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Rheological behavior and antiarthritic activity of *Pterodon pubescens* nanoemulsion

Comportamento reológico e atividade antiartrítica da nanoemulsão de *Pterodon pubescens*

Comportamiento reológico y actividad antiartrítica de la nanoemulsión de *Pterodon pubescens*

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Abstract

Pterodon pubescens, popularly known as "sucupira", it is traditionally used as anti-inflammatory agent. This work aimed to evaluate the in vivo antiarthritic properties of a *P. pubescens* oil nanoemulsion and the rheological behavior of the developed system. The viscoelastic properties, creep and recovery were evaluated by dynamic oscillatory tests. The antiarthritic activity of the nanoemulsion was evaluated by the zymozan-induced arthritis model, at three different doses (25, 50 and 125 mg/kg/day). *P. pubescens* oil nanoemulsion has been shown to decrease cell recruitment to a joint cavity and increased cartilaginous regeneration at the end of treatment of the dose of 50 mg/kg. The evaluation of the behaviour of deformation allowed to observe that the *P. pubescens* nanoemulsion presents predominantly elastic characteristic. These findings demonstrate the potential of *P. pubescens* and nanotechnology in the development of new antiarthritic drugs.

Keywords: Antiarthritic activity; Creep and recovery; Sucupira; Viscoelasticity.

Resumo

Pterodon pubescens, popularmente conhecido como "sucupira", é tradicionalmente utilizado como anti-inflamatório. Este trabalho teve o objetivo de avaliar as propriedades antiartríticas *in vivo* de uma nanoemulsão contendo o óleo de *P. pubescens* e o comportamento reológico do sistema desenvolvido. As propriedades viscoelásticas, fluência e recuperação foram avaliadas por testes oscilatórios dinâmicos. A atividade antiartrítica da nanoemulsão foi

avaliada pelo modelo de artrite induzida por zimozan, em três diferentes concentrações (25, 50 e 125 mg/kg/dia). Demonstrou-se que a nanoemulsão contendo o óleo de *P. pubescens* na dose de 50 mg/kg diminui o recrutamento de células para uma cavidade articular e aumenta a regeneração cartilaginosa no final do tratamento. A avaliação do comportamento de deformação permitiu observar que a nanoemulsão de *P. pubescens* apresenta característica predominantemente elástica. Esses achados demonstram o potencial da aplicação de *P. pubescens* na nanotecnologia para o desenvolvimento de novos medicamentos antiartríticos.
Palavras-chave: Atividade antiartrítica; Fluência e recuperação; Sucupira; Viscoelasticidade.

Resumen

Pterodon pubescens, conocido popularmente como "sucupira", se usa tradicionalmente como antiinflamatorio. Este trabajo tuvo como objetivo evaluar las propiedades antiartríticas in vivo de una nanoemulsión que contiene lo aceite de *P. pubescens* y el comportamiento reológico del sistema desarrollado. Las propiedades viscoelásticas, fluencia y recuperación se evaluaron mediante ensayos oscilatorios dinámicos. La actividad antiartrítica de la nanoemulsión se evaluó mediante el modelo de artritis inducida por zimozan, en tres concentraciones diferentes (25, 50 y 125 mg/kg/día). Se ha demostrado que la nanoemulsión que contiene aceite de *P. pubescens* en la dosis de 50 mg/kg disminuye el reclutamiento de células a la cavidad articular y aumenta la regeneración del cartílago al final del tratamiento. La evaluación del comportamiento de deformación permitió observar que la nanoemulsión de *P. pubescens* tiene una característica predominantemente elástica. Estos hallazgos demuestran el potencial del uso de *P. pubescens* en nanotecnología para el desarrollo de nuevos fármacos antiartríticos.
Palabras clave: Actividad antiartrítica; Fluidez y recuperación; Sucupira; Viscoelasticidad.

