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Biological and anti-microbial activity of irradiated green tea polyphenols

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Abstract

Polyphenols of green tea leaf were separated and irradiated at 40 kGy to investigate the effect of irradiation on changes of biological and anti-microbial activities. The major antioxidative activities, including electron donating, inhibition of xanthine oxidase, metal ion chelating, and inhibition of lipid oxidation were maintained through irradiation, except for superoxide dismutase (SOD)-like activity at the 200 ppm level. The anti-microbial activities against *Staphylococcus aureus* and *Streptococcus mutans* were higher in the irradiated sample, which showed inhibition of microorganisms tested at a lower concentration than those of the non-irradiated sample. Ranges of the inhibition zone for growth of *Escherichia coli*, *S. aureus*, *S. epidermidis* and *S. mutans*, at 1 mg/disc, were 9.3, 10.1, 22.5 and 9.3 mm in non-irradiated control but 10.8, 11.0, 25.0, and 11.7 mm in irradiated samples, respectively. Results indicated that irradiation of polyphenols, the major bioactive compounds in green tea, may maintain the biological activities and even increase the anti-microbial activity. The results also demonstrated that irradiation of green tea polyphenols, for removal of dark colour, may be applicable in the food or cosmetic industries.

Keywords: Green tea; Polyphenols; Biological activity; Anti-microbial; Irradiation

1. Introduction

Separations of bioactive compounds from natural resources, which have been consumed safely for a very long time have been actively studied (Choi, Shin, Chang, & Shin, 1989). Among such sources, green tea has the longest history in the world and used in about 160 countries everyday for drinking. Green tea is one of the three most popular beverages, besides coffee and cocoa (Kim, 1996). Green tea is composed of about 30% of polyphenols (dry basis), such as flavanols, flavandiols, flavonoids, and phenol acids. Polyphenols have been well-known to have various excellent biological activities, for example, inhibition of tooth decay (Sakanaka, Kim, & Yamamoto, 1989), inhibition of allergy (Yeo, Ahn, Lee, Lee, Park, & Kim, 1995), reduction of blood pressure (An, 1998), prevention of gout (An, Bae, & Choi, 1996) and inhibition of oxidation. Especially, the inhibition effect of green tea polyphenol on lipid oxidation was higher than that of the synthetic antioxidant, butylated hydroxytoluene (BHT) (Chen, Chan, Ma, Fung, & Wang, 1996; Wanasundara & Shahidi, 1998). Furthermore, the dark colour problem, on industrial application of green tea leaf extract, can be solved or minimized by irradiating the green tea leaf extract without compromising its biological activities (Son, Jo, Kim, Kim, & Byun, 2001).

The international consultative group of food irradiation (ICGFI) concluded that irradiation of food at a dose level of 10 kGy or below was toxicologically safe and nutritionally adequate (WHO, 1981). On the other

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hand, many reports have been published on anti-microbial effects of green tea polyphenols but knowledge of the effect of irradiated green tea polyphenols on growth of the microorganisms related to skin inflammation, such as *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus mutans*, is very limited.

The present study was designed to investigate the effect of irradiation on biological and anti-microbial activity of polyphenol separated from green tea leaf for further application in the food or cosmetic industries as a natural resource. Irradiation was performed at a dose (40 kGy) above the CODEX presently approved level (10 kGy) for better understanding of the effect of irradiation on the polyphenol.

2. Materials and methods

2.1. Chemicals

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH), xanthine, xanthine oxidase, pyrogallol, tris(hydroxymethyl)aminomethane (TRIZMA), fish oil, 2-thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Nutrient medium, tryptic soy medium, brain heart infusion medium, and YM medium for anti-microbial test in skin or oral cavity were purchased from Difco Lab. (Sparks, MD, USA).

