Anticancer activity of litchi fruit pericarp extract against human breast cancer in vitro and in vivo

Xiujie Wang a,⁎, Shulan Yuan a, Jing Wang a, Ping Lin a, Guanjian Liu b, Yanrong Lu a, Jie Zhang a, Wendong Wang c, Yuquan Wei d,⁎

a Division of Experimental Oncology, National Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, P.R. China
b Department of Clinical Epidemiology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, P.R. China
c Department of Pathology of Public Health School, Sichuan University, Chengdu 610041, Sichuan Province, P.R. China
d Division of Biotherapy, National Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, P.R. China

Received 8 October 2005; revised 23 January 2006; accepted 8 February 2006
Available online 23 March 2006

Abstract

Litchi fruit pericarp (LFP) extract contains significant amounts of polyphenolic compounds and exhibits powerful antioxidative activity against fat oxidation in vitro. The purpose of this study is to confirm the anticancer activity of LFP extract on human breast cancer in vitro and in vivo, and to elucidate the mechanism of its activity. Human breast cancer cells were tested in vitro for cytotoxicity, colony formation inhibition, BrdU incorporation, and gene expression profiling after treatment with LFP extract. Seven nude mice bearing human breast infiltrating duct carcinoma orthotopically were tested for its anticancer activity and expression of caspase-3 in vivo by oral administration of 0.3% (0.3 mg/ml) of LFP water-soluble crude ethanolic extract (CEE) for 10 weeks. LFP extract demonstrated a dose- and time-dependent inhibitory effect on cell growth (IC50 = 80 μg/ml), and it significantly inhibited colony formation and BrdU incorporation of human breast cancer cells. Oligonucleotide microarray analysis identified 41 (1.22%) up-regulated and 129 (3.84%) down-regulated genes after LFP water-soluble CEE treatment; the predominantly up-regulated genes were involved in various biological functions including cell cycle regulation and cell proliferation, apoptosis, signal transduction and transcriptional regulation, and extracellular matrix/adhesion molecules; and down-regulated genes were mainly associated with adhesion, invasion, and malignancy of cancer cells. A 40.70% tumor mass volume reduction and significant increase of casepase-3 protein expression were observed in vivo experiment. The findings in this study suggested that LFP extract might have potential anticancer activity on both ER positive and negative breast cancers, which could be attributed, in part, to its DNA damage effect, proliferating inhibition and apoptosis induction of cancer cells through up-regulation and down-regulation of multiple genes involved in cell cycle regulation and cell proliferation, apoptosis, signal transduction and transcriptional regulation, motility and invasiveness of cancer cells; ADP-ribose transferase (NAD+; poly(ADP-ribose)) polymerase-like 1 (ADPRTL1), Cytochrome P450, subfamily I (CYP1A1) and Hyaluronan-mediated motility receptor (HMMR) might be the main molecular targets at which LFP water-soluble CEE acted.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Litchi fruit pericarp extract; Anticancer activity; Breast cancer; Growth inhibition; Apoptosis

Introduction

Breast cancer is one of the main life-threatening diseases that a woman may have to face during her lifetime (Angelopoulos et al., 2004). The increasing incidence of breast neoplasia reported over the last a few decades has led to development of new anticancer drugs, drug combinations, and chemotherapy strategies by methodical and scientific exploration of enormous pool of synthetic, biological, and...
natural products (Mukherjee et al., 2001). In light of the continuing need for effective anticancer agents, and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly being considered as sources of anticancer drugs (Ferguson et al., 2004); there is a large amount of scientific evidence showing that fruits and vegetables lower the risk of cancer (Chen et al., 2004), and medicinal plants constitute the main source of new pharmaceuticals and healthcare products, including medications for ethnoveterinary medicine (Ivanova et al., 2005). Recently, cancer chemoprevention with strategies using foods and medicinal herbs has been regarded as one of the most visible fields for cancer control (Jo et al., 2004); however, whether fruit, vegetable, and antioxidant micronutrient consumption is associated with a reduction in breast cancer incidence remains unresolved (Gaudet et al., 2004).

