Antioxidant and protective effects of Sumac Leaves on chondrocytes

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In this study we evaluate in vitro antioxidant and chondroprotective effects of a lyophilised hydroalcoholic extract from Sumac leaves. The extract contains many constituents as flavones, tannins, anthocyanins and organic acids with several known biological effects. The antioxidant activity of extract was assayed employing 1, 1-diphenyl-2-picrylhydrazyl radical scavenging method (DPPH). The antioxidative/chondroprotective effect was evaluated on the production of key molecules released during degenerative osteoarthritis disease (OA) such as nitric oxide (NO), reactive oxygen species (ROS), glycosaminoglycans (GAGs), and prostaglandins (PGE2) in human chondrocyte cultures, stimulated with pro-inflammatory cytokine interleukin-1β (IL-1β). As result, the extract from Sumac leaves was effective in reducing the stable free radical DPPH, and in vitro was able to contrast the harmful effects of IL-1β.

Key words: DPPH, interleukin-1β, chondrocytes, nitric oxide, prostaglandins, glycosaminoglycans, reactive oxygen species.

INTRODUCTION

Sumac (Rhus coriaria L., family Anacardiaceae) is a well known, popular spice and has been utilized extensively for medicinal and other purposes. Its leaves have been used as a tanning agent for their high tannin content. The berries have diuretic properties, and are used in bowel complaints and for reducing fever. Other reports indicated that the extract of Sumac is used in traditional medicine as a medicinal herb for its antimicrobial and wound healing activity (Rayne and Mazza, 2007).

Recently, phytochemical studies of this plant reported that its leaves contained phenolic acids (Mavlyanov et al., 1995) (gallic acid, protocatechuic acid, p-OH-benzoic acid and vanillic acid), anthocyanins (cyanidin, peonidin, pelargonidin and petunidin), hydrolysable tannins (Mavlyanov et al., 1997) and gallic acid derivatives, condensed tannins, several flavonoids as quercetin and kaempferol glycosides (Zalacain et al., 2003). These substances have gained interest because may reduce the risk of chronic diseases reinforcing the defences against free radical species.

Osteoarthritis (OA) is a chronic disease in which the principal target of the pathogenic process is the articular cartilage. Cartilage consists of a relatively small number of chondrocytes and many extracellular matrix (ECM) components. Chondrocytes synthesize and catabolize ECM macromolecules. The normal structure and function of articular cartilage are the result of a precisely balanced interaction between anabolic and catabolic processes. When this process is altered, a series of changes occurs in the morphological and biomechanical characteristics of cartilage. The destructive effects of substances, such as nitric oxide (NO) and reactive oxygen species (ROS), inflammatory cytokines (e.g. IL-1β and TNF-alpha) and metalloproteinases (MMPs) determine a progressive damage of the articular cartilaginous tissue (Amin et al., 1997). Nitric oxide is a free radical that is synthesized as a result of the oxidation of the amino acid L-arginine.
through the enzyme nitric oxide synthase (NOS). It promotes vasodilatation and permeability in joints by increasing the secretion of TNF-α and IL-1β (Mathy-Hartert et al., 2002). The secretion of nitric oxide can act on articular cartilage inhibiting the synthesis of collagen and proteoglycans, stimulating the synthesis of metalloproteinases and inducing apoptosis of the chondrocytes. Recent evidence indicates that most of the cytotoxicity attributed to NO is rather due to peroxynitrite, produced from reaction between NO and another free radical, the superoxide anion. Peroxynitrite interacts with lipids, DNA and can play a significant role in cartilage loss and chondrocyte death during the development of arthritis. Inflammatory cytokine IL-1β is a pivotal driving force in inducing and substaining cartilage damage that usually leads to the loss of sulfated glycosaminoglycans (GAGs). It induces a catabolic response, stimulates metalloproteinase expression, inhibits the proliferation of chondrocytes, and the synthesis of collagen II and proteoglycans, besides determines high levels of prostaglandins E₂ (PGE₂) and nitric oxide (NO) (Moncada et al., 1995).

