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Title: Influence of gamma irradiation on microbial load and antioxidative characteristics of Polygoni Multiflori Radix

Article Type: Full Length Article

Keywords: Polygoni Multiflori Radix; Gamma irradiation, Antiseptic effect; Antioxidation

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Abstract: Gamma radiation is a physical process commonly used for the eradication of microorganisms distributed in food ingredients, medicinal plants and other bioresearches. The aim of this study was to investigate the effect of radiation dosage on the microbial load, chemical compounds and antioxidative characteristics of Polygoni Multiflori Radix (POMU). Ten commercial POMUs were purchased from different herbal markets and treated with 2 kGy, 4 kGy, 6 kGy, 8 kGy and 10 kGy gamma radiation doses to evaluate the microbial burdens of irradiated and unirradiated POMUs.
Our results confirmed that 2 kGy was sufficient for the inactivation of enterobacteria; at 4 kGy, mold and yeast counts were obviously reduced; and at 6 kGy, neither yeasts nor fungi were observed any longer.
The antioxidative effects and major antioxidant components of 0 kGy, 5 kGy, 10 kGy and 15 kGy irradiated POMU samples were also examined. Our results confirmed that 5 kGy irradiated POMU had both the highest antioxidative activity and lowest value in IC50 of DPPH radical-scavenging activity. The content of total phenols had no statistically significant changes. Therefore gamma irradiation at 5 kGy could be a potential method for decontaminate the microbial load of POMU to prolong shelf life and to improve hygienic quality.

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November 30, 2010

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Editor, Process Biochemistry

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Dear Dr. Zhong:

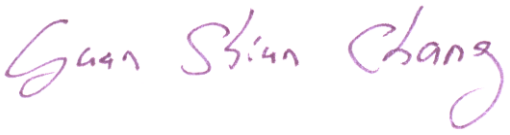
This is to resubmit our revised manuscript (Ms. Ref. No.: PRBI-D-10-00505) entitled Influence of gamma irradiation on microbial decontamination and antioxidative characteristics of Radix Polygoni Multiflori for publication in Process Biochemistry

First, I like to thank you and the reviewers for detailed and helpful comments. We had revised the manuscript accordingly. The response to the reviewers' comments is provided in a separated sheet. I also like to apologize for the delay of the resubmission of our revised manuscript due to a two week long overseas travel during the periods.

We wish the revised manuscript will be considered for publication in Process Biochemistry. Thanks again for all your help. We look forward to hearing from you again very soon.

The corrected manuscript has been resubmitted to your Journal. Any additional comments and suggestions concerning this manuscript will be highly appreciated. Thank you very much.

Yours sincerely



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Enclosure

Detailed Answers to the Editors' Comments of Manuscript PRBI-D-10-00505

November 30, 2010

Reviewer #1:

The present data provide important information for the use of Gamma radiation in microbes elimination of Radix Polygoni Multiflori, though Gamma radiation have been widely used for the antisepsis of herbs. The MS gave the appropriate irradiation dose and demonstrated that, after gamma radiation treatement, the antioxidative activity was not affected and the content of bioactive component was not decreased. But some questions were important, which need to be clarified:

1. Orinigal source or place of product of materials should be given: 10 samples were assayed in table 1, but samples were unknown for table 2, table 3, table 4, Figure 1, and Figure 2.

Ans: The source or place of product of materials had been added. "Ten samples of Polygoni Multiflori Radix were used throughout the manuscript. The samples were purchased from 8 traditional Chinese medicine importers, 1 manufactory and 1 Chinese drugstore, of which all herbs originated from Mainland China." was added in line 16 on page 5. " The POMU sample which purchased from traditional Chinese medicine importer" was added in line 9 on page 7.

2. I do not know whether the active compounds and antioxidant activity of materials from different original place of product have the same response pattern after gamma irradiation.

Ans: In this manuscript, we had repeated the experiments for three times. We found that the active compound of THSG and antioxidant activity of materials from different original place of product have the same response pattern after gamma irradiation.

3. Why select the antioxidant activity?

Ans: According to Chinese medicine theory, Polygoni Multiflori Radix counteracts toxicity, cures carbuncles and relaxes the bowels whereas processed Polygoni Multiflori Radix replenishes the liver and kidney with vital essence and blood, blackens the hair and strengthens the tendons and bones. The antioxidant activity is an easy method to detect the tissue or cell damage after gamma irradiation and THSG is the active compound with strong antioxidant activity, so we

selected it for comparison..

4. Was other important activity related to main effectiveness of Polygoni Multiflori Radix decreased after gamma irradiation?

