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Original Article

Effects of Gamma Irradiation on Microbiological, Phytochemical Content, Antioxidant Activity and Inhibition of Angiotensin Converting Enzyme (ACE) Activity of *Peperomia pellucida* (L.) Kunth

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ABSTRACT

Objective: Gamma irradiation is an effective technique can be used to reduce contaminants in herbal products. *Peperomia pellucida* (L.) Kunth belongs to Piperaceae family has some biological activity, such as antioxidant and ACE inhibitor. The aimed of this research was to determine the effects of gamma irradiation on microbiological, phytochemical content, antioxidant and ACE inhibition activity. **Methods:** Sample was irradiated at a various dose of 0, 2.5, 5, 7.5 and 10 kGy. Microbiological was determined as total aerobic plate count (TAPC), and total yeast and mold (TYC), total phenolic content (TPC), total flavonoid content (TFC), antioxidant and ACE inhibition activity were investigated by various *in vitro* colorimetric methods. **Results:** Irradiation dose of 10 kGy reduced TAPC and TYC. Irradiation dose of 10 kGy slightly increased TPC, antioxidant, and ACE inhibition activity ($p<0.05$) but reduced TFC significantly ($p<0,05$) and caused degradation of flavonoids in this plant. Correlation analysis showed that phenolic compounds have a role as antioxidant and ACE inhibitor. **Conclusion:** gamma irradiation was found to be sufficient to ensure microbiological safety, increase TPC, the antioxidant and ACE inhibitory activity but caused degradation of flavonoids on *Peperomia pellucida* (L.) Kunth.

Key words: ACE inhibitor, Antioxidant, Gamma irradiation, Microbiological, *Peperomia pellucida* (L.) Kunth, Total phenolic, Total flavonoid.

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INTRODUCTION

Raw plant materials usually carry a great number of bacteria and fungi that come from the soil. The current process like harvesting, handling, storage, and production also can cause additional contamination and microbial growth.[1] This can lead to a shorter shelf life and in some cases, it can cause disease.[2] One way can be used to overcome this problem is decontamination process. Conventional decontamination by using heat and chemicals can reduce the bioactivity of herbal products.[3] Gamma irradiation is an environmentally friendly technology that can be used to reduce contaminants in herbal products.[2, 3]

Available reports have clearly demonstrated that exposure of food commodities to Gamma Irradiation. Exposure of gamma irradiation at 1.4 kGy on *Ginkgo biloba* (L.) and *Paullinia cupana* (HBK) reduced the numbers of microbial load, the number of yeast and mold without cause degradation of flavonoid and caffeine.[1] Gamma irradiation on *Pennisetum glaucum* reduced microbial contamination as well as yeast and mold and didn't provide significant change in phenolic compounds.[4]

Peperomia pellucida (L.) Kunth belongs to Piperaceae family has activity as antioxidant and angiotensin converting enzyme (ACE) inhibitor.[5, 6] Ethanolic extract of this plants have ACE inhibitory activity with IC_{50} of 7.17 µg/ml.[7] Phenolic compounds and flavonoids contained in this plant is reported to have antioxidant activity and ACE inhibitors.[5, 7]

The aim of this research was to evaluate the effects of gamma irradiation in reducing microbia contamination, and also of antioxidant activity, ACE inhibitor activity, total phenol content and total flavonoid content, as well as changes in the chemical components by thin layer chromatography profiling on *Peperomia pellucida* (L.) Kunth.

MATERIALS AND METHODS

Chemicals and reagents

Petri film aerobic count plate and Petri film yeast and mold count plate were purchased from 3M company (USA), Folin-Ciocalteu reagent (Merck, Germany), DPPH (2,2-diphenyl-1-picrylhydrazyl) (Wako, Japan), gallic acid (Sigma-Aldrich, USA), quercetin (Sigma-Aldrich, USA), captopril (Zhejiang Huahai Pharmaceutical, China) and ACE Kit-WST test kit (Dojindo Laboratories, Japan). All other reagents were of analytical reagent grade.

Plants materials

Peperomia pellucida (L.) Kunth was obtained from the Research Institute for Medicinal Plants and Aromatic (Balitro), Bogor, West Java, Indonesia. This plants packed hermetically in polyethylene bags (each 50 g) before irradiation treatment and storage. These samples were irradiated using cobalt-60 as the source of irradiation to dose 2.5, 5, 7.5, and 10 kGy at the dose rate 7.290 kGy/h. Irradiation was conducted at the Center for Isotope and Radiation Technology Applications (PATIR), National Nuclear Energy Agency (BATAN), Pasar Jumat, South Jakarta.

