Research Article

Effects of D-002 and Grape Seed Extract on Monoiodo-Acetate Induced Osteoarthritis in Rats

Sarahi Mendoza*, Miriam Noa, Rosa Mas, Maikel Valle, Nilda Mendoza
Centre of Natural Products, National Centre for Scientific Research, Havana, Cuba.
***Corresponding author's E-mail:** sarahi.mendoza@cnic.edu.cu

Accepted on: 06-12-2014; **Finalized on:** 29-02-2016.

ABSTRACT

Osteoarthritis (OA), the most common age-related degenerative joint disease in adults, is characterized by a progressive cartilage loss which produces debilitating chronic pain in affecting individuals and tends to worsen over time as cartilage wears away. Non-pharmacologic interventions are the cornerstone of OA management. Nevertheless, optimal treatment should combine non-pharmacological and pharmacological modalities. Numerous studies have demonstrated that oxidative stress has been implicated in OA which support the evaluation of antioxidants for management of OA. D-002 is a mixture of six high molecular weight aliphatic alcohols purified from beeswax that has been shown to be effective in experimental models of acute and chronic inflammation and in experimental models of OA. The aim of this study was to compare effects of D-002 and Grape seed extract (GSE) in mono iodoacetate induce OA in rats. Male wistar rats were distributed into 6 groups: one negative control and five groups with MIA induce OA: a positive control group and four groups treated with D-002 (200 and 400 mg/kg) or GSE (200 and 400 mg/kg). OA was induced by MIA. Substances were administered during 10 days. Cartilage changes were measured by using histological and Mankin modified score. D-002 (200 and 400 mg/kg) and GSE (400 mg/kg) significantly prevented the increase on total histological score induced by the injection of MIA into the knee joint of the animals. Effects of D-002 were significantly higher than GSE ones in the reduction of total histological score. D-002 (200 and 400 mg/kg) significantly reduce histological parameters of the score except cellular abnormalities and osteoclasts presences. On its side, GSE was no effective on extension to damage, cellular abnormalities, bone loss and panus formation and osteoclast presence. D-002 was more effective to GSE in preventing cartilage damage in MIA- induced OA model in rats.

Keywords: Osteoarthritis, D-002, mono iodoacetate, rats.

INTRODUCTION

Osteoarthritis (OA) is the most common age-related degenerative joint disease in adults, involving not only the articular cartilage, but the whole joint tissues such as subchondral bone, ligaments, muscles, menisci, synovium, capsule and joint fluid.[1-3] OA is characterized by a progressive cartilage loss which produces debilitating chronic pain in affecting individuals and tends to worsen over time as cartilage wears away.[4]

Non-pharmacologic interventions are the cornerstone of OA management.[5] Nevertheless, optimal treatment should combine non-pharmacological and pharmacological modalities.[6,7] Since disease modifying anti-OA drugs (DMOADs) are still lacking, treatment targets have mainly focused on reducing pain.[4] In such regard, current guidelines recommend the use of analgesics, such as acetaminophen (paracetamol) as the first line of therapy.[6,7]

However, some evidences support that inflammation may be implicated in the development and progression of OA. Moreover, proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-are involved in disturbed homeostasis in OA cartilage.[4]

Then, non-steroidal anti-inflammatory drugs (NSAID) are the second line of therapy to provide symptom relief, despite they do not solve the underlying causal pathological process.[6,7]

Despite their leading place to treat pain and inflammation, traditional NSAIDs (t-NSAID) (non selective inhibitors of cyclooxygenase –COX- 1 and 2) and second-generation NSAIDs (COX2- inhibitors) use is relatively hampered by their adverse side effects, so that the t-NSAIDs-elicited inhibition of COX-1 in gastrointestinal cells leads to an increased risk of serious gastrointestinal adverse events, while the specific inhibition of COX-2 in vascular cells contributes to develop atherothrombosis and hypertension.[9,10]

