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Antioxidant and Antiproliferative Activities of Non-Edible Parts of Selected Tropical Fruits

(Aktiviti Antioksidan dan Antiproliferatif Bahagian Tidak Boleh Dimakan Buah-buahan Tropika Terpilih)

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ABSTRACT

*It is of interest that seeds and pericarps of tropical fruits contain phytochemicals being the components of various biological activities for beneficial health effects. This study was aimed to evaluate antioxidant and anticancer activities of the methanolic extracts from seeds and pericarps of three selected tropical fruits including Rambutan (*Nephelium lappaceum *L.), Litchi (*Litchi chinensis *Sonn.) and Tamarind (*Tamarindus indica *L.). Total phenolic content was determined by using the Folin-Ciocalteu method. Antioxidant capacity was evaluated based on the ability of the fruit extracts to scavenge ABTS and DPPH radicals. MTT reduction assay and Annexin V-FITC/PI staining were carried out for cytotoxicity and apoptosis induction, respectively. Total phenolic contents of the seeds and pericarps of the tropical fruits ranged from 104.60 to 501.95 mg/g DW. All extracts were found to have significant antioxidant activities. Among them, tamarind seed extract contained the highest total phenolic contents and possessed the highest antioxidant capacities. Tamarind seed extract showed the highest cytotoxicity to human mouth carcinoma (CLS-354) cells and had no toxicity to PBMCs. Staining with annexin V-FITC/PI showed that this apoptosis occurred early in this cell type with 10.0% of the cells undergoing apoptosis. Tamarind seed extract might have potential anticancer activity which could be attributed, in part, to selectively inhibit the growth of CLS-354 cells and induce apoptosis. This research finding would be valuable information to identify major constituents of the extracts and mechanisms underlying anticancer activity which could be attributed to dietary health supplements or cancer chemoprevention from fruits.*

Keywords: Antioxidant capacity; antiproliferation; non-edible parts; tropical fruits

ABSTRAK

*Benih dan perikarpa buah-buahan tropika mengandungi fitokimia yang menjadi komponen pelbagai aktiviti biologi yang berfaedah kepada kesihatan. Kajian ini adalah bertujuan untuk menilai antioksidan dan aktiviti anti-kanser ekstrak metanolik daripada benih dan perikarpa 3 jenis buah-buahan tropika termasuk Rambutan (*Nephelium lappaceum *L.), Litchi (*Litchi chinensis *Sonn.) dan Asam (*Tamarindus indica *L.). Jumlah kandungan fenol ditentukan melalui kaedah Folin-Ciocalteu. Kapasiti antioksidan dinilai berdasarkan keupayaan ekstrak buah-buahan untuk menggarut ABTS dan radikal DPPH. Kesan pengurangan asai MTT dan pewarnaan Annexin V-FITC/PI masing-masing dijalankan untuk sitotoksisiti dan apoptosis aruhan. Jumlah kandungan fenol benih dan perikarpa buah-buahan tropika adalah daripada 104.60 ke 501.95 mg/g DW. Semua ekstrak didapati mengandungi aktiviti antioksidan bererti. Dalam kalangan mereka, ekstrak biji Asam mengandungi jumlah fenol tertinggi dan mempunyai keupayaan antioksidan tertinggi. Ekstrak biji Asam menunjukkan sitotoksisiti yang tertinggi untuk sel karsinoma mulut manusia (CLS-354) dan tidak mengandungi ketoksikan untuk PBMCs. Pewarnaan dengan annexin PI/V-FITC menunjukkan bahawa apoptosis berlaku awal dalam jenis sel ini dengan 10.0% daripada sel mengalami apoptosis. Ekstrak biji Asam mungkin mempunyai potensi aktiviti anti-kanser dengan sebahagiannya menghalang pertumbuhan sel CLS-354 dan mendorong apoptosis. Keputusan kajian ini akan menjadi maklumat penting untuk mengenal pasti juzuk utama ekstrak dan mekanisme yang mendasari aktiviti anti-kanser yang disebabkan oleh suplemen pemakanan kesihatan atau kanser kemocegahan daripada buah-buahan.*

Kata kunci: Antipercambahan; bahagian yang tidak boleh dimakan; buah-buahan tropika; kapasiti antioksidan