Microbiological analysis

Total Aerobic Count Plate (TAPC)

TAPC were determined by using Petri film aerobic count plate method. Samples solution was prepared by diluting 1 g of dry samples then dissolved in 10 mL of saline solution as 10^{-1} dilution, and made dilutions 10^{-2}, 10^{-3}, 10^{-4} by using 1 ml of diluents in 9 ml saline solution. Top of the film was lifted, and then 1 ml of each dilution samples were pipetted into the bottom of the film and then closed again. Samples were dispersed using the plastic spreader and let the film for 1 minute to for gel to solidity. Plate was incubated for 48 hours at a temperature of 35 ± 1°C and count the number of bacterial colonies growing.[8]

Total Yeast and Mold Count (TYC)

TYC were determined by using Petri film yeast and mold count plate. The sample dilutions were prepared at the same as TAPC. The top of the film was lifted, and then 1 ml of each dilution samples were pipetted into the bottom of the film and then closed again. Samples were dispersed using the plastic spreader and let the film for 1 minute to for gel to solidity. Plate was incubated for 5 days at a temperature of 25 ± 1°C and count the number of bacterial colonies growing.[9]

Preparation of extracts

Dry powder of samples (50g) were extracted by reflux method for 30 minutes using 500 ml of 80% ethanol. The extract was concentrated by using rotary vacuum evaporator (Buchi, Switzerland) to give crude extracts. Extraction yield was calculated based on dry weight. Extracts were stored at 4°C until used.

Determination of the total phenolic content (TPC)

Total phenolic content was measured by Folin-Ciocalteu method. The extract (25 mg) was diluted in 25 ml of methanol, 0.5 ml aliquot of diluted was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent, vortexed and incubated at 25°C. After 2 minutes, 2 ml of 7.5% Na_2CO_3 was added. The mixture was vortex and incubated for 60 minutes at 25°C. The absorbance of the mixture was measured at 765 nm with a UV–VIS spectrophotometer.[10] Gallic acid was used as a reference standard, and the results were expressed as mg of gallic acid equivalents (GAE) per g of the extract (mg GAE/g extract).

Determination of the total flavonoid content (TFC)

Total flavonoid content was measured using 10% $AlCl_3$ solution with slight modification.[11] The extract (25 mg)was diluted in 25 ml of methanol, 0.5 ml aliquot of diluted was mixed with 1.5 ml methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1 M sodium acetate solution, and 2.8 ml distilled water. The mixture was vortex and incubated for 30 minutes at 37°C. The absorbance of the mixture was measured at 415 nm with a UV–VIS spectrophotometer. Quercetin was used as a reference standard, and the results were expressed as mg of quercetin equivalents (QCTE) per g of the extract (mg QCTE/g extract).

Determination of free radical scavenging activity by the DPPH method

Antioxidant activity was determined by using DPPH method.[12, 13] tanukimame Dry extract was dissolved in methanol with different concentrations. The sample (1ml) was reacted with 1 ml DPPH and 2 ml methanol. The mixture was vortex and incubated in the dark for 30 minutes at 37°C. The absorbance was measured with spectrophotometer at 515 nm. Quercetin was used as a reference standard.

Enzyme assay

Dry extracts were dissolved in distilled water and made in a final concentration of 100 µg/ml. Each extract was subjected to ACE inhibitory assay by using Dojindo ACE Kit-WST test kit as instructed in the manufacturer's protocol.[14] This assay used 3-hydroxybutyrate glycyl glycylglycine (3HB-GGG) as substrate for screening of the ACE inhibitory. The absorbance value of the assay reaction was measured at 450 nm using microplate reader. Captopril was used as a positive control in this study.

Thin layer chromatography (TLC) profile

TLC profile was performed in thin layer chromatography method using silica plate as the stationary phase and dichloromethane-methanol (90:10) as eluent. TLC profile was analyzed using densitometer at wavelength of 254 nm and 366 nm.

Statistical analysis

Data presented for TAPC, TYC, antioxidant activity, and ACE inhibition activity were obtained from triplicate measurements, and result was expressed as mean ± standard deviation. The data were analyzed using one-way ANOVA followed Bonferroni and Tukey's posttest to assess significant differences ($p<0.05$) between the samples. Correlation between TPC, TYC, antioxidant activity, and ACE inhibition activity analyzed using Pearson correlation.