To help minimize these risks, public health advisory have recommend that NSAIDs should be given at the lowest effective dose for the shortest duration.[11]

On its side, acetaminophen, although safe at therapeutic doses, may cause liver toxicity and increased risk of haematological malignancies, which limit its chronic use.[12,13]

This background supports the search of effective and safer treatments for OA. In this sense, numerous studies have demonstrated that oxidative stress has been implicated in OA.[8]

In conjunction with metalloproteinases, reactive oxygen species (ROS) can work to degrade matrix components. ROS directly oxidizes transcriptional factors, intracellular

and extracellular components resulting in cell death and matrix components breakdown.[4] These facts support the evaluation of potential benefits of the use of antioxidants for management of OA.

Grape Seed Extract (GSE) contains lipids, proteins, carbohydrates and polyphenols.

Proanthocyanidins are potent natural antioxidants and are the most abundant phenolic compound in GSE, being polymers of high molecular weight compound of dimers or trimers of catechins and epicatechins.[14] There evidences supporting the efficacy of GSE in animal models of diseases related to oxidation disorders such as models of tumors, atherosclerosis, gastric ulcers, cataracts, diabetic retinopathy and rheumatoid arthritis.[15] Similarly, Woo and cols (2011) demonstrated that GSE is antinociceptive and it is protective against joint damage in the monosodium iodoacetate (MIA)-induced OA model in rats.[16]

D-002 is a mixture of six high molecular weight aliphatic alcohols (C_{24}, C_{26}, C_{28}, C_{30}, C_{32}, C_{34}) purified from beeswax[17] that inhibits both COX and 5-LOX activities.[18,19] Consequently, D-002 has been shown to be effective in experimental models of acute and chronic inflammation[20-23] and in experimental models of OA, wherein it has shown to protect against cartilage degeneration as well.[24,25] Also, oral administration of D-002 (50 mg/day) for short-term (6-8 weeks) has demonstrated to reduce OA symptoms.[26,27] On the other hand, D-002 has antioxidant effects characterized for a reduction of MDA In light of these issues this study was undertaken to compare effects of D-002 and GSE on MIA induced OA in rats.

MATERIALS AND METHODS

Substances and chemicals

D-002 (Plant of Natural Products, Havana, Cuba) and GSE (Blackmore, Australia) were prepared as suspensions at the required concentrations in a Tween 20 / H_2O vehicle (2%). Adjusts according doses (200 and 400 mg/kg) and body weight of the animals were done. Animals were administered daily for 10 days by oral route at 1 mL per 100g of body weight.

Monoiodoacetato (Sigma) was used for induction of the damage. The substance was prepared dissolved in physiological saline solution 40 mg/mL.

Animals

Male Wistar rats weighing 240~280 g were purchased from Centre for Laboratory Animal Production (CENPALAB, Havana, Cuba). Before the experiment, rats were housed three per cage in a room with controlled temperature (20-25°C), humidity (60 ± 10%) and lighting (12-h light/12-h dark cycle) for 7 days. Food and water were supplied *ad libitum*. The study was conducted according to the Cuban Guidelines for the Care of Laboratory Animals (Regulatory Board for the Public Health Protection, 2004). An institutional committee for the use and care of laboratory animals (Natural Products Research Centre, Havana, Cuba) approved the use of rats and the study protocol.

Treatment and experimental design

Rats were distributed into six groups of 10 animals each one: one negative control group without damage and five groups with MIA-induced OA which were one positive control of vehicle and four groups treated with D-002 (200 and 400 mg/kg) and with GSE (200 and 400 mg/kg).

Doses selected of D-002 have resulted effective in experimental models of OA.[24,25] GSE doses used are in the range of effective doses in this model.[16]

Induction of OA: After anesthetization with isoflurane, 50 μL of MIA solution (2 mg) were injected into the intra-articular space of the left knee of the animals using a 26.5-G needle inserted through the knee patellar ligament.