2.2. Sample preparation

The schematic diagram of sample preparation is shown in Fig. 1. Dried green tea (10 kg) was extracted using 60% acetone (50 l) in an extractor for 24 h at room temperature. The extract was centrifuged (Hitachi, CR21, Tokyo, Japan) at 8000*g* for 30 min and the upper layer was collected. 60% acetone (50 l) was added to the pellet and the extraction process was repeated three times. The collected upper layer was evaporated (N-N SERIES, Eyela, Tokyo, Japan) and filtered (No. 2, Advantec, Tokyo, Japan) to remove chlorophyll. The remainder was used for sample separation. The stationary phase for chromatography was prepared as by Nonaka (1989). The sample was loaded into a Sephadex LH-20 column (Pharmacia biotech, Stokholm, Sweden) and progressed and separated by using a ratio of methanol to acetone of 0–1 through 1–0. Each fraction of polyphenol was developed using silicagel thin-layer chromatography (TLC) and lyophilized (FD5510, Ilshin Lab. Co., Gyeonggi, Korea). The content of polyphenol was quantified in lyophilized sample compared to the (+)-cathechin standard (Sigma Co. Ltd., St. Louis, MO, USA). The sample was defined to have 60% of polyphenol content and was used for the experiment.

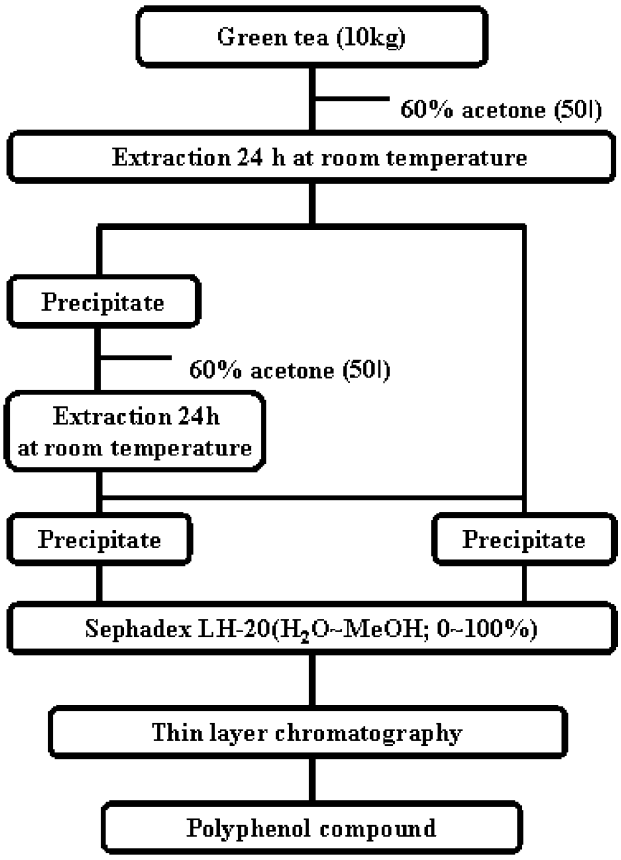

Fig. 1. The schematic diagram of isolation of polyphenol compounds from green tea.

2.3. Irradiation

The lyophilized sample, in tightly capped containers (each 100 g), was irradiated in a cobalt-60 irradiator (point source, AECL, IR-79, Nordion, Canada) with 0 and 40 kGy absorbed doses. The source strength was approximately 100 kCi with a dose rate of 10 kGy/h at 13 ± 0.5 °C. Dosimetry was performed using 5-mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany), and the free radical signal was measured using a Bruker EMS 104 EPR Analyzer. The actual dose was within $\pm 2\%$ of the target dose. Samples were turned 360° continuously during the irradiation process to achieve uniform target dose and the non-irradiated control was placed outside of the irradiation chamber to have the same temperature effect as the irradiated sample.

2.4. Electron donating ability

Electron donating ability (EDA) ability was measured by the Blois' (1958) method. The extract (1 ml) and DPPH solution (1 ml, 2×10^{-4} M) were vigorously vortexed and stood for 30 min at room temperature and measured using a spectrophotometer (AAS, Hitachi Z-

6000, Tokyo, Japan) at 517 nm. The electron donating ability was calculated as

EDA (%) =[1 − (absorbance value of testing solution/absorbance value of control solution)] × 100.

2.5. Xanthine oxidase inhibition effect

Xanthine oxidase inhibitory effect was assayed by the method of Stirpe and Corte (1969). The reaction mixture, containing 0.2 ml substrate (2 mM xanthine) in a 0.1 M potassium phosphate buffer (pH 7.5), 0.1 ml (0.2 Unit/ml) of enzyme solution and 0.1 ml of extract solution, was reacted at 37 °C for 5 min. The control solution was prepared by adding 0.1 ml distilled water instead of extracts. The reaction was stopped by adding 1 ml of 1 N HCl. The formation of uric acid by reaction was measured at 292 nm using a spectrophotometer. The inhibition of xanthine oxidase activity was expressed as

Inhibitory effect (%) = [1 − (uric acid of the reaction/uric acid of the control)] × 100.