1. Introduction

Rheumatoid arthritis is a chronic inflammatory disease, of autoimmune origin and unknown etiology, characterized by progressive inflammation of the synovial capsule. This results in joint destruction, cartilaginous and bony erosions, pain, morning stiffness and limited mobility (Neumann et al., 2010; Smyrnova, 2014). Pharmacological therapies, generally, include the use of non-steroidal anti-inflammatory drugs, corticosteroids, disease modifying drugs, and immunosuppressive agents. The goal of treatment is to prevent or delay joint damage, and complete remission is rarely achieved. Thus, the discovery of new therapeutic agents is a promising and interesting strategy (Henrique da Mota et al., 2013)

Therefore, it is very important to develop new and more effective pharmaceutical systems for the treatment of inflammation and pain (Hoscheid et al., 2015).

Fruits of species of *Pterodon* genus, popularly known as "sucupira-branca" or "faveira", are commonly used in folk medicine, in the treatment of rheumatism (Sabino et al., 1999; Coelho et al., 2005), sore throats, respiratory dysfunctions (bronchitis and tonsillitis), in addition to its depurative, tonic activities (Arriaga et al., 2000; Agra et al., 2008), anti-inflammatory (Carvalho et al., 1999; Dutra et al., 2009; Hoscheid et al., 2013) and antinociceptive (Spindola et al., 2010; Spindola et al., 2011). Scientific evidence of the anti-inflammatory activity of the hexanic fraction of the hydroalcoholic extract of *P. pubescens* fruits has already been demonstrated (Hoscheid et al., 2013), also formulations from extracts of *P. pubescens* has demonstrated anti-inflammatory activity (Hoscheid et al., 2017). These results allow a good perspective for the development of pharmaceutical products than can be applied in this field.

Nanoemulsions (NE) are a promising system of drug delivery for lipophilic compounds (Solans et al., 2005). The study of the rheological behavior of these systems can predict physical stability, behavior during administration, scattering, and recovery of the initial structure (De Araújo Pereira et al., 2013). Therefore, the understanding of how the elastic properties are influenced by the components of the emulsions can allow the improvement of the formulation and the physical stability of the systems produced, which stabilization is due to the formation of network between the particles. Previous studies have already shown that this system presents spherical-shaped nanosized micelles, exhibited shear-thinning behavior (pseudoplastic flow), allowing the adequate syringeability. It was also demonstrated the physicochemical stability and biocompatible components for parenteral administration (Hoscheid et al., 2015; Hoscheid et al., 2017).

Thus the objective of this work was to evaluate the antiarthritic activity of a NE containing the hexanic fraction of the ethanolic extract of *P. pubescens* by zymozan-induced arthritis model, as well as to evaluate the rheological behavior of the formulation.

2. Materials and Methodology

2.1. Chemicals

Polyethylene glycol hydrogenated castor oil/sorbitan oleate (PEG-40H) were kindly provided by Oxiteno. Purified soybean lecithin (Phospholipon 90G) was obtained from

Lipoid. Ultra-purified water (Milli-Q® Plus) was used for preparation of all aqueous solutions. Zymozan (purchased from Sigma) was used as a phlogistic agent to induce inflammation.

2.2. Oil extraction

P. pubescens fruits were obtained from Nossa Senhora do Livramento city, Mato Grosso state, Brazil (15°89' S; 56°41' W). The taxonomic identity was confirmed by Dr. Germano Guarim Neto, and a voucher specimen (no. 20502) was deposited in the Herbarium of the State University of Maringá.

Oil extraction was performed and chemical characterized as previously reported (Hoscheid et al., 2012). Briefly, *P. pubescens* oil were extracted with ethanol by turbo extraction (Ultra-Turrax UTC115KT, IKA Works, USA) and partitioned with hexane. The organic solvent was evaporated in a vacuum rotary evaporator (Büchi®R-210, Flawil, Switzerland) at 40°C until the solvent evaporated completely. Chemical characterization was performed by GC-MS.

2.3. NE preparation

NE was prepared at the optimal conditions that have been previously described (Hoscheid et al., 2015). The oil phase consisting of *P. pubescens* oil (5.0%, w/w) and a lipophilic emulsifier (soybean lecithin 5.0%, w/w) was previously homogenized and injected into the aqueous phase consisting of water and a hydrophilic emulsifier (PEG-40H, w/w), in a high-speed shear apparatus (IKA®T10 basic, USA) at 14500 rpm for 15 min. The pH was adjusted to 7.4 with NaOH solution (1 M).

