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Chemistry

Chemical profile and therapeutic potential of the essential oil and nanoemulsions of *Citrus x sp* (Tanja Lemon)

Perfil químico e potencial terapêutico do óleo essencial e nanoemulsões de *Citrus x* sp (Limão Tanja)

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ABSTRACT

The objective of this study was to evaluate the chemical profile and therapeutic potential of the essential oil and nanoemulsions of *Citrus x sp* (Tanja Lemon). Hydrodistillation was used to extract the essential oil. Gas Chromatography coupled to Mass Spectrometry (GC/MS) was used for the analysis of chemical constituents. The phenolic content (CFT) was analyzed by the Folin-Ciocalteau assay, and flavonoids (CFLT) by complexation with aluminum. The nanoemulsions were formulated by the phase inversion method. The antioxidant activity was evaluated by a hydroxyl radical assay and the anti-inflammatory activity by protein denaturation, and antiarthritic activity by a cyclooxygenase inhibition assay in bovine albumin serum. By means of GC/MS, limonene was identified as the major component (70.25%). The determination of CFT and CFLT quantified 227.645 mg EAT g^{-1} and 86.57 mg EQ g^{-1}. For antioxidant capacity, nanoemulsions have EC_{50} values of 9.10-11.28 mg L^{-1}. In anti-inflammatory activity, synergies quantified 4.63-11.03 mg L^{-1}. For the antiarthritic activity, it is noted that among the nine synergies formulated, some manifested excellent antiarthritic activity, with EC_{50} values of 1.9-1.98 mg L^{-1}. It can be affirmed that the formulations produced from *Citrus x sp* presented satisfactory results, evidencing the efficacy of their properties.

Keywords: Nanoemulsions; Activity; Essential oil

RESUMO

Este estudo teve por objetivo avaliar o perfil químico e potencial terapêutico do óleo essencial e nanoemulsões de *Citrus x sp* (Limão Tanja). Para extração do óleo essencial foi aplicada a técnica de

hidrodestilação. Para análise dos constituintes químicos foi utilizada a Cromatografia Gasosa acoplada a Espectrometria de Massas (CG/EM). O conteúdo fenólico (CFT) analisado pelo ensaio de Folin-Ciocalteau e flavonoides (CFLT) por complexação com alumínio. As nanoemulsões foram formuladas por método de inversão de fases. A atividade antioxidante foi avaliada através do ensaio de radicais de hidroxila e a anti-inflamatória por desnaturação proteica e antiartrítica por ensaio de inibição de ciclooxigenase em soro de albumina bovina. Por meio da CG/EM identificou-se o limoneno como componente majoritário (70,25%). A determinação de CFT e CFLT quantificou 227,645 mg EAT g^{-1} e 86,57 mg EQ g^{-1}. Para a capacidade antioxidante as nanoemulsões apresentam valores de CE_{50} de 9,10-11,28 mg L^{-1}. Na atividade anti-inflamatória as sinergias quantificaram 4,63-11,03 mg L^{-1}. Para a atividade antiartrítica, nota-se que dentre as nove sinergias formuladas, algumas manifestaram excelente atividade antiartrítica, com valores para CE_{50} de 1,9-1,98 mg L^{-1}. Pode-se afirmar que as formulações produzidas a partir de *Citrus x sp* apresentaram resultados satisfatórios, evidenciando a eficácia das propriedades que estes apresentam.

Palavras-chave: Nanoemulsões; Atividade; Óleo essencial

1 INTRODUCTION

Plants are indispensable sources for human life on earth, playing a fundamental role from perfumery to the cure and treatment of diseases in ancient times. These medicinal properties are related to the presence of bioactive substances in the composition of these species, from secondary metabolism capable of developing Only in natural species (Cunha et al., 2016).

The importance of medicinal plants is characterized by their applicability through therapeutic methods in folk medicine. It is known that this efficacy from different parts of the plant, such as leaves, stems, roots, flowers, fruits, and seeds, may indicate different medicinal properties (Taiz et al., 2017).

Complex mixtures of volatile compounds present in plant organs can be synthesized. Among these products from plants, essential oils (EOs) stand out (Ramos da silva et al., 2019). EOs have a wide application, known for their important biological properties in the control of pathogens, making several studies possible due to their properties and benefits for human health (Contrucci, 2019).

Its benefits have effects in several dimensions and depend on the composition and concentration of the components present in the essential oils of plants.

Therapeutic activities related to EOs, specifically antiviral, anxiolytic, antidepressant, anti-inflammatory, antioxidant and anticarcinogenic, are the most studied today (Maleck et al., 2021).

Among the plants that produce EOs, the *Citrus* genus, belonging to the Rutaceae family, is considered one of the most important, and its consumption is mainly as fresh fruit or juice, characterized by its *Citrus* flavor and nutritional value (Patrocínio, 2020). Brazil has become one of the largest producers of citrus fruits, and the residues from these fruits are of great interest for use in the food industry (Bizzo et al., 2016).

