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An Antioxidative and Antiinflammatory Agent for Potential Treatment of Osteoarthritis from *Ecklonia cava*

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Osteoarthritis is thought to be induced by the ageing-related loss of homeostatic balance between degeneration and repair mechanism around cartilage tissue in which inflammatory mediators such as reactive oxygen species, cytokines and prostaglandins are prone to overproduction under undesirable physiological conditions. Phlorotannins are unique polyphenolic compounds bearing dibenzo-1,4-dioxin skeleton which are not found in terrestrial plants but found only in some brown algal species such as *Ecklonia* and *Eisenia* families. Phlorotannin-rich extracts of *Ecklonia cava* including LAD103 showed significant antioxidant activities such as DPPH radical scavenging, ferric ion reduction, peroxynitrite scavenging, and inhibition of LDL oxidation, indicating their possible antioxidative interference both in onset and downstream consequences of osteoarthritis. LAD103 also showed significant down regulation of PGE_2 generation in LPS-treated RAW 246.7 cells, and significant inhibition of human recombinant interleukin-1α-induced proteoglycan degradation, indicating its beneficial involvement in pathophysiological consequences of osteoarthritis, the mechanism of which needs further investigation. Since LAD103 showed strong therapeutic potentials in arthritic treatment through several *in vitro* experiments, it is highly encouraged to perform further mechanistic and efficacy studies.

Key words: *Ecklonia cava*, Phlorotannin, Antioxidant, Antiinflammatory, Osteoarthritis

INTRODUCTION

Cartilage is a dynamic tissue in a constant state of flux, regulated by chondrocytes under the influence of various cellular mediators such as reactive oxygen species, prostaglandins, cytokines, etc (Corvol, 2000; Martel-Pelletier *et al.*, 1999; Mathy-Hartert *et al.*, 2002). In osteoarthritis, this equilibrium is disturbed to result in a dramatic increase in inflammatory mediators (Schuerwegh *et al.*, 2003), reactive oxygen species (Dalle-Donnea *et al.*, 2003; Evans and Stefanovic-Racic, 1996) and degradative enzymes (Marinia *et al.*, 2003; Morisset *et al.*, 1998), leading to cartilage degradation and subsequent loss of joint function (Sandell and Aigner, 2001). Recent findings on an interesting category of polyphenolic compounds derived from brown algae indicate their potentials to be used for modulating the pathophysiological processes of arthritis in a multifactorial manner.

Brown algae, mostly used as sources of alginic acid industrially are also widely consumed as food in many Asian countries followed by several European countries. Health benefits of their various polysaccharide components such as alginates, laminarin and fucoidans have been reported in many scientific literatures, while their polyphenolic compounds have been rarely studied in terms of medicinal applications. Among several categories of algal polyphenols, pharmacologically notable one is "phlorotannin" (Fig. 1). This unique polyphenolic category is characterized by its dibenzo-1,4-dioxin unit in the molecular skeleton (Glombitza and Gerstberger, 1985), which is found only in some specific alga such as Eisenia and Ecklonia species. Its a_2-antiplasmin activity (Fukuyama *et al.*, 1989, 1990; Nakayama *et al.*, 1989) and antioxidant activities (Kang *et al.*, 2003; Nakamura *et al.*, 1996) have

