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DNA Protection and Antitumor Effect of Water Extract from Residue of Jelly Fig (*Ficus awkeotsang* Makino) Achenes

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ABSTRACT

The present study investigated the effect of water extract from the residue of jelly fig achenes (WERJFA) on the protection of DNA against hydroxyl radical damage and on the growth inhibition of three cancer cell lines, including mouse colon cancer CT-26, human hepatoma HepG2 and breast cancer MCF-7. DNA protection against hydroxyl radical from hydrogen peroxide was assayed by agarose gel electrophoresis. Growth inhibition of the cell lines was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed that WERJFA was able to reduce the hydroxyl radical-induced DNA damage and to inhibit the growth of the three cancer cell lines with CT-26 being the most vulnerable one, HepG2 the second, while MCF-7 the least affected. The growth inhibition rates of WERJFA (200 µg total solids/mL) on CT-26 and HepG2 were 85.4% and 60.0%, respectively. MTT assay was also applied in the rat hepatocyte Clone 9 cytotoxicity test. No cytotoxicity of WERJFA (0-200 µg total solids/mL) was observed. These lines of evidence support that WERJFA has the potential to be an antitumor agent. Ultrafiltration enhanced the growth inhibitory effect of WERJFA. Among all the fractions of filtrate, the 3-10 kDa fraction had the highest inhibition on HepG2 cells, reaching 73.5% reduction of growth when added at 200 µg total solids/mL and incubated for 72 hr.

Key words: jelly fig, antitumor, DNA damage, cancer cell

INTRODUCTION

Chemoprevention is among the most promising areas in current cancer research. An attractive strategy is to attenuate malignant cells through apoptosis by dietary treatment⁽¹⁾. Therefore, biologically active ingredients from mushrooms, fungi, yeasts, algae, lichens, herbs and other plants attracted much attention in medical applications for their antitumor abilities⁽²⁻⁴⁾.

Carcinogenesis can be induced by free radicals, and be inhibited by free radical scavenging compounds⁽⁵⁾. Natural materials with free radical scavenging activity play a critical role in the chemoprevention of cancer⁽⁶⁾. The formation of hydrogen peroxide is involved in the mechanism for free radicals to damage genetic material⁽⁷⁾. Fenton reaction on calf thymus DNA is usually applied to evaluate the potential of biologically active ingredients to act against hydroxyl radical-induced damage⁽⁸⁾.

Jelly fig is a woody vine that grows on hillsides in Taiwan. Its achenes are rich in pectin, and can be used to prepare a fruit jelly curd as an ingredient in traditional

summer drinks. The residue is commonly regarded as waste. Recently, the water extract from the residue of jelly fig achenes (WERJFA) was found to have the potential in reducing the methanol content in carambola wine via the inhibition of pectic enzymes⁽⁹⁾, in preserving food as an antimicrobial and antioxidation additive⁽¹⁰⁾, and in inhibiting the proliferation of human leukemic U937 cells through the induction of apoptosis⁽¹¹⁾. Its effects on colon, breast and liver cancers have not been reported yet, although each of these cancer types occurs more frequently than leukemia⁽⁶⁾ in the world. Cytotoxicity study is well recognized as a prerequisite in the development of drugs or health food. The cytotoxicity of WERJFA has not been reported either. Liver is the major metabolism organ for various chemical compounds⁽¹²⁾. Culturing of rat hepatocyte clone 9 *in vitro* is thus commonly performed in the evaluation of liver function and cytotoxicity of chemicals^(13,14).

The present study was to investigate the effect of WERJFA on the protection of calf thymus DNA against hydroxyl radical damage, on the growth inhibition of mouse colon cancer cell line CT-26, human hepatoma HepG2 and breast cancer MCF-7, and on the cytotoxicity towards rat hepatocyte clone 9. The results would be

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helpful in evaluating the potential of WERJFA to be an antitumor agent.