INTRODUCTION

Oral cancer accounts for 2 to 3% of all cancers. Incidence rates of oral cancer vary internationally worldwide. In developing countries, incidence and mortality had been ranked as ninth and tenth, respectively (Jemal et al. 2011). Epidemiological studies have shown that high incidence rates were documented in Asian regions, including the South and Southeast Asia (Warnakulasuriya 2009). Lifestyle behaviors and socio-cultural habits have been associated with oral precancer and oral cancer (Petti 2009;

Zain 2001). The habits of tobacco smoking, betel-quid chewing and alcohol consumption have been shown as major risk factors of oral cancer (Ko et al. 1995; Petti 2009). Other factors include poor nutritional status, low intake of fruits and vegetables and drinking beverages at high temperatures (De Stefani et al. 2005; Islami et al. 2009). The exposure to those risk factors induces oxidative stress, a high level of reactive oxygen species (ROS) status, which implicates oral carcinogenesis (Ko et al. 1995; Petti 2009; Zain 2001). ROS such as superoxide anion (O2•–), hydroxyl radical (OH•) and peroxyl radical (ROO•) causes cellular macromolecule damages, including DNA, lipid and proteins, resulting in mutation, changes in cell physiology and subsequent cancer formation (Lopaczynski & Zeisel 2001).

It has been documented that plant antioxidants can help scavenging free radicals and oxidants against oxidative stress-related diseases including cancer. Fruits contain variety of phytochemicals which mainly are polyphenolic compounds, such as flavonoids and phenolic acids (Ka¨hko¨nen et al. 1999). Many fruit phenolic compounds have been a considerable attention for being the components of antioxidants (Lotito & Frei 2006). Interestingly, antioxidant compounds vary in different parts of fruits such as non-edible (seed and peel) and edible parts and also vary among cultivars and species (Abeysinghe et al. 2007; Khanizadeh et al. 2008; Singh et al. 2002). Currently, discovery of natural products exerting high antioxidants in order to search for a powerful chemotherapeutic agent is rising interest.

Rambutan (*Nephelium lappaceum* L.), Litchi (*Litchi chinensis* Sonn.) and Tamarind (*Tamarindus indica* L.) are the tropical fruits that have been widely consumed in Thailand and Asian countries that have significant economic impact. The fruits are either consumed fresh or as commercially prepared dried and canned products. Large quantities of waste products from non-edible parts such as seed and skin were produced from canning industry. It is of interest that non-edible parts of these three economic fruits contain antioxidant compounds and possess anticancer activity. Peel extracts of rambutan had been documented to have high antioxidant capacity, antimicrobial activity and cytotoxicity against colon cancer cells (Khonkarn et al. 2010; Thitilertdecha et al. 2008). The peel extract of litchi could inhibit proliferation of hepatocellular carcinoma cells (Wang et al. 2006a). Tamarind seed extract contained phenolic substances and possessed antioxidant activity (Sudjaroen et al. 2005). However, there is no report of anti-oral cancer activity from the seed and pericarp of these three tropical fruits. The aim of this study was to investigate antioxidant capacity and antiproliferative activity from non-edible parts of the three fruits in human oral cancer cells. Cytotoxicity to normal peripheral blood mononuclear cells was also investigated for screening non-toxic extracts in order to select a potentially chemopreventive agent against oral cancer in the further study.

MATERIALS AND METHODS

CHEMICALS

2, 2- diphenyl-1-picrylhydrazyl (DPPH) was purchased from Calbiochem (Germany). Folin–Ciocalteu reagent, 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium carbonate, gallic acid, ascorbic acid and Trolox were purchased from Sigma–Aldrich (USA). Dimethyl sulfoxide (DMSO) was obtained from LAB-Scan Corporation Ltd. (Thailand). Ficoll-Hypaque gradient reagent (Lymphoprep) was purchased from Robbins Scientific Corporation (Norway). Fetal bovine serum (FBS) was obtained from Biochrom AG (Germany). 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), RPMI 1640, Phosphate buffer saline, trypsin–EDTA and penicillin–streptomycin were purchased from PAA (Austria).