2.6. Lipid oxidation

To assess the development of lipid oxidation, oil emulsion was prepared. After adding 0.1 M maleic acid buffer (8 ml), Tween 20 (50 μl), and fish oil (0.5 ml), the mixture was homogenized for 15 min. KOH (2 g) was added to the mixture and the pH adjusted to 6.5 by adding 0.1 N HCl.

2-Thiobarbituric acid-reactive substances (TBARS) were measured by the Buege and Aust (1978) method, using the oil emulsion. The samples (0.1 ml), with polyphenol concentrations of 1, 10, 50, 100 and 200 ppm, were added to the oil emulsion (0.5 ml) and deionized distilled water (DDW, 0.4 ml). The test tube with reaction mixture (1 ml) was placed in a water bath at 37 °C for 1 h and dibutylated hydroxytoluene (BHT, 7.2%) was added to stop the reaction. After vortexing the reaction mixture, 20 mM TBA in 15% TCA (2 ml) solution was added and it was heated in a boiling water for 15 min. Tap water was used to cool the reaction mixture for 10 min and it was centrifuged (VS-5500, Vision Scientific, Co., Seoul, Korea) at 2000*g* for 15 min. The upper layer was read by the spectrophotometer (AAS, Hitachi Z-6000, Tokyo, Japan) at 531 nm. The reading of absorbance was recorded.

2.7. Assay for superoxide dismutase (SOD)-like activity

Superoxide dismutase (SOD)-like activity was assayed by the method of Marklund and Marklund (1974). The reaction mixture was prepared by mixing 0.2 ml of the sample solution, 2.6 ml of the tris–HCl buffer (50 mM TRIZMA + 10 mM EDTA, pH 8.5), 0.2 ml of 7.2 mM pyrogallol and stood at 25 °C for 10 min. The oxidized pyrogallol was measured at 420 nm, using a spectrophotometer (AAS, Hitachi Z-6000, Tokyo, Japan), after stopping the reaction by adding 0.1 ml of 1.0 N HCl. The SOD-like activity was expressed as reduction rate of absorbance.

SOD-like activity (%) = [1 − (absorbance value of testing solution/absorbance value of control solution)] × 100.

2.8. Anti-microbial activity

2.8.1. Growth of microorganisms

The tested microorganisms for anti-microbial activity were selected from skin and the oral cavity and purchased from the Korean Collection for Type Cultures (KCTC) in the lyophilized state. Human skin-related, *S. epidermidis* (KCTC 1917) and *E. coli* (KCTC 1039) were grown at 37 °C for 24 h in nutrient medium and *S. aureus* (KCTC 1621) was grown in tryptic soy medium at 37 °C for 24 h. The microorganisms of the oral cavity, *S. mutans* (KCTC 3065) and *Candida albicans* (KCTC 7965), were grown in brain heart infusion medium and YM medium at 37 °C for 24 h, respectively. As a dilution solution, 0.1% peptone water was used. The samples were centrifuged at 4220*g* for 10 min at 4 °C in a refrigerated centrifuge (Union 5KR, Hanil, Seoul, Korea). Samples were washed twice with sterile normal saline. The pellet was finally suspended in sterile peptone water to give a cell density above 10^6 CFU/g.

2.8.2. Measurement of growth inhibition zone

The anti-microbial activity was measured by the agar diffusion method, using a paper disc. One colony of the microorganisms was selected and grown in 10 ml of each medium at the designated temperature for 24 h and inoculated again with cell suspension (0.1 ml) to 10 ml of the medium. Then, the inoculum (about 10^5 cells/ml) was uniformly and aseptically spread on the different media. It was kept in a sterile workstation for 30 min to allow it to be absorbed with the designated concentration of green tea polyphenols and the cells were grown at 35 °C for 24 h. The clear zone (mm) around the disc was measured and reported.

2.8.3. Measurement of minimum inhibitory concentration

Measurement of minimum inhibitory concentration (MIC) was measured by inoculation of one colony to the media with different polyphenol concentrations. The test microorganisms were grown for 48 h and the MIC was determined visually if no growth was found.

2.9. Statistical analysis

The data were collected and analysed by the Statistical Package for the Social Science (SPSS Inc., 10.0, 2000) programme. Differences among mean values were obtained by Duncan's multiple range test.