RESULTS

Microbiological analysis

Table 1 showed effect gamma irradiation on total aerobic plate count (TAPC) and total yeast count (TYC). In this study, gamma irradiation reduced the level of bacteria, yeast, and mold of the samples. TAPC was higher than TYC on all examined samples. The total microbial count was decreased while the increase of irradiation dose. There were no detected bacteria at doses 10 kGy, while at dose of 7.5 kGy was enough to damage all yeast and mold in the sample.

Determination of total phenolic content and total flavonoid content

Table 2 showed the effect of gamma-irradiation on TPC and TFC of *P. pellucida*. Gamma-irradiation until 10 kGy didnot change TPC, but interestingly the TFC exhibited a different result in contrast with TPC after irradiation. Comparing TFC of control with the gamma irradiated samples demonstrate that the gamma irradiated samples exhibited significantly lower TFC than the control

Determination of DPPH free radical scavenging and ACE inhibitory activity

Table 3 showed DPPH free radical scavenging and ACE inhibitory activity. DPPH free radical scavenging activity increased after gamma irradiation. Statistical results showed a significant increase in IC_{50} value by increased irradiation dose. Variation dose of gamma irradiation lead to differences in ACE inhibitory activity. Results of statistical analysis results showed a significant increase ($p <0.05$) on the inhibitory activity after irradiation up to a dose of 7.5 kGy, however at dose of 10 kGy, ACE inhibitory activity did not change significantly ($p> 0.05$) compare to sample irradiated with a dose of 7.5 kGy.

Thin layer chromatography (TLC) profile

TLC profile of the *P. pellucida* extracts can be seen in Figure 1 and Figure 2. There is a reducing in the intensity of spot number 3 after gamma irradiation (Figure 2), and is accompanied by increasing intensity of spot

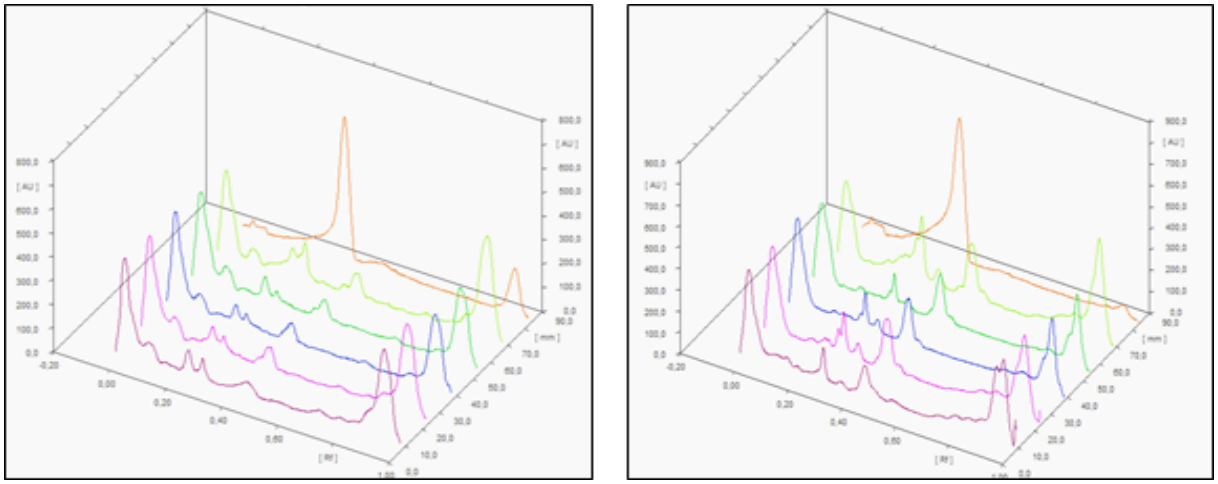

Figure 1: Thin layer chromatography profile of *P. pellucida* extract using densitometry (A) 254 nm wavelength (B) Wavelength 366 nm. (■) 0 kGy ; (■) 2.5 kGy ; (■) 5 kGy; (■) 7.5 kGy; (■) 10 kGy; (■) quercetin

