Chiang Mai J. Sci. 2018; 45(3) : 1345-1358
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

# Aqueous and Ethanolic Extracts of Mangosteen Peels as Natural Antimicrobial/anticancer Materials Against Pathogenic Microbes and B16F10 Murine Melanoma

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Received: 10 December 2016
Accepted: 6 February 2017

## ABSTRACT

The antimicrobial, anticancer and cytotoxic activities of crude extracts of the pericarps of mangosteen fruits, generated using water or ethanol as the extractant, were evaluated and compared with those of α-mangostin. Due to higher total mangostin content in the ethanolic extract (EEM), this extract exhibited considerably stronger activities against pathogenic microbes and skin cancer cells than the aqueous extract (AEM) did. *Staphylococcus aureus* and *Escherichia coli* showed high sensitivity to EEM, with minimum inhibitory concentrations (MICs) of 31.25 and 125 μg/ml, respectively. Selected bacteria and fungi exhibited good *in vitro* susceptibility to EEM, with minimum bactericidal and fungicidal concentrations (MBC and MFC) of less than 0.5 mg/ml. Shrinkage and lysis of bacteria were clearly observed after treatment with EEM or α-mangostin. EEM and α-mangostin also caused swelling and lysis of *Candida albicans*. EEM showed a great cytotoxic effect on B16F10 murine melanoma cells at 24, 48, and 72 h judged by $IC_{50}$ values of less than 25 μg/ml, which were 15-100 times lower than $IC_{50}$ values of AEM, but 4-10 times higher than those of α-mangostin. It was shown that the cell membranes of B16F10 cells were damaged after being treated with EEM and α-mangostin for 24 h.

**Keywords:** mangosteen, α-mangostin, antimicrobial properties, melanoma

## 1. INTRODUCTION

Mangosteen (*Garcinia mangostana*) is extensively planted in Thailand as well as in other countries in tropical zones. In addition to its tasty flesh, its trunk, branch, leaf, and peel contain phenolic compounds that are the secondary metabolites involved in the

plant's natural defense process against insect infestation and disease. Extracts from those parts of mangosteen have been utilized in traditional therapy for skin infections/wounds and in cosmetic products in Thailand.

A wide range of biological properties, including anti-inflammatory, antibacterial, antifungal, antioxidant [1], antitumor [2], and anticancer [3-6] activities, has been reported for phenolic compounds in mangosteen, such as α-mangostin, β-mangostin and γ-mangostin. Among these diverse phenolic compounds, α-mangostin (the chemical structure is shown in Figure 1) presumably can serve as a marker for the biological benefits of mangosteen extracts due to its relatively high activity. For instance, high inhibitory activity against leukemia HL60, NB4 and U937 cells, but no cytotoxicity against normal peripheral blood lymphocytes at the same concentrations [3], and anticancer activity toward colon cancer cells [4], prostate carcinoma cells [5] and breast cancer cells [6] have been established. According to the study of antimicrobial and antitumor activity of mangosteen cultivated in Southeast Brazil [7], the ethanolic extract of mangosteen obtained in the pericarp, resin, leaf and fruit showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, whereas the ethanolic extract mangosteen fruit exhibited a genotoxic and induced apoptosis in the B16-F10 melanoma cell line. Nonetheless, studies on the effect of mangosteen extract on skin cancer activity have been quite limited. Skin infection and skin cancer (melanoma) are health problems in humid and sunny areas, e.g., Thailand [8]. It is known that one of the main risk factors is exposure to a high level of ultraviolet (UV) radiation without protection. Although melanoma is not common compared with other types of cancer, such as liver and cervical cancer, which are frequently found in Thai males and females, respectively [9], it is the major cause of death related to skin cancer [10].

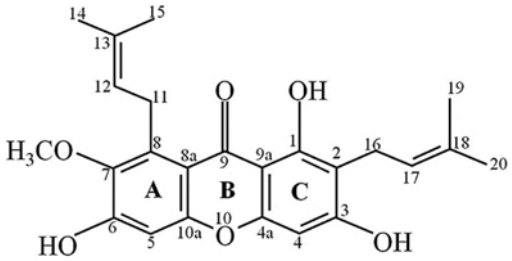


**Figure 1.** Chemical structure of α-mangostin ($C_{24}H_{26}O_6$) or 1,3,6-Trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-en-1-yl)-9H-xanthen-9-one [12].

In this study, mangosteen peel, which is basically solid waste, was used as a high-value natural resource. Crude extracts of mangosteen peels were prepared by using water or ethanol as solvents. The extracts underwent a drying process to remove the solvents before determination of the total phenolic and total mangostin content as well as the biological properties. The antimicrobial activity was investigated by broth dilution and agar diffusion methods to obtain minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) values, respectively. The cytotoxic effects toward Vero monkey kidney and B16F10 murine melanoma cell lines were determined. Moreover, the morphological changes in those cells after exposure to the prepared extracts were studied by scanning electron microscopy (SEM).