3. Results and discussion

3.1. Electron donating ability

Electron donating ability of polyphenols is shown in Fig. 2. EDAs of irradiated polyphenols at 40 kGy and non-irradiated controls were not different at all concentrations tested. This accorded with the conclusions of Son et al. (2001) and Jo, Son, Lee, and Byun (2003) that 20 kGy of γ-irradiation did not affect EDA of green tea leaf extract. The 1 ppm polyphenol concentration showed less than 20% of EDA but the 5 ppm concentration showed more than 70%. Mahoney and Graf (1986) reported that, in the EDA, the reduction capacity from donating hydrogen or electrons to ROO·, R·, or RO· radicals is very important but it cannot explain all the general antioxidative function.

3.2. Xanthine oxidase inhibition effect

Xanthine oxidase relates to purine metabolism and it converts xanthine or hypoxanthine to uric acid. It has been reported that the increase of uric acid and accumulation in blood and bone, resulting in gout and accumulation, occasionally induces kidney trouble (Stirpe & Corte, 1969; Storch & Feber, 1988).

Fig. 3 shows the effect of polyphenols separated from green tea, on inhibition of xanthine oxidase. At 5 and 10 ppm of sample concentration, the inhibitions of xanthine oxidase of irradiated samples were 10.0% and 14.6%, which were higher than those of non-irradiated control (0.44% and 4.27%, respectively) ($P < 0.01$). However, there was no difference between irradiated and non-irradiated samples at concentrations of 50 and 100 ppm. Byun, Son, Yook, Jo, and Kim (2002) reported that the inhibition of xanthine oxidase in traditional soybean-based fermented food, *Doenjang*, was decreased by irradiation at 20 kGy, which was not consistent with our results.

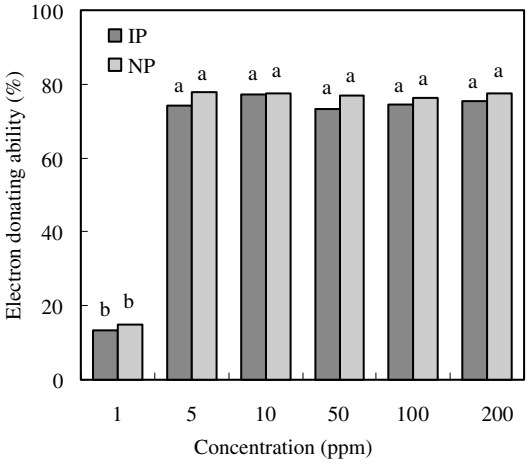

Fig. 2. Electron donating ability of irradiated polyphenols isolated from green tea. IP: irradiated polyphenols (40 kGy); NP: nonirradiated polyphenols. Values are means of five replicates and those with different alphabet letters are significantly different at $p < 0.01$.

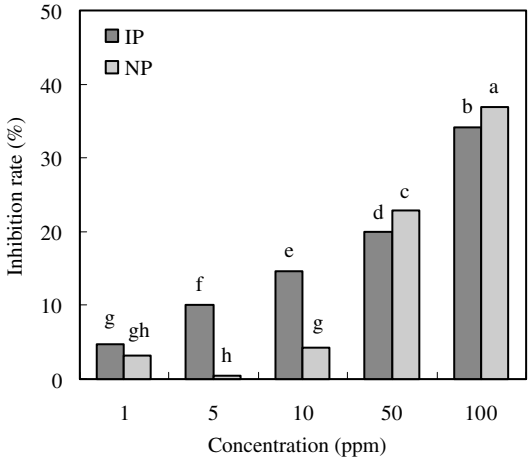

Fig. 3. Inhibition rate of irradiated polyphenols isolated from green tea on xanthine oxidase. IP: irradiated polyphenols (40 kGy), NP: nonirradiated polyphenols. Values are means of five replicates and those with different alphabet letters are significantly different at $p < 0.01$.

3.3. Inhibition of lipid oxidation

The inhibition effect (of polyphenols separated from green tea) on lipid oxidation was measured using fish oil (Fig. 4). The addition of polyphenol, regardless of irradiation, resulted in lower absorbance than the control, suggesting that the polyphenols from green tea had an antioxidative property. After 48 h of incubation, the absorbance of the irradiated sample at 5 ppm was 0.693 but the non-irradiated sample was 0.728. High antioxidative capacity was detected above 50 ppm, which showed half of the absorbance of the control sample (about 0.4 vs 0.835). Results indicate that irradiation has no effect on antioxidatve properties of polyphenols separated from green tea at various concentrations. This agreeds with the report that irradiation of oriental medicinal herbs did not change peroxide value on EDA (Byun, Yook, Kim, & Chung, 1999).

