

1 Anti-inflammatory properties of natural ingredients used in combinations on adjuvant induced
2 arthritis in rats

3 Amélie St-Pierre^a, Véronique Desrosiers^a, France Dufresne^a and Pierre U. Blier^{a*}

4

5 ^a Université du Québec à Rimouski, Département de biologie, Rimouski, Québec, G5L3A1

6 *Corresponding author: Pierre_Blier@uqar.ca

7

8 Abstract

9 Background: Rheumatoid arthritis has seen a significant increase in both incidence and prevalence
10 and its treatments show limited efficiency due to their undesirable effects on patient health.
11 Therefore, major interests lie in the development of treatments with drugs derived from plants or
12 other natural sources with little adverse effects as an alternative to current treatments.

13 Hypothesis/Purpose: The present study evaluates the therapeutic effects of glucosamine against
14 rheumatoid arthritis in combination with hyaluronic acid, resin extract of *Boswellia serrata* or a
15 bark extract of *Salix alba* on an animal model. We suggest that combinations with plants could
16 improve the attenuation of arthritis symptoms and articular inflammation.

17 Study design: We used Freund's complete adjuvant on rats as models of rheumatoid arthritis.
18 Individuals were separated into eight experimental groups: a control group without arthritis, one
19 with arthritis and without treatment, and six other groups receiving a daily therapeutic treatment
20 from days 14 to 29.

21 Methods: Hind-paw thickness and arthritis scores were measured at days 0, 3, 6 and 9 post-
22 induction, and then every day from days 12 to 29 with a digital caliper and a score system
23 respectively. At the end of the treatment, the mRNA content of three pro-inflammatory cytokines
24 from cartilage was measured using real-time PCR. The total antioxidant activity was evaluated with
25 an Antioxidant Assay Kit.

26 Results: Treatments with *Boswellia serrata* and *Salix alba* (*Glu+Hyal A+Bosw*, *Glu+Bosw+Sal*,
27 *Glu+Bosw* and *Glu+Hyal A+Sal*) saw significant reductions in hind-paw thickness and arthritis
28 scores at the end of the experiment when compared to the untreated group. Expression of pro-
29 inflammatory gene *IL 17A* was also reduced, but only the *Glu+Hyal A+Sal* combination
30 significantly decreased the expression of *IL-1 β* and *TNF- α* . The total antioxidant activity in blood
31 plasma significantly increased in groups treated with plant extracts.

32 Conclusion: The addition of *Boswellia serrata* and/or *Salix alba* attenuates clinical signs of
33 rheumatoid arthritis in Freund's complete adjuvant-induced arthritis in rats likely due to both their
34 anti-inflammatory and antioxidant properties.

35

36 Keywords: Rheumatoid arthritis, *Boswellia serrata*, *Salix alba*, Freund adjuvant, cytokines,
37 antioxidants

38

39 Abbreviations: BS, *Boswellia serrata*; SA, *Salix alba*; GS, Glucosamine sulfate; HA, Hyaluronic
40 acid; RA, Rheumatoid arthritis; NSAID, nonsteroidal anti-inflammatory drugs; ROS, Reactive
41 oxygen specie; SCW, Streptococcal cell wall; CIA, Collagen-induced arthritis; CFA, Complete
42 Freund adjuvant.

43 Introduction

44 Rheumatoid arthritis is an inflammatory autoimmune disorder. Its incidence and prevalence
45 increase considerably all over the world. In North America and North-European countries, its
46 incidence varies between 20 and 50 per 100,000 population (Fazal et al., 2018). It causes
47 inflammation in joints that leads to pain, stiffness, swelling and cartilage damage (Arthritis Society,
48 2018). Current treatments do not usually regenerate damaged cartilage or slow the degeneration,
49 but relieve symptoms instead (Arthritis Society, 2018). Treatments using steroids, nonsteroidal
50 anti-inflammatory drugs (NSAIDs), topical anti-inflammatories, biological agents (TNF- α and IL-
51 1 antagonists), acetaminophen and injection of corticosteroids and hyaluronic acid are used against
52 joint diseases but show limited efficiency due to their undesirable adverse effects on patient health
53 (Fan et al., 2005; Zheng et al., 2014). These pharmaceutical drugs can provoke gastrointestinal
54 disturbances (ulcers and perforations), cardiovascular complications, reproductive toxicity, loss of
55 bone mass, and topical applications can be of no benefit when the target joints are too deep (Fan et
56 al., 2005; Umar et al., 2014; Zheng et al., 2014). Due to these limitations, there is an important
57 incentive for the development of biomolecules derived from plants or natural sources without
58 adverse effects as an alternative to NSAIDs and other treatments. Among these biomolecules,
59 glucosamine sulfate (GS), hyaluronic acid (HA), resin extracts of indian frankincense (*Boswellia*
60 *serrata* Roxb. Ex Colebr., BS) and bark extracts of white willow (*Salix alba* L., SA) are four natural
61 ingredients that are individually considered as efficient against arthritis by regulatory agencies (for
62 example see the monograph of Natural and Non-prescription Health Products Directorate
63 (NNHPD) in Canada (Health Canada, 2018)).

64 GS is an important component of cartilage and is naturally synthesized in the body. This amino
65 monosaccharide stimulates the biosynthesis of glycosaminoglycan chains, giving the cartilage its
66 strength, flexibility and elasticity, all the while possessing anti-inflammatory properties (Singh et
67 al., 2007). HA is a large viscoelastic glycosaminoglycan present in the synovial fluid, and is
68 responsible for its viscoelastic properties (Moreland, 2003). It also confers good protective
69 properties including shock absorption, protective coating of the articular cartilage surface, and
70 lubrication. BS and SA have both anti-inflammatory and analgesic properties due to the presence
71 of boswellic acid and salicin respectively (Kimmatkar et al., 2003; Shara and Stohs, 2015).
72 Boswellic acid reduces pain and swelling, has antioxidant and free radical-scavenging properties,
73 and appears as a potential new treatment of inflammatory disorders like rheumatoid arthritis and
74 osteoarthritis (Umar et al., 2014). It reduces glycosaminoglycan degradation, keeping the cartilage
75 in good condition unlike NSAIDs that can induce the disruption of the glycosaminoglycan
76 synthesis, accelerating articular damage (Kimmatkar et al., 2003). Salicin has anti-inflammatory
77 and anabolic effects, as shown in canine joints (Shara and Stohs, 2015). Benefits of these natural
78 ingredients have so far only been studied separately, and their potential synergistic effects need to
79 be assessed, as a combination of ingredients can improve their therapeutic effects at the low doses
80 recommended by the health regulation agencies.