## 2. MATERIALS AND METHODS

### 2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Modified Eagles Medium (MEM), Fetal Bovine Serum (FBS), and Antibiotic-

Antimycotic (100X) ) containing 10,000 units of penicillin G, 10,000 µg of streptomycin, and 25 µg of amphotericin B (Fungizone® Antimycotic) in 1 ml of 0.85% saline were purchased from Gibco, Inc. Unless otherwise specified, all other chemicals used were of analytical grade, obtained from Sigma-Aldrich, Inc. Cancer cell lines and selected microbes were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The microbial activity in this study was evaluated against *Escherichia coli* (*E. coli*; ATCC 25922), *Staphylococcus aureus* (*S. aureus*; ATCC 12600), *Aspergillus niger* (*A. niger*; ATCC 16404), *Staphylococcus epidermidis* (*S. epidermidis*; ATCC 14990), *Propionibacterium acnes* (*P. acnes*; ATCC 6919), and *Candida albicans* (*C. albicans*; ATCC 10231). The cytotoxic activity was performed on Vero monkey kidney cells (ATCC® CCL-81™) and B16F10 murine melanoma cells (ATCC CRL-6475).

**2.2 Preparation of the Crude Mangosteen Extracts**

Dark-purple mature fruits of mangosteen were obtained from a local market in Bangkok, Thailand. After removing the arils, the peels were cleansed, chopped, and hot-air dried at 50 °C for a week. The dried pieces were ground into smaller pieces (~ 2 mm in diameter) by a mill (Thomas Wiley Laboratory Mill Model 4, USA) and then 800 g of the ground pieces were decocted in deionized water (4 l) for an hour to prepare the aqueous extract. The obtained crude extract was filtered through filter cloths, concentrated by a rotary vacuum evaporator (BUCHI, Switzerland) at 50 °C for18-19 h, and lyophilized using a freeze dryer (Labconco, SN 7960034, USA). For the preparation of the ethanolic extract, the ground pieces of dried mangosteen peels (800 g) were soaked in 95% ethanol (4 l) with gently shaking (20 rpm) in an orbital shaker (GFL 3005, Germany) under ambient condition for 3 days. The ethanol mixture was then filtered through a coarse sieve, filter cotton and filter paper (Whatman No.1), respectively. The filtrate was dried by the rotary vacuum evaporator at 50 °C for 18-19 h. The yields of dried compounds of the aqueous and ethanolic extracts were determined. The resulting crude aqueous and ethanolic extracts of mangosteen peels (referred to as AEM and EEM, respectively) were stored in dark seal containers at 4 °C before use.

**2.3 Phenolic Compounds Analysis**

The total phenolic compound content was evaluated using Folin-Ciocalteu method [10]. Briefly, 1 mg of mangosteen extracts (AEM and EEM) were dissolved in 1 ml of DI water and ethanol (95% vol), respectively. Then, 0.2 ml of each solution was firstly mixed with 1.6 ml of sodium bicarbonate aqueous solution (7.5%w/v). A 1.0 ml of the Folin-Ciocalteu reagent (previously diluted at 1:10 with DI water) was then added into the mixture. The mixture was then thoroughly shaken and incubated at room temperature (about 30 °C) for 30 minutes before measuring the absorbance at 765 nm by a UV-Vis spectrophotometer (Shaimadzu UV-2550, Japan). The content of total phenolic compounds was calculated in terms of mg of gallic acid equivalent (GAE)/g of dried crude extract. The results were expressed as mean values ± standard deviation (SD) (n = 5).

**2.4 Analysis of Total Mangostin Content**

The conditions for the analysis of total mangostin content were developed by Pothitirat and Gritsanapan [11]. The absorbance values of the aqueous extract (AEM) and the ethanolic extract (EEM), generated in dilute solutions of deionized water and absolute ethanol, respectively,

were directly measured at a wavelength of 320 nm by spectrophotometry. The total mangostin content was calculated as mean values ± SD (n=5) and expressed as mg of α-mangostin/g of dried crude extract. The standard α-mangostin used in the calibration curve was purchased from ChromaDex (Irvine, CA, USA).

**2.5 Antimicrobial Activity**

Antimicrobial activity was assessed by means of the broth dilution method [13]. Briefly, stock solutions were prepared at a concentration of 32 mg/ml in sterile deionized water for AEM and at concentration of 4 and 0.125 mg/ml in 10 % dimethyl sulfoxide (DMSO)/sterile phosphate-buffered saline (PBS) solution for EEM and α-mangostin, respectively. The stock solutions were 2-fold serially diluted in broth medium to obtain 10 concentrations of the stocks. Each type of microorganism, at a density of $10^8$ colony-forming units (CFU)/ml, was added to the same volume of growth medium containing AEM, EEM, or α-mangostin. It was noted that the final concentrations of the samples were half of the initial concentrations. After 24 h of incubation at 37 °C, the lowest concentrations, which were able to inhibit any visible bacterial growth (no turbidity), were recorded as the MIC values.

Agar diffusion was further applied to determine MBC/MFC values by subculturing the test dilutions of the suspension on nutrient agar. After 24 h of incubation at 37 °C, the lowest concentrations, which were able to kill at least 99.9 % of initial numbers of particular bacteria or fungi were recorded as the MBC or MFC values, respectively.

