Original

Functional Evaluation of the Ethanol Extracts from *Rosmarinus officinalis* L. (Rosemary)

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Abstract: Rosemary is used as an herb in cooking and aromatic oils, and has long been used as a medicinal herb because of its functional components. According to folklore, monks recommended a remedy of rosemary in alcohol ("Hungarian water") to a Hungarian queen suffering from limb numbness, resulting in instant recovery and rejuvenation. This is why the queen married a 20-year-old king of Holland while in her seventies. In order to clarify the "rejuvenation" mechanisms of traditional Hungarian water, we examined the anti-oxidant activity, anti-glycation activity, inhibition of melanin production and suppression of tumor growth of ethanol extracts of rosemary in this study. It was found that the ethanol extract had high anti-oxidant activity, high anti-glycation activity, high inhibition of melanin production and high suppression of tumor cell growth.

Key words: Rosemary, Anti-oxidant activity, Melanin production, Anti-glycation activity, Tumor cell growth

Introduction

Rosmarinus officinalis L. (Rosemary: drops of the sea) is primarily produced in France, Spain, Tunisia and Morocco, and has long been used for perfumes and medicine. One of the most interesting folklore stories involves Elisabeth I, the Queen of Hungary in the 14th century, who lost the king at a young age, worked hard on her duties, and received the trust of the people. When the busy queen was over 70 years old she suffered from rheumatism and became bedridden. However, the queen was completely cured and regained her beauty by applying an alcohol tincture of rosemary herbs (either as a bath or consumed), according to the teaching of a monk. Furthermore, there is a remarkable anecdote that she was subsequently beloved by the young king of Poland¹⁾.

The essential oil of rosemary, which has a clear scent and strong vitality, has many effects²⁾. Essential rosemary oil is thought to exert effects on the respiratory system, stiff shoulders, neuralgia, and rheumatoid arthritis, as well as effects on skin including tightening pores to refresh and rejuvenate loose skin. It is also well known for its effects on hair, and is said to strengthen hair that has lost its elasticity and restoring the original color of gray hair. In the olden days, the pharmacopoeia of the Lorche Monastery in Germany around the 8th century described a prescription for fatigue and pain, which was reported to grow on the farm of Charlemagne in western Rome at that time. It was also popular to use rosemary herb for treating fatigue and pain, gastrointestinal upset, asthma and rheumatism, and rejuvenation.

Rosemary extract is considered to be one of the safest and most effective antioxidants³⁾. Rosemary extract has higher antioxidant activity than vitamin C and vitamin E (tocopherol). Furthermore, it is said that it

has the effect of suppressing deterioration attributable to both internal factors (oil, enzymes) and external factors (light, heat), which are the two causes of food oxidation. With respect to oils, rosemary extract is effective in preventing oxidative deterioration of fats and oils, preventing pigment fading, suppressing odors from milk fat deterioration and oxidative odors, and has excellent thermal stability. Moreover, rosemary extract does not have adverse effects on foods, such as taste and smell. It also has the effect of improving the shelf life of various foods. Importantly, the European Union (EU) approved the use of rosemary extract (E 392) as an antioxidant in the filling material of stuffed dry pasta.

Rosemary extract is also used to maintain the quality of pet foods. Since dogs are carnivorous by nature, dog foods contain high amounts of meat. Meats contain oils that are easily oxidized. Oils immediately oxidize when exposed to light or oxygen, resulting in the loss of flavor, deterioration of quality, and oxidized oils induce vomiting and diarrhea. These consequences can be prevented by antioxidants. Rosemary extract is a naturally occurring antioxidant that does not affect the taste and smell of foodstuffs and is resistant to heat. Heat-resistant antioxidants are very useful for dog food, the production of which often involves heating at high temperatures during manufacturing. Also, when ingredients are cooked at 120°C or higher, the harmful chemical substance acrylamide is generated; rosemary extract has the effect of suppressing this effect.