On the basis of this consideration and evaluating the interesting properties of the active compounds contained in *R. coriaria* L., in the present work we estimated the free radical scavenging activity (DPPH assay) of Sumac leaves extract and assessed if the pool of active compounds contained in a lyophilised extract of these leaves might inhibit the production of key molecules released during chronic oxidative/inflammatory events, such as NO, PGE₂, ROS and GAGs employing an *in vitro* model based on human chondrocytes cultures stimulated with IL-1β. This model is particularly useful to reproduce the mechanisms involved in degenerative arthropathies, as osteoarthritis, where oxidative-inflammatory stress determines a progressive destruction of the articular cartilaginous tissue (Panico et al., 2007).

**MATERIALS AND METHODS**

**Materials**

All chemicals were purchased from Sigma Aldrich Co (Milano, Italy). All the chemicals and reagents were of analytical grade.

**Plant**

Preparation of the lyophilised extract of Sumac (*Rhus Coriaria* L.) leaves: Leaves of Sumac (*R. coriaria* L.) were obtained from a commercial plantation in Catania (Sicily). Leaves (25 g) were extracted with 150 ml of solvent (ETOH) by maceration (24 h x 3 times). The obtained extract was separated from the residue by filtration through Whatman No. 14. After the removal of ethanol under vacuum, the solid residue obtained was suspended in water and then lyophilised. For the *in vitro* tests the lyophilised was reconstituted in the suitable ETOH/H₂O solution (1:1).

**DPPH Assay**

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging activity of natural antioxidants (Brand-Williams et al., 1995).

**Human articular chondrocyte culture**

Human articular cartilage was obtained at replacement surgery from patients (aged 20 - 40) with femoral neck accidental fractures and that informed consent was obtained. The isolation procedure was conducted under aseptic conditions. The cartilage was cut into small fragments and carefully washed using Dulbecco’s Modified Eagle’s Medium (DMEM) culture medium containing NaHCO₃, 25 mM Hepes, 1 mM sodium piruvate, 50 mg/mL gentamycin, 100 U/mL penicillin, 100 mg/mL streptomycin and 2.5 mg/mL amphotericin B. Chondrocytes were isolated through 3 sequential passages of enzymatic digestion of the extracellular matrix. Incubation with 0.1% hyaluronidase type III (1 mg/mL for 100 mg of cartilage), for 30 min at 37°C; incubation with 0.5% pronase type XIV (5 mg/mL for 100 mg of cartilage), for 60 min at 37°C; finally incubation with 0.2% collagenase type IA (2 mg/mL for 100 mg of cartilage), for 45 min at 37°C. The cellular suspension obtained was filtered (filters from 100 and 70 mm) to eliminate the residues of the digestion and the cellular aggregates and to obtain a monocollecular suspension of chondrocytes. This was washed 3 times with DMEM supplemented with 10% foetal calf serum (FCS), and was subjected to vital coloration method staining with eosin in order to determine the number and the vitality of recovered cells. After 24 h the medium was removed and cells were treated as follows: a) Control, b) IL-1β (10 ng/ml), c) *R. Coriaria* extracts (10, 100 and 200 μg/ml) + IL-1β (10 ng/ml), d) Quercetin (10,100, 200 μM) + IL-1β (10 ng/ml). Quercetin was used as reference compound. After 120 hr the supernatants of chondrocytes culture were collected for different assays.

**Cell viability assay**

The cytotoxic effect of the experimental substances was evaluated by a cell viability test based on the cleavage of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells (Mossmann, 1983).