Ans: We did not know whether other important activity related to main effectiveness of Polygoni Multiflori Radix decreased after gamma irradiation. A previous study investigated the differences of chemistry between raw and processed Polygoni Multiflori Radix. Two anthraquinones, namely, emodin-8-*O*-(6′-*O*-malonyl)-glucoside and physcion-8-*O*-(6′-*O*-malonyl)-glucoside, either disappeared or greatly decreased. Similarly, the contents of 2,3,5,4′-tetrahydroxystilbene-2-*O*-*β*-*D*-glucopyranoside, emodin-8-*O*-*β*-*D*-glucopyranoside and physcion-8-*O*-*β*-*D*-glucopyranoside also decreased. Conversely, the contents of emodin and physcion generally increased. (Liang, et al., 2010). The processing Polygoni Multiflori Radix may change the contents and types of chemicals in it. These changes are probably responsible for the various pharmacological effects of raw and processed Polygoni Multiflori Radix as well as hepatotoxicity.

Reference:

Liang Z, Chen H, Yu Z, Zhao Z. Comparison of raw and processed Polygoni Multiflori Radix (Heshouwu) by high performance liquid chromatography and mass spectrometry. Chin Med. 2010; 5: 29.

5. The main effectiveness aspects of Polygoni Multiflori Radix as one kind herb should be stated directly.

Ans: The main effectiveness aspects of Polygoni Multiflori Radix had been added in line 7 on page 4 as follows: “POMU also counteracts toxicity, cures carbuncles and relaxes the bowels whereas processed POMU replenishes the liver and kidney with vital essence and blood, blackens the hair and strengthens the tendons and bones. [14]”.

Reviewer #2: Comments-

The full length article had addressed major aspects on gamma radiation processing of Radix Polygoni Multiflori viz. microbial decontamination, effect on TSHG and other antioxidant compounds. The article should be further improved in its presentation, sentences formation and clarity in results discussion. However, it has content that could be of interest to the readers of the journal. There are several minor issues to be rectified and few logical questions to be clarified by the authors.

1) Page 2, line no.2; Gamma radiation is not a "bio-process" it should be mentioned as physical process.

Ans: We corrected it as suggested, on page 2, line 2.

2) In the manuscript, instead of words like "antisepsis", "antiseptic level", words like "microbial decontamination", "microbial load" and "Hygienization" shall be used appropriately to convey the meaning more clearly.

Ans: We used the word "microbial load" instead of "microbial decontamination" on page 1, line1; page 18, line 14 and page 20, line 14.;using "microbial load" instead of "antiseptic level" on page 2, line 5; using " decontaminate the microbial load instead of "the microbial decontamination" on page 2, line 18; using " Hygienization " instead of " antisepsis " on page 18, line 12, 17 and 18, page 20, line 9 of this manuscript.

3) Page 2, line 12-14; the paragraph shall include control (non-irradiated) samples along with irradiated samples (5, 10 and 15 kGy) while comparing their antioxidative effects and major antioxidant components. (i.e. 5 kGy samples should be compared with both control and other irradiated samples)

Ans: We more described it as suggested, on page 2, line13.

4) Page 3; "Gamma irradiation" should be added as one of the key words.

Ans: We added "Gamma irradiation" as the key words as suggested, on page 3, line 1.

5) Page 3, line no.3; Clostridium is not an aerobic spore former, it is an anaerobic spore former.

Ans: We deleted Clostridium as suggested.

6) Page 3, line no.4; the word "fumigation" is the appropriate word instead of "gas".

Ans: We corrected the word "fumigation" as suggested, on page 3, line 8.

7) Page 4, line no.1; use the word "doses" instead of "power".

Ans: We used the word "doses" instead of "power" as suggested, on page 4, line 2.

8) Page 4, line no.14; include word "antioxidants" in the line, so that the sentence will be like, "whether they bring about any changes in the antioxidants composition of POMU and to find the…"Page 4, line no.15

Ans: We added "antioxidants" as suggested, on page 4, line 18.

9) Page 7, line no.5; "0 kGy" should be included, in different gamma radiation doses as (0 kGy, 5 kGy, 10 kGy and 15 kGy).

Ans: We added "0 kGy" as suggested, on Page 7, line 10.

10) Table.1, the values of microbial counts should be depicted uniformly. Same values of counts are given in tens of power in some places and as a mere number elsewhere. Also, an approximately narrow range like 10-20, 20-30, 30-40 and so on should be given, instead of using less than symbol (i.e. <), since this will not differentiate values like 10 and 50 if it is given as <70. Besides, no details on analyses of statistical significance between values were given. In sample No. 7, fungal counts are in ascending order i.e. control< 2 kGy <4 kGy in contrary to the logical expectation.

[**Ans:** We corrected the data expression form as suggested. The values of microbial counts was revised and depicted uniformly. It was given in tens of power. Three replicate experiments were done and the range of microbial count was shown in the Table 1.

11) Page 14, line no. 10 -16 and their corresponding values in table.3; the standard deviations of IC_{50} values of irradiated samples were very small with larger mean values, while the same for THSG and BHT were large with small means. Under this circumstance, the proposed significant differences between samples are questionable.

Ans: We have done this experiment again, and the result of the experiment was that we got the DPPH EC_{50} value for THSG and BHT about 21.51 ± 0.08 μg/mL and 23.46 ± 0.14 μg/mL. We corrected it in line 2 on page 15 in the manuscript.