Treatments (vehicle, D-002, GSE) were given by gastric gavage (1 mL/100 g bodyweight) once daily (9 to 10 am) for 10 days, starting immediately after MIA injection.

At treatment completion rats of all the groups were sacrificed at the same time point in ether atmosphere.

Histopathological study

The left knee joint was removed and preserved in formalin for 24 hours. Then samples were decalcified in 0.5 mol/L disodium EDTA (pH 7.4) dissolution at 4 °C for 4 weeks. After decalcification, the joint was sectioned in the longitudinal plane for 2 halves, and later included in paraffin, cut and stained with hematoxylin/eosin and toluidine blue to analyze the cartilage.[28] For each specimen serial sections were collected from two levels at 5 μm paraffin sections. The most central section of the medial femoro-tibial joint (with the least amount of meniscus) was selected for analysis as the major weight-bearing region where degradative change was most profound as Strassle.[29]

The depth and extent of cartilage damage was assessed by using a modified Mankin score.[29,30] The depth was scored from 0 to 5 (0 = normal, 1 = minimal impairment, affecting the superficial zone only, 2 = mild invasion into the upper middle zone only, 3 = moderate invasion into the middle zone, 4 = marked invasion into the deep zone but not to the tidemark, and 5 = severe full-thickness degradation to the tidemark). The degree of tibial plateau involvement was scored as 1 (minimal), 2 (moderate), or 3 (severe).

Cartilage structural changes were scored from 0 to 6 (0 = normal, 1 = irregular surface, including fissures into the radial layer, 2 = pannus, 3 =absence of superficial cartilage layers, 4 = slight disorganization evidenced by cellular row absent, some small superficial clusters), 5 = fissure into the calcified cartilage layer, and 6 =

disorganization, as per chaotic structure, clusters and osteoclasts activity).[29,30]

Cellular abnormalities in the cartilage were scored from 0 to 3 (0 =normal, 1 = hypercellularity, including small superficial clusters, 2 = clusters, and 3 = hypocellularity); and matrix staining from 0 to 4 (0 = normal/slight reduction of staining, 1 = staining reduced in the radial layer, 2 =staining reduced in the interterritorial matrix, 3 = staining present only in the pericellular matrix, and 4 = staining absent).[29,30]

Inflammation was scored from 0 to 4, based on the degree of cellular tissue infiltration, 0 being scored when infiltrates were not seen, and 1, 2, 3 or 4 scores corresponded to minimal, mild, moderate or marked inflammatory cell infiltrations, respectively. Pannus formation in the joint tissues and synovial lining cell hyperplasia were scored from 0 to 4 (0 = normal, 1 = minimal loss of cortical bone at a few sites, 2 = mild loss of cortical trabecular bone, 3 = moderate loss of bone at many sites, and 4 =marked loss of bone at many sites, with fragmenting and full-thickness penetration of the inflammatory process or the pannus formation into the cortical bone).[29,30]

Osteoclasts presence was scored from 0 to 4, 0 = normal condition (essentially no osteoclasts), 1 = few osteoclasts (lining <5% of most affected bone surfaces), 2 = some osteoclasts (lining 5–25% of most affected bone surfaces), 3 = many osteoclasts (lining 26–50% of most affected bone surfaces), and 4 = myriad osteoclasts (lining >50% of most affected bone surfaces). The mean of the scores of all histological parameters was calculated, and this value was designated as the histology score.

Statistical Analysis

Results were evaluated using the Mann Whitney test for between group comparisons. The level of statistical significance was chosen at $\alpha = 0.05$. Data were processed with the Statistic software package for Windows (Release 6.1, StatSoft Inc, Tulsa, OK, USA).

Histological score was performed by two researchers blinded to the treatment groups. Reliability was assessed by comparing scores from all observers for all histological specimens. Intra-class correlation coefficient (ICC) was determined. The inter-observer variability for the MANKIN system showed a good ICC (0.933; $p<0.001$).

