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Immunomodulatory and anticancer activities of phenolics from *Garcinia mangostana* fruit pericarp

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ABSTRACT

The methanolic extract of *Garcinia mangostana* fruit pericarp was partitioned into butanol and water fractions in this work. Three major phenolics were purified and identified as P_1 [1,3,6,7-tetrahydroxy-2,8-(3-methyl-2-butenyl) xanthone], P_2 [1,3,6-trihydroxy-7-methoxy-2,8-(3-methyl-2-butenyl) xanthone] and P_3 (epicatechin). Strong antioxidant activities were detected for P_1–P_3. *In vitro* cell proliferation trials indicated that P_1 and P_3 exhibited good immunomodulatory activities when 7.5 μg/ml was used. Furthermore, P_1 and P_3 showed good cytotoxicities against human breast cancer cells (MCF-7) and human colon cancer cells (LOVO). P_1 exhibited the maximal cytotoxicity of 73.06% against MCF-7 cells and of 46.27% against LOVO cells when 62.5 μg/ml was used. The cytotoxicities of P_1, P_2, P_3 and paclitaxel against normal embryonic lung fibroblast cells (HELF) were in a decreasing order: paclitaxel > P_3 > P_1 > P_2. These results suggested that P_1 and P_3 could be used as a potential anticancer agent.

1. Introduction

Breast cancer is the most commonly diagnosed invasive cancer in women and is considered to be one of the leading causes of death due to cancer. Breast cancer is extremely difficult to treat due to several distinct classes of tumors that exhibit different treatment responses (Middleton, Kandaswami, & Theoharides, 2000). Epidemiological reports have indicated that consumption of food rich in phenolics is associated with a lower incidence of degenerative diseases. In many studies of dietary prevention of cancer, a model of breast cancer has been established for assessing the impact of a wide variety of phenolics for their efficacy in inhibiting cancer (Lopez-Otin & Diamandis, 1998; Zhang, Yang, Coburn, & Morris, 2005). A decreased breast cancer incidence has been proven to be associated with a high intake of genistein and moderate consumptions of red wine (Tomera, 1999; Wang, Chan, Chen, & Leung, 2005). Paclitaxel, a secondary metabolite of plant, is also found useful for breast cancer treatment (Hoffman & Shahidi, 2009). At present, cancer treatments by chemotherapeutic agents, surgery and radiation have not proved effectively against the low survival rate for most of the cancer patients. The development of new therapeutic approach against breast cancer still remains as one of the most challenging area in cancer research.

Many tropical plants have important biological activities with potential therapeutic applications. *Garcinia mangostana* Linn. (GM) is a climacteric fruit, named as 'the queen of fruits'. It is widely distributed in subtropical regions, and well-accepted by consumers due to the delicious taste. GM is often used for traditional medicines including the treatment of abdominal pain, dysentery, diarrhoea, suppuration, infected wound, leucorrhoea, chronic ulcer and gonorrhoea (Linseisen, Piller, Hermann, & Chang-Claude, 2004). Furthermore, GM exhibits an anti-inflammatory, anti-tumor and antioxidant activities, as well as anti-bacterial activity against *Staphylococcus aureus* and *Helicobacter pyroli* (Sakagami, Iinuma, Piyasena, & Dharmaratne, 2005; Williams, Ongsakul, Proudfoot, Croft, & Bellin, 1995).

Functional food has been reported as the top trend facing the food industry, which provides humans with necessary nutrients, preventing nutrition-related diseases and increasing physical and mental well-being of consumers (Menrad, 2003). Plant secondary metabolites having beneficial effect on human health are important source of functional food (Schieber, Stintzing, & Carle, 2001). It has been revealed that GM pericarp is a good source of xanthone, mangostin and phenolics (Farnsworth & Bunyapraphatsara, 1992). It might have a good potential to be used as functional food material. However, little information is known regarding the bioactivities of these constituents till now. The objectives of this work were to isolate and purify three major phenolics from GM pericarp, and to investigate their antioxidant, immunomodulatory and anticancer activities.