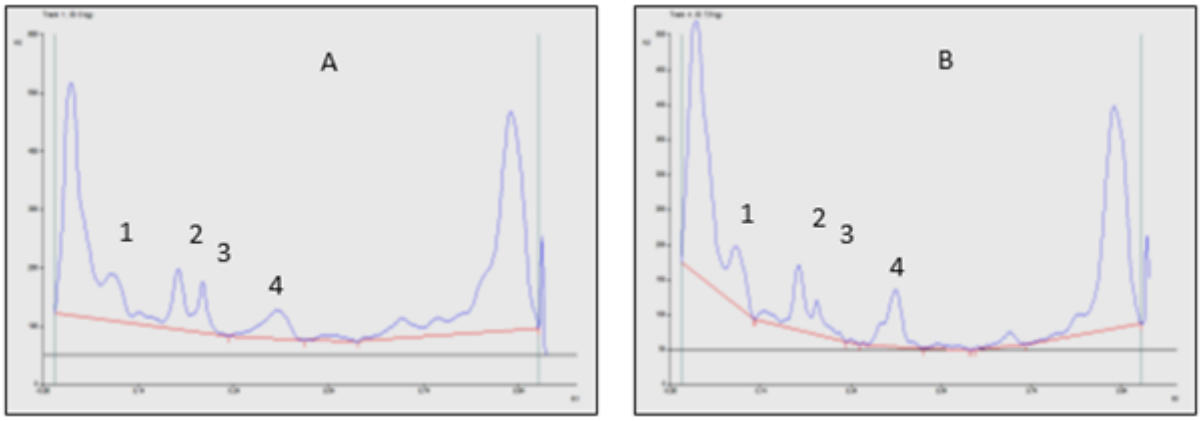

Figure 2: TLC profile of the *P. pellucida* extract with irradiation doses of (A) 0 kGy (B) 7.5 kGy.

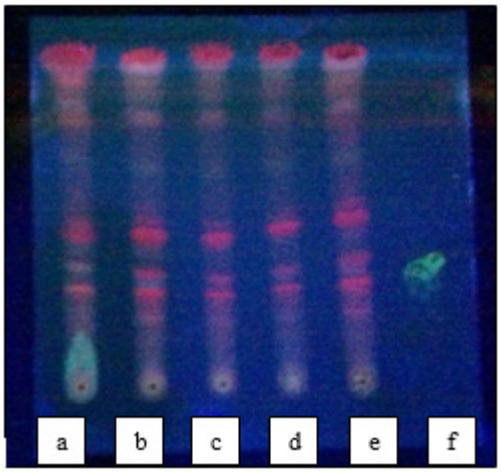

Figure 3: TLC profile after sprayed with 5% AlCl3 solution at wavelength of 366 nm. (a) 0 kGy (b) 2.5 kGy (c) 5 kGy (d) 7.5 kGy (e) 10 kGy (f) quercetin.

Table 1: Microbial decontamination level

Irradiation dose	TAPC (CFU / g)	TYC (CFU/ g)
0kGy	$5.3x\ 10^5 \pm 0.42$	$7.00\ x\ 10^2 \pm 2.64$
2.5 kGy	$7.7\ x\ 10^4 \pm 0.07$	$2.00\ x\ 10^1 \pm 1.73$
5 kGy	$3.35x\ 10^4 \pm 0.21$	3.33 ± 0.58
7.5 kGy	$2\ x\ 10^2 \pm 0.00$	ND
10 kGy	ND	ND

TAPC= Total aerobic plate count, TYC= Total yeast count, values are presented as mean+SEM from triplicate experiments.

number 4. Figure 3 showed TLC plat after sprayed by $AlCl_3$ reagent. Flavonoid was detected at non-irradiated extracts with strong intensity, while on the irradiated extracts spot flavonoid showed low intensity. This result corresponds with TFC after gamma irradiation.

DISCUSSION

Gamma irradiation is one effective method to reduce microbial load, to maintain quality and safety, and to extend the shelf life of natural products. However, there are several reports on effect of irradiation on sec-

Table 2: Total phenolic and total flavonoid content in *P. pellucida* after gamma-irradiation

Irradiation dose (kGy)	TPC (GAE mg / g extract)	TFC (QCTE mg / g extract)
0	26.29 ± 0.34	30.16 ± 0.18
2.5	29.41 ± 0.15*	25.09 ± 0.14*
5	31.46 ± 0.15*	28.47 ± 0.14*
7.5	34.41 ± 0.532*	25.02 ± 0.05*
10	29.36 ± 0.237*	25.38 ± 0.67*

Values are presented as mean ± SEM from triplicate experiments, TPC= total phenolic content, GAE= gallic acid equivalent, TFC= total flavonoid content, QCTE= quercetin equivalent, *) significantly different when compared with 0 kGy.

