RESEARCH PROJECT NO. 52-04 / SUB. NO. 2 / REP. NO. 1 (FINAL REPORT)

RESEARCH ON CHEMICAL CONSTITUENTS AND THEIR BIOLOGICAL PROPERTIES OF LONG-KONG FRUITS

THAILAND INSTITUTE OF SCIENTIFIC AND TECHNOLOGICAL RESEARCH

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by

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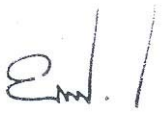

(Mr. Yongvut Saovapruk)

Governor

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การวิจัยทางเคมีและฤทธิ์ทางชีวภาพของสารสำคัญที่แยกได้จากผลลองกอง

ประไพภัทร คลังทรัพย์, ศรัญญา เหล่าวิทยางค์กูร, ธัญชนก เมืองมั่น, อุบล ฤกษ์อ่ำ, ภูษิตา วรรณิสสร, จีรายุ ทองดอนเอ, กฤติยา ทิสยากร, สรียา เรืองพัฒนพงศ์, ศรีศักดิ์ ตรังวัชรกุล และ ชุลีรัตน์ บรรจงลิขิตกุล

บทคัดย่อ

ลองกอง ชื่อวิทยาศาสตร์คือ *Lansium domesticum* Corr. อยู่ในวงศ์ Meliaceae เป็นพืชเศรษฐกิจที่ปลูกมากทางภาคใต้ของประเทศและเป็นผลไม้ที่คนไทยนิยมบริโภคอย่างมาก. วัตถุประสงค์ของโครงการวิจัยนี้เพื่อศึกษาองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของสารสกัดจากส่วนต่างๆ ของผลลองกอง ได้แก่ ฤทธิ์ต้านอนุมูลอิสระ, ฤทธิ์ต้านการก่อกลายพันธุ์ และความเป็นพิษต่อเซลล์. เตรียมสารสกัดจากผงแห้งจากเปลือกและเมล็ดของผลลองกอง โดยการสกัดด้วยแอลกอฮอล์ที่ความเข้มข้นร้อยละ 50 และ 95. นำไปสกัดแยกต่อโดยใช้ไดคลอโรมีเทนและตามด้วยเอทิลแอซีเทต ซึ่งทำให้ได้สารสกัดจำนวน 12 ตัวอย่าง. เมื่อทดสอบความเป็นพิษต่อเซลล์ด้วยเทคนิค MTT assay พบว่า ไม่ก่อความเป็นพิษทั้งในเซลล์มนุษย์ (TK6 cells) และเซลล์สัตว์ (V79 cells). จากนั้น นำไปทดสอบฤทธิ์ต้านอนุมูลอิสระซูเปอร์ออกไซด์ ($O_2^{-\bullet}$) ด้วยเทคนิค photochemiluminescence (PCL) ซึ่งสามารถตรวจสอบได้ทั้งสารต้านอนุมูลอิสระที่อยู่ในกลุ่มที่ละลายน้ำและละลายในไขมัน. จากนั้น ทดสอบฤทธิ์ต้านอนุมูลอิสระไฮดรอกซี ($OH^{-\bullet}$) และไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ด้วยเทคนิค deoxyribose assay และ comet assay ตามลำดับ. ผลการทดสอบบ่งชี้ว่า ตัวอย่างชั้นเอทิลแอซีเทตของสารสกัดจากเปลือกลองกองด้วย 50% เอทานอล (LDSK50-EA) มีฤทธิ์ต้านอนุมูลอิสระสูงที่สุด. โดยออกฤทธิ์ทั้งในกลุ่มที่มีคุณสมบัติละลายในน้ำและละลายในไขมัน. เมื่อนำไปทดสอบฤทธิ์ก่อกลายพันธุ์ด้วยเทคนิค micronucleus assay พบว่า สารสกัดลองกองดังกล่าว นอกจากจะไม่มีคุณสมบัติเป็นสารก่อกลายพันธุ์แล้ว ยังสามารถต้านการกลายพันธุ์ในดีเอ็นเอในเซลล์เม็ดเลือดขาวมนุษย์ (TK6, ATCC CRL-8015) โดยใช้ mitomycin C (MMC) เป็นตัวเหนี่ยวนำ. จากนั้น ได้ตรวจสอบกลุ่มสารสำคัญในตัวอย่างสารสกัดที่มีฤทธิ์ต้านอนุมูลอิสระดังกล่าวด้วยเทคนิค Thin Layer Chromatography (TLC) พบสารสำคัญคือ scopoletin (R_f 0.44), สาร rutin (R_f 0.34) และสาร chlorogenic (R_f 0.49). ผลการวิจัยครั้งนี้ เป็นประโยชน์ในการพัฒนาต่อยอดเป็นผลิตภัณฑ์เพื่อสุขภาพจากสารสกัดลองกอง เช่น ผลิตภัณฑ์เสริมอาหาร, ผลิตภัณฑ์เครื่องสำอางจาก LDSK50-EA, ซึ่งเป็นสารสกัดลองกองที่มีฤทธิ์เด่นในการต้านอนุมูลอิสระ, ไม่มีคุณสมบัติก่อกลายพันธุ์ และไม่เป็นพิษในเซลล์มนุษย์และเซลล์สัตว์ทดลอง.