**2.6 Anticancer and Cytotoxic Activities**

The cytotoxic effects of AEM, EEM, and α-mangostin were evaluated using the B16F10 murine melanoma cell line and the Vero cell line (Vero, ATCC CCL-81). The growth media (DMEM for B16F10 cells and MEM for Vero cells) were supplemented with 10 % FBS and 1 % antibiotic containing penicillin G of 100 units/ml, streptomycin of 100 µg/ml, and amphotericin B of 0.25 µg/ml. After the cells reached 80 % confluence in their growth media at 37 °C in a humidified atmosphere and 5 % $CO_2$, detachment was carried out using trypsin-EDTA. Single cells at a density of 3,000 cells/90 µl were seeded into each well of a 96-well polystyrene plate (3370, Costar, USA) and allowed to attach for 24 h. Next, 10 µl of AEM, EEM, or α-mangostin, previously prepared at multiple concentrations, was gently added, and the final concentrations were accordingly 10-3000, 1-300 and 0.1-30 µg/ml, respectively. After incubation for 24, 48 or 72 h, the cytotoxic effects were determined using the MTT assay. MTT solution was prepared at a concentration of 5 mg/ml in PBS before diluted into 0.5 mg/ml in serum-free media. After centrifugation at 1,200 rpm, 4 °C for 5 min, the culture media were aspirated and replaced with the diluted MTT solution. The cells were further incubated in an incubator for 4 h, after which centrifugation and media removal were performed before dissolving the complete formazan product in 100 µl of DMSO. The absorbance was immediately measured using a microplate reader (Bio-Rad Benchmark 550, USA) at 550 nm. The percent survival was calculated using the following equation:

$$\% \; survival = \frac{AB_t}{AB_u} \times 100$$

$AB_u$ and $AB_t$ denote the absorbance values of untreated and treated cells, respectively. The profiles of percent survival as a function of logarithm-transformed concentrations

were plotted and expressed in terms of the half-maximal inhibitory concentration ($IC_{50}$).

### 2.7 SEM Morphological Observations

The microbial cell suspensions were filtrated through 0.22-µm-pore-sized polycarbonate membrane filters. The cells attached on the filters were immediately fixed in 4 % glutaraldehyde in phosphate buffer solution (PBS) at 4 °C for an hour and rinsed a few times with PBS before dehydrated in a graded ethanol series: 30%, 50%, 70%, 90%, and 100%. Afterward, the cells were dried by a critical point dryer (a Tousimis Samdri-780, USA) using liquid carbon dioxide as a transitional fluid and sputtered with a thin layer of gold in a sputter coater (Balzers-SCD 040, Liechtenstein). Morphologies of cells were observed by scanning electron microscopy (SEM, JEOL JSM- 5410LV, Japan) subjected to the accelerating voltage of 15 kV.

B16F10 and Vero cells were cultured on a borosilicate-glass coverslip under the same cell density per working area and conditions as previously described (2.6). After 48 h of the incubation, the media were removed and the cells were immediately fixed by chemical fixation, rinsed, dehydrated and sputtered with gold. Their morphologies were also examined by SEM.

### 2.8 Statistical Analysis

The obtained data were expressed as an average from at least three independent experiments and statistically analyzed by student *t*-test: Two-sample assuming equal variances in Microsoft office 2010. The differences were considered statistically significance at the level of $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1 Total Phenolic Compound and Total Mangostin Content

In this study, solid waste consisting of the fruit peel of mangosteen at the mature stage was used as a source of antimicrobial and anticancer molecules. Mature peels with a dark-purple skin contain total mangostins at a level approximately two-fold higher than in young fruits [11]. Crude extracts of mangosteen peel, or AEM and EEM, were prepared using water and ethanol, respectively. After removal of the extractants by evaporation, yields of AEM and EEM were found as 1.3 and 8.8 %, respectively (Table 1). Total phenolic content and total mangostin content in the two crude extracts were analyzed by spectrophotometry [11,12]. The total phenolic content and total mangostin content presented in AEM and EEM are shown in Table 1. Since ethanol (95% vol) was more effective in extracting phenolic compounds than water, the ethanolic extract had higher yield than the aqueous extract. EEM contained the total phenolic compounds of 377 mg gallic acid/g dried crude extract (~63 % more than in AEM) and comprised 357.01 mg of α-mangostin/g of dried crude extract, which was nearly 3 times the amount contained in AEM. According to the result, 95 % of the phenolic compounds in EEM were total mangostin. Consistent with its chemical structure, α-mangostin should be more soluble in ethanol. The other phenols in AEM might have been tannins, flavonoid, and/or other phenolic compounds that can better dissolve in water than in less-polar extractants such as ethanol. Pothitirat et al. (2009) reported that the yield of crude ethanolic extract of mature mangosteen peels was about 26.58 % with α-mangostin content of 13.63% w/w of dried crude extract. The obtained yield of ethanolic extract from the procedure in this study was relatively lower; however, α-mangostin content was considerably higher (35.7% w/w of dried crude extract).