RESULTS AND DISCUSSION

This study demonstrated the protective effects of D-002 and GSE in MIA- induced OA, a model accepted for studying the etiology of this disease. MIA induces damage associated with the inhibition of chondrocytes metabolism and the reduction of proteoglycans in the joint tissue, which is evident, thought the absence of contrast after toluidine blue staining.[28] Progressive cartilage degeneration and functional disability after MIA injection are very similars to the clinical OA symptoms and signs in humans, there for, this model is suitable to assess the potential effects of any substance for preventing OA. Intraarticular injection of MIA inhibits the activity of glyceraldehyde- 3- phosphate dehydrogenase and hence the extent of glycolysis, inducing the chondrocytes death in the articular cartilage.[31]

No animals of the negative control group showed the expected damage pattern[25] while in animals of the positive control group a significant morphological damage was observed, characterized for the destruction of the articular space, extensive degeneration of cartilage with loss of chondrocytes from the femoral condyles and from the tibial plateaus, bone destruction and subchondral bone sclerosis, with partial replacement of the bone marrow by fibrotic tissue. In addition, the cartilage loss was almost complete or, in the areas where it was still present, the toluidine blue staining was very pale, indicating loss of proteoglycans. These differences confirm the validity of the study in our experimental conditions and indicate that the observed effects are attributable to the treatments.

D-002 (200 and 400 mg/kg) and GSE (200 and 400 mg/kg) significantly prevented the increase on total histological score induced by the injection of 2 mg MIA into the knee joint of the animals.

Effects of D-002 were significantly higher than GSE ones in the reduction of total histological score. In this sense D-002 (200 and 400 mg/kg) induces a reduction of 23.2 and 32.3 % respectively, different from those of GSE (200 and 400 mg/kg) with reductions of 11.3 and 21.9 %, respectively which are net differences of 10.4 and 11.9 % among treatments (Table 1). These results are consistent with previous ones obtained with D-002 in this experimental model in which D-002 (200 and 400 mg/kg) induce a total historical score reduction of 25.6 and 39.1%, respectively.[25]

The reduction on total score with D-002 400 mg/kg was higher than 200 mg/kg (32.3 vs 23.2 %, respectively). Similarly, GSE 400 mg/kg induces a reduction on total score significant higher than 200 mg/kg.

Table 2 shows effects on individual parameters of histological score. Effects on individual parameters are consistent with effect on total score. D-002 (200 and 400 mg/kg) significantly reduced the depth of damage (44% both), extent of damage (42.7-47.5%), cartilage changes (17.4-22.4%), matrix staining (26-56.6%) and extension of inflammation (24-36.1%). Bone loss was significantly reduced (29.6%) with 400 mg/kg. In particular, the reduction of matrix staining with D-002 was marked (> 50% with 400 mg/kg); indicating a protective effect of D-002 on proteoglycans destruction at the joint.

Theses results are consistent with those from previous study[24,25] and are in line with anti-inflammatory effects reported previously for D-002[20-23] and with its dual inhibitory effect on COX and 5-LOX enzymes,[18,19] keeping in mind that inflammation contributes to the joint damage[32-34] and that dual inhibition of these enzymes has

been associated with a reduction on the progression of experimental OA by suppressing the synthesis of collagenase 1 and interleukin-1ß.[35]

Moreover, since oxidative stress are closely related with cartilage damage in OA[4,8] it could be possible, that other effects of D-002, such as antioxidant effects, can also be related, at least in part, to its efficacy in this model and to its significant higher effects in relation with GSE. In such regard, D-002 orally given to rats for 2 weeks reduced the susceptibility of plasma lipoprotein to undergo lipid peroxidation (LP), lowering the levels of malondialdehyde (MDA), in rat plasma, stomach, liver and brain[36-38] and increased the activity of antioxidant endogenous enzymes.[38] In turn, D-002 (50 mg/kg) orally administered for 12 weeks has been shown to inhibit copper- induced LP of plasma lipoproteins, and plasma levels of MDA and total hydroperoxides (TOH) in healthy volunteers, middle-aged and older subjects,[39-41] and to increase plasma total antioxidant status (TAS).