2.4. Antiarthritic activity of intramuscular injection of Pterodon pubescens NE

2.4.1. Animals

The zymozan-induced arthritis experiment was conducted with Swiss mice (weighing 40–50 g). Mice were housed under controlled conditions (22 ± 2 ºC with a 12 h light/12 h dark cycle and free access to food and water). The experimental procedures were approved by the Ethics Committee of the Universidade Estadual de Maringá (protocol number 3968190615, date of approval August 14, 2015).

2.4.2. Treatment Protocol

In this experiment, the animals (n = 5 per group) were divided into 5 treatment groups: (Saline Group) control animals without arthritis induction which received only sterile solution of 0.9% sodium chloride; (Zymozan Group) animals with arthritis which received NE base (absent from *P. pubescens* oil); (1) animals with arthritis induced which received 25 mg/kg of *P. pubescens* NE; (2) animals with arthritis induced which received 50 mg/kg of *P. pubescens* NE; and (3) animals with arthritis which received 125 mg/kg of *P. pubescens* NE.

Treatment was performed daily by intramuscular administration (in the left pelvic limb) for a period of 7 days starting on the day of the intraarticular adjuvant injection. The doses were based on the results obtained with the crute extract (Hoscheid et al., 2013).

2.4.3. Induction and Assessment of zymozan-induced arthritis

Arthritis induction was performed 30 min. after NE treatment. The animals were anesthetized with halothane 3%, and an intraarticular injection was performed on the right femorotibiopatellar joint of 10 µL of zymosan suspension in sterile saline (at a concentration of 20 mg/mL, w/v) (Yamada et al., 2013; Milanova et al., 2014). The saline group received 10 µL sterile 0.9% sodium chloride solution.

2.4.4. Cell migration

The exudate of the articular cavity of the right knee of the animals was collected six hours and 7 days after the induction of arthritis. The joint cavity was then washed twice with 5 µl of 0.9% phosphate buffered saline (PBS) containing 1 mM ethylenediaminetetra acetic acid (EDTA). The sample was diluted to a final volume of 100 mL with PBS/EDTA. The number of leukocytes that migrated to the exudate was measured using a Neubauer chamber cell counting. For collection of the exudate, the animals were anesthetized with 3% halothane and euthanized.

2.4.5. Histological Analysis

The joints were removed and were preserved in a formalin solution for routine histological processing. Then, these were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological examination according to conventional histological methods.

2.4.6. Statistical analysis

Data are presented as means ± standard deviation. Results were statistically analyzed using GraphPad software (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by post hoc Tukey tests was performed. *P* values less than 0.05 were considered statistically significant.

2.5. Rheological behavior

Rheological studies were performed with a stress controlled rotational rheometer (MARS II Haake®, Thermo Fisher Scientific Inc., Germany) equipped with a thermostatic bath (Phoenix 2C30P, Thermo Fisher Scientific Inc., Germany). The rheological properties of *P. pubescens* NE were measured at 25.0 ± 0.05 °C, using cone–plate geometry (2º angle; 35 mm diameter; gap height 0.105 mm). Samples were allowed to equilibrate the temperature for 5 min prior the measurements.

2.5.1. Creep-Recovery Rheology

In a creep-recovery test, a constant stress of 0.025 and 0.10 Pa was applied to the NE, and the change in strain over time (called creep) was observed for 250 s. This stress was subsequently released, and the recovery response was recorded for 500 s. To identify the deformation pattern of the NE, a four-parameter Burger's model was applied to fit the experimental creep compliance curve (Steffe, 1996).

2.5.2. Oscillatory-Shear Rheology

The frequency dependence of storage (*G'*) and loss (*G"*) moduli were determined over an ascending frequency ramp from 0.1 to 10 Hz. These dynamic oscillatory assays were carried out within the linear viscoelastic range (LVR) that had been previously determined from an amplitude sweep (0.01 Pa – 20 Pa) and a constant frequency of 1 Hz.

