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Antioxidant activity and protective effects of *Trapa japonica* pericarp extracts against *tert*-butylhydroperoxide-induced oxidative damage in Chang cells

Yon-Suk Kim [a], Jin-Woo Hwang [a], Young-Ki Han [a], Hyuck-Ju Kwon [b], Heeok Hong [c], Ee-Hwa Kim [d], Sang-Ho Moon [e], Byong-Tae Jeon [e], Pyo-Jam Park [a,e,*]

[a] Department of Biotechnology, Konkuk University, Chungju, Chungbuk 380-701, Republic of Korea
[b] Korea Food Research Institute, Seong-Nam, Gyeonggi 463-746, Republic of Korea
[c] Department of Medical Science, School of Medicine Konkuk University, Seoul 143-701, Republic of Korea
[d] Department of Acupoint and Meridian, College of Oriental Medicine, Semyung University, Jecheon, Chungbuk 390-711, Republic of Korea
[e] Nokyong Research Center, Konkuk University, Chungju, Chungbuk 380-701, Republic of Korea

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ABSTRACT

In this study, the antioxidant properties of *Trapa japonica* pericarp extracts were evaluated through several biochemical assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), alkyl radical scavenging activity, hydroxyl radical scavenging, ferric reducing antioxidant power (FRAP) assay, ABTS radical scavenging activity and oxygen radical absorbance capacity (ORAC). The antioxidant activities were compared with other natural and synthetic antioxidants. The results showed that higher radical scavenging activity and antioxidant capacity in FRAP than those of vitamin C as a positive control. *T. japonica* pericarp extracts have antioxidant properties through its ability to prevent *tert*-butylhydroperoxide (*t*-BHP)-induced toxicity which enhance the cell viability, reduce reactive oxygen species (ROS) production, inhibits of oxidative damage and mitochondria dysfunction in Chang liver cells. Therefore, based on these finding, it seems reasonable to suggest that *T. japonica* pericarp extracts has the potential to protect liver against *t*-BHP-induced cell damage and should be considered as a potential functional food.

1. Introduction

Trapa japonica commonly known as water caltrop is an annual aquatic plant belonging to the family of Trapaceae. It is a free floating plant grown in shallow water fields, ponds or swampy lands in tropical and sub-tropical Asian countries (Singh et al., 2011). Mature *T. japonica* has one pair of spines in the shoulder and/or one pair of short spines in the abdomen (Ciou et al., 2011). The outer pericarp of *T. japonica* is hard, making it difficult to peel off to obtain the white edible fruit inside (Tulyathan et al., 2005). The meat of the fruit of the water chestnut is consumed primarily in a cooked form and is eaten raw at the tender age. The fruit meat contained about 80% starch, 5% protein and significant amount of vitamins (Kang et al., 2009) and the fruit shell contains hydrolyzable tannins such as trapain and eugenin (Nokata et al., 1981). Pericarp is always considered to be the waste of *T. japonica* fruit and is generally discarded or used as fertilizer (Chiang and Ciou, 2010).

It has been pointed out that polyphenolic compounds usually accumulate in the outer parts of plants such as shells, and skins (Bravo, 1998). Litchi pericarp tissues account for approximately 15% of the total weight of fresh fruit, and comprise of significant amounts of flavonoids (Zhao et al., 2007; Zhang et al., 2000). *T. japonica* pericarp amount is around 30–40% of dry weight. Thus, it can be taken as an importance source but, there is little information on the polyphenolic content of *T. japonica* pericarp (TJP).

tert-Butylhydroperoxide (*t*-BHP) is widely used to study the effect of free radicals on cellular functions *in vitro* (Tiwari and Kakkar, 2009). *t*-BHP triggers the generation of harmful free radical intermediates, such as, peroxyl and alkoxyl radicals, which readily cross cellular membranes and lead to the production of the highly reactive hydroxyl radical, which in turn, reacts with macromolecules, such as, DNA, proteins, and lipids, and thus, damages cells (Lima et al., 2007).

