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Antioxidant Activity of *Rubus crataegifolius* Bge. Fruit Extracts

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We investigated the fruits of *Rubus crataegifolius* Bge, a plant which has been traditionally used in Korea in phytotherapy, to describe antioxidant materials from plant sources. *R. crataegifolius* fruits were extracted with methanol and further fractionated into *n*-hexane, diethyl ether, and ethyl acetate. The antioxidant activity of each fraction and the residue was assessed using a 1,1-diphenyl-2-picrylhydrazyl (DPPH), H_2O_2 radical scavenging method, and their cytotoxicity on human primary kerationcyte (HK) was determined by an MTS assay. The *R. crataegifolius* fruit methanol extract showed strong antioxidant activity (75.04%, 50%) compared with vitamin C (79.9%, 54.1%) by the DPPH, and H_2O_2 method, respectively. The measured activity from the subsequent extracts of the methanol extract were 20.3% for *n*-hexane fraction (HF), 68.8% for diethyl ether fraction (DF), 67.1% for ethyl acetate fraction (EF), and 67.1% for the residue fraction (RE) by DPPH and 2.2% for HF, 1.6% for DF, 10% for EF, and 50% for the RE by H_2O_2 assay. An oxidative stress model of HK was established under a suitable concentration (1 mM). The cell viability of the RE treated group increased and the percentage of apoptotic cells decreased at concentrations of 0.005-0.02% RE compared with the H_2O_2 treated group. Fruit extracts of the medicinal plant *R. crataegifolius* showed potent antioxidant activity and the ability to relieve cell damage from H_2O_2 induced injury to HK.

Key words : Antioxidant activity, *R. crataegifolius*, fractionation, keratinocyte, skin disease

Introduction

Many naturally substances in plants have antioxidant activities. Antioxidants from natural sources have been used to increase the stability of foods by preventing lipid oxidation and to protect against oxidative stress living systems by scavenging reactive oxygen species (ROS) [10]. In fact, antioxidants have been used for the food, pharmaceutical and cosmetic industries because of similar functionality requirements for the products.

Rubus crataegifolius Bge. belongs to family *Rosaceae* and distributes widely in Southeast Asian countries, especially Korea, north-eastern China, Japan, and the Ussuri region of the Russian Far East [5]. For many years, *R. crataegifolius* has been used to alleviate rheumatic arthritis, hepatitis and lung cancer in China [12] and its fruit is used in edible food, juice and ice cream in Korea. Some small molecules in roots or leafs of *R. crataegifolius* have been identified to be bioactive phytochemicals [4,7,8,14].

However, little is known about the cytoprotective effects of *R. crataegifolius* fruit against oxidative stress. Oxidative stress has attracted great attention in the field of dermatology. It is believed that H_2O_2-induced oxidative condition of human keratinocytes (HK) can lead to many skin problems, such as vitiligo [3] and skin aging [9]. This study was undertaken in order to evaluate the antioxidant activity of *R. crataegifolius* fruit methanol extracts, as assessed by its ability to scavenge free radicals (DPPH, H_2O_2) and to protect HK from oxidative stress.

Materials and Methods

Chemicals and reagents

The *R. crataegifolius* fruits used here were collected in Kimhae, Busan, Korea on May 2009. Methanol, *n*-hexane, diethyl ether, and ethyl acetate (Merck, Germany) were used in fruit extraction and fractionation. DPPH (Tokyo Kasei Kogyo, Japan) was used in the DPPH assay.

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Cell culture and treatment

Normal HKs were purchased from ATCC cell bank (ATCC# PCS-200-011). HK was cultured in serum-free keratinocyte medium with epidermal growth factor at concentrations of 0.2% (v/v) of bovine pituitary extract, 5 g/ml bovine insulin, 0.18 g/ml hydrocortisone, 5 g/ml bovine transferring, 0.2 ng/ml human epidermal growth factor (EGF) at 37°C in a 5% CO_2 humidified atmosphere. The cultured HKs were randomly assigned to six groups; the control group and treated groups with different concentrations, 50, 100, 250, 500, and 1,000 μM of H_2O_2 (Sigma, USA) for 4 h. The proper concentration of H_2O_2 was identified to establish the oxidative stress model. In another set of experiments, HKs were randomly assigned to five groups: control, fractioned RCB extracts pretreated with final concentrations of 0.005%, 0.01%, 0.02%, 0.04%, and 0.08% + 1 mM H_2O_2, and H_2O_2 without pretreatment groups. After incubated with fractioned RCB extracts for 4 hr, the cells were washed with culture medium to remove the fractioned RCB extracts, and exposed to H_2O_2 before being introduced into different assays.

