Journal of Ethnobiology and Ethnopharmacology

ISSN: 2222-1271
www.ijscience.com

Investigation on Antioxidant, Antimutagenic and Cytotoxic Properties of Active Fractions of Thai Long-Kong (*Lansium domesticum* Corr.) Fruits

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ABSTRACT

Lansium domesticum Corr. (Meliaceae) is an economic plant and widely grown in the Southern, Eastern and Northern parts of Thailand. The fruits of the *L domesticum* (LD) are very popular in Thailand and commonly called "Long-Kong". This study was performed to investigate the biological activity of the *L. domesticum* fruits including anti-oxidant, anti-mutagenic and cytotoxic properties, in particular the skins and seeds which are considered as waste. The air-dried samples of skin (SK) and seed (SD) were extracted with 50% and 95% ethanol. The ethanolic extracts were partitioned between dichloromethane (DCM) and 50% aqueous ethanol. The aqueous phase was further extracted with ethyl acetate (EA) where twelve fractions named LDSK50-DCM, LDSK50-EA, LDSK50-H_2O, LDSK95-DCM, LDSK95-EA, LDSK95-H_2O, LDSD50-DCM, LDSD50-EA, LDSD50-H_2O, LDSD95-DCM, LDSD95-EA and LDSD95-H_2O were obtained. Their anti-oxidant capacity was firstly determined on superoxide anion ($O_2^{-\bullet}$) by photochemiluminescence (PCL) assay both lipid (ACL) and water (ACW) soluble substance systems. The deoxyribose assay was subsequently performed to assess their hydroxyl radical ($OH^{\bullet}$) scavenging activity. Our results suggested the LDSD50-EA fraction possesses a high antioxidant potential in both hydrophilic and lipophilic antioxidant systems. Then, the anti-mutagenic effect of LDSD50-EA fraction was investigated against mitomycin C (MMC) in TK6 human lymphoblasts using cytokinesis-blocked micronucleus (CBMN) assay for 4 and 24 h. The results of CBMN were analyzed on micronucleus (MN) frequencies in binucleated cells (BNC). We found that both treatment schedules (4 and 24 h) did not produce a significant increase in the MN frequency and also nearly to the spontaneous background MN frequency suggesting that LDSD50-EA was non-genotoxic to TK6 cells. The MMC-treated cells clearly exhibited a remarkably increase in MN frequency by 75% indicating its mutagenic activity. Interestingly, a significant reduction in MN frequency was obviously seen when TK6 cells were simultaneously treated with LDSK50-EA (25, 50, 100, and 150 µg/ml) and MMC (0.8µg/ml) for 24 h. Regarding the PCL, deoxyribose assay and CBMN results obtained in this study, we can summarize that LDSK50-EA fraction of *L. domesticum* fruits possesses potent antioxidant property and anti-mutagenic activity.

Keywords: *Lansium domesticum* Corr., antioxidant, PCL, antimutagenic, CBMN

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INTRODUCTION

Free radicals and reactive oxygen species (ROS) play a significant pathological role in human disease (Aruoma, 1995; Moskovitz *et al*., 2002; Curcio *et al*., 2009). Epidemiological evidences have supported the involvement of free radicals and ROS in cardiovascular disease, cancer, neurological disorders, diabetes, ischemia and ageing (Valko *et al*., 2007; Sen *et al*., 2010). Antioxidants prevent the onset of these deadly diseases by neutralizing free radicals and oxidizing them. Fruits and vegetables are good sources of natural antioxidants, such as carotenoids, vitamins, flavonoids, and other phenolics compounds (Sun *et al*., 2009; Girard-Lalancette *et al*., 2009; Yang *et al*., 2009). Over the past two decades, many peer-reviewed publications have demonstrated that daily consumption of fruits and vegetables is associated with reduced risks for chronic degenerative diseases and developing cancers (Steinmetz and Potter, 1996; Hashimoto *et al*., 2002). Therefore, the importance for utilizing antioxidants from plant origin has received much attention.

Recently, the trend to search for natural antioxidants has increased due to customers concern about the safety of synthetic antioxidants. Antioxidant products from natural sources particular fruits and vegetables are very attractive to the food industry and nutraceuticals (Thériault *et al*.,

2009). Various extracts from fruits have been recognized to possess beneficial effects against free radicals in biological systems as natural antioxidants (Guo *et al*., 2003). Many studies have shown positive correlation of the increased dietary intake of natural phenolic antioxidants with the reduced coronary heart disease and cancer mortality, as well as with longer life expectancy (Halliwell, 2007)

Lansium domesticum Corr belongs to the Maliaceae family. It originates from Southeast Asia and is also cultivated in Australia, Sri Lanka, India, and Puerto Rico. Although it is planted sporadically throughout the tropics, most of the commercial production is in Thailand, Malaysia, Indonesia, Philippines, and Vietnam (Lichanporn *et al*., 2009 and Tilaar *et al*., 2008). *L. domesticum* is known under a variety of common names in different countries and languages. In Thailand, the *L. domesticum* fruit is commonly called "long-kong" that is very popular and widely consumed.

The long-kong peels were formerly medicinally used against diarrhea and intestinal spasms, whereas the seeds were an effective remedy for fever and sickness. Previously, antimicrobial and antimalarial properties (Tilaar, 2008) of *L. domesticum* seeds were previously investigated. However, there is still little information on the antioxidant activity of this fruit. Anyhow, our preliminary experiment revealed a high sugar content (predominantly glucose) in the pulp of *L. domesticum* fruits and that this part was excluded from chemically purification step. The peels and seeds of the fruits were chosen in an attempt to identify the fractions with high antioxidant activity for further studies. Since peels and seeds are generally considered as waste, research on their toxicity has been scarce. Thus, we carried out this study to assess the antioxidant property, antigenotoxic and cytotoxic effects of skin and seed parts of *L. domesticum* fruits.

