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Gamma irradiation enhances biological activities of mulberry leaf extract

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ABSTRACT

The purpose of this study was to investigate the influence of irradiation on the anti-oxidative, anti-inflammatory and whitening effects of mulberry leaf extract. This was done by comparing the phenolic contents; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effects; 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) radical scavenging effects; *in vitro* tyrosinase inhibitory effects and the production of IL-6, TNF-α, PGE_2, and NO in lipopolysaccharide-stimulated RAW264.7 macrophages and the production of IL-6 and TNF-α in phorbol 12-myristate 13-acetate plus calcium ionophore A23187-stimulated HMC-1 cells, respectively. The results showed that irradiated mulberry leaf extract possesses more anti-oxidant, anti-inflammatory, and tyrosinase inhibitory activities than their non-irradiated counterpart, probably due to increase in phenolic contents induced by gamma irradiation at dose of 10kGy. This research stresses on the importance of irradiation in functional foods.

1. Introduction

It is now well known that reactive oxygen species (ROS) such as superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydroxyl (OH) radical and hydrogen peroxide (H_2O_2) are produced in the body either as by-products of biological activities or from exogenous factors like ionization radiation (Finkel and Holbrook, 2000). This ROS, though important to the normal functioning of the body such as re-establishing redox homeostasis (Dröge, 2002; Li and He, 2009), can also be detrimental to the body's cells. So its production must be tightly regulated by the body. Naturally occurring cellular enzymes such as the superoxide dismutase (SOD), glutathione peroxide (GP) and catalase (CAT) called antioxidant are well known to neutralize these ROS (Blois, 1960; Dalton et al., 1999; Diplock et al., 1998). However environmental stressors like UV or heat exposure have shown to significantly increase ROS generation thus resulting to damage to lipids, DNA, protein structures and the end result being cell death or dysfunction (Blumberg, 2004). Consequently, this has led to the search for new sources of antioxidant with low molecular weight to supplement the body's naturally occurring antioxidant.

Inflammation is essential in protecting the body against pathogens or dangerous cells. Injection or invasion of harmful stimuli such as viruses and bacteria into the body leads to an innate immune response by the various immune cells including macrophages, eosinophils and neutrophils (Nagata, 2005). For example, macrophages activate inflammation-related genes *via* signaling pathways of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK). NF-κB translocates into the nucleus and binds to the promoter regions of the target genes that will synthesize cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, interleukin (IL)−1β, IL-6 which will elicits immune responses (Baeuerle and Baltimore, 1996; Fujiwara and Kabayashi, 2005). As a result, these immune cells produce excessive amount of inflammatory mediators such as prostaglandin E_2 (PGE_2), nitric oxide (NO) (Lee et al., 2008) and pro-inflammatory cytokines such as IL-1, IL-6, interferon (IFN)-γ and TNF-β (Dinarello, 2000). This can lead to chronic inflammation which is the cause of numerous human diseases such as cancer, atherosclerosis, arthritis, septic shock and atopic dermatitis (Ferencík et al., 2007; Hogg, 1998; Kim et al., 2012; Nomura et al., 2003). Therefore modulating inflammatory status can be targeted for therapeutic purposes to improve abnormal metabolic process.

Melanogenesis, a process that involves the production of skin pigment-melanin in the dermal melanocytes (Chang, 2009) though very important in photo-protective functions of the skin can also result to undesirable pigmented skin patches (Lin et al., 2011) and the production of ROS like hydroxyl radicals (Perluigi et al., 2003). The pathway of melanin synthesis includes a copper containing monooxygenase-tyrosinase which catalysis the melanin synthesis in melanocytes (Sturm et al., 2001) by hydroxylating tyrosine to o-diphenol product (L-dopa) and oxidizing the dopa to produce reactive inter-

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mediates that results in the formation of melanin by free radical-coupling pathway (Wang et al., 2006). Hydroquinone is considered as a potent inhibitor of the tyrosinase enzymes including other inhibitors like corticosteroid but their usage is commonly associated with side effects like skin-lightening, skin irritation and contact dermatitis (Zhu and Gao, 2008). Therefore the search for potent tyrosinase inhibitors from natural sources that are not associated with cytotoxicity is of utmost importance.

Mulberry (*Morus alba* L.) belongs to the family Moraceae (Hamza and Mekawey, 2013) and is widely distributed in Europe, Asia, north and south American and Africa. It has been used widely in oriental medicine in several countries for the treatment of human cancer, diabetes, cardiovascular diseases and obesity and researchers widely believe its action is due to its polyphenolic contents including but not limited to flavonoids which have good antioxidant and anti-inflammatory properties (Krishnaswamy and Raghuramulu, 1998; Lin and Tang, 2007; Sass-Kiss et al., 2005; Zadernowski et al., 2005). Over the past decade, researches have shown that mulberry does not only have anti-oxidative, anti-inflammatory, anti-tumor and anti-diabetic properties but do have cardiovascular, hepato and neuro-protective properties (Andallu et al., 2001; El-Beshbishy et al., 2006; Huang et al., 2008; Isabelle et al., 2008; Kimura et al., 2007; Wang et al., 2000).