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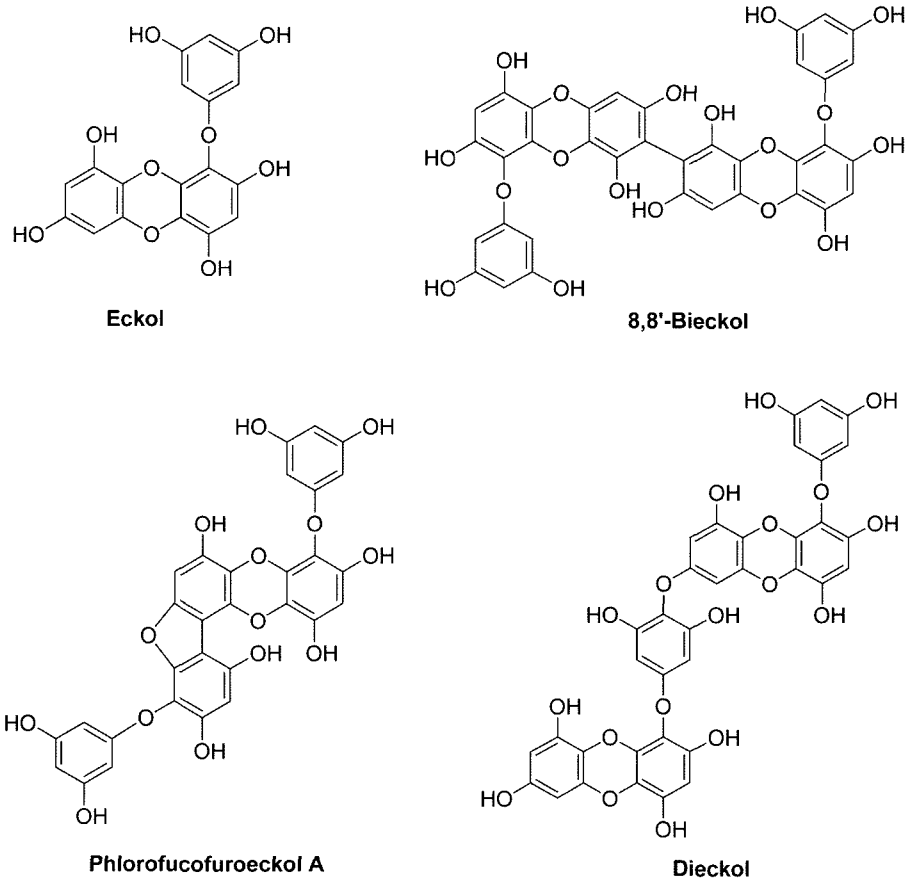

Fig. 1. Structures of Phlorotannins from brown alga

been reported. Recently, several studies supporting the potential use of these compounds for osteo- and rheumatic arthritis have been reported: inhibitory effects of phlortannins on hyaluronidase (Shibata *et al.*, 2002), secertory phospholipase A_2, cyclooxygenases, and lipoxygenases (Shibata *et al.*, 2003).

In this article, we report the several therapeutic potentials of LAD103, a phlorotannin-rich extract of a brown algae *Ecklonia cava*, in arthritis treatment. We report its specific antioxidant activities against peroxynitrite and LDL as well as general antioxidant activities such as radical scavenging activity and reducing power, its down-regulation of LPS-induced PGE_2 production in cultured rat macrophage, and demonstration of its effect on proteoglycan degradation in cartilage explant culture.

MATERIALS AND METHODS

Preparation of *Ecklonia cava* extracts

Freshly collected *Ecklonia cava* (Jeju island, Korea) was washed and freeze-dried, milled into small pieces and stored at -80°C until use. Water extract was prepared by extracting the dry seaweed with boiling water for 1 h followed by filtration and evaporation *in vacuo* to dryness. For ethanolic extract, the pretreated seaweed was extracted by 30% (v/v) ethanol at 50°C for 2 h. After the extract was filtered and evaporated *in vacuo*, the residue was extracted with 95% ethanol at 50°C for 2 h. The resulting extraction mixture was filtered and evaporated *in vacuo*. For LAD103, the 30% ethanolic extract as above was partitioned between ethyl ether and water. The ether layer was evaporated *in vacuo* and lyophilized for a complete removal of the residual solvent to yield a greenish brown powder.

Determination of phlorotannin content

The phlorotannin content of each extract was determined spectrophotometrically using Folin-Ciocalteu reagent from Sigma Co. (St. Louis, MO, U.S.A.). Folin-Ciocalteu reagent was diluted with distilled water. Each extract (50 μL) was added to Folin-Ciocalteu reagent (250 μL) and mixed thoroughly. After 1 min, 750 μL of 0.5 N sodium bicarbonate solution was added followed by thorough mixing. The mixture was allowed to stand at 37°C for 24 h. The absorbance of mixture was measured with spectrophotometer at 725 nm. Phlorotannin content was expressed in g/100 g of each extract's dry weight based on a standard curve using phloroglucinol.