The total phenolic contents in aqueous and ethanolic extracts from this study were quite high compared to previous work. According to Suttirak & Manurakchinakorn (2014), aqueous and ethanolic extracts from mangosteen peel contained ~ 20 and ~ 150 mg gallic acid/g dried crude extract, respectively [14]. The raw material and extraction procedure should be the main factors that significantly affect the yield and composition of the extracts.

**Table 1.** Total phenolic and total mangostin contents presenting in AEM and EEM.

| | Yield of crude extract (% g of extract/ g of dried peel) | Total phenolic (mg of GAE/ g of dried crude extract) | Total mangostin (mg of α-mangostin/ g of dried crude extract) |
|---|---|---|---|
| AEM | 1.3±0.0 | 238.40±5.48 | 139.77±1.36 |
| EEM | 8.8±1.5 | 377.01±4.80 | 357.01±2.52 |

**3.2 Antimicrobial Activity**

The crude extracts (AEM and EEM) and α-mangostin were quantitatively assessed for the inhibition of selected pathogenic microbes in terms of MIC and MBC/MFC values [13]. The microbial cells involved in food spoilage and human illness were used. The gram-negative and gram-positive bacteria that cause food poisoning are represented by *E. coli* and *S. aureus*, respectively [15]. *S. epidermidis* and *P. acnes* are bacteria that trigger inflammation in acne [16]. *A. niger* is a mold fungus involved in the spoilage of bakery products [17]. *C. albicans* causes mouth and skin infections (candidiasis) in most clinical cases [18]. The MIC and MBC values of the crude extracts (AEM and EEM) and pure α-mangostin for all of those strains are presented in Table 2. EEM showed higher antimicrobial activities against all tested microorganisms than AEM did. AEM had a stronger ability to inhibit the growth of the gram-positive bacteria *S. aureus*, *S. epidermidis*, and *P. acnes* than to inhibit the growth of both the gram-negative bacterium *E. coli* and fungi (*A. niger* and *C. albicans*). However, the microbicidal activities were generally low. Both the gram-negative bacterium *E. coli* and fungi appeared to be more resistant to AEM, as indicated by MIC and MBC/MFC values ≥ 8 mg/ml. Low anti-acne activity of AEM with MIC of 500 µg/ml and MBC of >500 µg/ml was also reported by Pothitirat et al. (2010) [19]. On the other hand, EEM exhibited greater inhibition of all tested microorganisms compared with AEM, corresponding to lower MICs and MBCs. The MICs and MBCs of EEM for all tested bacteria were in the range of 31-125 and 500 µg/ml, respectively. The results could be attributed to the significantly higher total mangostins content in EEM compared with that in AEM. EEM contained ≈ 357 mg of α-mangostin/g of dried crude extract (or 35.7% by weight of α-mangostin in dried crude extract); thus, antimicrobial activities of EEM appeared significantly different from that of pure α-mangostin (100% by weight).

**Table 2.** The MIC, MBC, and MFC in the unit of μg/ml of AEM, EEM, and α-mangostin.

| Microorganism strains | AEM | | EEM | | α-mangostin | |
|---|---|---|---|---|---|---|
| | MIC | MBC/MFC | MIC | MBC/MFC | MIC | MBC/MFC |
| *E. coli* | 8000[a] | >16000[+] | 125[b] | 500[*] | 15.625 | 15.625 |
| *S. aureus* | 125 [a] | 2000[+] | 31.25 [b] | 500[*] | 7.813 | 7.813 |
| *S. epidermidis* | 125 | 4000[+] | 125 [b] | 500[*] | 1.953 | 3.906 |
| *P. acnes* | 250 [a] | >16000[+] | 125 [b] | 500[*] | 0.977 | 0.977 |
| *A. niger* | > 16000 [a] | >16000[+] | 500 [b] | 500[*] | 7.813 | 62.5 |
| *C. albicans* | 16000 [a] | >16000[+] | 125 [b] | 125[*] | 7.813 | 7.813 |

[a] $p < 0.05$ versus MIC of EEM; [b] $p < 0.05$ versus MIC of α-mangostin
[+] $p < 0.05$ versus MBC/MFC of EEM; [*] $p < 0.05$ versus MBC/MFC of α-mangostin

In this finding, *P. acne* and *S. epidermidis* were susceptible to α-mangostin, with the MIC and MBC values of approximately 1 and 2-4 μg/ml, respectively. This result was in accordant with results reported by Pothitirat et al. (2009); α-mangostin exhibited strong anti-acne activity against *P. acne* and *S. epidermidis* with MIC/MBC of 1.95 and 3.91 μg/ml, respectively [11]. Moreover, this study showed that pure α-mangostin was also very effective against the other microorganisms, with MICs and MBCs below 20 μg/ml and an MFC of ~60 μg/ml.