3.4. Metal ion chelating effect

One of the traditional roles of antioxidant is chelation of metal ions and termination of radical reaction, by radical abstracting power from materials which have

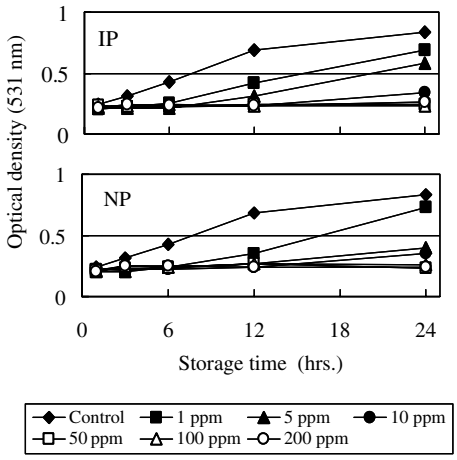

Fig. 4. Effect of irradiated polyphenols isolated from green tea on lipid oxidation in oil emulsion. IP: irradiated polyphenols (40 kGy); NP: nonirradiated polyphenols. Values are means of five replicates and those with different alphabet letters are significantly different at $p < 0.01$.

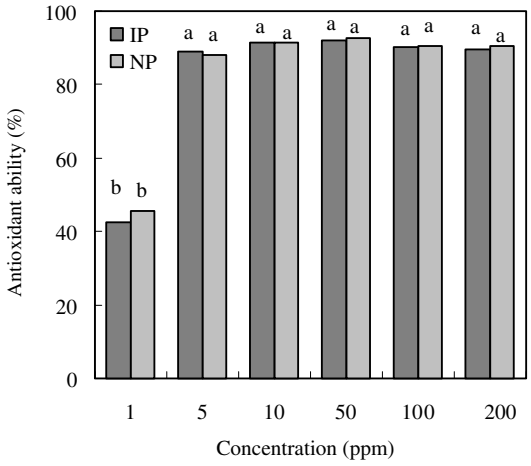

Fig. 6. Effect of irradiated polyphenols isolated from green tea and reacted with Cu^{2+} ion, on lipid oxidation in oil emulsion. IP: irradiated polyphenols (40 kGy), NP: nonirradiated polyphenols. Values are means of three replicates and those with different alphabet letters are significantly different at $p < 0.01$.

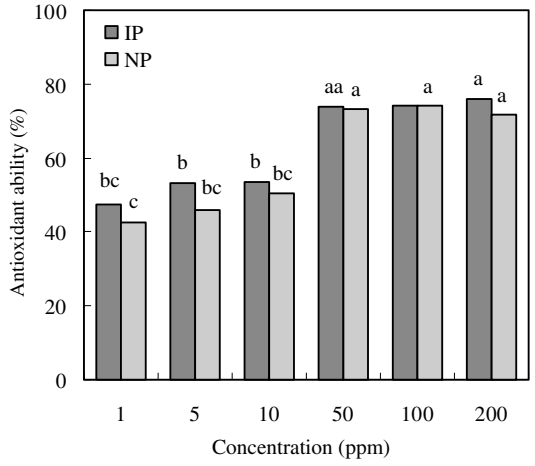

Fig. 5. Effect of irradiated polyphenols isolated from green tea and reacted with Fe^{2+} ion, on lipid oxidation in oil emulsion. IP: irradiated polyphenols (40 kGy), NP: nonirradiated polyphenols. Values are means of three replicates and those with different alphabet letters are significantly different at $p < 0.01$.