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2. Materials and methods

2.1. Plant materials

The GM fruit at the commercially mature stage was collected in the summer of 2006, from a supermarket in Guangzhou, China. The fruits were selected for the uniformity of size and color. They were peeled manually and then lyophilized.

2.2. Chemicals

Linoleic acid and ferrozine were purchased from Sigma, Aldrich (Steinheim, Germany). Vitamin E, vitamin C and ammonium thiocyanate were purchased from Nanjing Chemical Co. (Nanjing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), β-mercaptoethanol, L-glutamine, paclitaxel and rutin were purchased from Sigma Chemical Co (St Louis, MO, USA), while RPMI-1640 medium was purchased from GIBCO/BRL Invitrogen (Caithershurg, MD, USA). Fetal calf serum (FCS), penicillin and streptomycin were obtained from Zhongshan University (Guangzhou, China). All the other chemicals were of analytical grade.

2.3. Animals

Normal female BALB/c mice (2–3 month old) from Zhongshan University (Guangzhou, China) were housed under standard environmental conditions and fed with standard pellets and tap water.

2.4. Extraction and purification

According to the method of Yu, Zhao, Yang, Zhao, and Jiang (2007), the dried GM fruit pericarps were ground using a grinder and passed through a 60-mesh sieve. One hundred grams of pericarp powder were weighed precisely and extracted with 1000 ml of 70% aqueous methanol solution for 2 h at 50 °C. After filtering the extract through Whatman No. 1 paper (Beijing Weifang company, Shanghai, China), the residue was re-extracted and then filtered. Filtrates were combined and concentrated using a rotary evaporator at 40 °C. The methanolic extract (19.3 g) was re-dissolved in 100 ml of distilled water and then partitioned with 3 × 100 ml of butanol, which yielded butanol and aqueous fractions. The butanol fraction (4.75 g) was chromatographed on a silica gel column (Merck silica gel, mesh > 230) and then eluted with solvent systems of 50 ml of CH_2Cl_2/Me_2CO (10:1, v/v) and CH_2Cl_2/MeOH (10:1, v/v) to obtain fractions I and II. They were subjected to TLC analysis. TLC analysis was performed on a silica gel (Merck, Germany, Kieselgel 60 F254, 0.2 mm of thickness) using hexane/$CHCl_3$ as the mobile phase. Compounds were visualized by spraying the plates with 10% H_2SO_4 in ethanol under UV light (wavelengths at 254 and 365 nm). Fractions 1–17 were obtained from fraction I, while fractions 18–26 were from fraction II. Fractions 5–7 (1.02 g) were combined and rechromatographed through a silica gel column, followed by elution with hexane/EtOAc (5:1, v/v) to give P_2 (54 mg). Fractions 14–17 (1.73 g) were combined and rechromatographed through a silica gel column using hexane/$CHCl_3$ (1:3–7:3) as the eluent. Sub-fractions 1 and 2 were obtained after elution with 1:3 and 7:3 of hexane/$CHCl_3$, respectively. Subfraction 2 (0.77 g) was further subjected to Sephadex LH-20 and eluted with MeOH/H_2O (3:1) to yield P_1 (62 mg). Moreover, fractions 22–23 (0.74 g) were combined and further separated by a silica gel column, followed by eluted with CH_2Cl_2/MeOH (20:1) to yield two fractions (A and B). Fraction A (0.47 g) was rechromatographed on Sephadex LH-20 and eluted with MeOH/H_2O (3:1) to obtain P_3 (30 mg). The three compounds (P_1, P_2 and P_3) were collected and then freeze-dried. They were identified as 1,3,6,7-tetrahydroxy-2,8-(3-methyl-2-butenyl) xanthone (P_1), 1,3,6-trihydroxy-7-methoxy-2,8-(3-methyl-2-butenyl) xanthone (P_2), and epicatechin (P_3) by nuclear magnetic resonance spectrometry and mass spectrometry.