MATERIALS AND METHODS

Preparation of crude extracts

The fresh long-kong fruits of *L. domesticum* Corr. at the mature stage were purchased from the Talad-Thai market located in Bangkok, Thailand. After washing, skin and seeds of the fruits were manually separated, dried at 50ºC in a hot air oven for 48 h and finally were grounded into powder using a blender. The dried powder of skin (SK, 100 g) and seeds (SD, 100 g) were extracted separately with 50% and 95% ethanol. The extracts were then filtered and concentrated using a rotary evaporator. Four crude extracts were obtained and named LDSD50, LDSK50, LDSD95 and LDSK95, representing parts of *L. domesticum* fruits and their ethanolic extraction.

Partition of crude extracts

The four ethanolic extracts (prepared as described above) were partitioned between dichloromethane (DCM) and 50% aqueous ethanol. The obtained aqueous phase (H_2O) was further extracted with ethyl acetate (EA). The partition procedure resulted in a yield of 12 fractions namely; LDSK50-DCM, LDSK50-EA, LDSK50-H_2O, LDSK95-DCM, LDSK95-EA, LDSK95-H_2O, LDSD50-DCM, LDSD50-EA, LDSD50-H_2O, LDSD95-DCM, LDSD95-EA and LDSD95-H_2O. All these fractions were concentrated by a rotary evaporator at 45ºC. Then, all fractions were kept at 4ºC and protected from light until being used.

Antioxidant capacity determination of fractions

Superoxide anion radical ($O_2^{-\bullet}$) scavenging activity by PCL assay

The antioxidant capacity of twelve fractions of skins and seeds of *L. domesticum* fruits were assessed using Photochem® (Analytik Jena, Germany). The measurement was based upon the principle of photochemiluminescence (PCL) where superoxide anion radicals ($O_2^{-\bullet}$) are produced in the system by optical excitation of luminal, which is a photosensitizer substance (Popov, 1999). The antioxidant capacity of fractions was determined by comparing their inhibitory effect on luminescence generation to the standard anti-oxidants. The results were expressed in equivalent units (nmol) of ascorbic acid or trolox units, respectively for the antioxidative capacity of water soluble substances system (ACW) and lipid soluble substances (ACL) system. The *L. domesticum* samples were prepared by dissolving 10 mg of each fraction in 1 ml of reagent 1 of ACL or ACW kits before being sonicated and filtered through a 0.45 mm syringe filter. The filtrates were subjected to antioxidant capacity measurement following protocols provided by the manufacturer.

Hydroxyl radical ($OH^{-\bullet}$) scavenging activity by deoxyribose assay

The hydroxyl radical ($OH^{-\bullet}$) scavenging activity of *L. domesticum* fractions was assessed using the deoxyribose assay (Genaro-Mattos, 2009). The method was based upon determination of malondialdehyde (MDA) pink chromogen, which was a degradation product of 2-deoxyribose (2-DR) sugar by measurement of the condensation product with thiobarbituric acid (TBA). Typical reactions were started by the addition of 50 µM $FeCl_3$ to solutions (0.5 ml final volume), containing 5 mM 2-DR, 100 µM EDTA, 10 mM phosphate buffer (pH 7.2) and 0.5 mM H_2O_2 in presence of 100 µM ascorbic acid (reducing agent). Reactions were carried out for 10 min at room temperature and then stopped by the addition of 0.5 ml 2.8% trichloroacetic acid (TCA), followed by the addition of a 0.5 % TBA solution. After heating for 15 min, the solutions were allowed to cool down to room temperature, and the absorbance was read at 532 nm. The reagent blank contained buffer and 2-DR. Different concentrations of tannic acid (5-80 µg/ml) were used as the standard antioxidant. The inhibitory effect (%I) of deoxyribose degradation was calculated as given equation:

Inhibitory effect (%I)=($Abs_{control}$-Abs_{sample})/$Abs_{control}$ X 100

The IC_{50} value was determined by constructing a dose response curve between %I and concentration of test samples or the standard. The values were presented as means of triplicate analyses.

Cytotoxic determination of active fractions

Only the fractions exhibiting the highest $O_2^{-\bullet}$ scavenging activity of both ACL and ACW systems in

photochemiluminescence (PCL) assay, were chosen for further study on cytotoxic-property by the MTT method. The MTT assay measures the metabolism of 3-(4, 5-dimethylthiazol-2yl)-2, 5 - biphenyl tetrazolium bromide to form an insoluble formazan precipitate, by mitochondrial dehydrogenases, only present in viable cells (Oka, 1992). TK6 cells ($2x10^5$cells/ml) were seeded onto 24 well-plate and treated with *L. domesticum* fractions dissolved in RPMI at final concentrations of 500, 700, 800, 900 and 1,000µg/ml. Cells were incubated for 4 hr at 37ºC in a 5% CO_2 incubator. After treatment, cells were then collected by centrifugation and washed twice with HBSS and re-suspended in 1 ml of 0.625 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The tubes were kept in darkness at 37ºC in a 5% CO_2 incubator for 3 hr. After this incubation period, the crystals fomazans were washed with HBSS and dissolved in 200 µl of dimethyl sulfoxide (DMSO). The amount of formazan was evaluated by measuring absorbance at 540 nm by the micro-plate reader system.

Antimutagenic determination of active fractions

The micronucleus (MN) test was performed according to the cytokinesis-block micronucleus (CBMN) test described by Fenech (2008), with a slight modification. Briefly, TK6 cells (2×10^5cells/ml) were cultured in RPMI containing LDSD50 and LDSD95 at 100, 250, 500 and 1,000 µg/ml in 12-well plates with a total volume of 2 ml each in each well. Simultaneously cells were treated to, as controls, a known mutagen mitomycin C (MMC 1.5 µg/ml), culture medium and a culture medium containing 1% DMSO. After 4 hr of incubation time, cells were washed twice with HBSS, they were then further incubated in fresh RPMI containing cytochalasin B (3 µg/ml) for 18 hr, to allow an accumulation of cells at binucleated (BNC) stage. Cells were prepared onto microscope slides, fixed with cold methanol and stained in 10% Giemsa solution. The incidence of micronuclei was determined after counting 1,000 BNC of each treatment under a light microscope (40x magnification).