RESEARCH ON CHEMICAL CONSTITUENTS AND THEIR BIOLOGICAL PROPERTIES OF LONG-KONG 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 *L domesticum* (LD) are very popular in Thailand and commonly called "Long-Kong". This study was performed to investigate the biological activities of the fruits including anti-oxidant, anti-mutagenic and cytotoxic effects. The dried powder of skins (SK) and seeds (SD) of the long-kong fruits 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) which twelve fractions were obtained. Their anti-oxidant capacities were firstly determined on superoxide anion ($O_2^{-\bullet}$) radicals by photochemiluminescence (PCL) assays of both lipid (ACL) and water (ACW) soluble substance systems. Subsequently, the deoxyribose assays and comet tests were performed to assess their hydroxyl radical ($OH^{-\bullet}$) and hydrogen peroxide (H_2O_2) scavenging activities, respectively. Among 12 fractions, the results revealed a high antioxidant potential of the LDSK50-EA fraction in both hydrophilic and lipophilic antioxidant systems. Moreover, its anti-mutagenic effect was investigated against mitomycin C (MMC) in TK6 human lymphoblasts using cytokinesis-blocked micronucleus (CBMN) assay. Lastly, thin layer chromatography (TLC) technique was conducted in order to identify some phytochemical substances. The TLC results exhibited the presence of scopoletin (R_f 0.44), rutin (R_f 0.34) and chlorogenic (R_f 0.49) in LDSK50-EA fraction. The fruitful results generated in this study led to the development of healthy products *i.e.* healthy drinks and cosmetics containing the active antioxidant and antimutagenic *L domesticum* fraction.

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CHAPTER I

INTRODUCTION

Nowadays, the trend of using natural anti-oxidants has markedly increased due to the concern about the safety of synthetic anti-oxidants. Consequently, fruit is considered to be an important source of natural anti-oxidant, especially part of peel (skin) and seed which become wastes unless recycled or applied to use. Thailand has a variety of fruits; however, only some of them are widely consumed. Among these, fruit of *Lansium domesticum* Corr. or "longkong" has been very popular in Thailand and surrounding countries in Southeast Asia. It belongs to the Meliaceae family and is known in numerous common names such as langsat, duku or kokosan in Indonesia or as langsat, lansa, langseh or langsep in Malaysia. In the Philippines it is known as lansones and as bòn-bon in Vietnam (Tilaar *et al*. 2008). The well-known and economic fruit longkong is much cultivated in Thailand peninsular, especially in the southern region. Longkong develops between 15 and 25 fruits per bunch with little non-sticky sap on the skin. The appearance of longkong fruit is globular in shape with an average size of 1.2-2.4 inches in diameter. It has a soft and rough skin. It is almost seedless with five segments of white translucent flesh.