12) Page 16, lines 1-10; rewrite the paragraphs in plain sentences explaining the differences, instead of giving them in decreasing orders using > symbol. Since some values are not significantly different from others in the column, it cannot be written plainly that one value is less than or greater than the other.

Ans: We rewrote the paragraphs in plain sentences and explaining the differences on page 16, line 7~9

and 11~13.

13) Page 18, line no. 3; change words "insect infection" to "insect infestation".

Ans: We changed words "insect infection" to "insect infestation" on page 18, Line 3.

14) Page 20, line no. 3; it is "physicochemical" instead of "physiochemical"

Ans: We used "physicochemical" instead of "physiochemical" on Page 20, line 10.

15) Table 4, values significantly differing from others at 5% level should be marked from others.

Ans: We added "[d] values significantly differing from others at 5%" in Table 4.

16) Figure 2, the caption should be 'Quantitation of THSG in the methanol extracts of Polygoni Multiflori Radix by HPLC." Also give legends for * and ** in the figure.

Ans: We corrected the caption to "Quantitation of THSG in the methanol extracts of Polygoni Multiflori Radix by HPLC."

17) Figure 1. may be removed since the written part on this matter is sufficiently conveying the results.

Ans: We deleted it as suggested.

Besides, Radix Polygoni Multiflori had been corrected as Polygoni Multiflori Radix based on the format of PRC Pharmacopeia, 2010. Other minor typing errors were also corrected. All corrections were marked with color shadow in the revised manuscript for easy comparison.

Influence of gamma irradiation on microbial load and antioxidative characteristics of Polygoni Multiflori Radix

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Running Title: Radiation effects on Polygoni Multiflori Radix

[1] These two authors contributed equally to this work

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Abstract

Gamma radiation is a physical process commonly used for the eradication of microorganisms distributed in food ingredients, medicinal plants and other bioresearches. The aim of this study was to investigate the effect of radiation dosage on the microbial load, chemical compounds and antioxidative characteristics of Polygoni Multiflori Radix (POMU). Ten commercial POMUs were purchased from different herbal markets and treated with 2 kGy, 4 kGy, 6 kGy, 8 kGy and 10 kGy gamma radiation doses to evaluate the microbial burdens of irradiated and unirradiated POMUs.

Our results confirmed that 2 kGy was sufficient for the inactivation of enterobacteria; at 4 kGy, mold and yeast counts were obviously reduced; and at 6 kGy, neither yeasts nor fungi were observed any longer.

The antioxidative effects and major antioxidant components of 0 kGy, 5 kGy, 10 kGy and 15 kGy irradiated POMU samples were also examined. Our results confirmed that 5 kGy irradiated POMU had both the highest antioxidative activity and lowest value in IC_{50} of DPPH radical-scavenging activity. The content of total phenols had no statistically significant changes. Therefore gamma irradiation at 5 kGy could be a potential method for decontaminate the microbial load of POMU to prolong shelf life and to improve hygienic quality.

Keywords: Polygoni Multiflori Radix; Gamma irradiation, Antiseptic effect; Antioxidation

1. Introduction

Herbs quite commonly harbor large quantities of bacteria, fungi and spoilage-inducing organisms [1]. The most common bacteria are aerobic sporeformers, such as Bacillus and Salmonella species [2-4]. Today, three major methods are in use for the antisepsis of herbs, namely steam, fumigation (ethylene oxide and propylene oxide) and irradiation [5]. However, steam degrades light-weight leafy herbs, and ground products are difficult and sometimes impossible to handle in the steam system [5-8]. As for ethylene oxide gas, such disinfection method has been banned in the European Union and many other countries [9]. Gamma radiation within 3 to 10 kGy proved to be a viable alternative to fumigation or steam to ensure the hygienic quality of herbs; and there has been a steady increase of radiation utilization in the last 10 years after the banning of ethylene oxide [1, 10-12].

According to the standards established by world health organization (WHO) [13], most untreated herbs, harvested and handled under hygienic conditions and tested by appropriate methods of sampling and examination, should contain no more than 1 x 10^4 bacteria cfu/g. However there are no literatures available on POMU regarding its

variance in microbial burdens and chemical contents due to different doses of irradiation, especially when high doses (up to 15 kGy) are employed.

Polygoni Multiflori Radix (POMU), “He Shou Wu” in Chinese, is the dried root of *Polygonum multiflorum* Thunb. (Polygonaceae). It is an herb that has been used in traditional Chinese medicine for the treatment of liver diseases, anemia, and hypopigmentary skin diseases, as well as for the prevention of hair-graying and other diseases associated with aging. POMU also counteracts toxicity, cures carbuncles and relaxes the bowels whereas processed POMU replenishes the liver and kidney with vital essence and blood, blackens the hair and strengthens the tendons and bones. [14]. 2,3,5,4′-tetra-hydroxystilbene-2-O-glucoside (THSG), a water-soluble active component extracted from dried tuberous root of POMU, can promote the release of nitric oxide (NO) from vascular endothelial cells and has a strong antioxidative effect [15]. Structurally, THSG belongs to hydroxystilbene, and its structure is similar to that of resveratrol in red wine which has significant protective effects on myocardial ischemia-reperfusion injury.