Table 3: DPPH free radical scavenging and ACE inhibitory activity of *P. pellucida* extract after gamma-irradiation

Irradiation Dose (kGy)	DPPH free radical scavenging activity ($IC_{50,}$ ppm)	ACE inhibition Percentage (%)
0	244.92 ± 0.65	50.07 ± 2.61
2.5	191.74 ± 1.96*	59.70 ± 4.07 *
5	174.81 ± 0.72*	52.16 ± 3.59,
7.5	166.20 ± 0.72*	63.71 ±3.17 *
10	188.67 ± 0.75*	57.60 ± 2.00

Values are average from triplicate experiments,*) significantly different when compared with 0 kGy.

ondary metobolites. In this study, the microbial count decreased while increasing of irradiation dose. Irradiation treatment damaged microbial cells by break down the DNA, photons induced the death of the organism and caused some organisms can not reproduce.[4, 15]

There are several reports on effect of gamma irradiation on total phenolic content (TPC) and total flavonoid content (TFC). The gamma irradiation increased TPC in clove, nutmeg and skin of almond after irradiation.[2, 16] This increase may be caused by the degradation of tannins into a simple phenol compound which was contributed to increasing the total phenolic contents.[17] Gamma irradiation is also known to increase the of phenylalanine ammonia lyase activity which is responsible for synthesized of polyphenol acid.[18] In this study, irradiation decreased TFC. This decrease may be attributed to the change of flavonoid structure after irradiation that can't be extracted or not dissolve in the used solvent for the extraction.[19] The previous study showed the different result in *Centella asiatica*, irradiation caused increasing TFC compared with the nonirradiated.[20]

Polyphenols have been known to be responsible for the antioxidant activity of the products. Their free radical scavenging activity via electron donation to DPPH radical. The previous study reported that antioxidants activity of oat β-glucan increase by increasing irradiation dose.[10] On the polysaccharides, like ergosan reported that antioxidant activity increased with increasing irradiation dose.[21] Increasing of the antioxidant activity may be caused due to fragmentation of hydroxyl group from the sample, hydrogen atoms reacted with free radical, and they convert it into a more stable product.[22]

In the previous studies reported that the gamma irradiation treatment caused degradation of phenolic compounds with large into small molecule of phenolic compounds.[23,24,25] In another study, irradiation treatment caused degradation of tannin into phenolic compounds, so that total phenolic contents increased in the extract.[17] The increasing in total phenolic content is believed to cause an increasing in the inhibition of ACE activity. Phenolic compounds have been reported to have efficacy as angiotensin converting enzyme inhibitor.[7,26,27] ACE is a dipeptidyl carboxypeptidase with Zn metal in the active site, free OH groups of phenolic compounds form chelates with Zn ion, causing ACE becomes inactive.[26] But, further investigation is needed to confirm this speculation.

This because treatment of gamma irradiation up to10 kGy caused degradation of chemical components of the extracts and the degradation of product likely join the subsequent spot thus causing the rise the other spots intensity.[28] It can be concluded that irradiation treatment caused degradation of flavonoids on *P.pellucida*. Similarly, the amount of total flavonoids in *Amygdalus communis* was decreased after irradiation treatment.[24]

In this study, there is correlation between DPPH free radical scavenging activity with total phenol content (r=-0.903; p <0.05). These results are consistent with the strong correlation between TPC and antioxidant activity in Pomegranate peels.[17] TFC and IC_{50} of sample showed that there is no correlation between TFC and IC_{50} of samples (r=0.687, p=0.05). This indicated that flavonoid in the sample doesn't provide DPPH free radical scavenging activity.

ACE inhibition activity also correlated with TPC (p<0.05, r= 0.608), similarly correlation between TPC and ACE inhibition *Centella asiatica*.[29] The ACE inhibition activity showed significant correlation with TFC (p<0.05) but has negative correlation (r= -0.805). This result suggested that non-flavonoid compound plays a role as ACE inhibitor.

CONCLUSION

The result of this study indicated that gamma irradiation doses up to 10 kGy were found to be sufficient to ensure the microbiological safety of *Peperomia pellcida*. Gamma irradiation increase TPC, the antioxidant and ACE inhibitory activity, but caused degradation of flavonoids on *Peperomia pellucida* (L.) Kunth.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

ABBREVIATIONS USED

TAPC: Total Aerobic Count Plate; **TYC:** Total Yeast and Mold Count; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoid Content; **ACE:** Angiotensin Converting Enzyme.

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