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Differential Radiation Sensitivity of Shikonin Derivatives from *Lithospermum erythrorhizon* S.

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Differences in radiation sensitivity and color degradation of shikonin derivatives between the extracts of *Lithospermum erythrorhizon* root and authentic compounds were investigated. The degradation of shikonin derivatives in both root extracts and authentic compounds were drastically increased to above 8 kGy. HPLC analysis revealed radiation sensitivities of root extracts and authentic compounds differed significantly. Both shikonin and deoxyshikonin were the most sensitive with a degradation rate of 87-91%, while other derivatives in the extracts were the most resistant with a rate of 44-67%. Mixture of shikonin and deoxyshikonin showed a degradation rate of 20-24% at 4 kGy and around 60% at 8 kGy, whereas other shikonin derivatives showed a 48-62% degradation rate at 4 kGy and an 89-94% degradation at 8 kGy. The degradation pattern of shikonin derivatives in the mixture exhibited a similar pattern to those of the extract, but showed opposite pattern in individual derivative. The order of radiation sensitivity of shikonin derivatives are as follows: shikonin, deoxyshikonin>acetylshikonin, dimethylacrylshikonin, isobutyrylshikonin, isovalerylshikonin, 2-methyl-*n*-butyrylshikonin>hydroxyisovalerylshikonin in authentic compounds and isobutyrylshikonin, acetylshikonin, hydroxyisovalerylshikonin, isovalerylshikonin, 2-methyl-*n*-butyrylshikonin>deoxyshikonin, dimethylacrylshikonin>shikonin in root extracts.

Key words: gamma-ray, *Lithospermum erythrorhizon* S., radiation sensitivity, shikonin

Shikonin, a red naphthoquinone derivative, is a secondary metabolite that specifically occurs in boraginaceous plants and the active component of the medicinal plant *Lithospermum erythrorhizon*. Shikonin derivatives have wound-healing, anti-inflammation, anti-bacterial, anti-tumor, anti-diabetes, anti-viral activities, as well as beneficial proliferation of the granulation tissue. Due to these various beneficial effects, shikonin derivatives are used in pharmaceutical preparations,as food colorants and additives, and as ingredients in cosmetic formulations [Tomoyuki *et al*., 1995; Brigham *et al*., 1999; Yamamoto *et al*., 2000; Bulgakov *et al*., 2001; Kim *et al*., 2001; Yazaki *et al*., 2001; Kazufumi *et al*., 2002; Staniforth *et al*., 2004; Chung *et al*., 2006; Hu *et al*., 2006].

Recent trend of using natural products in industries tends toward multifunctional, high quality, and high-priced value foods and cosmetics. In order to meet the needs of consumers, cosmetics, medicine, and foods should contain the proper amount of natural products. Although the color removal processes such as filtration and absorption by clay are still useful, these procedures are difficult, time-consuming, and costly [Jo *et al*., 2003a].

Recently, Jo *et al*. [2003b] described a new irradiation method for processing bright-colored bio-materials. They tested the effects of gamma irradiation on the color characteristic and biological activities of various extracts such as green tea leaves, persimmon leaves, and licorice root extract. This technique is interesting, because it

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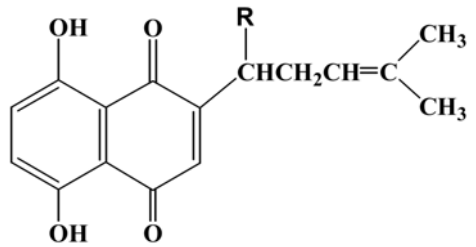
offers the possibility of substituting synthetic compounds with natural products for the production of high quality food and cosmetic products.

Even though various industrial applications of irradiated natural products have received considerable attention [Jo *et al*., 2003b], there is limited information on the radiation sensitivity of natural products as related to color characteristics, especially shikonin. Therefore, in the present study the effects of gamma irradiation on the shikonin derivative radiation sensitivity were investigated using both authentic compounds and root extracts. This study provides deeper insight into the mechanism of color change as well as valuable information on the radiation sensitivity of shikonin derivatives.

