Potential Ptereostilbene and Anti Inflammatory Effect on Rheumatoid Arthritis Therapy in *Rattus norvegicus* Rat

ABSTRACT

Introduction: Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory diseases, which primarily involves the joints and may also include extra-articular manifestations, such as nodules, pulmonary involvement, vasculitis, and systemic comorbidities. Pterostilbene (PTE) is responsible for the process of angiogenesis and contributes with its anti-inflammatory properties to the rheumatoid arthritis process. **Objective:** To verify whether oral Pterostilbene solution use has an effect on the acute phase of the inflammatory process in the treatment of rheumatoid arthritis. **Methodology:** This experimental study was composed of 45 *Rattus Norvegicus* rats, Wistar strain, divided into three groups corresponding to the Control Group (CG) with no lesion and no treatment; the SHAM Group (SHAM) with lesion but no treatment, and the Pterostilbene Group (PG) with lesion and treatment. The results were evaluated by histology and flow cytometry. **Results:** The lesions were characterized by a diffuse acute inflammatory process with mild to moderate intensity. It was observed that increasing proliferative activity of fibroblasts and angiogenesis occurred, besides the modulation of inflammatory cytokines, which corroborated the histological findings. **Conclusion:** It can be concluded that pterostilbene, due to its anti-inflammatory activities, can control inflammation so that there are no exacerbated symptoms and lesions, in addition to stimulating structural restoration by increasing fibroblast production and angiogenesis.

Keywords: Joint; rheumatoid arthritis; inflammation; Polyphenol

1. INTRODUCTION

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory diseases, which primarily involves the joints and may also include extra-articular and systemic manifestations. About 1% of the population is affected by RA, which can be present at any age, and is more prevalent in women [1-3]

Immunological mediators are usually detected in the pre-RA phase and precede the appearance of overt clinical symptoms. As the disease

progresses, chronic inflammation and associated tissue damage occur [4]. The most commonly reported symptoms are joint pain, stiffness, swelling, and reduced joint motion.

As it is an autoimmune disease characterized by a failure of the inflammatory process, a better understanding of the immunoregulatory signaling pathways may contribute to new therapeutic strategies. Since characteristic phenomena of RA is the overproduction of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and the reduction of anti-inflammatory cytokines (IL-10), the balancing of the action of these cytokines can settle the inflammation in RA [5,6].

In the context of new approaches for the treatment of RA, there is the option of phenolic compounds, secondary metabolites present in different plants, which have antioxidant activities and modulators of molecular and cellular signaling pathways. Anti-inflammatory activities have been attributed to polyphenols' capacity to inhibit the transcription and expression of pro-inflammatory cytokines [6].

Among the polyphenols, there is the pterostilbene (PTE), a dimethylated stilbenoid analog of resveratrol. It possesses antioxidant, anti-inflammatory, anticancer, antihypertensive, and antidiabetic properties that contribute to the prevention of human diseases, pterostilbene is more lipophilic due to its structure (FIGURE 1 SUPPLEMENTARY MATERIAL); it can be found in blueberries and grapes, from plants belonging to the families *Vitis* and *Vaccinium*, shrubs that produce edible fruit commonly found in the Northern Hemisphere, having greater availability *in vivo* [711]. Given the anti-inflammatory effect of pterostilbene, the objective was to verify this action in the acute phase of rheumatoid arthritis in an animal model.

2. METHODOLOGY

2.1 Drug acquisition

The drug was purchased from "Active Pharmaceutica - Importer and Distributor of Pharmaceutical Supply" of Palhoça - SC, Brazil, with 99% purity; molecular formula: $C_{16}H_{16}O_3$; molecular weight: 256.3 g/mol; CAS: 537-42-B; manufacturer: Hanzhong TRG Biotech.

2.2 Experimental Groups

The animals were divided into 3 groups, one group with 5, another with 10, and another with 30 rats corresponding, respectively, to the Control Group (CG); SHAM Group (SHAM), and Pterostilbene Group (PG). The SHAM and PG groups were divided into rats with joint injury without treatment and rats

with injury and treatment by oral Pterostilbene solution; the groups were subdivided into 0 (with euthanasia 12 hours after arthritis induction) and 3 days (with euthanasia 72 hours after induction) (TABLE 1 SUPPLEMENTARY MATERIAL).

2.3 Formulation

The drug was suspended in Carboxymethylcellulose (CMC) (1%) at different concentrations (4 mg/kg; 200 mg/kg; 400 mg/kg) to be administered orally [12,13]. For PG1, 20 mg of PTE was weighed and suspended in 10 mL of CMC; for PG50, 500 mg of PTE was weighed in 5 mL of CMC; for PG100, 1 g of PTE was weighed in 5 mL of CMC; these were the total volume used throughout the experiment. It is important to point out that the drug was suspended at the time of administration and had an approximate volume of 0.5 mL for each animal.