MTS assay as a measure of cell viability

Cell viability was determined using CellTiter 96 Aqueous One Solution Reagent from Promega (Genesearch. madison, US). Briefly, cells (2×10^5) were placed in 96-well plastic culture plates and incubated at 37°C in 5% CO_2 for 24 hr, at which point 100 μl of 0.5 mg/ml MTS solution was added to each well and incubated for 4 hr at 37°C. Formazan absorbance was read at 490 nm using a plate reader. To determine cell viability, the MTS assay was used with hydrogen peroxide (H_2O_2) used as a positive control.

Extraction and solvent fractionation

One kilogram of fresh fruits of *R. crataegifolius* was extracted with 10 l of methanol at room temperature for 24 hr, filtered using filter paper (Whatman 2; Sigma-aldrich, Germany) and the filtrate dried under vacuum by rotary evaporation, producing 177.2 g of extract. A 177.2 g portion of the extract was suspended in a ratio of 1:2 methanol:water (v/v) and partitioned into *n*-hexane, diethyl ether, and ethyl acetate. The rotary evaporated *n*-hexane, diethyl ether, ethyl acetate soluble fractions, and the water soluble residue massed 10.5 g, 3.5 g, 4.70 g, and 63.77 g, respectively.

Antioxidant assay

One mg of sample was first dissolved in 1 ml of DMSO and used for experimentation in a concentration of 0.025%. An assay using a DPPH radical scavenging method was performed as previously described by Arung et al [1]. For hydrogen peroxide scavenging activity, RCB was added to 10 mM H_2O_2 and 0.1 M phosphate buffer. The reaction mixture was pre- incubated for 5 min at 37°C. Then 1.25 mM ABTS with 30 μl peroxidase in 0.1 M phosphate buffer was added. After those were incubated for 10 min at 37°C and then absorbance was measured at 405 nm immediately. The content of hydrogen peroxide was calculated with absorbance and expressed as a percentage.

Statistical analysis

All statistical analyses were performed by SPSS13.0 software (SPSS Inc., USA). Measurement data were expressed as mean±standard deviation (SD). Differences between multiple groups were analyzed by analysis of variance (ANOVA), while that between two groups were analyzed by *t* test. $p<0.05$ was considered statistically significant.

Results

Effect of RCB fruit extracts on DPPH scavenging activity

To best of our knowledge, this is the first time that *R. crataegifolius* fruit extract has been demonstrated for DPPH antioxidant activity. The DPPH antioxidant activity of the methanol extract (75.04%) was similar than that of vitamin C (79.9% at 0.1%) as a positive control. The *n*-hexane layer, diethyl ether layer, ethyl acetate layer, and residue layers all represented scavenging activity (Fig. 1), with the diethyl ether layer showing scavenging activity (68.8% at 0.025%)

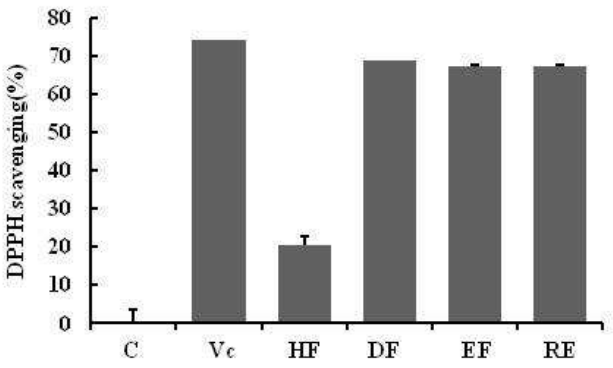

Fig. 1. Effect of RCB fruit extracts on DPPH scavenging activity. C=control; Vc=vitamin C; HF=*n*-hexane fraction; DF=diethyl ether fraction; EF=ethyl acetate fraction; RE=residue fraction.

higher than the *n*-hexane (20.3%), ethyl acetate (67.1%), and residue (67.1%) layers.

Effect of RCB fruit extracts on H_2O_2 scavenging activity

Assessment of the H_2O_2 antioxidant effect of the fractionated methanol extracts showed that induced scavenging activity produced effects of the *n*-hexane (2.2%), diethyl ether (1.6%), ethyl acetate (10%) and residue (50%) at 0.025% concentration (Fig. 2), respectively, using for a positive control vitamin C (54.1%). This means that the water soluble fraction of RCB fruit methanol extract contained anti-oxidantor against DPPH and H_2O_2 species.