PREPARATION OF TROPICAL FRUIT EXTRACTS

Three species of tropical fruits including Rambutan (*Nephelium lappaceum* L. cv. Rong Rean), Litchi (*Litchi chinensis* Sonn. cv. Hong Huay) and Tamarind (*Tamarindus indica* L. cv. Thailand Sweet) were collected from local markets in Thailand. Fruit pericarps and seeds were separated and air-dried. Dried materials were blended to powder before extraction. Extraction of fruit materials was modified previously (Sudjaroen et al. 2005). Air-dried material (50 g) was extracted with hexane in a soxhlet apparatus (3 h) to remove lipid. After drying, the solid was extracted with methanol (3 × 3 h). Organic solvent was removed by rotary evaporation at 35°C in vacuum. The extracts of methanolic fraction were dissolved in DMSO and kept at -20°C until use.

MEASUREMENT OF TOTAL PHENOLIC CONTENT

The total phenolic content was measured using Folin-Ciocalteau assay modifying by the method of Singleton et al. (1999). Folin-Ciocalteau reagent (phosphomolybdic phosphotunstic acid reagent) together with fruit extracts (1.0 mg/mL) were added into 96-well plate. The mixture was incubated at room temperature for 6 min. After incubation, 7% sodium carbonate were then added and further incubated at 45°C for 45 min. The absorbance was taken to 765 nm by using microplate reader. The standard curve was linear between 50-250 mg/L gallic acid. Total phenolic content was expressed as milligrams of gallic acid equivalent (mg GEA) per 1 g of dried extract.

DPPH RADICAL SCAVENGING ASSAY

The DPPH radical scavenging assay was performed by using DPPH assay kit. The 2, 2- diphenyl-1-picrylhydrazyl (DPPH) was dissolved in methanol to a final concentration of 0.2 mM. The extract solutions (0.2-0.25 mg/mL) were mixed with 150 mL of DPPH solution in 96-well plate. The mixture was incubated at room temperature for 30 min. Then

the absorbance was taken at 515 nm using a microplate reader. The standard curve was linear between 2 and 10 mg/100 mL ascorbic acid (vitamin C). The percentage of DPPH discoloration was calculated following below equation. DPPH radical scavenging activity was expressed as milligrams of vitamin C equivalent antioxidant capacity (VCEAC) per 1 g of dried extract.

$$\% \text{ radical scavenging activity} = \left(1 - \frac{ODsample}{ODblank}\right) \times 100.$$

ABTS CATION RADICAL SCAVENGING ASSAY

2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) solution was prepared following the method of Re et al. (1999). Briefly, the stock solution containing 7.4 mM ABTS and 2.6 mM potassium persulfate was prepared in deionized water and was allowed to stand for 12 h in the dark at 4°C before conducting the experiment. The working $ABTS^{\bullet+}$ solution was prepared by mixing the stock solution with deionized water until the absorbance was 0.7±0.02 at 734 nm. Fruit extracts (0.1 mg/mL) were mixed with 180 µL of working $ABTS^{\bullet+}$ solution. The mixture was incubated in the dark for 3 min. Then the absorbance was read at 734 nm using a microplate reader. The standard curve was linear using Trolox between 0.5 and 5.0 mg/100 mL. The percentage of ABTS radical scavenging activity was calculated. The unit of radical scavenging activity was defined as Trolox equivalent antioxidant capacity (TEAC) per 1 g of dried extract.

CULTURE OF HUMAN MOUTH CARCINOMA CELLS (CLS-354)

CLS-354 cells (Cell Line Service) at passage number 28-33 were cultured in complete media containing RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2 mM stable glutamine for 48 h at 37°C in humidified 5% CO_2 atmosphere. Cells were then trypsinized and plated at a density of 1.6×10^4 cells/cm^2 in order to perform cytotoxicity test.

PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

EDTA blood was collected from 4 healthy volunteers. All the subjects consented to take part in the study. This project has been reviewed and approved by Committee on Human Rights Related to Researches Involving Human Subjects, Walailak University (No. 52/036). Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll-Hypaque gradients technique and cells were suspended in complete media. PBMCs were then seeded at a density of 5.0×10^4 cells/cm^2 and incubated at 37°C in humidified 5% CO_2 atmosphere for cytotoxicity test.

