascaes et al., 2021; Ferreira et al., 2022), being extracted by steam distillation and cold pressing process (A. de Groot & Schmidt, 2016b). EOs are defined as complex mixtures of approximately 20 to 60 components, with two or three of them found in high concentrations (20-70 %) (Aziz et al., 2018; Bakkali et al., 2008; Dehsheikh et al., 2020). The chemical composition of volatile fractions mainly includes mono and sesquiterpenes, followed by several oxygenated derivatives, alcohols, aliphatic aldehydes and esters. On the other hand, the non-volatile fractions comprise 1-10 wt % of EO being composed of carotenoids, fatty acids, flavonoids and waxes (Aziz et al., 2018). Generally, they are lipophilic, organic solvents soluble and water immiscible products (Asbahani et al., 2015).

Considering its wide chemical diversity, EOs have a lot of properties being used since the Ancient times as medicine. Among its several activities, it can be mentioned antioxidant, anti-inflammatory, antibiotic, antiviral, antifungal, anti-parasitical, insecticidal, anticancer, wound healing, antihypertensive, analgesic and other clinical uses, such as sedative, spasmolytic, analgesic, anaesthetic and anxiolytic (Ishfaq et al., 2018; Ramsey et al., 2020). Furthermore, global EO industry is valued at 18.6 billion US\$ and expected to reach 35.5 billion by 2028. This exponential growth can be attributed to increasing preference for a healthy lifestyle. The commercial EO use in aromatherapy, (mainly in spa and massage therapies) is the dominant application segment, followed by food and beverages industries (Ridouh & Hackshaw, 2022). Pharmaceutical fields have also great interest in EOs properties, as rich sources of therapeutic compounds.

EOs are considered promising candidate to the cancer treatment through antioxidant, antimutagenic, antiangiogenic, antiproliferation, enhancement of immune system, enzyme induction and modulation of multidrug resistance mechanisms (Bhalla et al., 2013; Gautam et al., 2014). In PCa cells (Figure 1), EOs were demonstrated to act on PI3K/AKT signaling and Ubiquitin-Proteasome (UPS) pathway resulting in apoptosis. Moreover, EOs are able to induce mitochondrial stress leading to changes in expression of Bcl-2/Bax genes and membrane depolarisation resulting in increased release of cytochrome-C to the cytoplasm and induction of apoptosis. In addition, EOs are able to increase intracellular reactive

oxygen species (ROS) levels resulting in apoptosis of PCa cells. (Gautam et al., 2014). All of these signaling pathway may be targets for novel therapeutic drugs for PCa treatment.

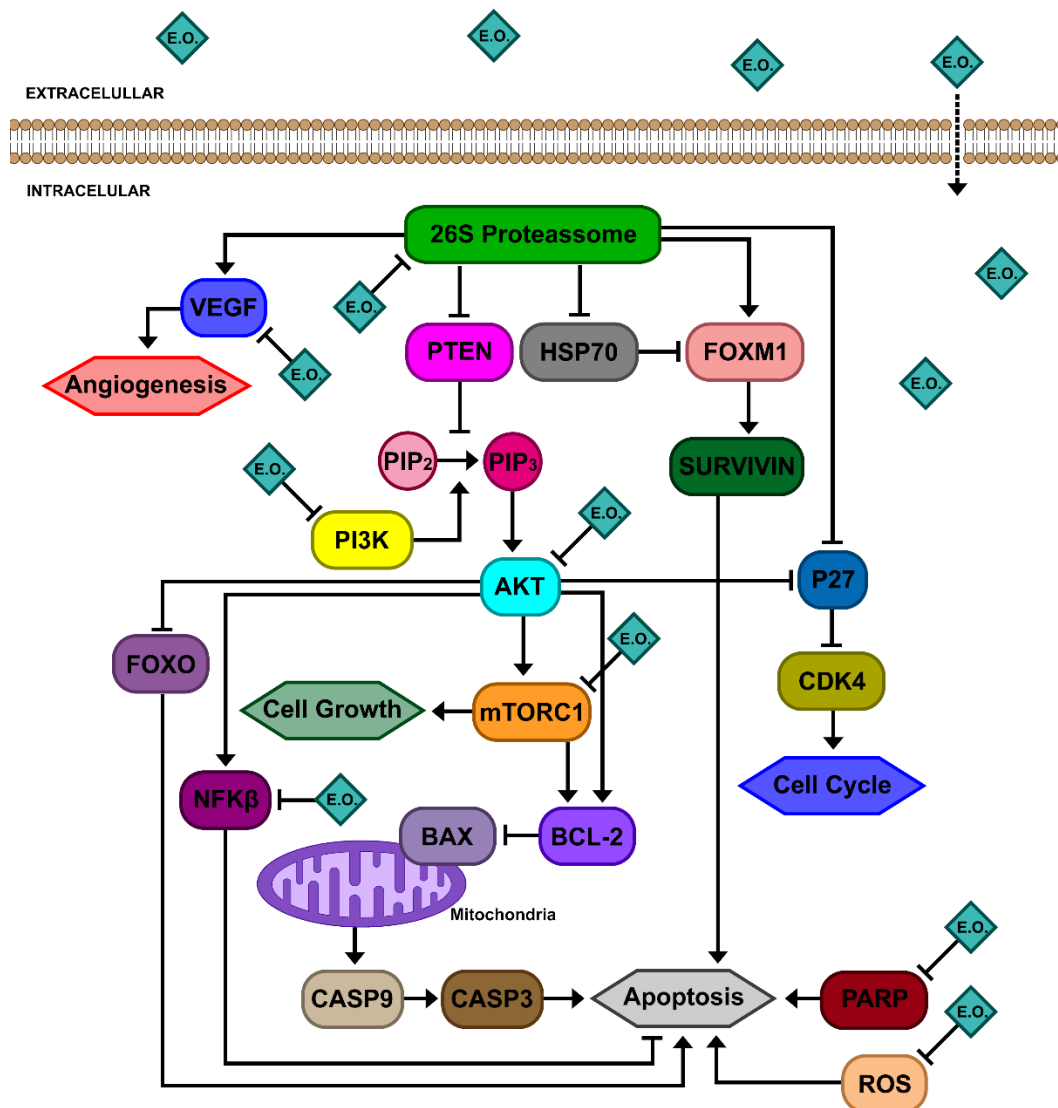


Figure 1. EOs and their bioactive compounds act in multiple pathways in PCa cells. EOs have cell membrane permeability and interact with several cellular targets involved in different pathways. EOs are able to interact with different targets of PI3K/Akt pathway leading to cell cycle arrest, apoptosis, inhibition of cell growth and proliferation. EOs can also play as proteasome inhibitors resulting in apoptosis, decrease in cell proliferation and suppression of angiogenesis. EOs modulate DNA damage repair mechanisms by acting as DNA polymerase inhibitors, which leads to PARP cleavage, resulting in apoptosis. EOs were demonstrated to induce mitochondrial stress leading to changes in expression of Bcl-2/Bax genes and membrane depolarisation resulting in increased release of cytochrome-C to the

cytoplasm and induction of apoptosis. In addition, EOs increase intracellular ROS levels resulting in PCa cells apoptosis.

In this context, the present work carried out a integrative review on anticancer effects of EOs from several plant species, also focusing on their main bioactive compounds as effective treatments against prostate cancer cells. Table 1 listed the succesful *in vitro* studies of EOs extracted from several plants against different prostate cancer cell lines. Table 2 displayed *in vitro* results of the major EO compounds.

2. Chamomile

Chamomile is one of the most common and well-known herbs widely used in medicinal applications from Asteraceae family. It has been traditionally used to treat several diseases due to its antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic, insecticidal, antidiabetic and anti-tumoral activities. Chamomile EO is obtained from flowers by steam distillation. (el Mihaoui et al., 2022; Srivastava et al., 2010). German chamomile (*Matricaria chamomilla*) and Roman chamomile (*Chamaemelum nobile*, also called *Anthemis nobilis*) EOs contains mainly the terpenoids α -bisabolol and its oxides A and B and chamazulene (Chauhan et al., 2022; Srivastava et al., 2010).

In a previous report, the cytotoxicity of chamomile (*Anthemis nobilis*) EO was evaluated in PC3 PCa cell line and it was found a dose-dependent decrease in the survival of cells with an IC₅₀ value of 0.071 % (v/v). At a concentration of 0.2 % (v/v) the EO exhibited strong cytotoxicity, showing cell viability lower than 4 % (Zu et al., 2010). A comparative work has evaluated the viability of PC3, DU145 and LNCaP human PCa cells exposed to chamomile (*Matricaria chamomilla*) aqueous and methanolic extracts. The aqueous extract treatment resulted in a time and dose-dependent reduction in cell viability for all the cell lines studied, with IC₅₀ values ranging from 2000 to 3000 μ g/mL after 72 h of treatment. Similar results were found in all the cells lines after treatment with methanolic extract, with IC₅₀ values ranging from 100 to 200 μ g/mL after 24 h treatment. In addition, exposure to both chamomile extracts for 48 h lead to apoptosis in all PCa cell lines with formation of internucleosomal DNA fragments. Still, such treatment did not reduce viability nor induced apoptosis in virally transformed normal human prostate epithelial PZ-HPV-7 cells (Srivastava & Gupta, 2007).

Apigenin, a natural flavonoid present in chamomile, oregano and other plants, inhibited growth of PC3, DU145 and LNCaP cells after 24 h of treatment in a dose-dependent manner (Srivastava & Gupta, 2007). The anti-proliferative effect of apigenin in PC3 and LNCaP cells can be explained by the reduced of net gain of DNA content, cell cycle arrest at G₂ phase and reduced cyclin B1, Polo-like kinase 1 (PLK1) and Aurora A at both protein and mRNA levels. CDK1 were also reduced in PC3 cells (Hnit et al., 2022). Apigenin treatment in transgenic adenocarcinoma of the mouse prostate (TRAMP) has suppress prostate tumorigenesis by activating FoxO3a and upregulation of FoxO-responsive proteins BIM and p27/ Kip1, resulting in cell cycle arrest at G₀/G₁ phase and reduced proliferation in prostate tumors. Similar results were observed in PC3 and LNCaP calls treated with apigenin (S. Shukla et al., 2014).

3. Cinnamon

The *Cinnamon* genus belongs to Lauraceae family and includes more than 250 species of evergreen trees. It is a common spice that possess several biological properties such as antioxidant, anti-inflammatory, antibacterial, antifungal, anti-parasitic, antidiabetic and antitumor (Ranasinghe et al., 2013; Yanakiev, 2020). Cinnamon (*Cinnamomum zeylanicum*, also called *Cinnamomum verum*) EO is obtained by steam distillation, usually from the bark, and the main components are cinnamaldehyde and eugenol (Denkova-Kostova et al., 2021; European Medicines Agency, n.d.-a).

The viability of PC3 PCa cells treated with cinnamon EO was determined by MTT essay. Results reveled a dose-dependent decrease in the survival of cells with an IC₅₀ value of 0.012 % (v/v) (Zu et al., 2010). Another work demonstrated that aqueous cinnamon extract and procyanidin B2 (PCB2), a hydrophilic component, induced time and dose-dependent decrease in viability of PC3 and LNCaP cells. Moreover, apoptosis occurred by inhibition of 26S proteasome activity, induction of a dose-dependent increase in caspase-3 activity, decrease in Akt protein levels and decrease of anti-apoptotic (Survivin and XIAP) and angiogenic (VEGF and VEGF receptor) gene markers expression in both cancer cells lines (Gopalakrishnan et al., 2018). HPLC analysis of this aqueous cinnamon extract revealed the presence of high amounts of cinnamaldehyde (Gopalakrishnan & Ismail, 2021).

Other relevant compounds that have been isolated from cinnamon include eugenol and cinnamic acid. Cinnamaldehyde, eugenol and cinnamic acid compounds were able to inhibit proteasome activity in both PC3 and LNCaP cells, resulting in an increase in the expression

of chaperone protein (Hsp70), which negatively regulate the expression of FoxM1, an oncogenic transcription factor. In addition, cinnamon compounds downregulated the expression of angiogenic factors (VEGF and VEGFR), acting as antiangiogenic agents. Treatment of tumor cells with cinnamon compounds also led to a dose-dependent decrease in cell viability with IC₅₀ values of 14.3 μ M for cinnamaldehyde, 73.2 μ M for eugenol and 6.2 μ M for cinnamic acid (Gopalakrishnan & Ismail, 2021). Eugenol also inhibit growth of DU145 cells (Carrasco et al., 2008). Other authors reported that cinnamaldehyde inhibited the growth of PCa-associated fibroblasts (CAFs) in a dose-dependent manner, showing an IC₅₀ value of 74.66 μ M. Prostate CAFs play an important role in promoting carcinogenesis and progression of prostate cancer. Besides that, cinnamaldehyde induced apoptosis by cell cycle arrest at G₂/M phase, increased ROS generation, decreased Glutathione (GSH) levels and caspase activation on prostate CAFs (Han et al., 2020). It also relieves the immunosuppressive effects on prostate CAFs in a Toll-Like Receptor 4 (TLR4)-dependent manner (Mei et al., 2020).

β -Caryophyllene oxide, a bicyclic sesquiterpene isolated from several EOs including cinnamon EO, inhibited growth of PC3 PCa cells in a time and dose-dependent manner. The sesquiterpene have also induced apoptosis by inhibiting PI3K/AKT/mTOR/S6K1 signaling, reduced mitochondrial membrane potential, cytochrome c release, activating caspase-3, cleavage of PARP, ROS generation, downregulation of anti-apoptotic proteins (Bcl-2, Bcl-xL, survivin, IAP-1 and IAP-2), cell cycle protein cyclin D₁ and upregulation of the tumor suppressor protein p53 and CDK inhibitor p21. In addition, β -Caryophyllene oxide downregulated proteins linked with metastasis and angiogenesis (COX-2 and VEGF) (Delgado et al., 2021; K. R. Park et al., 2011). Another report showed that β -Caryophyllene oxide inhibited cell growth, invasion and constitutive STAT3 activation in DU145 cells. Constitutive activation of STAT3 is often active and linked with proliferation, survival, invasion, metastasis and angiogenesis in tumor cells (C. Kim et al., 2014).

4. *Citrus* species

The genus *Citrus* (Rutaceae family) is one of the most ancient and popular crops (Gloria A. Moore, 2001). Citrus fruit are a significant source of vitamins, as ascorbic acid. Biological activities of citrus fruit include antioxidant, anti-inflammatory, antibacterial, antifungal, antitumor and others. The use of EO as natural preservatives in food industry has been extensively studied (Dosoky & Setzer, 2018a). Generaly, EOs of citrus fruit are obtained by

cold pressing method from different parts of plants such as fruit peel and leaves, mainly composed by limonene (ben Hsouna et al., 2017; A. C. de Groot & Schmidt, 2016; Deng et al., 2020; Denkova-Kostova et al., 2021; Dosoky & Setzer, 2018a).

Mandarin (*Citrus reticulata*) EO treated PC3 PCa cells exhibited a dose-dependent decrease in viability with an IC₅₀ value of 10.97 µg/mL. In addition, it induced apoptotic DNA fragmentation, ROS generation and decrease the expression of B-cell lymphoma 2 (Bcl2) and Murine Double Minute 2 (MDM2) genes, while it has increased the expression of p53 and Bax genes (Hussien & Mohamed, 2021). Lemon (*Citrus limon*) and Grape fruit (*Citrus paradisi*) EO treatment in PC3 cell line revealed a dose-dependent decrease in the survival of cells with an IC₅₀ values of 0.083 % and 0.094 % (v/v), respectively (Zu et al., 2010). Navel Orange (*Citrus sinensis*) EO treatment revealed time and dose-dependent inhibition of proliferation of 22RV1 cells by inducing apoptosis. IC₅₀ values at 24, 48 and 72 h were 45.74, 42.83, and 39.79 µg/mL, respectively (C. Yang et al., 2017). Kumquat (*Fortunella margarita*) EO showed a time and dose-dependent antiproliferative activity against LNCaP cells by inducing apoptosis and inhibition of inflammation. The treatment increased Bax/Bcl2 ratio and induced cleavage of caspase-3 indicating induction of apoptosis. Besides, it decreased expression of inflammatory transcription factor NFκB and Cox-2, a downstream product of NFκB, indicating inhibition of inflammation (Jayaprakasha et al., 2012).