Concerning skin, a previous study using a commercially available essential rosemary oil also suggested that it inhibited melanin production with a slight antioxidant activity⁴⁾. However, what was actually used by the Queen was a tincture of rosemary extracted with alcohol, which proved to be different from steam distillation of essential oil. Therefore, in this study, we measured the anti-oxidant activity, anti-glycation activity, inhibition of melanin production, and inhibition of pro-

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liferation of rodent cells by an ethanol extract of rosemary for the purpose of obtaining results by a method similar to the Hungarian tradition.

Materials and Methods

Preparation of samples

Rosemary (*Rosmarinus officinalis* 'Tuscan Blue') leaves were collected from rosemary plants grown in a kitchen garden. The leaves were cut in strip about one hour after leaf collection. Extraction was performed with distilled water, 50% ethanol, 70% ethanol or 100% ethanol. For extraction, 100 ml of each solvent was added to 10 g of rosemary leaves. The extraction was performed for 20 min at 4°C, the supernatant was centrifuged (3,000 rpm, 10 min) and the supernatant was used as the test sample.

Measurement of DPPH radical erasure activity⁵⁾

Radical scavenging activity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Merck KGaA, Darmstadt, Germany). A 0.5 ml aliquot of 200 μ M DPPH ethanol solution, 1.3 ml of ethanol and 0.2 ml of samples (diluted 5-fold with water) (0.2 ml of diluted water was used as a control) were mixed, and the absorbance at 720 nm was measured using a 96-well microplate reader (Multiskan Ascent, Thermo Fisher Scientific Inc., Waltham, MA).

Anti-glycation test⁶⁾

A mixture of distilled water (100 ml), phosphate buffer (600 ml), 2 M glucose solution (100 ml), 8 mg/ml albumin solution (100 ml) and samples (100 ml) were mixed well and incubated at 60°C for more than 40 hours to evaporate to dryness. An aminoguanidine hydrochloride (AG) (Fujifilm Wako Chemicals Corp., Tokyo, Japan) solution was used as a positive control solution. Then resulting residues were dissolved at 1 μ g/ml (samples) or 100 μ g/ml (control) in water, diluted 8 times with water and heated at 37°C for 2 hours. Fluorescence (ex: 370 nm, em: 465 nm) was measured with a plate reader.

Measurement of melanin production inhibition⁷⁾

A 0.1 ml sample consisting of 0.025 ml of PBS and 0.05 ml of tyrosinase (mushroom origin, 100 U/ml) (Merck KGaA, Darmstadt, Germany) was prepared. Thereafter, 0.05 ml of 5 mM L-DOPA (Cayman Chemical Company, Ann Arbor, MI) was added to the mixture, and absorbance at 475 nm was measured every 30 seconds. The results were indicated by every 30 seconds from the start of the assay vs increased absorbance during 30 seconds. Three samples were tested: water vs rosemary extracted by water; 50% ethanol vs rosemary extracted by 50% ethanol; 70% ethanol vs rosemary extracted by 70% ethanol.

Inhibition of tumor cell growth

Three tumor cell lines were evaluated in this study: MCF7 cells (human breast adenocarcinoma cell line), MDA231 cells (human breast adenocarcinoma cell line) and SW982 cells (human synovial sarcoma cell line). Tumor cells were plated in 96-well plates (MS8096F, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of 4,000 cells/well in 100 μ l of culture medium. After 24 hours, 50 μ l samples (water extract or 50% EtOH extract) diluted to one quarter were added to the wells. After 20 hours of incubation, an XTT assay was performed to estimate cell numbers. Each well was washed with medium and 100 μ l of medium and 50 μ l of XTT (AppliChem GmbH, Darmstadt, Germany) were added to the well. Two hours later, absorbance at 450 nm was measured with a spectrophotometer.

Results

DPPH radical scavenging activity

Fig. 1 shows the radical scavenging activities of the four samples. Compared to the water control, rosemary extracted with water scavenged about 52% of DPPH radical activity, rosemary extracted with 50% ethanol scavenged about 75% of DPPH radical activity and rosemary extracted with 70% ethanol scavenged about 63% of DPPH radical activity indicating that there are both water soluble and lipophilic components that show radical scavenging activity in rosemary. The lipophilic component seemed to be more effective or was present in higher amounts in rosemary than the water soluble component. Another hy-

Figure 1. Measurement of DPPH radical scavenging activity. A 0.5 ml aliquot of 200 μ M DPPH ethanol solution, 1.3 ml of ethanol and 0.2 ml of samples (diluted 5-fold by water) (in the case of control, 0.2 ml of diluted water was added instead of samples) were mixed, and absorbance (517 nm) was measured using a 96-well microplate reader. The ordinate indicates absorbance values at 517 nm. W: water; RM/W: rosemary extracted by water; RM/50Et: rosemary extracted by 50% ethanol; RM/70Et: rosemary extracted by 70% ethanol.