Statistical analysis

The computer software program SPSS 10.0 was used to analyze the data. The statistical significance of the effects of *L. domesticum* extracts on micronucleus (MN) frequencies of all concentrations, was analyzed by a one-way ANOVA (one-way analysis of variance). The significant difference between means at level of 0.05 (*p*-value ≤0.05) was considered ~~as~~ to be significant.

RESULTS

PCL assay

It can be seen that all twelve *L. domesticum* fractions including LDSK50-DCM, LDSK50-EA, LDSK50-H_2O, LDSK95-DCM, LDSK95-EA, LDSK95-H_2O, LDSD50-DCM, LDSD50-EA, LDSD50-H_2O, LDSD95-DCM, LDSD95-EA and LDSD95-H_2O~~)~~, exhibited the $O_2^{-\bullet}$ scavenging activity at different degree of activity for both ACL (Fig.1) and ACW (Fig.2) measurement systems. Results of the ACL demonstrated the overall anti-oxidant capacity of twelve fractions ranged from 0.380 to 6.625 nmol of Trolox when all samples were tested at 10 µg/ml concentration. Among these, the LDSK50-EA possessed highest anti-oxidant activity with an equivalent to 6.625 nmol of Trolox whereas other fractions exhibited a slight difference in anti-oxidant capacity (Fig. 1). Interestingly, the anti-oxidant capacity of ACW system (Fig. 2) indicated that 50% ethanol extract of peels (LDSK50) still had high anti-oxidant capacity. A wide range of anti-oxidant capacity of all fractions was found from -0.065 to 98.733 nmol of ascorbic acid when all samples were tested at 10 µg/ml concentration. The highest anti-oxidant activity was found in the fraction of LDSK50-H_2O (98.733 nmol of ascorbic acid) followed by the LDSK50-EA (54.660 nmol of ascorbic acid) (Fig. 2).

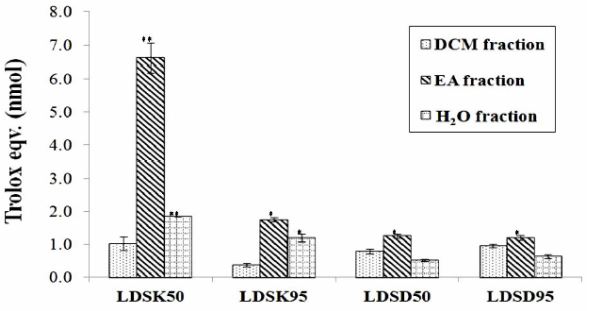

Fig. 1: Lipid-soluble antioxidant capacity (ACL) of twelve *L. domesticum* fractions by PCL assay. Results were expressed as means ± SD (*n*=3). *Significant difference was detected from the lowest activity fraction of same part-extraction ($p\leq0.05$). **Significant difference was detected from all fractions of same part-extraction ($p\leq0.05$).

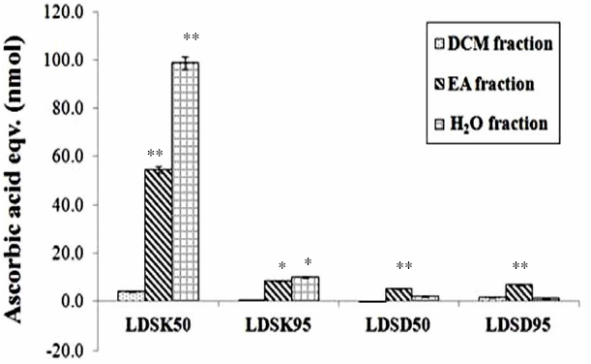

Fig. 2: Water-soluble antioxidant capacity (ACW) of twelve *L. domesticum* fractions by PCL assay. Results were expressed as means ± SD (*n*=3). *Significant difference was detected from the lowest activity fraction of same part-extraction ($p\leq0.05$). **Significant difference was detected from all fractions of same part-extraction ($p\leq0.05$).

Deoxyribose assay

The method is based on the determination of malondialdehyde (MDA) pink chromogen which is a degraded product of deoxyribose damaged by hydroxyl radical ($OH^\bullet$). Inhibitory effect of *L. domesticum* fractions on 2-deoxyribose degradation was determined by measuring the competition between 2-deoxyribose and sample fractions for the $OH^\bullet$ generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The anti-oxidant

activity of $OH^{\bullet}$ scavenging expressed as % inhibition of 2-deoxyribose degradation for the test sample of 0.5, 1.0 and 2.0 mg/ml. As shown in Fig. 3, the results of deoxyribose assay exhibited a wide range of $OH^{\bullet}$ scavenging activity demonstrated from 0.50 ± 0.12 to 93.44 ± 0.84 in % inhibition effect. The maximum inhibitory (%I) effectiveness of LDSK50-H_2O fraction was found up to 93.44 % when tested at 2,000 µg/ml. This effectiveness level was equal to that of tannic acid (reference standard anti-oxidant) at 80 ug/ml concentration (data not shown).

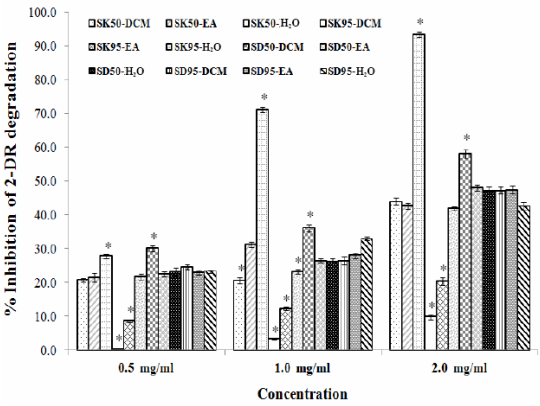

Fig. 3: Inhibitory effect (%I) of deoxyribose degradation of twelve *L. domesticum* fractions by deoxyribose assay. Results were expressed as means ± SD (n=3).* Significant difference was detected from all fractions of same concentrations (0.5, 1.0 and 2.0 mg/ml) ($p \leq 0.05$).