MATERIALS AND METHODS

I. Preparation of WERJFA

Jelly fig achenes were purchased from a farm in Fengshan City of Kaohsiung Prefecture. WERJFA was prepared according to the method described by Chang *et al.*⁽¹¹⁾. Briefly, jelly fig achenes were mixed thoroughly before random sampling in 50 g batches. Each batch of the achenes was repeatedly washed with 4% NaCl solution to remove the pectin, followed by homogenization in a blender and extraction in 15 parts (v/w) of distilled water (Milli-Q System, Millipore, Osaka, Japan) for 24 hr. The extract was centrifuged (at 10,000×g, 50 min, 4°C), filtered through Whatman No. 1 filter paper, and then heated in boiling water for 15 min to denature the residual enzymes to obtain WERJFA. It might be further vacuum concentrated in a rotary evaporator, dehydrated using a lyophilizer, and then kept in powder form at -20°C before use. The recovery of WERJFA powder from jelly fig achenes was approximately 2.3% in dry weight.

II. Ultrafiltration of WERJFA

Membrane ultrafiltration of WERJFA was conducted using an Amicon apparatus (Model 8200, Millipore, Bedford, MA, USA). The filtrate was separated into four fractions, containing 0.5-3, 3-10, 10-70, and >70 kDa solids, respectively. Each fraction was lyophilized and stored at -20°C before use.

III. Culturing of Cell Lines

Cell lines including rat hepatocyte clone 9, mouse colon cancer CT-26, human hepatoma HepG2 and breast cancer MCF-7 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The rat hepatocyte clone 9 and human hepatoma HepG2 were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, Gaithersburg, MD, USA). Mouse colon cancer CT-26 and human breast cancer MCF-7 were cultured in RPMI 1640 Medium (Gibco) and in Minimum Essential Medium (Gibco), respectively. All cell lines were incubated at 37°C in a medium containing 10% fetal bovine serum (FBS) in a fully humidified atmosphere of 5% CO₂. Cell lines were subcultured every 2 or 3 days to be maintained in the exponential growth phase.

IV. Evaluation of Hydroxyl Radical-induced DNA Damage

Fenton reaction can generate hydroxyl radicals⁽¹⁵⁾. The radicals would attack the deoxyribose elements of DNA molecules, degrade the molecules by the release

of purine and pyrimidine bases, and produce mutagenic sites⁽¹⁶⁾. By assaying the retention of intact DNA molecules, the protection effect of WERJFA on DNA damage was evaluated.

Each 45 µL aliquot of a reaction mixture, which was a blend of WERJFA (0-200 µg total solids/mL), 5 µL calf thymus DNA solution (25.0 A₂₆₀ unit/mL) (Amersham Biosciences, Piscataway, NJ, USA), 0.9 µL 180 mM FeSO₄ and 3.6 µL 600 mM hydrogen peroxide, was incubated at room temperature for 15 min. After then, 10 µL of 1 mM EDTA was added to stop the reaction. The blank was the calf thymus DNA solution. The control was the reaction mixture without WERJFA. Each 10 µL aliquot of the reaction mixture was applied on 1% agarose gel containing 0.1% ethidium bromide. The electrophoresis was conducted in TBE buffer (10 mM Tris-boric acid-EDTA, pH 7.4) for 5 min. The gel was then visualized under UV illumination.

V. Inhibition of Cell Growth

Growth inhibition of the cells was determined using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay⁽¹⁷⁾ with the chemicals purchased from Sigma Chemicals (St. Louis, MO, USA). In brief, cells were cultured in 96-well plates at an initial concentration of 1 × 10⁵ cells/mL for 1 to 3 days. MTT solution [3 mg MTT/mL PBS (phosphate-buffered saline, 8g NaCl/1.15 g Na₂HPO₄/0.2 g KH₂PO₄/0.2 g KCl/L)] was added to each well. The cells were incubated at 37°C for 4 hr, allowing mitochondrial dehydrogenases in viable cells to reduce MTT to a purple formazan product. The MTT-formazan product was then dissolved in MTT lysis buffer. The absorbance at 570 nm was measured, using an ELISA plate reader (OPTImax™ Tunable Microplate Reader Molecular Devices Co., Toronto, CA, USA). Growth inhibition was calculated as the follows: growth inhibition (%) = [1 - (absorbance of treatment / absorbance of control group)] × 100%.