Figure 2. Anti-glycation activity. A mixture of distilled water (100 ml), phosphate buffer (600 ml), 2 M glucose solution (100 ml), 8 mg/ml albumin solution (100 ml) and samples (100 ml) were mixed well and incubated at 60°C for more than 40 hours to evaporate to dryness. The residues were dissolved at the indicated concentrations in water, diluted 8 times with water and heated at 37°C for 2 hours. Fluorescence (ex: 370 nm, em: 465 nm) was measured with a plate reader. The ordinate indicates fluorescence (Ex/Em 370/465) observed in each sample. ** $p < 0.05$. W(NC): water (negative control); AG (PC): aminoguanidine hydrochloride (positive control); RM/50Et: rosemary extracted by 50% ethanol; RM/70Et: rosemary extracted by 70% ethanol. The concentrations of the residues are indicated below.

Figure 3. Measurement of melanin production inhibition. A 0.1 ml sample consisting of 0.025 ml of PBS and 0.05 ml of tyrosinase (mushroom origin, 100 U/ml) was prepared. Thereafter, 0.05 ml of 5 mM L-DOPA was added to the mixture, and absorbance (475 nm) was measured. The abscissa indicates elapsed time (sec) from the start of the reaction and the ordinate indicates the rate of change in absorbance (OD 475/min) per minute (the rate of change in absorbance in each 30 seconds was indicated as the change in one minute). a: water vs rosemary extracted by water; b: 50% ethanol vs rosemary extracted by 50% ethanol; c: 70% ethanol vs rosemary extracted by 70% ethanol. Closed symbols indicate samples and open symbols indicate control.

pothesis is that the water-soluble and lipophilic components are the same material that exhibits differential extraction efficiency in water and ethanol. The latter hypothesis may be more likely because the radical scavenging activity extracted with 50% ethanol is higher than the activity extracted with 70% ethanol, indicating that the material is neither fully lipophilic or water soluble.

Figure 4. Inhibition of tumor cell growth. The cells were plated in 96-well plates at a density of 4,000 cells/well in 100 μ l of culture medium. After 24 hours, 50 μ l samples (either water extract or 50% EtOH extract) diluted to one quarter were added to the wells. After 20 hours incubation, the XTT assay was performed to estimate cell numbers. a: MCF7 cells; b: MDA231 cells; c: SW982 cells. The ordinate indicates absorbance (λ 450-500 nm). MC: medium control; W: water, 50Et: 50% ethanol; RM/W: rosemary extracted by water; RM/50Et: rosemary extracted by 50% ethanol.

Anti-glycation activity

Fig. 2 shows the anti-glycation activity of rosemary extracted with water, 50% ethanol or 70% ethanol. The positive control of aminoguanidine hydrochloride (AG) showed about 68% inhibition of anti-glycation activity compared with the negative control. The three rosemary samples showed 56% (water extracted), 32% (50% ethanol extracted) and 83% (70% ethanol extracted) inhibition of anti-glycation activity compared with the negative control, indicating that rosemary extracts exhibit strong inhibitory effects on anti-glycation activity. However, the 70% ethanol extracted samples showed lower inhibition activity than the 50% ethanol extracted samples. Because there is an elimination step of sugars in the anti-glycation activity assay, remaining sugars in the sample were not effectively eliminated by 70% ethanol. This may be one of the reasons that this sample showed lower activity than the 50% ethanol extracted samples.