The results of this study could be supported by previous studies on antimicrobial screening of extracts that mostly contained phenolic compounds. A study by Rauna et al. showed that the gram-positive bacteria *S. aureus* and *S. epidermidis* were more sensitive, whereas the gram-negative bacterium *E. coli*, the mold *A. niger* and the yeast *C. albican*s were resistant to the majority of 29 phenolic compound-containing plant extracts [20]. The results of Shan et al. also confirmed the higher susceptibility of gram-positive bacteria than gram-negative ones based on an antibacterial activity evaluation of 46 rich-phenolic extracts from Chinese herbs and spices. It was shown that antibacterial activity, expressed in terms of clear zone formation, was highly linearly correlated with the total phenolic content [21]. Effective antimicrobial responses to phenolic compounds extracted from various plant sources have been reported among gram-positive bacteria, but these compounds were less active against gram-negative bacteria and fungi [22]. Clinically, *S. aureus* has strong susceptibility to hydrophobic drugs such as erythromycin, which is widely utilized in treating gram-positive bacterial infections [23]. In a similar way, the inhibition of gram-positive bacteria could be affected by the hydrophobicity of α-mangostin. Gram-negative bacteria were more resistant to the extracts and α-mangostin, which might be related to the outer membrane acting as a selective permeability barrier [24] to protect the cells from interfering substances.

To provide more insight into the extracts' mechanism of action against microorganisms, the SEM images of the morphological alterations of the microbial cells due to treatment with the crude extracts and α-mangostin are shown in Figure 2. The SEM images (Figure 2 a, e, and i) show

the normal conditions of untreated *S. aureus*, *S. epidermidis*, and *C. albicans* cells, respectively. Slight changes in *S. aureus* and *S. epidermidis* in AEM-containing media can be noted in Figure 2 b and f, respectively. In Figure 2 c and g, shrinkage and lysis of cells by the treatment with EEM can be observed. Greater cell shrinkage and lysis were observed following treatments with α-mangostin (Figure 2.d and h). The action mode of antimicrobial agents including phenolic compounds and α-mangostin might be related to the cell structures. The antibacterial action of phenolic compounds evidently degrades the cell wall by interacting with protein; changes the phospholipid and fatty acid components; interferes with the cell wall's integrated enzymes, resulting in cellular component leakage [21, 25]; and/or inhibits the formation of peptidoglycan, an important component of the cell wall of bacteria [26].

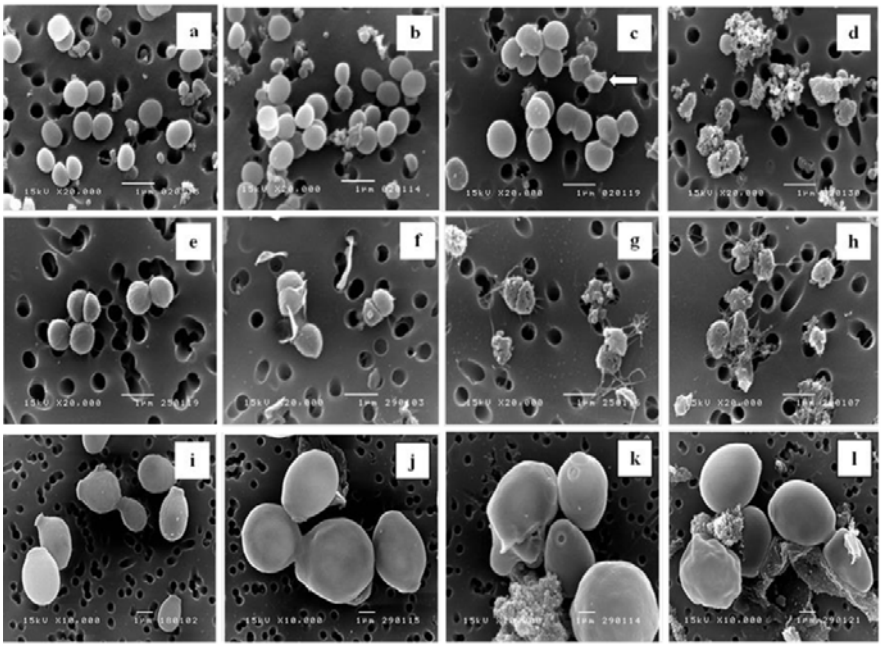


**Figure 2**. SEM images of *S. aureus* (a-d), *S. epidermidis* (e-h) and *C. albican* (i-l) after incubating in the growth medium and the growth medium containing AEM, EEM and α-mangostin at the minimal inhibitory concentration (MIC) as shown in Table 2 for 24 h. The cell morphologies were visualized at the magnification of 20,000× (a-h) and 10,000× (i-l).

EEM and α-mangostin have the potential to inhibit *C. albicans* and *A. niger*, although the effects on fungal cells and the antifungal actions might be different from the activity against bacteria. According to previous studies, chemical antifungal agents mostly target the interior of cells, such as the formation or function of ergosterol, an essential constituent of the fungal cell membrane [25]. In the present study, as shown in Figure 2.j, k and l, *C. albicans* cells became swollen after being treated with AEM, EEM or α-mangostin. Death of living cells caused by cell swelling and cell membrane rupture (cell lysis) was clearly observed in medium containing EEM or α-mangostin.