2.4 Sample

Forty-five Rattus *Norvegicus rats*, Wistar strain, from the Animal Facility of the State University of Maringá (UEM), weighing between 200 and 250 grams, were used. They were housed in groups of 5 in acrylic cages, with free access to water and feed, a 12-hour light/dark period, and room temperature of 23 ± 1 °C.

2.5 Experimental test

The animals were previously anesthetized with 80 mg/Kg ketamine hydrochloride and 15 mg/Kg xylazine hydrochloride. After verifying the anesthetic state of the animals by the manual compression test of the lower third of the tail, rheumatoid arthritis was induced through an intra-articular injection in the right knee of 50 µL of Freund's complete adjuvant. Twelve hours later, the injured joint was observed and palpated to assess the presence of the signs of arthritis, such as erythema and joint swelling, thus being indicative of the presence of local inflammation [14].

2.6 Treatment

The treatment was carried out orally by gavage. In the control and SHAM groups, no treatment was performed, and in the PG, the administration of the formulation containing pterostilbene as the active ingredient was performed. One dose of the drug was administered two hours before the induction of arthritis to all groups; groups 0 were euthanized 12 hours after induction; groups 3 received daily treatment (every 24h) with doses of 0.5 mL at the given concentrations (4 mg/kg; 200 mg/kg; 400 mg/kg), they were euthanized 72h after induction.

2.7 Euthanasia

In the subsequent treatment periods, the animals were anesthetized with 80 mg/kg ketamine and 20 mg/kg xylazine; after the anesthetic status was verified, they received 175 mg/kg pentobarbital (vial with 50 mL and 100 mg/mL) intraperitoneally for lethal dose [15]. After verifying the animal's death *status* by faded eye color, lack of spontaneous breathing, and lack of response to pain, the joint was removed. After removing the samples, they were included in 10% formaldehyde to maintain the morphological characteristics.

2.8 Flow cytometry (TABLE 2 SUPPLEMENTARY MATERIAL)

Two mL of blood was collected from each animal; subsequently, it was centrifuged at 1500 rpm for 10 minutes at room temperature. The blood was collected two times: first before the induction of arthritis, and the second collection occurred before euthanasia. After centrifugation, the supernatant (serum) was pipetted and separated for analysis. The kits used for the analysis were BD™ Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (Becton Dickinson, USA) and the BD™ Cytometric Bead Array Mouse Inflammation Kit (Becton Dickinson, USA); the cytokines analyzed were TNF; INF-γ; IL-2; IL-4; IL-5; IL-6; IL-10; IL-12p70 and MCP-1.

The samples were analyzed on the BD™ Flow Cytometer, Accuri C6 (Becton Dickinson, USA), according to the manufacturer's instructions. 50 µL of the cytokine beads mix, 50 µL of the sample (serum), and 50 µL of detection reagent were placed in a 2.0 mL Eppendorf for each sample; the tubes were placed in the dark for two hours at room temperature. After the two hours, 1 mL of the wash buffer was added to each Eppendorf and centrifuged at 200 g, 4°C, for five minutes. After centrifugation, the supernatant was carefully removed and discarded from each sample, and then 300 µL of the wash buffer was added to each tube to resuspend the samples.

The reading in the cytometer was performed manually by acquiring 10,000 events from each sample. The flow cytometry data were analyzed in FCap 3.0 Array software (Becton Dickinson, USA), and the results were plotted in graphs of means and standard deviations of the mean.

2.9 Histology (TABLE 2 SUPPLEMENTARY MATERIAL)

For morphological analysis, the paraffin blocks of articular tissues were cut into three sections of 2 µm thickness, with a distance of 50 µm between them, mounted on histological slides and submitted to hematoxylin/Eosin staining, and later examined under conventional light microscopy. From these sections, a descriptive analysis of the histopathological aspects of the

tissue was performed; the characteristics concerning the inflammatory infiltrate (the type and quantity of inflammatory cells) were recorded.

The criteria for morphological analysis included records for the epithelial lining of the presence/absence and amount of inflammatory changes observed under light microscopy using the following semiquantitative scale, based on Kerppers' study [16]:

- 0: normal epithelium (no changes observed, or the changes caused by inflammation are present in less than 10% of the epithelial surface area);
- 1: mild changes (in 10 to 25% of the surface area);
- 2: moderate changes (in 26 to 50% of the surface area);
- 3: intense changes (in more than 50% of the surface area).

The inflammatory infiltrate was analyzed by recording the amount of inflammatory cells, which were categorized according to the semiquantitative scale:
- 0: absence of inflammatory infiltrate (no inflammatory cells observed);
- 1: sparse inflammatory infiltrate (up to 25% of connective tissue occupied by inflammatory cells);
- 2: moderate inflammatory infiltrate (26 to 50% of the connective tissue occupied by inflammatory cells);
-3: intense inflammatory infiltrate (more than 50% of the connective tissue occupied by inflammatory cells).