Measurement of inhibition of melanogenesis

Fig. 3 shows that rosemary extracts exhibit strong inhibition of melanin production. Water extracted rosemary samples produced about 65% inhibition of melanogenesis over the initial 30 seconds of the assay. The 50% ethanol extract produced about 50% inhibition of melanogenesis over the initial 30 seconds of the assay. The 75% ethanol extract produced about 53% inhibition of melanogenesis over the initial 30 seconds. All samples effectively inhibited melanogenesis at all time points of the assay. Ethanol extracted rosemary samples showed relatively high inhibition of melanogenesis compared with the water extracted sample, indicating that the inhibitory components in rosemary may be relatively lipophilic.

Cancer cell growth inhibition tests

Fig. 4 shows the inhibitory activities of rosemary extracts toward cancer cell growth. The effect of two extracts on three cancer cell lines were assessed. The most effective inhibitory activity was observed in SW982 synovial sarcoma cells using the 50% ethanol rosemary extract, with cell numbers about 50% of the control. In MCF7 and MDA231 breast cancer cells, the cell numbers were 89% and 67% of control, respectively. The water extracted sample produced no effect on growth inhibition in all three cell lines. These results indicated that anti-proliferative substances in rosemary extracts may be highly lipophilic.

Discussion

In this study, we measured the anti-oxidant activity, anti-glycation activity, inhibition of melanin production, and inhibition of proliferation of cancer cells by water and ethanol rosemary extracts for the purpose of obtaining results by methods similar to the tradition and different from today's commercial ways meaning that demonstrating the biological activity of rosemary extracts using validated traditional assay systems.

It has been confirmed that ethanol extracts of rosemary exhibit all four of the activities measured in this study. Moreover, water extracts of rosemary were confirmed to have antioxidant activity, anti-glycation activity and inhibition of melanin production indicating that they can be expected to eliminate browning and sagging of the skin and returning firmness to the skin. Furthermore, it seemed that the effects of traditional Hungarian water (the ability to reduce stains, relieve skin tension, heal joint pain, and reduce the likelihood of neck irritation) are caused by various effects of rosemary extracts shown in this study.

Of note, we found that the water and ethanol extracts produced different effects. Furthermore, 50% ethanol extracts and 70% ethanol ex-

tracts showed somewhat different effects in this study. In the antioxidant activity assay, the magnitude of the effect of the 50% ethanol extract was about twice that of the water extract. In contrast, the effect of the 70% ethanol extract was about 70% of the 50% ethanol extract, indicating that the presence of lipophilic and water-soluble components that have antioxidant effects, or antioxidant components that have both lipophilic and water-soluble nature. Moreover, 50% ethanol may be optimal for extracting the most effective component(s). Similarly, in the anti-glycation activity assay, 50% ethanol extraction appeared to be optimal for extracting the most effective components. In this assay, the effect of 70% ethanol extracts were very weak and very close to the effect of the water control, which may be caused by the insufficient elimination of sugars in the sample. In the melanin production assay, all three samples produced an inhibitory effect; the 50% ethanol extract was the most effective and the 70% ethanol extract produced an effect similar to water extracts, suggesting that the effective components are assay specific. It has already been shown that antioxidant activity by CAA (cellular antioxidant activity) can contribute to the suppression of melanogenesis, and that Caffeic acid and Citrus depressa juice, which has demonstrated inhibitory effects on melanogenesis, has high antioxidant activities but has no inhibitory effects on tyrosinase activity. Their inhibitory effects on the melanogenesis was increased by inhibition of catalase activity⁸⁾. Thus, there appears to be several mechanisms by which melanogenesis can be inhibited. In the cancer cell proliferation assay, the water extract was not effective, indicating that the active component is lipophilic, as has been observed in tomato and cucumber⁹⁾. Moreover, the observed effects were different between the three cell lines tested. SW982 cells were the most sensitive to the ethanol and 50% ethanol extracts. It is thought that the effective component is likely to be an inhibitor of signal transduction or DNA replication.

Recipes for floral water obtained by a steam distillation method are common¹⁰⁾. Notably, this study confirmed that strong effects in every assay cannot be obtained from the water-soluble components and obtained from lipophilic components. Moreover, it is expected that lipophilic components will produce the strongest effects in eliminating browning and sagging of the skin and returning firmness to the skin.

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Conflict of Interest

The authors have declared that no COI exists.

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