Citrus fruits are among the main species that have sources of polyphenolic compounds, which give existence to one of the largest classes of secondary metabolites present in plants that have a wide variety of structures (Sousa, 2019). The main component of the EO of the *Citrus* genus is limonene, which is found in the peel of *Citrus* fruits and provides the characteristic bitter taste (Oliveira, 2020).

Species in the *Citrus* genus are rich in flavonoids. Their volatile constituents are a mixture of monoterpene and sesquiterpenes, hydrocarbons, and their oxygenated derivatives, which vary with the specific *Citrus* cultivated, as well as the extraction and separation methods (Tao et al., 2014). There is evidence that proves the use of products with biological functions for the *Citrus* genus, such as *Citrus limon*, commonly used in folk medicine as anti-inflammatory, bactericidal, antirheumatic, and antiseptic (Campelo, 2013).

Among the species of the *Citrus* genus widely recognized for their antioxidant properties capable of acting as reductants, it is known that the essential oils of *Citrus x sp*, commonly identified as a mixture of lemon and tangerine, present sources of phenolic compounds that inhibit free radicals, its study is of great value to the pharmaceutical and food nanotechnology industries (Luzia, 2010).

Nanostructured systems applied in the cosmetology and pharmaceutical industry have advantages because they present the diffusion of hydrophilic and lipophilic actives in the same

formulation, and increase the performance against microorganisms, in addition, their use is a more economically viable factor because their use requires a small amount of oil (Rosani, 2011).

These bioproducts are promising in cosmetic science due to their high hydration power and stability. The advancement of research in the area of innovation in the market of nanotechnological products is noted, seeking to improve their application in industries, especially for human health, representing a great potential under study (Felizardo et al., 2021). Thus, this study aimed to evaluate the chemical profile and biotechnological potential of EO and nanoemulsions of *Citrus x* sp.

2 METHODOLOGY

2.1 Collection of plant material

The collection of plant material was carried out in May 2021, in the morning time in São Luís (moisture content 75.01%, chlorophyll content 0.433 g/g). The *Citrus x sp* bark exsiccate was identified by the Herbarium Ático Seabra of the Federal University of Maranhão, registered under voucher n°11170. After collection, samples of plant material were transported to the Laboratory for Research and Application of Essential Oils (LOEPAV/UFMA), where they were weighed, crushed, and stored for essential oil extraction.

2.2 Essential oil extraction

To extract the essential oil, the hydrodistillation technique was applied with a glass Clevenger extractor coupled to a round-bottomed flask packed in an electric blanket as a source of heat. Used 120 g of the crushed vegetable peels, adding distilled water (1:8) to the vegetable material contained in the flask.

Hydrodistillation was carried out at 100°C for 3h and soon after, the extracted essential oil was collected. The essential oil was dried by percolation with anhydrous sodium sulfate (Na_2SO4). The samples were stored in amber glass vials under refrigeration at 4°C. Subsequently submitted to analyses.

2.3 Chemical constituents

The constituents of essential oil were identified by gas chromatography coupled with mass spectrometry (GC-MS). 1.0 mg of the sample was dissolved in 1000µL of dichloromethane (99.9% purity).

The analysis conditions were as follows: Method: Adams (2017); Injected volume: 0.3µL; Column: HP-5MS capillary (5% diphenyl, 95% dimethyl polysiloxane) (DB-5MS equivalent or CP-Sil 8CB LB/MS), in dimensions (30m x 0.25 mm x 0.25 µm); Carrier gas: He (99.9995); 1.0 mL.min^{-1}; Injector: 280ºC, Split mode (1:10); Oven: 40°C (5.0 min.) to 240°C at a rate of 4°C min^{-1}, from 240°C to 300°C (7.5 min) at a rate of 8°C.min^{-1}; tT=60.0 min; Detector: EM; EI (70 eV); Scan mode (0.5 sec scan^{-1}); Mass range: 40 –500 daltons(one); Transfer line: 280 ºC.; Filament: off 0.0 to 4.0 min; Linear quadrupole mass spectrometer. To identify the compounds in the sample, the program AMDIS (Automated Mass Spectral Deconvolution Mass & Identification System) was used.

2.4 Spectrophotometric Determination of Total Phenolic Content (TPC)

The determination of the total phenolic compounds of the essential oil was carried out by adapting the Folin-Ciocalteu method (Waterhouse, 2002). 5 mg of essential oil diluted in 1 mL of 70% ethanol was used. To this solution was added 7 mL of distilled water, 800 µL of the Folin-Ciocalteu reagent and 2.0 mL of 20% sodium carbonate. After two hours, the reading was performed in a UV-VIS spectrophotometer at a length of 760 nm. The standard curve was expressed in mg L^{-1} equivalent to tannic acid.