The present work was mainly undertaken to investigate the effect of different doses of gamma radiation on the elimination of microbes; whether they bring about any changes in the antioxidants composition of POMU and to find the lowest possible effective radiation dosage for POMU.

2. Materials and Methods

2.1 Materials

BHT, GSH, potassium peroxodisulfate ($K_2S_2O_8$), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), Tris (hydroxylmethyl) aminomethane, trypsin, potassium ferricyanide ($K_3Fe(CN)_6$), TCA, ferric chloride ($FeCl_3$), catechin, MTT, aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), rutin, 2,2'-azinobis-(3-ethylbenzothiazoline) -6-sulphonic acid (ABTS), sodium bicarbonate (Na_2CO_3), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu solution and 95% ethanol were purchased from Merck Co. (Santa Ana, CA, USA). THSG was purchased from the National Institute for the Control of Pharmaceutical and Biological Products.

2.2 Sampling and irradiation of Polygoni Multiflori Radix

Ten samples of Polygoni Multiflori Radix were all used in this manuscript. The samples were purchased from 8 traditional Chinese medicine importers, 1 manufactory and 1 Chinese drugstore, of which all herbs originated from Mainland China. POMU was packaged as 15 g samples in PVC (poly-vinyl chloride) containers

for irradiation. Gamma irradiation was performed at National Tsing Hua University according to the method of Wen *et al*. (2008) with slight changes [16]. Samples were placed on a hot cell stand and irradiated with cobalt-60 at room temperature. The irradiated stand rotated 10 rounds per minute to ensure a well-distribution of radiation applied to each sample. The samples were treated for various time intervals in order to achieve absorbed doses of 2, 4, 6, 8 and 10 kGy. Additional samples were treated with 5, 10 and 15 kGy of gamma radiation in the same way to evaluate any changes in antioxidant activities and active compounds. The absorbed dose was measured with silver dichromate dosimeter [17].

2.3 Enumeration of microbes

The microbial contents of POMU samples were measured immediately after irradiation. Direct observation and spread plate method were applied for the quantification of microorganisms [18]. The solid culture media used in this study included plate count agar (PCA; Difico, Detroit, USA), potato dextrose agar (PDA; Difico, Detroit, USA) and violet red bile glucose agar (VRBGA; Difico, Detroit, USA), and the liquid culture medium used in this experiment was plate count broth (PCB, Difico, Detroit, USA).

Appropriate dilutions of the homogenates with PBS were spread and plated onto

PCA culture plates for total aerobic bacterial enumeration. Molds and yeasts were enumerated by the spread plate method using PDA. Each sample was examined on violet red bile glucose agar (VRBGA, Difico, Detroit, USA) to determine the total number of *Enterobacteriaceae* bacteria. Counts were recorded in colony forming units (cfu/g). The presented data was the average count in three petri dishes for each diluted suspension [16].

2.4 Preparation of the methanol extract of Polygoni Multiflori Radix

The POMU sample which purchased from traditional Chinese medicine importer was treated with different gamma radiation doses (0 kGy, 5 kGy, 10 kGy and 15 kGy) and macerated with methanol for 24 hours at room temperature. Filtration and collection of the extracts were done three times. The methanol extracts were dried in vacuum at 40℃. The dried extracts were weighed and dissolved in methanol (stock 10 mg/mL) and stored in -20°C for further use.

2.5 Determination of antioxidant activity by $ABTS^{\cdot+}$

$ABTS^{\cdot+}$ scavenging ability was determined according to the method of Chang *et al.* (2009) [19]. Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 hours in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm

(Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 μL) of each sample (125 μg/mL) was mixed with 180 μL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as the reference standard. A standard curve was constructed for Trolox at 0, 15.625, 31.25, 62.5, 125, 250, 500 μM concentrations. TEAC was expressed as millimolar concentration of Trolox solution with the antioxidant equivalent to a 1000 ppm solution of the sample under investigation.

2.6 Determination of antioxidant activity by DPPH radical scavenging ability

The effect of crude extracts and positive controls (GSH and BHT) on DPPH radicals was estimated according to the method of Huang *et al*. (2005) [20]. 20 μL of sample extract was mixed with 100 mM Tris-HCl buffer (80 μL, pH 7.4) and 100 μL of DPPH in ethanol to a final concentration of 250 μM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm. The percentage of DPPH decolorization was calculated according to the equation: % decolorization = [1 - (ABS_{sample}/$ABS_{control}$)] × 100. IC_{50} value is the effective concentration by which 50% of DPPH radicals are scavenged and was obtained by interpolation with linear regression analysis. A lower IC_{50} value indicated a greater

antioxidant activity.