Limonene, a hydrocarbon monoterpene present in several citrus oils, inhibited growth of DU145 cells in a dose-dependent manner with an IC₅₀ value of 2.8 nM. In combination with docetaxel, limonene has enhanced cell growth inhibition. Moreover, the combination treatment induced ROS generation and enhanced apoptotic cell death by inducing cytoplasmic histone-associated DNA fragmentation, caspase-3 and caspase-9 cleavage, up regulation of p21, p53 and Bad, downregulation of Bcl-xL and cleavage PARP protein. No effect on caspase-8 cleavage and in the expression of Bax and Bcl-2 were observed (Rabi & Bishayee, 2009).

5. Turmeric

The genus *Curcuma* from Zingiberaceae family is constituted by perennial rhizomatous herbs and includes about 100 species (Dosoky & Setzer, 2018b). Turmeric (*C. longa*) is widely explored as coloring and flavoring agents, as well as in pharmaceutical industry due to its antioxidant, anti-inflammatory, antimicrobial, antiviral, antitumor, antidiabetic, antiasthmatic, hypoglycaemic, neuro and dermoprotective and others properties. The EO is

obtained by rhizome and is mainly composed by α and β -turmerone (Dosoky & Setzer, 2018b; Ibáñez & Blázquez, 2021).

Turmeric EOs obtained by *C. longa* rhizomes collected from 20 different habitats was investigated by its cytotoxicity activity against LNCaP cells. It was revealed an inhibition of cell growth with IC₅₀ values ranging from 16.41 to 124.27 $\mu\text{g/mL}$ (Zhang et al., 2017). Turmeric EO treated PC3 prostate cancer cells revealed inhibition of cells growth with IC₅₀ value of 97.94 $\mu\text{g/mL}$ (E. Essien et al., 2015).

β -elemene reduced PC3 and DU145 cell growth in a time and dose-dependent manner, with IC₅₀ values of 105, 102 and 96 $\mu\text{g/mL}$ at 24, 48 and 72 h; and 75, 70 and 66 $\mu\text{g/mL}$ at 24, 48 and 72 h, respectively. Treatment with β -elemene also induced apoptosis in a time and dose-dependent manner through cleaved caspase-3, caspase-9 and PARP increasing and Bcl-2 downregulation (Li et al., 2010).

6. *Cymbopogon* species

The *Cymbopogon* genus belongs to Andropoganeae family and includes more than 144 species of grass aromatic plants comprising lemon grass (*C. citratus*), tsauri grass (*C. giganteus*) and citronella (*C. nardus*). *Cymbopogon* species has several biological activities such as antioxidant, anti-inflammatory, antibacterial, antifungal, insecticidal and antitumor among others. *Cymbopogon* EOs are extensively used in fragrance, cosmetic, food and flavor industries. It is obtained by hydrodistillation from the leaves and the main components are geraniol, citral and citronellal depending on the specie (Tibenda et al., 2022).

Lemon grass and tsauri grass EOs treatment in PC3 and LNCaP cells showed to act as antiproliferative agents. IC₅₀ values of lemon grass were 32.1 $\mu\text{g/mL}$ for PC3 cells and 6.34 $\mu\text{g/mL}$ for LNCaP. Tsauri grass exhibited values round 303.2 $\mu\text{g/mL}$ for PC3 cells and 160.1 $\mu\text{g/mL}$ for LNCaP (Bayala et al., 2018). Citronella EO also has antiproliferative activity in LNCaP cells (IC₅₀ values of 58 $\mu\text{g/mL}$), caused by cell cycle arrest at G₂/M phase and changes in cell morphology (Bayala, Coulibaly, et al., 2020).

Geraniol, an acyclic monoterpene, inhibited cell growth of PC3 in the range of 0.25-1 mM. In the cells treated with geraniol, it was observed increased LDH and caspase-3 activity, induced mitochondrial membrane depolarization and cell cycle arrest at G₁ phase. Besides, expressions of four cyclin isotypes (cyclin A, B, D, and E), two of CDK family (CDK1 and CDK4), and two anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-w) were reduced while expressions of two CDK inhibitory proteins (p21 and p27) and two pro-apoptotic Bcl-2

family members (Bax and BNIP3) were noticeably elevated in cells treated with geraniol. In addition, geraniol treatment in PC3 cell xenograft tumor in nude mice has efficiently suppressed tumor growth by inducing apoptosis and cell cycle arrest (S. H. Kim et al., 2011). Geraniol treated PC3 cells also had autophagy induced, AKT/mTOR signaling inhibited (S. H. Kim et al., 2012) and downregulated the E2F8 transcription factor (Lee et al., 2016). Citral, an acyclic monoterpene, treatment in PC3 and PC3M PCa cells has inhibited cell viability in a dose-dependent manner with IC₅₀ values of 10 and 12.5 µg/mL. Citral treatment also reduced clonogenic potential, induced morphological alterations and expulsion of lipid droplets by activation of AMPK protein expression and consequently downregulation of AMPK pathway genes such as SREBP1, ACC and HMGR. In addition, Citral treatment induced apoptosis by DNA fragmentation, upregulation of Bax and downregulation of Bcl-2 expression (Balusamy et al., 2020).

7. Ginger

Ginger (*Zingiber officinale*), member of Zingiberaceae family, is a common spice used as a flavoring agent in beverages and food preparations. Ginger is also known to possess many therapeutic uses such as antioxidant, anti-inflammatory, anti-microbial and antitumor (Y. Shukla & Singh, 2007; Zheng et al., 2016). EO of ginger is obtained from rhizome and contains mainly zingiberene, curcumene and farnesene molecules in this complex mixture (Y. Shukla & Singh, 2007).

Ginger EO treatment in PC3 cells exhibited a dose-dependent cytotoxicity, showing IC₅₀ value of 0.077 % (v/v) (Zu et al., 2010). Another work also showed time and dose-dependent antiproliferative activity of ginger EO in PC3 and LNCaP cell lines, with IC₅₀ value of 0.42 mg/mL and 0.38 mg/mL, respectively (Bayala et al., 2014). Moreover, ginger extract treatment was able to inhibit proliferation of PC3 (IC₅₀ of 250 µg/mL), LNCaP (IC₅₀ of 75 µg/mL), DU145 (IC₅₀ of 95 µg/mL), C4-2 (IC₅₀ of 512 µg/mL) and C4-2B (IC₅₀ of 240 µg/mL) PCa cells while no effect was observed in normal prostate epithelial cells (PrEC). In addition, the treatment with ginger extract led to cell cycle arrest in PC3 cells by decreasing cyclin D1, cyclin E and CDK4 levels, and increasing p21 and CDK4 inhibitor. Besides, ginger extract induced apoptosis in PC3 cells by increasing Bax and decreasing Bcl2 expressions, releasing cytochrome c, increasing caspase-3 activity and cleaved PARP protein levels. In LNCaP cells, ginger extract also led to cell cycle arrest and increase

caspase-3 activity. Mice treated with ginger extract have tumor tissue suppressed and no detectable toxicity in normal tissues was reported (Karna et al., 2012).

8. Lavender

The genus *Lavandula* (Lamiaceae family) comprise herbs popularly known for relieving stress, anxiety and depression. It also has other important therapeutic properties, such as antioxidant, anti-inflammatory, antibacterial and antitumor (Fahmy et al., 2022; Ridouh & Hackshaw, 2022; Zhao et al., 2017). Lavender EO is obtained from flower heads and foliage by steam distillation and the main components are linalyl acetate and linalool molecules (Cavanagh & Wilkinson, 2002; A. de Groot & Schmidt, 2016a).

The cytotoxicity activity of lavender (*L. stoechas*) EO was evaluated in PC3 PCa cells. It was found a dose-dependent decrease in the survival of cells with IC₅₀ value of 0.05 % (v/v) (Zu et al., 2010). *L. stoechas* EO also was cytotoxic in LNCaP PCa cells (Gören et al., 2002). PC3 cells was also treated with lavender (*L. officinalis*) EO and showed a slight reduction on viability of cells (Fahmy et al., 2022). Lavender (*L. angustifolia*) EO treatment in PC3 and DU145 cells revealed a time-dependent decrease on viability of cells with IC₅₀ values of 0.037 % and 0.199 % (v/v), respectively. The treatment with the main components. linalyl acetate and linalool, have also showed a potent cytotoxicity against both cell lines, with IC₅₀ values of 4.98 µM and 11.74 µM in PC3 and 3.06 µM and 7.22 µM in DU145 cells, respectively. In addition, EO, linalyl acetate and linalool treatments inhibited migration and induced apoptosis by cell cycle arrest at G₂/M phase in PC3 and at S phase in DU145 PCa cell lines. Lavender EO and its components have also inhibited tumor growth of human PCa xenografts in mice (Zhao et al., 2017). Moreover, linalool treatment inhibited cell proliferation of 22Rv1 PCa cells in a dose-dependent manner, showing an IC₅₀ value of 3.38 mM. Linalool have also induced apoptosis, cell cycle arrest at G₀/G₁ phase and significant increase in expression of Bax, Bcl-2, p53, TRAIL receptors 1 (DR4), TRAIL receptors 2 (DR5) and cleaved caspases. The treatment with linalool suppressed significantly tumor growth of human PCa xenografts in mice by inhibiting tumor cell proliferation and apoptosis (Zhao et al., 2020).

9. *Mentha* species

The genus *Mentha* belongs to Lamiaceae family and possess more than 25 species including *M. arvensis* (menthol mint), *M. piperita* (peppermint), *M. longifolia* (wild mint) and *M.*

spicata (spearmint). EOs and extracts from *Mentha* species have been used since old times for treatment of several gastrointestinal system diseases. EO is obtained from leaves and flowering aerial parts and the main components are carvone, limonene, menthone, menthol and piperitone oxide molecules (A. de Groot & Schmidt, 2016a; Hussain, Anwar, Nigam, et al., 2010; Mahboubi, 2021).

It has been evaluated the cytotoxicity of the 4 mentha species-based EO in LNCaP PCa cells. All of them showed strong cytotoxicity against the cancer cell line, with an IC₅₀ value of 55.7, 95.7, 52.0 and 90.0 µg/mL, respectively (Hussain, Anwar, Nigam, et al., 2010). Another work has evaluated the cytotoxicity of spearmint EO in PC3 cells and it was found a dose-dependent decrease in the survival of cells with an IC₅₀ value of 0.088 % (v/v) (Zu et al., 2010).

Menthol can evoke cold sensation mediated by cold-sensitive transient receptor potential melastatin 8 (TRPM8). This receptor was found to be expressed at high levels in several tumors, including PCa tissue and PC3, LNCaP and DU145 culture cell lines (Nazıroğlu et al., 2018). Researchers evidenced a dose-dependent decrease on viability of PC3, LNCaP and DU145 cells treated with menthol (S. H. Kim et al., 2009; Nazıroğlu et al., 2018; E. J. Park et al., 2009; Y. Wang et al., 2012). Menthol also inhibits DU145 cell migration and induce cell cycle arrest at G₀/G₁ phase (Y. Wang et al., 2012). On the other hand, PC3 and LNCaP cell cycle was not affected by menthol treatment (S. H. Kim et al., 2009). Although all the three tested cell lines have expressed TRPM8 receptor, menthol activity seems to be not mediate by TRPM8 pathway (S. H. Kim et al., 2009; Nazıroğlu et al., 2018).

10. Rosemary

Rosemary (*Rosmarinus officinalis*), member of Lamiaceae family, has been used as stimulant, analgesic and anti-inflammatory compound. Besides, pharmacologically properties of rosemary include antioxidant, antibacterial, antifungal, antiviral and antiproliferative. Such EO is mainly composed of eucalyptol, α-pinene and camphor (Christopoulou et al., 2021).

A report has evaluated the cytotoxicity of rosemary EO in LNCaP cell line and an IC₅₀ value of 180.9 µg/mL has been found (Hussain, Anwar, Chatha, et al., 2010). Rosemary extract treatment in LNCaP and 22Rv1 prostate cells inhibit cell growth with (IC₅₀ of 27 and 13.3 µg/mL, respectively), induced cell cycle arrest at G₁/G₂ phase and G₂ phase, respectively, and induced apoptosis by increasing expression of Bax and cleaved caspase-3

(Petiwala et al., 2014). Rosemary extract also inhibited cell growth, survival and migration, inducing apoptosis in PC3 PCa cells, while it had no significant effect on proliferation of PNT1A normal PCa cells (Jaglanian et al., 2020).

α -pinene, one of the most common terpenes of rosemary EO, possess a strong inhibitory effect on growth of PC3 and DU145 PCa cells with IC₅₀ values of 2.9 and 5.8 μ M, respectively. In addition, α -pinene induced apoptosis and cell cycle arrest at S phase in PC3 cells and at G₂/M phase in DU145. In xenograft tumors, α -pinene suppress significantly its growth and induces apoptotic cells death (Zhao et al., 2018).

Rosmarinic acid, a phenolic compound, decreased viability of PC3 and DU145 PCa cells in a dose-dependent manner. Treatment with rosmarinic acid also induced apoptosis with DNA fragmentation, increased p53, Bcl-2 and caspase-3 expression (Jang et al., 2018).

Carnosic acid, a polyphenolic diterpene, inhibited cell growth in PC3 and DU145 PCa cells. In PC3 cells, carnosic acid has induced apoptosis with DNA fragmentation, caspase activation, PARP cleavage, upregulation of Bax, downregulation of Bcl-2 and increased cytochrome c release. In addition, carnosic acid inhibits constitutive IKK/NF- κ B signaling and Akt signaling pathway in PC3 cells (Kar et al., 2012).

12. *Salvia* species

Salvia genus (Lamiaceae family) comprising about 1000 species are herbaceous, suffruticosus or shrubby perennial plants. Sage EO is commonly used as analgesics, anti-inflammatory, anti-viral, antitumor, in the treatment of cardiovascular and liver diseases, in food and cosmetics industries among other applications. Sage EO is obtained by steam distillation process and the main components are linalyl acetate and linalool (Kumar Singh et al., 2021; Ovidi et al., 2021).

A group of researchers studied the effects of *S. aurea*, *S. Judaica* and *S. viscosa* EOs in DU145 PCs cells. Treatment with the three EOs in the range of 12.5-50 μ g/mL induced a dose-dependent decrease in cell growth. However, only at 50 μ g/mL it was observed increase in lactate dehydrogenase (LDH), indicating induction of necrosis cell death at this concentration. Furthermore, at 12.5 and 25 μ g/mL the EOs treatment caused increase in DNA fragmentation, caspase activity and Bax/Bcl-2 ratio and at all three concentrations caused increase in ROS generation and decrease in GSH levels in a dose-dependent manner (Russo et al., 2018). Although *S. officinalis* EO treatment in the range of 5-400 μ g/mL in

LNCaP cells has no significant effect, treatment with the sesquiterpene α -humulene showed high cytotoxicity with IC₅₀ value of 11.24 μ g/mL (LOIZZO et al., 2007).

12. Thyme

Thymus genus from Lamiaceae family contains about 400 species of perennial aromatic, evergreen or semi-evergreen herbaceous plants (Borugă O et al., 2014; Kowalczyk et al., 2020). *Thymus* species have been used to treat several cardiorespiratory and gastrointestinal diseases due to their antioxidant, antibacterial, antifungal, antiviral and antispasmodic, among other properties. Moreover, thyme EO is used in food and cosmetics as antioxidant and preservative (Salehi et al., 2018). It is a product obtained from flowering top of *Thymus vulgaris* or *Thymus zygis* or both species by steam distillation. Main components found in thyme EO are thymol and carvacrol (Borugă O et al., 2014; European Medicines Agency, n.d.-b; Paiano et al., 2020).

A previous work has evaluated the cytotoxicity of Thyme (*T. vulgaris*) EO in PC3 cell line and found a dose-dependent decrease in the survival of cells with an IC₅₀ value of 0.01 % (v/v). At a concentration of 0.2 % (v/v) the essential oil exhibited strong toxicity showing cell viability lower than 4 %. Compared to other nine EOs evaluated on this study, thyme EO showed the strongest cytotoxicity (Zu et al., 2010).

Studies reported that thymol, a monoterpene phenol, has cytotoxic effect on viability of PC3 and DU145 PCa cells. Thymol treated cells after 24, 48 and 72 h showed time and dose-dependent decreases in cancer cell growth, exhibiting IC₅₀ values of 711, 601 and 552 μ M for PC3, and 799, 721 and 448 μ M for DU145, respectively. Thymol also has induced apoptosis on both cell lines in a dose-dependent manner, but the mechanism of this action was not elucidated yet. (Elbe et al., 2020).