2.10 Statistical analysis

The data were analyzed using GraphPad Prism 6.0® software. For statistical analysis, the Kruskal-Wallis test and Dunn's post-hoc test were used with a statistical significance level of P= .05.

3. RESULTS AND DISCUSSION

It was observed that the lesions were characterized by an acute inflammatory process and presented a diffuse and high intensity distribution.

In the groups 12 hours after the induction of rheumatoid arthritis, it was observed that the groups treated with pterostilbene - pre-induction with a dose of 4 mg/kg and 200 mg/kg - had a classification 1 in the semiquantitative evaluation scale of epithelial changes, which were mild, and the 400 mg/kg groups had a classification 2, which were moderate (Table 1). A demonstrative of the histology is demonstrated on Supplementary figure 2. In the classification of inflammatory infiltrate, PG1 had a classification 1, in which the inflammatory infiltrate was scarce, and both the PG50 and PG100

had a classification 2, in which the inflammatory infiltrate was moderate; The SHAM group had a classification 2 in both evaluations.

Table 1: Epithelial Changes Classification and Inflammatory Infiltrate Classification

Groups	Epithelial classification	Epithelial change	Infiltrated classification	Infiltrated
CG	0	Normal Epithelium	0	Absence of Infiltrate
SHAM 12h	2	Moderate	2	Moderate
SHAM 72h	1	Mild	3	Intense
PG1 12h	1	Mild	1	Sparse
PG1 72h	1	Mild	3	Intense
PG50 12h	1	Mild	2	Moderate
PG50 72h	1	Mild	3	Intense
PG100 12h	2	Moderate	2	Moderate
PG100 72h	2	Moderate	3	Intense

In the groups 72 hours after the induction of rheumatoid arthritis, it was observed that they presented an intense inflammatory process, with classification 1 for the evaluation of the epithelium in groups SHAM, PG1, and PG50, and classification 2 in PG100; and all groups 72 hours had classification 3 for the evaluation of the level of inflammatory infiltrate.

Table 1 shows the classifications with regard to epithelial changes and inflammatory infiltrate from the morphological analysis of the joint tissues and Table 2 shows the histological analysis for the presence of cells, neovascularization, and fibroblasts; with respect to their concentrations, being classified as absent, mild, moderate, intense, or severe.

Table 2: Histological analysis for presence of cells, neovascularization, and fibroblasts.

Groups	Mono-nuclear Cells	Poly-morphonuclear Cells	Neo-vascularization	Fibroblasts
CG	Absent	Absent	Mild	Mild
SHAM 12h	Moderate	Moderate	Mild	Mild
SHAM 72h	Moderate	Mild	Mild	Mild
PG1 12h	Mild	Moderate	Mild	Absent
PG1 72h	Mild	Moderate	Mild	Mild
PG50 12h	Mild	Moderate	Mild	Mild
PG50 72h	Moderate	Moderate	Mild	Moderate
PG100 12h	Mild	Moderate	Mild	Moderate
PG100 72h	Mild	Moderate	Mild	Intense

Caption: 0: Absent; 1-500: Mild; 501-1000: Moderate; 1001-1500: Intense; >1500: Severe.

The lesions in all groups that underwent joint injury are characterized by a diffuse acute inflammatory process with mild to moderate intensity, as described in table 2.

In the 12h groups, the treated groups presented a mild amount of mononucleated inflammatory cells, while the SHAM group, in which the joint inflammatory process was induced but had no treatment, presented a moderate level. Regarding polymorphonuclear cells, all groups also showed a moderate level. It is observed that there are proliferative phenomena with an increasing the amount of fibroblasts even in the group treated with 400 mg/kg.

In the 72h groups, it was observed that the groups treated with 4 mg/kg and 400 mg/kg showed a moderate level in the concentration of polymorphonucleated inflammatory cells and a mild concentration when concerning mononucleated cells. The group treated with 200 mg/kg showed a moderate concentration of both cell forms. The SHAM group, in contrast, showed a moderate concentration of mononucleated and a mild concentration of polymorphonucleated. There was also an increasing proliferative phenomenon in the amount of fibroblasts as the concentration of the treatment increased.

Regarding neovascularization, it is observed that a mild angiogenesis process occurred in all groups.

The flow cytometry data encompasses results from different analysis pathways, being Th1/Th2. The results presented in figures 1 and 2 were discussed based on the information described in table 3 of the supplementary material.