81 Thus, the aim of this study is to examine the potency of different combinations of natural
82 ingredients to limit arthritis symptoms and articular inflammation on an animal model of
83 rheumatoid arthritis. In this context, we used rats previously injected with Freund's complete
84 adjuvant. Therefore, quantifying the modulation of inflammation might represent the extent to
85 which hyaluronic acid, *Boswellia serrata* or *Salix alba* extract combined to glucosamine can
86 improve the therapeutic efficiency of glucosamine alone.

87

88

89 Materials and methods

90 Animals

91 Adult female Lewis rats (10 weeks old) were obtained from Charles River Laboratories (Montreal,
92 QC, Canada). Animals were kept at Université du Québec à Rimouski (UQAR) in controlled
93 experimental conditions ($23 \pm 1^\circ\text{C}$, relative humidity 40-60%, 12h light/dark cycles, water and
94 LabDiet 5002 *ad libitum*). They were acclimated during 1 week before the experiment. Animal
95 manipulation was conducted in accordance with the Institutional Animal Care Committee of
96 Université du Québec à Rimouski (protocol #CPA-66-16-178).

97 Adjuvant Induction

98 Arthritis was induced by subcutaneous injection of 60 μl of Freund's adjuvant, a solution of
99 *Mycobacterium tuberculosis* inactivated by heat (Chondrex, Inc. Redmond, WA, USA, 10 mg/ml),
100 at the base of the tail. First symptoms of arthritis appeared 12 days after induction.

101 Evaluation of Clinical Signs of Arthritis

102 Arthritis symptoms were examined at days 0, 3, 6 and 9 post-induction, and then every day from
103 days 12 to 29. Hind-paw thickness was measured with a digital caliper. Arthritis scores were
104 determined by a score system: for each of hind paw, a scale of 0-4; 0, no macroscopic sign; 1,
105 irritation (swelling and redness) at one joint; 2, irritation at more than one joint and/or ankles; 3,
106 irritation at many joints and moderate swelling at the ankle; 4, irritation at many joints and severe
107 swelling at the ankle. For each forepaw, a scale of 0-3 was used; 0, no macroscopic sign; 1, irritation
108 at one joint; 2, irritation at many joints and/or wrist; 3, irritation at all joints and moderate to severe
109 swelling at the wrist. The final score was calculated by adding the individual score of each paw for
110 a maximal result of 14 (Aghazadeh-Habashi et al., 2014).

111 Therapeutic Ingredient Administration

112 Rats were separated randomly in eight different groups: a control group without arthritis (control),
113 another one with arthritis and no treatment (CFA, Freund's complete adjuvant) and six other groups
114 receiving a daily therapeutic treatment from days 14 to 29. The temporal experimentation plan of
115 animal manipulations is presented in Fig 1. Six therapeutic treatments were administered to the
116 rats: GS (*Glu*); GS and HA (*Glu+Hyal A*); GS, HA and BS (*Glu+Hyal A+Bosw*); GS, HA and SA
117 (*Glu+Hyal A+Sal*); GS and BS (*Glu+Bosw*); and GS, BS and SA (*Glu+Bosw+Sal*).

118 Each ingredient was administered individually, even when the therapeutic treatment had many
119 compounds, and orally (back of the mouth) with a pipette. For each ingredient, the daily dose
120 corresponded to the maximal recommended dosage for humans by Natural and Non-prescription
121 Health Products Directorate (NNHPD) of Health Canada (2014) in its monograph titled «Multiple
122 ingredient joint health products». The dosages for rats were calculated considering an average
123 human weight of 60 kg (weight approved by Food and Drugs Administration (FDA) for safety
124 studies) and a normalization that takes into account the body surface (Reagan-Shaw et al., 2007).
125 The daily dosages are presented in Table 1. This formula represents the allometric conversion:

126 Animal equivalent dose (mg/kg) = human equivalent dose (mg/kg) / [Factor k_m rat/Factor k_m
127 human]

128 where Factor k_m = body mass (kg) / total body surface (m^2)

129 and k_m rat = 6

130 k_m human = 37

131 Solutions of therapeutic ingredients were made daily as follows: GS (Novel Ingredients, NJ, USA),
132 200 mg/mL of water; HA from bacterial fermentation (A&A Pharmachem Inc, Ontario, Canada),
133 10 mg/mL of water; BS (40% of boswellic acid) (Dolcas Biotech, NJ, USA), 200 mg/mL of organic
134 canola oil; SA (25% of salicin) (Novel Ingredients, NJ, USA), 50 mg/ml of water. Due to the
135 specificity of BS's solvent, all rats in groups not receiving treatment with BS received an equivalent
136 daily volume of canola oil (100 μ L). At the end of the experiment, all rats were euthanized by
137 injection of a lethal dose of pentobarbital. A blood sample and knee cartilage of the two hind paws
138 were collected. Plasma was extracted, samples were rapidly frozen by liquid nitrogen and preserved
139 at -80 °C for future assays.

140 Expression of Pro-Inflammatory Genes and Cartilage Degradation

141 Cartilage samples were reduced to powder with liquid nitrogen. RNA was extracted with *Pure*
142 *LinkRNA Mini Kit* (Life Technologies, CA, USA; cat# MAN0000406, protocol with Trizol and
143 DNase). Extraction purity was validated using spectrophotometry (absorbance ratio 260 / 280 nm).
144 Inverse transcription was carried out on 400 ng of RNA for each extract according to *high capacity*
145 *cDNA reverse transcription kit* method (Applied Biosystems, CA, USA; cat# 4368814). Obtained
146 complementary DNA was used for real-time polymerase chain reaction essays (rt-PCR). Real-time
147 PCR was performed with *SensiFAST SYBR No-ROX* kit from Bioline and with a LightCycler 480
148 from Roche (Mississauga, Canada). Three cytokines responsible for pro-inflammatory processes
149 were targeted: interleukin-1 (IL-1 β), interleukin-17 (IL-17A) and tumor necrosis factor (TNF- α).
150 Primer sequences used for amplification are shown in Table 2. and were purchased from Sigma
151 Aldrich (Oakville, Ontario, Canada). Gene expression was quantified by Cycle Treshold method
152 (Ct). Amplification standard curve of each gene was performed and the specific amplification
153 efficiency was verified with a minimal threshold of 1,8 (maximum 2). In order to standardize and
154 compare the different assays, a pool of cDNAs of all groups was used as an internal calibrator.
155 Gene quantification values were expressed relative to the gene quantification of two endogenous
156 references, β -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ratio expressions
157 for both references were similar; only results with the expression of β -actin are shown in this study.
158 The specificity of PCR products was confirmed by migration on electrophoresis gel and melting
159 curve analysis.

160 Total Antioxidant Activity

161 Total antioxidant activity of plasma was measured with the Antioxidant Assay Kit from Cayman
162 Chemical (Ann Arbor, MI, USA; cat# 709001). Values were expressed in equivalent values of
163 Trolox.