VI. Protein Assay

Protein concentrations in the ultrafiltered fractions were assayed according to Bradford⁽¹⁸⁾ method using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin (0.05-0.4 mg/mL) was used to construct the standard curve.

VII. Carbohydrate Assay

Total carbohydrate content was quantified by phenol-sulfuric acid method⁽¹⁹⁾. D-glucose at various concentrations (20-100 mg/mL) was used to construct the calibration curve. Color development at 490 nm was monitored using a spectrophotometer (Helios Alpha, Spectronic Unicam, Cambridge, UK).

VIII. Total Polyphenol Assay

The total polyphenol content was assayed by referring to the Folin-Ciocalteu method by Spanos and Wrolstad⁽²⁰⁾. The results were expressed as gallic acid equivalents. Gallic acid (0.2-1 mg/mL) from Sigma Chemicals was used as standards. Color development at 750 nm was monitored by the spectrophotometer.

IX. Statistical Analysis

The experiment data were expressed as means \pm SD and analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. $P < 0.05$ was considered as statistically significant. Linear regression was applied wherever applicable. The coefficient of determination (R^2) was calculated.

RESULTS AND DISCUSSION

I. Protection by WERJFA Against Hydroxyl Radical-induced DNA Damage

The oxidative damage of DNA is one of the most important mechanisms in the initiation of cancer. The damage is usually caused by hydroxyl radicals⁽²¹⁾. The activity of these radicals can be reduced by natural antioxidants found in plants including herbs⁽⁶⁾. The Fenton reaction involves the reaction between hydrogen peroxide and Fe^{2+} to form hydroxyl radicals. Scavengers of hydroxyl radicals inhibit this reaction through the reduction of Fe^{2+} ⁽¹⁵⁾. In previous study, WERJFA was found to have reducing capacity and superoxide anion radical scavenging activity. Phenolic compounds accounted for approximately 13% of the total solids in WERJFA⁽¹⁰⁾. The present study found that WERJFA at 200 μg total solids/mL concentration was able to reduce the hydroxyl radical-induced damage in calf thymus DNA by approximately 30% (Figure 1). These results suggest that WERJFA protects DNA through the antioxidant activity.

II. Growth Inhibition of HepG2, CT-26, and MCF-7 Cells by WERJFA

Growth inhibition of HepG2, CT-26, and MCF-7 cells in culturing with different concentrations of WERJFA is shown in Figure 2. Culturing with WERJFA at 200 μg total solids/mL concentration for 3 days resulted in 85.4% and 60% growth inhibition on CT-26 and HepG2 cell lines, respectively. The inhibition on the viabilities of HepG2 and CT-26 cell lines by WERJFA in 0-200 μg total solids/mL concentration was found to be concentration-dependent with $R^2 = 0.9859$ and 0.9723 , respectively. There was no sign of inhibition on MCF-7 cell line by WERJFA at 200 μg total solids/mL, indicating that this cell line is the least vulnerable among all the three cell

lines tested. The IC_{50} values for HepG2 and CT-26 were 158.4 and 125.2 μg total solids/mL, respectively, indicating that CT-26 is the most vulnerable one.

WERJFA was previously reported to inhibit the growth and proliferation of human leukemia U937 cell. The growth rate of U937 cells was reduced by about 90.0% in a medium containing 50 μg total solids/mL of WERJFA in 3 days⁽¹⁰⁾. In the present study, culturing in a medium containing WERJFA at 200 μg total solids/mL for 3 days exhibited 85.4% and 60.0% growth inhibition on CT-26 and HepG2 cell lines, respectively, while culturing in a medium containing WERJFA at 400 μg total solids/mL exhibited 19.4% growth inhibition on MCF-7 cell line. WERJFA looks more effective in the inhibition on U937 cells than the three cell lines tested in the present study, possibly because U937 cells are more sensitive than others as described in a previous report⁽²¹⁾. At 100 μg total solids/mL concentration, the growth inhibi-

