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The Effects of Rosemary *(Rosmarinus officinalis L.)* Extract on the Oxidant Stress Indexes and Proliferation Capacities of SW1353 Chondrosarcoma Cells

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Abstract: Arthritis is characterized by symptoms such as joint pain, swelling, and limitation of movement. The main objectives of treating the disease are eliminating pain and inflammation and protecting joint functions. Several herbal remedies have been reported to be applied in specific forms to treat arthritis or prevent some ailments. In this study, the effects of rosemary extract (RE) were examined in terms of proliferation capacity, total antioxidant status (TAS) and total oxidant status (TOS) and oxidative stress index (OSI) on SW1353 cell line to see whether rosemary extract can be used in the treatment of arthritis. The cytotoxic effect of RE, which appears in high doses, draws attention to its pharmacovigilance in its use. Our findings indicate that RE at low concentrations, regardless of its antioxidant properties, may be utilized to treat arthritis associated with cartilage damage.
Keywords: Antioxidant, chondrosarcoma, oxidative stress index, rosemary extract

Biberiye *(Rosmarinus officinalis L.)* Ekstraktının SW1353 Kondrosarkom Hücrelerinin Oksidan Stres İndeksleri ve Çoğalma Kapasiteleri Üzerindeki Etkileri

Öz: Artrit, eklem ağrısı, şişme ve hareket kısıtlılığı gibi semptomlarla karakterizedir. Hastalığın tedavisinde temel amaç ağrı ve iltihabı ortadan kaldırmak ve eklem fonksiyonlarını korumaktır. Artriti tedavi etmek veya bazı rahatsızlıklarını önlemek için belirli formlarda çeşitli bitkisel ilaçlar uygulanır. Bu çalışmada biberiye ekstraktının SW1353 hücre hattı üzerindeki proliferasyon kapasitesi, toplam antioksidan seviyesi (TAS), toplam oksidan seviyesi (TOS) ve oksidatif stres indeksinin (OSİ) etkileri incelenmiştir. Yüksek dozlarda ortaya çıkan biberiye ekstraktının sitotoksik etkisi, farmakovijilansı kullanımında oldukça dikkat çekmektedir. Bulgularımız, antioksidan özelliklerinden bağımsız olarak düşük konsantrasyonlarda biberiye ekstraktının artritten kaynaklı kıkırdak hasarı tedavisinde kullanılabileceğini göstermektedir.
Anahtar kelimeler: Antioksidan, biberiye özü, kondrosarkom, oksidatif stres indeksi

Introduction

Arthritis can be defined briefly as the inflammation or swelling of one or more joints. Along with joints and neighboring tissues, it may also affect other connective tissues leading to the impairment of internal organs, such as the heart, lungs and kidneys, in conditions such as rheumatoid arthritis, lupus and fibromyalgia (Garcia, 2019; Giles et al., 2020). When cartilage is damaged, the ends of the bones become unprotected, resulting in pain, swelling, and stiffness in the joints (Chua et al., 2019; Kolasinski et al., 2020). In recent years, the developments and inventions observed in pharmacognosy and food science have provided opportunities using scientific methods to support the idea that taking certain natural substances, extracts, or chemicals beneficial for our body in specific forms has effects on preventing discomfort (Ng and Azizudin, 2020; Rehman et al., 2019). Today, several herbal-treatment methods, under the name of alternative medicine, are widely used in treating arthritis and relief of symptoms with both local and systemic applications, in addition to existing modern treatment methods (Ko, 2019; Kose et al., 2019).

While osteoarthritis (OA) is the most common form of arthritis defined by joint degeneration and cartilage loss, rheumatoid arthritis (RA) is a chronic, inflammatory, multi-systemic, and autoimmune disease that affects the joints and can progress with disfigurement. Even though these two diseases have very different etiopathogenesis and reveal other clinical manifestations, they are both characterized by the destruction of connective tissue, especially cartilage

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tissue, leading to inflammation and pain shows similarities in both diseases (Chua et al., 2019; Schroeder et al., 2020).

Free radicals and inflammation cause premature apoptosis of cartilage cells in the joint and slow down the damaged cartilage tissue regeneration, and the disease becomes chronic and permanent (Lepetsos et al., 2019; Saxena and Batra, 2020). An increase in proinflammatory cytokines in chronic inflammatory manifestations causes an increase in oxygen free radicals (OFRs). OFRs are highly reactive compounds that affect macromolecules such as lipids, proteins, DNA and carbohydrates, causing deterioration in their structure (Lepetsos et al., 2019; Pradhan et al., 2019; Saxena and Batra, 2020). Free radicals such as reactive oxygen species, superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$), peroxyl radical ($ROO^{\cdot\,\cdot}$), and non-radical hydrogen peroxide (H_2O_2) in biological systems are among the most critical causes of oxidative stress (Hatai and Banerjee, 2019; Miao et al., 2020). While OFRs are generated endogenously in physiological conditions, their formation increases with exogenous factors such as increased immobility, malnutrition, environmental pollution, pesticides, UV, radiation and X-ray (Wu and Pan, 2019; Zahan et al., 2020).