Epidemiological studies suggested that antioxidant supplements might reduce the risk of breast cancer recurrence or breast cancer-related mortality (Fleischauer et al., 2003), and consuming food and beverages rich in polyphenols (e.g., catechins, flavones, and anthocyanines) is associated with a lower incidence of cancers (Naasani et al., 2003). Experimental investigations demonstrated that many naturally occurring agents and plant extracts have shown antioxidant and anticancer potential in a variety of bioassay systems and animal models, having relevance to human disease (Aziz et al., 2003), e.g., Crude methanolic extract (CME) from the pericarp of *Garcinia mangostana* (family Guttiferae) has antiproliferative, apoptotic, and antioxidant activities against human breast cancer cell line in vitro (Primchani et al., 2004). The antioxidant and anticancer activity of the extracts from medical plants and herbs was associated with their components of phenolic compounds; the major types of phenolic compounds included phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids (Cai et al., 2004).

*Litchi* (*Litchi chinensis*, Sapindaceae) is a tree that originates from China and is cultivated for its sweet fruits all over the world in warm climates (Gontier et al., 2000). Pharmacological studies showed the petroleum ether extract of leaves of the plant *Litchi chinensis Gaertn* possess anti-inflammatory, analgesic, and antipyretic activity without toxicity (Besra et al., 1996).

*Litchi* fruit pericarp (LFP) contains significant amounts of polyphenolic compounds, the principal characteristic of the polyphenolic compounds is their ortho-diphenolic structure, which gives them high oxidability; the major components of fresh LFP extract were condensed tannins (polymeric proanthocyanidins), epicatechin, and procyanidin A2 (Sarni-Manchado et al., 2000). The main components of mature and premature LFP extract were phenolic compound and flavonoids, which exhibited powerful antioxidative activity against fat oxidation in vitro (Zheng et al., 2003).

Based on the main components of LFP extract and its antioxidant properties (Sarni-Manchado et al., 2000; Zheng et al., 2003), we hypothesized that LFP extract may have anticancer activities against some cancer cell lines in vitro and animal models in vivo. However, there are no such reports. To confirm this hypothesis, the inhibitory effect of LFP extract on the growth of human breast cancer cells in vitro and the growth of human breast infiltrating ductal carcinoma (IDC) in vivo, and the mechanism of its activity were investigated in this experimental study.

### Materials and methods

**Reagents.** RPMI-1640 was purchased from GIBCO/BRL Invitrogen (Catterarb, MD). Fetal bovine serum (FBS) was purchased from Huxi Biology Institute (Chengdu, People’s Republic of China). Trypsin, methylene-zolylidiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from the Sino-American Biotechnology Company of Beijing (Beijing, People’s Republic of China). 5-Bromo-2-deoxy-uridine (BrdU) was purchased from Roche (Nutley, NJ), and S-P immunohistochemical staining kit (SP9001) was purchased from Beijing Zhongshan Biological Technology Ltd. (Beijing, People’s Republic of China). All of other chemicals and reagents were obtained from Sigma (St. Louis, MO).

**Preparation of extracts.** Mature Baila litchi (*L. chinensis*, Sapindaceae in Guangdong, China) fruit pericarp was collected, dried at room temperature naturally, and powdered. The powdered material (100 g) was extracted with 95% ethanol (800 ml, two times) for 48 h at room temperature, blended with magnetic force stirrer continuously. The extracts were filtered and concentrated to remove the solvent at 75 °C for 4 h and freeze dried, and more than 22.0 g of crude ethanolic extract (CEE) was yielded eventually, the percentage yield was higher than 22.0%. Of the extract, 60% was water-soluble, 40% was soluble in ethanol and acetone.

**Cell line and culture.** Human breast cancer MCF-7 (GDC055) and MDA-MB-231 (HTB-26) cell lines were obtained from China Center for Type Culture Collection (Nanjing, People’s Republic of China). The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 50 μg/ml CO₂ at 37 °C.

**In vitro assay for cytotoxic activity (MTT assay).** The cytotoxicity of LFP water-soluble CEE on both MCF-7 and MDA-MB-231 cells was determined by the MTT assay (Selvakumaran et al., 2003). Cells (3 × 10⁵/well) were plated in 100 μl of medium/well in 96-well plates (Costar Corning, Rochester, NY). After incubation overnight, LFP water-soluble CEE was added in various concentrations (20, 40, 80, 160, 320 μg/ml) without cytotoxicity to human normal liver cell line L-02 (reported in elsewhere); 5 wells were included in each concentration. After treatment with LFP water-soluble CEE for 1, 2, 3, 4, and 5 days, 20 μl of 5 mg/ml MTT (pH 4.7) was added well and cultivated for another 4 h, the supernatant fluid was removed, 100 μl DMSO was added per well and shaken for 15 min. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Caithershurg, MD). Fetal bovine serum (FBS) was purchased from Huaxi Biology Institute (Chengdu, People’s Republic of China). Trypsin, methylthiouracil (MTT) was purchased from the Sino-American Biotechnology Company of Beijing (Beijing, People’s Republic of China), Trypsin, methylthiouracil, and acetone.