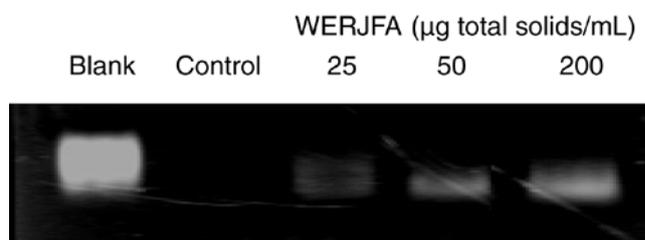


Figure 1. WERJFA protected calf thymus DNA against hydroxyl radical-induced damage. Samples were a blend of WERJFA, calf thymus DNA solution, FeSO_4 , and hydrogen peroxide. Blank was calf thymus DNA solution. Control was the reaction mixture containing no WERJFA.

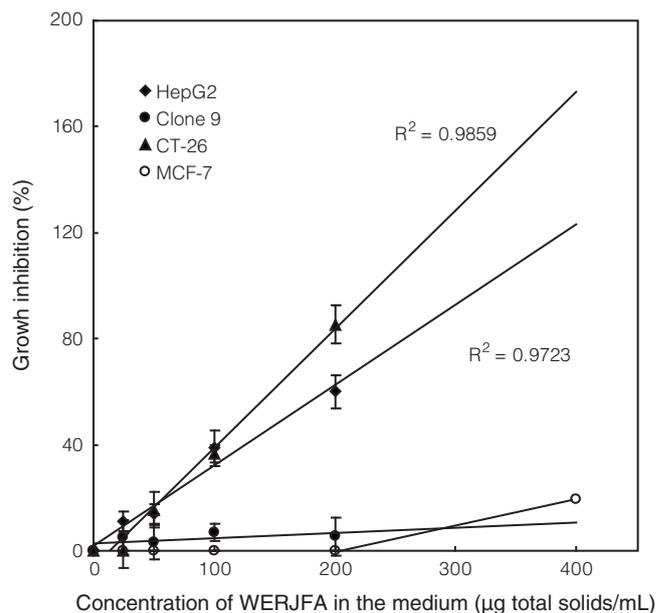


Figure 2. Effect of WERJFA on the growth of rat hepatocyte Clone 9, human hepatoma HepG2, mouse colon CT-26, and human breast MCF-7 cancer cell lines in 3-day cultivation.

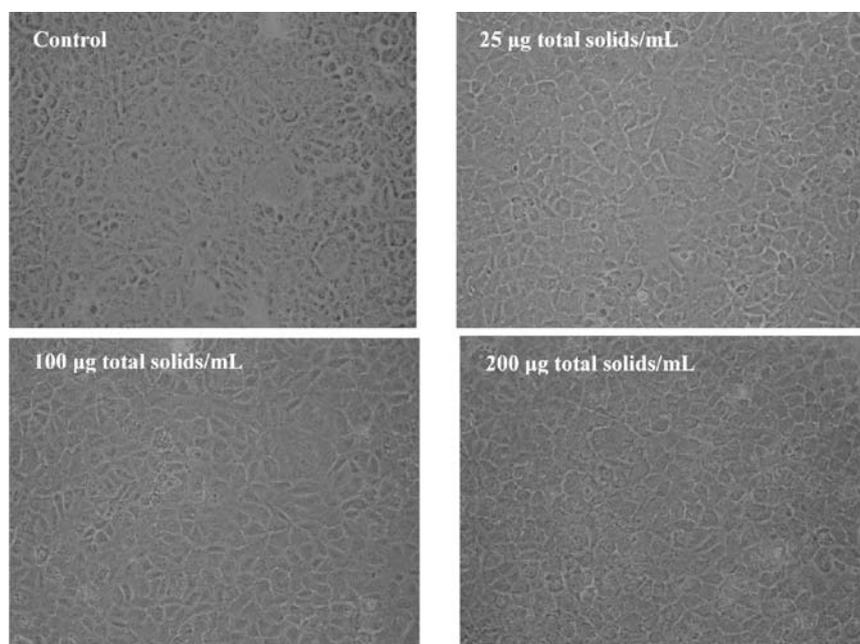


Figure 3. Morphology of rat hepatocyte clone-9 cells after culturing in a medium containing various concentrations of WERJFA for 3-days. Control: no WERJFA in the culture medium.