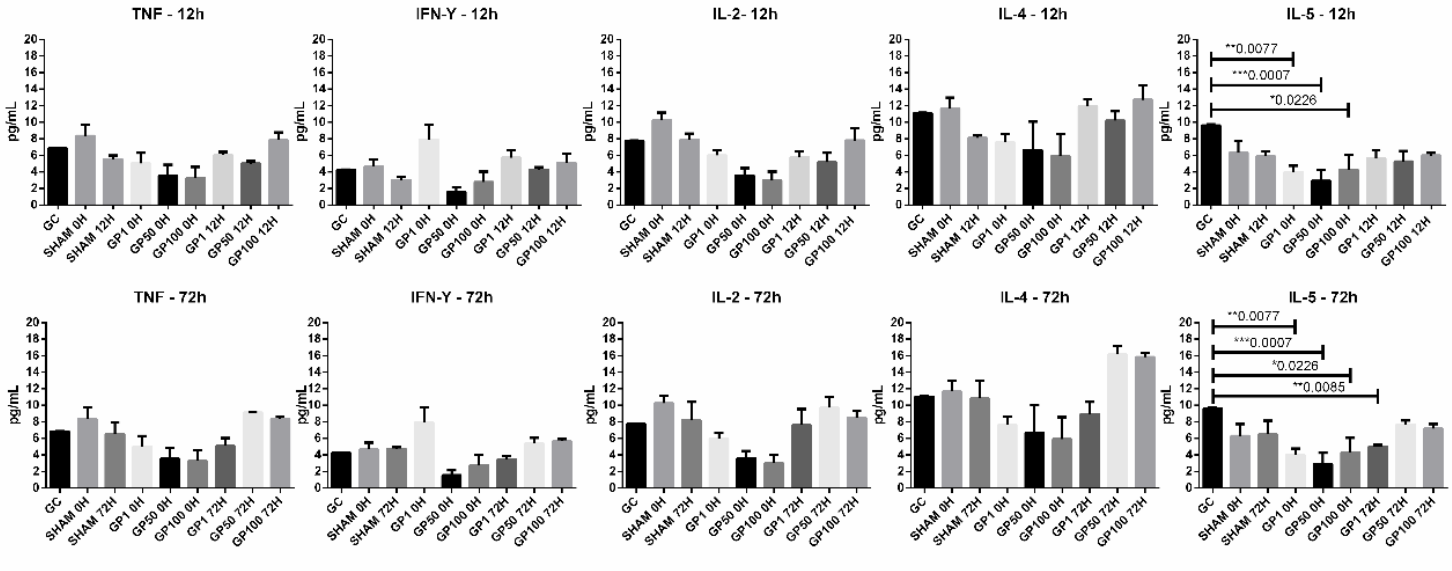


Figure 1: Mean and standard deviation of the mean of TNF, IFN-γ, IL-2, IL-4, and IL-5 levels (pg/mL) analyzed by the TH1/Th2 kit. The bars show the significant difference between the groups. Kruskal-Wallis test with Dunn's post-hoc test with $p<0.05$.