3. Results and Discussion

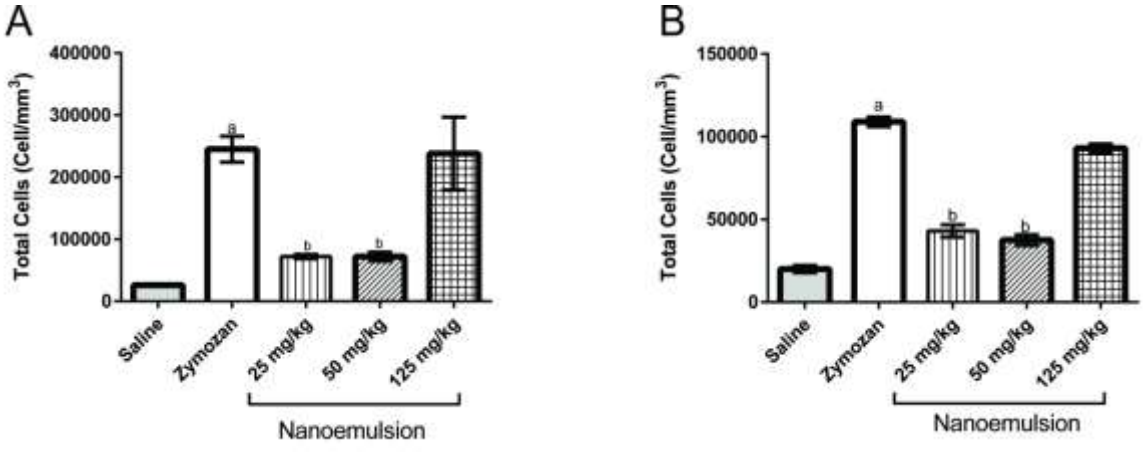
3.1. Antiarthritic activity of intramuscular injection of *Pterodon pubescens* NE

An inflammation is a response of vascularized tissues against a woven lesion or infection, involving actions coordinated by the immune system. Tissue damage and cellular necrosis are potent stimuli for inflammation. This occurs in response to cell death to neutralize damage and promote healing. However, the acute response of neutrophils may exacerbate a tissue injury and cause greater tissue damage (McDonald & Kubes, 2011). Thus the recruitment and migration of leukocytes to the focus of injury is an important step in the inflammatory response and these act as the first line defense in the initial phase persisting until resolution of the process (Souto et al., 2011). Leukocyte migration is a multifactorial process involving endothelial cells from blood vessels, adhesion molecules and inflammatory mediators which activate the sequence of migration events (Huang et al., 2016).

Zymosan, a cell wall polysaccharide of *Saccharomyces cerevisiae*, has been used in the study of innate immune responses and is capable of stimulating arthritis by intra-articular injection (Frasnelli et al., 2005), with a morphologically similar response to that seen in humans.

The Figure 1 show the cell counting data in the right femorotibiopatellar joint cavity after 6 hours and 7 days of the zymosan-induction arthritis.

Figure 1 - Cell counting data in the right femorotibiopatellar joint cavity after 6 hours (a) and 7 days (b) of the zymosan-induction arthritis.



Note: Values expressed as mean ± standard deviation (n = 5). [a]*P*<0.05 compared to the Saline group; [b]*P*<0.05 compared to the Zymosan and 125 mg/kg groups. One-way ANOVA post hoc Tukey test. Source: Authors.