tory effects of WERJFA on CT-26 (36.6%) and HepG2 (38.9%) are close to those of the extracts from brown alga *Ecklonia cava* (about 34%)⁽²¹⁾ and herb *Perilla frutesces* (about 47%)⁽²²⁾, respectively. The extract of herb *Platycodon grandiflorum* at 200 µg total solids/mL concentration was reported to inhibit the growth of HepG2 cell line by 10% only⁽²³⁾. WERJFA is much more effect, being 38.5% in the same experiment condition. In conclusion, WERJFA appears to have a decent potential in inhibiting the growth of many types of cancer cell lines.

III. Cytotoxicity Towards Clone 9 Cells

Low cytotoxicity in normal cell lines is an important prerequisite in the development of chemopreventive agents⁽²¹⁾. WERJFA cytotoxicity has not been reported previously. Figure 2 show the effect of WERJFA on the growth of rat clone 9 cells. Statistical analysis revealed no occurrence of significant growth inhibition. No morphological changes were noticed either (Figure 3). The results indicate that WERJFA has no cytotoxicity towards rat hepatocyte clone 9 cells, and therefore is safe to be developed into a chemopreventive agent.

IV. Growth Inhibition of HepG2 Cells by Ultrafiltrated WERJFA Fractions

Rajapakse *et al.*⁽²⁴⁾ applied ultrafiltration technique in the separation of giant squid muscle protein and reported that fractions with a molecular size smaller than 3 kDa have higher antioxidation activity. Jiang *et al.*⁽²⁵⁾ ultrafiltrated WERJFA and found that the 3-10 kDa fraction has higher pectinesterase inhibition activity. The result of present study showed that no growth inhibition

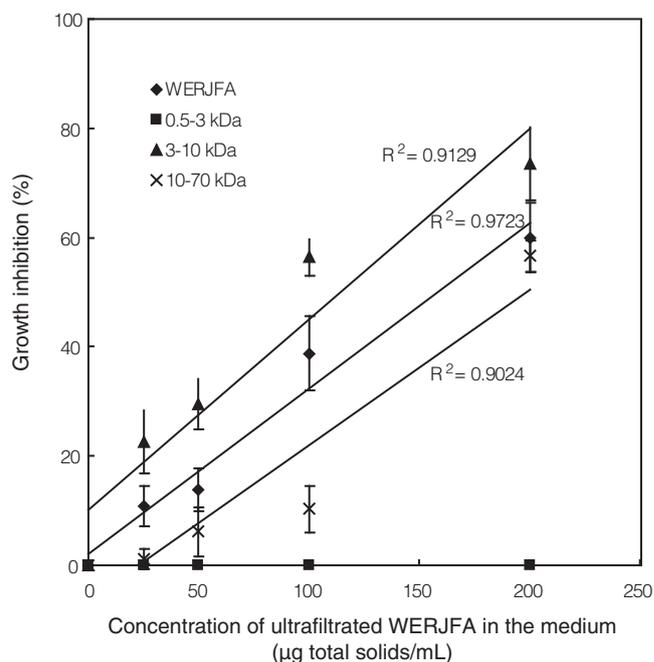


Figure 4. Growth inhibition on HepG2 cancer cells by culturing with different ultrafiltrated WERJFA fractions for 3 days.

on HepG2 cells occurred in culturing with the 0.5-3 kDa membrane cut-off fraction for 3 days (Figure 4). Linear concentration-dependency with a R^2 value higher than 0.900 was observed in the growth inhibition of HepG2 cells by treating with 3-10 or 10-70 kDa fraction in 0-200 µg total solids/mL concentration range. The growth inhibitory effect on HepG2 cells by the 3-10 kDa fraction of WERJFA (73.5% inhibition, $IC_{50} = 158.4$ µg total solids/mL) was higher than total WERJFA (60.0% inhi-

Table 1. Chemical composition and inhibition effect of ultrafiltrated WERJFA fractions

