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Anti-skin cancer properties of phenolic-rich extract from the pericarp of mangosteen (*Garcinia mangostana* Linn.)

Jing J. Wang [a,b], Qing H. Shi [a,c], Wei Zhang [a,b], Barbara J.S. Sanderson [a,*]

[a] Level 4, Health Science Building, Department of Medical Biotechnology, Flinders Medical Sciences and Technology, School of Medicine, Faculty of Health Science, Flinders University, Registry Road, Bedford Park, Adelaide 5042, Australia
[b] Level 4, Health Science Building, Flinders Centre for Marine Bioproducts Development (FCMBD), Flinders University, Registry Road, Bedford Park, Adelaide 5042, Australia
[c] Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

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ABSTRACT

Skin cancers are often resistant to conventional chemotherapy. This study examined the anti-skin cancer properties of crude ethanol extract of mangosteen pericarp (MPEE) on human squamous cell carcinoma A-431 and melanoma SK-MEL-28 lines. Significant dose-dependent reduction in% viability was observed for these cell lines, with less effect on human normal skin fibroblast CCD-1064Sk and keratinocyte HaCaT cell lines. Cell distribution in G_1 phase (93%) significantly increased after 10 μg/ml of MPEE versus untreated SK-MEL-28 cells (78%), which was associated with enhanced $p21^{WAF1}$ mRNA levels. In A-431 cells, 10 μg/ml MPEE significantly increased the sub G_1 peak (15%) with concomitant decrease in G_1 phase over untreated cells (2%). In A-431 cells, 10 μg/ml MPEE induced an 18% increase in early apoptosis versus untreated cells (2%). This was via caspase activation (15-, 3- and 4-fold increased caspse-3/7, 8, and 9 activities), and disruption of mitochondrial pathways (6-fold decreased mitochondrial membrane potential versus untreated cells). Real-time PCR revealed increased Bax/Bcl-2 ratio and cytochrome *c* release, and decreased Akt1. Apoptosis was significantly increased after MPEE treatment of SK-MEL-28 cells. Hence, MPEE showed strong anti-skin cancer effect on these two skin cancer cell lines, with potential as an anti-skin cancer agent.

1. Introduction

Skin cancer, including non-melanoma and melanoma, is a growing public health problem due to a dramatically increasing incidence (Trakatelli et al., 2007; Doan, 2008). This increase in the incidence of skin cancer is expected to continue due to the aging population and the greater levels of UV radiation reaching the earth surface as a result of the ozone layer depletion (Johnson et al., 1998; Miller and Weinstock, 1994). Therefore, it is important to develop novel effective chemopreventive agents that can reduce or control skin cancer.

More than 50% of anti-cancer drugs currently in clinical trials are derived from, or inspired by, natural products, especially terrestrial plants (Cragg and Newman, 2000; Nuijen et al., 2000; Gordaliza, 2007). Mangosteen (*Garcinia mangostana* Linn.) is a tropical fruit available in south-east Asia. Its pericarp has been used for centuries as traditional medicine by Southeast Asians and South Americans for a great variety of medical conditions, for example, treatment of skin infections, wounds and diarrhea (Mahabusarakam et al., 1987; Pedraza-Chaverri et al., 2008; Obolskiy et al., 2009). A 50% ethanol extract of mangosteen pericarp had antioxidative and neuroprotective activities in NG108-15 neuroblastoma cells against H_2O_2-induced oxidative stress (Weecharangsan et al., 2006). Another ethanol extract of mangosteen pericarp inhibited histamine release, prostaglandin E2 synthesis (Nakatani et al., 2002), and HIV-1 protease (Chen et al., 1996). A methanol extract of mangosteen pericarp displayed antiproliferative, apoptotic and antioxidative activities on SKBR3 human breast cancer cell line (Moongkarndi et al., 2004a). Xanthones and anthocyanins are found in the pericarp of mangosteen, with xanthones being the major bioactive compounds (Nguyen et al., 2005). The antioxidant activity and cytotoxicity of xanthones on some cancer cells (e.g. colorectal cancer, hepatoma, leukemia, and small cell lung cancer) have been reviewed extensively (Akao et al., 2008; Pedraza-Chaverri et al., 2008; Obolskiy et al., 2009). We previously demonstrated that α-mangostin, γ-mangostin, and 8-desoxygartanin isolated from the pericarp of mangosteen exhibited significant anti-cancer effect on human melanoma SK-MEL-28 cell line via cell cycle arrest in G_1 phase and apoptosis induction (Wang et al.,

* Corresponding author. Tel.: +618 7221 8556; fax: +618 7221 8555.
E-mail addresses: wang0524@flinders.edu.au (J.J. Wang), qhshi@tju.edu.cn (Q.H. Shi), wei.zhang@flinders.edu.au (W. Zhang), Barbara.sanderson@flinders.edu.au (B.J.S. Sanderson).