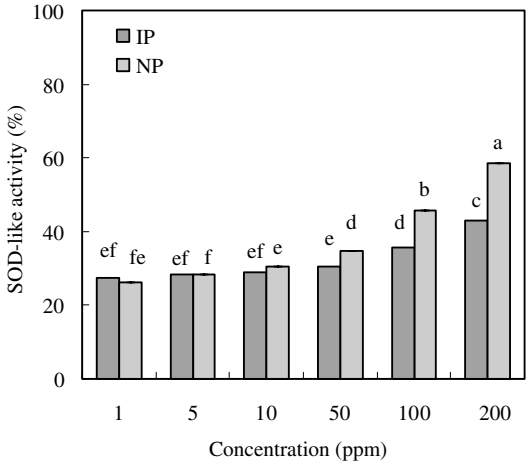

Fig. 7. SOD-like activity of irradiated polyphenols isolated from green tea. IP: irradiated polyphenols (40 kGy); NP: nonirradiated polyphenols. Values are means of five replicates and those with different alphabet letters are significantly different at $p < 0.01$.

enzyme or enzyme-like activity (Chan, Decker, Lee, & Butterfield, 1994). Thus, the metal chelating effects of oxidation-accelerating metal ions, Fe^{2+} and Cu^{2+}, were observed (Figs. 5 and 6). The metal chelating effects of the irradiated or non-irradiated polyphenols isolated from green tea were higher for Cu^{2+} than for Fe^{2+}. Over 80% of Cu^{2+}-chelating effect was determined with 5 ppm, or above, polyphenol concentration but about 70% of Fe^{2+}-chelating effect with 50 ppm or above. There was no difference found by irradiation treatment.

3.5. Superoxide dismutase (SOD)-like activity

Superoxide dismutase (SOD)-like activity of polyphenols separated from green tea, which is the important self-defence mechanism of body cell against oxidative damage (Ji, 1993), is shown in Fig. 7. As concentration of the polyphenol increased, the SOD-like activity increased ($P < 0.01$). Irradiated samples showed higher SOD-like activity than non-irradiated above 50 ppm but, at 200 ppm, the irradiated and non-irradiated polyphenols showed 59.4% and 43.1%, respectively, indicating lower activity in irradiated sample.

As described earlier, irradiation did not affect electron donating activity, inhibition of xanthine oxidases, lipid oxidation, or metal-chelating effect of polyphenols separated from green tea leaf. The only exception was shown in SOD-like activity at 200 ppm. Thus, irradiation of green tea extract may increase industrial applicability by improving the colour of the extract for further processing without affecting its natural biological activities.

Table 1
Minimum inhibitory concentrations (MIC) of irradiated polyphenols isolated from green tea on microbial growth

Strains	MIC (ppm)	
	Green tea	Irradiated green tea
Escherichia coli	500	500
Staphylococcus aureus	250	125
Staphylococcus epidermidis	<1	<1
Streptococcus mutans	500	250
Candida albicans	>2000	>2000

3.6. Anti-microbial activity

The minimum inhibitory concentrations (MIC) of polyphenols separated from green tea against human skin- or oral cavity-presented microorganisms are shown in Table 1. Irradiated polyphenols had lower MIC against *S. aureus* and *S. mutans*. Generally, MIC values of non-irradiated sample, against *E. coli*, *S. mutans*, *Staphylococcus aureus* and *S. epidermidis*, were 500, 500, 250, and less than 1 ppm, respectively, and the MIC values of irradiated sample against these microorganisms were 500, 125, 250, and less than 1 ppm, respectively.

Growth inhibition zones of green tea polyphenols against human skin- or oral cavity-presented microorganisms are shown in Table 2 and Fig. 8. Non-irradiated samples with 1 mg/disc resulted in 9.3, 10.05, 22.5, and 9.3 mm for *E. coli*, *S. aureus*, *S. epidermidis,* and *S. mutans*, respectively, while in irradiated sample, with 1 mg/disc, these were 10.8, 11.7, 25.0, and 11.7 mm, in the same order. Results indicate that the irradiated samples showed a higher anti-microbial activity than did the non-irradiated ($P < 0.05$). Generally, the polyphenol is very stable except for thiolysis, reaction with strong acid (McGraw, Steynberg, & Hemingway, 1993). The irradiated polyphenol might not change its chemical structure but some change in activity is expected of functional groups such as –OH or –COOH, resulting in higher anti-microbial activity. However, there was no detectable growth inhibition zone for *C. albicans,* regardless of irradiation treatment. Kim and Han (1998)

Table 2
Anti-microbial activity of irradiated polyphenols isolated from green tea on different microorganisms

Strains	Green tea (mg/disc)			Irradiated green tea (mg/disc)		
	0.25	0.5	1	0.25	0.5	1
Escherichia coli	–[a]	8.3 ± 0.53[b]	9.3 ± 0.29	–	9.3 ± 0.45	10.8 ± 0.76
Staphylococcus aureus	–	8.6 ± 0.29	10.0 ± 0.5	–	8.7 ± 0.29	11.0 ± 1.00
Staphylococcus epidermidis	17.0 ± 0.87	19.3 ± 1.53	22.5 ± 2.12	18.3 ± 2.75	22.7 ± 1.61	25.0 ± 3.12
Streptococcus mutans	–	–	9.3 ± 0.29	–	–	11.7 ± 1.04
Candida albicans	–	–	–	–	–	–

[a] No inhibition.
[b] Diameter of inhibition zone (mm).