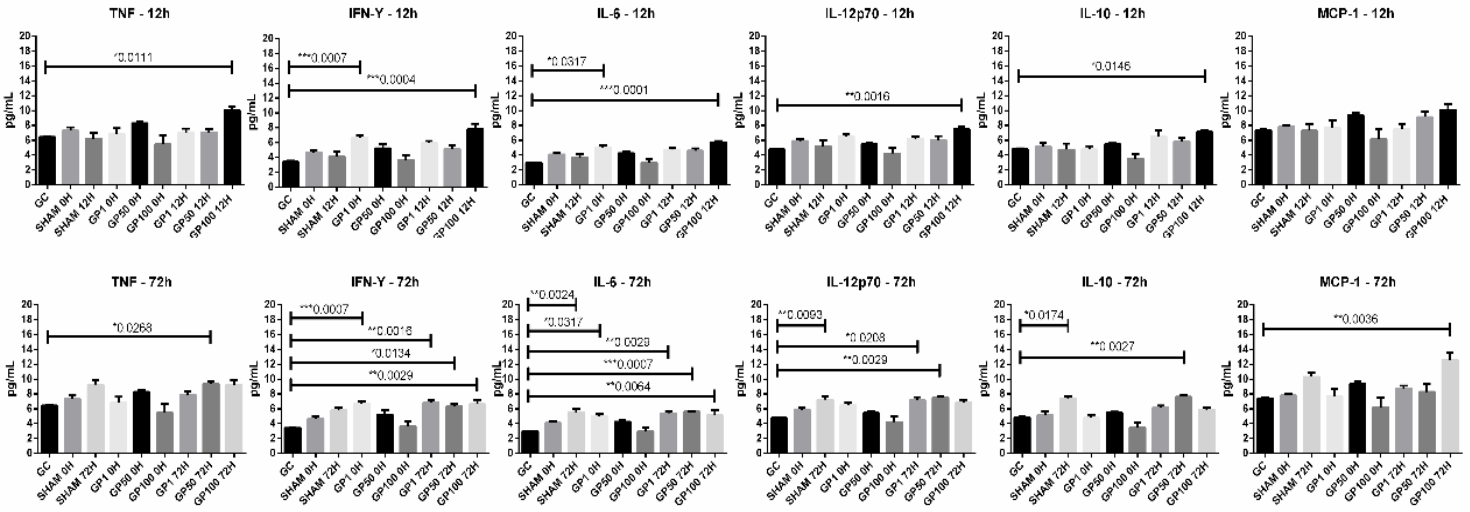


Figure 2: Mean and standard deviation of the mean of TNF, IFN-γ, IL-6, IL-12p70, IL-10, and MCP-1 levels (pg/mL) analyzed by the Inflammation kit. Bars show the significant difference between the groups. Kruskal-Wallis test with Dunn's post-hoc test with $p<0.05$.

In this study, the concentration of TNF remained slightly increased, which is characteristic of the pathophysiology of RA, and also in agreement with the findings of the histological analysis, in which mild to moderate amounts of mononuclear cells, such as macrophages, and also moderate amounts of polymorphonuclear cells, such as neutrophils, were found, which are involved in the process of stimulating the production of TNF.

There was a slight increase in the proportions of IFN, which corroborates with the histological findings, in which a mild to moderate amount of mononuclear cells, such as macrophages and T cells, that induce IFN production were found; as well as a large amount of polymorphonuclear cells, such as neutrophils, that are activated by IFN.

The IL-2 concentrations can be explained by stimulated T-cell production and also by the concentration of IL-12, which is linked to the production of pro-inflammatory cytokines by stimulation of the Th1 pathway.

IFN can inhibit the synthesis of IL-4; however, IFN did not have increased levels, which may indicate that the drug may have increased the production of this interleukin by stimulating the Th2 system to produce anti-inflammatory interleukins. Moreover, the histological findings are also related to these concentrations since there was a moderate amount of polymorphonuclear cells that are part of the process of stimulating the production of this

interleukin. In addition, IFN can also inhibit the synthesis of IL-5. The lower levels found may be due to this action.

In this study, there was an increase in IL-6 levels, corresponding to what is expected in cases of RA. PTE proved to be effective in controlling and not exacerbating the concentration of this pro-inflammatory interleukin since it is a necessary interleukin for the promotion and maturation of neutrophils, macrophage maturation, and differentiation and maintenance of T lymphocytes and NK cells, and its levels corroborate with the histological findings. The production of IL-6 can be influenced by TNF, which can stimulate its synthesis, and by IFN, which can inhibit it.

As expected in the RA inflammatory process, IL-12 levels are increased, and it participates in the Th1 activation cascade, which is pro-inflammatory, thus PTE proved capable of promoting a modulation of the inflammatory process. Moreover, IL-12 can also be inhibited by the action of IL-10.

In this work, an increase in IL-10 levels occurred, especially in the group treated with 200 mg/kg after 72h; its levels were not higher in all groups due to the probable inhibition that may occur by the action of IL-10 itself, IL-4, and IFN-γ.

There was a significant increase in the concentration of MCP-1 in the group treated with 100 mg (400 mg/kg) of PTE, which also corroborates the results found in the histological analysis, which showed an intense amount of fibroblasts after 72h; PTE may be correlated with the stimulation of fibroblast production.

The effects of stilbenoids on the development of adjuvant arthritis was studies in Lewis rats [17], but, in this case, the action of both PTE and pinosylvine (PIN) was evaluated. Treatment consisted of administration of PTE and PIN at a dose of 30 mg/kg, orally, daily for 28 days, and the parameters evaluated were: hind paw volume change (HPV) on days 14, 21, and 28, luminol-enhanced chemiluminescence (CL) of the joint and myeloperoxidase (MPO) activity in hind paw joint homogenates (day 28). Arthritic animals treated with PIN had reduced HPV, decreased joint CL, and joint homogenate MPO activity. However, PTE treatment had no effect linked to HPV and MPO in hind paw joint homogenates, and the effect on CL was only partial.

The pathogenesis of chronic diseases, such as rheumatoid arthritis, is linked to inflammatory processes and oxidative stress. In the study by Yu et. al (2018) PTE inhibited these processes by regulating advanced glycation end products and systems related to MAPK and NF-kB in RAW264.7 cells. The results demonstrated that PTE can interact with NF-kB and reduce the

production of reactive oxygen species, in addition, the data also demonstrated a regulation in the levels of the protein p38 MAPK and p-ERK1/2, which are proteins that play the function of inflammatory regulation and progression [18]. Therefore, the main effects of PTE are linked to the suppression of p38 and ERK in the MAPK signaling and NF-kB phosphorylation pathways and that it inhibited, as a result of the decrease in signaling, pro-inflammatory cytokines such as TNF-a, IL-1B, IL-6, MCP-1.