Cytotoxicity of RCB methanol extract on human primary keratinocyte

Assessment of the cytotoxic effect on HK showed that the RE was little cytotoxicity compared with the various concentration of RE fractions (0.5%, 1%, 1.5% and 2%, respectively, Fig. 3).

Cell viability of human primary keratinocytes (assayed by MTS)

H_2O_2 (50-1,000 μM) decreased the cell viability of the HK in a concentration-dependent manner ($p<0.05$). The cell viability in the 250 μM and 1,000 μM H_2O_2 groups were significantly lower than in the other groups ($p<0.05$) (Fig. 4A). The cell viability was increased in the RE groups compared with the H_2O_2 group ($p<0.05$, Fig. 4B). The cell viability in 0.005-0.02% RE groups showed a concentration-dependent increase, but a little difference was found in the 0.04% and 0.08% groups without a statistical difference compared with the 0.02% group ($p>0.05$). These results indicate that RE could increase the cell viability compared with that in the

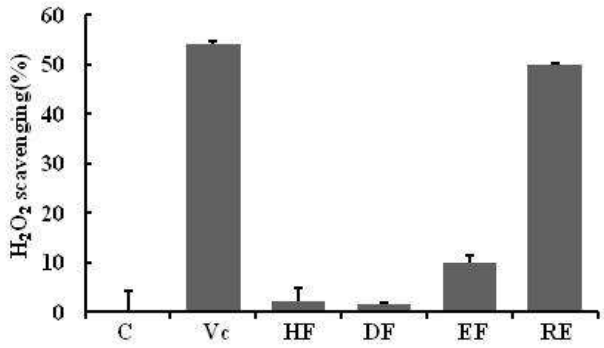

Fig. 2. Effect of RCB fruit extracts on H_2O_2 scavenging activity. C=control; Vc=vitamin C; HF=*n*-hexane fraction; DF=diethyl ether fraction; EF=ethyl acetate fraction; RE=residue fraction.

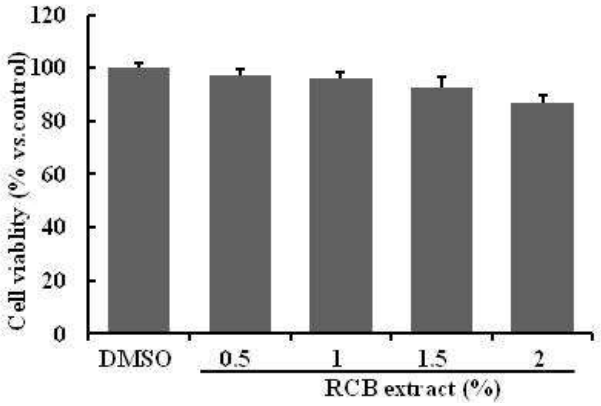

Fig. 3. Cytotoxicity of RCB methanol extract on human primary keratinocyte. DMSO=vehicle

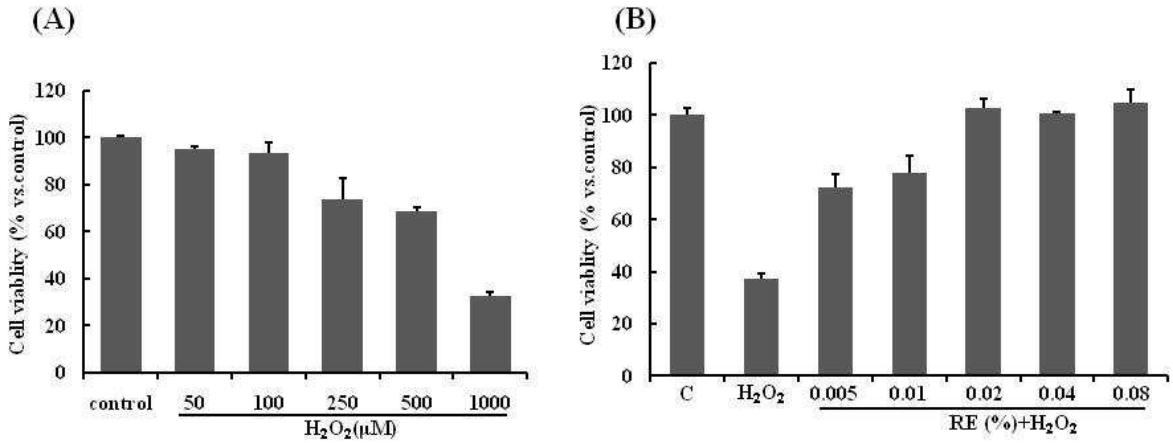

Fig. 4. Cell viability of human primary keratinocytes (assayed by MTS). **A:** H_2O_2 significantly decreased cell viability in the primary kerationocytes in a concentration-dependent manner compared with the control group ($p<0.05$). **B:** The cell viability increased significantly in the RE group compared with that in H_2O_2 group (1 mM) ($p<0.05$).

control group.