2.5. Determination of antioxidant activity with the ferric thiocyanate (FTC) method

The antioxidant activity of sample on inhibition of linoleic-acid peroxidation was determined by the thiocyanate method (Mitsuda, Yasumoto, & Iwani, 1996). A methanolic solution (2.5 ml) of each sample was mixed with 2.5 ml of 0.02 M linoleic-acid emulsion in phosphate buffer (pH 7.0). The reaction mixture was incubated at 37 °C to accelerate the oxidation process. Ethanol (75%, 9.7 ml) and ammonium thiocyanate (30%, 0.1 ml) were added to 0.1 ml of this solution. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% (w/v) hydrochloric acid to the reaction mixture, the peroxide value was determined by reading the absorbance at 500 nm for every 12 h until the absorbance of the blank (without sample) reached maximum. The solutions without sample were used as the control. The antioxidant activities of vitamins E and C as reference antioxidants were also assayed. The percent inhibition of lipid peroxidation of linoleic acid emulsion was calculated:

$$\text{Inhibition of lipid peroxidation } (\%) = (1 - A_1/A_0) \times 100\%,$$

where A_0 was the absorbance of the control, while A_1 was the absorbance in the presence of the samples. All the tests were carried out in triplicates.

2.6. Cell line and culture

Human breast cancer MCF-7 and human embryonic lung fibroblast (HELF) cell lines were obtained from the immune system analysis laboratory of Zhongshan University (Guangzhou, China). The cells were maintained in the RPMI-1640 complete medium in a humidified 5% CO_2 atmosphere at 37 °C.

Mice were killed by cervical dislocation and spleens were removed aseptically. Single cells were prepared by mincing spleen fragments and pressing them through a stainless 200-mesh screen in the RPMI complete medium (Fernandez et al., 1998). The RPMI-1640 complete medium was supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml). An amount of 1×10^7 cells was placed in a 16-mm well and incubated for 3 h in a humidified 5% CO_2 at 37 °C. The supernatant together with the non-adherent cells were collected by centrifugation at 630*g* for 10 min. The cell pellets were re-suspended in the RPMI complete medium and then adjusted to 1×10^6 cells/ml (Manosroi, Saraphanchotiwitthaya, & Manosroi, 2006).

2.7. In vitro proliferation assay

The proliferation stimulatory effects of phenolics of GM hull on the splenocytes and the cytotoxicity against both MCF-7 and HELF cells were evaluated by the MTT assay method described previously (Sun, Qin, & Pan, 2005). Briefly, twenty microliters of samples, paclitaxel and rutin at 0–500 μg/ml, which were dissolved in ethanol diluted with RPMI 1640 and then added in the wells, was added to 20 μl of cell suspension (1×10^6 cells/ml) and 40 μl of RPMI in a 96-well flat-bottom plate. Control cells were incubated in a medium containing an equivalent solvent amount without the test materials. The final solvent concentrations were in the range of 0.5% to 1.0%. This concentration level did not alter cell growth in this work. The plate was incubated at 37 °C in a humid-

ified 5% CO_2 atmosphere. After 44 h of incubation, MTT solution (5 mg/ml, 50 μl) was added to each well and then incubated for 4 h. The result showed that the incubation for 4 h was sufficient for cells to reduce MTT to formazan crystals (brown color). The supernatant was removed carefully by pipetting from wells without disturbing the attached cells and formazan crystals were solubilized by adding 200 μl of dimethyl sulphoxide (DSMO) to each well and shaken for 15 min. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Richmond, CA, USA), using wells without cells as control. The cytotoxicity (%) of samples against the proliferation of MCF-7 and HELF was calculated from the following formula: (A570 of control cells – A570 of treated cells)/A570 of control cells × 100%, while the stimulatory effect (%) of samples on the proliferation of splenocytes was calculated as (A570 of treated cells – A570 of control cells)/A570 of control cells × 100%. All the experiments were performed in triplicate.