The effects of PTE on neutrophil activity in an experimental model of arthritis was studied, due to these cells playing a crucial part in the innate immune system [19]. In the study, the authors used treatment at a dose of 30 mg/kg daily, orally. Neutrophil quantification and activity were measured weekly, and the total radical scavenging potential in plasma was measured at the end of the experiment (21 days). It was observed that PTE significantly reduced the blood neutrophil count at 14 and 21 days, but there was no negative regulation of neutrophil oxidative burst. Also, the total radical scavenging potential in RA animals was increased, but without significant results.

The role of neutrophils in the pathogenesis of rheumatoid arthritis is still not fully explored, but it is known that they have the function of mediating inflammation and joint damage; and its activation linked to the amount of circulating MPO. In the study carried out by Odobasic et al. (2014), the authors observed that reducing the amounts of MPO would attenuate the level of worsening of arthritis such as K/BxN, as in studies with models of collagen-induced arthritis. Therefore, treatments that influence the concentration of MPO would be linked to the activity and concentration of neutrophils in the region affected, whether by invading microorganisms or autoimmune diseases [20].

The results found [17-18] oppose each other regarding the significant activity of PTE treatment in a model of RA; however, they suggest that PTE may act by different mechanisms, indicating that one of those responsible for the positive effects of PTE can be attributed to the regulation of neutrophil numbers. In relation to the present study, in which only the acute response to treatment was tested, the absence of better and more significant results can be attributed to the treatment time, and a positive response would be obtained with a longer treatment time as in Perecko's study [19]. Furthermore, due to the lipophilic character of the drug, an improvement in the solubilization and consequently in absorption could occur when ingested in a fed state.

4. CONCLUSION

It can be concluded that pterostilbene can control the inflammatory process, thus preventing exacerbation of symptoms and injuries and stimulating structural restoration by increasing fibroblast production and angiogenesis. However, there are still doubts about the doses and pharmaceutical forms that have the best effectiveness in the treatment.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee. This study was approved by the Ethics Committee On Animal Use (Comitê De Ética Em Uso Animal, Ceua) Of Midwestern State University, under the number: 007/2020.

REFERENCES

[1] Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT Bingham CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/ European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;69(9):1580-8. DOI: 10.1136/ard.2010.138461

[2] Gaujoux-Viala C, Nam J, Ramiro S, Landewé R, Buh MH, Smolen JS, Gossec L. Efficacy of conventional synthetic disease-modifying antirheumatic drugs, glucocorticoids and tofacitinib: a systematic literature review informing the 2013 update of the EULAR recommendations for management of rheumatoid arthritis. Ann Rheum Dis. 2014; 73(3):510-5. Doi: 10.1136/annrheumdis-2013-204588.

[3] Nam JL, Ramiro S, Gaujoux-Viala C, Takase K, Leon-Garcia M, Emery P, et al. Efficacy of biological disease-modifying antirheumatic drugs: a systematic literature review informing the 2013 update of the EULAR recommendations for the management of rheumatoid arthritis. Ann Rheum Dis. 2014;73(3):516-28. Doi: 10.1136/annrheumdis-2013-204577

[4] McInnes, IB, Schett G. Pathogenetic insights from the treatment of rheumatoid arthritis. Lancet. 2017;389(10086):2328-37. DOI: 10.1016/S0140-6736(17)31472-1.

[5] Chen Z, Bozec A, Ramming A, Schett G. Anti-inflammatory and immune-regulatory cytokines in rheumatoid arthritis. Nat Rev Rheumatol. 2019;15(1): 9–17. DOI: 10.1038/s41584-018-0109-2

[6] Christman LM.; Gu L. Efficacy and mechanisms of dietary polyphenols in mitigating rheumatoid arthritis. J Funct Foods. 2020;71:104003. DOI: 10.1016/j.jff.2020.104003

[7] Estrela JM, Ortega A, Mena S, Rodriguez ML, Asensi M. Pterostilbene: biomedical applications. Crit Rev Clin Lab Sci. 2013;50(3):65-78. DOI: 10.3109/10408363.2013.805182

[8] McCormack D, McFadden D. A review of pterostilbene antioxidant activity and disease modification. Oxid Med Cell Longev. 2013; 575482. DOI: 10.1155/2013/575482

[9] Kapetanovic IM, Muzzio M, Huang Z, Thompson TN, McCormick DL. Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats. Cancer Chemother Pharmacol. 2011;68(3):593–601. DOI: 10.1007/s00280-010-1525-4

[10] IPNI. Vaccinium vitis-idaea L. Available at: <https://www.ipni.org/n/293975-2>. Accessed: 06/06/2023.

[11] ROYAL BOTANIC GARDENS. Vaccinium vitis-idaea. Available at: <http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:293975-2>. Accessed: 06/06/2023.