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2011). However, no information is available on the anti-skin cancer activity of mangosteen pericarp crude extracts.

In this study, we investigated the antioxidant properties of ethanol extracts of mangosteen pericarp and its anti-skin cancer activity in human squamous cell carcinoma (non-melanoma) A-431 and melanoma SK-MEL-28 cell lines and the underlying cellular and molecular mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetic acid, and ethanol were of analytical grade from Merck (Australia) and all the other reagents were purchased from Sigma–Aldrich (St. Louis, USA) unless stated otherwise.

2.2. Extraction

Fresh mangosteen fruits were purchased from the local market in Adelaide, South Australia, Australia. The standardized extraction method as described previously (Shan and Zhang, 2010) was used to produce the extracts with quality consistency and reproducibility. Briefly, the mangosteen fruit was cleaned with MilliQ water. The pericarp was peeled from fruit and ground into fine powder in a juice blender with a cross blade. The extraction was conducted by mixing mangosteen pericarp with absolute ethanol at 75 °C for 1 h with a weight-to-volume ratio of 1:10. After extraction, the mixture was centrifuged at 10,000*g* for 10 min. The supernatant was filtered through 0.22 μm filter (Minisart, Sartorius Stedim Biotech, Germany) and then vacuum dried to yield crude ethanol extract (MPEE). The green tea (*Camellia sinensis*) sample branded as "Chinatea" was purchased as ground dried plant leaves from a local store and used as an antioxidant comparison standard and its extraction was carried out by mixing green tea powder with MilliQ water at 100 °C for 1 h with a weight-to-volume ratio of 1:10. The remaining steps were the same as for the mangosteen pericarp extraction, except that the supernatant was freeze dried instead of vacuum dried. The extract was stored in a desiccators at −20 °C. When required, the dried MPEE extract was redissolved in ethanol and green tea extract was redissolved in MilliQ water. The major xanthone compounds in the MPEE were identified and quantified by HPLC as α-mangostin (321 mg/g MPEE), β-mangostin (3.88 mg/g MPEE), γ-mangostin (81.3 mg/g MPEE), 8-desoxygartanin (10.7 mg/g MPEE), 9-hydroxycalabaxanthone (4.84 mg/g MPEE), and gartanin (18.7 mg/g MPEE) (Shan and Zhang, 2010).

2.3. Total phenolics measurement

Total phenolics content of MPEE or green tea extract was determined by the method as described previously (Singleton and Rossi, 1965) with minor modification. Briefly, 1 ml MilliQ water was added into the 200 μl of the extract followed by adding 50 μl Folin–Ciocalteu reagent. The mixture was vortexed and incubated for 7 min at room temperature (RT). Then 290 μl of sodium carbonate (20%) was added and mixed evenly. The absorbance was measured at 760 nm by UV–vis Spectrophotometer (UV mini 1240, Shimadzu Corporation, Japan) after 1 h incubation. Quantitation of total phenolics content was based on the standard curve of gallic acid. Results were expressed in terms of gallic acid equivalence (GAE) in mg per dry pericarp weight in gram.

2.4. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The extract was tested for its ability to scavenge DPPH· radicals by DPPH radical scavenging assay as described previously (Blois, 1958). Three milliliters of 0.1 mM DPPH· in ethanol was added to 0.5 ml of MPEE extract or green tea extract and incubated for 30 min in the dark at RT. Absorbance was read at 517 nm using a UV–vis Spectrophotometer. The percentage of free radical scavenging activity was calculated using the formula

$$\%\text{DPPH inhibition} = (1 - \text{Absorbance in the presence of extract}/\text{Absorbance in the absence of extract}) \times 100$$

The antioxidant activity of the extract was expressed as IC_{50} (the concentration of the extract required to inhibit DPPH radicals by 50%), as calculated using GraphPad Prism 5 software (San Diego, CA, USA).

