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Antioxidant Activity of (8*E*,13*Z*,20*Z*)-Strobilinin/(7*E*,13*Z*,20*Z*)-Felixinin from a Marine Sponge *Psammocinia* sp.

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Abstract – During the course of our screening for bioactive metabolites from marine sponges, EZZ, the inseparable 1:1 mixture of (*8E,13Z,20Z*)-strobilinin and (*7E,13Z,20Z*)-felixinin has been found to deliver significant cytotoxicity against some cancer cell lines. In this study, the antioxidant activity of EZZ was first time evaluated by a series of antioxidant assays. It was found that EZZ was weak in scavenging the stable free radical 1,1-diphenyl-2-picrylhyrazyl (DPPH), but it was comparable to ascorbic acid in scavenging ABTS and superoxide radicals. In addition, EZZ could protect DNA from hydroxyl radical-induced strand cleavage. The findings of the present study suggest that EZZ possess certain antioxidant activity, which might help to prevent occurrence of cancer by alleviating the oxidative stress in cells.
Keywords – furanosesterterpenes, antioxidant, radical scavenger

Introduction

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen, and hydrogen peroxide are often generated as byproducts of biological reactions or from exogenous factors (Cerutti, 1991). ROS are essential for life because they are involved in cell signaling and are used by phagocytes for their bactericidal action. However, nonessential production of ROS, for example, oxidative stress, is suggested to be strongly associated with aging process and certain degenerative diseases including various cancers, cognitive dysfunctions, and coronary heart disease (Finkel *et al.*, 2000). Among the possible causes of cancer, damage to DNA and other molecular molecules by ROS, ranks high as a major culprit in the onset and development of the disease. In clinic, many cancer patients who are undergoing therapy take antioxidant supplements in an effort to alleviate treatment toxicity and improve long-term outcome (Borek, 2004). However, the antioxidant supplements may interfere with the mechanism of therapy, causing diminished treatment effect and protecting of tumor tissue (Block, 2004). Therefore, we have a great interest to look for both anticancer and antioxidant compounds from nature.

In the course of screening for bioactive metabolites from marine sponges, we have found that marine sponges of the order Dictyoceratida contain various linearfura nosesterterpene derivatives. Characterized by a furan group and a terminal tetronic acid moiety, these linear furanosesterpenes displayed a wide arrange of bioactivities including antiviral, antibacterial, anti-inflammatory, anti tumor, and protein phosphatase inhibitory activity and toxicity to sea urchin and starfish eggs (Choi *et al.*, 2004a). We previously reported that most of the furanosesterterpenes isolated from *Psammocinia* sp. displayed significant cyto toxicity against some cancer cell lines (Choi *et al.*, 2004b). Among them, EZZ, the inseparable 1:1 mixture of (*8E,13Z,20Z*)-strobilinin and (*7E,13Z,20Z*)-felixinin (Fig. 1) showed a rather high potency. The mechanism of EZZ based cytotoxicity and cell cycle arrest has been studied (data not shown). In this study, we evaluated the antioxidant activity of EZZ. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Trolox equivalent antioxidant capacity (TEAC) assay for the free radical scavenging activity, nitrobluetetrazolium (NBT) assay for the superoxide radical scavenging activity, and the inhibitory effect of hydroxyl radical-induced DNA damage were used.

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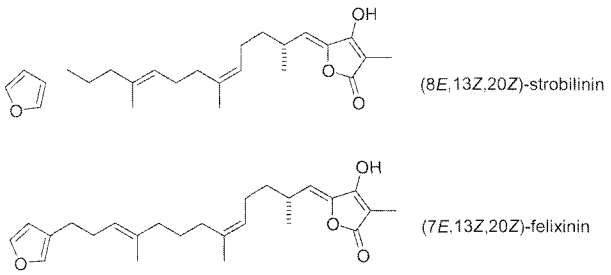

Fig. 1. Chemical structure of EZZ.