164 Statistical Analysis

165 The results are shown as mean \pm standard errors. They were analyzed using JMP Pro (SAS, Cary,
166 NC, USA). One-way ANOVAs followed by a Tukey's test were used to determine if there were
167 differences between groups. The homogeneity of variance and the normality of data were tested
168 using a Shapiro-Wilk and Bartlett's test respectively. Two groups were statistically different if the
169 p-value is lower than 0,05

170

171 Results

172 Effect on Clinical Signs of Arthritis

173 Measurement of hind-paw thickness and evaluation of the arthritis scores quantified the
174 development of clinical symptoms in rats during the experiment. First signs of arthritis appeared
175 on day 12 after the injection of Freund's adjuvant (Fig. 2). On day 19, hind-paw thickness of the
176 CFA group increased significantly (6.19 ± 0.90 mm) compared to the control group (3.59 ± 0.17
177 mm). Treatments with combinations of three ingredients (*Glu+Hyal A+Bosw*; *Glu+Hyal A+Sal*;
178 *Glu+Bosw+Sal*) and *Glu+Bosw* limited articular swelling and significantly reduced hind-paw
179 thickness during the treatment. At the end of the experiment (day 29), hind-paw thickness of these
180 groups was significantly inferior to the CFA group (Fig. 2A) (*Glu+Hyal A+Sal*: 4.50 ± 0.78 mm;
181 *Glu+Hyal A+Bosw*: 4.35 ± 0.65 mm; *Glu+Bosw*: 3.85 ± 0.63 mm; *Glu+Bosw+Sal*: 3.84 ± 0.48
182 mm; CFA: 5.84 ± 0.97 mm, $p < 0.05$). Severity of arthritis was evaluated by visual inspection
183 through a score system which reflects the number of affected joints and the swelling intensity in
184 digits and wrists/ankles. Arthritis scores increased from days 12 to 18 post-induction. At day 18,
185 the maximal score was reached for the CFA group (Fig. 2B). A significant decrease of arthritis
186 scores was observed for *Glu+Hyal A+Bosw*, *Glu+Bosw+Sal*, *Glu+Bosw* and *Glu+Hyal A+Sal*
187 treatments in comparison to the CFA group at days 23, 25, 26 and 27 respectively. At day 29,
188 arthritis scores of these groups were significantly inferior to the CFA group (*Glu+Hyal A+Bosw*:
189 5.33 ± 3.61 ; *Glu+Bosw+Sal*: 5.17 ± 4.07 ; *Glu+Bosw*: 5.17 ± 3.06 ; *Glu+Hyal A+Sal*: 5.83 ± 2.14 ;
190 CFA: 11.42 ± 2.31 , $p < 0.01$). Treatments with only glucosamine (*Glu*) and both glucosamine and
191 hyaluronic acid (*Glu+Hyal A*) yielded no significant improvement of the clinical symptoms. The
192 hind-paw conditions of each group at the end of the experiment are shown in Fig. 3.

193 Effect on Expression of Pro-inflammatory and Cartilage Degradation Genes

194 At day 29, expression of cytokines *IL-17A* and *IL-1 β* of CFA group was significantly greater than
195 the control group (Fig. 4A-B). *Glu+Hyal A+Sal* treatment significantly reduced the expression of
196 *IL-17A* and *IL-1 β* compared to the CFA group. A decrease in the expression of *IL-17A* by
197 *Glu+Bosw* and *Glu+Bosw+Sal* was also noticed. *Glu+Hyal A+Bosw* tended to limit *IL-17A* and
198 *IL-1 β* expressions, but the difference with the CFA group wasn't significant. *TNF- α* expression of
199 CFA group was not superior to the control group (Fig 4C). *Glu+Hyal A+Sal* treatment inhibited
200 *TNF- α* compared to both the control and the CFA group.

201 Effect on Total Antioxidant Activity of Plasma

202 Fig. 5 shows that all treatments with combinations of two or three ingredients significantly
203 increased the antioxidant capacity of blood, compared to the CFA group and control group
204 (*Glu+Hyal A*: $4,16 \pm 0,14$; *Glu+Hyal A+Bosw*: $4,51 \pm 0,07$; *Glu+Hyal A+Sal*: $3,81 \pm 0,18$;
205 *Glu+Bosw*: $4,71 \pm 0,27$; *Glu+Bosw+Sal*: $3,80 \pm 0,06$; CFA: $2,87 \pm 0,16$; Heal: $3,24 \pm 0,13$, $p <$
206 $0,05$).

207

208 Discussion

209 The aim of this study was to evaluate if glucosamine sulfates therapeutic effects against rheumatoid
210 arthritis could be enhanced through a combination with hyaluronic acid, *Boswellia serrata* extract

211 or *Salix alba* extract. First symptoms of arthritis were evaluated by measuring the thickness of hind
212 paws and by a visual examination (arthritis score). The combinations with BS and/or SA
213 significantly reduce hind-paw thickness and arthritis scores during the treatment. *Salix nigra* bark
214 methanol extract (100 mg/kg/day) has inhibited the progression of collagen-induced arthritis in rats
215 at the end of the experiment by leaving arthritis scores and paw swelling close to healthy control
216 (Sharma et al., 2011). In other studies, boswellic acid extract (total acid content: $93 \pm 3\%$) from BS
217 (250 mg/kg) was more efficient than glucosamine (250 mg/kg) to reduce inflammation in
218 Mycobacterium-induced arthritis in acute and chronic model of inflammation in rats (Singh et al.,
219 2007). Furthermore, in the same study, the combination of these two ingredients have shown a
220 significant synergistic effect on chronic inflammation with a dose of 125 mg/kg for boswellic acid
221 and 125 mg/kg for glucosamine. We suspect that the synergistic effect result from the combination
222 of the different metabolic targets by which the bioactive molecules reduce inflammation. Anti-
223 arthritic proprieties of each ingredient might have amplified the therapeutic effect of treatments.
224 Glucosamine and hyaluronic acid have major structural roles in articular cartilage. Glucosamine
225 stimulates the production of glycosaminoglycans that provide strength and elasticity to cartilage
226 and connective tissues by holding joint tissue together and giving shock-absorbing properties
227 (Singh et al., 2007). HA is a component of the synovial fluid and confers viscosity, as well as shock-
228 absorbing and lubricating abilities (Moreland, 2003)., *Boswellia spp.* and *Salix spp.* do indeed
229 produce active compounds like boswellic acid and salicin that show anti-inflammatory activities
230 (Kimmatkar et al., 2003; Shara and Stohs, 2015). They directly target the inflammatory mediators
231 such as interleukins and metalloproteinases (Umar et al., 2014; Sharma et al., 2011). BS extracts
232 inhibit the 5-lipoxygenase which contributes to the progression of chronic inflammation through
233 greater recruitment of white blood cells at inflammatory sites (Kimmatkar et al., 2003). BS and SA
234 also have ROS-scavenging properties and can have a certain control on antioxidant enzymes
235 (Sharma et al., 2011; Umar et al., 2014). Many studies, including ours, have concluded that
236 combinations of glucosamine with either BS and/or SA are a promising strategy for limiting clinical
237 signs of arthritis (Umar et al., 2014; Sharma et al., 2011; Kimmatkar et al., 2003). In our study, GS
238 alone or in combination with HA did not result in any improvement of the arthritis symptoms. A
239 previous study demonstrated that glucosamine can inhibit swelling in joints and reduce arthritic
240 scores in rat adjuvant arthritis (Hua et al., 2005). However, the dose that they used was much higher
241 than the equivalent dose usually administered to humans. It happens frequently in animal studies
242 that the administered dose is higher than the recommended equivalent (when corrected for
243 allometry) for humans. These doses could not be applied to humans according to the severe
244 legislation in different countries. Maximal dosages of natural products are highly regulated to avoid
245 adverse effects. We can therefore suspect that maximal recommended dosages for humans may not
246 result in significant inhibition of the clinical signs of arthritis in both rats and humans. For example,
247 a meta-analysis including 10 trials with an average of at least 100 patients concluded that
248 glucosamine (1500 mg/kg/day: recommended dosage) was not effective against osteoarthritis,
249 having no relevant clinical effect on pain or structure of affected joints (Wandel et al., 2010). Then,
250 it becomes clearly advantageous to use combinations of ingredients to compensate for the small
251 effect of a single ingredient (at recommended doses), and to rely on the synergetic effect of
252 combinations without exceeding the recommended dosages of individual biomolecules.