MTT assay

The MTT (methylthiazol tetrazolium) assay was chosen for determining cytotoxic-effect of the two active fractions; LDSK50-H_2O and LDSK50-EA. These two fractions exhibited the greatest antioxidant activity, determined by PCL assay. The MTT assay is a well-established assay and has widely been used to assess mitochondrial competence. Thus valuable information on the presence and absence of cytotoxicity as well as the potentially identification of the cellular site of the toxicity, in case there is evidence of cytotoxicity, will be obtained. The dose causing 50% inhibition, IC_{50}`s of a 4-h post incubation period of LDSK50-EA (Fig. 4) and LDSK50-H_2O (Fig. 5) fractions, was greater than 1,000 µg/ml.

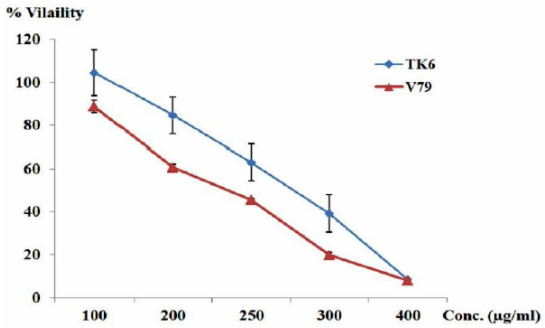

Fig. 4: Concentration-viability curve of LDSK50-EA treated TK6 and V79 cell lines for 24 h expeosure and determined by MTT assay.

We carried out the MTT assay by incubating small amounts of cells in the presence of LDSK50-EA and LDSK50-H_2O in small volume microplate wells. Following incubation, cell viability was determined by measuring their ability to reduce tetrazolium salts into formazan crystals. Under the condition used in our study, it was noticed that V79 cells were more sensitive to LDSK50-EA than TK6 cells (Fig. 4). This observation was supported by the IC_{50} (inhibitory concentration inhibited cell growth by 50%) value of LDSK50-EA in TK6 cells which was 280 µg/ml whereas in V79 cells it was 231 µg/ml. No cell proliferation inhibition activity was found for LDSK50-H_2O. This was evident by % cell viability greater than 80% at concentrations up to 5,000 µg/ml. The values of IC_{50} of LDSK50-H_2O were 4,309 µg/ml inV79 cells and were greater than 5,000 µg/ml in TK6 cells (Fig. 5).

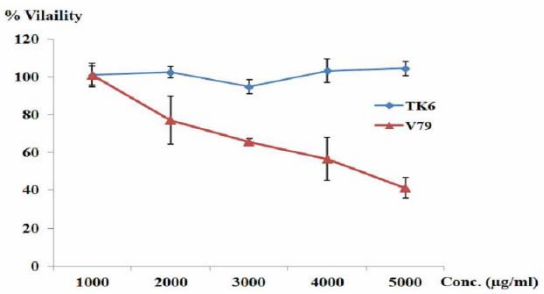

Fig. 5: Concentration-viability curve of LDSK50-H_2O treated TK6 and V79 cell lines for 24 h expeosure and determined by MTT assay.

Micronucleus assay

Micronucleus (MN) is generated during cellular division by late chromosomes or by chromosome fragments, following an exposure to DNA damaging agents. It is an indicator of genotoxicity. We performed this assay (Fig. 6) to determine mutagenic as well as anti-mutagenic activity of the LDSK50-EA fraction only due to its most potent in free radical scavenging property. The micronucleus was conducted on TK6 cells using the cytokinesis-block micronucleus (CBMN) test. Briefly, the cells were simultaneously treated with known mutagen mitomycin C (MMC, 0.8 µg/ml) and LDSK50-EA (25, 50, 100 and 150 µg/ml) for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. The concentrations of LDSK50-EA were designed based upon results obtained from the cytotoxicity test. The results were expressed as number of micronucleated cells (MNC) per 1,000 binucleated cells (BNC) in means ± SE (n=3). The survival rates of TK6 cells of all treatments were monitored and there was focused on survival rates greater than 70% in order to discriminate cytotoxic effect of LDSK50-EA.

Results of CBMN were demonstrated in Fig. 7. The mean value of micronucleus (MN) formation (number of MNC per 1,000 BNC cell scored) of untreated TK6 cells (receiving RPMI) was 11.33 ± 1.86 and 13.00 ± 1.53 respectively for 4 h and 24 hr treatment times. The MN formations were markedly increased to 74.67 ± 2.96 (for 4 h treatment) and 78.67 ± 3.84 (for 24 h treatment) by MMC at 0.8 µg/ml at p<0.05 (ANOVA). Therefore, the