Free radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydarzyl) radical scavenging effect was measured according to the method first employed by Blois (1958). The sample solution (100 μL) was added to 900 μL of DPPH (Sigma) solution in ethanol (1.01×10^{-4} M). After incubation at room temperature for 30 min, the absorbance of this solution was determined at 518 nm using a spectrophotometer and the remaining DPPH was calculated. All experiments were carried out in triplicate. Results are expressed as the percentage decrease with respect to control values. Each fraction was evaluated at the final concentration of 100 μg/mL in the assay mixture.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed as previously described by Benzie and Strain (1999). The experiment was conducted at 37°C under pH 3.6 condition with a blank sample in parallel. In this assay, reductants ("antioxidants") in the sample reduce the Fe^{3+}/TPTZ (tripyridyltriazine, Sigma) complex present in stoichiometric excess, to the blue colored ferrous form, with an increase in absorbance at 593 nm. The difference in absorbance between the blank and each sample is proportional to the total ferric reducing antioxidant power (FRAP value) of the antioxidants in the sample. Aqueous ascorbic acid solutions of known concentrations were used for calibration. The final results were expressed as micromolar (μM) ascorbic acid equivalent. The assay was evaluated at sample concentrations of 100 μg/mL.

Measurement of peroxynitrite scavenging activity

Peroxynitrite scavenging activity was measured by monitoring the oxidation of dihydrorhodamine123 (DHR 123, Calbiochem, U.S.A.) according to a modification of the method of Kooy *et al.* (1994). DHR 123 (5 μM) dissolved in dimethylformamide was purged with nitrogen and stored at -80°C as a stock solution. A working solution with DHR 123 (final concentration, 5 mM) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 μM diethylenetriaminepentaacetic acid (DTPA, Sigam-Aldrich) was purged with nitrogen and placed on ice before use. Peroxynitrite scavenging by the oxidation of DHR123 was measured with a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, Inc., U.S.A.) with excitation and emission wavelengths of 485 and 530 nm, respectively, at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with or without authentic peroxynitrite (10 μM) in 0.3 N sodium hydroxide solution.

Inhibition of LDL oxidation

Oxidation of LDL (5 mg protein/mL) was carried out in a water bath at 37°C. 100 μL of LDL (5 mg/mL, Sigma) was incubated with 800 μL of 30 μM $CuSO_4$ (in phosphate buffer, pH 7.4) and 100 μL of each sample (2 mg/mL) at 37°C for 4 h. After reaction, 300 μL of 100 μM EDTA was added to the mixture solution to stop the reaction. A mixture of 1 mL of the TBA-TCA-HCl reagent (0.375% thiobarbituric acid, 15% trichloroacetic acid, 0.25 N hydrochloric acid), 0.5 mL of post-incubated sample mixture was placed in a test-tube with a screw cap. The tube was placed in boiling water for 30 min. After cooling, the reaction mixture was centrifuged at 3,000 rpm for 15 min. The absorbance of the supernatant was measured at 535 nm. The malondialdehyde concentration of the sample can be calculated using tetramethoxypropane (Sigma-Aldrich) as a standard (Buege and Aust, 1987).

Measurement of prostaglandin E_2 (PGE_2) in RAW 264.7 cells

The murine macrophage cell line RAW 264.7 (from Korean Cell Line Bank) was cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone Laboratories, Inc., U.S.A.). Macrophages were grown at 37°C and with 5% CO_2 in fully humidified air. Macrophages were transferred to 96-well plates (1×10^6/well, 200 μL) and were incubated with LPS (*E. coli*, serotype 055:B5, Sigma, 1 μg/mL) and various concentrations of samples for 18 h. All test samples were first dissolved in dimethyl sulfoxide (DMSO) and further diluted with medium and stored at -20°C. PGE_2 concentration in supernatants was measured by EIA according to manufacturer's guide (from Cayman Chemical Co., U.S.A.).

Cartilage explant culture

Articular cartilages from hock joints of 5-week-old rabbits (Samtako, Osan, Korea) were removed immediately after each animal was sacrificed. The care and handling of the animals were in accordance with National Institutes of Health guideline. The articular cartilage explants were obtained by following the method described by Sandy *et al.* (1978). Briefly, after the articular surfaces were exposed surgically under sterile conditions, approximately 200-220 mg of articular surfaces per joint were dissected and submerged into complete medium (DMEM, supplemented with heat inactivated 5% FBS; penicillin 100 U/mL; streptomycin 100 μg/mL). They were then rinsed several times with the complete medium and incubated for 1 to 2 days at 37°C in a humidified 5% CO_2/95% air incubator for stabilization. The complete medium was replaced with a basal medium (DMEM, supplemented with heat-inactivated 1% FBS, 10 mM HEPES, and penicillin 100 U/mL streptomycin 100 μg/mL). Approximately 50 to 60 mg

cartilage pieces were placed in 24-well plates and treated with given concentrations of test agents. After pretreatment for 1 h, 5 ng/mL of rhIL-1α was added to the culture medium and further incubated at 37°C in a humidified 5% CO_2/95% air incubator. The culture medium was collected 60 h later and stored at -20°C until assay.