GSE 400 mg/kg was effective since produces a significant reduction of depth of the damage (41.2%), cartilage changes (20%), matrix staining (26%) and extension of inflammation (24%). These results are consistent with those reported for other authors for GSE in this model.[16] Other antioxidant substances have been effective too.[4]

However, there evidence about similar antioxidant effects of D-002 and GSE (25-250 mg/kg) on plasma oxidative markers and liver MDA levels.[42] Then, the remarkable differences among D-002 and GSE, observed in this study, suggests a possible combination of antinflammatory and antioxidant effects in MIA-induced OA, but further studies are necessary to confirm such hypothesis.

D-002 and ESU unaffected the presence of osteoclasts in the joint of rats with MIA induced OA, indicating that they are no effectives for ameliorating the bone destruction associated to this model.

Table 1: Effects of D-002 on total histological score (X ± SD)

Treatments	Histological score	Inhibition (%)
Negative control	0^{+++}	
Positive Control	3.28 ± 0.25	
D-002 200 mg/kg + MIA	$2.52 \pm 0.23^{+++bb}$	23.2
D-002 400 mg/kg + MIA	$2.22 \pm 0.26^{+++ab}$	32.3
GSE 200 mg/kg + MIA	$2.91 \pm 0.11^{++}$	11.3
GSE 400 mg/kg + MIA	$2.56 \pm 0.19^{+++aa}$	21.9

+p<0.05; ++ p< 0.01; +++ p< 0.001, Comparisons vs positive control; ^{a}p<0.05; ^{aa}p< 0.01; Comparisons between 200 and 400 mg/kg; ^{b}p<0.05; ^{bb}p< 0.01; Comparisons between similar doses of D-002 and GSE; Mann Whitney U Test

Table 2: Effects of D-002 and GSE on histological parameters (X ± SD)

Treatments	Depth of damage		Extent of damage		Cartilage changes		Cellular abnormalities	
	X ± SD	%	X ± SD	%	X ± SD	%	X ± SD	%
Negative control	0^{+++}		0^{+++}		0^{+++}		0^{+++}	
Positive Control	4.25 ± 0.46		2.38 ± 0.52		5.00 ± 0.53		2.88 ± 0.35	
D-002 200 mg/kg + MIA	$2.38 \pm 0.52^{+++}$	44	$1.38 \pm 0.52^{++b}$	42	$4.13 \pm 0.64^{+}$	17.4	2.75 ± 0.46	4.5
D-002 400 mg/kg + MIA	$2.38 \pm 0.52^{+++}$	44	$1.25 \pm 0.46^{++}$	47.5	$3.88 \pm 0.64^{++}$	22.4	2.38 ± 0.74	17.4
GSE 200 mg/kg + MIA	$2.63 \pm 0.52^{+++}$	38.1	2.13 ± 0.64	10.5	4.38 ± 0.52	12.4	2.75 ± 0.46	4.5
GSE 400 mg/kg + MIA	$2.50 \pm 0.53^{+++}$	41.2	1.88 ± 0.64	21	$4.00 \pm 0.53^{++}$	20	2.50 ± 0.53	13.2
Treatments	**Matrix satining**		**Extent of inflammation**		**Bone loss and pannus formation**		**Osteoclasts presence**	
	X ± SD	%	X ± SD	%	X ± SD	%	X ± SD	%
Negative control	0^{+++}		0^{+++}		0^{+++}		0^{+++}	
Positive Control	2.88 ± 0.38		3.13 ± 0.64		3.38 ± 0.52		2.38 ± 0.52	
D-002 200 mg/kg + MIA	$2.13 \pm 0.35^{+}$	26	$2.38 \pm 0.52^{+bb}$	24	2.63 ± 0.74	22.2	2.38 ± 0.52	0
D-002 400 mg/kg + MIA	$1.25 \pm 0.71^{+++ab}$	56.6	$2.00 \pm 0.53^{++}$	36.1	$2.38 \pm 0.52^{++}$	29.6	2.25 ± 0.46	5.5
GSE 200 mg/kg + MIA	2.38 ± 0.52	17.4	3.50 ± 0.53	11.8	3.13 ± 0.64	7.4	2.38 ± 0.52	0
GSE 400 mg/kg + MIA	2.13 ± 0.64+	26	$2.38 \pm 0.52^{+aa}$	24	2.75 ± 0.71	18.6	2.38 ± 0.52	0