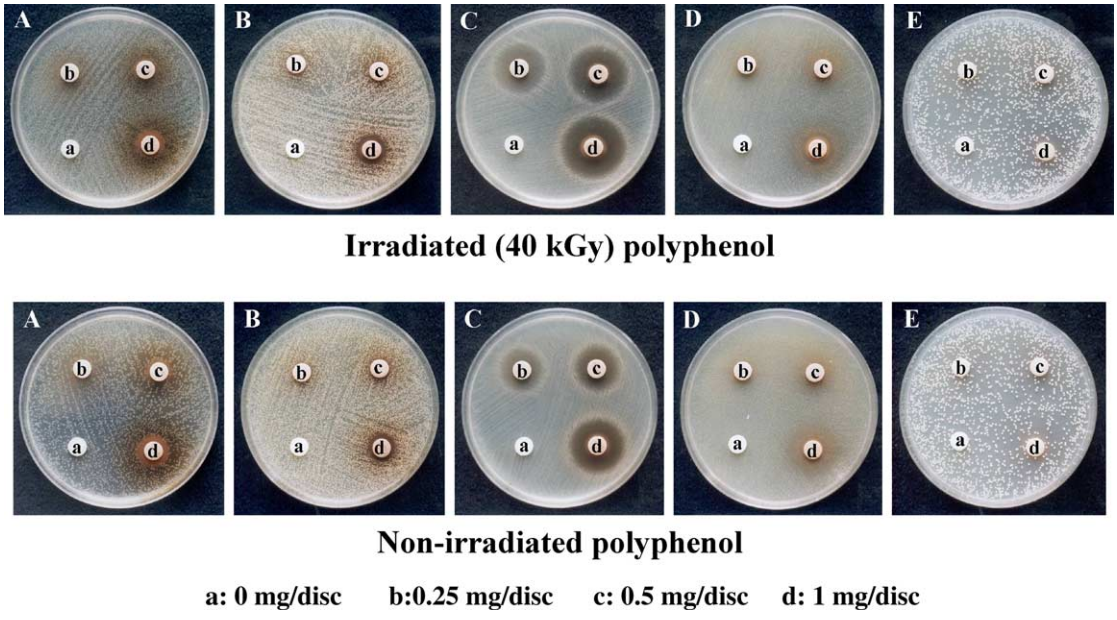

Fig. 8. Inhibition zone of irradiated polyphenols isolated from green tea with different microorganisms. A: *Escherichia coli*, B: *Staphylococcus aureus*, C: *Staphylococcus epidermidis*, D: *Streptococcus mutans,* E: *Candida albicans*.

reported that a chloroform fraction of dandelion showed 11 and 9 mm of inhibition zone at 1.5 mg/disc for *S. aureus* and *E. coli,* respectively. Ethanol extract of leaf mustard (*Brasica juncea* Coss) inhibited the growth of *S. aureus* and *E. coli* at the concentration of 2 mg/disc (Kang, 1995). These previous results indicate that the polyphenols of green tea inhibit the growth of those microorganisms at a lower concentration. Also, results indicate that, among the major microorganisms presented in human skin or the oral cavity, *S. epidermidis,* which is closely associated with skin eczema or development of pimples, was effectively inhibited by the green tea polyphenols. Therefore, the green tea polyphenols can be used as an antiseptic aid in food or cosmetics.

In conclusion, biological and anti-microbial activities of the polyphenol isolated from green tea leaf were not changed by irradiation at 40 kGy. Furthermore, the fact that the irradiated polyphenols seem to have a higher anti-microbial effect than the non-irradiated, in all strains tested, indicates that irradiation technology is very useful and applicable to the food or cosmetic industries in addition to removal of the dark colour of the green tea leaf extract or polyphenol.

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