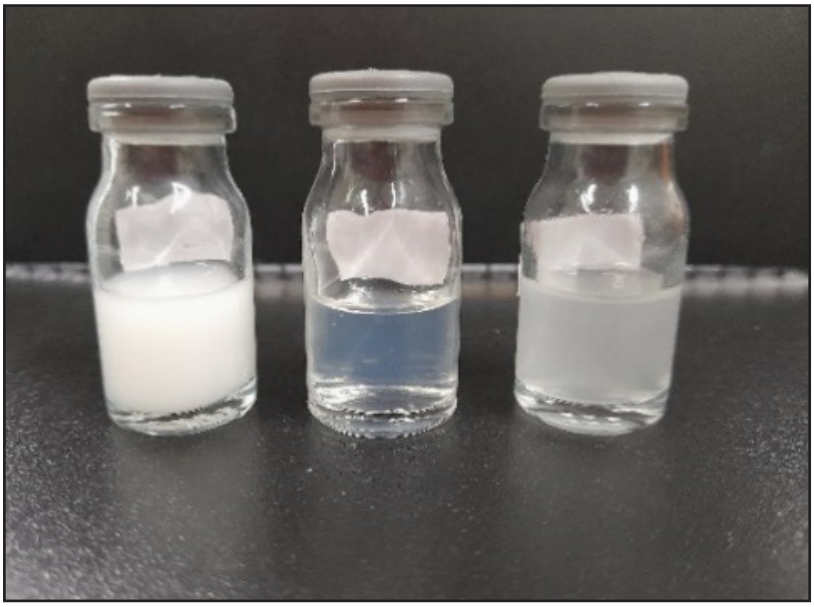
2.5 Spectrophotometric determination of total flavonoids

The determination of total flavonoids in the essential oil was performed by a spectrophotometric method using aluminum chloride. 5 mg of essential oil diluted in 4mL of 70% methanol was used. 0.4 mL of 2% aluminum chloride was added to the solution. After two hours, the reading was performed in a UV-VIS spectrophotometer at a wavelength of 510 nm. The standard curve was expressed in mg L^{-1} equivalent to quercetin.

2.6 Formulation of nanoemulsions

For the formulation of nanoemulsions, the adapted methodologies described by Lima et al. (2020), Sugumar et al. (2014), Kubitschek et al. (2014) and Rodrigues et al. (2014). Three oil-in-water nanoemulsions were prepared, with each oil, a different non-ionic surfactant and water, called NEO1 - nanoemulsion 1 (5% essential oil + 2.5% Tween 20 + 92.5% distilled H_2O); NEO2 - nanoemulsion 2 (5% essential oil + 2.5% Tween 80 + 92.5% distilled H_2O) and NEO 3 - nanoemulsion 3 (5% essential oil + 2.5% DMSO + 92.5% distilled H_2O). The required amounts of each constituent of the oil phase (essential oil + surfactant) were heated to 65 ± 5 °C. The aqueous phase was heated separately to 65 ± 5°C, providing a primary formulation, by the phase inversion method.

Figure 1 – Oil-in-water nanoemulsion



Source: Authors (2022)

The synergies (S1 to S9) were elaborated from the nanoemulsions called NEO1, NEO2 and NEO3. For each nanoemulsion, mixtures were made from the following proportions described in table 1. NEO 1+NEO 2+NEO 3

Table 1 – Formulation of synergies and their proportions

Synergies	nanoemulsions	Proportion
S1	NEO 1 + NEO 2	1:1
S2	NEO 1 + NEO 3	1:1
S3	NEO 1+NEO 2+NEO 3	1:1:1
S4	NEO 1+NEO 2+NEO 3	2:1:1
S5	NEO 1+NEO 2+NEO 3	1:2:1
S6	NEO 1+NEO 2+NEO 3	1:1:2
S7	NEO 1+NEO 2+NEO 3	3:1:1
S8	NEO 1+NEO 2+NEO 3	1:3:1
S9	NEO 1+NEO 2+NEO 3	1:1:3

Note: NEO1 – nanoemulsion (5% essential oil + 2.5% Tween 20 + 92.5% distilled water; NEO 2 - nanoemulsion (5% essential oil + 2.5% Tween 80 + 92.5% distilled water; NEO 3 - nanoemulsion (5% essential oil + 2.5% DMSO + 92.5% distilled water).
Source: Authors (2022)

2.7 Determination of the refractive index of essential oil

The refractive index was determined with the aid of the Abbé Refractometer device, initially calibrated according to the refractive index of distilled water at 25ºC, informed by the manufacturer, of 1.332.

2.8 Determination of antioxidant activity

The antioxidant activity was determined by the spectrophotometric method of scavenging salicylic acid hydroxyl radicals. according to the methods described by Smirnoff & Cumbes (1989) and Sundarajan et al. (2016).

