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Hepatoprotective Effect of Solvent Fractions from *Raphiolepis indica* against Oxidative Stress

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Abstract : *Raphiolepis indica* (*R. indica*) is one of evergreen shrubs belonging to the Rosaceae and is grown wildly in Jeju. This study was performed to evaluate the hepatoprotective effect of different fractions (n-hexane, dichloromethane, ethyl acetate, butanol, water) from *R. indica*. Anti-oxidative effects were determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and total phenol contents. Hepatoprotective effect was identified by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay in Huh7 cells. Among various fractions, ethyl acetate and butanol fractions showed the lowest DPPH remained rate reaching approximately 78.7 and 65.5% at 400 μg/mL. Ethyl acetate and butanol fractions showed the total phenolic content at 164.5 and 137.3 mg GAE/g extract. The ethyl acetate and butanol fractions were resistant against oxidative stress in MTT assay and showed higher hepatoprotective effect than other fractions. Therefore, these results suggest that the ethyl acetate and butanol fractions of *R. indica* might have therapeutic value in liver damage.

Keywords : Anti-oxidative effect, Hepatoprotective effect, Jeju natural extract, Raphiolepis indica

1. Introduction

There are reactive oxygen species (ROS) in the cells, and ROS induce the peroxidation of membrane lipids[1]. The human have mechanism for defense to protect the human body from ROS. Defense mechanisms are known to include anti-oxidative enzymes[2]. However, excessive ROS and low anti-oxidant defense system continue to increase oxidative stress[3]. As a result, oxidative stress is related with various chronic inflammatory diseases such as cancer, hepatitis, and early aging as well as various diseases[4,5]. With the increasing number of various diseases caused by oxidative stress, research on anti-oxidant agent is increasing as well.

As synthetic anti-oxidant agents including butylated hydroxyanisole and butylated hydroxytoluene, have side effects which include liver damage and the production of cancer, antioxidant agents from natural extracts are active research field of pharmacy, currently[6]. Materials from natural extracts are sources of

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many medical supplies which are currently applied in clinic therapy.

Diverse flora are distributed on Jeju Island, and widely used as medical plants[7]. *Rhaphiolepis indica* (*R. indica*), which is an evergreen, belongs to Rosaceae. There are more than 3,000 species in Rosaceae, many of which are economically instrumental fruit trees like apple, apricot, cherry, peach and pear[8]. In the field of studies about *R. indica*, as physiological effects has not been researched enough except anti-inflammative effects of Triterpenoids, Biphenyls, Dibenzofurans extracted from the roots of *R. indica*, research on physiological effects are necessary[9,10].

In this study, the experiment was preformed in order to confirm anti-oxidative effects using solvent fractions from *R. indica* leaves, to prove cytoprotective effect after treating the extracts to hepatocyte, and to be used as base data for development of natural anti-oxidant drug using natural extracts.

2. Materials and Methods

2.1. Extraction of plants

Leaves of *R. indica*, were collected in Jeju, where *R. indica* grow naturally. Collected material was washed two or three times, dried for two weeks, and pulverized to be used as extract material. After dried material was extracted twice with 80% ethanol, the filtrated extract was decompression-condensed and dried by a freeze drier. And then the production of fraction of *R. indica* leaves includes extracting process, using different polarities of solvents such as n-hexane, dichloromethane, ethyl acetate, butanol and water in sequence.

2.2. DPPH radical scavenging

Anti-oxidative effects of the fractions from *R. indica* was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging effects. After preparing natural materials at 25, 100 and 400 μg/mL, 10 μL material was dispensed in a 96 well plate and 190 μL of 200 μM DPPH (Sigma-Aldrich, St. Louis, MO, USA) in ethanol was applied. In this process, after 30 minutes at 37℃, optical density (550 nm) were measured using Biotrak II Plate reader (Amersham Life Science, Buckinghamshire, UK).

2.3. Total phenolic content

Total phenolic content was carried out in the Folin-Denis method. Mixing a 0.1% fractions (50 μL), D.W (1.65 mL), and Folin-Denis reagent (100 μL), 200 μL of 1 N Na_2CO_3 was added, and the mixture compound reacted for 2 hours at room temperature. Then, the optical density was measured at 750 nm using spectrophotometer (Milton Roy Company, New York, USA). Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) was measured in the same method as that used for the samples. After it, the standard curve was made. Total phenolic contents ware indicated as gallic acid equivalents per gram fractions sample (mg GAE/g fractions).