Measurements of glycosaminoglycans (GAG)

The amount of sulphated GAGs in the medium at the end of reaction reflecting the amount of proteoglycan (PG) degradation was determined through 1,9-dimethy-methylene blue method using a commercially available kit (The Blyscan proteoglycan & glycosaminoglycan assay kit, BioColor Ltd., Ireland) according to the instructions of the manufacturer.

Statistical analysis

Data are expressed as mean ± SD of three different measurements. Statistical significances were confirmed by Student's *t*-test. Differences were designated as significant at $p < 0.05$.

RESULTS AND DISCUSSION

We tested the DPPH radical scavenging activity and ferric reducing antioxidant power of several extracts of *Ecklonia cava* in comparison with well-known synthetic and natural antioxidants (Table I). Considering the polyphenolic content, the preparations showed excellent activities compared with those of the well-known antioxidants such as BHA and catechin. The activity of each extract was in accordance with its phlorotannin content.

The peroxynitrite scavenging activities of each extract was measured and compared with that of penicillamine, a well-known peroxynitrite scavenging agent, as a positive control. The oxidation of DHR 123 to fluorescent rhodamine 123 mediated by authentic peroxynitrite was determined in the presence of each sample at three different concentrations (Fig. 2). All the extracts showed significant activities at physiologically relevant concentrations. Especially, LAD103 showed comparable activity to penicillamine. The extracts' activity also correlated well with phlorotannin content. High level of nitrite/nitrate has been found in synovial fluid, serum and urine of patients with rheumatoid arthritis and osteoarthritis, suggesting the involvement of reactive nitrogen species such as NO and peoxynitrite in the pathophysiology of these diseases (Kaur and Halliwell, 1994). These radicals are implicated in the physical degradation of cartilage not only by inhibiting cartilage matrix synthesis but also by promoting breakdown of cartilage matrix (Li *et al.*, 1997; Morisset *et al.*, 1998). We demonstrated that the *Ecklonia cava* extracts could efficiently scavenge the peroxynitrite radical in a similar manner and potency to that of penicillamine. It is noteworthy that the phlorotannin-rich extracts showed potent peroxynitrite scavenging activities in addition to general antioxidant activities because, in green tea polyphenols, the peroxynitrite scavenging activity was reported to be highly dependent on the presence of a gallate ring in their structures regardless of other antioxidant activity (Haenen *et al.*, 1997; Ahmed *et al.*, 2002). Therefore, it is worth investigating the mechanism of peroxynitrite scavenging by gallate-free phlorotannins.

The *Ecklonia cava* extracts also showed efficient inhibition of LDL oxidation in $CuSO_4$ mediated oxidation of LDL. All the *Ecklonia cava* extracts showed substantial inhibition (Fig. 3). BHA showed highest activities followed by LAD103, the ethanolic extract, the water extract and

Table I. Free radical scavenging activities and ferric reducing antioxidant power of Ecklonia cava extracts with different polyphenol contents.

Sample	Phlorotannin content (wt%)	DPPH scavenging % Scavenging(100μg)	FRAP value (μM of Vit C)
Water extract	20.7 ± 0.9	48.4 ± 2.8	210 ± 1
30% Ethanol extract	45.3 ± 1.6	58.3 ± 1.2	238 ± 6
LAD103	65.2 ± 1.3	83.4 ± 0.7	388 ± 8
BHA	-	83.4 ± 0.2	1212 ± 23
catechin	-	84.6 ± 1.4	748 ± 19

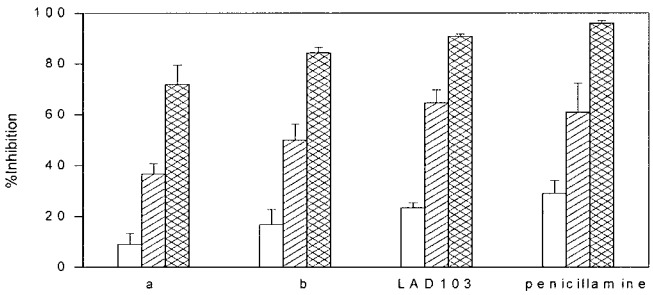

Fig. 2. Peroxynitrite scavenging activity. (a) water extract, (b) 30%-ethanolic extract. For each sample, concentration (from left to right) was 0.1, 1, and 10 μg/mL.