**Clonogenic survival determination.** MCF-7 and MDA-MB-231 cells were assayed for colony-forming ability by replaying them in specified numbers (300–400/well) in 6-well plates and treated with 40, 80, and 160 μg/ml of LFP water-soluble CEE, respectively. After 12 days of incubation, the cells were stained with 0.5% crystal violet in absolute ethanol and colonies with > 50 cells were counted under dissection microscope.

**BrdU incorporation in vitro.** Cells were seeded onto glass coverslips at an initial density of 4.0 × 10⁵ cells/cm² and allowed to grow for 12 h, and then treated with 100 μg/ml of LFP water-soluble CEE for 48 h. The cells were incubated with BrdU (20 μg/ml) for 12 h. At the appropriate time, the cells were fixed in methanol at −20 °C for 1–2 min, allowed to air dry, then stored at −20 °C until all coverslips were ready for processing. The cells were rehydrated in PBS for 5 min followed by immersion in 2 N HCl for 1 h at room temperature. The cells were incubated in 0.1 M borate buffer (pH 8.5, 0.1 M boric acid,
25 mM Na₂B₄O₇, and 75 mM NaCl) twice for 5 min each, followed by three times of 10 min washes in PBS. Next, cells were incubated with BrdU mouse monoclonal antibody (11B5, Zymed Laboratories, USA) at a dilution of 1:100, overnight at 4 °C, with biotyled second antibody for 20 min, and with streptavidin/peroxidase at 30 min at room temperature. Subsequently, the sections were subjected to color reaction with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.005% H₂O₂ in PBS (pH 7.4), and were counterstained with hematoxylin lightly. A percentage of BrdU-labeled cells were determined by counting several fields of 200 cells (in areas of the slide containing the highest labeling cells) (Thor et al., 1999).

Oligonucleotide microarray analysis. Human breast cancer MCF-7 cells were treated with 100 μg/ml of LFP water-soluble CEE for a required time (48 h). Total RNA was extracted from the treated and untreated cells using the Qiagen (Valencia City, CA) RNeasy Minikit. cDNA was synthesized from total RNA using cDNA Synthesis System (Roche), cRNA probes were created from cDNA with cy3-UTP using MEGAscrip T7 Kit (Ambion, Austin, TX) and hybridized to HO4 ExpressChip (Mergen, San Francisco, CA) containing 3360 genes (www.mergen.ltd.com), according to the manufacturer’s instructions. After a series of washes, the hybridized slides were scanned with GMS418 Array Scanner (Affymatrix, Santa Clara, CA). The signal intensity of each pair of spots was quantified using ImaGene4.0 software (BioDiscovery, El Segundo, CA) by subtracting the local regional background intensity from each spot; the two slides were normalized to each other by using the signal of global normalization gene (Glyceraldehyde-3-phosphate dehydrogenase, GAPD) provided by the manufacturer. Fold changes were calculated and expressed relative to untreated control sample for each pair of spots; the results were confirmed by duplicate spots of cRNA.

RT-PCR. To verify the microarray results, the representative up-regulated (ADPRTL1, CYP1A1) and down-regulated (HMMR) genes in response to LFP water-soluble CEE treatment were confirmed experimentally by semi-quantitative RT-PCR (Morandi et al., 2006). The primers for each gene were designed with Primers Express software (Applied Biosystems) according to ExpressChip Gene Database of the manufacturer (Mergen, San Francisco, CA). Primer sequences and the length of amplified products were as follows:

ADPRTL (accession number: AF057160, product size: 477 bp)
Fw 5′-gaagaacttaggttagcgtcc-3′ Rew 5′-ccctggatgaaagcctcatc-3′
CYP1A1 (accession number: K03191, product size: 486 bp)
Fw 5′-acactctactctacctctctc-3′ Rew 5′-agtgctccttgaccatcttctg-3′
HMMR (accession number: U29343, product size: 390 bp)
Fw 5′-gataactatgtctgtctgccgtc-3′ Rew 5′-cttagccatcatacccctcatc-3′
β-actin (accession number: BC013380, product size: 695 bp)
Fw 5′-ccacccacactctagc-3′ Rew 5′-ggatgtcattcttcagtccgt-3′