**3.3 Anticancer and Cytotoxic Activities**

The survival of B16F10 melanoma cells after exposure to AEM, EEM, or α-mangostin was assessed by MTT assay. The survival profiles are illustrated in Figure 3. The number of viable cells is proportional to the amount of purple formazan obtained from the reaction between MTT and succinate-dehydrogenase mitochondrial enzyme in living cells [27]. At low concentrations, ranging from 10-100 µg/ml for AEM (Figure 3a), 1-10 µg/ml for EEM (Figure 3b), and 0.1-1 µg/ml for α-mangostin (Figure 3c), the numbers of surviving B16F10 cells were constant or increased at 24 h of treatments, with relative survival in the range of 100-200 %. However, at 48 and 72 h of treatments, survival of the B16F10 cells tended to be lower than those at 24 h. The numbers of living cells were dramatically decreased after treatments with AEM, EEM, or α-mangostin at concentrations greater than 100, 10, and 1 µg/ml, respectively.

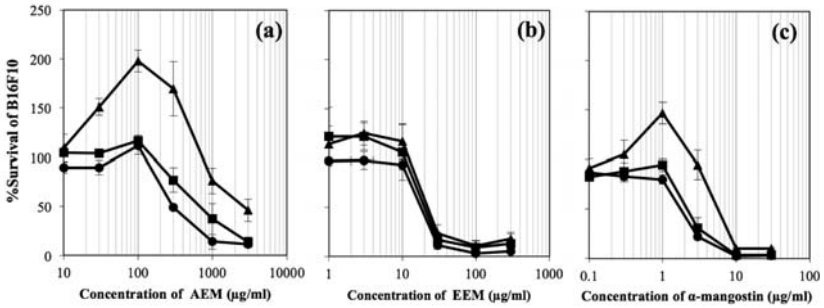


**Figure 3**. Survival profiles of B16F10 murine melanoma cells after incubating in DMEM containing AEM (a), EEM (b), or α-mangostin (c) for 24 (▲), 48 (■), and 72 h (●).

The effect on Vero monkey kidney cells was relatively similar to that previously observed among B16F10 cells (Figure 4). The number of Vero cells could be increased at 24 h after treatments with a low concentration of AEM, EEM, or α-mangostin but was decreased after treatments with AEM, EEM, or α-mangostin at concentrations of 100, 10, and 1 µg/ml, respectively. The percent survival was also decreased when the treatment time was prolonged to 48 or 72 h. The results suggested that the dose and exposure time strongly mediated the potency of the treatments. It has been reported that at low concentrations, α-mangostin protects the mitochondria in mammalian cells from peroxidative damage but causes a dramatic reduction of respiration at high concentrations [28].

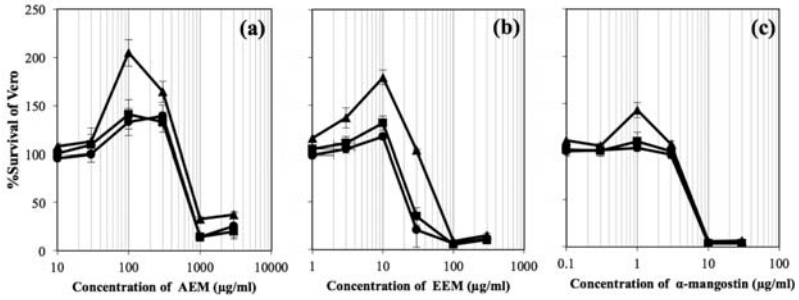


**Figure 4.** Survival profiles of Vero monkey kidney cells after incubating in MEM containing AEM (a), EEM (b), or α-mangostin (c) for 24 (▲) 48 (■), and 72 h (●).

The effectiveness of AEM, EEM, and α-mangostin was further verified based on $IC_{50}$ values, which were obtained from the interpolation of survival profiles (Figure 3 and 4). The $IC_{50}$ values are summarized in Table 3. The $IC_{50}$ values after 72-h treatment of B16F10 cells were the lowest, indicating the greatest inhibition. The $IC_{50}$ value of AEM at 24 h was significantly greater than those at 48 and 72 h ($p < 0.05$), whereas the $IC_{50}$ values of EEM at 24, 48, and 72 h ranged from 20-25 µg/ml (no significant difference). Accordingly, this finding suggested that the treatment with EEM at a concentration of 25 µg/ml for 24 h was sufficient to inhibit B16F10 cells, with 50 % survival. Pure α-mangostin exhibited a strong inhibitory effect on B16F10 cells, with an $IC_{50}$ value of less than 7 µg/ml. From Table 3 (b), although the survival profiles of Vero cells were relatively similar to those of B16F10 cells, the $IC_{50}$ values of all treatments appeared significantly greater among Vero cells than among B16F10 cells ($p < 0.05$).