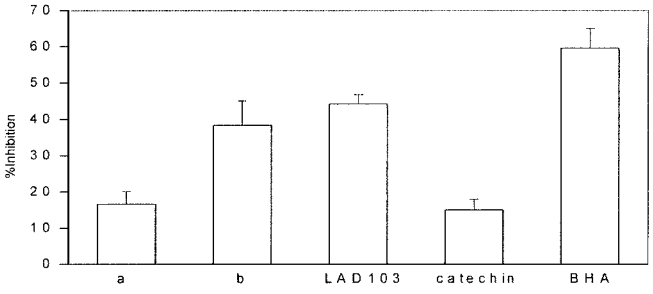

Fig. 3. Inhibition of LDL oxidation. (a) water extract, (b) 30%-ethanolic extract. For each sample, concentration in the reaction mixture was 200 μg/mL.

catechin. Phlorotannin content correlated well with the inhibition activity. Recently, lectin-like ox-LDL receptor was discovered in cartilage of arthritic rat, and was found that the induction of the expression of lectin-like oxidized LDL receptor was accompanied by the accumulation of ox-LDL in chondrocytes, suggesting the possible interaction of ox-LDL with lectin-like oxidized LDL receptor in cartilage (Nakagawa *et al.*, 2002). It was also observed that the blocking of the interaction suppressed joint swelling, leukocyte infiltration and cartilage degradation in rat zymosan-induced arthritis suggesting a new beneficial method for treating joint diseases such as rheumatic arthritis or osteoarthritis. Therefore, it is also important to evaluate the ability to inhibit LDL oxidation. Although LAD103 showed lower ferric reducing ability than catechin, it showed much higher inhibition than catechin in LDL oxidation. Phlorotannin compounds possess a unique structure which is not found in terrestrial plants (Shibata *et al.*, 2003). Especially the compounds with dibenzo-1,4-dioxin skeleton which are only found in limited algae species are the most interesting ones in medicinal sense because of their small size (Mw 300-800) and their rigid structure owing to dibenzo-1-4-dioxin linkage, enabling them to strongly interact with various biological molecules (Kang *et al.*, 2003; Glombitza and Gerstberger, 1985). Furthermore, these compounds are of polyphenolic nature to provide strong antioxidant characteristics.

We examined the influence of LAD103 in LPS-induced generation of PGE_2 using RAW 246.7 cells. While PGE_2 was barely detectable in non-stimulated cells, more than hundred-fold PGE_2 was detected in the stimulated cells. LAD103, celecoxib and aspirin all showed significant inhibition of PGE_2 generation in the concentration range tested (10~100 mg/mL). LAD103 showed inhibition of 61%, 85%, 92%, and 99% at concentration of 10, 30, 60, and 100 mg/mL, respectively, showing similar activity to celecoxib which showed 65%, 79%, 85%, and 96%, respectively. (Fig. 4) While LAD103 and celecoxib showed similar dose-dependent inhibition, aspirin showed somewhat anomalous fashion at the tested concentration range.

Rabbit articular cartilage explant culture was treated with recombinant human interleukin 1α (rhIL-1α) to induce proteoglycan degradation. The amount of glycosaminoglycan released into the medium was measured as an index of proteoglycan degradation. When the rabbit cartilage explants were treated with rhIL-1α for 60 h, the amount of released glycosaminoglycan into the culture medium increased significantly compare to the vehicle treated group (1.44 ± 0.06 μg/mg vs. 0.30 ± 0.01 μg/mg, Fig. 5). 10 μM (3.2 mg/μL) diclofenac which is known as a selective COX-2 inhibitor was used as a positive control. LAD103 significantly interfered with the rhIL-1α-mediated degradation of proteoglycan in all concentrations tested ($p < 0.001$). It showed 53%, 79%, 81%, and 70% of inhibition at 1, 3, 10, and 30 mg/mL concentration, respectively.