2.7 Determination of antioxidant activity by reducing power measurement

The reducing power of the crude extracts and BHT was determined according to the method of Yen and Chen (1995) [21]. POMU samples (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) and BHT were each mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50℃ for 20 min, during which time ferricyanide was reduced to ferrocyanide. Then an equal volume of 1% trichloroacetic acid was added to the mixture before centrifugation at 6,000 *g* for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ at a ratio of 1:1:2, then the absorbance was measured at 700 nm to determine the amount of ferric ferrocyanide (Prussian Blue) formed. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

2.8 Determination of total polyphenol content

The total polyphenol content of the crude extracts was determined according to the method of Huang *et al.,* (2004) [22]. 20 μL of sample extract (125 μg/mL) was added to 200 μL distilled water and 40 μL of Folin-Ciocalteu reagent. The mixture was

allowed to stand at room temperature for 5 min, and then 40 μL of 20% sodium carbonate was added to the mixture. The resulting blue complex was measured at 680 nm. (+)-Catechin was used as standard for the calibration curve. The polyphenol content was calibrated using a linear equation based on the calibration curve. The total polyphenol content was expressed as μg (+)-catechin equivalent/mg dry weight (μg CE/mg/). The dry weight indicated was the sample dry weight.

2.9 Determination of total flavonoid content

The total flavonoid content of the crude extracts was determined according to the method of Huang *et al*. (2004) [22]. 1.5 mL aliquot of each extract was added to an equal volume of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 mL methanol) solution. The mixture was vigorously shaken, and the absorbance at 430 nm was read after 10 min of incubation. Rutin was used as the standard for the calibration curve. The total flavonoid content was calibrated using a linear equation based on the calibration curve. The total flavonoid content was expressed as μg rutin equivalent/mg dry weight (μg RE/mg). The dry weight indicated was the sample dry weight.

2.10 Determination of total flavonol content

The total flavonol content of the crude extracts was determined according to the method of Chang *et al*. (2007) [23]. 200 μL of sample extract was added to 1 mL of 0.1% *p*-dimethylaminocinnamaldehyde (DMACA) in methanol/HCl (3:1, v/v). The mixture was vigorously shaken, and the absorbance was read after 10 min of incubation at 640 nm. (+)-Catechin was used as standard for the calibration curve. The total flavonol content was calibrated using a linear equation based on the calibration curve, and expressed as μg (+)-catechin equivalent/mg dry weight (μg CE/mg).

2.11 Analyses of THSG by HPLC

Moderate amount of the methanol extracts from POMU was weighed and dissolved in methanol. At first, the solutions were filtered through 0.45 μm PVDF filters. HPLC (Waters 2695 separations module; detector: Waters 996 photodiode array detector) analysis was carried out under the following conditions: Waters XTerra RP18 column (5 μm, 4.6 × 250 mm) was used with deioned water as mobile phase A, and acetonitrile was used as mobile phase B; in a ratio of 25:75 and ran for 30 min at a flow rate of 0.6 mL/min. The injection volume was 10 μL, and a wavelength of 320 nm was used for detection. THSG was also analyzed by HPLC under the same conditions, and the retention time was used to identify THSG contents in the samples.

2.12 Statistical analyses

Experimental results were presented as the mean ± standard deviation (SD) of three parallel measurements. Statistical analyses were performed by one-way ANOVA, followed by Dunnett's *t* test. The difference was considered to be statistically significant when *p* value was less than 0.05.

3. Results

3.1 Effect of gamma irradiation on the microbial burden of Polygoni Multiflori Radix

The microbial loads in POMU specimens of different origins were analyzed immediately after irradiation by the spread plate method. The viability of microorganisms in POMU specimens irradiated with different gamma-ray doses of 0, 2, 4, 6, 8 and 10 kGy are shown in Table 1. POMU specimens contained total mesophilic bacterial counts of 5.0×10^1 to 2.9×10^3 cfu/g, and mould and yeast counts of 1.0×10^1 to 6.2×10^2 cfu/g. Total bacterial counts were much higher than the mould and yeast counts in all examined samples. Enterobacteria were also found in seven of the ten POMU specimens, and ranged from not detected to 1.0×10^1 cfu/g. Although the microbial profiles of specimens from different sources varied markedly, following irradiation at 2 kGy, enterobacteria were inactivated; at 4 kGy, mold and yeast counts were obviously reduced; and yeast and fungi were no longer observed in POMU

specimens after irradiation at 6 kGy. As the radiation dosage increased, microbial profiles in the POMU specimens changed dramatically; the microbial counts decreased as the irradiation dose increased.

3.2 Detecting changes in antioxidant activity after gamma irradiation by ABTS assay

ABTS assay is often used for the evaluation of total antioxidant power of single compounds and complex mixtures of various plants [24]. In this assay, ABTS radical monocation was generated directly in stable form from potassium peroxodisulfate. Radicals were generated before antioxidants were added; this modification made the assay less susceptible to interruptions and prevented overestimation of antioxidant power [25]. The sample was added to the reaction medium when stable absorbance was obtained, and the antioxidant activity was measured in terms of decolorization.