Total RNA was isolated from the control and treated cells as described above. One microgram of total RNA was retro-transcribed to cDNA using 100 Units of Rever Tra Ace (Toyobo, Japan), 10 μM Oligo(dT) primer, and 10 ml dNTP mix. PCR amplification was performed with Taq PCR MasterMix (Tiangen Biotech, Beijing, People’s Republic of China) using the following conditions: denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C, and elongation for 45 s at 72 °C for 30 cycles. PCR products were analyzed on 1.5% agarose gel and visualized with ethidium bromide. Image were acquired and quantified using the ChemiDocTM XRS (Bio-Rad).

In vivo tumor growth inhibition study. To confirm the anticancer activity of LFP water-soluble CEE on human breast IDC, an in vivo experiment was carried out using an ER negative human breast IDC-xenografted animal model (Chen et al., 2003). Thirteen 6-week-old female nude mice (Experimental Animal Center, Sichuan University) were xenotransplanted with human breast infiltrating duct carcinoma F35 orthotopically (Chen et al., 2003). The procedures involving animals and their care were conducted in accordance with institutional guidelines for Laboratory Animal Care of Experimental Animal Center, Sichuan University. At 2 weeks, tumors reached 50 mm³ in volume, the tumor bearing mice were randomized into two experimental groups. The treatment group animals (n = 7) were fed with 0.3% (0.3 mg/ml) of LFP water-soluble CEE through drink water ad libitum for 10 weeks, the control animals (n = 6) were without treatment. Mice were weighted, and tumor volume was assessed by measuring two perpendicular dimensions (long and short) using a caliper and calculated using the formula (a × b³) / 2, where a is the larger and b is the smaller dimension of the tumor (Zhang et al., 2003).

Immunohistochemical analysis of tumors for caspase-3 protein expression. Tumor samples from mice were fixed in 4% paraformaldehyde for 24 h and processed conventionally. Caspase-3 protein expressions were detected immunohistochemically with SP kit (Zymed); briefly, tissue sections were de-paraffinized and rehydrated through graded alcohols. Antigen retrieval was performed by microwave oven heating in 0.1 mM citrate buffer (pH 6). Then, endogenous peroxidase activity was blocked with 0.3% H₂O₂, and after treatment with normal goat serum, the tissue sections were incubated with caspase-3 rabbit polyclonal antibody (Boster Biotechnology, Wuhan, People’s Republic of China) at a dilution of 1:100, overnight at 4 °C, with biotyled second antibody 20 min, and with streptavidin/peroxidase 30 min at room temperature. Subsequently, the sections were subjected to color reaction with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.005% H₂O₂ in PBS (pH 7.4) and were counterstained with hematoxylin lightly. Caspase-3 positive cells were determined by counting several fields of 200 cells, Caspase-3 labeling index (LI) was calculated using the formula Caspase-3 labeling index (LI) = Number of activated caspase-3-positive cells / Total number of nuclei × 100% (Duan et al., 2003).

Statistical analysis. The statistical significance of difference between control and LFP-extract-treated groups was determined by one-way ANOVA followed by Tukey test for multiple comparisons. Dunnett’s t tests (2-sided) were employed, as needed, and result was considered significant at P < 0.05.

Results

Cytotoxic activity of LFP water-soluble CEE against human breast cancer cells

LFP water-soluble CEE showed a dose- and time-dependent inhibitory effect on the growth of MCF-7 and MDA-MB-231 breast cancer cells (P < 0.05). IC₅₀ was 80 μM/ml, and the maximal inhibition of cell growth (>80%) was obtained at 320 μg/ml. The result of cytotoxic activity of LFP water-soluble CEE against human breast cancer cells is shown in Fig. 1.