Carvacrol, a monoterpene phenol, was found to have antiproliferative and apoptotic action against human PCa DU145 cells in a time and dose-dependent manner, showing IC₅₀ values of 84.39 μ M and 42.06 μ M, after 24 and 48 h of treatment, respectively. Carvacrol has also induced apoptosis by caspase-3 activation, increase of ROS generation and cell cycle arrest at G₀/G₁ phase (Khan et al., 2017). In PC3 cells, carvacrol revealed similar effects inhibiting proliferation, migration and invasion. Apoptosis acted in reducing cell viability with induced activation of caspase-3, 8 and 9, induced high levels of ROS, disrupted of mitochondrial membrane potential, arrested cell cycle at G₀/G₁ phase, upregulated Bax and downregulated

Bcl-2 expression. In addition, carvacrol decreased expression of Notch1, Jagged-1, cyclin D1 and CDK4 and increased expression of p21 (Khan et al., 2019).

Carvacrol also inhibit cell growth, migration and invasion in both PC3 and DU145 cell lines by decreasing TRPM7-like current and reducing Matrix metalloproteinase-2 (MMP-2) protein expression and F-actin dynamics. In addition, both the PI3K/Akt and MEK/MAPK signaling pathways seems to be involved in anticancer effects of carvacrol (Heidarian & Keloushadi, 2019; Luo et al., 2016). Trindade et al. successfully complexed carvacrol with β -cyclodextrin in order to enhance its solubility and anticancer activity. The resulting inclusion complex reduced cell viability and migration of PC3 PCa cells in a dose-dependent manner (Trindade et al., 2019).

13. Other species

Jasmine (*Jasminum grandiflorum*) and Rose (*Rosa centifolia*) EOs treated PC3 cells revealed a dose-dependent decrease in the survival of cells with IC₅₀ value of 0.022 % and 0.04 % (v/v), respectively (Zu et al., 2010). Oregano (*Origanum vulgare*) EO, which has a high content of carvacrol, cymene and linalool, was successfully encapsulated into a nanoemulsion and inhibited significantly the growth of PC3 PCa cells in a dose-dependent manner, with an IC₅₀ value of 13.82 μ g/mL. The treatment with the oregano-based nanoemulsion has also induced apoptosis by causing cellular damage, DNA fragmentation, enhanced Bax expression, cytochrome c release and caspase-3 activation and decrease in Bcl2 expression (Perumalsamy et al., 2022).

Bayala et al., evaluated the cytotoxicity of several EOs in PC3 and LNCaP cell lines. In addition to ginger already mentioned here, *Ocimum basilicum*, *Lippia multiflora* and *Ageratum conyzoides* EOs treatment showed strong antiproliferative activity against both PCa cell lines. In PC3 cells IC₅₀ values were 0.45, 0.30 and 0.49 mg/mL, respectively, and in LNCaP cells were 0.46, 0.58 and 0.35 mg/mL, respectively. However, *Ocimum americanum*, *Hyptis spicigera* and *Eucalyptus camaldulensis* EOs have showed no antiproliferative effects (Bayala et al., 2014). *Hyptis suaveolens* EO treated LNCaP cells have showed a dose-dependent decrease in cell viability with an IC₅₀ value of 163.01 μ g/mL and cell cycle arrest at G₀/G₁ phase (Bayala, Nadembega, et al., 2020).

Zataria Multiflora EO, which is mainly composed of carvacrol, terpinene, cymene and thymol, has inhibited cell viability of PC3 PCa cells with an IC₅₀ value of 26.3 μ g/mL after 48h of treatment. It also has induced apoptosis by increasing ROS generation, DNA

fragmentation, cell cycle arrest at G₀/G₁ phase, caspase activation, upregulation of Bax and downregulation of Bcl-2 expression. Combined treatment with the EO and doxorubicin improved the effects on PC3 cells in comparison with pure drug (Zare et al., 2021).

EO from *Xylopi frutescens* leaves, which is rich in E-caryophyllene and commonly known as embira, showed cytotoxic activity against PC-3M metastatic PCa cells with an IC₅₀ value of 40 µg/mL (Ferraz et al., 2013). EO from leaves of *Guatteria pogonopus*, which is mainly composed of γ-patchoulene, (E)-caryophyllene and β-pinene, showed similar results with an IC₅₀ value of 17.0 µg/mL (Fontes et al., 2013).

Pinus mugo EO, which has a high content of β-caryophyllene, bornyl acetate and α-pinene, inhibited viability of DU145 cells in time and dose-dependent manner with an IC₅₀ value less than 50 µg/mL. Constitutive STAT3 activation signaling cascade was down-modulated, which decreased the expression of anti-proliferative as well as anti-apoptotic genes and proteins such as cyclin D1, Bcl-2, survivin, XIAP, Cox2 and IL-6. It has also induced a quick decrease in GSH levels and an increase in ROS generation. In addition, apoptotic death was induced by caspase-3 and PARP cleavage induction and cell migration was inhibited in a dose-dependent manner (Thalappil et al., 2022).

Abies balsamea (Legault et al., 2003), *Symphyopappus itatiayensis*, *Myrciaria floribundus*, *Talauma ovata*, *Psidium cattleianum*, *Nectandra megapotamica* (Apel et al., 2006), *Amomum tsao-ko* (Y. Yang et al., 2010), *Solanum erianthum*, *Solanum macranthum* (E. E. Essien et al., 2012), *Achillea wilhelmsii* (Ahmadi-jouibari et al., 2013), *Annona sylvatica* (Formagio et al., 2013), *Hypericum hircinum* (Quassinti et al., 2013), *Anaxagorea brevipes* (de Alencar et al., 2016), *Guatteria elliptica* (Rajca Ferreira et al., 2018), *Bursera glabrifolia* (Villa-Ruano et al., 2018), *Iryanthera polyneura* (Martins et al., 2019), *Aloysia polystachya* (Moller et al., 2021) and *Euodia ruticarpa* (Yeh & Lin, 2021) EOs revealed antiproliferative activity in PC3 PCa cells. In contrast, Emani et al., evaluated *Nepeta cataria* EO (15-500 µg/mL) treatment in PC3 and DU145 PCa cells and no significant effect was observed (Emami et al., 2016). Clove (*Syzygium aromaticum*) EO, which has a high content of eugenol and β-caryophyllene, also showed no significant effect on DU145 cell line in the range of 100-300 µl/mL (Dwivedi et al., 2011). The same was found to *Anemopsis californica* EO treatment on PC3 cells (Medina-Holguín et al., 2008).

14. Conclusion

PCa is the second most prevalent cancer in men worldwide and treatment options remain limited. EOs are natural products that have been used in medicine since the Ancient times. Due to a wide chemical diversity, it possesses several therapeutic properties including anticancer activity. EOs and its constituents can be promising candidates as cancer treatments since they have potent therapeutic effects and are biocompatible, abundant and cheaper than current chemotherapeutics. In addition, novel anticancer compounds can be found in EOs and further be exploited in pharmaceutical preparations for prostate anticancer therapies.

15. Perspectives

There are a lot of works that provide relevant results regarding on the EOs effects in the treatment of several diseases but few clinical tests. As observed for PCa, there are very few *in vivo* tests and none clinical trials. The most challenging aspects about EOs which limit its clinical uses is its high lipophilic nature, physicochemical instability, photosensitivity and high volatility. The processing of EO-based products in pharmaceutical field may be a good alternative to prevent EOs limitations, resulting in benefit from its therapeutic properties. New technologies of EO-based drug delivery system, such as its nanoencapsulation, may be considered to ensure its high bioavailability and, consequently, the therapeutic effects. Different nanosystems can be developed, such as nanoemulsions, liposome, lipid nanoparticles (SLN/NLC), polymer nanoparticles and cyclodextrin. Although they are different nanocarriers, all of them have compatibility to upload hydrophobic molecules with success. Therefore, they can offer a physical protection of EO together with their nanometric particle sizes, which improve EO solubility, prevent its degradation, oxidation and evaporation, allowing its safe and efficient administration for different routes. However, it is still necessary more efforts in order to develop stable, scaling up, biocompatible and efficient EO-based nanosystems. We hope that this review can stimulate further researches in this field, in order to provide promising PCa treatments in a close future.

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Conflict of interests

The authors have declared no conflict of interest.

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Table 1. Essential oils studied for anti-prostate cancer potential *in vitro* and major observations reported.

Common name	Scientific name	Cell line	Effects	References
Balsam fir	<i>Abies balsamea</i>	PC3	Inhibition of cell growth	(Legault et al., 2003)
Basil	<i>Ocimum basilicum</i>	PC3 and LNCaP	Inhibition of cell growth	(Bayala et al., 2014)
Chamomile	<i>Anthemis nobilis</i>	PC3	Inhibition of cell growth and induction of apoptosis	(Zu et al., 2010)
Cinnamon	<i>Cinnamomum zeylanicum</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
Citronella	<i>Cymbopogon nardus</i>	LNCaP	Inhibition of cell growth, cell cycle arrest and changes on cells morphology	(Bayala, Coulibaly, et al., 2020)
Embira	<i>Xylopia frutescens</i>	PC-3M	Inhibition of cell growth	(Ferraz et al., 2013)
Ginger	<i>Zingiber officiale</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
		PC3 and LNCaP	Inhibition of cell growth	(Bayala et al., 2014)
Grape fruit	<i>Citrus paradisi</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
Jasmine	<i>Jasminum grandiflora</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
Kumquat	<i>Fortunella margarita</i>	LNCaP	Inhibition of cell growth, induction of apoptosis, increased Bax/Bcl2 ratio, induced caspase-3 cleavage, inhibition of inflammation by decreased expression of NFκB and Cox-2	(Jayaprakasha et al., 2012)
		PC3	Inhibition of cell growth	(Zu et al., 2010)
		LNCaP	Inhibition of cell growth	(Gören et al., 2002)
Lavender	<i>Lavandula angustifolia</i>	PC3 and DU145	Inhibition of cell growth and migration; induction of apoptosis and cell cycle arrest	(Zhao et al., 2017)
	<i>Lavandula officinalis</i>	PC3	Slight Inhibition of cell growth	(Fahmy et al., 2022)
Lemon	<i>Citrus limon</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
Lemon grass	<i>Cymbopogon citratus</i>	PC3 and LNCaP	Inhibition of cell growth	(Bayala et al., 2018)
Lippia	<i>Lippia multiflora</i>	PC3 and LNCaP	Inhibition of cell growth	(Bayala et al., 2014)

Table 1. Continued.

Common name	Scientific name	Cell line	Effects	References
Mandarin	<i>Citrus reticulata</i>	PC3	Inhibition of cell growth, induction of apoptotic DNA fragmentation and ROS generation; alterations in expression of apoptotic and anti-apoptotic genes	(Hussien & Mohamed, 2021)
Menthol Mint	<i>Mentha arvensis</i>	LNCaP	Inhibition of cell growth	(Hussain, Anwar, Nigam, et al., 2010)
Mentrasto	<i>Ageratum conyzoides</i>	PC3 and LNCaP	Inhibition of cell growth	(Bayala et al., 2014)
Mullein nightshade	<i>Salanum erianthum</i>	PC3	Inhibition of cell growth	(E. E. Essien et al., 2012)
Navel Orange	<i>Citrus sinensis</i>	22RV1	Inhibition of cell growth and induction of apoptosis	(C. Yang et al., 2017)
Oregano	<i>Origanum vulgare</i>	PC3	Inhibition of cell growth, induction of apoptosis, cellular damage, DNA fragmentation, enhanced Bax expression, cytochrome c release and caspase-3 activation and decreased Bcl2 expression	(Perumalsamy et al., 2022)
Peppermint	<i>Mentha piperita</i>	LNCaP	Inhibition of cell growth	(Hussain, Anwar, Nigam, et al., 2010)
Potato tree	<i>Salanum macranthum</i>	PC3	Inhibition of cell growth	(E. Essien et al., 2015)
Rose	<i>Rosa centifolia</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
Rosemary	<i>Rosmarinus officinalis</i>	LNCaP	Inhibition of cell growth	(Hussain, Anwar, Chatha, et al., 2010)
	<i>Salvia aurea</i>	DU145	Inhibition of cell growth, induction of apoptosis, DNA fragmentation, increased LDH, caspase activity, Bax/Bcl-2 ratio and ROS generation and decreased GSH	(Russo et al., 2018)
Sage	<i>Salvia Judaica</i>	DU145	Inhibition of cell growth, induction of apoptosis, DNA fragmentation, increased LDH, caspase activity, Bax/Bcl-2 ratio and ROS generation and decreased GSH	(Russo et al., 2018)
	<i>Salvia viscosa</i>	DU145	Inhibition of cell growth, induction of apoptosis, DNA fragmentation, increased LDH, caspase activity, Bax/Bcl-2 ratio and ROS generation and decreased GSH	(Russo et al., 2018)

Table 1. Continued.

Common name	Scientific name	Cell line	Effects	References
Thyme	<i>Thymus vulgaris</i>	PC3	Inhibition of cell growth	(Zu et al., 2010)
Tsauri grass	<i>Cymbopogon giganteus</i>	PC3 and LNCaP	Inhibition of cell growth	(Bayala et al., 2018)
Turmeric	<i>Curcuma longa</i>	PC3	Inhibition of cell growth	(E. Essien et al., 2015)
		LNCaP	Inhibition of cell growth	(Zhang et al., 2017)
Wild mint	<i>Mentha longifolia</i>	LNCaP	Inhibition of cell growth	(Hussain, Anwar, Nigam, et al., 2010)
Wild Spikenard	<i>Hyptis suaveolens</i>	LNCaP	Inhibition of cell growth and cell cycle arrest	(Bayala, Nadembega, et al., 2020)
	<i>Achillea wilhelmsii</i>	PC3	Inhibition of cell growth	(Ahmadi-jouibari et al., 2013)
	<i>Aloysia polystachya</i>	PC3	Inhibition of cell growth	(Moller et al., 2021)
	<i>Amomum tsao-ko</i>	PC3	Inhibition of cell growth	(Y. Yang et al., 2010)
	<i>Anaxagorea brevipes</i>	PC3	Inhibition of cell growth	(de Alencar et al., 2016)
	<i>Annona sylvatica</i>	PC3	Inhibition of cell growth	(Formagio et al., 2013)
	<i>Bursera glabrifolia</i>	PC3	Inhibition of cell growth	(Villa-Ruano et al., 2018)
	<i>Euodia ruticarpa</i>	PC3	Inhibition of cell growth	(Yeh & Lin, 2021)
	<i>Guatteria elliptica</i>	PC3	Inhibition of cell growth	(Rajca Ferreira et al., 2018)
	<i>Hypericum hircinum</i>	PC3	Inhibition of cell growth	(Quassinti et al., 2013)
	<i>Iryanthera polyneura</i>	PC3	Inhibition of cell growth	(Martins et al., 2019)
	<i>Symphyopappus itatiayensis</i> , <i>Myrciaria floribundus</i> , <i>Talauma ovata</i> , <i>Psidium cattleyanum</i> , <i>Nectandra megapotamica</i>	PC3	Inhibition of cell growth	(Apel et al., 2006)
	<i>Guatteria pogonopus</i>	PC-3M	Inhibition of cell growth	(Fontes et al., 2013)

Table 1. Continued.

Common name	Scientific name	Cell line	Effects	References
	<i>Pinus mugo</i>	DU145	Inhibition of cell growth and constitutive STAT3 activation; decreased the expression cyclin D1, Bcl-2, survivin, XIAP, Cox2 and IL-6; decrease in GSH levels and increase in ROS generation; induced caspase-3 and PARP cleavage and inhibition of cell migration	(Thalappil et al., 2022)
	<i>Zataria Multiflora</i>	PC3	Inhibition of cell growth, induction of apoptosis, DNA fragmentation, cell cycle arrest, increased ROS generation and caspase activation, upregulation of Bax and downregulation of Bcl-2 expression	(Zare et al., 2021)

Table 2. Essential oils constituents studied for anti-prostate cancer potential *in vitro* and major observations reported.