Essential oil, nanoemulsions and synergies at different concentrations from 10-500 mg L^{-1} were dissolved in 0.2% DMSO and distilled water, respectively. 1 mL of salicylic acid (9 mM), 1 mL of ferrous sulfate (9 mM) and 1 mL of hydrogen peroxide (9 mM) were added to these concentrations. Ascorbic acid was used as a positive standard. The reaction mixture was incubated for 60 min at 37 °C in a water bath; after incubation, the absorbance of the mixtures was measured at 510 nm using a UV/VIS spectrophotometer and the EC_{50} was calculated.

2.9 Determination of anti-inflammatory activity by protein denaturation

Anti-inflammatory activity assessment (Padmanabhan & Jangle, 2012). The reaction mixture (5 mL) consisted of 2 mL of different concentrations of essential oil (10-500 mg L^{-1}), 0.2% DMSO and 2 mL of a 10% solution of albumin diluted in PBS. For the nanoemulsions and synergies, distilled water and 2 mL of a 10% albumin solution diluted in PBS were added. Samples were incubated at (37±1) °C for 15 minutes.

Denaturation was induced by keeping the reaction mixture at 60°C in a water bath for 10 minutes. After cooling, the absorbance was measured at 660 nm. Inhibition of protein denaturation was expressed in percentage and the 50% Efficient Concentration (EC_{50}) capable of inhibiting 50% of denaturation was expressed in mgL^{-1}.

2.10 Determination of antiarthritic activity

In the reaction mixture (0.5 mL) were added 0.45 mL of bovine serum albumin (BSA, 5%) and 0.05 mL of different concentrations (10-100 µg mL^{-1}) for the essential oil, nanoemulsions and synergies. Each solution was adjusted to pH 6.3 with glacial acetic acid. Samples were incubated at 37°C for 20 minutes and heated at 57°C for 3 minutes. 0.45 mL of 5% BSA was used as a reference for test control. Then, PBS (2.5 mL) was added and the absorbance was measured at 660 nm using a spectrophotometer (Hasan et al., 2015, 808-809). Cyclooxygenase inhibition (an enzymatic mechanism) was expressed as a percentage and the Efficient Concentration of 50% (EC_{50}) was expressed as mg L^{-1}.

3 RESULTS AND DISCUSSION

3.1 Identification of the Chemical composition

The analysis of the constituents was performed in GC/MS equipment. According to the results obtained, in the essential oil of *Citrus x sp* bark, ten (10)

chemical constituents were identified, with limonene being the major component (70.25%, monoterpene) followed by β-pinene (21.12%, monoterpene), α-pinene (3.01%, monoterpene), β-bisabolene (2.10%, sesquiterpene), linalool (1.00%, monoterpene), terpinene-4ol (0.98%, monoterpene), β-myrcene (0.81%, monoterpene), pinocarvone (0.41%, monoterpene), caryophyllene oxide (0.22%, terpenoid) and carvone (0.10%).

For the analysis of the chemical constituents of the essential oil of the leaves and peels of the fruit of *Citrus medica* L., Dos Santos et al. (2016) quantified 12.35% of dry leaves and 85.35% of fruit peels for limonene, which is the major constituent. Campelo et al. (2013) obtained 52.77% for limonene extracted from the essential oil of *Citrus limon* leaves.

A study by Kummer et al., (2013) for the essential oil of *Citrus latifolia* Tanaka shows the predominance of limonene in this species (62%), followed by γ-terpinene (14.2%), β-pinene (12, 2%) and α-pinene (2.8%). Leite et al. (2008) identifies limonene (96.24%), α-pinene (0.53%) and linalool (0.44%) for the species *Citrus aurantium*. On the other hand, Taghvaeefard et al., (2021) quantified limonene (48.59%) to a lesser extent and linalool (22.98%).

Sousa (2019) evaluated different species of the *Citrus* genus, including the essential oil of *Citrus x* sp (Limão Tanja), which quantified 44.75% for limonene, in addition to 23.01% for β-pinene and 2.4% for α-pinene.

The essential oils of *Citrus* fruits have a high content of limonene, being widely studied in species of the genus *Citrus* to evaluate their behavior in combating diseases (Bitencourt, 2020). Linalool is a compound that gives citrus fruit essential oils their intense floral aroma, having an organoleptic property capable of differentiating them from other essential oils (Simas et al., 2015).

Several industries choose to reuse *Citrus* fruit peel residues for the development of a range of products, extracting the raw form of constituents such as limonene, citral, citronellal, and eugenol (Barbosa et al., 2017).

3.2 Total Phenolic Content

The total content of phenolic compounds found was expressed in equivalents of tannic acid per gram (mg ETA g^{-1}). Table 2 below presents the Total Phenolic Content (TPC) for the essential oil of *Citrus x sp*.

Table 2 – Total Phenolic Content for the EO of *Citrus* x sp

Essential oil	TPC (mg EAT g^{-1})	Equation	R^2
Citrus x sp	227.645	y=0.0586x + 0.06	0.9998

Source: Authors (2022)

According to Table 2, the essential oil of *Citrus x sp* presented a quantity for phenolic compounds of 227.645 mg ETA g^{-1}.