253 Inhibition of pro-inflammatory cytokines can reduce clinical signs of arthritis. We therefore
254 analyzed the mRNA content of three pro-inflammatory cytokines. IL-1 β and TNF- α , two major
255 pro-inflammatory cytokines, are both known to be present at high concentrations in serum and
256 synovial fluid in patients with RA (Umar et al., 2014). IL-1 β and TNF- α stimulate their own

257 production and the production of other cytokines, amplifying the inflammation process (Moreland,
258 2003) and contribute synergistically to produce the inflammasome (Gaffen, 2009).

259 In our study, *TNF- α* and *IL-1 β* expression was significantly reduced by only *Glu+Hyal A+Sal*. A
260 previous study, on synovial-cell cultures from patients with RA, demonstrated that blocking the
261 activity of *TNF- α* significantly reduced the production of IL-1, IL-6 and IL-8 (Butler et al., 1995).
262 Thus, blocking *TNF- α* may have a greater overall effect on inflammation than only blocking IL-1.
263 Moreover, we suspect that this reduction of *TNF- α* partly results from SA activity. In a recent
264 review, Shara and Stohs (2015) came to the conclusion that the anti-inflammatory activity of SA is
265 associated with down regulation of the pro-inflammatory effect of *TNF- α* . In addition to salicin,
266 SA has other active compounds like polyphenols and flavonoids which may also play a role in the
267 therapeutic action of SA (Dragos et al., 2017). On the other hand, HA didn't seem to have any
268 impact on the level of *TNF- α* in RA rats. Injection of intra-articular HA in rat antigen-induced
269 arthritis did show no significant changes in the level of *TNF- α* in short-term and in long-term
270 experiments (Roth et al., 2005). Thus, we suspect that SA has an important role in the anti-
271 inflammatory properties of a *Glu+Hyal A+Sal* treatment.

272 At the end of the experiment, three treatments containing BS and/or SA were able to inhibit *IL-17A*
273 expression compared to *TNF- α* that was only inhibited by *Glu+Hyal A+Sal*. Furthermore, we did
274 not observe a difference in the level of *TNF- α* expression between the control and CFA group.
275 These results suggest that *TNF- α* had a weaker role in chronic inflammation than *IL-17A*, at least
276 at the sampling periods of our experimentation. *IL-17A* is also over-expressed in RA (Dudler et al.,
277 2000). It has been shown that *TNF- α* may be important in the onset of the arthritis induction, but it
278 gradually loses its dominance with the progression of the inflammation (Joosten et al., 1996). They
279 showed that anti-TNF α treatment was efficient shortly after the collagen-induced arthritis (CIA) in
280 DBA/1 mice, reducing cartilage destruction, but that it had little effect when CIA is fully
281 established (Joosten et al., 1996). To notice a difference in *TNF- α* levels, measurements should
282 have then been performed at the beginning of the inflammatory phase. We suggest that *TNF- α*
283 played a lesser role in the late phase of our experiment and had lower involvement in inflammatory
284 modulation than *IL-1 β* and *IL-17A*.

285 As mentioned previously, our treatments had a stronger impact on *IL-17A* expression than in the
286 expression of the two other cytokines. *IL-17* is involved in inflammation by stimulating other pro-
287 inflammatory cytokines and metalloproteinases in synoviocytes and chondrocytes (Dudler et al.,
288 2000). For example, it stimulates secretion of *IL-1 β* and *TNF- α* by macrophages (Jovanovic et al.,
289 1998). Two studies came to the conclusion that arthritis treatments involving inhibition of *IL-17*
290 could be as efficient as blocking *IL-1* and *TNF- α* . They showed that *IL-17* expression and activity
291 is partly independent of these two cytokines under arthritis conditions (Koenders et al., 2005;
292 Koenders et al., 2006). It can, for example, aggravate joint inflammation and cartilage destruction
293 on its own without the increase of *IL-1* or *TNF- α* . Also, blocking *IL-1* in *TNF-deficient* mice was
294 not sufficient to reduce *IL-17* effects in streptococcal cell wall-induced (SCW) arthritis model
295 (Koenders et al., 2006). *IL-17* has the capacity to partly supplant the functions of *IL-1* since these
296 two have many overlapping responses and functions even if they are not from the same cytokine
297 family, as shown in SCW-induced arthritis and *IL-1-deficient* mice (Koenders et al., 2005). We
298 conclude that *IL-17A* might partly lead the inflammatory process at the end of our experimental
299 arthritis and that treatments with plants were effective to decrease the expression of this cytokine.
300 Anti-inflammatory properties of BS and SA successfully reduced the *IL-17A* effect according to
301 our results.