Experimental

Materials – pBR322 DNA was Purchased from Takara Shuzo Co., Japan, and agarose was from Promega Co., USA. Xanthine oxidase, DPPH, Trolox (6-hydroxy-2,5,7,8-tetra methylchroman-2-carboxylic acid), ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and NBT were from Sigma Co., USA. All other chemicals were Durchased of analytical reagent grade. Stock solution of (*8E,13Z,20Z*)-strobilinin and (*7E,13Z,20Z*)-felixinin (EZZ) was Durchased prepared in methanol and kept at −20°C. Further dilutions were made immediately prior to each experiment.

DPPH free radical scavenging activity – The potential antioxidant activity of EZZ was assessed on the basis of its scavenging activity of the stable DPPH free radical (Cottelle *et al.*, 1996). Reaction mixtures containing 200 μL of 0.1 mM DPPH-ethanol solution, 90 μL of 50 mM Tris-HCl buffer, and 10 μL of methanol (as control) or test sample EZZ. After 30 min incubation at room temperature, absorbance (517 nm) of the reaction mixtures was taken. The inhibitory ratio (%) was calculated as follows:

[(Absorbance of control-absorbance of test sample)/ absorbance of control] × 100

Measurement of TEAC – The Trolox equivalent antioxidant capacity of the EZZ was determined according to the published procedure (Re *et al.*, 1999). Ascorbic acid was used as an antioxidant reference. ABTS, was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS \cdot ^+$), the blue/green chromophore, was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 hr before use. $ABTS \cdot ^+$ solution was diluted with 5 mM PBS to obtain about 0.70(0.02 unit of absorbance at 734 nm. After addition of 1.0 mL of diluted $ABTS \cdot ^+$ solution to 10 μL of EZZ (concentrations were 0.63, 1.25, 2.5, 5, 10, and 20 mM) or Trolox standards in ethanol the absorbance reading was taken at 30°C up to 6 min. Antioxidant activities of EZZ and ascorbic acid were expressed as TEAC using the calibration curve plotted with different amounts of Trolox.

NBT (Superoxide scavenging) assay – Measurement of superoxide radical scavenging activity was carried out according to the published by Kirby and Schmidt (1997) with slight modifications. Reagents in this study were prepared in 50 mM KH_2PO_4/KOH buffer (pH 7.4). The reaction mixture contained 20 μL of 15 mM Na_2EDTA, 50 μL of 0.6 mM NBT, 30 μL of 3 mM hypoxanthine, 50 μL of xanthine oxidase (1 unit in 10 mL of buffer), 5 μL of EZZ in methanol (final concentrations of EZZ were 0.2, 0.4, and 0.8 mM), and 145 μL of buffer in 96-well microplates. The reactions were initiated by the addition of xanthine oxidase at 25°C, and absorbance values (570 nm) were recorded every 20 sec up to 5 min using a plate reader. The control was 5 μL of methanol instead of EZZ. Ascorbic acid was used as a positive control. Results were expressed as percentage of inhibition relative to the control, given by

[(Rate of control - rate of sample reaction)/rate of control] × 100%

Inhibitory effect on hydroxyl radical-induced DNA damage – This assay was done according to the method of Keum *et al.* (2000) with minor modifications. The reaction mixture (30 μL) contained 0.15 μg of pBR322 plasmid DNA, 30 mM H_2O_2, and 10 mM Tris-EDTA buffer (pH 8.0). Various amounts of EZZ dissolved in 5 μL of methanol (final concentrations of EZZ were 0.25, 0.5, and 1.0 mM) were added prior to H_2O_2 addition. Hydroxyl radicals were generated by irradiation of the reaction mixtures at a distance of 20 cm with a 12 W UV lamp. After incubation at room temperature for 30 min, the reactions were terminated by the addition of a loading buffer (0.25% bromophenol blue tracking dye and 50% sucrose), and the mixtures were then analyzed by 0.8% submarine agarose gel electrophoresis. The gels were stained with ethidium bromide, destained in water, and photographed on a transilluminator.