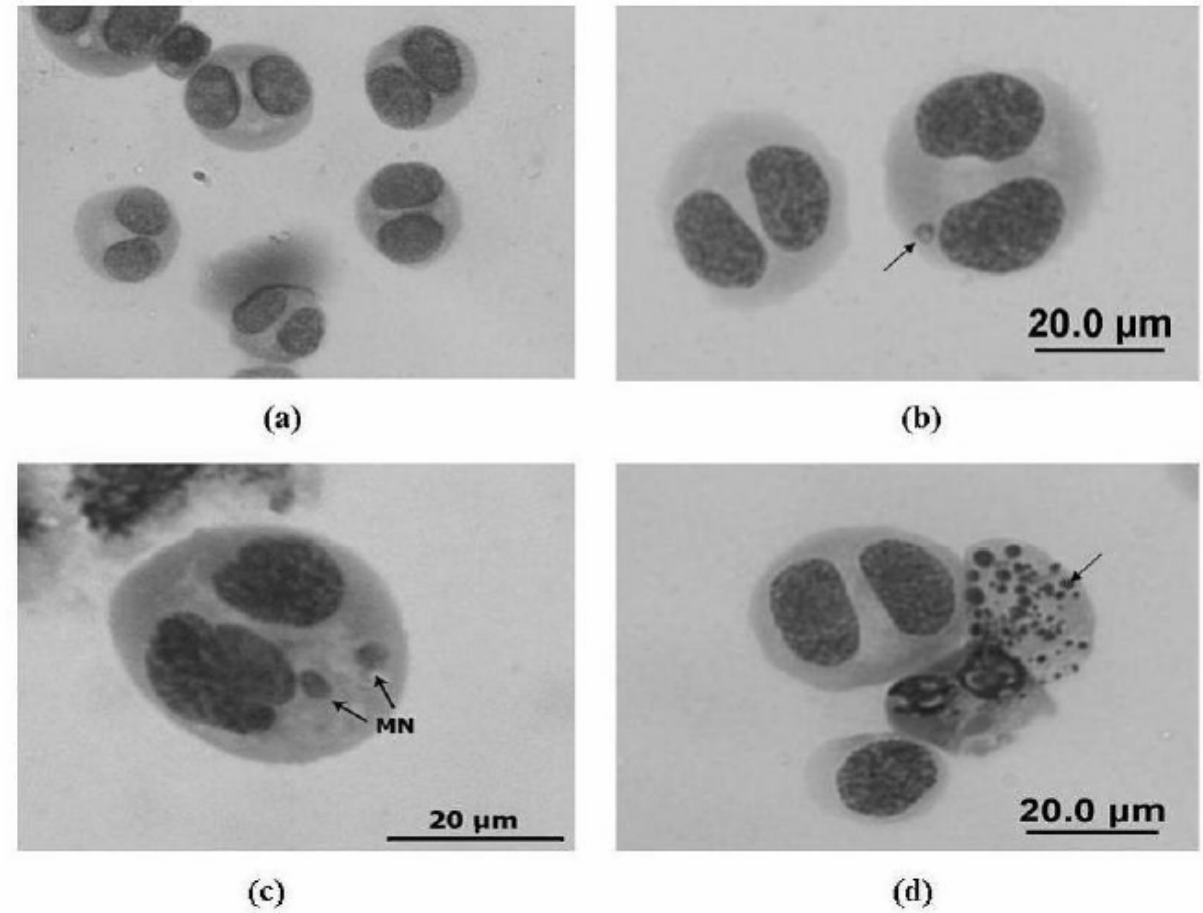

Fig. 6: Micronucleated (MNC) cells of TK6 in the CBMN assay: **(a)** an accumulation of binucleated cells (BNC) following cytochalasin B treatment, (**b**) BNC with one micronuleus (arrow), **(c)** BNC with two micronuclei (arrows), (**d**) apoptotic cell (arrow, not included in micronucleus scoring).

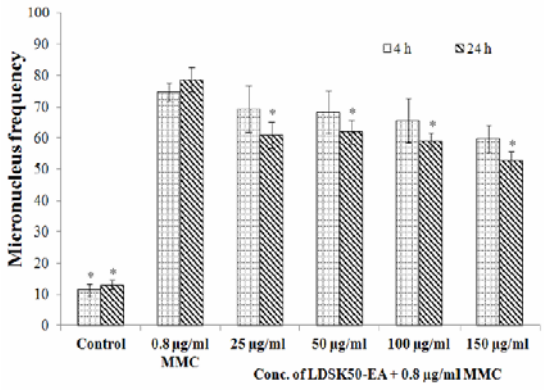

Fig. 7: Micronuclei frequency (number of MNC per 1,000 BNC scored) in TK6 cells after combination treatments of LDSK50-EA (at 25, 50, 100 and 150 µg/ml) and MMC (standard mutagen, 0.8 µg/ml) for 4 and 24 h. Results were expressed as means ± SE values of three independent experiments. *Significant difference was detected from each MMC treatment groups at $p \leq 0.05$ (ANOVA).

reproducibility of the CBMN test was ascertained. Interestingly, the MN formation in BNC was suppressed in the presence of LDSK50-EA. At 24-h treatment time, the suppressive effect of LDSK50-EA against MMC-induced MN formation at concentrations of 25, 50, 100, and 150 µg/ml was 69.33 ± 7.51, 68.33 ± 6.74, 65.67 ± 6.94 and 59.67 ± 4.33 MNC cells for 1,000 BNC cell scored, respectively**.** However, the anti-mutagenic effect of LDSK50-EA in TK cells was not observed at 4 h-treatment time at all concentrations (25, 50, 100 and 150µg/ml) tested. This was indicated by no significant difference in MN frequency of LDSK50-EA treated groups in comparison to the MMC positive control group (p<0.05, ANOVA) **(**Fig. 7).

DISCUSSION

Several epidemiological studies suggest the importance of a high consumption of secondary plant products widely distributed in fruits and vegetables in reducing the incidence of many degenerative diseases (de Kok *et al*., 2008; Comhair & Erzurum, 2010). Parts of peels (skins) and seeds of fruit are considered to be an important source of natural anti-oxidants.

At present, there is an increasing trend to replace synthetic antioxidants, out of safety concern, with natural antioxidants available from plant extracts or isolated products of plant origin. Although Thailand has a variety of fruits, only some of them are widely consumed. Among these, the fruits of the long-kong *Lansium domesticum* Corr. have been very popular in Thailand and many Southeast Asian countries. We carried out this current

study to investigate the biological activity of long-kong fruits. The study focused on certain anti-oxidant mechanisms using the cell-based (anti-oxidative DNA damage activity) and non-cell based systems (ROS scavenging property) as well as toxicity evaluation of active fractions of long-kong fruit extracts.

Among many ROS, the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are considered to be highly reactive chemical species. They can react to every biological molecule such as lipid, polypeptides, protein and DNA. In comparison with many other radicals, $O_2^{-\bullet}$ is unreactive, but it can be converted into highly reactive species such as $HO^{\bullet}$, peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals. Moreover, the dismutation of $O_2^{-\bullet}$ can lead to the formation of H_2O_2 which is the main source of $OH^{\bullet}$ through Haber-Weiss and Fenton reaction (Kohen & Nyska, 2002; Valko *et al*., 2006; Ďuračková & Gvozdjáková, 2008).