2.8. Statistical analyses

All the data were expressed as means ± standard deviations (SD) of three replicated determinations. Statistical calculations by OriginPro Version 7.5 software (OriginLab Corporation, Northampton, MA, USA) were carried out. One way of variance analysis was applied for determining differences between results. Values of $P < 0.05$ were considered as significantly different.

3. Results and discussion

3.1. Assay of antioxidant activity with the ferric thiocyanate (FTC) method

Their lipid inhibitory abilities of three phenolic compounds were compared with selected standard antioxidants (BHA, Trolox and α-tocopherol), by the FTC method of measuring the amount of peroxide produced during the initial stage of oxidation. During linoleic-acid oxidation, peroxides are formed which can oxidize Fe^{2+}into Fe^{3+}, resulting in a maximum absorbance at 500 nm. Low absorbance value indicates a high level of antioxidant activity (Duh, Tu, & Yen,1999). As shown in Table 1, the samples and standard antioxidants exhibited good antioxidant activity. At 40 μg/ml, P_1 exhibited 83.2% of inhibition capacity against the peroxidation of linoleic acid, which was significantly ($P < 0.05$) higher than a-tocopherol (78.5%) and Trolox (56.2%). Otherwise, the antioxidant activities of BHA, P_2 and P_3 at the same concentration were 90.9%, 62.4% and 57.2%, respectively.

3.2. Immunomodulatory activity of phenolics

Splenocyte proliferation from female BALB/c mice species cultured in the presence of different samples was used to evaluate cell stimulatory effects on the splenocyte growth. As shown in Fig. 1, all the samples used in this work had good stimulatory effects. When the concentrations of epicatechin and P_1 were 7.5 μg/ml, the lowest concentration used in this study, significantly ($P < 0.05$) stimulatory effects on splenocyte proliferation were observed. The concentrations of epicatechin, P_1 and P_2 to obtain 50% of stimulatory effect were 84, 62 and 376 μg/ml, respectively. There was a 265.7% of stimulatory effect when P_1 (250 μg/ml) was used. P_2 had a significantly ($P < 0.05$) stimulatory effects when the concentration used was up to 125 μg/ml. It exhibited a little weaker stimulatory effect when comparing with epicatechin and P_1. The reference, rutin, had a significantly ($P < 0.05$) stimulatory effect when the concentration was up to 50 μg/ml. Previous studies indicated that many plant extracts could stimulate the proliferation of splenocyte (Kong, Hu, Rui, Wang, & Li, 2004). Propolis flavones could also promote the lymphocyte proliferation (Lin, Chiang, & Lin, 2005). The phenolics in these substances are responsible for the stimulatory effect. In the present study, strong stimulatory effects of these three compounds on splenocyte proliferation further confirmed the good bioactivity of phenolics. It also indicated that the extract of GM hull had the potential to be used as an additional drug ingredient against some inflammations or other diseases.

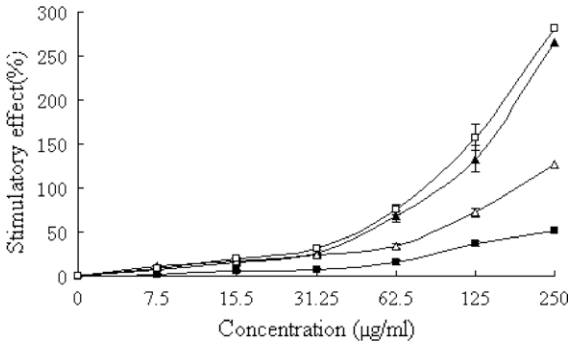

Fig. 1. The stimulatory effects of P_1 (□), P_2 (▯), P_3 (▲),and rutin (■) on the proliferation of splenocytes. Results were represented as means ± SD ($n = 3$).