Inhibition of colony formation

Untreated MCF-7 and MDA-MB-231 cells produced 235 ± 16 and 205 ± 12 colonies, respectively; the colony numbers of MCF-7 cells were suppressed to 145 ± 20 (P < 0.05), 70 ± 13 (P < 0.01), and 28 ± 9 (P < 0.01) with 40, 80, and 160 μg/ml of LFP water-soluble CEE treatment, respectively; the colony numbers of MDA-MB-231 cells were suppressed to 118 ± 16 (P < 0.05), 62 ± 11 (P < 0.01), and 21 ± 7 (P < 0.01) after the treatment of 40, 80, and 160 μg/ml of LFP water-soluble CEE, respectively; and a dose-dependent colony-forming inhibition effect on both cell lines were observed (Fig. 2).

Inhibition of cell proliferation

The inhibitory effect of LFP water-soluble CEE on MCF-7 cells was further confirmed using BrdU incorporation into the
untreated and treated (100 μg/ml) breast cancer cells in vitro (Fig. 3). BrdU-labeled cells in LFP water-soluble CEE-treated cells were 15.20 ± 1.30%, and lower than that in untreated controls (33.50 ± 2.18%), a statistical significance was found ($P < 0.05$).

**Change of gene expression profile**

Out of 3360 genes studied, 170 differential genes were identified from oligonucleotide microarray in which gene expression was increased or decreased more than 2-fold in cells treated with 100 μg/ml of LFP water-soluble CEE compared with control cells cultured under identical condition without treatment; 41(1.22%) were up-regulated (Table 1) and 129 (3.84%) were down-regulated (Table 2). Eight genes showed more than 3-fold up-regulation, and 38 showed more than 3-fold down-regulation, two genes were up-regulated for more than 5-fold, i.e., Cytochrome P450, subfamily IIA polypeptide 3 (CYP3A3), were down-regulated for more than 5-fold (Table 2, Fig. 4). The range of fold regulation varied widely with ADP-ribosyltransferase (ADPRTL1) exhibiting the maximum up-regulation (29.25-fold) and Hyaluronan-mediated motility receptor (RHAMM) exhibiting the maximum down-regulation (~15.48-fold).

**Confirmation of the representative genes by RT-PCR**

To assess the reliability of the microarray results, 3 representative genes with increased or decreased expression were analyzed by semi-quantitative RT-PCR using β-actin as the reference gene to normalize expression data. A good qualitative correlation was observed in the results obtained by the two techniques, with all 3 genes tested showing similar trends; however, quantitative differences in regulation are present (Fig. 5).
Inhibition of tumor growth in human breast IDC-xenografted nude mice

The average tumor volume at the end of experiment was 738.8 ± 328.11 mm³ in the treated mice and 1232.8 ± 424.65 mm³ in the control mice; tumor growth inhibitory rate was 40.70% \( (P < 0.05) \). The tumor volume in the treated mice increased 8.80 mm³ everyday, 1.67 times slower than that in controls (14.68 mm³). The experimental finding showed that there is a significant inhibition of tumor growth in the treated mice as compared with the control mice (Fig. 6). No evidence of drug-related toxicity was identified in the treated animals by comparing the body weight increase, histopathological changes of major organs, and blood biochemistry analysis of both group animals (data not shown).

Increase of caspase-3 protein expression in tumors

The antibody specific for activated caspase-3 selectively labeled the cytoplasm of cells that had morphology consistent with apoptosis, as well as the cytoplasm of some morphologically healthy-looking cells; occasional nuclear staining was observed (Fig. 7). Caspase-3 labeling index (LI) in the tumor tissue of the treated mice was 29.45 ± 5.81% significantly higher than that (5.86 ± 2.20%) in the control mice \( (P < 0.05) \).

Discussion

*Litchi* fruit pericarp (LFP) contains significant amounts of polyphenolic compounds, including condensed tannins (polymeric proanthocyanidins), epicatechin, procyanidin A2, and flavonoids (Sarni-Manchado et al., 2000; Zheng et al., 2003). LFP extract has been shown to have antioxidant properties against fat oxidation in vitro (Zheng et al., 2003), but no other bioactivity of LFP extract was reported. In vitro cytotoxicity assay, some plant extract exhibited potential antioxidant and anticancer properties (Primchanien et al., 2004; Ju et al., 2004) and inhibited proliferation of multiple human cancer cells (Magiatis et al., 2001; Kazi et al., 2003). Animal studies have demonstrated that a dietary polyphenol...
Table 2 (continued)