302 Reactive oxygen species (ROS) can also contribute to matrix component degradation (Campo et
303 al., 2008). ROS are generated at high rates in synovial neutrophils from RA patients (Sato et al.,
304 1988). Synovial fluid and HA are respectively susceptible to degradation and depolymerization by
305 a high level of ROS (Sato et al., 1988). These processes promote loss of viscosity in the joint as
306 well as osteoclast activation (Filippin et al., 2008). Different antioxidants (polyphenols, tannins,
307 etc.) are found in natural ingredients (Sato et al., 1988) which have been reported to partly protect
308 and limit damage to cartilage (Venkatesha et al., 2011). All combinations with HA, BS and/or SA
309 have significantly increased the total antioxidant level in the plasma of our experimental rats.
310 Combinations without SA appear to have more impact on antioxidant levels than those with SA. In
311 a previous study, it has been demonstrated that CIA in rats increases the activity of three important
312 enzymes involved in oxidative stress management in plasma: superoxide dismutase, glutathione
313 peroxidase and catalase (Sharma et al., 2011). They showed that these enzymes respond naturally
314 to a higher concentration of ROS after arthritis induction, increasing their activities to protect
315 tissues in the joint. The antioxidant properties of biomolecules in the present study may have helped
316 to attenuate oxidative stress. Another study demonstrated that GS reduces superoxide radicals in a
317 dose-concentration manner which partly explains its antioxidant activity (Xing et al., 2009).
318 Synovial fluid and endogenous HA usually protect the articular tissues from oxidative damage.
319 Excessive ROS decrease the HA content of articulation while addition of exogenous HA can
320 decrease ROS levels in synovial cells of RA and buffer the impact on HA oxidation and decline
321 (Sato et al., 1988). It has also been observed that extract of BS has improved the antioxidant level
322 in CIA rat models, significantly decreasing ROS (Umar et al., 2014). *Salix nigra* bark methanol
323 extract has contributed to attenuate oxidative stress in CIA rats (Sharma et al., 2011). BS and SA
324 also has a phenolic compound showing antioxidant activities (Kokkiripati et al., 2011; Dragos et
325 al., 2017). We suggest that a combination of antioxidant properties of these natural ingredients
326 increases the antioxidant capacity in plasma of our experimental rats. We also suggest that this rise
327 in antioxidant capacity can partly be responsible for the reduction of the clinical signs of arthritis.

328 Conclusion

329 We conclude that the addition of *Boswellia serrata* and/or *Salix alba* attenuates clinical signs of
330 rheumatoid arthritis in Freund's complete adjuvant-induced arthritis in rats likely due to both their
331 anti-inflammatory and antioxidant properties. Combinations with these plants have decreased hind-
332 paw swelling and improved arthritis scores. Our results on clinical symptoms have been confirmed
333 by some of our molecular markers. Treatments with BS and/or SA help to reduce inflammation and
334 cartilage degradation by reducing effectively *IL-17A* expression and to a lesser extent, the
335 expression of *IL-1 β* . BS and SA have helped to create a redox status that might buffer oxidative
336 stress through higher antioxidant capacity in plasma. This capacity may be partly responsible for
337 the amelioration of clinical symptoms of arthritis.

338 Acknowledgements

339 We are thankful to Samuel Fortin (Ph.D.) and Caroline Morin (Ph.D.) to their help for the
340 familiarization with the arthritic model of Freund's complete adjuvant.

341 Funding

342 This work was supported by the Industrial Research Assistance Program (IRAP) of National
343 Research Council Canada (NRCC).

344 References

- 345 Aghazadeh-Habashi, A., Gilzad Kohan, M.H., Asghar, W. & Jamali, F., 2014. Glucosamine
346 Dose/Concentration-Effect Correlation in the Rat with Adjuvant Arthritis. *J. Pharm. Sci.* 103, 760-
347 767.
- 348 Arthritis Society. Arthritis Type – Arthrose, Arthritis Society. [Consulted on April 18, 2018].
349 <https://www.arthritis.ca/>
- 350 Butler, D.M., Maini, R.N., Feldmann, M. & Brennan, F.M., 1995. Modulation of proinflammatory
351 cytokine release in rheumatoid synovial membrane cell cultures: comparison of monoclonal anti
352 TNF- α antibody with interleukin1 receptor antagonist. *Eur. Cytokine Netw.* 6, 225-30.
- 353 Campo, G.M., Avenoso, A., Campo, S., D'Ascola, A., Traina, P. & Calatroni, A., 2008.
354 Chondroitin-4-sulphate inhibits NF- κ B translocation and caspase activation in collagen-induced
355 arthritis in mice. *Osteoarthr. Cartilage* 16, 1474-1483.
- 356 Dragos, D., Gilca, M., Gaman, L., Vlad, A., Iosif, L., Stoian, I. & Lupescu, O., 2017.
357 Phytomedicine in joint disorders. *Nutrients* 9, 70.
- 358 Dudler, J., Renggli-Zulliger, N., Busso, N., Lotz, M. & So, A., 2000. Effect of interleukin 17 on
359 proteoglycan degradation in murine knee joints. *Ann. Rheum. Dis.* 59, 529-532.
- 360 Fan, A.Y., Lao, L., Zhang, R.X., Zhou, A.N., Wang, L.B., Moudgil, K.D., Lee, D.Y.W., Ma, Z.Z.,
361 Zhang, W.Y. & Berman, B.M., 2005. Effects of an acetone extract of *Boswellia carterii* Birdw.
362 (Burseraceae) gum resin on adjuvant-induced arthritis in lewis rats. *J. Ethnopharmacol.* 101, 104-
363 109.
- 364 Fazal, S.A., Khan, M., Nishi, S.E., Alam, F., Zarin, N., Bari, M.T & Ashraf, G.M., 2018. A clinical
365 update and global economic burden of rheumatoid arthritis. *Endocr. Metab. Immune Disord. Drug*
366 *Targets* 18, 98-109.
- 367 Filippin, L.I., Vercelino, R., Marroni, N.P. & Xavier, R.M., 2008. Redox signalling and the
368 inflammatory response in rheumatoid arthritis. *Clin. Exp. Immunol.* 152, 415-422.
- 369 Gaffen, S.L., 2009. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. *Curr.*
370 *Rheumatol. Rep.* 11, 365-370.
- 371 Health Canada. Multiple ingredient joint health products, Health Canada. [Consulted on April 18,
372 2018]. <http://webprod.hc-sc.gc.ca/nhpid-bdipsn/atReq.do?atid=multiple.joint.health&lang=eng>
- 373 Hua, J., Suguro, S., Hirano, S., Sakamoto, K. & Nagaoka, I., 2005. Preventive actions of a high
374 dose of glucosamine on adjuvant arthritis in rats. *Inflamm. Res.* 54, 127-132.
- 375 Joosten, L.A., Helsen, M., van de Loo, F.A. & van den Berg, W.B., 1996. Anticytokine treatment
376 of established type II collagen-induced arthritis in DBA/1 mice: A comparative study using anti-
377 TNF α , anti-IL-1 α/β , and IL-1Ra. *Arthritis Rheumatol.* 39, 797-809.
- 378 Jovanovic, D.V., Di Battista, J.A., Martel-Pelletier, J., Jolicoeur, F.C., He, Y., Zhang, M., Mineau,
379 F., & Pelletier, J.P., 1998. IL-17 stimulates the production and expression of proinflammatory
380 cytokines, IL- β and TNF- α , by human macrophages. *J. Immunol.* 160, 3513-3521.
- 381 Kaur, A., Nain, P., & Nain, J., 2012. Herbal plants used in treatment of rheumatoid arthritis: a
382 review. *Int. J. Pharm. Pharm. Sci.* 4, 44-57.