Gamma irradiation is being used worldwide as a phytosanitory food and herbal material treatment. It reduces microbial contamination, insect damage and is currently used by the food and pharmaceutical industry (Farkas, 1998). Over the past years, researchers have started looking into the influence of irradiation processes on anti-oxidative and anti-inflammatory activities. A dose dependant increase in phenolic and anti-oxidant contents of gamma irradiated tomato pomace has been reported (Khalaf et al., 2014). Lee et al. (2003, 2005) reported the release of low-molecular weight phenolics with antioxidant activity in rice hulls and sesame meal by far-infrared radiation. Gamma irradiation did also increased the phenolic contents in cinnamon and clove (Variyar et al., 1998) However the influence on bioactive material is not very clear as several studies have shown that it has both positive and negative effects on biomaterial (Ahn et al., 2005; Jo et al., 2003; Lampart-Szczapa et al., 2003).

Although many researches has been done on the influence of irradiation on plant antioxidant activities, little or no research has been conducted on irradiated mulberry leaf especially to compare the antioxidant, anti-inflammatory and anti-tyrosinase activities of irradiated mulberry leaf and mulberry leaf side by side. Our work is the first of its kind to compare the antioxidant, anti-inflammatory and tyrosinase inhibition activities of gamma-irradiated mulberry leaf and mulberry leaf itself. The aim of this study was therefore to evaluate the influence of irradiation on the anti-oxidative, anti-inflammatory and whitening (through tyrosinase inhibition) activities of gamma-irradiated mulberry leaf extract (IMLE) and to compare their effects with their non-irradiated counterpart (MLE).

2. Materials and methods

2.1. Chemicals

TNF-α, IL-6, and PGE_2 ELISA kits were purchased from R & D System (Minneapolis, MN, USA). DMEM and IMDM were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals used in this study were purchased from Sigma-Aldrich chemical co. Ltd. (St. Louis, MO, USA) unless otherwise stated.

2.2. Plant materials

The leaves of mulberry were collected on the 29 June 2015 from Bugwi-myeon, Jinan-gun, Jeollabuk-do, Republic of Korea. The plant was identified and authenticated by Prof. Hong-Jun Kim, at the College of Oriental Medicine, Woosuk University. A voucher specimen has been deposited in the Department of Health Care & Science, College of Medical Science, Jeonju University.

2.3. Extract preparation

The dried mulberry leaves (100 g) were extracted in 2 L of 70% ethanol at room temperature for 5 days. The extract was filtered using ADVANTEC 5 A followed by ADVANTEC 2 filter paper purchased from Toyo Roshi Kaisha (Tokyo, Japan). The filtrate was then divided into two parts (50% each). One part was evaporated in a vacuum at 40 °C and further concentrated under reduced pressure to yield the dry extract of 8.9 g. The other part was irradiated at a dose of 10 kGy in a cobalt 60 irradiator (point source AECL, IR-79; MDS Nordion International Co., Ltd, Ottawa, Ontario, Canada) equipped with a 11.1 PBq source strength at 10 ± 0.5 °C and operated at a dose rate of 10 kGy/h at Korea Atomic Energy Research Institute. The irradiated sample was then concentrated under reduced pressure to yield the dry extract of 6.6 g. The two extracts were placed in separate plastic bottles labeled MLE (non-irradiated mulberry leaf extract) and IMLE (gamma-irradiated mulberry leaf extract) and stored at −20 °c until used.

2.4. Determination of total polyphenol

The total polyphenol (TP) contents were determined according to Peterson et al. (2001) with slight modifications. Briefly, 20 mg/mL of MLE and IMLE were made with DMSO as solvent and thereafter serial dilutions (with distilled water) of the extract and standard (gallic acid) were prepared 0–1000 μg/mL) in ependolf tubes. 0.1 mL each of the extract and standard was mixed with 0.1 mL Folin-Ciocalteu's phenol reagent. After 5 min, 1 mL of 4% Na_2CO_3 was added and the mixture allowed standing at room temperature and pressure for 30 min. All determinations were performed in triplicates. The absorbance was read at 600 nm and the total polyphenol concentration was calculated from a calibration curve (r^2 =0.996) using gallic acid as standard.

2.5. HPLC analysis

HPLC (high performance liquid chromatography) was performed using an Agilent 1100 series (Santa Clara, CA, USA), equipped with a binary pump delivery system, a degasser (G1379A), an auto sampler (G1313A) and a diode array detector (G1315B). The separation of compounds was performed on Agilent Eclipse XDB-C18 column (4.6×250 mm, 5 μm particles) through the gradient elution with 0.5% aqueous formic acid (A) and acetonitrile (B): 0 min, 5% B; 10 min, 10% B; 50 min, 40% B; 54 min, 100% B, and then held for 10 min before returning to the initial conditions. The mobile phase was retained at a flow rate of 1 mL/min and the column oven was set at a temperature of 30 °C. Ten microliters of the extracts have been injected and the UV detection was monitored at 280 nm. All standards (chlorogenic acid, isoquercetin, astragalin and rutin) were identified based on retention times and concentrations were calculated based on comparisons of sample peak areas with standards and isolated compounds. Stock solutions (1,000 μg/mL) were prepared using methanol. Calibration curves were constructed for each standard using 6 different concentrations (6.25, 12.5, 25, 50, 100, and 200 μg/mL). A degree of high linearity (r^2 > 0.994) was obtained for each standard curve. The integration of each component on the chromatograms was processed using software Agilent Chemstation.

2.6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The radical scavenging activity by DPPH was analyzed using the method of Blois (1958). Briefly, 0.1 mL of each extract was mixed with 0.1 mL of 0.3 mM DPPH solution and allowed standing at room temperature in the dark for 30 min and absorbance was measured at