The results of ABTS assay were expressed in TEAC values. A higher TEAC value meant that the sample had a stronger antioxidant activity. TEAC values determined from the calibration curve for POMU are shown in Table 2. Antioxidant activities of the POMU extracts were in the following decreasing order: 5 kGy (63.62 ± 0.84 μM /mg extract) > 10 kGy (59.77 ± 1.03 μM /mg extract) > 15 kGy (55.92 ± 0.80 μM /mg extract) > 0 kGy (52.08 ± 0.79 μM /mg extract). The antioxidant potency of THSG (positive control) was 1,871.53 ± 15.38 μM/mg extract.

ABTS assay was used to estimate the total antioxidant power because it is quick and simple to perform, and reactions are reproducible and linearly related to the molar concentration of antioxidants [26]. Furthermore it can also be used to measure the antioxidant capacity of a wide range of biological samples, pure compounds, fruits, wines, and animal tissues [27].

3.3 Detecting the effect of gamma irradiation on antioxidant activity by DPPH assay

The relatively stable organic radical DPPH is widely used in modeling systems to investigate the scavenging activity of several natural compounds, such as phenolics and anthocyanins, or crude mixtures. DPPH radical is scavenged by antioxidants through the donation of a proton to form reduced DPPH. The color changes from purple to yellow after reduction, and can be quantified by the decrease in absorbance at wavelength 517 nm. Table 3 shows IC_{50} values for the radical-scavenging activities of POMU, THSG, GSH, and BHT using the DPPH colorimetric method. It was found that 0 kGy had the lowest IC_{50} value (513.04 ± 0.01 μg/mL), followed by 5 kGy (514.28 ± 0.01 μg/mL), 10 kGy (526.79 ± 0.01 μg/mL) and 15 kGy (546.03 ± 0.02 μg/mL). The four extracts showed significant differences ($p < 0.05$) in radical-scavenging activity. As shown from the above results, the sample without any treatment of gamma radiation (0 kGy) had the highest DPPH radical scavenging

activity. However, its capacity was still much lower compared to the positive controls of BHT and THSG (23.46 ± 0.14 and 21.51 ± 0.08 μg/mL).

3.4 Detecting changes in antioxidant activity after gamma irradiation by reducing power measurement

We investigated Fe^{3+}-Fe^{2+} transformation in POMU samples for the measurement of their reducing capacity. The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity [28]. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging [29]. In this assay, potassium ferricyanide was added to POMU samples and positive controls of THSG and BHT for the determination of any changes in reducing power after gamma irradiation. The mixtures were allowed to incubate during which time ferricyanide was reduced to ferrocyanide.

The reducing power was measured in terms of absorbance of the reaction mixtures at 700 nm. A higher absorbance indicated a stronger reducing power, vice versa. The reducing powers of the samples were in the following decreasing order: 0 kGy (0.79 ± 0.02) > 5 kGy (0.55 ± 0.02) > 10 kGy (0.52 ± 0.01) > 15 kGy (0.46 ±

0.01) at the dose of 1 mg/mL of POMU. The reducing powers of THSG and BHT were 1.98 ± 0.04 and 1.30 ± 0.02 respectively (data not shown).

3.5 Effect of gamma irradiation on total polyphenol, flavonoid, and flavonol contents

The total polyphenol, total flavonoid, and total flavonol contents of POMU are shown in Table 4. The total polyphenol content of POMU extracts ranged from 26.82 to 28.21 μg CE/mg. 0 kGy was 27.84, 5kGy was 28.21, 10kGy was 27.77 and 15kGy was 26.82μg CE/mg. 5 kGy had the highest polyphenolic content and 15kGy was the lowest.

The total flavonoid content was expressed as μg rutin equivalent/mg dry weight, and ranged from 7.14 to 5.12 μg RE/mg.0 kGy was 6.99, 5kGy was 7.14, 10kGy was 7.01 and 15kGy was 5.12μg RE/mg. 5 kGy had the highest flavonoid content. 15kGy was the lowest and significant different from others.

The total flavonol content was expressed as μg catechin equivalent/mg dry weight. The total flavonol content of POMU extracts varied slightly and ranged from 5.79 to 5.71 μg CE/mg.

Both flavonoids and flavonols are polyphenolic compounds. Polyphenolic compounds have important roles in stabilizing lipid oxidation and are associated with antioxidant activities [30]. Phenolic compounds may contribute directly to

antioxidative actions [19]. It is suggested that when as much as 1.0 g of polyphenolic compounds is ingested from a daily diet rich in fruits and vegetables, there may be inhibitory effect on mutagenesis and carcinogenesis in humans [31]. The antioxidative activities observed could be ascribed both to variant mechanisms exerted by different phenolic compounds and to their synergistic effects. The antioxidant assays used in this study measured the oxidative products at the early and final stages of oxidation. Antioxidants have different functional properties, such as reactive oxygen species scavenging, e.g. quercetin, rutin, and catechin [32]; and inhibition of free radical generation and chain-breaking activity, e.g. *p*-coumaric acids [33] and metal chelation [34]. These compounds are normally phenolic compounds, which are effective proton donors, such as tocopherols, flavonoids and other organic acids. However, the components responsible for the antioxidative activity of POMU are still currently unclear.