+p<0.05; ++ p< 0.01; +++ p< 0.001, Comparisons vs positive control; ^{a}p<0.05; $^{a\,a}$p< 0.01; Comparisons between 200 and 400 mg/kg. ^{b}p<0.05; ^{bb}p< 0.01; Comparisons between similar doses of D-002 and GSE; Mann Whitney U Test

CONCLUSION

D-002 was more effective to GSE in preventing cartilage damage in MIA-induced OA model in rats.

REFERENCES

1. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, Bridgett L, Williams S, Guillemin F, Hill CL, Laslett LL, Jones G, Cicuttini F, Osborne R, Vos T, Buchbinder R, Woolf A, March L, The global burden of hip and knee osteoarthritis: estimates from the Global Burden of Disease 2010 study, Ann Rheum Dis, 2014, [Epub ahead of print].
2. Litwic A, Edwards MH, Dennison EM, Cooper C, Epidemiology and burden of osteoarthritis, Br Med Bull, 105, 2013, 185-199.
3. Aigner J, Cook JL, Gerwin N, Glasson SS, Laverty S, Little C, McIlwraith W, Kraus VB, Histopathology atlas of animal model systems – overview of guiding principles, Osteoarthritis and Cartilage, 8 suppl 3, 2010, S2-S6.
4. Moon SJ, Jeong JH, Jhun JY, Yang EJ, Min JK, Choi JY, Cho ML, Ursodeoxycholic Acid ameliorates pain severity and cartilage degeneration in monosodium iodoacetate-induced osteoarthritis in rats, Immune Netw, 14(1), 2014, 45-53.
5. Lee YC, Shmerling RH, The benefit of non-pharmacologic therapy to treat symptomatic osteoarthritis, Curr Rheumatol Rep, 10, 2008, 5-10.
6. Nelson AE, Allen KD, Golightly YM, Goode AP, Jordan JM, A systematic review of recommendations and guidelines for the management of osteoarthritis: The Chronic Osteoarthritis Management Initiative of the U.S, Bone and Joint Initiative, Semin Arthritis Rheum, 2013, Epub ahead of print.
7. McAlindon TE, Bannuru RR, Sullivan MC, OARSI guidelines for the non-surgical management of knee osteoarthritis, Osteoarthritis Cartilage, 22, 2014, 363-88.
8. cott JL, Gabrielides C, Davidson RK, Swingler TE, Clark IM, Wallis GA, Boot-Handford RP, Kirkwood TB, Taylor RW, Young DA, Superoxide dismutase downregulation in osteoarthritis progression and end-stage disease, Ann Rheum Dis, 69, 2010, 1502–1510.
9. Wallace JL, Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? Physiol Rev, 88, 2008, 1547–1565.
10. Fine M, Quantifying the impact of NSAID-associated adverse events, Am J Manag Care, 19(14 Suppl), 2013, s267-272.
11. Fine M, Quantifying the impact of NSAID-associated adverse events, Am J Manag Care, 19(14 Suppl), 2013, s267-272.
12. Graham GG, Davies MJ, Day RO, The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings, Inflammopharmacology, 21, 2013, 201-232.
13. Walter RB, Brasky TM, White E, Cancer risk associated with long-term use of acetaminophen in the prospective VITamins and lifestyle (VITAL) study, Cancer Epidemiol Biomarkers Prev, 20, 2011, 2637-2641.