The presence of phenolic compounds *for Citrus sinensis* in a study by Storck et al. (2013), was 631.25 mg EGA g^{-1} using methanol as solvent and 31.76 mg ETA g^{-1} using ethanol as solvent (Júnior, 2014). In a study carried out by Luzia et al. (2010) the TPC concentration for *Citrus limon* was 76 mg EGA g^{-1}.

For the essential oil of *Citrus nobilis* and *Citrus sinensis*, the TPC results found by Melo et al. (2008) were 146.30 and 208.10 µg mL^{-1} from acetone extract and from aqueous extract the results were 409.0 and 465.25 µg mL^{-1}, respectively.

Rodrigues et al. (2021) obtained results for the TPC of *Citrus tahiti* essential oil of 1.64 mg EGA g^{-1}. In contrast, Johnson (2022) obtained results ranging from 144 to 197 mg EGA g^{-1} of *Citrus australasica*. In a study carried out by Ghasemi (2009), it is noted that the value for phenolic compounds is similar to that found in this study, of 226.2 mg EGA g^{-1} for *Citrus reticulata var*.

Xi et al. (2017) analyzed the phenolic composition of different parts of the fruit of 5 cultivars of *Citrus limon* Burm, such as peel, pulp, seed, juice and whole fruit, concluding that higher levels of phenolics are present in the peel than in other specific parts. of the fruit. Marcucci et al. (2021) state that the main sources of phenolic compounds are in citrus fruits such as lemon, orange and tangerine.

3.3 Total flavonoid content

Table 3 shows the quantification of the total flavonoid content in mg of quercetin g^{-1}, present in the essential oil of *Citrus x sp*.

Table 3 – Total Flavonoid Content for the EO of *Citrus* x sp

Essential oil	TFC (mg EQ g^{-1})	Equation	R^2
Citrus x sp	86.57	y=0.00332x + 0.00058	0.9998

Source: Authors (2022)

According to the results presented in Table 3, the essential oil of *Citrus x sp* quantified 86.57 mg EQ g^{-1} for total flavonoids.

In a study by Tenorio-Dominguez (2016) for *Citrus reticulata x Citrus paradisi*, the values for TFC were 81.1727 mg EQ g^{-1} in aqueous extract and 49.9399 mg EQ g^{-1} in methanolic extract.

Studies found in the literature point to the *Citrus* genus as a rich source of phenolic compounds, referring to flavonoids as one of the main constituents (Anagnostoulou et al., 2006; Ojito et al., 2012)

Among the main phenolic compounds present in natural products, there are flavonoids. Flavonoids are among the bioactive compounds that act in the elimination of free radicals that play an anti-inflammatory and antioxidant action (Pereira et al., 2018).

The results obtained corroborate the studies found in the literature, in addition to proving the importance of the role that flavonoids play in the species of *Citrus x sp* and *Citrus* genus in general (Pereira, 2015).

The phytochemical compounds present in *Citrus* fruits are responsible for their functionalities such as antioxidant, anti-inflammatory, and antimicrobial activity attributed to this species (Araújo et al., 2019). Therefore, further studies of the toxicological and pharmacological properties are necessary, characteristics of flavonoids because they have a wide cultural diversity (Cocco et al., 2010).

3.4 Determination of the refractive index

The results of the refractive index analysis are described in Table 4 for the essential oil of *Citrus x sp*, according to the acceptable limits by the quality standards adopted by the Brazilian Pharmacopoeia.

Table 4 – Refraction index (nD 25°C) for the essential oil of *Citrus x sp*

Essential oil	Value found	Standard value
Citrus x sp	1.475	1.475 - 1.476

Source: Authors (2022)

The essential oil under study is within the standards adopted by the Brazilian Pharmacopoeia and ISO (Martins et al., 2011). The refractive index is a parameter used not only to identify substances but also to determine the purity of volatile oils, that is, it is useful to detect the presence of impurities in oils (Anvisa, 2010).

Comparing the results described in the literature, the essential oil studied has a value similar to that found by Oliveira (2012), obtaining a refractive index of 1.4753 for the essential oil of *Citrus limon Linneo*. For the work carried out by Assunção (2013), the essential oil of *Citrus sinensis L*. Osbeck) had a refractive index of 1.471 and 1.476 for fresh and dry peels, respectively.

Teixeira et al. (2017) analyzed different genotypes of *Citrus limon Burm*. and the refractive index analysis results range from 1.4710 to 1.4735 for the respective essential oils. In a study by Borges (2021), four *Citrus* fruit species obtained results for the refractive index in the value of 1.4755 for *Citrus latifolia*, 1.4725 for *Citrus limonia*, 1.4742 for *Citrus deliciosa* and values ranging from 1 .4715 to 1.4725 for cultivars of *Citrus sinensis*.

Through the refractive index it is possible to identify the characteristics of each oil and the degree of unsaturation of the connections (Radünz et al., 2018). The refractive index is considered an important quality parameter for oils and fats, being proportional to the increase in the iodine index, acting as an object of physical-chemical quality control (Uliana, 2017).