Several assays of anti-oxidant capacity determination are available and are differently employed regarding to their principles. In our study, the photochemiluminescence (PCL) was chosen. The PCL could measure the potential anti-oxidant property of *L. domesticum* fractions by two different protocols including ACW and ACL, meant to measure the anti-oxidant capacity of the water and lipid soluble components, respectively (Popov & Lewin, 1999). The anti-oxidant property of compounds is quantified and expressed in equivalent concentration units of ascorbic acid and trolox equivalent for water and lipid soluble systems, respectively (Besco *et al*., 2007).

Regarding PCL results generated in our study, it could be considered that peels of *L. domesticum* fruits possessed $O_2^{-\bullet}$ scavenging activity at a greater level than seeds. The LDSK50-EA (skin extracted with 50% aqueous ethanol and partitioned with ethyl acetate) was considered the most potent $O_2^{-\bullet}$ scavenger. In the other hand, the results of ACL and ACW suggested that the $O_2^{-\bullet}$ scavenger in LDSK50-EA fraction was present in both polar and non-polar phytochemical groups.

Subsequently, we further determined the hydroxyl ($HO^{\bullet}$) radical scavenging activity of *L. domesticum* fractions by the deoxyribose assay, which is another cell-free radical generating system. This assay determined an inhibitory effect of *L. domesticum* fractions on 2-deoxyribose degradation by measuring the competition between 2-deoxyribose and sample fractions for the $OH^{\bullet}$, generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. $OH^{\bullet}$ radicals were formed in the solution were detected by their ability to degrade 2-deoxyribose into fragments that on heating with TBA at low pH form a pink chromogen (Gutteridge & Halliwell, 1988; Okezie, 1994). The absorbance read at the end of the experiment was used for the calculation of the percentage inhibition of 2-deoxyribose degradation by the test samples (Genaro-Mattos *et al*., 2009)

It was postulated that when *L. domesticum* fractions were added to the reaction mixture, they removed $OH^{\bullet}$ from the sugar and prevented the degradation. Hence, the scavenging effect of *L. domesticum* fractions on $OH^{\bullet}$ was determined by monitoring the reduction of deoxyribose degradation. Results would be expressed as % inhibition of 2-deoxyribose degradation. As demonstrated in Fig. 3, upon the presence of *L. domesticum* fractions (0.5, 1.0 and 2.0 mg/ml concentration), a wide range of $OH^{\bullet}$ scavenging activity was found from 0.50 ± 0.12 to 93.44 ± 0.84. The LDSK50-H_2O fraction clearly demonstrated the most effective inhibitor of the $OH^{\bullet}$ by 93.44 ± 0.84. However, the wide range of % inhibition values among various *L. domesticum* fractions was possibly affected by their solubility character in water which was the solvent mostly used in the deoxyribose assay.

The overall anti-oxidant capacity of the *L. domesticum* fractions evaluated by ACL, ACW and deoxyribose assays, revealed the greatest $O_2^{-\bullet}$ and $OH^{\bullet}$ scavenging activity of LDSK50-EA and LDSK50-H_2O. Hence, these two fractions were classified as active fractions in this study. While active fractions possess high anti-oxidant capacity, it should be noted that safety is even more important. Therefore, the MTT assay was performed on LDSK50-EA and LDSK50-H_2O fractions to determine their cytotoxic property. The MTT is a colorimetric assay whose principle is based on the reduction of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide] by cellular mitochondrial dehydrogenase to a purple formazan product (Plumb, 1999; Ozdemir *et al*., 2009). The MTT was conducted on TK6 human lymphoblasts and V79 Chinese hamster lung cells due to their high sensitivity in cytotoxicity and genotoxicity testing (Honma, *et al*., 1997; Hartmann *et al*., 2001; Guillamet *et al*., 2008).

We carried out the MTT assay by incubating small amounts of cells in the presence of LDSK50-EA and LDSK50-H_2O in small volume microplate wells. Following incubation, cell viability was determined by measuring their ability to reduce tetrazolium salts into formazan crystals. Under the condition used in our study, it was noticed that V79 cells were more sensitive to LDSK50-EA than TK6 cells (Fig. 4). This observation was supported by the IC_{50} (inhibitory concentration inhibited cell growth by 50%) value of LDSK50-EA in TK6 cells was 280µg/ml whereas in V79 cells it was 231 µg/ml. According to the classification of the cytotoxicity for natural ingredients (Gad, 1999) the LDSK50-EA could be categorized as potentially harmful substance. Its degree of cytotoxicity suggested that LDSK50-EA could be applied for cancer treatment application. For this purpose, a cytotoxicity assay on cancer cell lines is needed. In contrast to LDSK50-EA, the LDSK50-H_2O (Fig. 5) was classified as potentially non-toxic. No cell proliferation inhibition activity was found for LDSK50-H_2O. This was evident by percent cell viability greater than 80% at concentrations up to 5,000 µg/ml. The values of IC_{50} of LDSK50-H_2O were 4,309 µg/ml in V79 cells and greater than 5,000 µg/ml in TK6 cells.

Results generated by the CBMN assay demonstrated that a combination treatment of LDSK50-EA (25, 50, 100, 200 µg/ml) and MMC (0.8 µg/ml) for 24 h exhibited a clear anti-mutagenic effect in TK6 cells than at a 4 h treatment time. The mean value of micronucleus (MN) formation (number of MNC per 1,000 BNC cell scored) of untreated TK6 cells (receiving RPMI) was 11.33 ± 1.86 and 13.00 ± 1.53 for 4 h and 24 hr treatment times, respectively. This MN formations were markedly

increased to 74.67 ± 2.96 (for 4 h treatment) and 78.67 ± 3.84 (for 24 h treatment) by adding MMC at 0.8 µg/ml at $p<0.05$ (ANOVA). This suggested the mechanism of MMC through DNA cross-link formation which led to the occurrence of chromosome breakage and originated MN formation (Lawley and Phillips, 1996; Tomasz & Palom, 1997; OECD, 2009). Thus, we could call it a substance that is able to produce MN from a lagging acentric chromosome fragment as a clastogen (Lorge *et al*., 2006; Kayani and Parry, 2010).