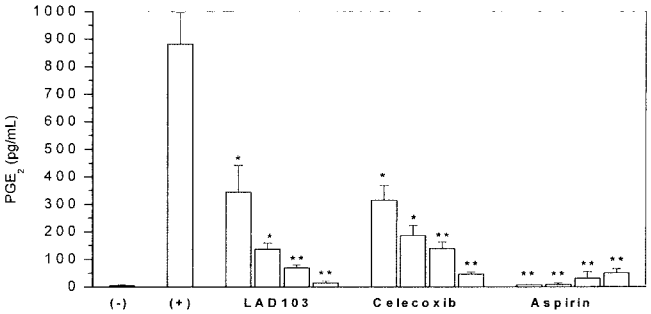

Fig. 4. Influence of LAD103 on LPS-induced PGE_2 generation in RAW 264.7 cells. RAW 264.7 cells were incubated with LPS and test samples. For each tested sample, concentration was, from left to right, 10, 30, 60, 100mg/mL. $^{*}P < 0.05$, $^{**}P < 0.01$ versus positive control using Student's t-test.

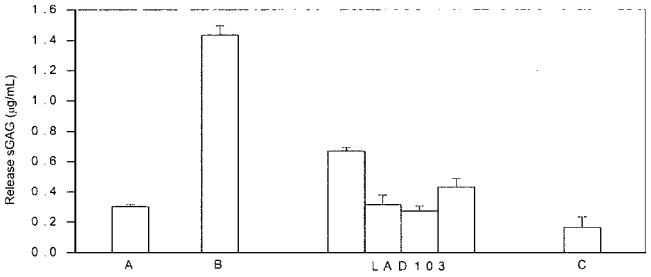

Fig. 5. rhIL-1α induced degradation of proteoglycan in rabbit cartilage explant culture. (A) no rhIL-1α, (B) rhIL-1α, (C) rhIL-1α + diclofenac (10 μM), For LAD103, rhIL-1α + 1, 3, 10, and 30 μg/mL (from left to right).

IL-1 is known to be a key cytokine in the pathogenesis of arthritis. IL-1 is known to generate reactive oxygen species and promote PGE_2 production in human chondrocytes (Mathy-Hartert *et al.*, 2002). IL-1-mediated induction of COX-2 produces high levels of PGE_2, which mediates cartilage resorption by decreasing proliferation of chondrocytes, enhancing matrix metalloproteinase activity, and inhibiting aggrecan synthesis in chondrocytes. (Taskiran *et al.*, 2000) We examined the influence of LAD103 in human recombinant IL-1α induced proteoglycan degradation in explant culture of rabbit articular joint cartilage in order to evaluate its protective effect against degradation of cartilage under arthritic situations (Choi *et al.*, 2002). LAD103 significantly reduced proteoglycan degradation at 1~30 mg/mL. This result can be attributed to its multifactorial features such as antioxidant capacity against diverse ROSs, down-regulation of PGE_2 generation, inhibition of PLA_2. The reported inhibitory activities of some phlorotannin compounds against LOX and hyaluronidase may additionally contribute to this protective effect. ACECLO which is also known to exert its effect *via* multifactorial fashion (Mathy-Hartert *et al.*, 2002) acts not only by inhibiting

preferentially the COX-2 activity, but also by decreasing proinflammatory cytokines synthesis and by scavenging peroxynitrite radical. Since cartilage is an avascular tissue, chondrocytes are under an environment prone to repeated ischemia and reperfusion, under which high oxidative stress can be generated (Henrotin *et al.*, 2003). Therefore, it is considered beneficial to add appropriate antioxidants in the current arthritic treatments. Phlorotannin-rich extracts from *Ecklonia cava* showed strong activities in both DPPH radical scavenging and FRAP tests, suggesting their potential as a new category of antioxidants for this purpose.

In a clinical sense, suppression of the upstream stimulation of proinflammatory factors through neutralization of excessive ROS together with inhibition of the downstream degenerative inflammatory consequences by a natural nontoxic agent in combination with current modalities of treatment can be of therapeutic value for effective treatment of both inflammatory and degenerative joint disorders.

In conclusion, since it was demonstrated that phlorotannin-rich *Ecklonia cava* extracts including LAD103 could efficiently reduce several key risk factors involved in the pathogenesis and pathophysiology of osteoarthritis, and was also demonstrated that LAD103 could actually reduce the proteoglycan degradation in the joint explant culture, they are considered as promising candidates for the therapeutic or prophylactic agents for arthritic treatment, encouraging further studies for revealing its mechanism of action and confirming its clinical efficacy.

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