- 383 Kimmatkar, N., Thawani, V., Hingorani, L. & Khiyani, R., 2003. Efficacy and tolerability of
384 *Boswellia serrata* extract in treatment of osteoarthritis of knee - A randomized double blind placebo
385 controlled trial. *Phytomedicine* 10, 3-7.
- 386 Koenders, M.I., Lubberts, E., Oppers-Walgreen, B., van den Bersselaar, L., Helsen, M.M., Kolls,
387 J.K., Joosten, L.A. & van den Berg, W.B., 2005. Induction of cartilage damage by overexpression
388 of T cell interleukin-17A in experimental arthritis in mice deficient in interleukin-1. *Arthritis*
389 *Rheumatol.* 52, 975–983.
- 390 Koenders, M.I., Lubberts, E., van de Loo, F.A., Oppers-Walgreen, B., van den Bersselaar, L.,
391 Helsen, M.M., Kolls, J.K., Di Padova, F.E., Joosten, L.A. & van den Berg, W.B., 2006. Interleukin-
392 17 acts independently of TNF- α under arthritic conditions. *J. Immunol.* 176, 6262-6269.
- 393 Kokkiripati, P.K., Bhakshu, L.M., Marri, S., Padmasree, K., Row, A.T., Raghavendra, A.S. &
394 Tetali, S.D., 2011. Gum resin of *Boswellia serrata* inhibited human monocytic (THP-1) cell
395 activation and platelet aggregation. *J. Ethnopharmacol.* 137, 893-901.
- 396 Moreland, L.W., 2003. Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of
397 osteoarthritis: mechanisms of action. *Arthritis Res. Ther.* 5, 54-67.
- 398 Reagan-Shaw, S., Nihal, M. & Ahmad, N., 2007. Dose translation from animal to human studies
399 revisited. *FASEB J.* 22, 659–661.
- 400 Roth, A., Mollenhauer, J., Wagner, A., Fuhrmann, R., Straub, A., Venbrocks, R.A., Petrow, P.,
401 Bräuer, R., Schubert, H., Ozegowski, J., Peschel, G., Müller, P.J. & Kinne, R.W., 2005. Intra-
402 articular injections of high-molecular-weight hyaluronic acid have biphasic effects on joint
403 inflammation and destruction in rat antigen-induced arthritis. *Arthritis Res. Ther.* 7, R677-R686.
- 404 Sato, H., Takahashi, T., Ide, H., Fukushima, T., Tabata, M., Sekine, F., Kobayashi, K., Negishi, M.
405 & Niwa, Y., 1988. Antioxidant activity of synovial fluid, hyaluronic acid, and two subcomponents
406 of hyaluronic acid: Synovial fluid scavenging effect is enhanced in rheumatoid arthritis
407 patients. *Arthritis Rheumatol.* 31, 63-71.
- 408 Shara, M. & Stohs, S.J., 2015. Efficacy and safety of white willow bark (*Salix alba*)
409 extracts. *Phytother. Res.* 29, 1112-1116.
- 410 Sharma, S., Sahu, D., Das, H.R. & Sharma, D., 2011. Amelioration of collagen-induced arthritis
411 by *Salix nigra* bark extract via suppression of pro-inflammatory cytokines and oxidative
412 stress. *Food Chem. Toxicol.* 49, 3395-3406.
- 413 Singh, S., Khajuria, A., Taneja, S.C., Khajuria, R.K., Singh, J. & Qazi, G.N., 2007. Boswellic acids
414 and glucosamine show synergistic effect in preclinical anti-inflammatory study in rats. *Bioorg.*
415 *Med. Chem. Lett.* 17, 3706-3711.
- 416 Umar, S., Umar, K., Sarwar, A.H.M.G., Khan, A., Ahmad, N., Ahmad, S., Katiyar, C.K., Husain,
417 S.A. & Khan, H.A., 2014. *Boswellia serrata* extract attenuates inflammatory mediators and
418 oxidative stress in collagen induced arthritis. *Phytomedicine* 21, 847-856.
- 419 Venkatesha, S.H., Berman, B.M. & Moudgil, K.D., 2011. Herbal medicinal products target defined
420 biochemical and molecular mediators of inflammatory autoimmune arthritis. *Bioorgan. Med.*
421 *Chem.* 19, 21-29.
- 422 Wandel, S., Jüni, P., Tendal, B., Nüesch, E., Villiger, P.M., Welton, N.J., Reichenbach, S. & Trelle,
423 S., 2010. Effects of glucosamine, chondroitin, or placebo in patients with osteoarthritis of hip or
424 knee: network meta-analysis. *Brit. Med. J.* 341, c4675.

- 425 Xing, R., Liu, S., Wang, L., Cai, S., Yu, H., Feng, J. & Li, P., 2009. The preparation and antioxidant
426 activity of glucosamine sulfate. *Chin. J. Oceanol. Limn.* 27, 283-287.
- 427 Zheng, C.J., Zhao, X.X., Ai, H.W., Lin, B., Han, T., Jiang, Y.P., Xing, X. & Qin, L.P., 2014.
428 Therapeutic effects of standardized *Vitex negundo* seeds extract on complete Freund's adjuvant
429 induced arthritis in rats. *Phytomedicine* 21, 838-846.
- 430

431 **Table 1.** Daily doses of therapeutic ingredients recommended for human and equivalent doses for
432 rat.

THERAPEUTIC INGREDIENTS	MAXIMAL RECOMMENDED DAILY DOSE FOR HUMAN (mg)	EQUIVALENT DAILY DOSE FOR RAT (mg/kg corporeal)
Glucosamine (sulphated form)	1500	154
Hyaluronic acid (from bacterial fermentation)	200	21
Extract of <i>Boswellia serrata</i> (normalized at 40% of boswellic acid)	1000	103
Salicine (active ingredient of <i>Salix alba</i> extract)	240	25

433

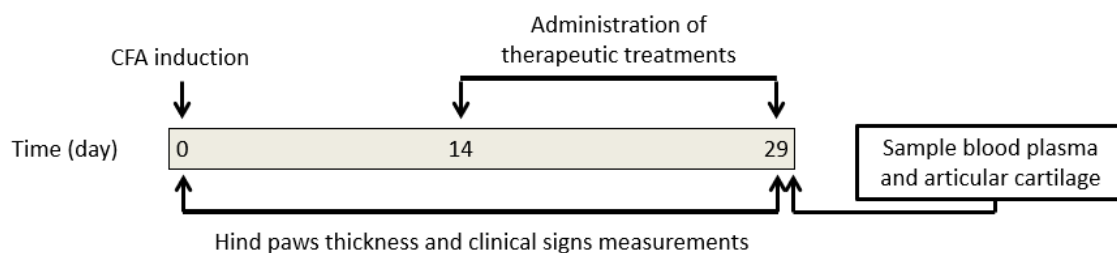
434 **Table 2.** Primer sequences used for real-time PCR analysis.