2.5. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed as previously described (Benzie and Strain, 1996) to test the reducing activity of MPEE and green tea extract. Briefly, the extract was centrifuged for 10 min at 2830*g* prior to use and the supernatant was saved. FRAP reagent consisted of 25 ml of 300 mM acetate buffer at pH 3.6, 2.5 ml of 10 mM 2,4,6-tripyridyl-*s*-triazine and 2.5 ml of 20 mM $FeCl_3$. FRAP reagent (900 μl) was heated at 37 °C for 10 min, then 90 μl of Milli-Q water and 30 μl of sample were added to it and mixed well. The absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37 °C for 4 min. $FeSO_4{\cdot}7H_2O$ of 1 mM was used as the standard solution (standard solution ranged from 0 to 1 mM). The antioxidant activity of the tested MPEE or green tea extract was expressed as FRAP value/g dry pericarp weight (DW) as shown in the equation below:

$$\text{FRAP value } (\mu\text{mol/L FeSO}_4 \text{ equivalents (FE)/g DW}) = [(\text{Absorbance at 593 nm/slope}) \times \text{dilution factor}] \times \text{volume of supernatant (ml)/equivalent DW (g)/1000}$$

2.6. Oxygen radical absorbance capacity (ORAC) assay

The assay was carried out as described previously (Huang et al., 2005) with minor modification. The ORAC assay was carried out on a microplate reader (DTX 880, Beckman Coulter, USA). Trolox, a water-soluble analog of vitamin E, was used as a control standard. Briefly, the extract and trolox were diluted in a 96-well microplate. Trolox was prepared in concentrations of 100, 75, 50, 25, 12.5 and 0 μM while the dilution factors of 20, 200, 400, 800, 1600 and 3200× were applied for the extract sample. In the analysis, 180 μl of fluorescein working solution, 30 μl of phosphate working buffer and 30 μl of diluted sample/trolox were loaded into each well. The reaction was carried out at 37 °C and the plate was incubated for 10 min before adding 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). After adding AAPH, the plate was shaken to mix the reagents in each well before recording the initial fluorescence (f0). Fluorescence readings were taken at 0 s (s) (f0) and then every 90 s thereafter (f1, f2, f3, ...) for 50 cycles. The final ORAC values were calculated by using a regression equation between trolox concentration and the net area under the curve by MATLAB program and were expressed as micromole trolox equivalents (TE) per gram of dry pericarp weight (μmol TE/g DW). All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample.

2.7. Cell culture

Human melanoma SK-MEL-28, squamous cell carcinoma A-431, and skin fibroblast CCD-1064Sk cell lines were purchased from the American Type Culture Collection. Human normal keratinocytes (HaCaT) cell line was a kind gift from the Department of Haematology, Flinders University, Adelaide, Australia. SK-MEL-28 and A-431 cells were cultured in DMEM, HaCaT in RPMI 1640, and CCD-1064Sk in IMEM, all supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corporation, Australia) in the presence of 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Scientific, Melbourne, Australia). All the cultures were maintained in a fully humidified incubator with 5% CO_2 at 37 °C. Cells used in experiments were under the passage number of 20 and free of mycoplasma contamination as detected by PCR (data not known).

2.8. Treatment preparation

MPEE was dissolved in absolute ethanol, and diluted with the respective media to the desired treatment concentrations. Ethanol was used as vehicle control and its volume was consistently kept as 1% (v/v) for each treatment.

2.9. Cell proliferation assay

Crystal violet assay was carried out to measure the cell viability as described previously (Wang et al., 2011). EC_{50} was determined as 50% viability relative to the untreated control, as calculated using GraphPad Prism 5 software. 5-Fluorouracil (5-FU) and dacarbazine (DTIC) were used as positive control for A431 and SK-MEL-28, respectively, because 5-FU is a commonly used drug for human squamous carcinoma, and DTIC is the only drug for melanoma approved by both the United States Food and Drug Administration and the European Agency for the Evaluation of Medicinal Products (Tsao et al., 2004; Garbe and Eigentler, 2007). The morphological changes of cells were observed under the microscope and recorded by the Olympus 1X71 phase contrast inverted fluorescence microscope and analySIS image capture software (magnification 100×).

2.10. DNA cell cycle analysis

Cell cycle distribution of the untreated/treated cells was measured by flow cytometry using propidium iodide staining as described previously (Wang et al., 2011).