3.3. Anticancer activity of phenolics

Anticancer activities of P_1, P_2 and P_3 were evaluated in terms of MCF-7 and LOVO proliferation. Figs. 2 and 3 present the cytotoxicity on MCF-7 and LOVO of P_1, P_2, P_3 and paclitaxel at various concentrations after treatment for 72 h. As shown in Figs. 2 and 3, P_1 and P_3 were highly active in the MCF-7 cell and LOVO cytotoxicity assays. When the concentrations were up to 7.5 μg/ml, the lowest concentration used in this study, significantly ($P < 0.05$) cytotoxicities against MCF-7 and LOVO cells was observed for P_1 and P_3. The inhibition effect was enhanced with the increased concentration

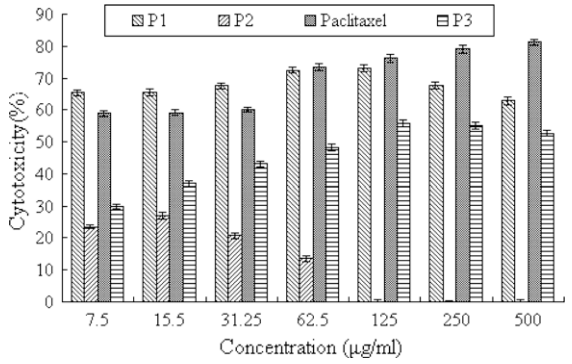

Fig. 2. Cytotoxicity of P_1, P_2, P_3 and paclitaxel against human breast cancer MCF-7 cells. Data were represented as means ± SD ($n = 3$).

Table 1
Total antioxidant activities of P_1, P_2, P_3 and standard antioxidants (α-tocopherol, BHA and trolox)*.

Samples	Inhibition of lipid peroxidation (%)	Sample	Inhibition of lipid peroxidation (%)
P_1	82.2 ± 0.1	Trolox	56.2 ± 1.3
P_2	62.4 ± 1.8	a-Tocopherol	78.5 ± 2.0
P_3	57.2 ± 0.3	BHA	90.9 ± 0.8

* The concentration of all the tested samples was 40 μg/ml. P_1, 1,3,6,7-tetrahydroxy-2,8-(3-methyl-2-butenyl) xanthone; P_2, 1,3,6-trihydroxy-7-methoxy-2,8-(3-methyl-2-butenyl) xanthone; P_3, epicatechin.

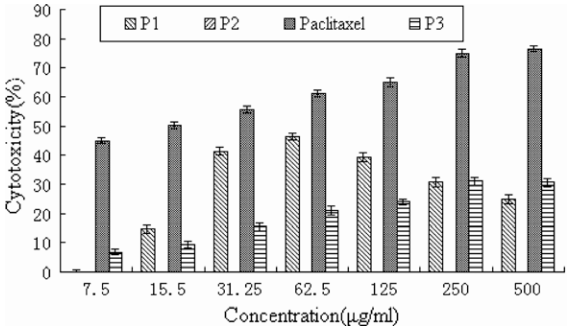

Fig. 3. Cytotoxicity of P_1, P_2, P_3 and paclitaxel against LOVO cells. Data were represented as means ± SD (n = 3).

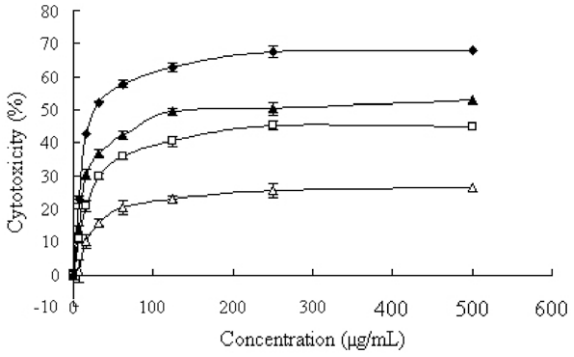

Fig. 4. Cytotoxicity of P_1 (□), P_2 (△), P_3 (▲) and paclitaxel (♦) against HELF cells. Data were represented as means ± SD (n = 3).