GENE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	FRAGMENT LENGTH (base pair)
IL-17A ¹	GTGAGCCGGCAGAAGCAGGA	GGCTCCGCCCAACCCAAGAT	107
IL1- β ¹	GGGATTTTGTCTGCTTGCTGTC	TGCAGGCTTCGAGATGAAC	147
TNF- α ¹	CTTCTGTCTACTGAACTTCGGG	GCTACGGGCTTGTCACCTC	146
MMP 2 ²	AGGAGGGCACTGGTGGCTCA	GCCAGGGCAGCCGTAAGGGA	104
MMP 9 ¹	GGAGACGGCAAACCCTGCGT	GTGGTGGCGCACCAGCGATA	104
MMP 13 ¹	AGCTTGGCCACTCCCTCGGT	TGAACGTCATCATCTGGGAGCA	112
β -actin ^{1,2}	TCACTATCGGCAATGAGCG	GGCATAGAGGTCTTTACGGATG	143
GAPDH ^{1,2}	GCCAAGGCTGTGGGCAAGGT	GCAGGTTTCTCCAGGCGGCAT	119

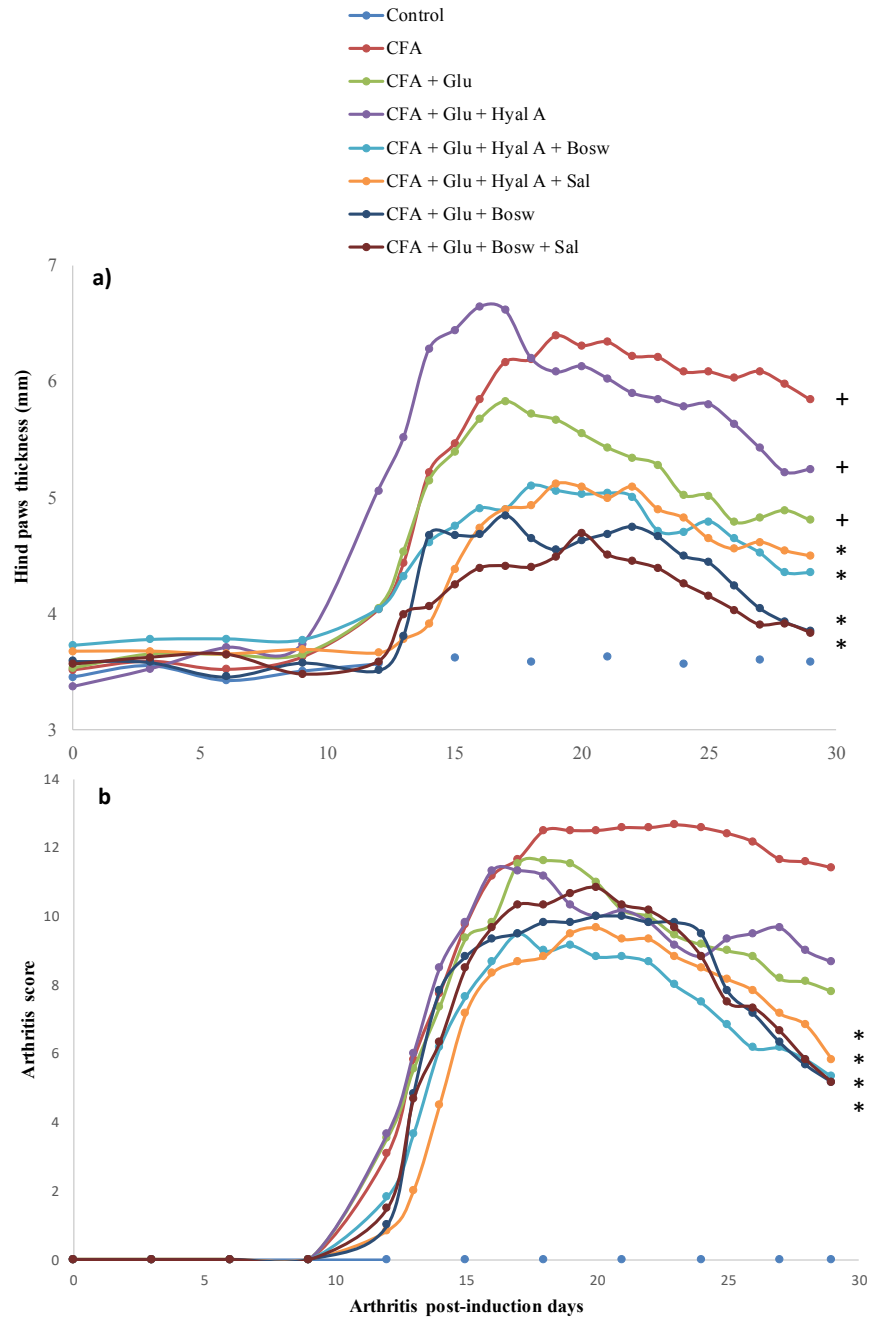
435 ¹ Amplification cycle: 1) initial activation at 95°C for 2 minutes, 2) 2-step: [95°C for 15 sec followed by
436 60°C for 30 sec] X 40 amplification cycles.

437 ² Amplification cycle: 1) initial activation at 95°C for 2 minutes, 2) 2-step: [95°C for 15 sec followed by
438 63°C for 30 sec] X 40 amplification cycles.