CYTOTOXICITY TEST

CLS-354 cells and PBMCs were treated with crude methanolic extracts at various concentrations. For vehicle control, cells were treated with varied volumes of DMSO. Cells were incubated for 24 h at 37°C in humidified 5% CO_2 atmosphere. After the treatment, spent medium was removed and replaced with complete RPMI-1640 for overnight. Cell viability was performed by MTT assay based on the conversion of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals by mitochondrial dehydrogenases from viable cells. Briefly, MTT was dissolved in cultured medium to a final concentration of 0.5 mg/mL. After spent medium was removed, 500 µL of MTT solution was added into each well and incubated at 37°C in CO_2 incubator for 4 h. The solution was removed and 500 µL of DMSO was then added to dissolve the formazan crystals. Absorbance was read at 560 nm with subtraction of background at 670 nm using a microplate reader.

APOPTOSIS ASSAY

Cells at a density of 2.4×10^4 cells/cm^2 were incubated with Tamarind seed extract at 75 µg/mL for 24 h. All adherent and floating cells were harvested and washed twice with PBS before transferred to a sterile centrifuge tube. The cell pellet was then suspended in binding buffer at a concentration of 0.5×10^6 cells/mL. A sample of this cell suspension (2×10^5 cells) was transferred to a 5 mL tube, the cells were stained with annexin V-FITC and PI according to the manufacturer's instructions (Roche, Germany). The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Fluorescence intensity was immediately determined by flow cytometry (BD, USA). For each measurement, at least 15000 cells were counted. The results were analysed using CellQuest Pro software.

STATISTICAL ANALYSIS

All measurements were performed in three independent experiment in triplicate. The results were expressed as the mean ± standard deviation. One-Way ANOVA was analyzed by using SPSS version 16.0. Significant differences were considered at $p<0.05$.

RESULTS AND DISCUSSION

Oral carcinogenesis is associated with oxidative stress, induced by the exposure to risk factors, such as tobacco, smoking, alcohol drinking and betel-quid chewing (Ko et al. 1995; Petti 2009; Zain 2001). The increasing risk of oral cancer has been related to low intake of fresh fruits and vegetables (Cartmel 2005; De Stefani et al. 2005). The consumption of fruits and vegetables has been shown to reduce the risk of developing variety of cancers (Kris-Etherton et al. 2002; Surh 2003). Currently, it is of interest that a number of non-edible parts of fruits contain antioxidant compounds that possess anticancer activity (Akhtar et al. 2009; Ka¨hko¨nen et al. 1999; Xiao et al. 2009).

In this study, methanolic extracts from seeds and pericarps of the three selected tropical fruits including Rambutan, Litchi and Tamarind were subjected for their antioxidant and anticancer properties. The percent yield of

methanolic extracts from Tamarind, Litchi and Rambutan were shown in Table 1. The highest yield (12.93%) was obtained from Tamarind seeds, while the lowest yield (5.47%) was obtained from Rambutan pericarps.

Phenolic compounds is purposed to be the major constituents of fruit extracts. Tamarind seed extract contained the highest levels of total phenolic content (501.95 ± 3.40 mg GAE/g) (Table 2) using Folin-Ciocalteau assay. Rambutan pericarp extract showed the lowest levels in phenolic content (104.60 ± 0.87 mg GAE/g), while others showed moderate levels of total phenolic content.

Methanolic fruit extracts potentially possessed free radical scavenging activities, which was an important mechanism of antioxidants. As compared to others, Tamarind seeds extract contained the highest antioxidant activity, which was determined by ABTS assay (1105.49 ±17.45 mg TEAC/g) and DPPH assay (535.85 ± 5.88 mg VCEAC/g) as shown in Table 2. Rambutan pericarp extract showed the lowest antioxidant activities (167.17 ± 6.86 mg TEAC /g and 334.77 ± 1.51 mg VCEAC /g). In this present study, it was found that the amount of total phenolic content was consistent with yield of the extracts and antioxidant activities. This result indicated that phenolic compounds may play an important role in antioxidant capacity of the extracts.