Materials and Methods

Chemicals. The authentic compounds, shikonin (S), deoxyshikonin (DS), 2-methyl-n-butyrylshikonin (MBS), dimethylacrylshikonin (DAS), isovalerylshikonin (IVS), and isobutyrylshikonin (IBS), were purchased from TCI (Tokyo, Japan). Acetylshikonin (AS) and hydroxy-isovalerylshikonin (HIVS) were purchased from Wako (Tokyo, Japan). The structures of shikonin derivatives are shown in Fig. 1.

Plants and gamma irradiation. *Lithospermum erythrorhizon* roots were purchased from the Geumsan area (Chungnam, Korea). Roots (5 g) were extracted three times with 500 mL of 100% methanol for 24 h with constant stirring at ambient temperature in the dark. After evaporation, root extract (1 mg) was dissolved in 1 mL of 100% methanol, and all authentic shikonin derivatives were made at the same concentration (0.2 mM). Samples (5 mL) in tightly capped vials were irradiated in a ^{60}Co irradiator (point source, ACEL, IR-79, Nordion, Canada) with 0.5-10 kGy absorbed doses. Digital photos were taken of the vials after gamma irradiation, and 20 mL each of the samples were analyzed by HPLC for color changes in both the authentic and root extract samples.



Shikonin derivatives	R
Shikonin	OH
Deoxyshikonin	H
Acethylshikonin	$OCOCH_3$
Dimethylacrylshikonin	$OCOCH_2(CH_3)_2$
Isobutyrylshikonin	$OCOCH(CH_3)_2$
Isovalerylshikonin	$OCOCH_2CH(CH_3)_2$
Hydroxyisovalerylshikonin	$OCOCH_2C(CH_3)_2OH$
2-methyl-n-butrylshikonin	$OCOCHCH_2(CH_3)_2$

Fig. 1. The structures of shikonin derivatives.

Measurement of shikonin content. The contents of shikonin derivatives were determined by HPLC. HPLC was performed on a C18 60-Å 4-mm column (3.9×300 mm, Nova-Pak, Waters, Massachusetts, USA) fitted to a 1200 Series HPLC System (Agilent Technologies, Waldbronn, Germany). The isocratic solvent system used was CH_3CN:H_2O:CH_3COOH:Et_3N (630:370:3:3, v/v). Chromatography was performed at a flow rate of 1.0 mL min^{-1}, injection volume of 20 mL, pressure of 140 bar, and a column temperature of 23°C. The wavelength of detection was set at 520 nm [Brigham *et al.,* 1999]. The linearity of the method was evaluated by processing a 5-point calibration curve ranging from 0.01 to 1 mM. Coefficients of determination (r^2) were greater than 0.995.

Results and Discussion

The color changes of shikonin derivatives in 100% methanol solution after gamma irradiation are shown in Fig. 2. The red color of the shikonin derivatives disappeared dose-dependently. The red color disappeared at doses above 8 kGy for the HIVS, MBS, IVS, IBS, DAS, AS, and Shikonin (S) compounds, whereas color of DS disappeared at doses above 1 kGy. This result indicates that DS in the methanol solution was the most sensitive toward gamma rays among the authentic shikonin derivatives. In order to confirm the disappearance of the red color, HPLC analysis was performed on the gamma-irradiated authentic compounds, and the results were compared to the color change results.