2.4. Cell Culture

The cells used in the experiment was Huh7 hepatoma cells. These cells were dispensed by the Korean Cell Link Bank (KCLB) in South Korea. Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) were used as the medium for the culture of cells. The cell line was cultured in a 5% CO_2 incubator at 37℃ by adding 10% fetal bovine serum (Hyclone, Logan, UT, USA), and anti-biotics such as penicillin-streptomycin (GIBCO, Rockville, MD, USA).

2.5. Cytotoxic effects using hepatocytes

The cytotoxic effects of solvent fractions from *R. indica* were identified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay [11]. After the culture of Huh7 cells, cell

counting was performed. The cells were adjusted to $1X10^5$/mL. After each 100 μL of cells was dispensed in a 96 well micro plate, the content was cultured for 24 hours at 37℃. After the fractions were dispensed in the respective cells (25, 50, 100, 200, 400 μg/mL), the content was cultured for 48 hours at 37℃ and 5% CO_2. MTT (Ambresco, Ohio, USA) solution was each 20 μL was dispensed. The materials were cultured at 37℃ and 5% CO_2 for 2 hours. After the medium was removed, each 200 μL of dimethyl sulfoxide (DMSO) was dispensed. The optical density at 560 nm was identified, ELISA reader (Amersham Life Science, Buckinghamshire, UK).

2.6. Cytoprotective effects for hepatocytes

The cytoprotective effects of solvent fractions from *R. indica* were identified using a MTT assay when hydrogen peroxide (H_2O_2) and t-butyl hydroxy peroxide (t-BHP) were treated respectively. After the culture of Huh7 cells, cell counting was performed. The cells were adjusted to $1X10^5$/mL. After 100 μL of cells were dispensed in a 96 well micro plate, the cells were cultured for 24 hours at 37℃. After each 100 μL of the extract fractions were dispensed in the respective cells at 25, 50, 100 and 200 μg/mL, the materials were cultured for 24 hours at 37℃ and 5% CO_2. Then 100 mM H_2O_2 and t-BHP were prepared and each 20 μL was dispensed in the 96 well plates of the H_2O_2 and t-BHP treatment group, and the materials were cultured for 24 hours. MTT solution was each 20 μL was dispensed. The content was cultured at 37℃ and 5% CO_2 for 2 hours. After mediums were removed as much as possible, each 200 μL of DMSO was dispensed. The optical density at 560 nm was measured, ELISA reader (Amersham Life Science, Buckinghamshire, UK).

2.7. Statistical analysis

Statistical analysis was conducted with one-way ANOVA.

3. Results and Discussion

3.1. DPPH scavenging activity

The results of measuring the DPPH scavenging activities of solvent fractions from *R. indica* showed dose-dependent anti-oxidative effect. Among various fractions, ethyl acetate and butanol fractions from *R. indica* showed the lowest DPPH remained rate reaching approximately 78.7 and 65.5% at 400 μg/mL. Quercetin used as the control group showed DPPH remained rate reaching approximately 47.0%, which were relatively lower than ethyl acetate and butanol fractions from *R. indica*(Fig. 1). As free radicals can be scavenged by compounds that can donate hydrogen atom[12], a number of research about anti-oxidative effects are conducted using radical scavenging test. About 18% of DPPH radical scavenging was identified in the extracts of *Bonnemaisonia hamifera at* 200 μg/mL[13] and a similar level of DPPH scavenging activity (21%) at 400 μg/mL was identified by the ethyl acetate fractions from *R. indica*.

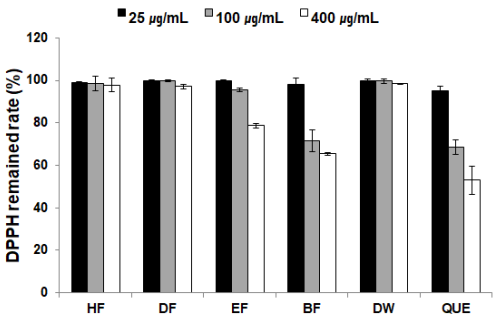

Fig. 1. DPPH radical remained rate of Solvent Fractions from *Raphiolepis indica* and quercetin. Abbreviation: HF, hexane fraction; MF, methylene chloride fraction; EF, ethyl acetate fraction; BF, butanol fraction; DW, DW fraction; QUE, quercetin.