Constituent	Plant	Cell line	Effects	References
α -humulene	<i>Salvia</i> species	LNCaP	Inhibition of cell growth	(LOIZZO et al., 2007)
α -pinene	Rosemary, lavender and others	PC3 and DU145	Inhibition of cell growth, induced apoptosis and cell cycle arrest	(Zhao et al., 2018)
β -Caryophyllene oxide	Cinnamon, oregano, clove, and black pepper	PC3	Inhibition of cell growth, induced apoptosis by inhibiting PI3K/AKT/mTOR/S6K1 signaling, reduced mitochondrial membrane potential, cytochrome c release, activating caspase-3, cleavage of PARP, ROS generation, downregulation of Bcl-2, Bcl-xL, survivin, IAP-1 and IAP-2, cyclin D1, and upregulation of p53 and p21; downregulation of COX-2 and VEGF	(Delgado et al., 2021; K. R. Park et al., 2011)
		DU145	Inhibition of cell growth and invasion and constitutive STAT3 activation	(C. Kim et al., 2014)
β -elemene	Curcuma and <i>Cymbopogon</i> species	PC3 and DU145	Inhibition of cell growth, induced apoptosis through cleaved caspase-3, caspase-9 and PARP increasing and Bcl-2 downregulation	(Li et al., 2010)
		PC3, DU145 and LNCaP	Inhibition of cell growth	(Srivastava & Gupta, 2007)
Apigenin	Chamomile, oregano, some citrus fruits and others	PC3 and LNCaP	Anti-proliferative activity by reducing net gain of DNA content, cell cycle arrest and reduced cyclin B1, PLK1 and Aurora A levels	(Hnit et al., 2022)
		PC3 and LNCaP	Suppressed tumorigenesis by activating FoxO3a, upregulation of FoxO-responsive proteins and cell cycle arrest	(S. Shukla et al., 2014)
Carnosic acid	Rosemary	PC3 and DU145	Inhibition of cell growth, induced apoptosis with DNA fragmentation, caspase activation, PARP cleavage, upregulation of Bax, downregulation of Bcl-2 and increased cytochrome c release	(Kar et al., 2012)

Table 2. Continued.

Constituent	Plant	Cell line	Effects	References
Carvacrol	Thyme, Oregano and others	DU145	Inhibition of cell growth, induction of apoptosis, cell cycle arrest increased caspase-3 activation and ROS generation	(Khan et al., 2017)
		PC3	Inhibition of cell growth, migration and invasion; induction of apoptosis increased caspase activation and ROS generation; disruption of mitochondrial membrane potential, cell cycle arrest, upregulation of Bax and downregulation of Bcl-2 expression; decreased expression of Notch1, Jagged-1, cyclin D1 and CDK4 and increased expression p21	(Khan et al., 2019)
		PC3 and DU145	Inhibition of cell growth, migration, and invasion; decreased TRPM7-like current and reduced MMP-2 protein expression and F-actin dynamics; alterations on PI3K/Akt and MEK/MAPK signaling pathways	(Heidarian & Keloushadi, 2019; Luo et al., 2016)
		PC3 and LNCaP	Inhibition of cell growth and proteasome activity; upregulated Hsp70 and downregulated VEGF and VEGFR expression	(Gopalakrishnan & Ismail, 2021)
Cinnamaldehyde	Cinnamon	Prostate CAF	Inhibition of cell growth, induction of apoptosis induction, cell cycle arrest, increased ROS generation and caspase activation; decreased GSH levels	(Han et al., 2020)
Cinnamic acid	Cinnamon	Prostate CAF	Relieves the immunosuppressive effects in a TLR4-dependent manner	(Mei et al., 2020)
		PC3 and LNCaP	Inhibition of cell growth and proteasome activity; upregulated Hsp70 and downregulated VEGF and VEGFR expression	(Gopalakrishnan & Ismail, 2021)
Citral	<i>Cymbopogon</i> species	PC3 and PC3M	Inhibition of cell growth, reduced clonogenic potential, induced morphological alterations, expulsion of lipid droplets, activation of AMPK protein expression; induction of apoptosis, DNA fragmentation, upregulation of Bax and downregulation of Bcl-2 expression	(Balusamy et al., 2020)

Table 2. Continued.

Constituent	Plant	Cell line	Effects	References
Eugenol	Cinnamon, clove and others	DU145	Inhibition of cell growth	(Carrasco et al., 2008)
		PC3 and LNCaP	Inhibition of cell growth and proteasome activity; upregulated Hsp70 and downregulated VEGF and VEGFR expression	(Gopalakrishnan & Ismail, 2021)
Geraniol	<i>Cymbopogon</i> species	PC3	Inhibition of cell growth, increased LDH and caspase-3 activity; induced mitochondrial membrane depolarization and cell cycle arrest at G1 phase; reduced expressions of cyclin A, B, D, and E, CDK1 and CDK4, and Bcl-2 and Bcl-w; elevated expressions of p21 and p27 and Bax and BNIP3	(S. H. Kim et al., 2011)
			Inhibition of cell growth, induced autophagy and inhibited AKT-mTOR signaling	(S. H. Kim et al., 2012)
			Downregulated the transcription factor E2F8	(Lee et al., 2016)
Limonene	<i>Citrus</i> and <i>Mentha</i> species	DU145	Inhibition of cell growth, induction of apoptosis, ROS generation, DNA fragmentation, caspase-3 and caspase-9 cleavage, upregulation of p21, p53 and Bad, downregulation of Bcl-xL and cleavage of PARP protein	(Rabi & Bishayee, 2009)
Linalool	Lavender, sage and others	PC3 and DU145	Inhibition of cell growth and migration; induction of apoptosis and cell cycle arrest	(Zhao et al., 2017)
		22Rv1	Inhibition of cell growth, induction apoptosis, cell cycle arrest; increased expression of Bax, Bcl-2, p53, DR4, DR5 and cleaved caspases	(Zhao et al., 2020)
		PC3, LNCaP and DU145	Inhibition of cell growth	(Nazıroğlu et al., 2018)
Menthol	<i>Mentha</i> species	PC3 and LNCaP	Inhibition of cell growth	(S. H. Kim et al., 2009)
		LNCaP	Inhibition of cell growth	(E. J. Park et al., 2009)

Table 2. Continued.

Constituent	Plant	Cell line	Effects	References
Rosmarinic acid	Rosemary	PC3 and DU145	Inhibition of cell growth, induced apoptosis with DNA fragmentation, increased p53, Bcl-2 and caspase-3 expression	(Jang et al., 2018)
Thymol	Thyme	PC3 and DU145	Inhibition of cell growth and induction of apoptosis	(Elbe et al., 2020)

Chapter II – Antitumor activity of essential oils-based nanostructured lipid carriers on prostate cancer cells

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Abstract

Prostate cancer (PCa) is the second most frequent malignancy in men worldwide. Essential oils (EOs) are natural products which can act in cancer suppression by several mechanisms. In this work, a nanotechnological approach was used to develop and evaluate the antineoplastic effects of EOs loaded by nanostructured lipid carriers (NLCs). Three different NLC systems composed of cinnamon, sage or thyme EOs were optimized using factorial design (2³). The optimal formulations were characterized in terms of biophysical parameters, structure, stability, safety and efficacy. All optimized NLC formulations exhibited excellent structural properties and stability over a year (25 °C). They proved to be *in vitro* and *in vivo* biocompatible. on PNT2 normal prostate cells and on chicken embryos (CE), respectively. In PC3 PCa cells, optimized NLCs inhibited cell proliferation and migration and changed its morphology. In CE xenograft tumor, NLCs have inhibited tumor growth and angiogenesis. The results from this work suggested that all developed EO-based NLC formulations had their stability improved while the biological activity remains unchanged.

Key words: Cinnamon, sage, thyme, NLC, prostate cancer

1. Introduction

Prostate cancer (PCa) is the second most frequent men malignancy worldwide and the fourth overall (World Health Organization, 2022). The main risk factors are age, ethnicity, diet and genetic factors. The incidence increases with age, affecting 1 in 350 men under 50 years and nearly 60 % in men over 65 years (Rawla, 2019). PCa is known as a highly heterogeneous disease with subtypes poorly understood yet (Kaffenberger & Barbieri, 2016). Treatment options are still limited to chemotherapy based on synthetic drugs, that act not only on cancer cells, exhibiting severe side effects (Machado et al., 2022). In addition, traditional therapies have not achieved the expected results, since in most cases tumor recurrence and metastasis may occur (Sharifi-Rad et al., 2019). Therefore, searching of new cancer treatment candidates that have fewer or no side effects together with high efficiency is urgent.

Natural products have demonstrated potential to be employed as cancer treatment. Moreover, several drugs have been developed derived from plants. Essential oils (EOs) are natural products derived from secondary metabolites of aromatic plants (Cascaes et al., 2021; Ferreira et al., 2022), defined as complex mixtures of about 20-60 components with 2 or 3 of them found in high concentrations (Aziz et al., 2018; Bakkali et al., 2008; Dehsheikh et al., 2020). EOs have been used since the Ancient time in treatment of several diseases and, as a result of a wide chemical diversity, it possesses several biological properties, among them: antioxidant, anti-inflammatory, antibiotic, anticancer, wound healing properties and other therapeutic activities, such as sedative, analgesic, anesthetic and anxiolytic properties (Ishfaq et al., 2018; Ramsey et al., 2020). EOs can act in cancer suppression by antioxidant, antimutagenic, antiangiogenic, antiproliferation, enhancement of immune system, enzyme induction and modulation of multidrug resistance mechanisms (Bhalla et al., 2013; Gautam et al., 2014). Despite these useful biological activities, their application in pharmaceutical industries is still limited by its toxicity, instability, insolubility, volatility and photosensitivity (Guidotti-Takeuchi et al., 2022).

Hereupon, the development of nanostructured drug delivery systems (DDS) aims the improvement of physicochemical properties of EOs minimizing its intrinsic toxicity (L. N. M. Ribeiro et al., 2019). In this sense, nanostructured lipid carriers (NLCs), that has been firstly described in 1999, is the second generation of lipid nanoparticles and is composed of a blend of solid and liquid lipids as the structural matrix, stabilized by at least one surfactant (Müller et al., 2011). Different classes of drugs have been loaded by NLC for several applications (Rodrigues da Silva et al., 2020; Souto et al., 2020). In this sense, the use of

EOs as structural and bioactive excipient in NLC preparations can be a smart strategy to preserve its therapeutics properties while its degradation and volatilization are prevented, also ensuring the stability. Indeed, it can be already found some works that described different natural oils as active ingredients in NLCs with anti-inflammatory (Carbone et al., 2018), antioxidant (Badea, Lăcătușu, et al., 2015; Carbone et al., 2020), UV protection (Badea, Lăcătușu, et al., 2015), antiarthritic (Shakeel et al., 2021), insecticidal (Radwan et al., 2022), antifungal (Badea, Bors, et al., 2015; Baldim et al., n.d.; Carbone et al., 2019; Uchida et al., 2021), bactericidal (de Barros et al., 2021; L. N. de M. Ribeiro et al., 2021) and wound healing (Khezri et al., 2019, 2020) properties.

This work provides the development of optimized EO-based formulations as potential candidate against PCa. A screening of 10 EOs were performed regarding the cytotoxic effects on PCa cell line in order to select the most desirable structural and bioactive liquid lipids for subsequent NLC preparation. Using factorial design approach, 3 optimized NLC formulations were chosen for subsequent analyses of long-term physicochemical and thermal stability and structural characterization, followed by *in vitro* and *in vivo* bioassays, ensuring their safety and efficacy.

2. Materials and methods

2.1. Screening of essential oils

EOs of cinnamon (*Cinnamomum zeylanicum*), clove (*Eugenia caryophyllus*), copaiba (*Copaifera officinalis*), garlic (*Allium sativum*), lavender (*Lavandula angustifolia*), olibanum (*Boswellia carterii*), oregano (*Origanum vulgare*), sage (*Salvia sclarea*), tea tree (*Melaleuca alternifolia*) and thyme (*Thymus vulgaris*) were solubilized in surfactant P188 (3 %) and evaluated regarding the cytotoxic effects on PNT2 and PC3 cell lines in three independent experiments, which are detailed in the section 2.8 below. The samples that exhibited the highest toxicity in PC3 cells and lowest toxicity in PNT2 cells were selected to be used as liquid lipid excipients in NLC development.

2.2. Nanostructured lipid carrier preparation

NLC formulations were prepared by the emulsification-ultrasonication method (L. N. de M. Ribeiro et al., 2021). Briefly, the solid (shorea butter) and liquid lipids (cinnamon, sage or thyme) were heated at 10 °C above the melting point of solid lipid under magnetic stirring.

Simultaneously, an aqueous solution of surfactant P188 was heated at the same temperature of the lipid phase and added to the oily phase under high-speed agitation (10,000 rpm) for 2 min with an Ultra-Turrax blender (IKA WerkeStaufen, Germany). The resulting microemulsion was sonicated for 20 min in a tip sonicator (Hielscher Ultrasonic) operated at 500 W and 20 kHz, in alternating 30 s (on/off) cycles. Finally, the resultant formulations were immediately cooled to room temperature. Formulations with absence of EO were prepared by the same method to be used as control. Actually, these nanoparticles are defined as solid lipid nanoparticles (SLN), but here they are referred as Free NLC.

2.3. Factorial design

Three 2^3 factorial designs, with triplicates in the central point, were carried out in order to investigate the role of each excipient, as well as its interactions, on the desired responses resulting in optimized NLC formulations. The range of variation of each excipient was determined based on the previously developed NLC by our group (data not shown). The significant variables were determined by analysis of variance (ANOVA, 95 % confidence level) and polynomial equations were generated for these variables and lack of fit was tested, using Design Expert 9 software (Minneapolis, USA). Table 1 shows the factor levels in percentage of each component in the formulations. Table 2 lists the combination of excipients used, and Table 3 displays the critical quality attributes (CQAs) and their desired targets.

Table 1. Factor levels of the excipients of EO-based NLC formulations.

Factor (%)	-1	0	1
SL	10	12.5	15
EO	0	2.5	5
P188	1	2	3

SL = solid lipid; EO = Essential Oil; P188 = surfactant P188.

Table 2. 2^3 Factorial design for NLC formulations terms of solid lipid (SL), essential oil (EO), and surfactant (P188), with triplicate at the central points.

Formulations	SL	EO	P188
NLC 1	-1	-1	-1
NLC 2	1	-1	-1
NLC 3	-1	1	-1
NLC 4	1	1	-1
NLC 5	-1	-1	1

NLC 6	1	-1	1
NLC 7	-1	1	1
NLC 8	1	1	1
NLC 9	0	0	0
NLC 10	0	0	0
NLC 11	0	0	0

Table 3. Critical quality attributes (CQAs) and the targets to be achieved for the EO-based NLC formulations studied.

CQAs	Target	Reason
Size (nm)	<350 nm	Lower sizes
PDI	≤ 0.2	Homogeneous distribution
Zeta potential [mV]	≥ -25 mV	Nanoparticle stability

2.4. Particle size, polydispersity, zeta potential and pH determination

The hydrodynamic diameter (size), polydispersity index (PDI), and zeta potential (ZP) of nanoparticles were obtained by dynamic light scattering (DLS) using a Litesizer 500 Particle Analyzer (Anton Paar GmbH). Measurements were performed in triplicate at 25 °C. The pH values of the formulations were measured at room temperature with a calibrated pH meter (BioVera).

2.5. Long-term physicochemical and thermal cycles stability study

Long-term physicochemical stability of the optimized NLCs was monitored for a year at 25 °C. Thermal cycles stability of the same formulations was evaluated by three temperature cycles of four days at 40 °C plus three days at 15 °C. The parameters analyzed were nanoparticle size (nm), PDI, ZP (mV), pH and visual inspection of systems in both tests.

2.6. Structural characterization

Morphology of optimized NLCs were evaluated by Field Emission Scanning Electron Microscopy (FE-SEM) images from Tescan VEGA 3 LMU microscope.

Molecular interactions analyses of optimized NLCs, their respective controls and excipients was carried out by Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR). Measurements were performed on Cary 630 FTIR spectrometer (Agilent). The spectra of the samples were recorded in absorbance mode ranging between 4000 and 500 cm^{-1} .

Thermal analysis of optimized NLCs, their respective controls and excipients were analyzed by Differential Scanning Calorimetry (DSC). Measurements were performed on a cooled TA Q20 calorimeter system. Samples were placed in aluminum pans and thermal transitions were assessed in temperature range of 0-200°C, at a heating rate of 10°C/min, under nitrogen flow.

2.7. Cell culture

Normal human prostate epithelial (PNT2) and human prostate adenocarcinoma (PC3) cell lines were obtained from the American Type Culture Collection (ATCC®). These cell lines were grown and maintained at 37 °C in a humidified incubator containing 5 % CO₂ and cultivated in Roswell Park Memorial Institute Medium (RPMI) 1640 (Gibco) medium supplemented with 10 % fetal bovine serum (FBS) (Cultilab). All the cell lines were kept free of mycoplasma and confirmed by PCR.

2.8. Cell viability assay

Viability of cells was evaluated by the resazurin assay (Sigma-Aldrich®) according to the manufacturer's instructions. Briefly, PNT2 and PC3 cells (1×10^4 cells/well) were seeded into 96-well microplates and incubated for 24h at 37 °C in 5 % CO₂. After adhesion, cells were treated with different concentrations of EOs (emulsified in P188 3 %) and NLC formulations for 24, 48 and 72 h. Control were incubated only with medium. Subsequently, cells were incubated with resazurin dye solution 10 % of the culture medium volume for 4 h. Samples were measured fluorometrically at wavelength of 590 nm using an excitation wavelength of 560 nm (Perkin Elmer). Three independent experiments were performed in triplicates.