**Table 3.** Summary of $IC_{50}$ (µg/ml) of B16F10 (a) and Vero (b) after incubating in the growth medium containing AEM, EEM, or α-mangostin for 24 h, 48 h, and 72 h.

| (a) | 24 h | 48 h | 72 h |
|---|---|---|---|
| AEM | $3006.50 \pm 1005.15^{a,*}$ | $840.54 \pm 41.73^{b}$ | $309.53 \pm 29.59^{*}$ |
| EEM | $24.41 \pm 0.53^{*}$ | $22.22 \pm 2.53$ | $20.52 \pm 0.72$ |
| α-mangostin | $6.63 \pm 0.50$ | $2.38 \pm 0.27$ | $2.03 \pm 0.12$ |
| (b) | 24 h | 48 h | 72 h |
| AEM | $919.88 \pm 18.77^{+}$ | $788.59 \pm 14.83$ | $799.54 \pm 12.57$ |
| EEM | $59.53 \pm 0.21^{+}$ | $26.89 \pm 1.80$ | $26.99 \pm 4.38$ |
| α-mangostin | $6.98 \pm 0.01$ | $6.63 \pm 0.25$ | $6.58 \pm 0.11$ |

[a] $p < 0.05$ versus $IC_{50}$ of crude extracts against B16F10 at 48 h
[b] $p < 0.05$ versus $IC_{50}$ of crude extracts against B16F10 at 72 h
[*] $p < 0.05$ versus $IC_{50}$ of crude extracts against Vero at the same treatment time
[+] $p < 0.05$ versus $IC_{50}$ of crude extracts against Vero at 48 h

Previously, B16-F10 cells proliferation rate was reduced to 45% after being treated with 10 mg/mL ethanolic extract of mangosteen fruit for 48 h [7]. For other cell types such as prostate cancer cells (22Rv1 and LNCaP), Li et al. (2013) reported the effect of magosteen fruit extract containing ~ 35% total mangostin in dried extract, which was comparable to the value observed in EEM in this study. Their mangosteen extract exhibited anti-cancer activity against prostate cancer cells with the $IC_{50}$ values in the range of 15-20 µg/mL after 24 and 48 h of treatment, but the $IC_{50}$ value at 72 h appeared 2 times lower (~ 10 µg/ml) [29]. Kurose et al. (2012) and Moongkarndi et al. (2014) also reported inhibitory effect of α-mangostin extracted from mangosteen against breast cancer cells (MDA-MB231 and SKBR3, respectively) after treatment for 24 h. Based on the $IC_{50}$ data, 20 µM was determined to be the optimal concentration of α-mangostin for *in vitro* studies [30]. It was concluded that ethanolic extract contained mostly low-polar constituents, which exhibited cytotoxicity, apoptosis and antioxidative

activity in cancer cells, whereas water extract contained highly polar ones, which presented powerful antioxidant but very low cytotoxicity activity [31].

The morphologies of B16F10 and Vero cells (Figure 5a and b) after incubation in growth medium alone or growth medium containing AEM, EEM, or α-mangostin at their approximate individual $IC_{50}$ values (according to Table 3) were observed. The morphology of B16F10 cells in growth medium alone is shown in Figure 5 (a1 and a5). At 48 h, the AEM-treated cells (Figure 5 (a6)) appeared to have a slightly different shape, with a less-spread and more round morphology, compared with the untreated cells (Figure 5(a5)). Disruption of cell membranes and cell lysis were induced by EEM at an exposure time of 48 h (Figure 5(a7)) and by α-mangostin at 24 and 48 h (Figure 5(a4 and a8)). Previously, Membrane blebbing and cell shrinkage were also observed in SKBR3 cells after treatment with ethanoic extract and α-mangostin [31]. The morphologies of untreated and treated Vero cells are shown in Figure 5(b). No significant alteration in Vero cells was observed with AEM treatment at 24 or 48 h. However, the cytotoxic effects of EEM and α-mangostin on Vero cells were clearly observed, similar to those previously observed among B16F10 cells.