Rosemary (*Rosmarinus officinalis* L.) has been described as an aromatic self-growing plant and perennial herb, usually within the maquis flora. The chemical structure and antioxidant activity of rosemary extract (RE) has been investigated by various researchers (Cheung and Tai, 2007; Genena et al., 2008). Two main rosemary oils have been reported, the first comprises 1,8-cineole at a rate of more than 40%, and the second contains approximately equal amounts of 1,8-cineole, R-pinene, and camphor. There are potent antioxidants in rosmarinic acid and carnosic acid in rosemary leaves, carnosic acid being the most powerful antioxidant for animal fats. Abietatrien-derived diterpenes are responsible for 90% of the antioxidant effect of carnosic acid and carnosol in RE (Vallverdú-Queralt et al., 2014; González-Vallinas et al., 2015; Andrade et al., 2018). Both raw and refined extracts of rosemary leaves are found in trade. RE is commonly used today to preserve food products (Vlavcheski and Tsiani, 2018; Jaglanian and Tsiani, 2020). Studies showed that RE containing 20% carnosic acid added to the diet of rats reduced oxidative stress in elderly rats and RE reduced the antioxidant enzyme activity, lipid peroxidation and OFRs in the heart and brain tissue, and the OSI activity in the brain; hence, revealed its antioxidant protective effects. Also, RE reduces the neuronal damage caused by H_2O_2 and protects against neurodegenerative diseases due to oxidative stress and apoptosis. We aim to investigate the impact of RE, which can be taken orally with meals and/or applied locally, on articular joints, as a supplement to classical medical treatment methods in arthritis treatment and investigate its acting mechanisms.

Materials and Methods

Rosemary extract preparation

The plant materials used in the study were collected as leafy shoots in the Adana-Kozan region by cutting with scissors. The samples were kept in the shade for 8-10 days and dried. The leaves were separated from the dried leafy branches and the dry leaves were bagged and labeled and kept in a dry and cool place in the laboratory for distillation and extraction processes. Existing rosemary stocks were diluted with DMEM containing 20% dimethyl sulfoxide (DMSO). The final concentration of DMSO is 0.1%, and this concentration does not have a toxic effect on cells. Rosemary solutions were sterilized by passing through a 0.2mm filter and added to cell cultures. Dried rosemary leaves were ground and steeped overnight (16 hours) in a 1:1 mixture of dichloromethane and methanol, followed by filtration. After filtering, the solvent was set aside while the leaves were boiled in methanol for 30 min. After boiling, the solvent was combined with the filtered solvent. Rotary evaporation was used to remove the combined solvent from the final extract, and the green powder was collected and stored at -20^{0}C in a light-protected environment. Aliquots of DMSO were prepared to a final concentration of 100 mg/mL. RE powder was dissolved in DMSO to make a 100 mg/mL stock solution. The RE stock (400 µg/mL) was created by treating the cells with cell culture media (n=3) (Figure 1).

Figure 1. Oxidative stress and inflammatory response were examined by applying Lipopolysaccharide (L).

Cell culture and treatment

SW1353 chondrocyte cells (HTB-94, American Type Culture Collection-ATCC) were cultured in DMEM containing 100 units/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of Amphotericin B and FBS (10% v/v) with 5% CO_2, at 37^{0}C. A working stock of RE (400 g/mL in DMEM, stored at -20^{0}C in a light-protected environment) was used to treat the cells and the final concentration of DMSO in the RE-treated cells was less than 0.1%. The cells were indicated in DMSO at the same concentration as cells exposed to RE (vehicle control), which did not affect

the measurements (Ketsawatsakul, 2007).

Cell proliferation assay

SW1353 cell proliferation was evaluated by using WST-1 assay. Absorbance at 450nm was measured with a microplate reader (Synergy HTX, Biotek, US). After seeding cells, the colorimetric assay was performed for 48hr.