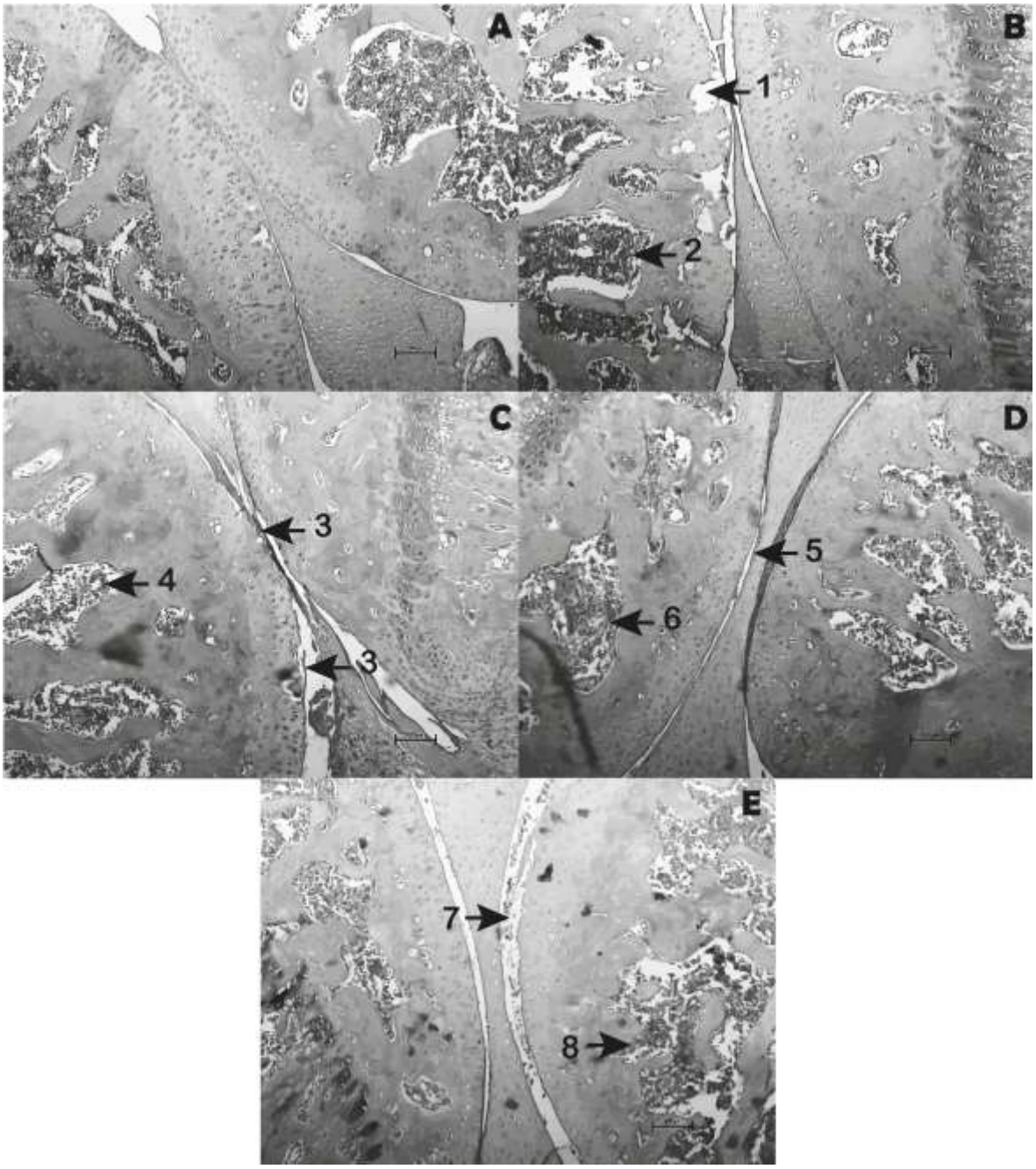
The zymosan group had significantly higher cellular recruitment than the saline group, demonstrating the efficacy of induction of the inflammatory process in the joints after 6 hours of induction. The 25 mg/kg and 50 mg/kg dose groups showed a significant decrease ($P<0.05$) in cell recruitment compared to the zymosan group, while the 125 mg/kg group was statically equal to the group zymosan (Figure-1a). The same can be observed after 7 days of the induction of arthritis by zymosan (Figure-1b).

The significant decrease in inflammatory cells in the groups treated with 25 mg/kg and 50 mg/kg of NE, observed in this study, evidenced a possible involvement of the formulation in the suppression of the immune response, which may be related to inhibition of lymphocyte proliferation, corroborating previous studies (Sabino et al., 1999; Hoscheid & Cardoso, 2015; Denny, 2002; Cardoso et al., 2008). The terpenes present in the oily extract of the genus *Pterodon* are the possible responsible for the anti-inflammatory action of this extract (Carvalho et al., 1999). Previous studies have shown that a fraction of *P. pubescens* extract containing cyclic diterpenes, epoxifarnesol and geranylgeraniol showed in vitro inhibition of NF-κB in leukemic cells (Pereira et al., 2012). In addition, inhibition of NF-κB was observed in rats treated with a dose of 160 mg/kg orally of geranylgeraniol isolated from the extract of *P. pubescens* (Espíndola et al., 2005). These results allow us to infer the possible relationship between the inhibitory action of cellular recruitment observed in this study and the inhibition of NF-κB and other pathways linked to the inflammatory process. However, it is worth noting that in this study the dose used was significantly lower (50 mg/kg) than the previous studies using the crude extract (250 mg/kg), which is due to the development of a nanoemulsive system for intramuscular administration.