and a dose-dependent effect was observed on cell viability and proliferation in the tested range. P_1 exhibited the maximal cytotoxicity of 73.06% against MCF-7 cells and of 46.27% against LOVO cells when 62.5 μg/ml was used, which was significantly ($P < 0.05$) higher than P_2 (13.65%) and P_3 (48.29%) against MCF-7. P_2 had a weak cytotoxicity against MCF-7 cells. Moreover, no cytotoxicity was observed against LOVO cells at all the tested concentrations for P_2. Paclitaxel, being approved by FDA of USA as an anticancer drug, has been confirmed to have good and broad-spectrum anti-cancer activities against breast, bladder and colon cancers (Gonçalves et al., 2000; Lück & Roché, 2002; Vaughn, 2000). Therefore, it was used as a reference in this work. The cell viability trials indicated that paclitaxel had a strong cytotoxicity against MCF-7, LOVO and HELF cells. When the concentration of paclitaxel used in this study was up to 7.5 μg/ml, the cytotoxicity against MCF-7 cells was beyond 50%. The cytotoxicities against MCF-7, LOVO and HELF were calculated to be 81.20%, 76.41% and 62.4%, respectively, when 7.5 μg/ml paclitaxel was used. These results suggested that paclitaxel had good inhibitory effect on cancer cell proliferation. However, it could also destroy normal cell seriously.

Fig. 4 presents the degree of cytotoxicity of various concentrations of P_1, P_2, P_3 and paclitaxel against HELF. As shown in Fig. 4, all the samples exhibited a different cytotoxicity to HELF cells and a dose-dependent effect on cell viability and proliferation at all the concentrations tested. The cytotoxicities of P_1, P_2, P_3 and reference anticancer agent were in a decreasing order: paclitaxel > P_3 > P_1 > P_2. These results suggested P_1, P_2 and P_3 might be used as a potential anticancer agent.

A number of studies have suggested that phenolics might play a protective role in preventing breast cancer (Cooray, Janvilisri, van Veen, Hladky, & Barrand, 2004; Marchand, 2002). In many reports, isoflavonoids have a significant effect on the prevention of breast cancer. However, information regarding the effect of phenolics on the breast cancer was relatively limited. Zhao et al. (2007) have indicated the good anti-breast cancer activity of epicatechin, procyanidin B_2 and B_4 which are purified from litchi pericarp. Rodgers and Grant (1998) have suggested that the possible mechanisms responsible for anti-breast cancer prevention by flavanol and flavonol which might involve in xenobiotic metabolising enzymes that alter metabolic activation of potential carcinogens. Birt, Hendrich, and Wang (2001) have mentioned that some phenolics can alter hormone production and inhibit aromatase to prevent the development of breast cancer cells. These hypothesis might explain the inhibitory mechanisms of P_1, P_2 and P_3 against the proliferation of breast cancer cells.

4. Conclusions

Significant amounts of phenolics were found in the GM hull. Three major phenolics (P_1, P_2 and P_3) were obtained. Their lipid inhibitory abilities were determined and compared with selected standard antioxidants, BHA, Trolox and α-tocopherol. By *in vitro* trials of mouse splenocyte proliferation, significantly stimulatory effects were found for these three phenolics. When the concentrations of P_1, P_2 and P_3 were 7.5 μg/ml, the lowest concentration used in this study, significantly ($P < 0.05$) stimulatory effect on splenocyte proliferation was observed, which was significantly ($P < 0.05$) higher than that of the reference, rutin. The assay of anti-breast cancer activities suggested that P_1 and P_3 had higher cytotoxicities against human breast cancer cell MCF-7 and human intestines cancer cell LOVO than P_2. A dose-dependent effect on cell cytotoxicity was observed for P_1, P_2, P_3 and paclitaxel. They were in a decreasing order: paclitaxel > P_3 > P_1 > P_2. It suggested that P_1 and P_3 can be employed as the potential components of anti-breast cancer drugs. However, further analysis on the precise mechanism of anticancer and immunostimulatory activities of these compounds are worthy to be done in the future work.

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