**Determination of reactive oxygen species (ROS)**

Reactive species production was estimated by using the fluorescent probe 2’-7’-dichlorodihydrofluorescein diacetate (DCFH-DA) purchased from Molecular Probes, Eugene, OR, USA. DCFH-DA diffuses through the cell membrane and is enzymatically hydrolysed by intracellular esterases to the non fluorescent DCFH. Intracellular ROS are able to oxidise DCFH to the fluorescent 2’-7’-dichlorofluorescin (DCF), whose intensity of fluorescence is directly proportional to the levels of intracellular ROS. Briefly, 5 mM DCFH-DA was added to the different groups of cultured chondrocytes and cells were kept in a humified atmosphere (5% CO₂, 37°C) for 30 min, washed in PBS, trypsinised, suspended in 1 ml PBS, centrifuged at 800 g for 10 min, and finally resuspended in 2 ml PBS. Fluorescence was measured as I. F. /mg protein, using a spectrofluorimeter (Perkin-Elmer) λex = 485 nm, λem = 525 nm. The amount of protein/sample was determined according to Bradford (Bradford 1976).

**Determination of nitrite levels**

Nitrite was determined by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 5% of hydrochloric acid) to 100 μl of samples (Green, 1982).
The optical density at $\lambda = 570$ nm was measured using a microtitre plate reader. Nitrite concentrations were calculated by comparison with respective optical densities of standard solutions of sodium nitrite prepared in medium.

**Determination of glycosaminoglycans (GAGs)**

The level of GAGs was measured by spectrophotometry with a solution of 1.9-dimethylmethylene blue at $\lambda = 535$ nm (Farndale et al., 1989). The amount of glycosaminoglycans was calculated from a standard curve obtained for shark chondroitin sulphate.

**Determination of prostaglandins (PGE$_2$)**

PGE$_2$ was determined in the culture supernatant by the enzyme immunoassay (EIA) system using a commercially available immunoassay kit (Amersham-Pharmacia, UK) according to the manufacture’s instructions. The detection limit is 1 ng/ml. The values were expressed as ng/ml PGE$_2$ released.

**Statistical analysis**

All the present results are means ± SEM of 3 experiments performed on quadruplicate samples. The Student’s t-test was used to evaluate the differences between the means of each group. p < 0.05 was considered to be statistically significant.

**RESULTS**

In the present study, the free radical scavenging activity of *R. coriaria* leaves extract was investigated using the DPPH test. The anti-inflammatory/chondroprotective effect of *R. coriaria* leaves extract on human chondrocytes was evaluated at different concentrations (10, 100 and 200 $\mu$g/ml) by determining in vitro cellular viability, NO, PGE$_2$, ROS production and GAGs release.

**Radical scavenging activities**

The radical scavenging activity of the different concentrations of *R. coriaria* leaves extract (10, 100, 200 $\mu$g/ml) and Quercetin (10, 100, 200 $\mu$M) are shown in Figure 2. The DPPH radical is a stable free radical and the DPPH radical-scavenging activity was determined by the decrease in absorbance at 515 nm. The DPPH radical-scavenging capacity, in this study, was reported after 10 min reaction time for each sample. The results were expressed as % inhibition DPPH.

The lyophilised extract showed potent free radical-scavenging activity on the DPPH radical compared to quercetin (Figure 1).

**Anti-oxidative /chondroprotective effects of sumac (Rhus Coriaria L)**

The anti-inflammatory/chondroprotective effects of Sumac (*R. coriaria* L) on human chondrocytes were evaluated at 10, 100, 200 $\mu$g/ml concentrations by determining in vitro cellular viability, NO, PGE$_2$ production and GAGs release after 120 hr after the treatment.

**Cell viability assay**

MTT assay showed that the tested compounds did not reduce the chondrocytes ability to metabolise tetrzolium salts, demonstrating that they did not interfere with cell viability (data not showed).

**Determination of nitrite (NO)**

The results, reported in Figure 3, showed that NO production in chondrocytes under basal conditions (untreated control) is very low and mainly due to activity of the constitutive nitric oxide synthetase (cNOS). When human chondrocytes were treated with IL-1$\beta$ (10 ng/ml) a substantial increase in NO production was observed. *R. coriaria* leaves extract, when combined with IL-1$\beta$, caused a dose-dependent inhibition in the production of NO, similar to quercetin combined with IL-1$\beta$. IL-1$\beta$, Quercetin 200 $\mu$g/ml + IL-1$\beta$, and Sumac extract 200 $\mu$g/ml + IL-1$\beta$ produced 42 ± 0.5, 12 ± 0.7 and 4 ± 0.2 mM respectively.