2.9. Cell migration assay

Migration of cells lines was evaluated by wound-healing assay. Briefly, PC3 cells (1×10^5 cells/well) were seeded into 24-well microplates and grown up to 80 % confluence. Then the wound was created in the monolayer using a standard 200 µL pipette tip and cells were washed once with PBS to remove the non-adherent cells. Subsequently, cells were treated with 0.6 % (w/v) of EOs (emulsified in P188 3 %) and optimized NLCs containing such EOs at the same concentrations. Control group cells were incubated only with medium. Photos were taken by an inverted phase contrast microscope (EVOS) immediately after the wound was created, and after 24 and 48 h of incubation. The distance between the

borderlines were measured by four different equidistant points in four independent fields. Three independent experiments were performed in triplicates.

2.10 Chicken embryos

All experimental procedures were performed in accordance with Brazilian government's ethical and animal experiment's regulations and approved by the Committee on the Ethics of Animal Experiments of the Federal University of Uberlândia under the protocol number 23117.023754/2022-40. The eggs of laying hens (*Gallus gallus*), lineage Hy-Line W-36 were donated by Hy-Line of Brazil (Uberlândia, Brazil). Pathogen-free embryos were maintained at 37.5 °C under 58 % relative humidity. Before experiments, the eggs were submitted to a light ovoscopy to ensure the quality and embryonic development.

2.11. *In vivo* toxicity assay

In vivo toxicity was evaluated in 7-days-old chicken embryos (CE) as previous described (L. N. de M. Ribeiro et al., 2020) with some modifications. Briefly, the eggs were disinfected, a square window were made in the center of the eggshell and EOs (emulsified in P188 3 %) and optimized NLC formulations (0.6 %) were inoculated via chorioallantoic membrane (CAM). Negative control group received only the surfactant P188 (3 %). After 10 days, mortality, weight and changes in embryo were evaluated. Also, the analytes alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and uric acid (UA) (Bioclin®, Minas Gerais, Brazil) were analyzed.

2.12. *In vivo* tumorigenesis and angiogenesis assay

Tumorigenesis and angiogenesis were evaluated on CAM with 7-days-old CE. Briefly, eggs were disinfected and a square window were made in the center of the eggshell. Then, a suspension of 1×10^6 PC3 cells in Matrigel (20 μ L) and EOs (emulsified in P188 3 %) or optimized NLC formulations (0.6 %) were inoculated. Positive control group received medium instead of treatment. Negative control group did not received cells or treatment. After 10 days, digital photographs of CAM were taken for each group and tumor tissue were recovered. Images were analyzed using ImageJ software with Vessels Analyses plugin. Tumor was fixed in 10 % buffered formalin and processed for histological slides stained with Hematoxylin and Eosin (HE).

2.13. Statistical analysis

The statistical analysis was carried out using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed by mean $\pm$ standard deviation (SD) of the mean (SEM). Differences between mean values were analyzed using ANOVA test and Tukey post-hoc. Differences were considered significant when $P < 0.05$.

3. Results and discussion

In this work, we developed three different optimized NLC formulations containing natural (liquid and solid) lipids as structural and bioactive components: cinnamon EO-based NLC (CI-NLC), sage EO-based NLC (SA-NLC) and thyme EO-based NLC (TH-NLC). All EOs were used at 5 % (w/v) with 15 % of shorea butter as solid lipid stabilized by 3 % of P188 in the formulations. We have successfully improved the stability and solubility of EOs used to compose the NLCs, without affecting their therapeutic efficacy, as discussed below. The selection of EOs considered its *in vitro* safety and antineoplastic activity in PNT2 and PC3 PCa cell lines, respectively. Considering the novelty of the developed formulations, it was fundamental to understand the role of each excipient, as well as their supramolecular organization, physicochemical stability and therapeutic effects. In this context, a detailed systems study was carried out, using factorial design approach, structural characterization methods, *in vitro* and *in vivo* tests to ensure their quality control, safety and efficacy.

3.1. Screening of essential oils

The screening of ten EOs preliminarily evaluated its cytotoxic effects on PNT2 normal prostate and androgen-resistant PC3 PCa cell lines (Figure 1). Both cell lines were exposed to increasing concentrations of EOs ranging from 0 to 5 % for 48 h. All EOs were emulsified in P188 3 % in order to increase its solubility, allowing its use as control groups in subsequent experiments. Although these resultant emulsions are also considered a DDS, several disadvantages are widely reported, such as: require cold storage, phase separation and polydisperse size distribution, justifying the development of innovator nanocolloids. In general, EOs were not cytotoxic to PNT2 cells (Figure 1A), except for the garlic and oregano EOs, which showed low viability at concentration of 1.25 and 0.15 %, respectively, ($p > 0.001$). The half maximal inhibitory concentration (IC_{50}) were 0.544 and 0.032 % (w/v) (Table 4), respectively.

On the other hand, all EOs exhibited significant dose-dependent cytotoxicity activities against PC3 PCa cells (Figure 1B) showing low IC₅₀ values (Table 4). Among the tested samples, thyme EO showed the strongest cytotoxicity against tumor cells with an IC₅₀ value of 0.052 % (w/v), followed by cinnamon and sage EOs with IC₅₀ values of 0.138 and 0.185 % (w/v), respectively. These results are in agreement with other studies that have found a decrease in the viability of PCa cells treated with cinnamon, sage and thyme EOs (LOIZZO et al., 2007; Russo et al., 2018; Zu et al., 2010).

Moreover, cinnamon, sage and thyme EOs, which exhibited the highest toxicity in PC3 PCa cells and lowest toxicity in PNT2 prostate normal cells, were selected to be used as liquid lipids and bioactive in the development of optimized NLC systems. Although oregano EO was highly toxic to PC3 cells, it was also highly toxic to PNT2 cells. For that reason, it was not selected to be used in NLC formulations.

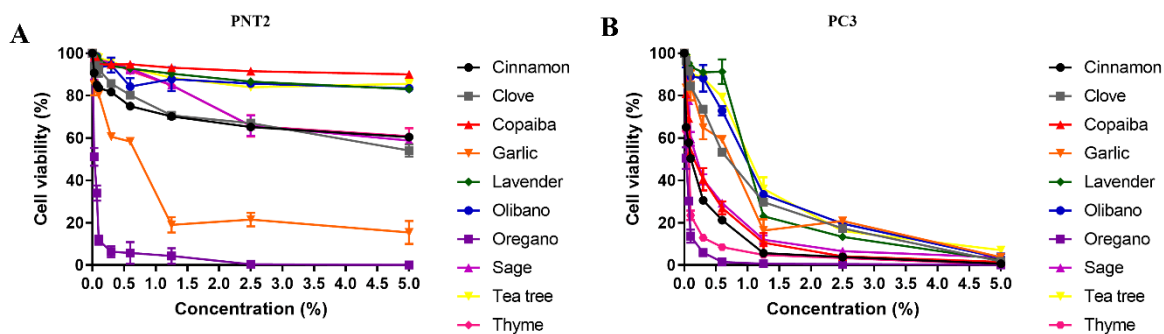


Figure 1. Cell viability of different essential oils on prostate cell lines. Viability of PNT2 normal prostate cells (A) and PC3 prostate cancer cells (B) exposed for 48h determined by resazurin assay. Values are expressed as means $\pm$ SD of three independent experiments.

Table 4. Half maximal inhibitory concentrations (IC₅₀, % w/v) values for ten essential oils on PC3 and PNT2 cell lines determined by the resazurin assay.

	Cinnamon	Clove	Copaiba	Garlic	Lavender	Olibanum	Oregano	Sage	Tea Tree	Thyme
PC3	0.138	0.884	0.204	0.774	0.963	0.976	0.031	0.185	0.992	0.052
PNT2	15.90	5.45	25.25	0.544	16.23	15.08	0.032	6.06	17.40	6.38

3.2. Factorial design

In the development of optimized NLC formulations it was used the systematic approach of Quality by Design (QbD) recommended by the Food and Drug Administration (FDA) and ICH (International Council for Harmonization of Technical Requirements for

Pharmaceuticals for Human Use) (L. N. M. Ribeiro et al., 2016). According to the QbD, critical quality attributes (CQAs) are important parameters in terms of security and efficiency of formulations, which can be directly influenced by experimental factors, known as critical process parameters (CPPs) (Rose et al., 2015). Employment of factorial design allows to minimize the number of required *in vitro* and *in vivo* tests (L. N. M. Ribeiro et al., 2017).

In this work, three 2^3 factorial design with triplicate in the central point were carried out for the NLC systems (CI-NLC, SA-NLC and TH-NLC) in order to select optimized formulations for the subsequent tests. The role of CPPs, solid lipid (SL), essential oil (EO), and surfactant (P188) concentrations, on the CQAs were evaluated for each system. The dependent variables (CQAs) was nanoparticle size (nm), PDI and ZP (mV). Lower sizes (< 350 nm), monodisperse distribution ($PDI \leq 0.2$), and colloidal stability ($ZP \geq -25$ mV) of the nanoparticles were the targets (Table 3). CQAs responses that were statistically affected by the experimental variables, had linear regression models generated through multiple linear regressions. The p-values of the coefficients of the regression models for cinnamon, sage and thyme EO-based NLC formulations are shown in Tables S1, S2 and S3, respectively.

Particle size is a mandatory parameter which influences stability, encapsulation efficiency, drug release, biodistribution and cellular uptake of lipid nanocarriers (Danaei et al., 2018). Carriers designed to have site-specific delivery for chemotherapeutics should present a size up to 300 nm, due to a high tendency to enter fenestrated capillaries, accumulation in tumor tissue and increased uptake by cells (Khosa et al., 2018; Obeid et al., 2018). In the CI-NLC factorial design, the particle size ranged from 270.2 to 857.2 nm. This response was not significantly modulated ($p = 0.1480$) for any coefficient tested. For SA-NLC, size varied from 215.9 to 857.2 nm. This response was significant ($p = 0.0003$) and the lack of fit was not significant ($p = 0.0872$), as expected for the generated model. Such response was negatively affected by EO and P188 coefficients. The increase of both excipients caused a decrease in size (Figure S1), as expected. Once the presence of liquid lipid decreases the viscosity and P188 decreases the interfacial tension of lipid nanoparticles, resulting in smaller and more stable nanoparticles (L. N. M. Ribeiro et al., 2016, 2017). On the other hand, for TH-NLC the size ranged from 318.1 to 857.2 nm. In such factorial, this response ($p = 0.0180$) and the lack of fit ($p = 0.0055$) were both significant, therefore, such model was not provided.

PDI is a theoretical parameter which provides information on the size distribution homogeneity in a system. PDI value ≤ 0.2 is requested indicating homogenous size distribution of particle size (Danaei et al., 2018). PDI values for CI-NLC ranged from 0.184 to 0.398 and this response was significantly ($p = 0.0401$) modulated by the interaction between EO and P188 ($p = 0.0300$), exerting a positive effect on PDI (Figure S2A). SA-NLC exhibited PDI values in the range of 0.107 to 0.398, such response was significant ($p = 0.0324$) and the lack of fit insignificant ($p = 0.5329$) for the generated model. Only P188 was able to modulate PDI values significantly ($p = 0.0206$) with a negative effect, similar to observed for size response in this factorial (Figure S2B).

On the other hand, TH-NCL exhibited PDI values in the range of 0.110 to 0.450, being significantly ($p = 0.372$) affected only by SL ($p = 0.0287$), which exerted a positive effect in this response. Increasing SL concentration on NLC was responsible for the increase of crystallinity of lipid matrix (L. N. M. Ribeiro et al., 2016), causing higher heterogeneity in size distribution (Figure S3).

ZP is a theoretical value of the electrostatic potential which is associated to the surface charge of nanoparticles (Obeid et al., 2018), allowing predictions about nanoparticle stability in colloidal aqueous systems (L. N. M. Ribeiro et al., 2017). ZP values $> |30|$ mV is desirable due to the suitable electrical repulsion of nanoparticles (Souto et al., 2004). ZP response was not significant for CI-NLC that presented values ranged from -27.7 to -34.6 mV. On the other hand, ZP was significant for SA-NLC and TH-NLC formulation, with values in the range from -27.8 to 38.6 and -27.8 to 33.4 mV, respectively. The only significant responses able to modulate ZP values were P188 ($p = 0.0405$; negative effect) and EO ($p = 0.0393$; positive effect) for SA-NLC and TH-NLC, respectively. Despite P188 is a non-ionic surfactant, there is some reports describing similar negative effect of ZP on NLC, probably caused by the P188 polarization together with the water adsorption molecules to NLC surface (Castro et al., 2021).

Design Space (DS) is a robust yellow region (Figure 2) obtained from the overlay of the statistically significant responses, defined by ICH as “multidimensional and interaction of input variables that have been demonstrated to provide assurance of quality of the product” (L. N. M. Ribeiro et al., 2017). There were considered size up to 300 and 350 nm for SA-NLC and TH-NLC, respectively, PDI up to 0.2 and ZP higher than 27.8 mV for both systems. The DS for SA-NLC (Figure 2 - left) and TH-NLC (Figure 2 - right) was provided,

identifying the range of each excipient able to be employed to prepare optimized NLC formulations. It was observed that to obtain optimized NLC formulations, the concentration of P188 would be lower than 3 %, which is interesting once the surfactant can be related to a potential toxicity of formulations. It is worth mentioning that it was not provided DS for CI-NLC, once only PDI response was significant.

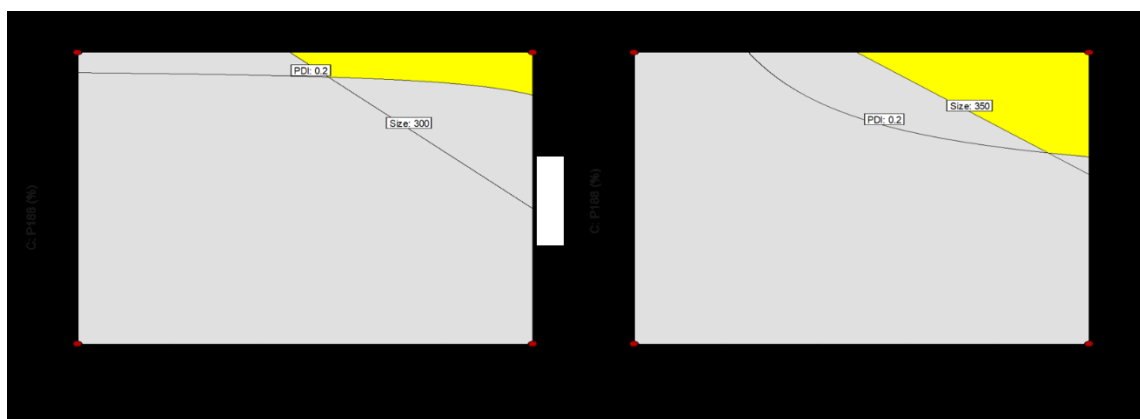


Figure 2. Design space (yellow region) of SA-NLC (left) and TH-NLC (right) formulations composed of 15 % solid lipid (SL), EO ranging from 1 to 5 % and surfactant (P188) from 1 to 3 %.

Based on the factorial analysis, the optimized formulation of each system was composed of 15 % SL, 5 % EO, and 3 % P188. Particle size of optimized CI-NLC, SA-NLC and TH-NLC was 270.3 nm, 233.7 and 347.2 nm, respectively. PDI values registered indicated homogeneous size distribution for all formulations, with values of 0.272 (CI-NLC), 0.227 (SA-NLC) and 0.110 (TH-NLC). ZP values obtained for all optimized formulations indicated desirable stability and anionic particles, with values of -27.8, -27.8 and -33.4 mV, for CI-NLC, SA-NLC and TH-NLC, respectively.