3.5 Antioxidant capacity

The determination of the antioxidant activity of the essential oil of *Citrus x sp*, of the nanoemulsions and synergies are presented in Table 5. From the equation of the straight line it is possible to calculate the effective concentration (EC_{50}), the factor responsible for the inhibition of 50% of the hydroxyl radicals.

Table 5 – EO antioxidant capacity of *Citrus x sp*, nanoemulsions and synergies

Sample	EC_{50}	line equation	R^2
EO	118.84	y=44.989x-43.351	0.9999
NEO 1	10.18	y= 45.186x+4.468	0.9996
NEO 2	11.28	y=33.289x+15.044	0.9978
NEO 3	9.10	y=15.412x+35.22	0.9996
S1	45.80	y=129.84x-165.65	0.9999
S2	38.84	y=56.958x-40.519	0.9990
S3	74.87	y=55.482x-53.95	0.9984
S4	42.00	y=152.17x-197.01	0.9742
S5	48.72	y=50.148x-34.637	0.9991
S6	74.82	y=39.296x-23.641	0.9993
S7	68.46	y=61.635x-63.127	0.9974
S8	45.23	y=183.52x-253.8	0.9306
S9	61.64	y=69.432x-74.276	0.9975

Note: EO – Essential Oil; NEO 1 - nanoemulsion (5% essential oil + 2.5% Tween 20 + 92.5% H_2O; NEO 2 - nanoemulsion (5% essential oil + 2.5% Tween 80 + 92.5% H_2O; NEO 3 - nanoemulsion (5% essential oil + 2.5% DMSO + 92.5% H_2O); S1 - NEO1 + NEO2 (1:1); S2- NEO1 + NEO3 (1:1); S3 - NEO1+NEO2+ NEO3 (1:1:1); S4 - NEO1 + NEO2 + NEO3 (2:1:1); S5 - NEO1 + NEO2 + NEO3 (1:2:1); S6 - NEO1 + NEO2 + NEO3 (1:1 :2); S7 - NEO1 + NEO2 + NEO3 (3:1:1); S8 - NEO1 + NEO2 + NEO3 (1:3:1); S9 - NEO1 + NEO2 + NEO3 (1:1:3);
Source: Authors (2022)

Campos et al. (2003) state that natural products with EC_{50} concentrations lower than 500 mg L^{-1} are classified as active. Regarding the effective concentration of 50% of inhibition (EC_{50}), it can be said that it is inversely proportional to the antioxidant capacity of a compound, that is, the lower the EC_{50}, the greater the antioxidant potential, as it expresses the amount of antioxidant needed to decrease the concentration of hydroxyl

radicals by 50% (Villaño et al., 2007). As the total phenolic content increases, the EC_{50} value decreases, consequently, the antioxidant capacity of a compound increases (Neves et al., 2022).

The results found for the calculation of EC_{50} of the essential oil of *Citrus x sp* quantified 118.84 mg L^{-1}. In a comparative study found in the literature for the genus *Citrus*, Pereira (2016) obtained results for EC_{50} of 264.160 and 395.451 mg mL^{-1} for the essential oils of *Citrus latifolia* and *Citrus limon*, respectively. According to the results obtained by Santos et al. (2016), the EC_{50} value of the essential oil of *Citrus aurantium var*. Dulcis was 63.12 µL mL^{-1} by the DPPH method.

The values for the EC_{50} of the synergistic effect of different concentrations (S1 to S9) were better than the EC_{50} of the essential oil of *Citrus x sp*, with values between 38.84 to 74.87 mg L^{-1}, of which the S2 synergy showed the lowest value of EC_{50}.

However, there is a significant increase in EC_{50} for synergism results. This factor can be explained by the proportion used when mixing the nanoemulsions (Pombo et al., 2018).

For the results of the nanoemulsions, it appears that they present the best results of EC_{50}, with values of $9.10 < 10.18 < 11.28$ mg L^{-1} for NEO3 (essential oil + DMSO), NEO1 (essential oil + Tween20) and NEO2 (essential oil + Tween80) respectively, giving them the better antioxidant capacity. Since synergies are mixtures between vegetable oils capable of potentiating their effects (Oliveira, 2020), it is observed that there is an improvement in the antioxidant capacity of synergies when compared to essential oil.

3.6 Anti-inflammatory capacity

Table 6 presents the EC_{50} values for the evaluation of the anti-inflammatory activity of the essential oil, nanoemulsions and synergies, in which, through the straight line equation, the effective capacity of 50% inhibition was calculated.

For the essential oil (EO) of *Citrus x sp*, the EC_{50} calculation quantified 21.53 mg L^{-1}, evidencing anti-inflammatory properties of the essential oil, also confirmed by several

studies found in the literature for the genus *Citrus* (Amorim et al., 2016; Ramón-laca, 2005; Haeffner et al., 2012). Bioactive compounds present in natural products are responsible for the anti-inflammatory and antioxidant action, giving this potential to act in the prevention of diseases (Morais, 2014).