It is believed that cartilage injury is mediated by an excess of synthesis and release of inflammatory and catabolic factors (Braza-Böíls et al., 2012). Another point to be highlighted is the control of angiogenesis in the inflammatory joint process. Antiangiogenic therapy may promote a more orderly cellular reconstruction, and compounds possessing this ability coupled with an anti-inflammatory action would be ideal for the treatment of joint lesions (Stupack et al., 1999). NF-κB inhibitors are well studied for the treatment of RA because of their antiangiogenic and anti-inflammatory characteristics (Keifer et al., 2001).

The Figure 2 show histological analysis of the right knee joint of animals after the seventh day of treatment of intramuscular administration of *P. pubescens* NE.

Figure 2 - Right knee joint of animals of the saline (a) and zymosan (b) groups, and of the daily intramuscular groups with 25 mg/kg (c), 50 mg/kg (d) and 125 mg/kg (e) of *P. pubescens* NE after the seventh day of treatment.



Note: (1) points to extensive cartilaginous degeneration; (2) indicates an extensive and marked mononuclear inflammatory reaction, with connective tissue encapsulation and mild angiogenesis; (3) point to the slight cartilaginous formation; (4) points to a mononuclear inflammatory reaction, with connective tissue encapsulation and discrete angiogenesis; (5) points out the marked cartilaginous formation; (6) points to a moderate focal mononuclear inflammatory reaction, evident angiogenesis presenting a young bone matrix formation at the extremities of the inflammatory reaction; (7) shows mild cartilaginous formation; (8) moderately/highly focally extensive mononuclear inflammatory reaction, forming in the surrounding tissues an encapsulation of connective tissue, joining two portions of connective tissue properly and a discrete angiogenesis. Source: Authors.

As expected, the saline group presents a histologically normal bone matrix, with no reparative inflammatory reaction as can be observed in the **Erro! Fonte de referência não encontrada.**2a, while the Zymozan group presented extensive cartilaginous lesions (Figure-2b). These animals also present an extensive and marked mononuclear inflammatory reaction, with predominance of reactive macrophages forming in the surrounding tissues an encapsulation of connective tissue, joining two portions of connective tissue proper and a discrete angiogenesis.

In contrast, animals treated with NE at a dose of 25 mg/kg had mild cartilaginous formation (Figure-2c). These animals presented a mononuclear inflammatory reaction with a predominance of reactive macrophages, forming in the surrounding tissues a connective tissue encapsulation and a discrete angiogenesis. A marked cartilaginous formation was observed at 50 mg/kg (Figure-2d). The animals in this group presented a moderate focal mononuclear inflammatory reaction, with predominance of reactive macrophages uniting two portions of connective tissue proper, with evident angiogenesis presenting a formation of young bone matrix at the extremities of the inflammatory reaction, with foci of penetration of bone matrix center of the lesion.

On the other hand, daily administration of 125 mg/kg of *P. pubescens* oil NE showed only mild cartilaginous formation (Figure-2e), with a moderately/highly focally extensive mononuclear inflammatory reaction, and a predominance of reactive macrophages forming in tissues surrounding an encapsulation of connective tissue, joining two portions of connective tissue properly and a discrete angiogenesis. These results are in line with those observed in cell migration, since the dosages that triggered lower cell migration (25 and 50 mg/kg/day) also showed lower cartilaginous matrix degradation. So, our results amplifly revious findings on the efficacy of formulations containing *P. pubescens* oil in the treatment of rheumatoid arthritis and suggest a improve clinical symptoms derived for cartilage defects at osteoarthritic knees.