3.6 Compositional changes of Polygoni Multiflori Radix after gamma irradiation

The effect of different doses of gamma radiation on the compositional changes of POMU was assessed. Quantitative analyses of POMU were conducted with HPLC-UV. THSG content of unirradiated and irradiated POMU was quantified based on a previously constructed HPLC-UV calibration curve for THSG standard

(R^2=0.996). The content ranged from 2.13 to 3.05 mg/g of dry weight (DW) for unirradiated or irradiated POMU. Irradiation treatment resulted in significant changes in THSG content at different doses.

In this database, THSG showed statistically significant decreases from 3.05 mg/g (0 kGy, p <0.05) to 2.60 mg/g after 5 kGy of irradiation (p <0.05), 2.50 mg/g at 10 kGy (p <0.05) and 2.13 mg/g at 15 kGy (p <0.01) (Figure 2; N = 3). Therefore, it was indicated that gamma irradiation caused damage to THSG content in POMU.

4. Discussion

POMU has long been used as a medicinal herb in Asia. Insect infestation and microbial contamination are serious problems for POMU storage. Gamma irradiation is able to penetrate deeply, and has been found to be useful for the hygienization of herbal products even when they are already in packages or sacks. The choice of a suitable radiation dosage is particularly important for decontaminate the microbial load of stored POMU. In this research, the optimal dosage for the inactivation of microorganisms in POMU was evaluated.

2 kGy was sufficient for the hygienization of enterobacteria, whereas a dosage of 6 kGy was required for the hygienization of yeasts and fungi. Since the dosage required removing microbial contamination is higher than that required killing insects,

insects would be killed at the same time [35].

Our results confirmed that at the dose of 4 kGy, mold and yeast counts were obviously reduced; and at 6 kGy, neither yeasts nor fungi were observed any longer in POMU specimens. Thus in our research, it was demonstrated that 4~6 kGy of treatment was effective for the inactivation of microorganisms and 8 kGy treatment could induce complete inactivation.

Phenols are regarded as main contributors to antioxidant activities, the measurements are based on radical scavenging. Antioxidant capacity depends on various factors, such as the number and location of hydroxyl groups on the aromatic ring, as well as their mutual positions [36]. Our results demonstrated that the irradiation dosage on POMU (5 kGy, 63.62 ± 0.84 μM/mg extract) had both the highest antioxidative activity and the lowest value in IC_{50} of DPPH radical-scavenging activity, and as expected, an increase of radiolytic products, which agreed with literary reports for other foods and medicines. In this study, 5 kGy γ-irradiation increased radiolytic products of POMU; however it seemed to increase the antioxidant effect of POMU in the ABTS assay.

The content of total phenols, expressed as μg (+)-catechin equivalent/mg dry weight value, did not vary distinctively in irradiated POMU samples. The values were slightly lower; however variations were not statistically significant (Table 4). THSG

content of POMU had decreased as the γ-radiation increased (Figure 1). However, the total phenolic compounds and antioxidative activities were increased, probably because other phenolic compounds were formed during irradiation. It has to be mentioned that irradiation of aqueous systems containing aromatic compounds can implicate the formation of phenols.

5. Conclusion

The results of this study indicated that 5 kGy of gamma irradiation was effective for the hygienization of POMU. It did not alter the appearance of POMU before and after gamma irradiation. Though, after 5 kGy of irradiation, some physicochemical properties were slightly changed or compensated for the improved hygiene of this medicinal herb, such as the decrease in THSG content. However, the total phenolic compounds and antioxidative activities were increased. Therefore gamma irradiation at 5 kGy could be a potential method for decontaminate the microbial load of POMU to prolong shelf life and improve hygienic quality.

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Figure Legends

Figure 1. Quantitation of THSG contents in the methanol extracts of Polygoni Multiflori Radix by HPLC.

Table 1

Table 1. Effect of gamma irradiation on the microbial count (CFU/g ± SD) of Polygoni Multiflori Radix samples.