14. Jia Z, Song Z, Zhao Y, Wang X, Liu P, Grape seed proanthocyanidin extract protects human lens epithelial cells from oxidative stress via reducing NF-κB and MAPK protein expression, Mol Vis, 20(17), 2011, 210-7.
15. Ariga T, The antioxidative function, preventive action on disease and utilization of proanthocyanidins, Biofactors, 21(1-4), 2004, 197-201.
16. Woo YJ1, Joo YB, Jung YO, Ju JH, Cho ML, Oh HJ, Jhun JY, Park MK, Park JS, Kang CM, Sung MS, Park SH, Kim HY, Min JK, Grape seed proanthocyanidin extract ameliorates monosodium iodoacetate-induced osteoarthritis, Exp Mol Med, 43(10), 2011, 561-70.
17. Mas R, D-002: A product obtained from beeswax, Drugs of the Future, 26, 2001, 731-744.
18. Perez Y, Oyarzabal A, Ravelo Y, Mas R, Jiménez S, Molina V. Molina V, Effect of D-002 on 5-lypooxygenase activity *in vitro*, Revista Cubana Farmacia, 46, 2012, 259–266.
19. Pérez Y, Oyarzábal A, Ravelo Y, Mas R, Jiménez S, Molina V, Inhibition of ciclooxygenase and 5-lipooxygenase enzymes by D-002 (beeswax alcohols), Top Current Nutraceutical Res, 2014 (in press).
20. Carbajal D, Molina V, Valdés S, Arruzazabala ML, Más R, Magraner J, Anti-inflammatory activity of D-002: an active product isolated from beeswax, Prostagl Leukotr Essent Fatty Acids, 59, 1998, 235-238.
21. Carbajal D, Ravelo Y, Molina V, Mas R, Arruzazabala ML. Effect of D-002 on models of acute inflammation. IJPSRR, 21(2), 2013, 62-67.
22. Ravelo Y, Molina V, Carbajal D, Fernández L, Fernández JC, Arruzazabala ML, Más R, Evaluation of antiinflammatory and antinociceptive effects of D-002 (beeswax alcohols), J Nat Med, 65, 2010, 330-335.
23. Molina V, Ravelo Y, Carbajal D, Mas R, Arruzazabala ML, Anti-inflammatory and gastric effects of D-002, aspirin and naproxen and their combined therapy in rats with cotton pellet-induced granuloma, Lat J Pharm, 30, 2011, 1709-1713.
24. Mendoza S, Noa M, Valle M, Mendoza N, Mas R, Effects of D-002 on formaldehyde-induced osteoarthritis in rats, IOSR PHR, 3, 2013, 9-12.
25. Mendoza S, Noa M, Valle M, Mendoza N, Mas R, Ameliorating effects of D-002, a mixture of beeswax alcohols, on monosodium iodoacetate-induced osteoarthritis in rats, Int J Pharm Sci Rev Res, 19, 2013, 10-15.
26. Rodríguez I, Mendoza S, Illnait J, Mas R, Fernández JC, Fernández L, Mesa M, Gámez R. Effects of D-002, a mixture of beeswax alcohols, on osteoarthritis symptoms: a randomized placebo-controlled study. IOSR Journal of Pharmacy, 2(6), 2012, 1-9.
27. Puente R, Illnait J, Mas R, Mas R, Carbajal D, Mendoza S, Fernández JC, Mesa M, Gámez R, Reyes P, Evaluation of the effect of D-002, a mixture of beeswax alcohols, on arthritic symptoms, KJIM, 29, 2014, 191–202.