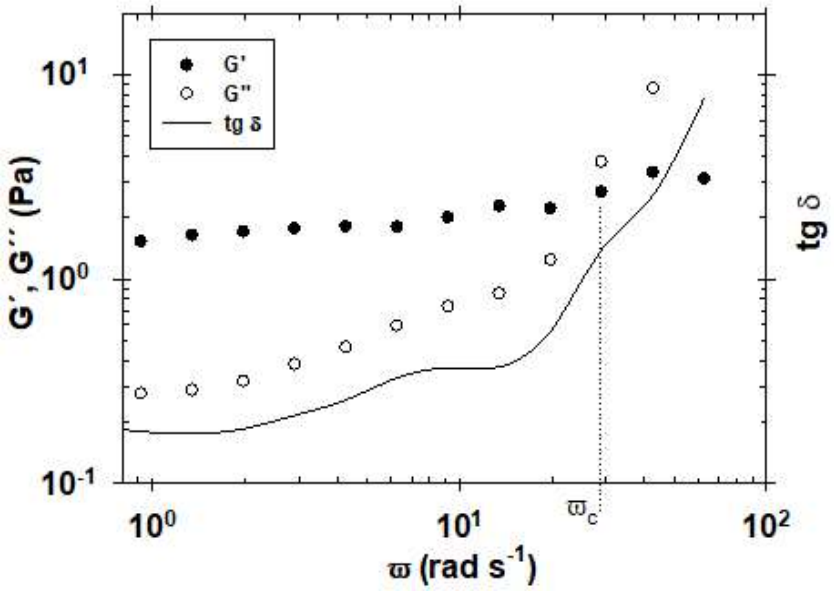
3.2. Oscillatory rheological measurements

Previous studies have already shown that *P. pubescens* oil NE system presents spherical-shaped nanosized micelles, exhibited shear-thinning behavior (pseudoplastic flow), allowing the adequate syringeability (Hoscheid et al., 2015). NE usually present elastic properties, originated by the interfacial energy of the droplets. In small volume, the interfacial tension guarantees the spherical shape of the droplets. However, in a denser and more

compact volume, the droplets deform - resulting in energy storage. The application of tension in an emulsion leads to a greater deformation of the dispersed phase, culminating in the elevation of the surface area and, consequently, in the elastic energy storage. In addition, the long-term stability of emulsions can be evaluated by means of dynamic rheological studies, since oscillatory measurements can be used to correlate with the phenomena of cremation, flocculation, coalescence and phase inversion (Tadros, 2004).

Figure 3 shows the elastic modulus (G') higher than viscous modulus (G") over range of ω=10 to ~29 rads-1.

Figure 3 - Frequency dependence of the loss (○) and storage moduli (●) of *P. pubescens* NE. Variation of loss tangent δ.



Source: Authors.

The elasticity behavior is linked to the microstructure formed by the stable oil droplets that form a closely packed structure revealing a three-dimensional connectivity of the elastic associative network dispersed of water phase of the NE (Esquenet et al., 2004; Hoscheid et al., 2017; Wilson & Baljon, 2017).

Parameters for the identification of a stable emulsion by means of oscillatory rheology are the magnitude of the quantities G 'and G "and the independence of the values in response to the applied frequency (ω). Thus, an emulsion which G' modulus has a value greater than G" is considered stable and both G' and G" are independent of the frequency, since they are

characteristic of the presence of an elastic gel. The greater G', the more elastic the deformation and therefore the greater its recovery (Torres et al., 2007; Marku et al., 2012).

At higher values, G" becomes larger than G', displaying the crossover frequency point (ω_c) at 28.876 rad s^{-1}. As the frequency is the inverse of the time, the relaxation time, defined as $\lambda_0 \sim 2\pi/\omega_c$, can be evaluated as 0.218 s. The relaxation time is related as the lifetime of the junctions of the formed network. Once this network break, the restructuration time is necessary for the reach the equilibrium and reorganization of the system (Metri et al., 2018).