<table>
<thead>
<tr>
<th>Gene description</th>
<th>UniGeneSymbol</th>
<th>UniGeneID</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein kinase C binding protein 1</td>
<td>PRKCBP1</td>
<td>Hs.75871</td>
<td>−4.26</td>
</tr>
<tr>
<td>Tousled-like kinase 2</td>
<td>TLK2</td>
<td>Hs.57553</td>
<td>−3.04</td>
</tr>
<tr>
<td>Polo (Drosophila)-like kinase</td>
<td>PLK</td>
<td>Hs.77597</td>
<td>−3.74</td>
</tr>
<tr>
<td>Ribosomal protein S6 kinase, 90 kDa, polypeptide 3</td>
<td>RPS6KA3</td>
<td>Hs.173965</td>
<td>−3.58</td>
</tr>
<tr>
<td>Hyaluronan-mediated motility receptor (RHAMM) A disintegrin and metalloproteinase domain 9 (melrin gamma)</td>
<td>HMMR</td>
<td>Hs.72550</td>
<td>−15.48</td>
</tr>
<tr>
<td>A disintegrin and metalloproteinase domain 9 (melrin gamma)</td>
<td>ADAM9</td>
<td>Hs.2442</td>
<td>−5.09</td>
</tr>
<tr>
<td>Tousled-like kinase 1</td>
<td>TLK1</td>
<td>Hs.18895</td>
<td>−3.37</td>
</tr>
<tr>
<td>Cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 3</td>
<td>CYP3A3</td>
<td>Hs.329704</td>
<td>−5.05</td>
</tr>
<tr>
<td>Splicing factor, arginine/serine-rich 11</td>
<td>SFRS11</td>
<td>Hs.11482</td>
<td>−3.05</td>
</tr>
<tr>
<td>ATP-binding cassette, subfamily E (OABP), member 1</td>
<td>ABCE1</td>
<td>Hs.12013</td>
<td>−4.40</td>
</tr>
</tbody>
</table>

known as tannic acid (TA) exhibits anticarcinogenic activity in chemically induced cancers (Gali-Muhtasib et al., 2001). Based on the potent antioxidant activity of LFP extract and its major components of polyphenolic compounds and flavonoids (Sarni-Manchado et al., 2000; Zheng et al., 2003), it could be assumed that LFP extract might have anticancer activity against some cancer cells and/or animal models. The present study confirmed that LFP water-soluble CEE has strong dose- and time-dependent anticancer activity against human breast cancer cells (Fig. 1), with the IC50 at 80 μg/ml, and inhibited the colony growth potential of cancer cells in a dose-dependent manner (Fig. 2).

Cultured cancer cells are valuable reagents for rapid screening of potential anticancer agents as well as for elucidation of mechanism of their activity. Prior to clinical trials, however, it is essential that the in vivo efficacy of potential anticancer agents is determined in a suitable animal model (Singh et al., 2004). It was well known that human breast cancer cell line MCF-7 is ER positive, but approximately one-third of breast cancers are ER negative, carrying a worse prognosis than the positive ones (Swami et al., 2003). Therefore, it is important to discover new agents that are effective on both ER positive and negative breast cancers. The results of this experimental study demonstrated that LFP water-soluble CEE not only had powerful inhibitory effect on the proliferation of both ER positive and ER negative human breast cells in vitro but also significantly inhibited ER negative human breast infiltrating duct carcinoma growth in vivo, without any untoward toxicity. These findings suggested that LFP water-soluble CEE might have potential anticancer activity on human breast cancer. There was no difference in antitumor activity of LFP water-soluble CEE in either presence or absence of estrogen, its action might be independent from estrogen-
regulatory mechanism, but further more experimental investigation is needed to confirm its antitumor activity against ER positive breast cancer model in vivo.

The anticancer activity of LFP water-soluble CEE might result, at least in part, from inhibition of DNA synthesis, proliferation, as well as apoptosis induction of cancer cells.

Fig. 4. Oligonucleotide microarray analysis. Human breast cancer MCF-7 cells were treated with 100 μg/ml of LFP water-soluble CEE for 72 h, total RNA was extracted from the treated and untreated cells, cRNA probes were created, and hybridized to HO4 ExpressChip, the hybridized slides were scanned with GMS418 Array Scanner. (A) Gene expression profiling of the untreated cells; (B) gene expression profiling of LFP water-soluble CEE-treated cells; (C) the histogram shows the up-regulation and down-regulation of more than 5-fold.