28. Bar-Yehuda S, Rath-Wolfson L, Del Valle L, Ochaion A, Cohen S, Patoka R, Induction of an antiinflammatory effect and prevention of cartilage damage in rat knee osteoarthritis by CF101 treatment, Arthritis & Rheumatism, 60, 2009, 3061–3071.

29. Strassle BW, Mark L, Leventhal L, Piesla MJ, Jian Li X, Kennedy JD, Glasson SS, Whiteside GT, Inhibition of osteoclasts prevents cartilage loss and pain in a rat model of degenerative joint disease, Osteoarthritis and Cartilage, 18, 2010, 1319–1328.

30. Mankin HJ, Dorfman H, Lippiello L, Zarins A, Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data, J Bone Joint Surg Am, 53, 1971, 523-537.

31. Cournil C, Liagre B, Grosin L, Vol C, Abid A, Overexpression and induction of heat shock protein (Hsp) 70 protects *in vitro* and *in vivo* from mono-iodoacetate (MIA)-induced chondrocytes death, Arthritis Res, 3 (Suppl 1), 2001, 41.

32. Martel-Pelletier, Pathophysiology of osteoarthritis, Osteoarthritis cartilage, 12 (Suppl A), 2004, S31- S33.

33. Goldring MB, Goldring SR, Osteoarthritis, J Cell Physiol, 213, 2007, 626-634.

34. Johanne J, Pelletier JP, Is osteoarthritis a disease involving only cartilage or other articular tissues? Eklem Hastahk Cerrahisi, 21, 2010, 2-14.

35. Jovanovic DV, Fernandes JC, Martel-Pelletier J, Jolicoeur FC, Reboul P, Laufer S, *in vivo* dual inhibition of cyclooxygenase and lipoxygenase by ML-3000 reduces the progression of experimental osteoarthritis: suppression of collagenase 1 and interleukin- 1 beta synthesis, Arthritis Rheum, 44, 2001, 2320-2330.

36. Menéndez R, Amor AM, González RM, Jiménez S, Más R, Inhibition of rat microsomal lipid peroxidation by the oral administration of D-002, Brazil J Med Biol Res, 33, 2000, 85-90.

37. Mendoza S, Noa M, Pérez Y, Mas R, Preventive effect of D-002, a mixture of long-chain alcohols from beeswax, on the liver damage induced with CCl4 in rats, J Med Food, 10(2), 2007, 379-83.

38. Pérez Y, Gonzalez RM, Amor AM, Jiménez S, Menéndez R, D-002 on antioxidant enzymes in liver and brain of rats, Rev CENIC Cien Biol, 33(1), 2002, 3-5.

39. Menéndez R, Mas R, Amor AM, Perez Y, González RM, Fernández JC, Jiménez S, Antioxidant effect of D-002 on the *in vitro* susceptibility of whole plasma in healthy volunteers, Arch. Med Res, 32, 2001, 436-441.

40. Menéndez R, Mas R, Illnait J, Pérez J, Amor AM, Fernández JC, González RM, Effects of D-002 on lipid peroxidation in older subjects, J Med Food, 4(2), 2001, 71-77.

41. López E, Illnait J, Molina V, Oyarzábal A, Fernández L, Pérez Y, Mas R, Mesa M, Fernández JC, Mendoza S, Gámez R, Jiménez S, Ruiz D, Effects of D-002 (beeswax alcohols) on lipid peroxidation in middle-aged and older subjects, LAJP, 27, 2008, 695-703.

42. Oyarzábal A, Molina V, Jiménez S, Curveco D, Mas R, Efectos del policosanol, el extracto de semillas de uva y su terapia combinada sobre marcadores oxidativos en ratas, Redvet, 13(4), 2012, 1-13.

Source of Support: Nil, **Conflict of Interest:** None.