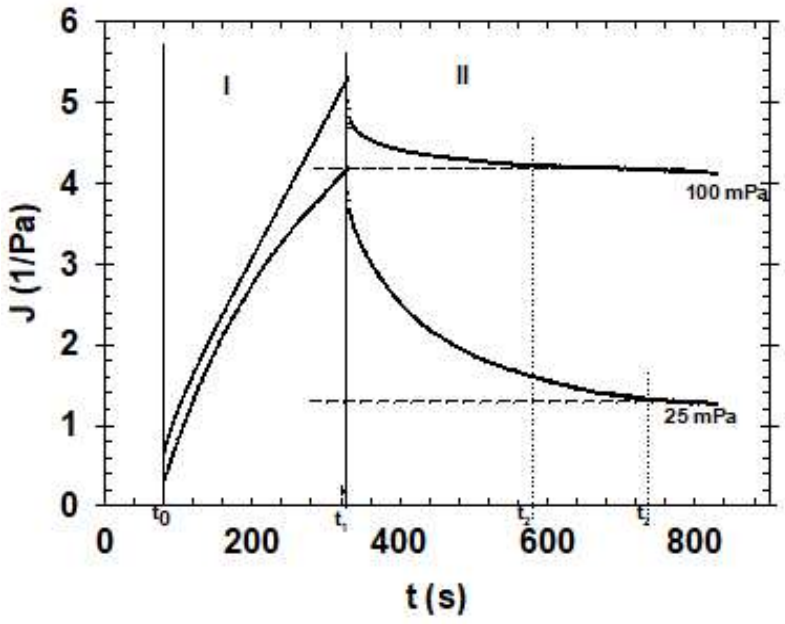
Other important parameter is the tanδ, obtained by the ratio of G" and G'. As displayed in Figure 3, the tan δ values were < 1 until the ω_c, confirming the elastic behavior for the *P. pubescens* NE (Syahariza & Yong, 2017; Ghosh et al., 2018).

3.3. Creep and recovery

The transient behavior of the viscoelastic features of the nanostructured system is further investigated by creep recovery tests. In a first time (creep) the sample is submitted to an instantaneous stress, with the strain increasing over the time. The second step is to remove instantaneously the stress to allow the sample to recover as function of their properties after the yield stress test and to reapply a constant load to the sample. Creep and recovery is widely used to evaluate the deformation mechanism of polymeric systems and, associated with other techniques, predict the behavior and workability characteristics of pharmaceutical systems (Cuciniello et al., 2018).

Figure 4 show the values of compliance $J=\gamma/\sigma$ as function of time, for creep and recovery tests of *P. pubescens* NE, performed at 25 and 100 mPa, in a time interval ranging of 0 to 800 s.

Figure 4 - Creep recovery tests for *P. pubescens* NE performed at 25 and 100mPa.



Source: Authors.

In the creep phase (section I), after 355 s of stress load, the system reached maximum deformation, J_{MAX} for that period of time, where was observed an instantaneous increase in strain (maximum creep, higher peak). In this phase, the curve profile reveal reversible flow for which elastic deformation is instantaneously removed. Sequentially, the stress was suppressed ($\sigma = 0$) and the measurements of compliance, $J = f(t)$, were performed reaching 800 s, that corresponds to the complete recovery test (section II). In this phase, the elastic response was followed by a viscoelastic response. The viscous flow is observed towards the end of the load application period. Removal of the load results in a rapid decrease in strain response, which is similar to the initial elastic response.

The recovery phase is related to time-dependent molecular relaxation required for the restructuration of the material. As expected, at 25 mPa, for the *P. pubescens* NE, the % recovered is higher when compared to the 100 mPa.

4. Final considerations

NE administration was shown to be effective in decreasing zymosan-induced cell recruitment of arthritis, whereas intramuscular 50 mg/kg dose demonstrated the greatest efficacy in the treatment of experimental arthritis, where it was possible to observe the decrease in cellular recruitment and better cartilaginous regeneration of the joint compared to the other groups analyzed. The evaluation of the behaviour of deformation allowed to observe that the *P. pubescens* NE presents predominantly elastic characteristic. When exposed to a amplitude sweep it undergoes deformation, however at the end of the application of the tension, the system returns to its initial elastic behavior, demonstrating its capacity to restructure and reorganize the system, physical characteristics desirable and compatible with intramuscular administration.

Thus, the results of this study amplify previous findings on the efficacy of formulations containing *P. pubescens* oil in the treatment of rheumatoid arthritis, and encourages investigations to confirm this possibility.

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