**Determination of glycosaminoglycans (GAGs)**

Figure 4 shows the levels of GAGs in the chondrocyte culture medium. As can be seen, the GAGs concentration was significantly reduced in cells exposed to IL-1$\beta$. Extracts of *R. coriaria* leaves or quercetin added to the chondrocytes prevented the inhibition of GAGs synthesis in dose dependent manner.IL-1$\beta$, Quercetin 200 $\mu$g/ml + IL-1$\beta$, and Sumac extract 200 $\mu$g/ml + IL-1$\beta$ produced 75 ± 2.1, 145 ± 4 and 140 ± 2 mg/ml respectively.

**Determination of prostaglandins (PGE$_2$)**

The effects of the extract of *R. coriaria* leaves on the prostaglandin E$_2$ (PGE$_2$) production induced by IL-1$\beta$ in human articular chondrocytes were presented in Figure 5. When human chondrocytes were treated with IL-1 $\beta$
(10 ng/ml) an increase in production was observed. All the samples, included ones treated with quercetin, when combined with IL-1β, exhibited significant reduction of PGE₂ production. IL-1β, Quercetin 200 µg/ml + IL-1β and Sumac extract 200 µg/ml + IL-1β produced 41 ± 2, 12 ± 1 and 15 ± 2 ng/ml, respectively.

**Determination of reactive oxygen species (ROS)**

In Figure 6 the levels of ROS production are reported. As expected, IL-1β treatment induced a massive ROS production compared to the untreated control. The treatment of extract of *R. coriaria* leaves at 10-100-200 µg/ml...
did not modify the production of ROS in comparison with the untreated group (1150 ± 30 I.F. /mg protein). The treatment of extract of *R. coriaria* leaves combined with IL-1β showed a significant and dose-dependent reduction of ROS levels with respect to IL-1β. IL-1β, Quercetin 200 µg/ml + IL-1β and Sumac extract 200 µg/ml + IL-1β produced, 5100 ± 50, 2000 ± 80 and 2700 ± 70 I.F./mg protein, respectively.

**DISCUSSION**

The importance of this study is that it gives new information on the application of a extract of Sumac leaves in the treatment of chronic diseases as osteoarthritis (OA). This latter is a crucial pathogenic aspect of oxidative-inflammatory stress and degenerative characterized by a progressive destruction of the articular cartilaginous tissue. This phenomenon represents a central moment of the pathogenetic course, where metabolic modifications of the cartilaginous tissue and chondrocytes have a fundamental role. OA appears to be the result of an imbalance between the destructive and reparative/synthetic processes of the articular cartilage due to the critical effects of free radicals (ROS and NO), inflammatory cytokines (e.g. IL and TNF-alpha) and metalloproteinases.

**Figure 4.** GAGs release (means ± S.E.M.) in the culture medium from articular chondrocytes 120 h after the addition of *Rhus coriaria* L. extract and Quercetin at 10, 100, 200 µg/ml with IL-1β. Values are expressed as µg /ml. *significantly different from IL-1β treated samples (p < 0.05).

**Figure 5.** PGE₂ production (means ± S.E.M.) in the culture medium from articular chondrocytes 120 h after the addition of *Rhus coriaria* L. extract and Quercetin at 10, 100, 200 µg/ml with IL-1β. Values are expressed as ng /ml. *significantly different from IL-1β treated samples (p < 0.05).
Figure 6. ROS production (means ± s.e.m.) in the culture medium from articular chondrocytes 120 h after the addition of *Rhus coriaria* L. extract and Quercetin at 10, 100, 200 μg/ml with IL-1β. Values are expressed as I.F./mg protein. *Significantly different from IL-1β treated samples (P < 0.05).

(MMP) (Cipoletta et al., 1998).

The cytokine IL-1β is responsible, at least in part, of the changes in osteoarthritic joint tissues (Martin et al., 1999).