All shikonin derivatives were completely degraded at 10 kGy (Fig. 3). S and HIVS exposed to 1 kGy showed the highest (85%) and lowest (29%) degradation rate, respectively, whereas the other derivatives had similar degradation rates, ranging 46-54%. When shikonin derivatives were exposed to up to 4 kGy, S gradually degraded from 87-91%, and the other derivatives showed 53-69% degradation. DS showed a particularly sharp increase of degradation from 48-93%. Based on the results of the visible color change and the HPLC analysis, the degradation rates of all shikonin derivatives were determined to be positively correlated with the dose level (0-8 kGy) of gamma irradiation (Figs. 4 and 5). The sensitivity among shikonin derivatives was clearly different. Although S and DS were very sensitive to

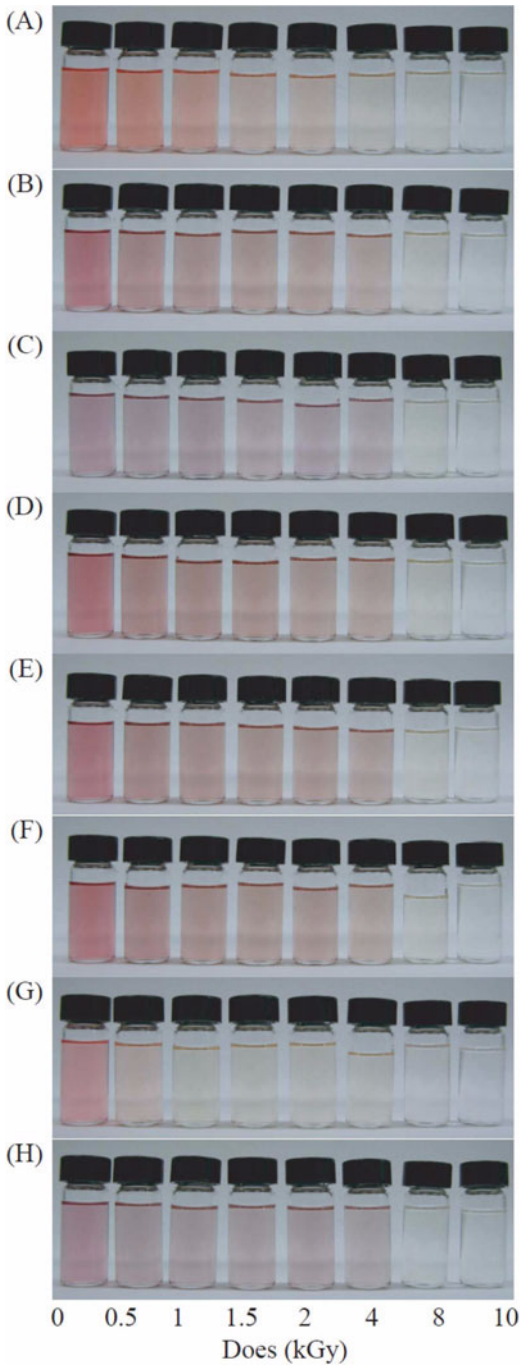


Fig. 2. The color change of shikonin derivatives after gamma irradiation treatment. (A), acetylshikonin; (B), dimethylacrylshikonin; (C), hydroxyisovalerylshikonin; (D), isobutyrylshikonin; (E), isovalerylshikonin; (F), 2-methyl-*n*-butyrylshikonin; (G), shikonin; (H), deoxyshikonin.

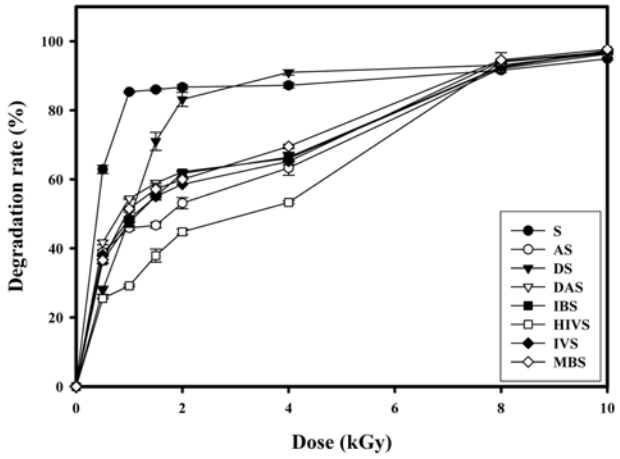


Fig. 3. The degradation of authentic compounds of shikonin derivatives after gamma irradiation treatment. The results are presented as the means±SE of three replicate experiments. "Rate" mean degradation per unit time. Delete "rate." Figure 2 and Figure 3 are basically same data. Recommend deleting Fig. 2.