Table 6 – EO anti-inflammatory capacity of *Citrus x sp*, nanoemulsions and synergies

Sample	EC_{50}	Equation	R^2
EO	21.53	y=76.921x-52.539	0.9991
NEO 1	404.14	y=111.14x-239.69	0.9976
NEO 2	158.27	y=83.482x - 133.61	0.9974
NEO 3	157.16	y= 82.824x - 131.91	0.9912
S1	91.03	y=14.057+22.46	0.9995
S2	31.34	y=25.301x+12.148	0.9949
S3	94.41	y=20.093x+10.316	0.9959
S4	4.63	y=30.024x+30.019	0.9976
S5	11.12	y=31.096x+17.465	0.9966
S6	14.16	y=25.382x+20.781	0.9951
S7	11.03	y=18.837+30.358	0.9874
S8	86.78	y=9.7198x+31.159	0.9976
S9	87.50	y=34.333x-16.675	0.9980

Note: EO – Essential Oil; NEO 1 - nanoemulsion (5% essential oil + 2.5% Tween 20 + 92.5% H_2O; NEO 2 - nanoemulsion (5% essential oil + 2.5% Tween 80 + 92.5% H_2O; NEO 3 - nanoemulsion (5% essential oil + 2.5% DMSO + 92.5% H_2O); S1 - NEO1 + NEO2 (1:1); S2 - NEO1 + NEO3 (1:1); S3 - NEO1+NEO2+ NEO3 (1:1:1); S4 - NEO1 + NEO2 + NEO3 (2:1:1); S5 - NEO1 + NEO2 + NEO3 (1:2:1); S6 - NEO1 + NEO2 + NEO3 (1:1 :2); S7 - NEO1 + NEO2 + NEO3 (3:1:1); S8 - NEO1 + NEO2 + NEO3 (1:3:1); S9 - NEO1 + NEO2 + NEO3 (1:1:3);
Source: Authors (2022)

Comparatively, the nanoemulsions quantified EC_{50} above the values found for the essential oil, however, they indicate anti-inflammatory activity. With values for EC_{50} of 157.16; 158.27 and 404.14 mg L^{-1} for NEO3, NEO2 and NEO1 respectively.

About synergies, their values for EC_{50} range from 4.63 to 94.41 mg L^{-1}. Synergies S4, S5 and S7 quantified 4.63; 11.12 and 11.03 mg L^{-1} respectively, with a considerable improvement in these values about the results of the essential oil and its nanoemulsions.

The formulated synergies have an anti-inflammatory effect. With this, it

was possible to observe the synergistic effect in the formulations between the nanoemulsions. The biological activity resulting from the mixture of two compounds is due not only to their concentration but also to the interaction that occurs between them (Souza, 2006). *Citrus* sources have a high content of limonene, making them effective in reducing oxidative stress due to their free radical scavenging properties (Ullah et al., 2014).

Queiroz (2021) evaluated the anti-inflammatory action related to the essential oil of *Citrus x sp*, which quantified 400.05 mg L^{-1}, attributing to the anti-inflammatory effect of the presence of terpenes in the composition of this species, since its main constituent is the limonene.

Several essential oils originating from *Citrus* fruits were evaluated for their anti-inflammatory action by Dosoky & Setzer (2018), among them are *Citrus sinensis, Citrus aurantium, Citrus limon, Citrus aurantifolia* and *Citrus reticulata*, which showed anti-inflammatory potential.

Studies that encompass the genus *Citrus* confirm the anti-inflammatory potential of essential oils from several species, which have important biological characteristics under study for application in the pharmaceutical industry (Ferreira, 2014).

For nanoemulsions and formulated synergies, this study is unprecedented.

3.7 Antiarthritic Activity

From the results expressed in Table 7, it is possible to observe, through the calculation for EC_{50}, the antiarthritic capacity that the essential oil of *Citrus x sp* presents, as well as the formulated nanoemulsions and their respective synergies.

Jonville et al. (2011) state that natural products with concentrations lower than 130 mg L^{-1} are considered active against inflammatory actions. According to Table 7, the effective concentration of EC_{50} is estimated at 16.58 mg L^{-1} for the essential oil of *Citrus x sp*. About nanoemulsions, it is noted that NEO1 presented better antiarthritic

performance with a value of 6.44 mg L^{-1}. Then there are NEO3 and NEO2 with EC_{50} values of 30.57 mg L^{-1} and 86.42 mg L^{-1}, respectively.