Fig. 5. Comparison of microarray results with those determined by RT-PCR. (A) Gel image of 3 representative genes confirmed by RT-PCR. The amplified fragments were quantified using β-actin for the normalization. (B) The correlation coefficient is shown in the graph.
Inhibition of DNA synthesis and proliferation of cancer cells were verified by its ability to reduce BrdU incorporation into cancer cells that correlates with decreased cell proliferation (Milosevic et al., 2002) after treatment with LFP water-soluble CEE, BrdU-labeled cells in the treated cells reduced significantly ($P < 0.05$), which indicated that LFP water-soluble CEE inhibited proliferation of cancer cells. Apoptosis induction was additionally determined by increased caspase-3 protein

Fig. 6. Inhibition of tumor growth in nude mice xenografted with human breast IDC by LFP water-soluble CEE. Two weeks after orthotopical inoculation of human breast IDC, the mice were randomly divided into two groups, six mice in control and seven mice in treatment group. In the treated mice, LFP water-soluble CEE (0.3 mg/ml) was given through drink water ad libitum for 10 weeks. A significant reduction of tumor volume was observed in the treated mice. (A) Tumor bearing nude mice of the untreated group (up row) and the treated group (down row); (B) tumor masses of the untreated group (up row) and the treated group (down row); (C) the histogram shows that there was a significant difference of tumor volumes between the untreated and treated mice, the single asterisk (*) indicates a significant difference from the control, one-way ANOVA, Tukey’s test ($P < 0.05$).

Fig. 7. Increase of caspase-3 protein expression by LFP water-soluble CEE. Caspase-3 protein expressions of tumor tissues derived from the untreated and treated mice were detected through immunohistochemical staining. (A) Representative photographs of caspase-3 protein expression of the untreated mouse tumor; (B) the treated mouse tumor; (C) the histogram shows that there was a significant increase of caspase-3 protein expression of LFP water-soluble CEE-treated mouse tumors, the single asterisk (*) indicates a significant difference from the control, one-way ANOVA, Tukey’s test ($P < 0.05$).
expression in tumors of the animals treated with LFP water-soluble CEE.

The anticancer mechanism of LFP extract was further investigated by oligonucleotide microarray; it was demonstrated that non-toxic dose of LFP water-soluble CEE affected the gene expression profile of cancer cells by up-regulation of 41 genes (1.22%) and down-regulation of 129 genes (3.84%), involved in various biological functions including cell cycle regulation and cell proliferation, apoptosis, signal transduction and transcriptional regulation, and extracellular matrix/adhesion molecules, etc. The range of fold regulation varied widely with ADPRTL1 exhibiting the maximum up-regulation (29.25-fold) and RHAMM exhibiting the maximum down-regulation (−15.48-fold).

The predominantly up- and down-regulated genes were mainly associated with apoptosis, cell–cell and cell–matrix interactions, motility, and invasiveness of cancer cells. CYP1A1, encoding Cytochrome P450, subfamily I, up-regulation is paralleled with cytotoxicity of aminoflavone in sensitive breast tumor cells (Loaiza-Perez et al., 2004a, b), resveratrol-induced differentiation and apoptosis of medulloblastoma cells (Liu et al., 2004), antiproliferative activity and apoptosis induction of aminoflavone analogue (AF) of human tumor renal cell carcinoma lines (Loaiza-Perez et al., 2004a, b), and CYP1A1 activation led to cytotoxicity of anticancer agent through DNA damage-induced apoptosis toxicity (Monks et al., 2003). ADPRT gene encodes a zinc-finger DNA-binding protein, poly (ADP-ribose) polymerase-1 (PARP-1), that modifies various nuclear proteins by poly (ADP-ribose)ylation and functions as a key enzyme in the base excision repair pathway; altered ADPRT/PARP-1 enzyme function was associated with response to oxidative damage (Griesenbeck et al., 1997; Lockett et al., 2004), during almost all forms of apoptosis, PARP is activated by caspases (Hatip-Al-Khatib et al., 2004), and inhibition of ADP-ribosyltransferase significantly reduced its ability to induce apoptosis of cancer cells (Hatip-Al-Khatib et al., 2004; Takamura-Enya et al., 2001), BIRC, encoding apoptosis inhibitory protein was associated apoptosis and cell cycles (Takita et al., 2004). Inhibitors of apoptosis (IAPs) antagonize cell death and regulate the cell cycle (Dong et al., 2002); decrease of c-IAP2 expression led to promotion of apoptosis (Nishihara et al., 2003). In the present study, the expressions of ADPRTL1 and CYP1A1 in LFP water-soluble CEE-treated breast cancer cells increased and the expression of BIRC3 decreased significantly; it could be inferred that LFP water-soluble CEE inhibited proliferation and induced apoptosis of breast cancer cells mainly through up-regulation expressions of CYP1A1 and ADPRTL1, and down-regulation of BIRC genes.