Preparation of cell lysates for oxidative stress analyses

A solution containing 15mM TRIS-HCl, 150mM NaCl, 0.1mM EDTA, and 0.5% Triton-X was prepared for lysing cultured cells. The pH was adjusted to 7.5 and the lysing solution was prepared fresh and kept at +4^{o}C until use. After the cells were washed with PBS, chilled lysis buffer was added to the wells. Plates were kept on ice for 10min and cell lysates were transferred into micro-centrifuge tubes by pipetting. Lysates were centrifuged at 5000rpm for 5min. The supernatants were stored at +4^{o}C until the TAS and TOS levels were measured. All biochemical analyzes were performed on the same day, within 1-2h.

Total antioxidant status measurement

The working principle of the method is that Fe2+ -*o*-dianicide complex generates an OH radical by generating a Fenton-type reaction with H_2O_2. This solid reactive oxygen type is reduced and reacts with the colorless *o*-dianicide molecule at low pH to generate yellow-brown dianicidil radicals. Dianicidil radicals increase color formation by participating in advanced oxidation responses. However, antioxidants overturn these oxidation reactions and stop color construction. This reaction calculates the optical density measured at 660nm spectrophotometrically (Rel Assay Diagnostics, Germany) (Erel, 2004). The blue-green colored reduced 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) generated during the response was calculated in mmolEq/L. Values for standardization were interpreted by proportioning to the total protein amounts of the cells in each application well.

Total oxidant status measurement

The method is based on the principle that oxidants oxidize the ferrous ion-*o*-dianicide complex to the ferric ion. The glycerol accelerates this reaction and triples it approximately. Ferric ions generate a colored complex with xylenol orange in an acidic environment. The color reaction is directly related to the number of oxidants determined by spectrophotometric evaluation. The number of total oxidant molecules generated by measurements at 530nm was expressed in H_2O_2 formed (mmolH_2O_2Eq/L) (Rel Assay Diagnostics, Germany) (Erel, 2005). Values for standardization were represented in proportion to the total protein amounts of the cells. Values for standardization were interpreted by proportioning to the total protein amount of the cells in the application well.

Calculation of oxidative stress index

Considering that it would be a more meaningful parameter in determining the degree of oxidative stress (Bolukbas et al., 2005; Demirbag et al., 2007).

OSI = [TOS (µmolEq/L) / TAS (mmolEq/L) x 100]

Statistical evaluation

The data are the mean±SD (Mean±Standard Deviation) error mean of the indicated number of independent experiments. All the data were subjected to statistical analyses using the ONE-Way ANOVA and Tukey's test for the multiple comparisons of means. The homogeneity of variances was analyzed according to the Brown-Forsythe and Bartlett test. The statistical significance level was assumed at P<0.05. Statistical tests were performed using GraphPad Prism software.

Results

Effects of RE on SW1353 chondrosarcoma cell proliferation

Treatment with RE resulted in a dose-dependent inhibition of cell proliferation. A significant increase in cell proliferation (P<0.001) was seen with 1 and 50 µg/mL doses of RE, while a maximum decrease was caught between 50µg/mL and C (P=<0.001), suggesting a toxic effect of RE on cells (Figure 2). Treatment of the cells with 1-10µg/mL did not result in any significant inhibition of cell proliferation. In contrast, treatment with 50µg/mL showed a considerable increase in cell proliferation compared to D and L (P< 0.05).

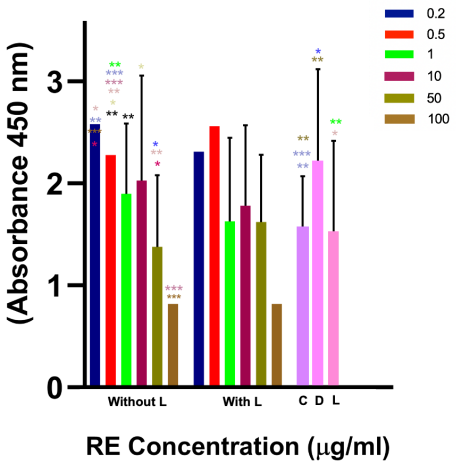

Figure 2. WST-1 cell proliferation assay with different doses with L (1-50µg/mL ***p=0.0005, 50-Cµg/mL ***p=0.0002, 50-Dµg/mL **p=0.0038, 50-Lµg/mL **p=0.0019).

Cells treated with 0.2-50mg/ml concentrations of RE for 48h showed no difference in TOS values compared to the control (C=no treatment), DMSO (D=0.1% DMSO) and L (L=0.1% Lipopolysaccharide) groups (Figure 3A). 100mg/ml dose of RE demonstrated the highest increase in TOS value either with/without L. A significant decrease in TOS activity was observed with RE (0.2 and 0.5mg/ml) at 48h compared to 100mg/ml concentration (P<0.001), both in groups treated with/without L, which correlated with the proliferation capacity of the cells.