The purpose of this research is to investigate the antioxidant effects of *T. japonica* pericarp through the antioxidant properties using various methods and protective effect against the oxidative

* Corresponding author at: Department of Biotechnology, Konkuk University, Chungju, Chungbuk 380-701, Republic of Korea. Tel.: +82 43 840 3588; fax: +82 43 852 3616.
E-mail address: parkpj@kku.ac.kr (P.-J. Park).

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stress in *t*-BHP induced cell damage. The results showed TJP exhibits powerful antioxidant activities and suggests that it could be useful as a potential natural antioxidant in the functional food and pharmaceutical industries.

2. Materials and methods

2.1. Materials

tert-Butyl hydroperoxide (*t*-BHP), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as diammonium salt (ABTS), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (4-pyridyl-1-oxide)-*N*-tert-butylnitrone (4-POBN), linoleic acid, ammonium thiocyanate, N-Acetyl-L-cysteine (NAC), 2′,7-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) and catechin, ferrous and ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). In addition, the *T. japonica* was obtained at Jecheon herbal medicine mall (Jecheon, Korea). All other reagents were of the highest grade available commercially.

2.1.1. Chemical analysis

Proximate compositions of *T. japonica* pericarp, including moisture, crude protein, crude fiber, crude fat and crude ash were analyzed according to AOAC methods (1990). Carbohydrate content was obtained by phenol–H_2SO_4 methods.

2.1.2. Preparation of extracts from the TJP

T. japonica pericarp was prepared using straw cutter and milling treatment. The TJP was extracted with water and 70% ethanol. TJP was extracted twice with 70% ethanol and was filtered with whatman filter paper No. 41 at room temperature (R.T). The filtrate was evaporated by an evaporator (EYELA, Tokyo, Japan) at 40 °C and then lyophilized in a freeze-dryer (Samwon, Busan, Korea). After evaporation, the water and ethanol extracts were lyophilized and stored at −20 °C until use.

2.2. Determination of total polyphenol contents

Total phenolic contents of TJP extracts were determined using Folin–Ciocalteu assay (Aline et al., 2005). Briefly, 10 mg of TJP extracts were individually dissolved in 10 mL of DW. Then, 0.1 mL of these solutions was mixed with 50 μL of 50% Folin–Ciocalteau reagent, and 150 μL of 20% sodium carbonate (Na_2CO_3). After incubation at R.T for 30 min, the absorbance of the reaction mixtures were measured at 760 nm by a spectrophotometer (SECOMAM, Ales, France). Gallic acid was used as a standard, and the total polyphenol contents of TJP extracts were expressed in milligram gallic acid equivalents (mg GAE/g extract).

2.3. Determination of total flavonoid contents

Total flavonoid contents were determined by the aluminium colorimetric method (Vábková and Neugebauerová, 2012) with slight modification. Briefly, the test samples were individually dissolved in D.W. Next, the sample solution (150 μL) was mixed with 45 μL 5% $NaNO_2$, and allowed to react for 5 min. Following this, 90 μL 10% $AlCl_3$ was added and the mixture was allowed to stand for further 5 min. Finally, 300 μL 1 M NaOH and 165 μL distilled water were added to the reaction mixture. After 10 min of incubation at ambient temperature, the absorbance of the supernatant was measured at 510 nm using a spectrophotometer. Three replicates were made for each test sample. The total flavonoid contents were expressed in milligram catechin equivalents per gram extract (mg CE/g extract).

2.4. Radical scavenging activity by ESR measurement

2.4.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine; JOEL, Tokyo, Japan) according to the technique described by Kim et al. (2008). Sixty microliters of each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μM) in ethanol. After 10 s of vigorous mixing, the solutions were transferred to Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adducts were determined by the ESR spectrometer exactly 2 min later under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2G, microwave power 5 mW, gain 6.3×10^5, and temperature 298 K.