3.3. Long-term physicochemical and thermal cycles stability study

Stability evaluation of NLC systems is essential to prevent future clinical failures caused by several processes, such as nanoparticle aggregation and drug expulsion. Long-term physicochemical stability studies can ensure the shelf-life of NLC formulations (L. N. de M. Ribeiro et al., 2021). The three optimized NLC formulations and NLC with no EO (Free NLC) were monitored at predetermined time intervals, by visual inspection, nanoparticle size (nm), PDI, ZP (mV) and pH (Figure 3), for a year at 25 °C. Appearance of all

formulations remained the same throughout the experimental period, maintaining the homogeneity and whiteness of the colloidal aqueous dispersions. All NLC formulations showed no statistically significant changes in particle size (Figure 3A) over time. At the end of the 12 months of storage, the final particle size for all optimized NLC formulations did not exceed 300 nm which is expected for NLC dispersions (Khosa et al., 2018). On the other hand, particle size of free NLC showed fluctuations exceeding 300 nm during the whole experiment. These results indicated that EO had a structural role in the lipid matrix of NLC, improving the structural properties during experimental time. Despite slightly fluctuations, PDI values (Figure 3B) showed no statistically significant changes over time for all NLC formulations. Moreover, the final PDI values obtained indicated monodisperse size distribution after a year.

ZP measurements (Figure 3C) revealed statistically significant changes ($p < 0.05$) over time for all optimized NLC formulations and free NLC. Fluctuations from - 37.0 to - 32.4, from - 29.3 to -25.5 mV, from -31.1 to -23.0 mV and from -33.9 to -29.4 mV were observed for free NLC, CI-NLC, SA-NLC and TH-NLC, respectively. However, ZP did not exceed values lower than $|20|$ mV which is expected for steric stabilization as in NLC dispersions (Khosa et al., 2018), indicating good stability after a year. pH values (Figure 3D) for CI-NLC and SA-NLC ranged from 4.6 to 3.9 and from 4.2 to 3.2, respectively, and exhibited no statistically significant changes. On the other hand, TH-NLC showed a significant ($p < 0.05$) decreasing pH values over time ranging from 6.1 to 3.5. Similarly, free NLC showed a significant ($p < 0.05$) decrease ranging from 6.1 to 4.3.

The set of data demonstrated that all optimized formulations remained stable up to 12 months at 25 °C, exhibiting desirable final properties in terms of particle size, homogeneity, surface charge and pH values.

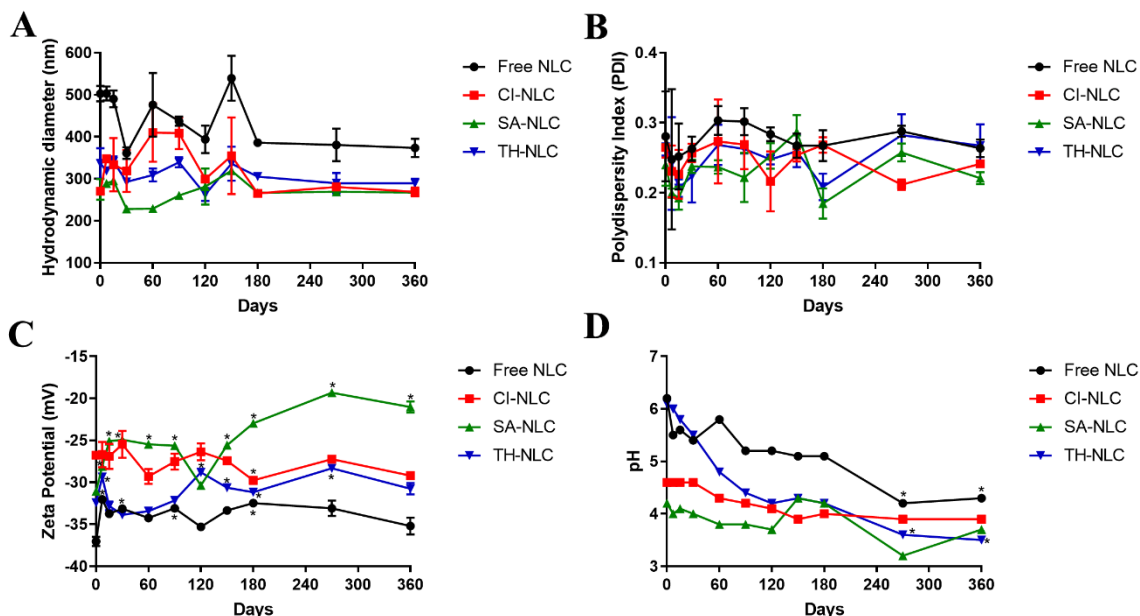


Figure 3. CI-NLC, SA-NLC and TH-NLC are physicochemical stable. Analyses of particle size (A), PDI (B), ZP (C) and pH values (D), monitored for a year (25 °C). *Statistically significant differences ($p < 0.05$).

The thermal cycles stability study is useful to predict biophysical changes of nanoparticles subjected to critical conditions during the transport and storage process, providing information of the temperature range limits of drug transport and distribution. In this study, thermal stability of optimized NLC formulations was evaluated by three temperature cycles of four days at 40 °C and 3 days at 15 °C. At the end of each cycle, visual inspection, nanoparticle size (nm), PDI, ZP (mV) and pH (Figure 4) was evaluated.

Appearance of optimized formulations remained the same during experimental period, maintaining the homogeneity and whiteness of the colloidal dispersions. Particle size (Figure 4A) of optimized CI-NLC and SA-NLC exhibited no statistically significant changes over time. On the contrary, TH-NLC showed significant ($p < 0.05$) fluctuations on particle size during the study, exceeding 400 nm at the end of the test. In addition, free NLC also showed significant ($p < 0.05$) fluctuations ranging from 378.43 nm to 555.30 nm. Moreover, PDI values (Figure 4B) showed no statistically significant changes over time for all NLC formulations, indicating monodisperse size distribution once after critical temperature conditions. These results indicate that even with changes in particle size for TH-NLC, the homogeneity of dispersion was not affected.

ZP measurements (Figure 4C) revealed statistically significant changes ($p < 0.05$) over time for all NLC formulations, similar to observed in the long-term stability study. Such fluctuations are compatible with nanocolloids, which present complex structural organization. ZP values ranged from -39.8 to -33.96, from -34.0 to -29.5 mV, from -26.4 to -23.7 mV and from -40.4 to -30.7 mV for free NLC, CI-NLC, SA-NLC and TH-NLC, respectively. However, ZP values were higher than $|20|$ mV for all evaluated systems, indicating desirable stability after thermal cycles experiment (Khosa et al., 2018). Finally, pH values (Figure 4D) for all optimized NLC formulations exhibited no statistically significant changes over time.

These data strongly suggested that all optimized formulations remained stable up to 12 months, exhibiting excellent properties in terms of particle size, homogeneity, surface charge and pH values at the end of experiment.

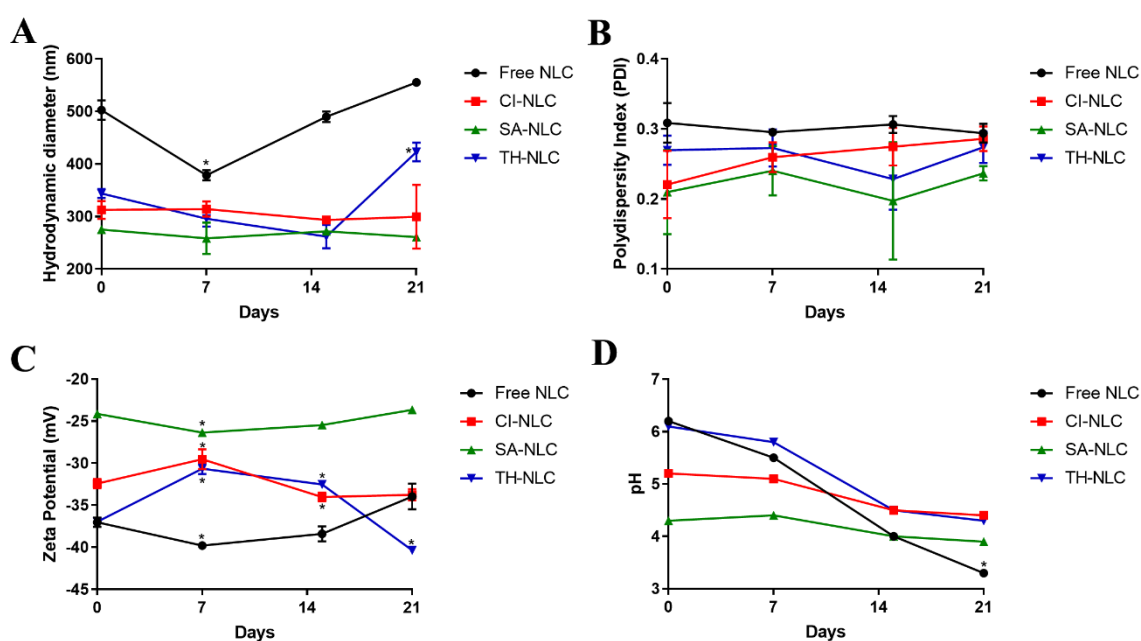


Figure 4. CI-NLC, SA-NLC and TH-NLC are thermal stable. Analyses of particle size (A), PDI (B), ZP (C) and pH values (D), monitored at the end of each temperature cycle. *Statistically significant differences ($p < 0.05$).

3.4. Structural characterization

Morphological aspects of CI-NLC, SA-NLC and TH-NLC were accessed by FE-SEM images (Figure 5). It was confirmed the existence of spherical nanostructures with smooth

surface, being homogeneously distributed for all formulations, as expected for NLCs (Araújo et al., 2010; L. N. de M. Ribeiro et al., 2021).

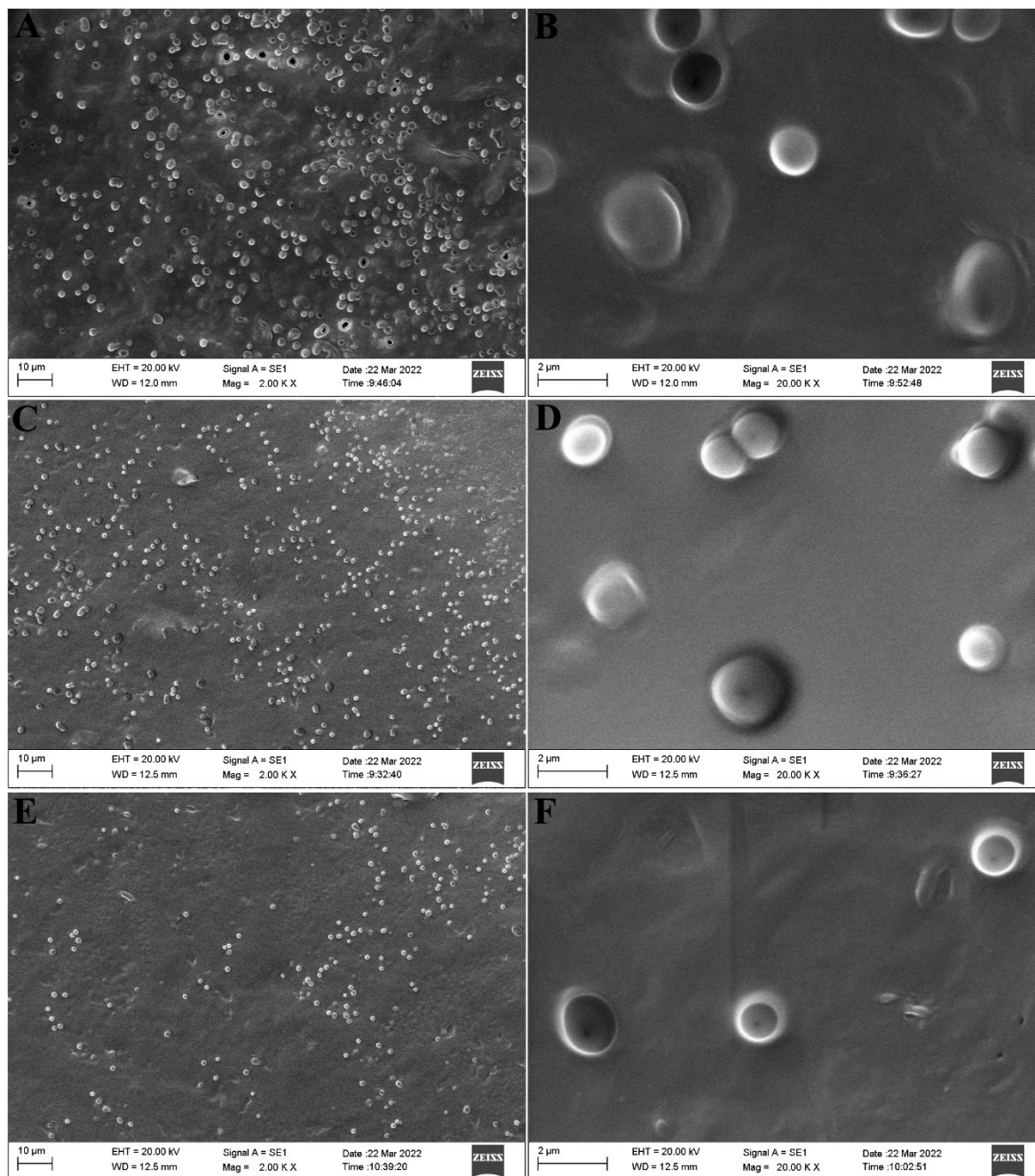


Figure 5. Morphological studies. FE-SEM images of CI-NLC (A and B), SA-NLC (F and G) and TH-NLC (J and K). Magnification: 2,000 X (first column) and 20,000 X (second column).

Infrared analysis is a very useful technique for the characterization of molecular interactions of the excipients and bioactive in NLC formulations (L. N. M. Ribeiro et al., 2017). ATR-

FTIR spectra were acquired for optimized CI-NLC, SA-NLC and TH-NLC formulations, its excipients and free NLC (with no EO) (Figure 6A-C). As previously reported for others NLC formulations (L. N. de M. Ribeiro et al., 2021; L. N. M. Ribeiro et al., 2016, 2017), it was revealed typical bands of their lipid components in the regions between 2850 and 2925 cm^{-1} ($\nu\text{O-CH}_2$ and νCH), 1620–1740 cm^{-1} ($\nu\text{C}=\text{O}$), and 1105–1160 cm^{-1} ($\nu\text{C}=\text{O}$). As expected, optimized NLCs spectra presented an overlapping of shorea butter and EOs bands, once they were the structural lipids of NLCs. In addition, EO encapsulation did not disturb any of the nanocarriers structure, displaying characteristic stretching vibrations of free NLC. Bands at 2965–3062 cm^{-1} that were assigned to the νCH and $\nu\text{N-H}$ of all EOs, completely disappeared in the spectra of optimized NLC formulations, suggesting a strong interaction and high solubility with solid lipid of NLC.

Thermodynamic properties were evaluated by DSC analyses for optimized CI-NLC, SA-NLC and TH-NLC formulations, its excipients and free NLC (Figure 6D-F). Shorea butter and P188 exhibited typical endothermic peaks at 37 °C and 54 °C, respectively, related to their respective melting points (L. N. M. Ribeiro et al., 2016). Thermogram of Free NLC exhibited similar peak at round 36 °C to the respective solid lipids (shorea butter), but it also presented thermal polymorphism with another peak at 51 °C, related to P188 contribution to the system. Moreover, CI-NLC, SA-NLC and TH-NL showed thermal polymorphism, due to the high internal disorder caused by EO to the lipid matrices in NLC formulations (L. N. M. Ribeiro et al., 2017).

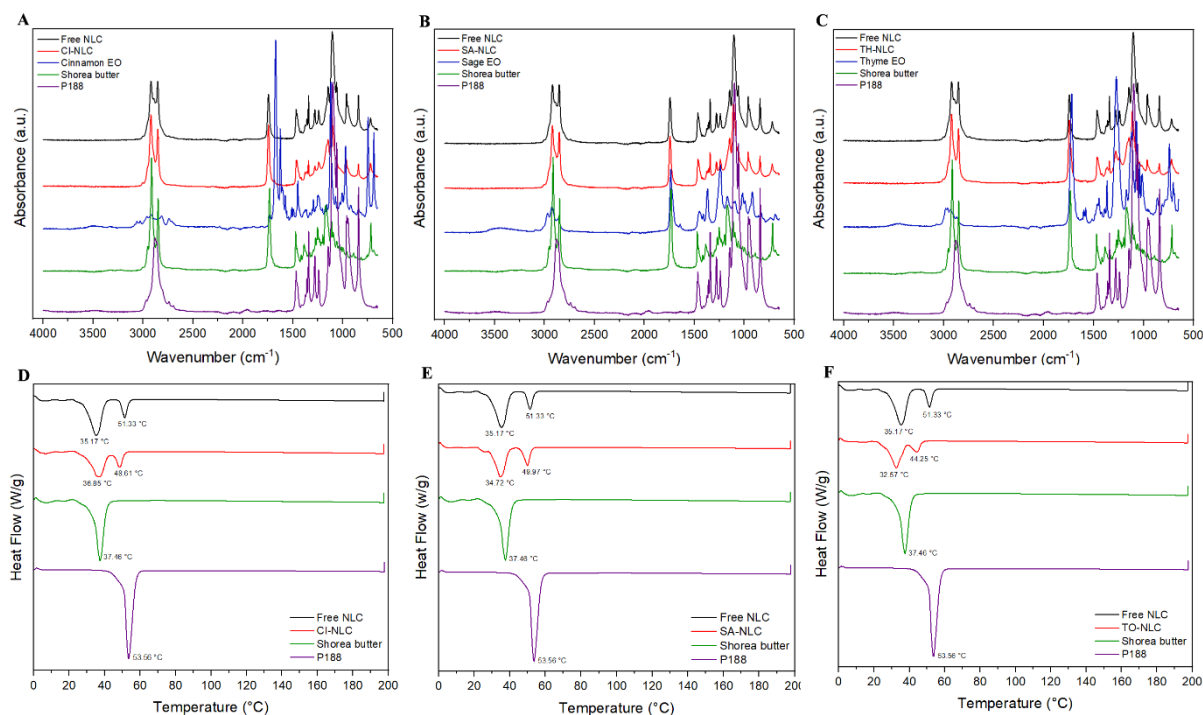


Figure 6. Structural and thermal studies. ATR-FTIR spectra of CI-NLC (A), SA-NLC (B) and TH-NLC (C) and their respective controls and excipients. DSC analyses of CI-NLC (D), SA-NLC (E) and TH-NLC (F) and their respective controls and excipients.