For other predominantly down-regulated genes, ADAM encoding a disintegrin and metalloprotease family contributes to regulation of the cell–cell and cell–matrix interactions that are critical determinants of malignancy; ADAM9 overexpression enhances cell adhesion and invasion of non-small cell lung cancer cells (Shintani et al., 2004), and is associated with poor differentiation and shortened survival of pancreatic cancer (Grutzmann et al., 2004). HMMR encoding the receptor for hyaluronan mediated motility (RHAMM), a hyaluronan (HA) binding protein, has been shown to play an important role in the motility and invasiveness of malignant cells (Kuwabara et al., 2004); expression and splicing of RHAMM are important molecular determinants of disease severity in multiple myeloma (Maxwell et al., 2004); and RHAMM blockade might be a potential therapeutic target for difficult-to-treat neoplasm (Tolg et al., 2003). The decreased expressions of ADAM9 (−5.09-fold) and RHAMM (−15.48-fold) in the treated cancer cells (Table 2, Fig. 4) suggested that LFP water-soluble CEE could inhibit the motility and invasiveness of cancer cells, and decreased their malignancy (Shintani et al., 2004; Grutzmann et al., 2004; Kuwabara et al., 2004; Maxwell et al., 2004; Tolg et al., 2003).

The most predominantly up-regulated gene after LFP water-soluble CEE treatment was ADPRTL1 (29.25-fold) which was associated with DNA repair, damage, and cell apoptosis (Griesenbeck et al., 1997; Lockett et al., 2004; Hatip-Al-Khatib et al., 2004; Takamura-Enya et al., 2001), and the most predominantly down-regulated gene HMMR (−15.48-fold) was associated with the motility and invasiveness of cancer cells. The findings in this study showed LFP water-soluble CEE resulting in DNA damage, proliferation inhibition and apoptosis of cancer cells through up-regulation of ADPRTL1 expression, and inhibition of the motility and invasiveness of cancer cells through down-regulation of HMMR expression. ADPRTL1 and HMMR might be the main drug target at which LFP water-soluble CEE acted, but this needs to be confirmed in normal and/or other cancer cells.

Although there was qualitative agreement between the cDNA microassay and PCR assay, with 90% of genes tested showing similar trend reported, false positive results were common in cDNA microarray (Swami et al., 2003). Therefore, 3 representative genes in response to LFP water-soluble CEE treatment were confirmed by semi-quantitative RT-PCR. A good qualitative correlation was observed in the results obtained by microarray and RT-PCR, but quantitative differences exist. As cDNA microarray tended to have greater interassay variation than quantitative RT-PCR (Swami et al., 2003), the existence of quantitative differences is inevitable, even reverse correlation is present (Swami et al., 2003; Morandi et al., 2006). The findings in this work could show the trend of gene express profile of cancer cells treated with LFP water-soluble CEE.

In conclusion, the potential anticancer activity of LFP extract against human breast cancer was investigated in this experimental study, for the first time. LFP extract exhibited a strong inhibitory effect on the proliferation of both ER positive and negative breast cancer cells in vitro and inhibited growth of ER negative breast cancer in vivo. The results of this experimental study suggested that Litchi fruit pericarp contains some constituents, which would be useful for anticancer drug discovery. The anticancer activity of LFP extract could be attributed, in part to its proliferating inhibition and apoptosis induction of cancer cells through up-regulation (CYP1A1, ADPRTL1) and down-regulation (BIRC3, ADAM9, HMMR) of multiple genes, which are involved in the cell cycle regulation and cell proliferation, apoptosis, signal transduction...
and transcriptional regulation, motility, and invasiveness of cancer cells. ADPRTL1, CYP1A1, and HMR1 might be the main molecular targets at which LFP water-soluble CEE acted.

References


Primchian, M., Nattavat, K., Sineenart, K., Omboon, L., Narongchai, P.,