2.4.2. Alkyl radical scavenging activity

Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures containing 40 mM AAPH, 40 mM 4-POBN and indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to 100 μL Teflon capillary tube. The spin adducts were recorded by an ESR spectrometer. The measurement conditions were as follows: central field 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

2.4.3. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated via the Fenton reaction, and reacted rapidly with nitrone spin trap DMPO. The resultant DMPO-OH adducts were detected using an ESR spectrometer. Reaction mixtures containing 100 μL of 0.3 M DMPO, 100 μL of 10 mM $FeSO_4$, and 100 μL of 10 mM H_2O_2 were mixed with the test samples, and then transferred to a Teflon capillary tube. The spin adducts were measured by an ESR spectrometer exactly 2.5 min later under the following measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2G, microwave power 1m W, gain 6.3×10^5, and temperature 298 K.

2.5. ABTS radical scavenging activity

For ABTS assay, the procedure followed the method of Erkan et al. (2008) with some modifications. The stock solutions included 7.4 mM $ABTS^{\cdot+}$ solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at R.T in the dark.

The mixture was diluted that its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. To determine the scavenging activity, 0.9 mL of ABTS reagent was mixed with 0.1 mL of extracts and the absorbance was measured at 734 nm after 6 min of reaction at R.T, using ethanol as a control. The antioxidant activities of TJP extracts were expressed by Trolox equivalents antioxidant capacity (TEAC), as mM Trolox equivalents/mg extract.

2.6. FRAP (ferric reducing antioxidant power) assay

FRAP assay was carried out by the method of Benzie and Strain (1996) with minor modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). To conduct the assay, a 3 mL aliquot of a FRAP reagent, a mixture of 0.3 M acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride (10:1:1 v/v/v), were combined with 1 mL of TJP extract. To determine the antioxidant capacity of the samples, the absorbance values were compared with those obtained from the standard curves of $FeSO_4$ (0–5 mM). The antioxidant capacity values were expressed as mM $FeSO_4$ equivalent in mg extract (mM $FeSO_4$ eq./mg extract).

2.7. Oxygen radical absorption capacity (ORAC) assay

The ORAC assay was based on a modified method of Ou et al. (2002). Samples and Trolox solutions were made in 75 mM phosphate buffer (pH 7.4). Fifty microliters of blank, Trolox standard or TJP extract (in triplicate) was mixed with 50 μL fluorescein (7.8 μM) solution and incubated for 15 min at 37 °C. At the injection of 25 μL 221 mM AAPH, the fluorescence was measured every 5 min for about 120 min (excitation wavelength 485 nm, emission wavelength 535 nm) using a fluorescence microplate reader (SpectraMax M2/M2e, CA, USA). The final ORAC values of the samples were calculated by using the net area under the decay curves (AUC) and were expressed as μmol Trolox equivalent (TE) per miligram extract (μmol TE/mg extract).

2.8. Cell culture

Chang liver cells purchased from American Type Culture Collection (ATCC CCL-13™) were cultured at 37 °C in humidified 5% CO_2, 95% air mixture in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fatal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin. Adherent cells were detached by trypsin–EDTA and plated onto 6- or 48-well plates at 70–80% confluence.

2.9. Cell viability

The cell viability was estimated by MTT assay. Chang liver cells were seeded in 48-well plate at a concentration of 7.0×10^3 cells/well. After 20 h, the cells were treated with different concentrations of various TJP extract, and incubated in a humidified incubator at 37 °C for 1 h. Then, 80 μM *t*-BHP was added as final concentration, and incubated for 24 h. Thereafter, a 100 μL of MTT stock solution (0.5 mg/mL) was added and incubated for 4 h. Then, the supernatants were aspirated and the formazan crystals in each well were dissolved in 150 μL of DMSO. Absorbance was measured by spectroflurometer (SpectraMax M2/M2e, CA, USA) at a wavelength of 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability.

2.10. Cell cycle analysis by flow cytometry

A total of 2×10^4 cells were plated per well in 6-well plates with 2 mL culture medium for 16 h and pretreat TJP ethanolic extract for 1 h before exposure to 80 μM *t*-BHP for 24 h. For flow cytometry cell cycle analysis, cells were harvested and