Table 6 – EO antiarthritic capacity of *Citrus x sp*, nanoemulsions and synergies

Sample	EC_{50}	Line equation	R^2
EO	16.58	y=22.031x+23.13	0.9999
NEO 1	6.44	y=7.9873x+43.538	0.9811
NEO 2	86.42	y=15.192x+20.579	0.9253
NEO 3	30.57	y=6.786x+39.921	0.9815
S1	1.9	y=5.7275x+48.399	0.9959
S2	9.0	y=9.864x+40.587	0.9997
S3	47.0	y=14.819x+25.221	0.9999
S4	6.28	y=15.819x+37.382	0.9984
S5	13.86	y=13.315x+34.797	0.9772
S6	1.69	y=11.006x+47.488	0.9923
S7	5.99	y=16.579x+29.471	0.9997
S8	1.98	y=43.22x-37.18	0.9999
S9	11.94	y=30.242x+17.434	0.9839

Note: EO – Essential Oil; NEO 1 - nanoemulsion (5% essential oil + 2.5% Tween 20 + 92.5% distilled water; NEO 2 - nanoemulsion (5% essential oil + 2.5% Tween 80 + 92.5% distilled water; NEO 3 - nanoemulsion (5% essential oil + 2.5% DMSO + 92.5% distilled water); S1 - NEO1 + NEO2 (1:1); S2- NEO1 + NEO3 (1:1); S3 - NEO1+NEO2+ NEO3 (1:1:1); S4 – NEO1 + NEO2 + NEO3 (2:1:1); S5 – NEO1 + NEO2 + NEO3 (1:2:1); S6 – NEO1 + NEO2 + NEO3 (1:1 :2); S7 – NEO1 + NEO2 + NEO3 (3:1:1); S8 – NEO1 + NEO2 + NEO3 (1:3:1); S9 – NEO1 + NEO2 + NEO3 (1:1:3);

Source: Authors (2022)

Table 7 shows that among the nine (9) synergies formulated, S1, S6, and S8 showed excellent antiarthritic activity, with EC_{50} values of 1.9 mg L^{-1}, 1.69 mg L^{-1} and 1 98 mg L^{-1}, respectively. Therefore, synergies S2, S4 and S7 quantified 9.0 mg L^{-1}, 6.28 mg L^{-1} and 5.99 mg L^{-1}. The synergies S3, S5 and S9 obtained values of 47.0 mg L^{-1}, 13.86 mg L^{-1} and 11.94 mg L^{-1}, showing potential for antiarthritic activity.

The results obtained in the study by Moraes (2012) that evaluated the essential oil (EO) of *Citrus aurantium* and its major compound (limonene) in the treatment of rheumatoid arthritis (RA), confirmed through their experimental results from induced edema in paws of rats the antiarthritic potential of EO, attributing to limonene the

ability to inhibit harmful actions. The antiarthritic action is observed for the EO of *Citrus x sp*, which also belongs to the *Citrus* genus in the work presented by Moraes (2012).

Starting from the comparison of the proven antiarthritic action for the genus *Citrus*, Schneider (2014) observed the action of the hydroalcoholic extract of *Citrus reticulata* in the treatment of RA in vivo, concluding that the phytoconstituents present inhibited symptoms of the pain of inflammatory origin.

Rheumatoid arthritis is an autoimmune disease that, due to the lack of drugs capable of inhibiting inflammation and pain, makes possible studies for the use of natural products with great antiarthritic potential having qualities of acting directly on inflammation linked to both bone damage and its mechanisms of action (Ponceano, 2020).

Essential oils from *Citrus* fruits have limonene as a major constituent. The monoterpenes present in these essential oils are reported to inhibit cyclooxygenase enzymes and their active ingredients have properties to fight inflammation (Romero, 2014).

Citrus flavonoids have beneficial effects that fight inflammation, inhibit inflammation and pain, and can be found in lemon and orange peels, in addition to preventing the action of free radicals (Pereira, 2015). The use of flavonoids and derivatives of phenolic compounds present in *Citrus* fruits are possible natural anti-inflammatories in the fight against acute inflammation caused by chronic and degenerative inflammation diseases such as Rheumatoid Arthritis (RA) (De Souza et al., 2022).

For the evaluation of the antiarthritic capacity, the results were satisfactory both for the EO under study and for the nanoemulsions and their synergies, stating that this unprecedented work, brings an alternative perspective to the use of natural biotechnological products in the application of diseases of inflammatory origin.

Therefore, the study of the antiarthritic activity of natural species for its application in chronic inflammation has grown nowadays (Pereira et al., 2021). In vitro and in vivo tests are used as a mechanism for action against inflammation and immune responses, classifying herbal medicines with great potential in studies for the treatment of chronic diseases (Schneider, 2014).

3 CONCLUSIONS

Based on the results obtained in this study, limonene was the major constituent of the essential oil. The formulated nanoemulsions showed stability and biotechnological potential. It was possible to conclude that the essential oil and the nanoemulsions of Citrus x sp presented antioxidant and anti-inflammatory potential, in addition to acting in the antiarthritic inhibition. The evaluation of the potential of nanoemulsions, as well as their synergies, show their effectiveness, preliminarily proven to the genus Citrus, demonstrating the importance of studying them for their application in the market and highlighting the diversified applicability attributed to this species.

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