3.5. Cell viability

The cytotoxicity of NLC formulations was accessed by the exposition of PNT2 and PC3 cells to increasing concentrations of CI-NLC, SA-NLC and TH-NLC, ranging from 0 to 2.5 % (v/v) for 24, 48 and 72 h (Figure 7). All formulations showed no cytotoxicity to PNT2 prostate normal cells (Figure 7A, C and E). On the other hand, all optimized NLC formulations exhibited strong time and dose-dependent cytotoxicity ($p < 0.05$) activities against PC3 PCa cells (Figure 7B, D and F) showing IC_{50} values lower than 0.23 % (Table 5). The cytotoxicity of optimized NLC formulations and EOs (emulsified in P188 3 %) were similar, proving that using EO as liquid lipid in NLC preparation maintained the therapeutic properties and improve the physicochemical properties and stability of them. Among the investigated formulations, TH-NLC exhibited strongest cytotoxicity against PCa cells. In addition, TH-NLC showed higher cytotoxicity than thyme EO (emulsified in P188 3 %) with an IC_{50} of 0.014 % at 24h after treatment. As expected, free NLC (with no EO) showed no significant cytotoxicity to PC3 cells, confirming the structural and bioactive role of EOs.

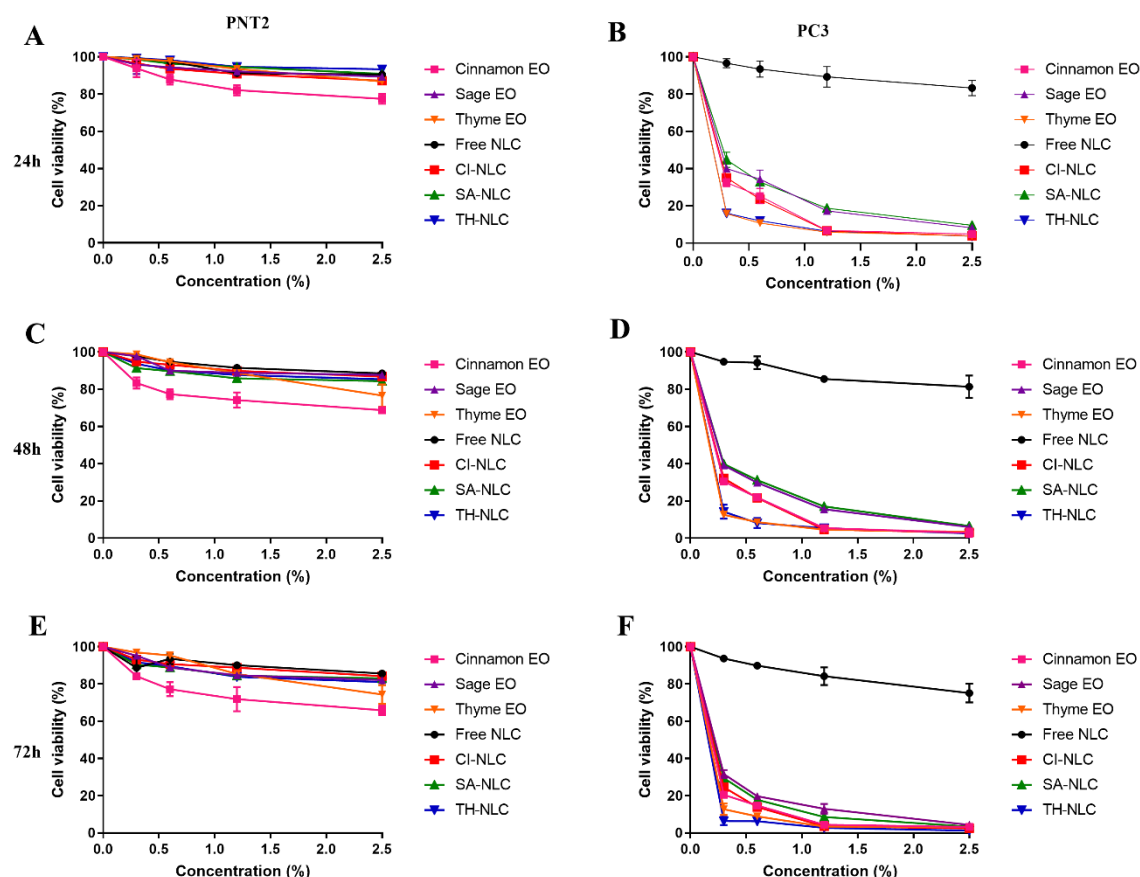


Figure 7. CI-NLC, SA-NLC and TH-NLC selective effects on cell viability of prostate cells. Increasing concentrations of NLC formulations in PNT2 normal prostate cells and PC3 prostate cancer cells after 24 (A and B), 48 (C and D) and 72 h (E and F) of exposure, respectively, determined by resazurin assay. Values are expressed as means $\pm$ SD of three independent experiments.

Table 5. Inhibition concentrations 50 % (IC_{50} , %v/v) values for CI-NLC, SA-NLC and TH-NLC on PC3 prostate cancer cell line as determined by the resazurin assay.

	Cinnamon	Salvia	Thyme	CI-NLC	SA-NLC	TH-NLC
24h	0.172	0.184	0.027	0.192	0.230	0.014
48h	0.156	0.180	0.019	0.170	0.220	0.010
72h	0.060	0.137	0.018	0.118	0.128	0.009

3.6. Cell migration

To investigate the ability of PC3 PCa cells to migrate we performed the wound healing assay (Figure 8). The wound was created in the monolayer of PC3 cells using a pipette tip and cells were treated with 0.6 % (w/v) of EOs (emulsified in P188 3 %) and optimized NLCs containing such EOs at the same concentrations. Untreated cells gradually migrated to the

cell-free wound area and after 72h the wound was closure, as expected (Zhao et al., 2017). On the other hand, treatment with 0.6 % of optimized CI-NLC, SA-NLC and TH-NLC completely inhibited wound closure when compared to untreated cells ($p < 0.05$) after 72 h, respectively. Emulsified cinnamon EO treated cells also showed inhibition of migration but the effect was enhanced when EO was part of NLC formulation. Emulsified sage EO (data not shown) after 24h already changed density, adherence and morphology (Figure 9) of cells. Emulsified thyme EO formed a crust in the wells (data not shown) and prevented analyses. It is worth mentioning that there are no reports evaluating the effect of these three EOs on PCa cells migration. However, a significant inhibition of migration of PC3 and DU145 cells treated with carvacrol, a monoterpene phenol found in thyme EO, was previously described (Khan et al., 2019; Luo et al., 2016; Trindade et al., 2019). In here, our results indicated that optimized NLC formulations have anti-migratory properties against PC3 PCa cells.

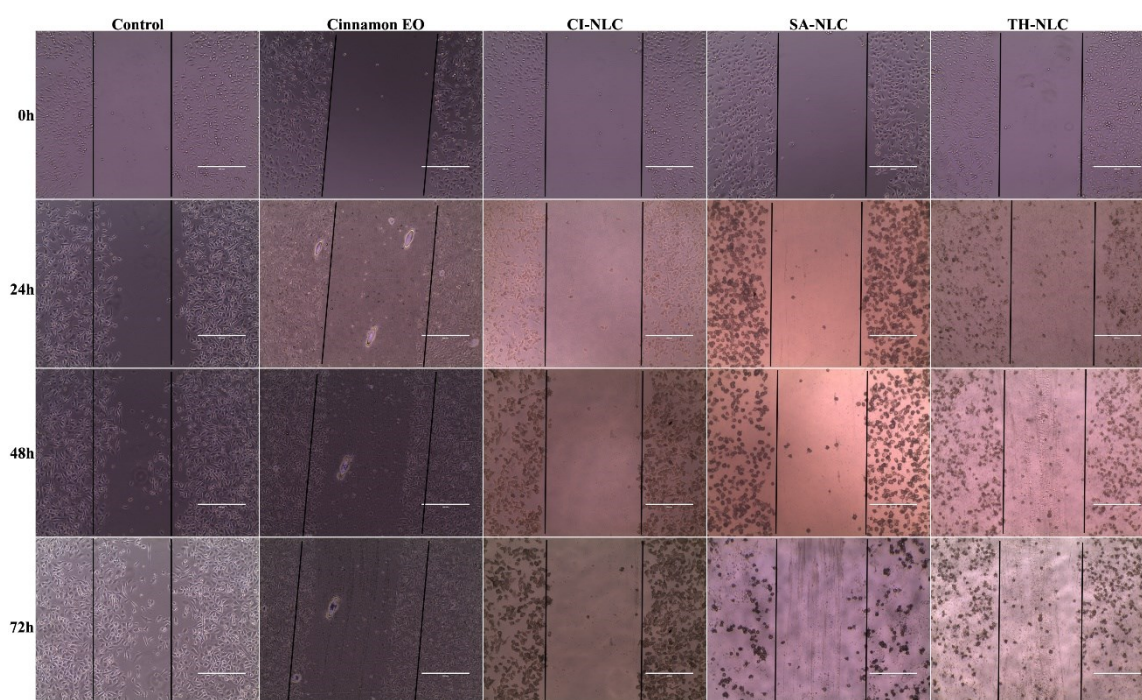


Figure 8. CI-NLC, SA-NLC and TH-NLC and essential oils inhibited migration of PC3 cells. Images from EVOS microscope of 0.6 % of essential oils (emulsified in P188 3 %) and optimized NLC formulations treated PC3 cells at 0, 24, 48 and 72 h after exposure.

Morphology of cells was routinely microscopically examined before and after treatments (Figure 9). Untreated cells showed no morphology changes during experiments, as expected. On the contrary, PC3 cells treated with 0.6 % of optimized CI-NLC, SA-NLC and TH-NLC

revealed significant changes in morphology displaying cells in round and shrunken shape with granulated cytoplasm. These changes were more pronounced in SA-NLC and TH-NLC formulations. Also, PC3 cells treated with 0.6 % of emulsified cinnamon and sage EOs have showed the same alterations in morphology except for the granulated cytoplasm for sage EO. Similar alterations were found in cells treated with carvacrol (Heidarian & Keloushadi, 2019; Khan et al., 2019). Visual inspection also revealed that sage EO strongly inhibit adherence of PC3 cells. These results indicate that changing morphology of cells may be a mechanism of antiproliferative and anti-migration of the EOs and optimized formulations evaluated.

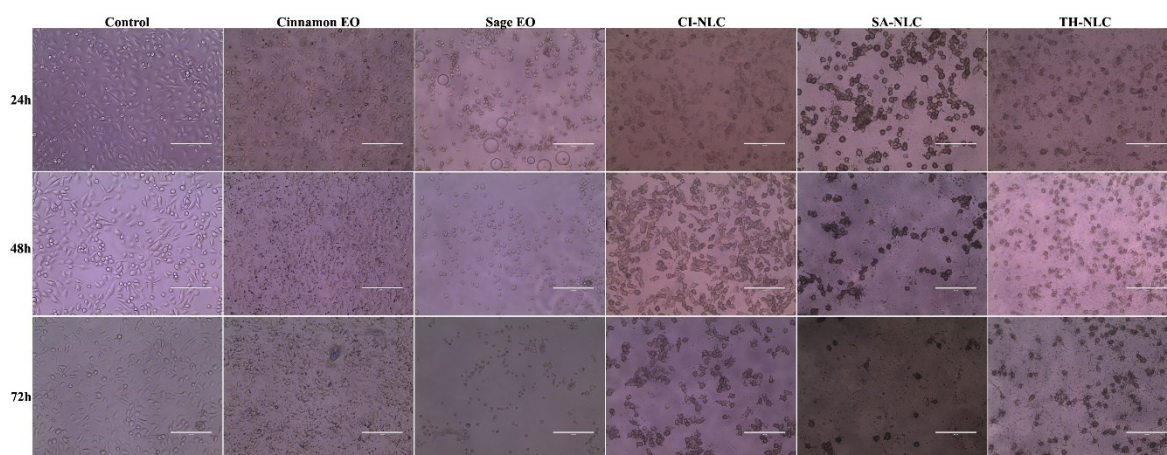


Figure 9. CI-NLC, SA-NLC, TH-NLC and essential oils changed PC3 cell morphology. Images from EVOS microscope of 0.6 % of essential oils (emulsified in P188 3 %) and optimized NLC formulations treated PC3 after 24, 48h and 72 h of exposure.

3.7. *In vivo* toxicity

The alternative biological CE model allows evaluation of efficacy and biocompatibility of a wide variety of drugs, bioactive and nanoparticles. In addition, FDA considers this model as a versatile alternative for preclinical evaluations (RIBEIRO et al., 2022). Safety of CI-NLC, SA-NLC and TH-NLC was carried out through *in vivo* CE nanotoxicity assay, analyzing weight changes, survival and biochemical markers in the serum (Figure 11). There were no significant changes in CE weight for any group compared to untreated group (Control) (Figure 11A). All emulsified EOs and optimized NLCs (0.6 %) have not induced mortality on CE (Figure 11B). On the other hand, free NLC (with no EO) showed strong mortality ($p < 0.005$).

Alterations of physiological enzymes levels can indicate how toxic is a compound to specific biological tissues. Increases in AST and ALT levels are related to liver injury but are not very specific (Sommerfeld et al., 2022). GGT levels is often increased in hepatocellular damage (Xing et al., 2022). Elevation of ALP levels can be caused by liver and bones diseases (Giannini et al., 2005). In this work, CE treated with emulsified EOs and optimized NLCs showed no significant difference for AST, ALT and GGT levels compared do control and free NLC groups. On the other hand, the treatments induced significant increase in ALP levels. These results indicate that the increasing in ALP levels may be caused by extrahepatic source. Increasing in UA levels are associated to kidney and cardiovascular diseases (Giordano et al., 2015; Saito et al., 2021). In this work, no significant difference was observed in UA levels compared do control and free NLC groups. These results suggested no damage on hepatic, renal and cardiovascular tissues. As expected, optimized NLCs showed desirable safety on CE.