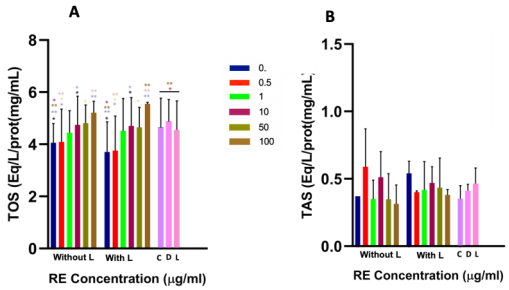

Figure 3. Expression of RE with/without L obtained from (A) TOS, (B) TAS in different doses comparison with C.

TAS assay measured whether RE could inhibit the antioxidant capacity of SW1353 cells. No statistically significant difference was observed in TAS values among the study groups (Figure 3B). In the group where 0.5 mg/ml RE dose was applied, an increase in TAS value was observed compared to C and L-treated groups. The TAS effect of 0.5mg/ml RE on cells was higher than the TAS effect of C and L-treated groups. Again, cells with 0.5mg/ml RE dose were more sensitive than the others.

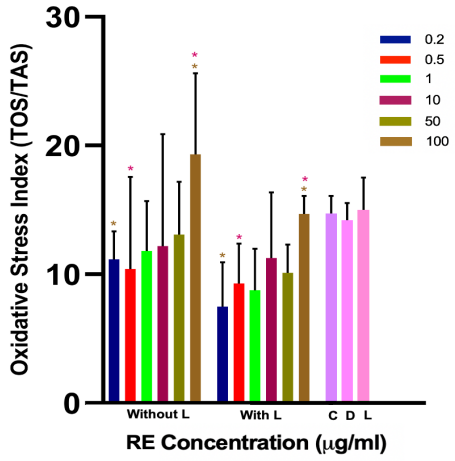

Figure 4. Expression of RE with/without L obtained from OSI in TOS/TAS in different doses comparison with C.

The effects of RE on oxidative stress in cells were evaluated by calculating the oxidative stress index (OSI=TOS/TAS) (Figure 4). When the index values were analyzed, 0.2 mg/ml RE treatment created the lowest ratio, whereas 50mg/ml RE treatment caused the highest TOS/TAS ratio. At dosages of 0.2 and 0.5, it was shown that the oxidative stress was lower compared to the C. OSI index, which seemed to be affected by inflammation. In the presence of inflammation, the L-treated group had decreased OSI, which was not anticipated. Additionally, the dose-dependent impact of RE becomes hazardous in higher doses compared to the C and low dose-treatment groups.

Discussion and Conclusion

Under normal conditions, certain levels of free radical formation exist in all aerobic cells and all cell sections. Free oxygen radicals generated in the organism are eliminated by intracellular free radical converting enzymes (superoxide dismutase, catalase, and glutathione peroxidase, etc.) or substances such as exogenous vitamin C (ascorbic acid) and vitamin E (alpha-tocopherol). While determining the oxidative stress, it is helpful to examine intracellular xanthine oxidase (Schmidt et al., 2019; Manivasagam et al., 2020), adenosine deaminase, SOD, GSH-Px, and CAT or vitamin C and E levels (Sinbad et al., 2019) to learn about antioxidant capacity while looking at the levels of free radicals (Suleman et al., 2019), thiobarbituric acid reactive products, nitric oxide (Poprac et al., 2017; Tejero et al., 2019).

The balance between oxidants and antioxidants is essential for cellular hemostasis. In recent years, the relationship between oxidant and antioxidant capacity and the role of imbalance in cell destruction has been frequently examined (Bagherifard et al., 2020). In particular, the importance of exogenous and endogenous free radicals generated in the cell in terms of toxicity and their roles in the pathogenesis of several diseases such as cancer, emphysema, hyperoxidation, bronchopulmonary dysplasia, arteriosclerosis and pancreatitis have been shown in many researches (Moussa et al., 2019; Neha et al., 2019).

Our work intended to question the mechanism of RE on arthritic chondrocytes by looking at the cells' proliferation capacity and antioxidant/oxidant status. It revealed that RE improves the antioxidant/oxidant status of SW1353 cells in both standard and inflammatory conditions. However, this effect is dose and condition dependent.

A subsequent investigation is whether RE can inhibit SW1353 cell proliferation was evaluated. A significant decrease in cell number was observed at 50mg/ml when the proliferation was individually compared to the C group. The proliferative capacity of the cells

was higher at 50µg/mL RE with L doses compared to the C.