Results and Discussion

A number of assays have been introduced for the measurement of the total antioxidant activity of food extracts (Rice-Evans *et al.*, 1995; Wang *et al.*, 1996), pure compounds (Rice-Evans *et al.*, 1996; Miller *et al.*, 1996) and body fluids (Miller *et al.*, 1993). Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range.

To evaluate the radical scavenging activities of EZZ,

various assay systems, namely, the DPPH, TEAC, and NBT assays, were performed and a well-known antioxidant, ascorbic acid was used as a reference. DPPH radical-scavenging assay is used to determine whether the compounds are acting as free radical scavengers. In this assay, antioxidants presented as free radical scavengers should pair up with the stable DPPH free radical forming 1,1-dipheny-2-picrylhydrazine. In Table 1, EZZ was shown to scavenge directly the stable DPPH radical over a concentration range of 0.01 mM to 0.8 mM. It scavenged the stable radical DPPH in a concentration-dependent manner. However, at concentration of 0.2 mM, ascorbic acid could reach almost the inhibition plateau while EZZ just showed about 15% inhibition. These values suggest that EZZ has weak free radical scavenge activity which is not comparable to that of ascorbic acid.

Generation of the ABTS radical cation was measured on the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of samples (Rice-Evans *et al.*, 1996). Addition of antioxidants to the pre-formed radical cation reduced ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration the reaction. Thus the extent of decolorization as percentage inhibition of the ABTS radical cation is determined as a function of concentration and calculated relative to the reactivity of Trolox as a standard under the same conditions (Re *et al.*, 1999). In TEAC assay (Fig. 2), EZZ exhibited the scavenging activity in a concentration-dependent manner over a concentration range of 0.63 to 20 mM. EZZ has similar antioxidative activity with ascorbic acid at low concentration (0.63~1.25 mM) while it showed lower capacity at higher concentration (2.5~20 mM) compared with ascorbic acid.

On the other hand, superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of cell-damaging free radicals and oxidizing reagents (Liu *et al.*, 2000). Moreover, xanthine oxidase is one of the main enzymatic sources of those ROS *in vivo*. Hypoxanthine-xanthine oxidase generates superoxide radicals, which reduce NBT to yield blue formazan. In NBT assay, EZZ dose-dependently inhibited the NBT reduction induced by xanthine-xanthine oxidase (Fig. 3). At the concentration of 0.8 mM, both

Table 1. Percent inhibition of DPPH radical scavenging effects of PSA and ascorbic acid as positive control

Sample \ Conc.(mM)	0.01	0.03	0.05	0.2	0.4	0.8
PSA	3.77 ± 1.46	7.36 ± 2.45	10.94 ± 2.78	15.09 ± 2.54	35.85 ± 4.21	58.49 ± 3.68
Ascorbic acid	28.54 ± 3.12	51.98 ± 3.04	81.28 ± 2.01	85.16 ± 2.97	87.64 ± 1.38	89.75 ± 2.81

Data are shown as means±SD of three independent experiments.

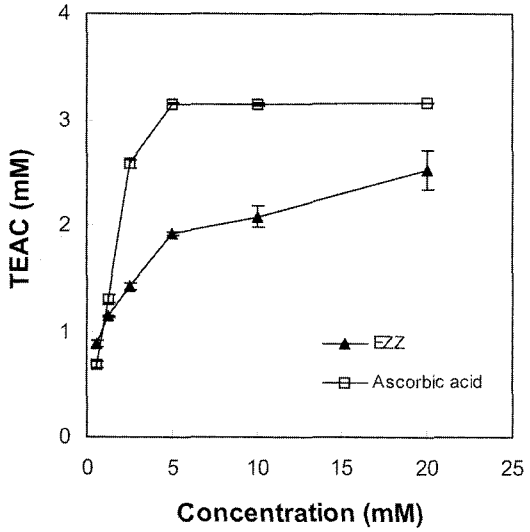

Fig. 2. Antioxidant capacity of EZZ as determined by the Trolox equivalent antioxidant capacity (TEAC) assay. Ascorbic acid was used as a reference. Data are shown as means±SD of three independent experiments.