In the light of our results, the MN formation in BNC was suppressed in the presence of LDSK50-EA **(**Fig. 7). When cells were treated for 24 h (long-term treatment), the suppressive effect of LDSK50-EA against MMC-induced MN formation was found in a dose-dependent manner. The highest anti-mutagenic of LDSK50-EA (59.67 ± 4.33 MNC cells per 1,000 BNC cell scored) was found when cells were treated at 150 µg/ml. However, at the short-term treatment (4 h), this anti-mutagenic effect was not observed. Under the condition used in this study, we found that the survival rates of TK6 cells after short-term (4 h) or long-term (24 h) treatments were greater than 70% in any LDSK50-EA concentrations. However, at higher concentrations (above 150 µg/ml) and for 24 h treatment, a combination of MMC and LDSK50-EA treatment resulted in cytotoxic effect (data not shown).

In order to assure that the treated TK6 cells had one undergone nuclear division (mitosis) during the experiment, we investigated the cytokinesis block proliferation index (CBPI) values in this study. The average CBPI of TK6 cells of RPMI treatment (negative control) for 4 and 24 h treatments were 1.91 and 1.90, respectively. On the other hand, the average CBPI of MMC (positive control) for 4 and 24 h treatments were reduced to 1.31 and 1.29, respectively. Similarly, the combination treatment of LDSK50-EA plus MMC exhibited CBPI value similar to MMC per se, was significantly lower than the CBPI value of the control group ($P\leq0.05$, ANOVA). This phenomenon suggested that the appearance of MMC interferes with the cell proliferation. This might confirm the clastogenic mechanism of MMC which leads to damage to the cell and is expressed as the reduction of BNC cell or cell division (Fenech, 2000; Fowler *et al*., 2010).

Regarding results of our study, it just corroborates stronger this hypothesis; the capacity of LDSK50-EA in binding to DNA damage inducing agents (MMC). A decisive fact for this hypothesis is the suppression of MN induced by MMC verified for the TK6 cells in the simultaneous treatments. It may be possible that the anti-mutagenic property of LDSK50-EA might be due to the scavenging effect to react with alkyl radical or due to blocking cross-link between MMC and DNA. However, it is still premature to conclude the study with respect to the mechanism of action of LDSK50-EA. It is necessary to obtain a better understanding of how this fraction of long-kong interacts with DNA or other possible mechanisms involved in DNA replication and repair, so that its anti-mutagenic capacity can be considered. To explain this, we are currently conducting anti-oxidative DNA damage experiment on LDSK50-EA using the single cell gel electrophoresis (SCGE) or comet assay. Several studies exhibited a close relationship between antioxidant activities and total phenolic content (Liu *et al*., 2008; Singh and Rajini, 2004). Thus, further investigations into the identification of phenolic compounds present in this *L. domesticum* active fraction will be undertaken by us to better elucidate its antioxidant activity.

Conclusion

This study generates new and updated information on biological activity of skins (peels) of long-kong *L domesticum* Corr. fruits that has not yet been published before. The fruitful results on free radical ($O_2^{-\bullet}$ and $OH^{\bullet}$) scavenging activity, non-genotoxic and anti-mutagenic property, will promote and strengthen utilization of *L. domesticum*. Also, it may lead to the discovery of a new candidate or an alternative substance used for anti-mutagenic and anti-oxidative stress. It will be beneficial for the utilization of natural substances from Thai fruits as a health promoter.

Acknowledgements

The study was funded during 2009-2012 by the Ministry of Science and Technology. It was carried out in collaboration between the Thailand Institute of Scientific and Technological Research (TISTR) and the Faculty of Pharmacy, Mahidol University.

REFERENCES

Aruoma I, 1995. Nutrition and health aspects of free radicals and antioxidants. Food Chem Toxicol, 32: 671–683.

Ballantyne B and J Cawley, 1999. Toxicology update. J Appl Toxicol, 19: 291-294.

Besco E, E Braccioli, S Vertuani, P Ziosi, F Brazzo, R Bruni and S Manfredini, 2007. The use of photochemiluminescence for the measurement of the integral antioxidant capacity of baobab products. Food Chem, 102: 1352-1356.

Comhair A and C Erzurum, 2010. Redox control of asthma: molecular mechanisms and therapeutic opportunities. Antioxid Redox Signal, 12: 93-124.

Curcio M, F Puoci, F Iemma, I Parisi, G Cirillo, G Spizzirri and N Picci, 2009. Covalent Insertion of Antioxidant Molecules on Chitosan by a Free Radical Grafting Procedure. J Agric Food Chem, 57: 5933-5938.

de Kok T, S van Breda and M Manson, 2008. Mechanisms of combined action of different chemopreventive dietary compounds. Eur J Nutr, 47: 51-59.

Ďuračková Z and A Gvozdjáková, 2008. Oxidants, Antioxidants and Oxidative Stress Mitochondrial Medicine. In A. Gvozdjáková (Ed.), Springer Netherlands, pp:19-54.

Fenech M, 2000. The *in vitro* micronucleus technique. Mutat Res, 455: 81-95.

Fenech M, P Chang, M Kirsch-Volders, N Holland, S Bonassi and E Zeiger, 2008. Human project: detailed description of the scoring criteria for the cytokinesis block micronucleus assay using isolated lymphocyte cultures. Mutat Res, 534: 65-75.

Fowler P, J Whitwell, L Jeffrey, J Young, K Smith and D Kirkland, 2010. Etoposide; colchicine; mitomycin C

and cyclophosphamide tested in the in vitro mammalian cell micronucleus test (MNvit) in Chinese hamster lung (CHL) cells at Covance laboratories; Harrogate UK in support of OECD draft Test Guideline 487.

Gad C, 1999. Alternatives to *in vivo* studies in toxicology, in: Ballantyne B, Marrs C and TLM Syversen (editors), General and applied toxicolog*,* London. New York, NY: Macmillan, Vol.1, pp. 178.

Genaro-Mattos C, T Dalvi, G Oliveira, S Ginani and M Hermes-Lima, 2009. Reevaluation of the 2-deoxyribose assay for determination of free radical formation. Biochim Biophys Acta, 1790: 1636-1642.