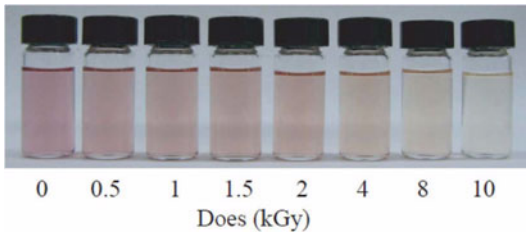


Fig. 4. The color change of root extracts after gamma irradiation treatment.

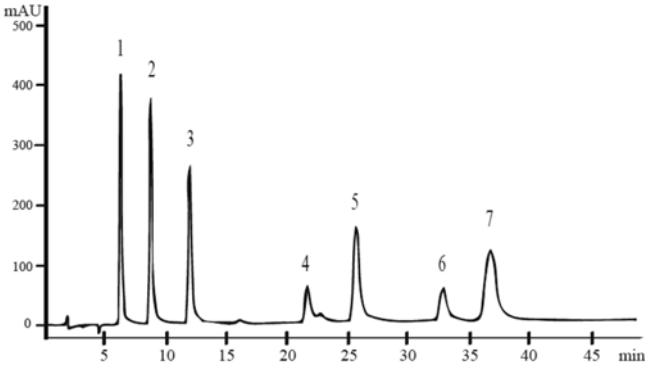


Fig. 5. High performance liquid chromatograms of authentic compound mixture. 1, shikonin; 2, hydroxyisovalerylshikonin; 3, acetylshikonin; 4, deoxyshikonin; 5, isobutyrylshikonin; 6, dimethylacrylshikonin; 7, isovalerylshikonin+2-methyl-*n*-butyrylshikonin. HPLC was performed on a C18 60-Å 4-mm column (3.9-3300-mm, Nova-Pak, Waters) fitted to a 1200 Series HPLC System (Hewlett Packard, Germany). The isocratic solvent used was CH_3CN:H_2O:CH_3COOH:Et_3N (630:370:3:3, v/v).

gamma rays, HIVS was relatively resistant.

The visible color changes of *Lithospermum erythrorhizon* S. root extract in 100% methanol solution after gamma irradiation are shown in Fig. 4. The disappearance of red color from shikonin derivatives from root extracts was also dose-dependent. The red color from root extracts disappeared at dose above 8 kGy, which is quite similar to the pattern of authentic shikonin derivatives. This

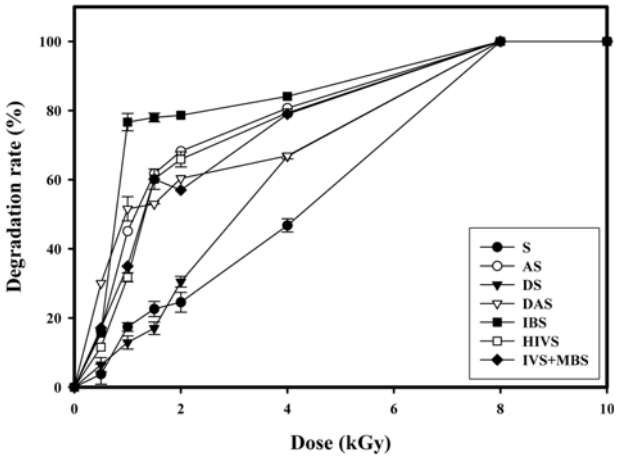


Fig. 6. The degradation of shikonin derivatives from root extracts after gamma irradiation treatment. The results are presented as the means±SE of three replicate experiments.