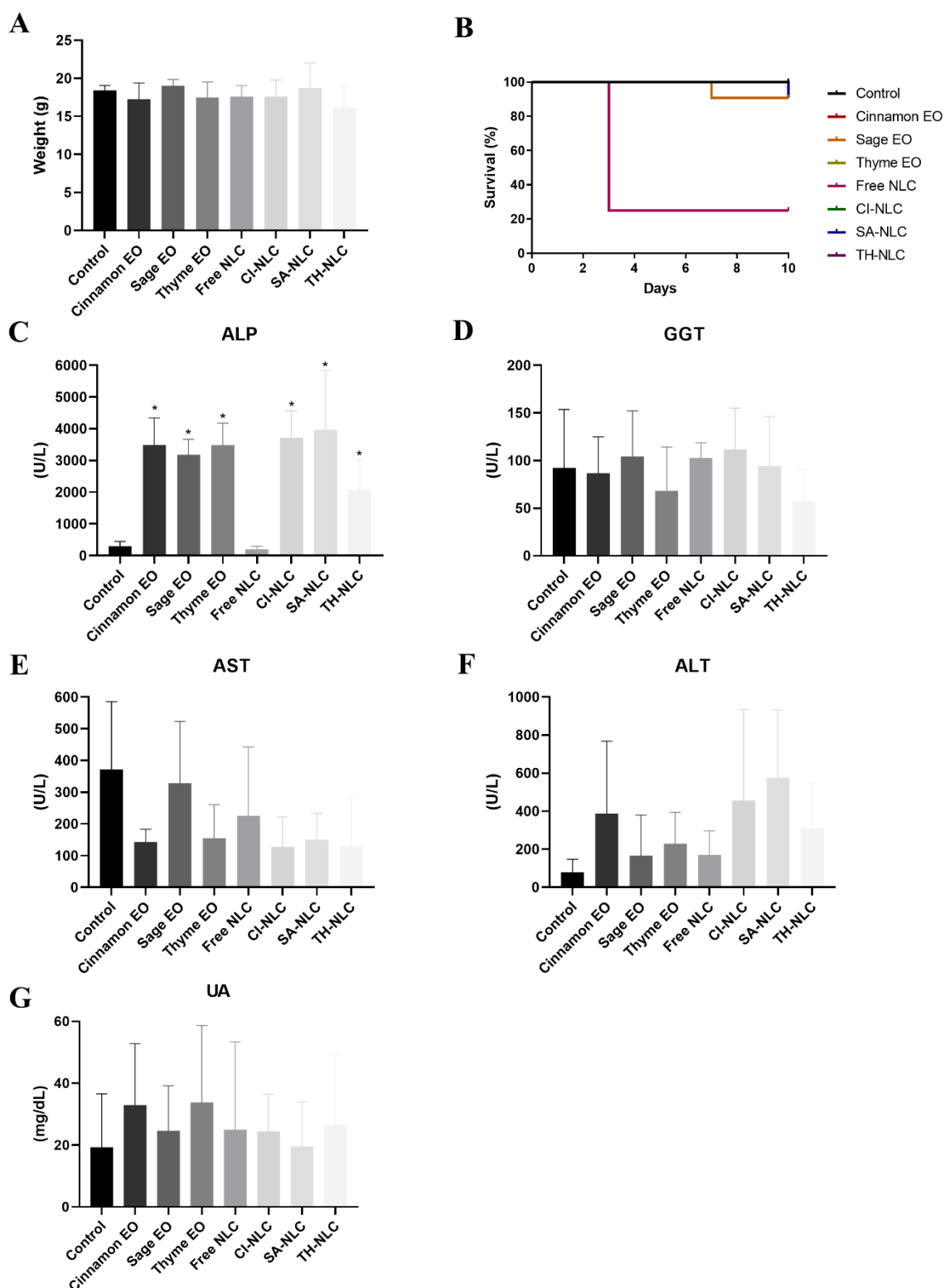


Figure 10. CI-NLC, SA-NLC and TH-NLC have no *in vivo* toxicity. Chicken embryos were treated with 0.6 % of essential oils (emulsified in P188 3 %), optimized NLC formulations or only surfactant P188 (3 %) (Control). Weight changes (A), survival (B), alkaline phosphatase (ALP) (C), gamma-glutamyl transferase (GGT) (D), aspartate aminotransferase

(AST) (E), alanine aminotransferase (ALT) (F) and uric acid (UA) (G). Values are expressed as means $\pm$ SD. *Statistically significant differences ($p < 0.05$).

3.8. *In vivo* tumorigenesis and angiogenesis

The rich vascularity and a poor immune system of CE 7-days aged makes it possible to grown xenografts tumors on CAM surface and to administrate active molecules in the tumor tissue (Preis et al., 2021). Here, it was successfully established PC3 microtumors in CAM (Figure 12B). Histologically, the PC3 microtumor could be easily distinguish from CAM tissue (Figure 12C), as expected (Kunzi-Rapp et al., 2001). Therefore, tumor tissue presented characteristic glandular-like structures (Figure 12C – gray arrows). Then, tumors implanted in CAM were treated with 0.6 % of EOs (emulsified in P188 3 %), 0.6 % of optimized NLCs or nothing (positive control). Tumor of all treated groups had a reduction in size ($p < 0.005$) of at least 62.19 % when compared to the positive control group (Figure 12J). Tumor of positive control presented an average size of 9.62 mm. Tumors of treated groups showed a reduction on size up to at least 3.56 mm. TH-NLC was the treatment that showed the highest decrease, with 89.65 % of reduction and an average size of 0.97 mm.

Angiogenesis is a biochemical process essential to development, invasion and metastasis of solid tumors. Prevention and treatment of many cancer have been based on anti-angiogenesis strategies (O. Adaramoye et al., 2017; O. A. Adaramoye et al., 2017). In this work, all treatments, except TH-NLC, were able to decrease angiogenesis ($p < 0.005$) to nearly 20 % compared to positive control group (Figure 12K), being similar to the negative control group (with no tumor). Despite no other works have evaluated the effect of cinnamon, sage and thyme EO on angiogenesis, it has been reported that some compounds found in these oils have antiangiogenic activity. Cinnamaldehyde, eugenol and cinnamic acid, monophenols of cinnamon EO, can act as proteasome inhibitors which led to downregulation of the expression of angiogenic factors (VEGF and VEGFR) in PCa cells (Gopalakrishnan & Ismail, 2021). β -Caryophyllene oxide, a bicyclic sesquiterpene isolated from cinnamon EO, is able to downregulated proteins linked with metastasis and angiogenesis (COX-2 and VEGF) and also induce apoptosis by inhibiting PI3K/AKT/mTOR/S6K1 signaling and others mechanisms in PCa cells (Delgado et al., 2021; Park et al., 2011). It can also inhibit STAT3 activity may leading to suppression of several gene involved in survival, invasion and angiogenesis (Kim et al., 2014). Carvacrol, a monoterpene phenol found in thyme EO, among several activities, has modulated both PI3K/AKT and MEK/MAPK signaling

pathways (Heidarian & Keloushadi, 2019; Luo et al., 2016). It has been described that inhibition of PI3K/AKT signaling decrease angiogenesis and tumor growth (Fang et al., n.d.) and may be an important strategy in treatment of PCa.

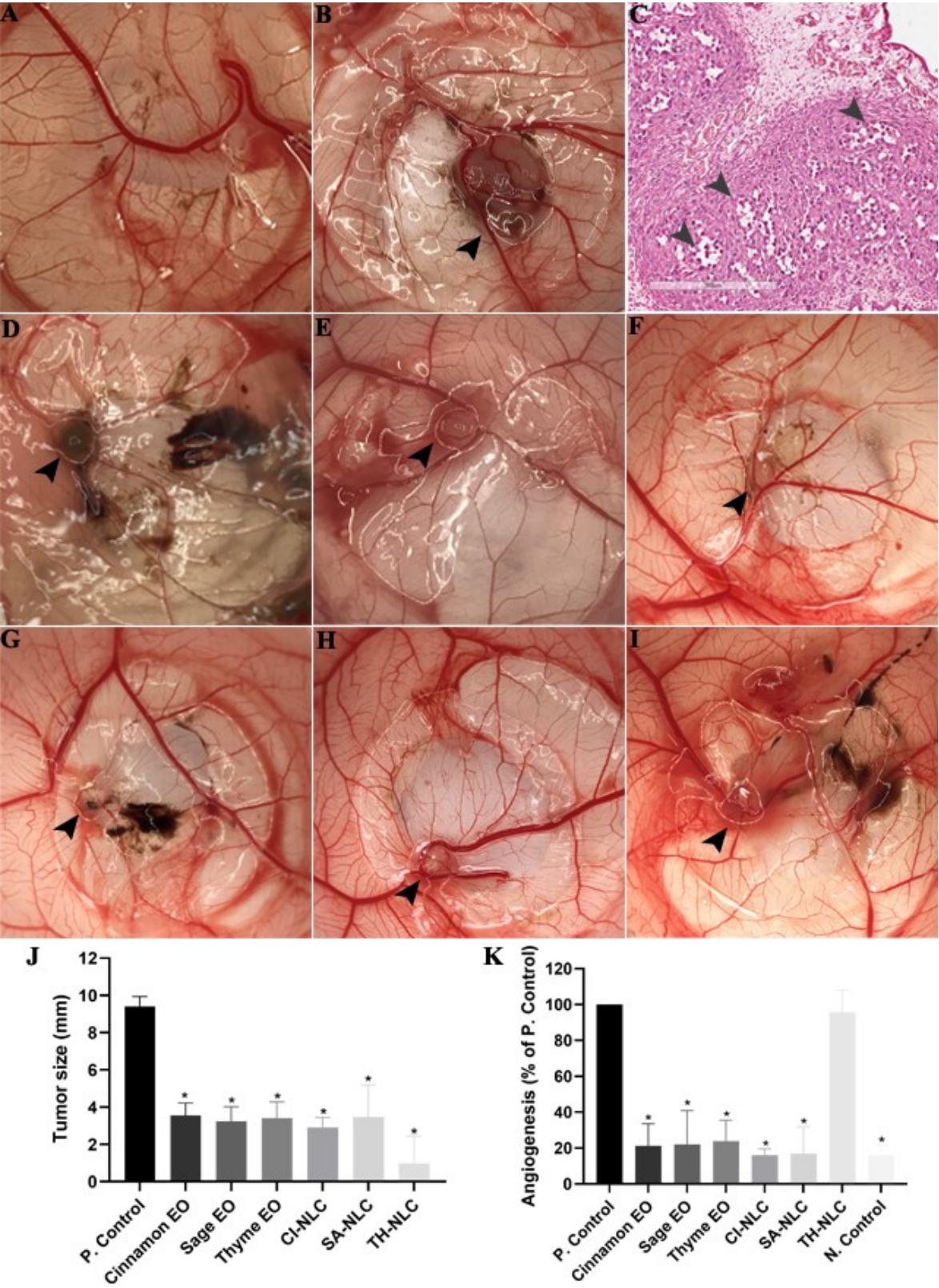


Figure 11. CI-NLC, SA-NLC and TH-NLC inhibition of tumor growth and angiogenesis. 1×10^6 PC3 cells with 0.6 % of essential oils (emulsified in P188 3 %) or optimized NLC formulations was inoculated in 7-days year old chicken embryos via CAM. Negative tumor control (A) and positive tumor control (B). Histopathology of tumor (C). Treatments with cinnamon EO (D), sage EO (E), thyme (F), CI-NLC (G), SA-NLC (H) and TH-NLC (I). Size of tumor (J) and angiogenesis (K). Black arrows indicate tumors and grey arrows indicate glandular-like structures. Values are expressed as means $\pm$ SD. *Statistically significant differences ($p < 0.05$).

Conclusion

This work was pioneer in the use of different EOs as structural and bioactive compounds in NLC formulations as promisor treatment candidate against prostate cancer. Optimized CI-NLC, SA-NLC and TH-NLC formulations through factorial design showed long-term physicochemical and thermal stability and structural properties. The safety of all optimized EO-based NLCs was confirmed by *in vivo* CE model. Such systems demonstrated *in vitro* inhibition of proliferation and migration, also changing the morphology of PC3 cells. In xenograft tumor, optimized NLCs were able to inhibit tumor growth and angiogenesis process. This approach in the development of NLC is promising for the treatment of cancers and other diseases, improving the solubility and stability of EO while their therapeutic activity remains unchanged. Such systems are able to be evaluated in efficacy tests through mammal models.

Competing interests

The authors declare no competing interests.

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Antitumor activity of essential oils-based nanostructured lipid carriers on prostate cancer cells

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Supplementary Data

Table S1: p-values of the coefficients of the regression models for cinnamon EO-based NLC formulations.

Coefficients	PDI (Y ₂)
SL% (A)	
EO% (B)	
P188% (C)	
AB	
AC	
BC	0.0300
ABC	

Table S2: p-values of the coefficients of the regression models for salvia EO-based NLC formulations.

Coefficients	Size (Y ₁)	PDI (Y ₂)	Zeta (Y ₃)
SL% (A)			
EO% (B)	0.0040		
P188% (C)	0.0041	0.0206	0.0405
AB			
AC			
BC			
ABC			

Table S3: p-values of the coefficients of the regression models for thyme EO-based NLC formulations.

Coefficients	Size (Y ₁)	PDI (Y ₂)	Zeta (Y ₃)
SL% (A)		0.0287	
EO% (B)	0.0372		0.0393
P188% (C)	0.0161		
AB			
AC			
BC			
ABC			

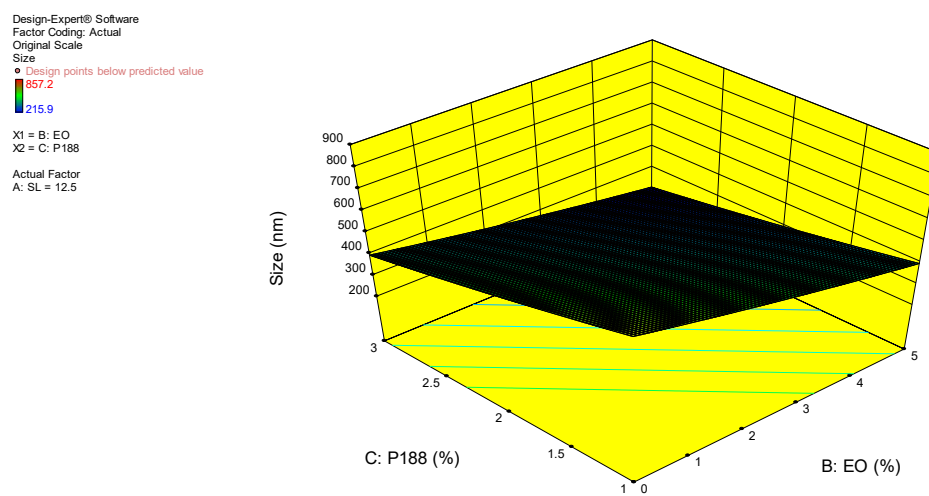


Figure S1: Surface size response of SA-NLC with 12.5 % of solid lipid (SL).

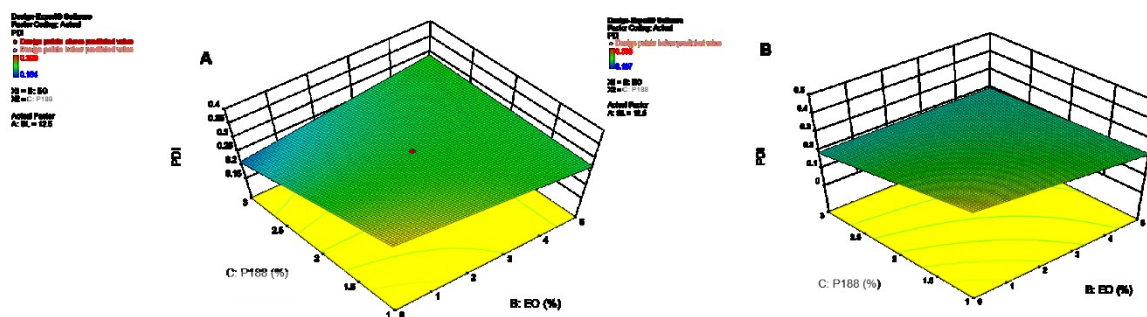


Figure S2: Surface PDI response of CI-NLC (A) and SA-NLC (B) with 12.5 % of solid lipid (SL).

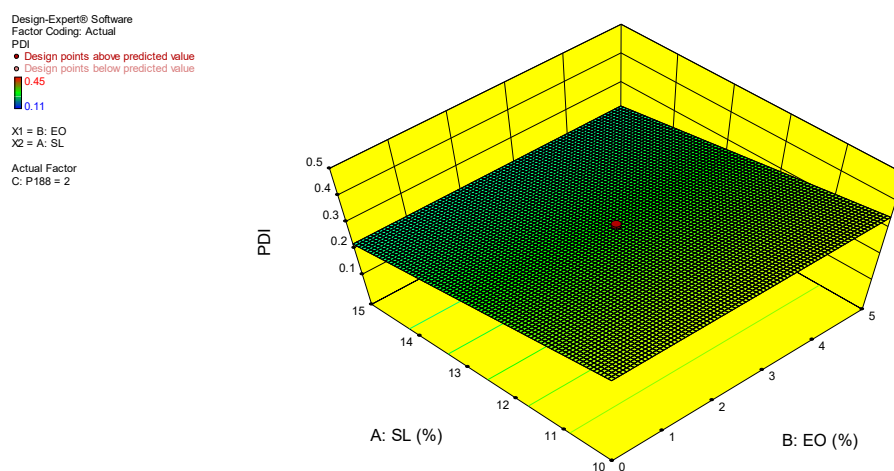


Figure S3: Surface PDI response of TH-NLC with 2.0 % of P188.