[12] Juan ME, Vinardell MP, Planas JM. The Daily Oral Administration of High Doses of trans-Resveratrol to Rats for 28 Days Is Not Harmful. J Nutr. 2002;132(2):257–60. DOI: 10.1093/jn/132.2.257

[13] Magaji MG, Iniaghe LO, Abolarin M, Abdullahi OI, Magaji RA. Neurobehavioural evaluation of resveratrol in murine models of anxiety and schizophrenia. Metab Brain Dis. 2017;32(2)437–42. DOI: 10.1007/s11011-016-9927-6

[14] Tartari APS, Moreira FF, Pereira MCS, Carraro R, Cidral-Filho FJ, Salgado AI, Kerppers II. Anti-inflammatory Effect of Ozone Therapy in an Experimental Model of Rheumatoid Arthritis. Inflammation. 2020;43(3):985-993. Doi: 10.1007/s10753-020-01184-2.

[15] Kerppers II, de Lima CJ, Fernandes AB, Villaverde AB. Effect of light-emitting diode (ʎ 627 nm and 945 nm ʎ) treatment on first intention healing: Immunohistochemical analysis. Lasers Med Sci. 2015;30(1):397–401. DOI: 10.1007/s10103-014-1668-3

[16] Ivo Ilvan Kerppers. Efeitos dos LEDs 627 nm e 945 nm associados ao extrato de uvarana na recuperação de feridas cutâneas em ratos. São Paulo: Universidade Camilo Castelo Branco, 2014.

[17] Macickova T, Drabikova K, Nosal R, Bauerova K, Mihalova D, Harmatha J, Pecivova J. In vivo effect of pinosylvin and pterostilbene in the animal model of adjuvant arthritis. Neuro Endocrinol Lett. 2010;31(2):91-5.

[18] Wenzhe Yu, Xiaoqian Hu, Mingfu Wang. Pterostilbene inhibited advanced glycation end products (AGEs)-induced oxidative stress and inflammation by regulation of RAGE/MAPK/NF-ÎºB in RAW264.7 cells. Journal of Functional Foods, 2018; 40:272-279. Doi: https://doi.org/10.1016/j.jff.2017.11.003

[19] Perecko T, Drabikova K, Lojek A, Ciz M, Ponist S, Bauerova K, et al. The Effects of Pterostilbene on Neutrophil Activity in Experimental Model of Arthritis. BioMed Res Int. 2013; 2013:1–7. DOI: doi: 10.1155/2013/106041

[20] Odobasic, D., Yang, Y., Muljadi, R.C.M., O'Sullivan, K.M., Kao, W., Smith, M., Morand, E.F. and Holdsworth, S.R. (2014), Endogenous Myeloperoxidase Is a Mediator of Joint Inflammation and Damage in Experimental Arthritis. Arthritis & Rheumatology, 66: 907-917. https://doi.org/10.1002/art.38299

[21] Varella PPV, Forte WCN. Citokines: a review. Rev Bras Alergia Imunopatol. 2001;24(4)146–54.

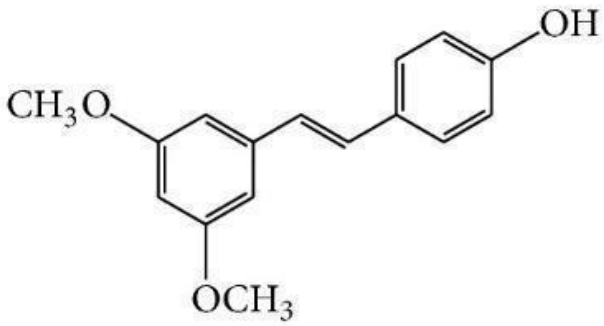
[22] Zhang J-M, AN J. Cytokines, Inflammation, and Pain. Int Anesthesiol Clin. 2007;45(2):27–37. Doi: 10.1097/AIA.0b013e318034194e

APPENDIX

SUPPLEMENTARY MATERIAL

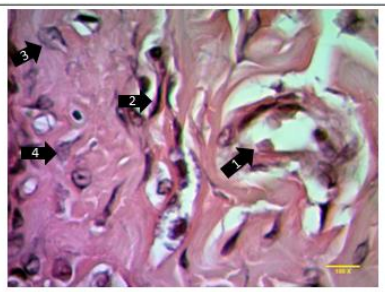
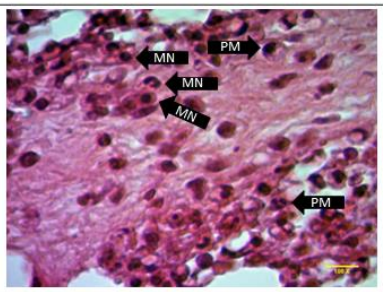
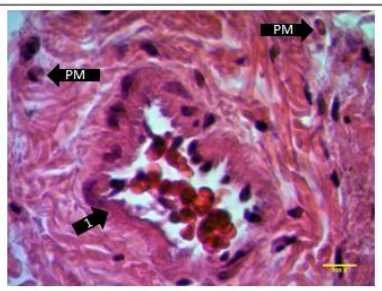
FIGURES

Figure 1. Chemical structure of Pterostilbene
(trans-3,5-dimethoxy-4'-hydroxy stilbene)



SOURCE: PERECKO, T. et al.[19]

Figure 2. Illustrative demonstration of histology studies.