Discussion

Previous studies have claimed that *R. crataegifolius* extracts have diverse pharmacological activities such as anti-inflammatory [4], and anti-cancer activities [8], and hepatitis [12]. In the present study, attempts were made first to assess and compare the anti-oxidant actions of the RCB fruit extracts. The results obtained are expected to serve as information for understanding their pharmacological effects, developing new drugs from RCB fruit extracts, searching natural anti-oxidants or expanding its uses as various forms of beverages. The anti-oxidant activity of RCB extract was assessed by oxidation reactions, DPPH, H_2O_2, OH radical, TBARS oxidation and reducing power in this research. Of the 5 reactions, RCB extracts inhibited the DPPH, H_2O_2 oxidant species. At present, we do not know the real compounds of RCB fruit extracts related to anti-oxidant activity. One of raspberries, *Rubus coreanus Miquel* contains an abundance of sugars, vitamins, minerals, and polyphenols [2,13] and was reported to have anti-inflammatory, anti-nociceptive, anti-gastropathic and anti-rheumatic effects [6,11]. The pharmacological properties of phenolic compounds such as caffeic acid conjugates, ellagic acid glycosides, and flavonol glycosides may be the major bioactive constituents responsible for antioxidant effects of RCB. As shown in Fig. 1, the result indicated that DPPH scavenging activity decreased after fractionation of the methanol extract and that compounds of the extract appeared to work synergistically to produce the overall methanol extract scavenging activity. In Fig. 2, these results indicate that the extract could be described as an industrial applicator such as cosmetics, drug development and tonic material. Further experiments are in progress to clarify the active phytochemical in these extracts. In conclusion, it is shown in this study that H_2O_2 can damage HK and cause cell apoptosis. RE fraction is an efficient reagent able to prevent HK oxidative damage induced by H_2O_2 exposure when used at certain concentrations. We do not know whether this action can occur in the clinical use of these extracts. Although we do not know its meaning or significance now, however, the anti-oxidant activity of these plants may be new information we should pay attention to. Considering the present results, RCB fruit methanol extract was found to be a good, potential candidate for future use as an antioxidant and industrial cosmetic materials in the treatment of oxidative damage related skin disease.

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초록 : *Rubus crataegifolius* Bge. 열매 추출물의 항산화 활성

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식물자원으로부터 항산화 물질을 확보 하기위해 한국 등에서 전통적으로 phytotherapy로 이용되어 온 *Rubus crataegifolius* Bge. 열매를 조사하였다. *R. crataegifolius*의 열매를 메탄올로 추출하였고 순차적으로 n-hexane, diethyl ether, and ethyl acetate로 분획화하였다. 각 분획물의 항산화 활성은 DPPH와 H_2O_2에 대항 인간 primary 세포인 keratinocyte (HK)를 이용하여 세포 독성 및 효능을 검증하였다. *R. crataegifolius* 열매 추출물은 비타민 C와 비슷한 강력한 DPPH (75.04%, 50%)와 H_2O_2 (79.9%, 54.1%) 소거능을 보였다. 분획물의 DPPP에 대한 소거능을 측정하였는데 n-hexane fraction (HF)은 20.3% , diethyl ether fraction (DF)은 68.8%, ethyl acetate fraction (EF)는 67.1% 그리고 residue fraction (RE)은 67.1%의 소거능을 보였으며 H_2O_2에 대해서는 2.2%, 1.6%, 10%, 그리고 50%로 각각 나타내었다. H_2O_2에 대한 HK의 세포 보호능을 확인하기 위해 산화적 스트레스 모델을 확립하였고(1 mM) 0.005-0.02%의 RE 분획물에서 H_2O_2에 대한 보호능을 발휘하였다. 따라서, *R. crataegifolius*의 열매 추출물은 H_2O_2유발 상처에 대하여 HK세포의 보호능을 가지며 강력한 항산화 활성을 가지고 있다.