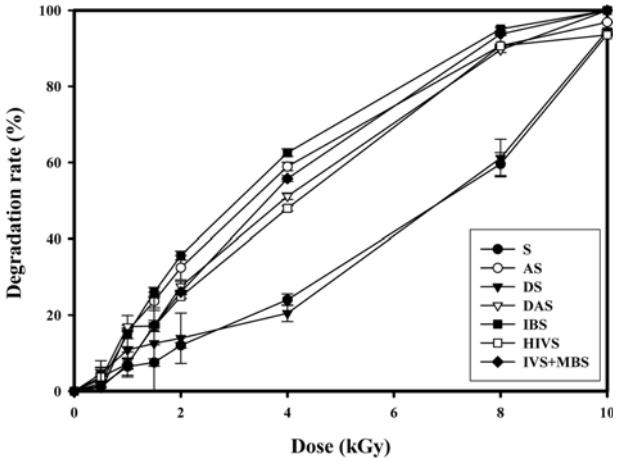


Fig. 7. The degradation of mixed authentic compounds of shikonin derivatives after gamma irradiation treatment. The results are presented as the means±SE of three replicate experiments.

phenomenon could be due to the gamma radiolysis of methanol, which can produce various molecular species and free radicals. Hydrogen (H·) and methoxy (CH_3O·) radicals are predominantly produced from methanol by the radiation, and these radicals are rapidly converted into a hydroperoxyl radical (HOO·) and an acetoxy radical (CH_3OOO·) in the presence of oxygen [Choi, 1970]. Although the details of the decay mechanism of chromophore by gamma-rays has yet to be determined, results of the present study suggest that the free radicals produced are capable of demolishing the chromophore group in all shikonin derivative compounds, resulting in the bleaching of the substrate solution.

Shikonin derivatives from root extracts treated at 4 kGy showed degradation rates ranging from 44-84%, much lower than those of the authentic compounds ranging from 53-91% (Fig. 3). The degradation rate of all shikonin derivatives from root extracts increased at 8 kGy. Isobutyrylshikonin (IBS), which showed an 84% degradation rate, was the most radiation-sensitive derivative of root extracts, whereas S and DS, which showed degradation rates ranging from 87-91%, were the most radiation-sensitive derivatives in the authentic compounds (Figs. 3 and 6). However, the tendency towards radiation sensitivity for S and HIVS between authentic and root extract samples was the opposite. S was the most sensitive with an 87% degradation rate, but the most resistant derivative with a 43% degradation rate in root extracts at 4 kGy. On the contrary, HIVS was the most resistant with a 53% degradation rate at 4 kGy, but it was sensitive in root extracts with a 79% degradation rate. In addition, the other derivatives such as IBS, DAS, IVS, and MBS showed similar degradation patterns both as pure forms (67-84% degradation) and the extracts (65-69%) at 4 kGy (Figs. 3 and 6).

The differences in radiation sensitivity between the pure form and the extracts were investigated using mixed authentic compounds. S and DS showed degradation rates of 20-24% at 4 kGy and around 60% at 8 kGy, whereas other shikonin derivatives showed 48-62% degradation rates at 4 kGy and 89-94% 8 kGy. These results obtained from a mixed solution of shikonin derivatives were similar to the degradation pattern of the root extracts, but in exact opposite pattern from the solution of individual derivative. From the results of different radiation sensitivity, we assumed that each shikonin derivative seemed to be affected by other shikonin derivatives against gamma irradiation through the complicated reciprocal reactions. However, we could not find any specific compound causing structural changes, as all of the shikonin derivatives had equal degradation in response to gamma-irradiation (data not shown).

In summery, the order of radiation-sensitivity of shikonin derivatives was as follows; 1) S, DS>others> HIVS in individual solution, 2) IBS, AS, HIVS, IVS, MBS>DS, DAS>S in root extracts, 3) others>S, DS in mixed solution. In addition, the inverse radiation sensitivity of S and DS in individual and mixed solutions of shikonin derivatives could be due to protective or degrading effects of other shikonin derivatives.

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