ILLUSTRATIVE MATERIAL OF HISTOLOGY EVALUATION			
	CG	SHAM	PG
12 HOURS			

1 - Blood Vessel; 2 – Myofibroblast; 3 – Fibroblast; 4 – Fibrocyte; MN - Mononuclear cells; PM - Polymorphonuclear cells

SUPPLEMENTARY TABLE 1

Experimental Groups

Control Group	CG N=5	No injury No treatment Euthanasia: 12h
SHAM Groups	SHAM - 12H N = 5	With injury No treatment Euthanasia: 12h
	SHAM - 72 H N = 5	With injury No treatment Euthanasia: 72h
Groups treated with PTE	PG1 - 12h N = 5	With injury With treatment Euthanasia: 12h
	PG1 - 72 h N = 5	With injury With treatment Euthanasia 72h
	PG50 - 12h N = 5	With injury With treatment Euthanasia: 12h
	PG50 - 72h N = 5	With injury With treatment Euthanasia: 72h
	PG100 - 12h N = 5	With injury With treatment Euthanasia: 12h
	PG100 - 72h N = 5	With injury With treatment Euthanasia: 72h

SUPPLEMENTARY TABLE 2

Analyses performed in the experimental study

Flow cytometry	1st blood collection before induction of arthritis (0h)	2nd blood collection before euthanasia (12h or 72h)
Histology	Classification of epithelial changes	0: Normal epithelium 1: Mild change 2: Moderate changes 3: Intense change
	Classification of the inflammatory infiltrate	0: Absence of infiltrate 1: sparse Infiltrate 2: Moderate infiltrate 3: Intense infiltrate
	Presence of mono- and polymorphonuclear cells, neovascularization and fibroblasts	0: None 1 - 500: Mild 501 - 1000: Moderate 1001 - 1500: Intense > 1500: Severe

SUPPLEMENTARY TABLE 3

Function and relationship of the analyzed interleukins.

System	Interleukin	Produced by:	Stimulates/functions:
TH1 Pro-inflammatory	IL - 2	Th0 and Th1 cells	T-cell, B-cell, NK-cell, and macrophage growth factor
	IL - 6	Macrophages, endothelial cells, and T cells	It promotes neutrophil maturation and activation, macrophage maturation, and differentiation/maintenance of cytotoxic T lymphocytes and NK cells.
	TNF	Monocytes, macrophages, and T-lymphocytes	Stimulates the migration of neutrophils and monocytes to the site of infection and activates these cells to destroy the microorganisms
	IL - 12 (p70)	Activated macrophages and dendritic cells and B cells	Eradication of intracellular microorganisms by stimulating INF-γ production and differentiation of CD4 helper T cells into TH1 cells; Stimulates NK cells.
	IFN - γ	NK cells, TH1 CD4 cells, and CD8 T cells	Activator of macrophages to destroy the phagocytose microorganisms. Inhibits IL-10, stimulates IL-2; Inhibits proliferation of cells that synthesize IL-4, IL-5, IL-6, IL-10, IL-13
TH2 Anti-inflammatory	IL-4	T-CD4 lymphocytes, mast cells, eosinophils, and basophils	It has an action on T and B lymphocytes, NK cells, mast cells, synoviocytes, and endothelial cells. It acts on activated macrophages by reducing the effects of the cytokines IL-1, FNTα, IL-6, and IL-8 and by inhibiting the production of oxygen free radicals.
	IL-5	TH2 subgroup of CD4 T cells, by activated mast cells, and the eosinophil.	It acts on eosinophils, stimulating their growth and differentiation. It also stimulates B-cell proliferation and IgA antibody production.
	IL-10	Activated CD8+ cells. LPS-activated Th0, Th1, Th2 cells, B lymphocytes, mast cells and monocytes.	It inhibits activated macrophages to terminate the immune system response as the microorganism is destroyed. It inhibits IL-12 production and expression of major histocompatibility complex (MHC) molecules. The main effect of IL-10 is to inhibit the synthesis of other cytokines, such as IFN-g, IL-2, IL-12, and TNF-β.
	MCP-1 (monocyte chemoattractant protein) or CCL2	Macrophages, epithelial cells, and endothelial cells; Fibroblasts	Chemokines that stimulate and regulate the migration of leukocytes from the blood to the tissues. Migration of monocytes and macrophages into the tissue.

Source: Adapted from VARELLA and FORTE [21]; ZHANG et al. (2007) [22].