Fraction	Protein (mg/mg total solids)	Carbohydrate (mg/mg total solids)	Polyphenol (mg/mg total solids)	Growth inhibition on HepG2 cells (%)*
0.5~3 kDa	0.043 ± 0.001 ^a	0.024 ± 0.001 ^a	0.279 ± 0.088 ^a	0.0 ± 0.0
3~10 kDa	0.209 ± 0.003 ^b	0.064 ± 0.002 ^b	0.387 ± 0.062 ^a	73.5 ± 6.7
10~70 kDa	0.243 ± 0.001 ^c	0.060 ± 0.006 ^b	0.684 ± 0.029 ^b	56.6 ± 3.0
> 70 kDa	0.127 ± 0.003 ^d	0.043 ± 0.003 ^c	0.386 ± 0.077 ^a	--

* HepG2 cells were cultured for 3 days with the fraction.

--: data not available.

^{a-d}Means across the same column with different superscripts are statistically different ($p < 0.05$).

bition, $IC_{50} = 114.0 \mu\text{g total solids/mL}$). Thus, ultrafiltrated WERJFA fraction displayed a stronger growth inhibition activity on HepG2 cancer cells. These results suggest that the 3-10 kDa fraction might contain certain components with high antitumor activity, and that antitumor activity could be boosted by ultrafiltration or further purification.

V. Chemical Composition of WERJFA Fractions

Phenolic substances are well-known to exert health-promoting effects such as antitumor, antimutagenic, anti-inflammatory, and antimicrobial activities^(26,27), reduction in risk of cardiovascular diseases^(28,29), inhibition of the oxidation of low-density lipoproteins⁽³⁰⁾, and anticarcinogenic effects^(31,32). Protein or peptides may also exhibit various bioactivities such as antioxidation⁽³³⁾, antitumor⁽³⁴⁾, antihypertensive⁽³⁵⁾ and immunomodulatory⁽³⁶⁾ effects.

As shown in Table 1, the 10-70 kDa fraction had the highest contents of protein (0.24 mg/mg total solids), carbohydrate (0.06 mg/mg total solids) and polyphenol (0.66 mg/mg total solids) among all these membrane cut-off fractions while the 0.5-3 kDa fraction had the lowest contents of protein (0.04 mg/mg total solids), carbohydrate (0.02 mg/mg total solids) and polyphenol (0.28 mg/mg total solids). However, the 3-10 kDa fraction contained less protein (0.21 mg/mg total solids) and polyphenol (0.38 mg/mg total solids) than the 10-70 kDa fraction while exhibited higher growth inhibition on HepG2 cells, suggesting again the presence of some components with higher bioactivity in the 3-10 kDa fraction.

The brown alga *Ecklonia cava*⁽²¹⁾, herb *Perilla frutescens*⁽²²⁾ and herb *Platycodon grandiflorum*⁽²³⁾ were reported to have high polyphenol contents and to show pronounced antitumor activity. The antitumor effect was also found in peptides from various natural materials, such as Lunasin from soybean protein⁽³⁷⁾, lactoferrin from bovine milk⁽³⁸⁾ and VR-3848 from tropical plants⁽³⁴⁾. The mechanism for the antitumor activities of polyphenols and peptides may probably involve the induction of apoptosis⁽⁶⁾. As a matter of fact, WERJFA has been

found to inhibit the proliferation of human leukemic U937 cells through the induction of apoptosis⁽¹¹⁾.

In the present study, the correlation coefficient was applied to elucidate the effects of protein and polyphenol on growth inhibition (Table 1). The results showed a high correlation ($R^2 = 0.8754$) between the growth inhibition and the content of protein in various fractions, versus a low correlation ($R^2 = 0.2812$) between the growth inhibition and the polyphenol content. Therefore, we propose that the protein in WERJFA is responsible for the antitumor activity.

CONCLUSIONS

WERJFA has reducing capacity and superoxide anion radical scavenging activity. It has also been proved to protect DNA against hydroxyl radical-induced damage. Through correlation analysis we found that proteins may probably be the active component to display antitumor activity toward cancer cells. WERJFA caused no cytotoxicity in the experiment. Further investigation in the development of WERJFA to be a chemopreventive agent would be worthwhile.

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