The effects of methanolic extracts from fruit seeds and peels on the growth of CLS-354 and PBMCs were examined by MTT assay. The percent viability of CLS-354 cells exposed to the extracts was decreased in a dose-dependent manner (Figure 1). The Tamarind seed extract had the highest cytotoxicity against CLS-354 cells with the lowest IC_{50} value of 100 μg/mL, followed by Litchi seed extract (135 μg/mL) (Table 3). The extracts from seeds of Tamarind and Litchi showed lower IC_{50} values than that of 5-FU (Table 3) indicating a potential cytotoxicity. Rambutan pericarp, Rambutan seed and Litchi seed showed weak cytotoxicity against CLS-354 cells. Moreover, the effects of the extracts on the growth of human peripheral blood mononuclear cells (PBMCs) were investigated. The extracts from Tamarind seed, Litchi pericarp and Litchi seed did not cause the cytotoxic effect in PBMCs, whereas the extracts of Rambutan seed and pericarp significantly reduced PBMCs viability (p<0.05) (Figure 1). It was indicated that the extracts of Rambutan had toxicity to PBMCs. Among the extracts tested, Tamarind and Litchi seed extracts appeared to be selectively cytotoxic to oral cancer cells and had no cytotoxic effect in normal cells.

Cells in early and late apoptosis have been extensively distinguished by using the combination of annexin V-FITC with propidium iodide. During early apoptosis, phosphatidylserine, which is usually located in the inner membrane of cells, is transported into the outer portion of the membrane and this can be detected by its strong affinity for annexin V-FITC, a phospholipid binding protein. Meanwhile, the dead cells can be detected by the binding of propidium iodide to the cellular DNA in cells where the cell membrane has been damaged. Viable cells were negative for both annexin V and PI (lower left quadrant); early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic displayed positive annexin V and PI (upper right quadrant); necrotic cells were negative for annexin V and positive for PI (upper left quadrant). Treatment with Tamarind seed extract at 75 μg/mL obviously increased the proportion of cells in early apoptosis with values of 11% compared to camptothecin, pro-apoptotic drug with values of 12% (Figure 2). The total apoptosis was

TABLE 1. Yields of methanolic extracts from seeds and pericarps of the three fruits

Extracts	g/g of dry weight	Yield (%, w/w)
Rambutan seed	0.1260	12.60
Rambutan pericarp	0.0547	5.47
Litchi seed	0.0577	5.77
Litchi pericarp	0.1231	12.31
Tamarind seed	0.1293	12.93

TABLE 2. Total phenolic content and free radical scavenging activities of methanolic extracts from the three fruits

Extracts	Phenolic content GAE (mg/g DW)[1]	$ABTS^{+\cdot}$ scavenging TEAC (mg/g DW)[2]	$DPPH^{\cdot}$ scavenging VCEAC (mg/g DW)[3]
Rambutan seed	124.14 ± 3.01[d]	172.09 ± 9.28[d]	383.38 ± 10.03[c]
Rambutan pericarp	104.60 ± 0.87[e]	167.17 ± 6.86[d]	334.77 ± 1.51[d]
Litchi seed	357.82 ± 6.97[b]	837.65 ± 8.63[b]	511.03 ± 1.64[b]
Litchi pericarp	271.72 ± 4.74[c]	529.00 ± 8.41[c]	512.29 ± 0.55[b]
Tamarind seed	501.95 ± 3.40[a]	1105.49 ± 17.45[a]	535.85 ± 5.88[a]

Data are expressed as means ± S.D., n =3. Means with different letters within the same column represent significant differences at p<0.05.
[1] GAE (mg/g DW); Garlic acid equivalent (mg of garlic acid/g dried weight of the extract)
[2] TEAC (mg/g DW); Trolox equivalent antioxidant capacity (mg of trolox/ g dried weight of the extract)
[3] VCEAC (mg/g DW); Vitamin C equivalent antioxidant capacity (mg of vitamin C/ g dried weight of the extract)