3.2. Total phenolic content

Total phenolic content in the solvent fractions from *R. indica* was measured using gallic acid as a reference material. Total phenolic content in the n-hexane, dichloromethane, ethyl acetate, butanol and water fractions were 22.7, 41.7, 164.5, 137.3 and 37.6 mg GAE/g fractions, respectively. Among various fractions, ethyl acetate and butanol fractions from *R. indica* showed higher total phenolic content(Table 1). The content of polyphenol, which plays an essential role as an antioxidant, has been discovered in various plants, and the total phenolic contents of guava (*Psidium Guayaba* L.), mango (*Mangifera indica* L.), and barbados cherry (*Malpighia glabra* L.) are 24.15, 44.18, and 49.21 mg GAE/g respectively. Ethyl acetate and butanol fractions from *R. indica* were confirmed to contain higher total phenolic contents than these three extract[14].

3.3. Cytotoxic effects using hepatocytes

To measure the cytotoxic effects of the solvent fractions from *R. indica* in hepatocytes according to concentrations, the solvent fractions from *R. indica* were treated using the Huh7 cells and their cytotoxic effects was measured in 48 hours. The n-hexane fractions in hepatocytes did not show cytotoxic effects when treated at 25 μg/mL or below. The dichloromethane fractions in hepatocytes did not show cytotoxic effects when treated at 50 μg/mL or below. The ethyl acetate fractions in hepatocytes did not show cytotoxicity when treated at 200 μg/mL or below. The butanol and water fractions in hepatocytes did not show cytotoxicity when treated at 400 μg/mL or below. Quercetin used as the control group showed cytotoxicity when treated at 400 μg/mL or below(Fig. 2).

3.4. Cytoprotective effects on hepatocytes against H_2O_2

To measure the cytoprotective effects of the solvent fractions from *R. indica* in hepatocytes regarding oxidative stress, the Huh7 cells were treated with H_2O_2, thereby inducing oxidative stress. After this process, the cytoprotective effects of the solvent fractions from *R. indica* were measured. n-hexane, dichloromethane and water fractions did not show cyto-protective effects. However, ethyl acetate fractions from *R. indica* identified approximately 43 and 47% of cell survival rates at 100 and 200 μg/mL. This means that ethyl acetate fractions of *R. indica* showed 13 and 17% higher cell survival rates than the negative control showed. Also, the butanol fractions from *R. indica* showed 10% higher cell survival rates than the negative control showed. Quercetin as positive control showed approximately 90% of cell survival rates. This means that quercetin showed 60% higher cell survival rates than the negative control showed. This result identified cytoprotective effects of ethyl acetate and butanol fractions from *R. indica*, though lower than the effects of quercetin(Fig. 3).

Table 1. Total phenolic content of solvent fractions from *Raphiolepis indica*

Fractions	Total phenolic content(mg GAE/g fractions)
n-Hexane	21.73 ± 1.51
Dichloromethane	41.73 ± 2.41
Ethyl acetate	164.53 ± 4.81
Butanol	137.33 ± 3.84
Water	37.60 ± 1.06