It is the major autocrine pro-inflammatory cytokine involved in the stimulation of catabolic factors. IL-1β, in fact, interferes in extracellular matrix turnover, accelerates the degradation of cartilage matrix, and induces chondrocytes apoptosis (Chu et al., 1992). Moreover, IL-1β is a pivotal driving force in inducing and sustaining cartilage damage that usually loading to the loss of sulfated glycosaminoglycans (GAGs), fundamental components of cartilaginous extracellular matrix (Blanco et al., 1995).

Additionally, it has been shown that IL-1 β induces the production of NO that reacts rapidly with superoxide anions (O₂⁻) to form peroxynitrite (ONO²⁻), and other oxidants (Moncada, 1995), such as hydrogen peroxide (H₂O₂) and hydroxyl radicals. Superoxide, hydroxyl and peroxyl radicals represent different forms of ROS. The catabolic effect of NO determines besides the inhibition of proteoglycans synthesis and stimulates in the chondrocyte production of proenzymes, which are converted into active enzymes, as metalloprotease (MMPs). In addition, during the inflammatory processes, ROS determine induction of the PGE₂ synthesis, and upregulate IL-1β (Manfield et al., 2000). The presence of ROS may affect on matrix more directly, resulting in the destruction of the integrity of its components, including proteoglycans, hyaluronic acid and collagen. During the inflammatory processes, PGE₂ production is intensified and this contributes to synovial inflammation by increasing local blood flow and by potentiating the effects of mediators such as bradykinin, responsible for relevant vasopermeability (Manfield et al., 2000).

Recent studies (Panico et al. 2005) showed that in plants there are bioactive compounds with human health benefits and that are considered to represent essential components and have gained increasing interest as potential therapeautic agents against a wide variety of disease.

In the last years, we looked for novel antioxidative/ antiinflammatory/chondroprotective substances from natural products as potential suppressor of mediators involved in cartilage destruction (Panico et al., 2007). Thus, several studies were conducted with human cultured chondrocytes stimulated with IL-1β that is particularly useful to reproduce the mechanisms involved in the pathogenesis of cartilage damage. In the present research, the experiments were carried out in presence of quercetin, used as control, because it represents the main polyphenolic component of *R. coriaria* leaves (Mavlyanov et al., 1995).

Our data demonstrate that *R. coriaria* Leaves extract could act on the three key pathogenetic mechanisms of degenerative joint disease: oxidative stress, inflammation and chondrodegeneration. In fact, the addition of this extract of *Sumac* leaves to human chondrocyte cultures reduced the production of NO, ROS and PGE₂ induced by IL-1β and increased the synthesis of GAGs in a dose dependent manner. The action exhibited by *R. coriaria* leaves extract under our experimental conditions could be partially due to the scavenger action of its polyphenolic active constituents, in particular flavonoids, such as
quercitin, and kaempferol glycosides (Zalacain et al., 2003), phenolic acids (Mavlyanov et al., 1995) (gallic acid, protocatechuic acid, p-OH-benzoic acid and vanillic acid) and hydroxycynamic acids (caffeic acid, ferulic acid, p-cumaric acid and cinamic acid). These poliphenolic in R. coriaria leaves extract once introduced into the lipid bilayer of the cell membranes performe an anti-apoptotic or cytoprotective action, which prevents cell death caused by reactive oxidative substances (ROS). The antioxidant effect of extract is also associated with an anti-inflammatory/chondroprotective action. The results of our study demonstrate that the addition of extract of Sumac leaves on human chondrocyte cultures reduces the production of NO and ROS and prevents the inhibition of GAGs synthesis and PGE₂ contrasting the IL-1β effects.

Conclusion

The importance of this study is that it gives new information on the application of a medicinal plant as Sumac leaves in the treatment of chronic diseases as osteoarthritis. Our data show that R. coriaria leaves which are rich in flavones, tannins, anthocyanins, and organic acids (Mavlyanov et al., 2001) would be a good source of bioactive antioxidant substances. Further studies are required on the antioxidant components derived from R. coriaria leaves and their potential application in joint disease therapy.

REFERENCES