SW1353 cells are commonly used as a model in the literature to evaluate response to inflammation and study chondrocyte matrix destruction. Nevertheless, the proliferation capacities of SW1353 cells increase in the presence of an inflammatory inflammation (Ngo et al., 2019; Zhao et al., 2019). RE has been reported to have an anti-inflammatory effect, especially by inhibiting the production of COX-2 (Mengoni et al., 2011). Therefore, the high dose of RE's antiproliferative impact in our study is consistent with the literature.

RE demonstrated an antiproliferative effect on SW1353 cells *in vitro*; its inhibitory role on proliferation was statistically significant in 0.2 and 0.5mg/ml RE treatment groups. TOS/TAS results indicated a statistically significant decrease for the 0.5mg/ml dose group. At higher concentrations, however, it was observed that RE led to a rise in both TOS and TAS values in SW1353 cells. Although not statistically meaningful, this increase was more pronounced in TOS values than in TAS values.

The antioxidant activity of RE also depends on how the extract is obtained. It is especially recommended to use dried RE as an antioxidant. When we calculated the OSI of cells treated with RE, OSI was highest with 100mg/ml RE concentration; it was observed that 0.2mg/ml significantly decreased cell proliferation compared to the others; this increase may be due to the increased TOS, causing a toxic effect on SW1353 cells *in vitro* with L, in other words, the decreased antioxidant status could not compensate this TOS activity. The antioxidant capacity was higher at lower doses of RE (0.2-0.5 ug/mL) treatment, which also impacted OSI and decreased stress, especially in the L-treated group. The benefit of assessing the oxidative status and antioxidant capacity together and expressing the differences in values with an index is obvious when evaluating the oxidant stress.

When the effects of RE on cell proliferation and OSI are evaluated together, it may be stated that the impact of RE in protecting cartilage cells at low concentrations (≤1mg/ml) and proliferation enhancement occurs independently of the antioxidant effect of the herbal extract. The decrease in cell proliferation at high concentrations is likely due to the increased oxidative effect of RE on cells. Indeed, in the literature, the antiproliferative effects of RE on cancer cells by blocking COX-2 expression have been reported (Moore et al., 2016; Scheckel et al., 2008). Since SW1353 cells originate from chondrosarcoma cells, these results may also indicate that applying a high dose of RE may be an additional/supplemental treatment method for existing cartilage-bone origin mesenchymal tumors. Similarly, studies report reduced proliferation capacity in cancer cells treated with 100mg/ml) RE. This unwanted effect at high doses can be prevented by topical -low-dose use of the RE in people with the arthritic disease.

Although it grows naturally on the western and southern coasts of Turkey, rosemary spreads widely in Çanakkale, Mersin, Adana, Tarsus and Hatay provinces, especially in Mersin and Adana region, in maquis flora, in forest spaces, on the edges of fields and vineyards, and in protected forestation areas (Malayoğlu, 2010). Furthermore, it is reported that RE originated from different regions and may have divergent chemical compound contents. The antioxidant, antiproliferative and anti-inflammatory effects of these substances have been emphasized in different studies (Oliviero et al., 2018). Researchers also emphasized that there might be differences in phenolic and chemotype compounds and therapeutic effects obtained from different rosemary species and herbs gathered at other times of the year (Tsiani, 2019; Shen et al., 2020).

The cytotoxic effect of rosemary appearing in high doses draws attention to the importance of the dose arrangement in its use. Current results indicate that rosemary at low concentrations may be a good candidate as an alternative medicine in the therapy of arthritis characterized by cartilage damage in terms of its antioxidant properties. On the other hand, the unexpected effect of RE observed in SW1353 chondrosarcoma cells brings out the possibility of using rosemary as an addition/supplement to existing treatments in high-grade tumors such as chondrosarcoma, which has limited medical treatment but can be treated only with surgical intervention.

When the OSI was calculated, although a change was observed in the groups where low RE concentrations (0.2-1mg/ml) were applied, this was not statistically significant compared to the C group. In comparison, OSI increased at the higher doses of RE treatment (50-100mg/ml). It is observed to decrease at low concentrations of RE treatment (0.2-0.5mg/ml) in SW1353 cells, compared to the C group. In conclusion, the findings indicate that having antioxidant properties at low concentrations, rosemary may be utilized in treating cartilage damage in arthritis. On the other hand, the cytotoxic effects of rosemary at high doses draw attention to the importance of tightly controlled usage of the herbal extract. This unexpected effect we observed in SW1353 cells suggests using rosemary as a supplement/support to the existing chemotherapeutic regimes in high-grade tumors such as chondrosarcoma, which has limited medical treatment but can be treated with surgical intervention.

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