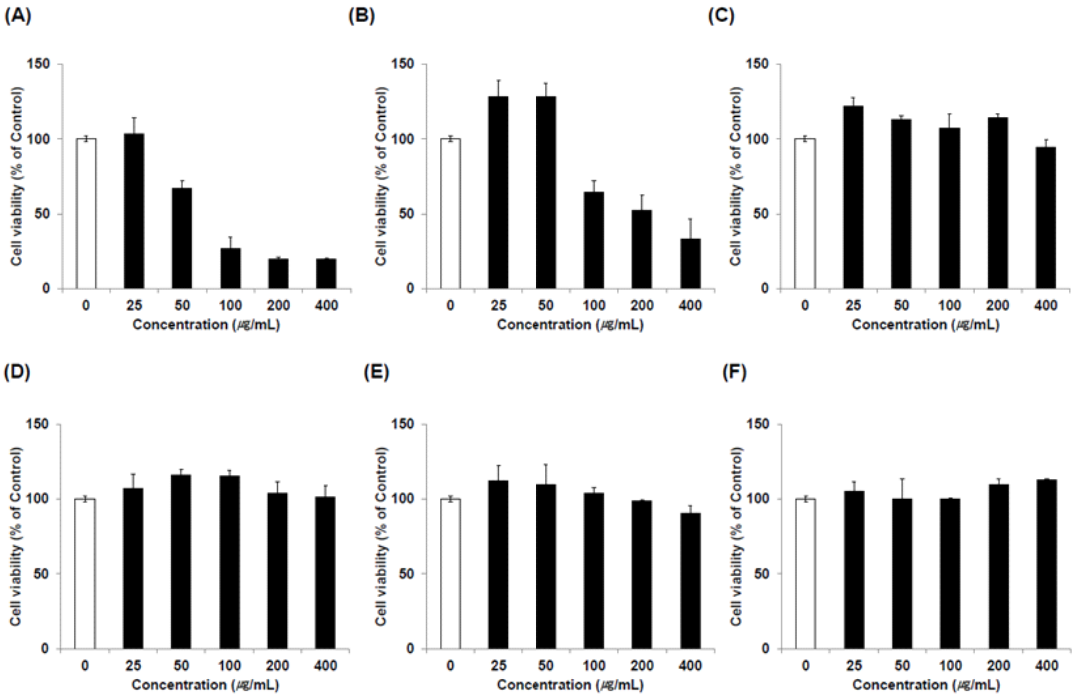

Fig. 2. Cell viability on Huh7 cells.
(A) hexane fraction, (B) dichloromethane fraction, (C) ethyl acetate fraction, (D) butanol fraction, (E) water fraction, (F) quercetin

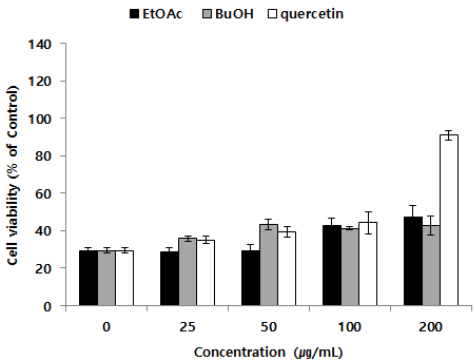

Fig. 3. Cytoprotective effects on Huh7 cells against hydrogen peroxide-induced oxidative damage. Abbreviation: EtOAc, ethyl acetate fraction; BuOH, butanol fraction.

3.5. Cytoprotective effects on hepatocytes against t-BHP

To measure the cytoprotective effects of the solvent fractions from *R. indica* in hepatocytes regarding oxidative stress, the Huh7 cells were treated with t-BHP, thereby inducing oxidative stress. After this process, the cytoprotective effects of the solvent fractions from *R. indica* were measured. n-hexane, dichloromethane and water fractions did not show cytoprotective effects. However, ethyl acetate and butanol fractions showed approximately 90% of cell survival rates at 25, 50, 100 and 200 ㎍/mL. This means that ethyl acetate fractions of *R. indica* showed 40% higher cell survival rates than the negative control showed. Quercetin as positive control showed 50% higher cell survival rates than the

negative control showed. This result identified cytoprotective effects of ethyl acetate and butanol fractions, though lower than the effects of quercetin. As studies on cytoprotective effects using various extracts have been conducted. The group treated with oxidative stress such as t-BOOH using *Pinus koraiensis* extracts protected hepatocytes and increased the cell survival rates[15]. And the group treated with oxidative stress such as t-BOOH using *Sutherlandia frutescens* protected pneumocytes[16]. This study confirmed cytoprotective effects against the oxidative stress in the hepatocytes. Hepatoprotective effects were identified with ethyl acetate and butanol fractions, which showed higher anti-oxidative effects.

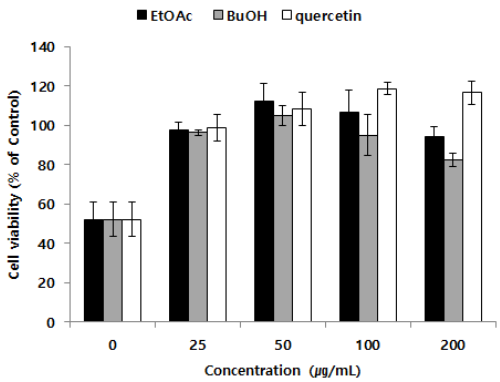

Fig. 4. Cytoprotective effects on Huh7 cells against tert-butyl hydroperoxide -induced oxidative damage. Abbreviation: EtOAc, ethyl acetate fraction; BuOH, buthanol fraction.

4. Conclusion

This study confirmed a high level of DPPH scavenging activity, total phenolic content and cyto-protective effects on Huh7 cells from ethyl acetate and butanol fractions from *R. indica*, and this conclusion can be used as basic data for development of a liver protectant.

Acknowledgements

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