Sample No.		Irradiation dose (kGy) 0	2	4	6	8
1	T[a]	(1.2 ± 0.4)x10^3	(1.3 ± 0.4)x10^2	(5.0 ± 0.6)x10^1	ND[b]	ND
	M[c]	(6.2 ± 0.7)x10^2	(4.0 ± 0.6)x10^1	(2.0 ± 0.3)x10^1	ND	ND
	E/Pa[d]	ND	ND	ND	ND	ND
2	T	(2.1 ± 0.3)x10^2	(2.0 ± 0.4)x10^1	(2.0 ± 0.5)x10^1	ND	ND
	M	(1.7 ± 0.4)x10^2	ND	ND	ND	ND
	E/Pa	ND	ND	ND	ND	ND
3	T	(5.1 ± 0.9) x10^2	(7.0 ± 1.4)x10^1	ND	ND	ND
	M	(1.3 ± 0.3) x10^2	(2.0 ± 0.4)x10^1	(1.0 ± 0.2)x10^1	ND	ND
	E/Pa	ND	ND	ND	ND	ND
4	T	(2.9 ± 0.3)x10^3	(3.5 ± 0.5)x10^2	(6.9 ± 1.1)x10^1	ND	ND
	M	(4.5 ± 0.4)x10^1	(1.9 ± 0.3)x10^1	(1.0 ± 0.2)x10^1	ND	ND
	E/Pa	ND	ND	ND	ND	ND
5	T	(6.5 ± 1.2)x10^1	(4.0 ± 0.7)x10^1	(2.5 ± 0.3)x10^1	ND	ND
	M	(5.0 ± 0.8)x10^1	(2.5 ± 0.2)x10^1	(1.5 ± 0.3)x10^1	ND	ND
	E/Pa	ND	ND	ND	ND	ND
6	T	(3.0 ± 0.4)x10^2	(7.5 ± 0.5)x10^1	(1.5 ± 0.2)x10^1	ND	ND
	M	(5.0 ± 0.9)x10^1	(1.5 ± 0.4)x10^1	ND	ND	ND
	E/Pa	(1.0 ± 0.4)x10^1	ND	ND	ND	ND
7	T	(5.0 ± 0.5)x10^1	ND	ND	ND	ND
	M	(2.5 ± 0.4)x10^1	(1.5 ± 0.2)x10^1	(1.0 ± 0.2)x10^1	ND	ND
	E/Pa	ND	ND	ND	ND	ND
8	T	(1.5 ± 0.3)x10^2	(5.0 ± 0.5)x10^1	ND	ND	ND
	M	(5.0 ± 1.3)x10^1	(2.0 ± 0.3)x10^1	ND	ND	ND
	E/Pa	ND	ND	ND	ND	ND
9	T	(5.0 ± 0.7)x10^1	(3.0 ± 0.4)x10^1	(2.0 ± 0.3)x10^1	ND	ND
	M	(5.0 ± 0.3)x10^1	(2.0 ± 0.3)x10^1	ND	ND	ND
	E/Pa	ND	ND	ND	ND	ND
10	T	(2.6 ± 0.4)x10^2	(5.0 ± 0.6)x10^1	(3.0 ± 0.3)x10^1	(2.0 ± 0.2)x10^1	ND
	M	(5.0 ± 0.5)x10^1	(2.0 ± 0.3)x10^1	ND	ND	ND
	E/Pa	ND	ND	ND	ND	ND

[a] T: Total aerobic microbes; [b] ND: No microbe detected on plates; [c] M: Molds;

[d] E: *Enterobacteriaceae*, Pa: *Pseudomonas aeruginosa*

Table 2. Total antioxidant activity assessed by TEAC

Samples and positive control	TEAC (μM Trolox/mg ± SD) *
0 kGy	52.08 ± 0.79
5 kGy	63.62 ± 0.84
10 kGy	59.77 ± 1.03
15 kGy	55.92 ± 0.80
THSG	1,871.53 ± 15.38
BHT	1,869.41 ± 34.63
GSH	1,827.68 ± 76.84

* All values are expressed as mean ± S.D. of triplicate tests (n = 3). Values represented mean ± S.D. of three parallel measurements ($p < 0.05$) when analyzed by ANOVA and Ducan's multiple-range tests.

Table 3

Table 3. IC_{50} values of the DPPH radical scavenging activities of the methanol extracts of Polygoni Multiflori Radix that had been irradiated with different dosages of gamma ray.

The dosage of gamma radiation	IC_{50} (μg/mL) *
0 kGy	513.04 ± 0.01
5 kGy	514.28 ± 0.01
10 kGy	526.79 ± 0.01
15 kGy	546.03 ± 0.02
THSG	37.51 ± 0.31
BHT	41.06 ± 0.76

* Values represented mean ± S.D. of three parallel measurements (P<0.05).

Table 4

Table 4. Total polyphenol, flavonoid and flavonol contents of the methanol extracts of Polygoni Multiflori Radix that had been exposed to different doses of gamma radiation.

Dose of gamma radiation	Total polyphenols (μg CE/mg)[b]	Total flavonoids (μg RE/mg)[c]	Total flavonols (μg CE/mg)[b]
0 kGy	27.84 ± 0.06	6.99 ± 0.02	5.74 ± 0.27
5 kGy	28.21 ± 0.02	7.14 ± 0.04	5.78 ± 0.06
10 kGy	27.77 ± 0.01	7.01 ± 0.03	5.79 ± 0.03
15 kGy	26.82 ± 0.01	5.12 ± 0.02[d]	5.71 ± 0.07

[a] Values represented mean ± S.D. of three parallel measurements.

[b] Data expressed in μg (+)-catechin equivalent / mg dry weight (μg CE/mg).

[c] Data expressed in μg rutin equivalent / mg dry weight (μg RE/mg).

[d] values significantly differing from others at 5%

Figure 1

Figure 1. Quantitation of THSG in the methanol extracts of Polygoni multiflori Radix by HPLC