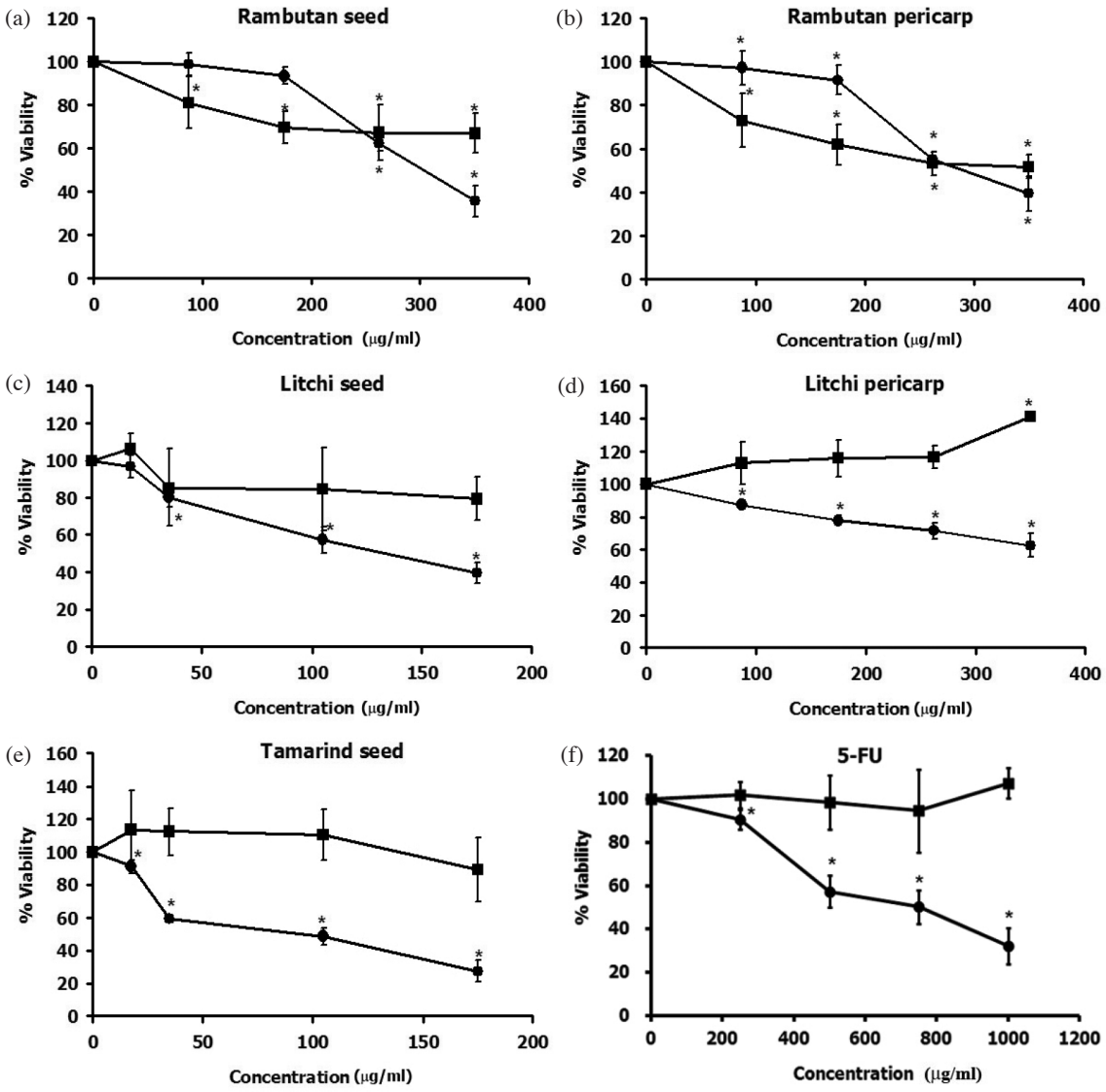

FIGURE 1. Cell viability of CLS-354 cells (•) and PBMCs (■) was determined by MTT assay after the exposure to methanolic extracts of pericarps and seeds of fruits at different concentrations for 24 h. Cells were treated with (a) Rambutan seed extract, (b) Rambutan pericarp extract, (c) Litchi seed extract, (d) Litchi pericarp extract, (e) Tamarind seed extract and (f) 5-FU. The data shown were expressed as the mean ± standard deviation of three-independent experiments in triplicate (n=9)
*, p<0.05 vs 0 μg/mL

TABLE 3. IC_{50} values of methanolic extracts for CLS-354 cells and PBMCs

Extracts	IC_{50} (μg/mL)	
	CLS-354	PBMCs
Rambutan seed	305	>350
Rambutan pericarp	292	>350
Litchi seed	135	>175
Litchi pericarp	> 350	ND
Tamarind seed	100	>175
5-FU	750	ND

IC_{50}: The half maximal inhibitory concentration of the extract (μg/mL), ND; IC_{50} was not detectable