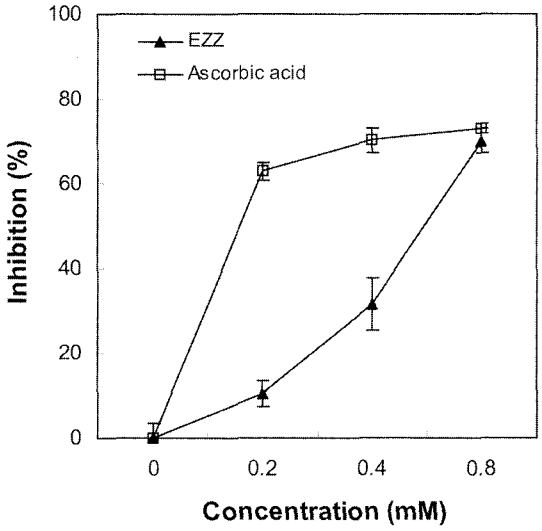

Fig. 3. Superoxide scavenging activity of EZZ. Ascorbic acid was used as a reference. Data are shown as means±SD of three independent experiments.

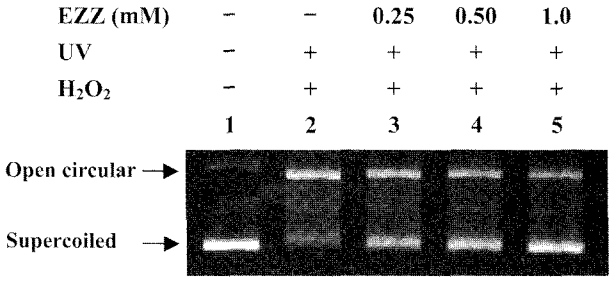

Fig. 4. Protective effect of EZZ on DNA strand scission induced by H_2O_2 and UV. Lane 1, native supercoiled DNA without any treatment; Lane 2, DNA treated with H_2O_2 + UV irradiation; Lane 3-5, DNA samples treated with H_2O_2 + UV irradiation in the presence of 0.25, 0.5, 1.0 mM of EZZ. Data are representative of three independent experiments.

EZZ and ascorbic acid could reach more than 70% of inhibition. However, it looks clear that EZZ works at a relatively higher concentration than ascorbic acid.

It has been well known that hydroxyl radical induces DNA base modification and strand breaking, which may cause serious diseases in connection with carcinogenesis (Hochstein *et al*, 1988). In biochemical systems, superoxide radicals is converted by superoxide dismutase to hydrogen peroxide, which can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions such as iron and copper, especially iron (Halliwell *et al*., 1981), or by UV photolysis (Keum *et al*., 2000). In this assay, the protective effect of EZZ on pBR322 DNA cleavage by hydroxyl radicals, generated from UV-irradiated H_2O_2, was evaluated. As shown in Fig. 4, the treatment of supercoiled pBR322 DNA with UV and H_2O_2 led to conversion of the DNA to open circular form (lane 2), whereas UV illumination alone did not cause DNA strand cleavage (data not shown). However, EZZ shows a dose-dependent protection of DNA under oxidative stress (lane 3-5). When the DNA was incubated with 0.25 mM of EZZ, the prevention of DNA strand scission was clearly observed (lane 3). There was significant protection by EZZ at a dose of 1.0 mM (lane 5), which shows good antioxidant activity *in vitro*. The working concentration is high in this assay. However, we still believe that EZZ could specifically protect DNA from hydroxyl radicals-induced breaks, since ascorbic acid has less significant effect in the same assay (data not shown).

From above four assays (DPPH, TEAC, NBT, and DNA cleavage protective assay) employed to evaluate the antioxidant activity of EZZ, we conclude that EZZ has obvious antioxidant activity. At present, it is not clear whether there is any relationship between its antioxidant activity and anticancer effect because of lacking definite evidences. However this is the first report about the antioxidant activity of EZZ. We suggest here that except being a potent anticancer candidate, EZZ might help to prevent occurrence of cancer by alleviating the oxidative stress in cells.

Acknowledgments

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