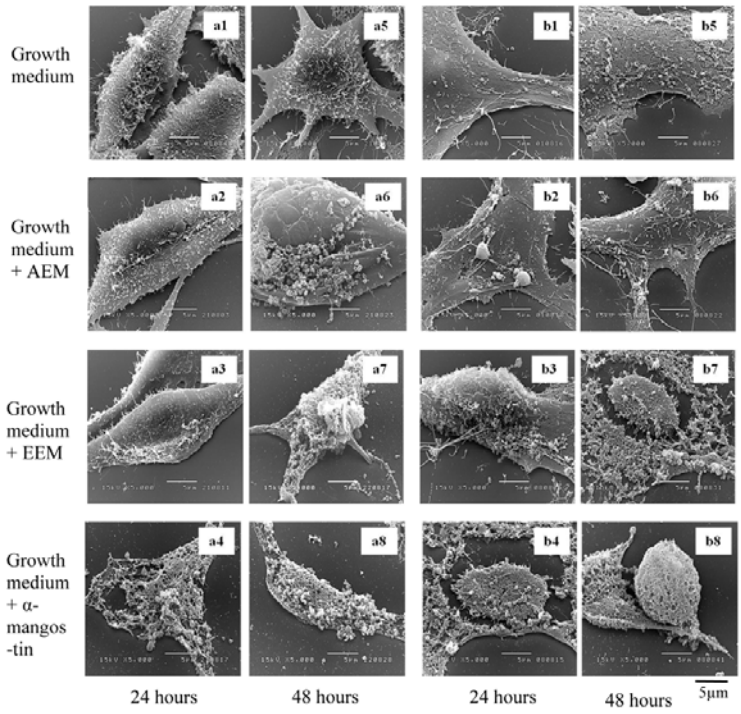


**Figure 5.** SEM images of murine melanoma B16F10 (a) and Vero monkey kidney cells (b) after incubating in the typical growth medium (1st row) and the growth media containing AEM (2nd row), EEM (3rd row), or α-mangostin (4th row) for 24 and 48 h. The concentrations of AEM, EEM and α-mangostin used in the experiment were based on their $IC_{50}$ at 24 and 48 h (Table 3) as follows: AEM, 3.00 and 0.90 mg/ml for B16F10 & 1.00 and 0.80 mg/ml for Vero; EEM, 0.024 and 0.022 mg/ml for B16F10 & 0.050 and 0.030 mg/ml for Vero; α-mangostin, 0.007 and 0.002 mg/ml for B16F10 & 0.007 and 0.007 mg/ml for Vero. The cell morphologies were visualized at the magnification of 15 kV × 5,000.

A previous study showed that mangosteen extract and α-mangostin strongly inhibited various cancer cell lines, such as leukemia (HL60, NB4, and U937) [3], human colon cancer (DLD-1 and HT-29) [4], and human prostate carcinoma (PC-3) [5]. The cytotoxic effects on cancer cells were reported to be related to the polyphenols and α-mangostin in the extract targeting the mitochondria, which are the core of cellular energy metabolism, by reduction of intracellular ATP generation and accumulation of reactive oxygen species (ROS) in the early phase. The mitochondria consequently failed to function, causing cell membrane damage. Different degrees of oxidative damage to the mitochondria were reported, such as proton leakage through the mitochondrial inner membrane and inhibition of respiration by disturbing protein-lipid interactions and other specific interactions with complex IV of the electron transport chain [28]. Moreover, apoptotic transduction activated by α-mangostin in mangosteen extract was reported. Mitogen-activated protein kinases (MAPKs) in the mitochondria interacted with α-mangostin, leading to the improper regulation of cell growth, proliferation, differentiation, and survival, and death of the cells eventually occurred [32]. The inhibitory effect was stronger than that of other phenolic compounds in mangosteen extract, such as β-mangostin, γ-mangostin and 8-deoxygartanin [33]. The biological activities of the bioactive compounds not only are influenced by the type of cell line but also are correlated with the chemical structure of those bioactive compounds. The highly active chemical structure of phenolic compounds/xanthone derivatives, providing efficient biological activity, contains a hydroxyl group at C-1 in the xanthone nucleus and tetra-oxygen functions with two $C_5$ units in rings A and C [33,34]. One of the derivatives that have that structure is α-mangostin.

The efficiency of certain chemical drugs for cancer might be stronger than that of the plant extract. It has been suggested that the combined use of chemical drugs for cancer and mangosteen extract could reduce chemical use, which might help to reduce chemical toxicity. It was also reported that the bioactivity of an anticancer drug was enhanced by α-mangostin, possibly by increasing absorption of the drug [4]. At high levels, EEM and α-mangostin also show cytotoxic effects against Vero cells. Unwanted side effects could be reduced by restricted distribution to specific target cells. Previously, delivery of extract from polymeric films to a target area showed good potential to improve efficacy and reduced side effects [35].

## 4. CONCLUSIONS

Crude extracts of mature mangosteen peels, which were prepared by using water or ethanol as the solvent, were evaluated for antimicrobial, anticancer and cytotoxic activities in comparison with α-mangostin. EEM contained higher levels of phenolic compounds and α-mangostin, resulting in greater antimicrobial and anticancer properties compared with AEM. All of the tested pathogenic bacteria, and especially the gram-positive bacterium *S. aureus*, and fungi showed a better response to EEM than to AEM. *A. niger* and *C. albicans* appeared to be resistant to AEM, whereas α-mangostin showed the greatest inhibitory effects on all microorganisms tested. Morphological changes in microbial cells after treatment with EEM or α-mangostin were clearly observed. Moreover, EEM exhibited potential anticancer activity against B16F10 murine melanoma. The anticancer effects of

EEM and α-mangostin were perhaps associated with the apoptotic process, causing damage to the cell membrane. It has been suggested that α-mangostin-rich EEM might also be used as an antimicrobial agent for skin infection and that the treatment could be effective within 24 h. EEM has shown cytotoxicity to Vero cells, similar to that to B16F10 cells; thus, selective distribution to target cells should be applied to reduce side effects.

## ACKNOWLEDGEMENTS

This work was supported by the National Research University Program (Health Cluster), Chulalongkorn University, contract Grant No. NRU59-016-HR.

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