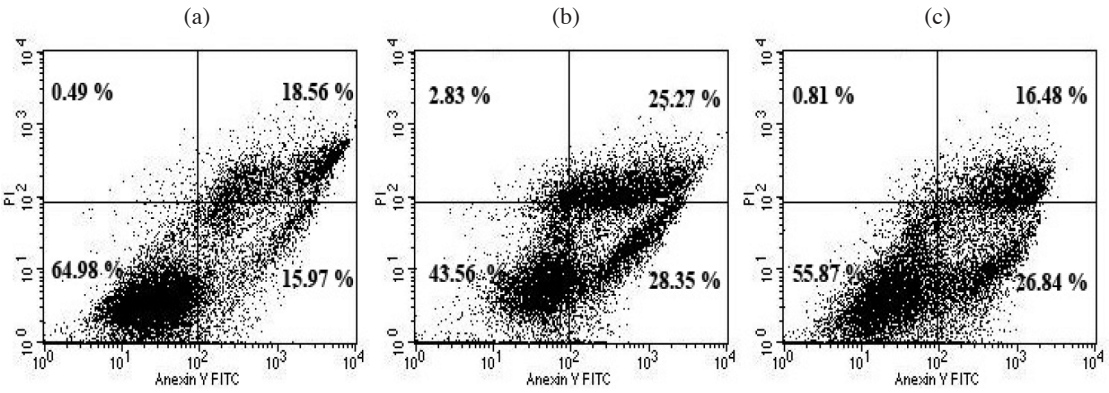

FIGURE 2. Apoptosis assay using flow cytometry after annexin V-FITC and propidium iodide (PI) staining. (a) untreated cells, (b) Camptothecin at 12 mM and (c) Tamarind seed extract at 75 μg/mL

increased up to 43 and 54% in Tamarind seed extract and camptothecin treatments, respectively. No significant apoptosis was demonstrated in Tamarind treated cells with IC_{50} values of 50 μg/mL (data not shown). This result is suggested that the antiproliferative effect of Tamarind seed extract is mediated by the induction of apoptosis.

Polyphenolic compounds are the major bioactive components responsible for antioxidation and antiproliferation. Natural antioxidants have been proved to inhibit tumor growth selectively, because of different redox status between normal cells and cancer cells (Nair et al. 2007). Methanolic extract of Tamarind seed contained many flavonoids such as (+) -catechin, procyanidin B2, epicatechin and polymeric procyanidins (Sudjaroen et al. 2005). These compounds have been demonstrated to inhibit cell growth of many cancer types (Al-Hazzani & Alshatwi 2011; Miura et al. 2008; Wang et al. 2008) and mediate apoptosis induction (Al-Hazzani & Alshatwi 2011; Miura et al. 2008). The changes in cellular redox status has been shown to influence cancer growth through activation of signaling pathways leading to proliferation or apoptosis (Jiang et al. 2011; Owuor & Kong 2002). These polyphenolic compounds may play a role in modulating cellular redox state via regulating signaling pathway mediated apoptotic induction. The major components of Litchi pericarp extract were condensed tannins, epicatechin and procyanidin A2, which possessed anticancer activity on human breast cancer and hepatocellular carcinoma (Wang et al. 2006a, 2006b). Ethanolic extract from Rambutan peel possessed free-radical scavenging power and exhibited non-toxic activity to both PBMCs and Caco-2 cells (Okonogi et al. 2007). Ethyl acetate fraction from Rambutan peel crude extract contained the highest levels of phenolic content (2.28 ± 0.02 GAE mg/mL) among other fractions and possessed antiproliferative property in KB and CaCo-2 cells with non-toxicity to PBMCs (Khonkarn et al. 2010). However, antioxidants and cytotoxic activities in different fruit extract materials were varied among cultivars (Rupasinghe & Clegg 2007), species (Abeysinghe et al. 2007) and extraction methods (Sun et al. 2002).

CONCLUSION

In conclusion, Tamarind seed extract contained the highest levels of total phenolic content and possessed the highest potential antioxidant capacity, but low levels obtained from the extracts from Rambutan seed and pericarp. The amount of total phenolic content in the investigated fruit extracts was related to their antioxidant capacity and cytotoxicity to oral cancer cells. The extracts of Tamarind could inhibit CLS-354 cell growth with the lowest IC_{50} values and had no toxicity in normal PBMCs. Moreover, cytotoxic effect of Tamarind seed extract was mediated by the induction of cells in early and late apoptosis. The materials of these fruits will be a potential source of antioxidant and chemopreventive agents. However, compound identification and mechanism(s) underlying antiproliferation have to be elucidated by our further investigation.

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