Girard-Lalancette K, A Pichette and J Legault, 2009. Sensitive cell-based assay using DCFH oxidation for the determination of pro- and antioxidant properties of compounds and mixtures: Analysis of fruit and vegetable juices. Food Chem*,* 115: 720–726.

Guillamet E, A Creus, M Farina, E Sabbioni, S Fortaner and R Marcos, 2008. DNA-damage induction by eight metal compounds in TK6 human lymphoblastoid cells: Results obtained with the alkaline comet assay. Mutat Res, 654: 22-28.

Guo C, J Yang, J Wei, Y Li, J Xu and Y Jiang, 2003. Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. Nutr Res, 23: 1719-1726.

Gutteridge M and B Halliwell, 1988. The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production. Biochem J, 253: 932-933.

Halliwell B, 2007. Dietary polyphenols: good, bad, or indifferent for your health? Cardiovasc Res, 73: 341–347.

Hansen L, H Vehof, O Dragsted, A Olsen, J Christensen, K Overvad and A Tjonneland, 2009. Fruit and vegetable intake and serum cholesterol levels: a cross-sectional study in the diet, cancer and health cohort. J Hort Sci Biotech, 42–46.

Hartmann A, E Kiskinis, A Fjällman and W Suter, 2001. Influence of cytotoxicity and compound precipitation on test results in the alkaline comet assay. Mutat Res, 497: 199-212.

Hashimoto K, S Kawamata, N Usui, A Tanaka and Y Uda, 2002. *In vitro* induction of the anticarcinogenic marker enzyme, quinone reductase, in human hepatoma cells by food extracts. Cancer Lett, 180: 1–5.

Honma M, M Hayashi and T Sofuni, 1997. Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells. Mutat Res, 374: 89-98.

Kayani A, and M Parry, 2010. The *in vitro* genotoxicity of ethanol and acetaldehyde. Toxicology in vitro : an international journal published in association with BIBRA, 24: 56-60.

Kohen R and A Nyska, 2002. Invited Review: Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification. Toxicol Pathol, 30: 620-650.

Lawley D and H Phillips, 1996. DNA adducts from chemotherapeutic agents. Mutat Res, 355: 13-40.

Lichanporn I, V Srilaong, C Wongs-Aree and S Kanlayanarat, 2009. Postharvest physiology and browning of longkong (Aglaia dookkoo Griff.) fruit under ambient conditions. Post Biol Tech*,* 52: 294-299.

Liu Y, Qiu X, H Ding and Q Yao, 2008. Polyphenols contents and antioxidant capacity of 68 Chinese herbals suitable for medical or food uses. Food Res Int, 41: 363–370.

Lorge E, V Thybaud, J Aardema, J Oliver, A Wakata, G Lorenzon and D Marzin, 2006. SFTG international collaborative study on in vitro micronucleus test I. General conditions and overall conclusions of the study. Mutat Res, 607: 13-36.

Moskovitz J, B Yim and B Chock, 2002. Free radicals and diseases. Arch Biochem Biophys, 397: 354–359.

OECD, 2009. Guildline for the testing of chemicals draft proposal for a new Guideline 487: *In Vitro* Mammalian Cell Micronucleus Test (MNvit). Retrieved 3 March 2012, from Draft test guideline http://www.oecd.org/dataoecd/45/51/43996258.pdf

Oka M, S Maeda, N Koga, K Kato and T Saito, 1992. A modified colorimetric MTT assay adapted for primary cultured hepatocytes: Application to proliferation and cytotoxic assay. Biosci Biotechnol Biochem, 56:1472-1473.

Okezie A, 1994. Deoxyribose assay for detecting hydroxyl radicals. In: P Lester (editor), Methods in Enzymology, Academic Press, pp: 57-66.

Ozdemir G, H Yilmaz and S Yilmaz, 2009. *In vitro* evaluation of cytotoxicity of soft lining materials on L929 cells by MTT assay. J Biomed Mater Res, 90: 82-86.

Plumb A, 1999. Cell sensitivity assays : the MTT assay. Methods Mol Med*,* 28: 25-30.

Popov I and G Lewin, 1999. Antioxidative homeostasis: characterization by means of chemiluminescent technique. Methods Enzymol*,* 300: 437-456.

Sanchez-Moreno C, 2002. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci Technol Int, 8: 121-137.

Sen S, R Chakraborty, C Sridhar, R Reddy and B De, 2010. Free radicals, antioxidants, diseases and phytomedicine: Current status and future prospect. Int J Pharml Sci Rev Res, 3: 91-100.

Singh N and S Rajini, 2004. Free radical scavenging activity of an aqueous extract of potato peel. Food Chem, 85: 611–616.

Steinmetz A and D Potter, 1996. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc, 96: 1027–1039.

Sun P, C Chou and C Yu, 2009. Antioxidant activity of lactic-fermented Chinese cabbage. Food Chem, 115: 912–917.

Thériault M, S Caillet, S Kermasha and M Lacroix, 2009. Antioxidant, antiradical and antimutagenic activities of phenolic compounds present in maple products. Food Chem*,* 98: 490–501.

Tilaar M, W Wong, S Ranti, M Wasitaatmadja and D Junardy, 2008. Review of *Lansium domesticum* Corrêa and its use in cosmetics. Bol Latinoam Caribe Plant Med Aromaticas, 7: 183-189.

Tomasz M and Y Palom, 1997. The mitomycin bioreductive antitumor agents: Cross-linking and alkylation of DNA as the molecular basis of their activity. Pharmacol Ther, 76: 73-87.

Valko M, D Leibfritz, J Moncol, T Cronin, M Mazur and J Telser, 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol, 39: 44-84.

Valko M, J Rhodes, J Moncol, M Izakovic and M Mazur, 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact, 160: 1-40.

Vinson A, J Jang, A Dabbagh, M Serry and S Cai, 1995. Plant polyphenols exhibit lipoprotein-bound antioxidant activity using an in vitro oxidation model for heart disease. J Agric Food Chem, 43: 2798–2799.

Yang J, E Martinson and H Liu, 2009. Phytochemical profiles and antioxidant activities of wine grapes. Food Chem, 116: 332–339.