439



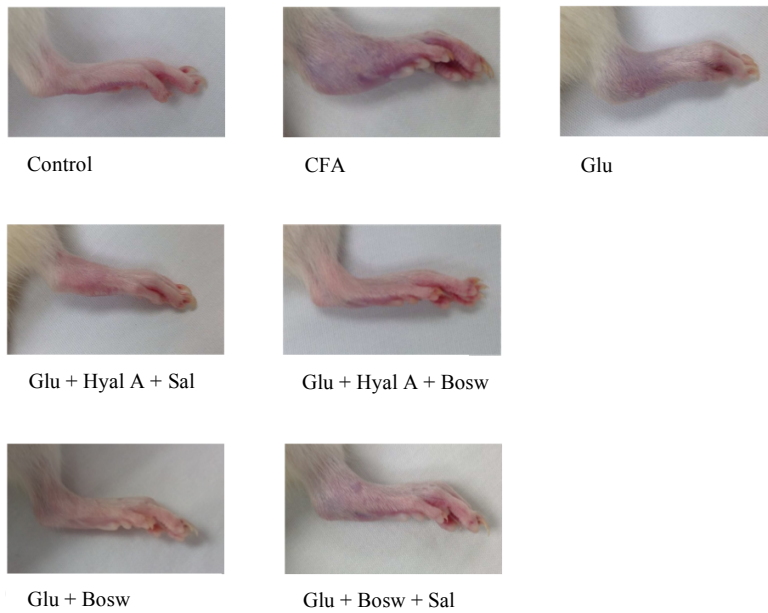
440 **Fig 1.** Temporal experimental plan of animal manipulations.



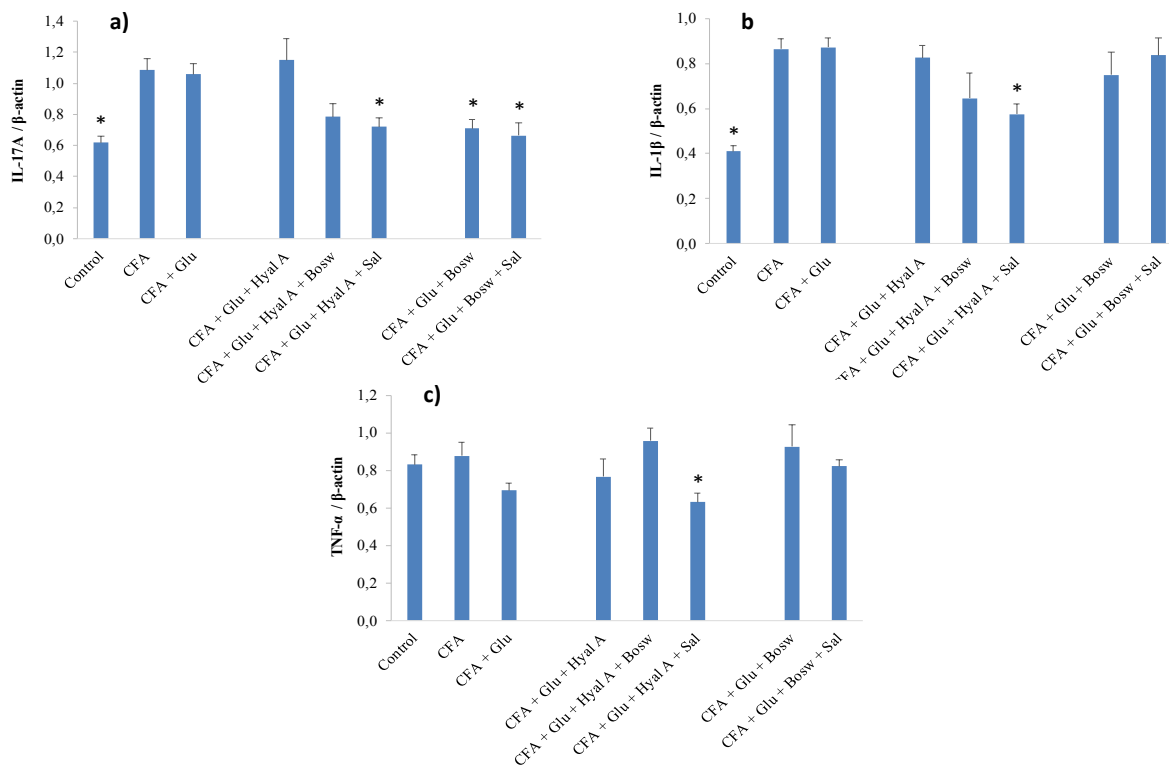
441

442 **Fig 2.** Effect of treatments on severity of arthritis. **a)** Hind paws thickness (mm) and **b)** arthritis
443 score in time (number of arthritis post-induction days). Treatments have been daily administered
444 starting at day 14 to day 29. Each circle is a mean \pm SEM (control and CFA groups: $n = 12$; *Glu*: n
445 $= 11$; *Glu+Hyal A*, *Glu+Hyal A+Bosw*, *Glu+Hyal A+Sal*, *Glu+Bosw* and *Glu+Bosw+Sal*: $n = 6$).
446 Significant differences with CFA group (*) and control group (+) at day 29 are shown in **a)** $p <$
447 0.05 and in **b)** $p < 0.01$.

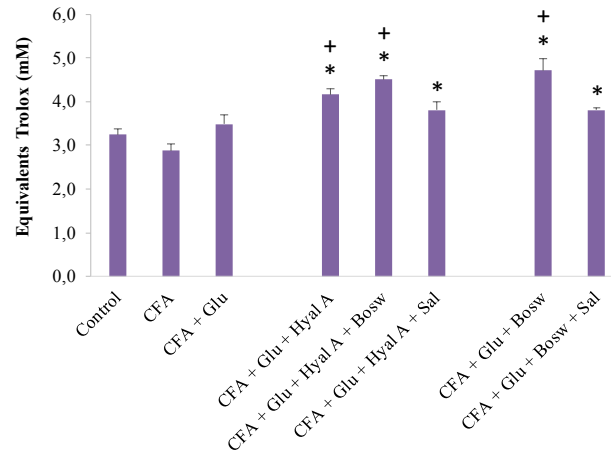
448



449 **Fig 3.** Clinical condition of hind paws at the end of the experiment (after treatment of 15 days).



450 **Fig 4.** Effect of treatments on genes expression of pro-inflammatory cytokines. Genes expression
 451 of **a) IL-17A**, **b) IL-1 β** and **c) TNF- α** were measured in articular cartilage of hind paws at day 29
 452 (15e day of treatment). Each circle is a mean \pm SEM (control and CFA groups: n = 10-12; *Glu*: n =
 453 = 9-11; *Glu+Hyal A*, *Glu+Hyal A+Bosw*, *Glu+Hyal A+Sal*, *Glu+Bosw* and *Glu+Bosw+Sal*: n =
 454 5-6). Significant differences with CFA group (*) at day 29 are shown (p < 0.05).



455 **Fig 5.** Effect of treatments on plasma content as total antioxidants. The content was measured in
456 blood plasma at day 29 (15e day of treatment). Each circle is a mean \pm SEM (control group: n = 8;
457 CFA groups: n = 10-11; *Glu*: n = 9-10; *Glu+Hyal A*, *Glu+Hyal A+Bosw*, *Glu+Hyal A+Sal*,
458 *Glu+Bosw* and *Glu+Bosw+Sal*: n = 5-6). Significant differences with CFA group (*) and control
459 group (+) at day 29 are shown ($p < 0.05$).