The bark of *L. domesticum* is used traditionally as an anti-malarial remedy by the native people of Borneo (Leaman *et al*. 1995). The leaves have been used by indigenous people in the Philippines for the control of mosquitoes (Monzon *et al*. 1994). Previous phytochemical studies on peels and seeds of *L. domesticum* had several types of triterpenoids (Tanaka *et al*. 2002). Saewan *et al.* (2006) reported anti-malarial activity of tetranortriterpenoids, domesticulide A, C, B and other triterpenoids isolated from the seeds of *L. domesticum*. The peel of this fruit is traditionally known to be toxic to domestic animals. Phytochemical investigations of the peels revealed the presence of triterpene glycosides and seco-onoceranoids such as lansic acid (Mayanti *et al*. 2011). Moreover, numerous researches have

reported the anti-oxidant property of this fruit peels in many models, such as the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, the tyrosinase activity, the iron (III) reducing assay, the ABTS (2,2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid]) assay, etc (Huang *et al*. 2010 and Okonogi *et al*. 2007).

However, there is little information concerning the biological activity particularly anti-oxidant activity of peels and seeds of longkong fruits. Therefore, this study was carried out with following objectives:

- To prepare the fraction extracts from peels and seeds of longkong fruits using ethanol extraction and sub-fractionation procedure.
- To determine free radical scavenging activities of the fraction extracts.
- To evaluate the cytotoxic property of the fraction extracts, particularly those fraction extracts yielded strong activities in anti-oxidant assays, called active fraction(s).
- To determine anti-oxidative DNA damage activity of the active fraction(s).
- To determine anti-mutagenic property of the most active fraction that possessed highest anti-oxidative DNA damage activity.
- To analyze the presence of phytochemical contents of the most active fraction.

For sample preparation, peels and seeds of longkong fruits were dried and extracted with 50% and 95% ethanol by maceration method and further fractionated with ethyl acetate (EA) and dichloromethane (DCM) where 12 fractions were obtained. The free radical scavenging screenings in these 12 fractions were performed using photochemiluminescence (PCL) and deoxyribose assays which were respectively applied for superoxide anion radical ($O_2^{-\bullet}$) and hydroxyl radical ($OH^{\bullet}$) scavenging activities. As of PCL system, $O_2^{-\bullet}$ radicals were generated about 1,000 times greater than that occurred in normal cells by optical excitation of

photosensitizer substance (luminol). These $O_2^{-\bullet}$ radicals were partially eliminated by reaction with the anti-oxidants in the test samples. Then, the remaining $O_2^{-\bullet}$ radicals caused the detector substance to emit luminescence. Therefore, the anti-oxidant capacities of samples could be determined by their inhibitory effect on luminescence generation. The results of PCL assays indicated the $O_2^{-\bullet}$ radical scavenging activity of 12 fractions of longkong fruit extracts both obtained from the lipid and water soluble solvent systems possessed anti-oxidant capacities.

The principle of deoxyribose assay is based on the determination of malondialdehyde (MDA) pink chromogen which is a degradation product of 2-deoxyribose (2-DR). The $OH^{\bullet}$ is formed in the reaction between iron (III)-EDTA and H_2O_2 in the presence of ascorbic acid (reducing agent) and attacking the deoxyribose sugar to form products that on heating with thiobarbituric acid (TBA) at low pH yields a pink chromogen which can be determined using a spectophotometer at 532 nm absorbance. Thus, longkong extract fractions that possessed $OH^{\bullet}$ scavenging activity could inhibit the deoxyribose degradation and yield less chromogen than the one absent of anti-hydroxyl radical activity.

On the next step, the highly anti-oxidant activity fractions (active fractions) were selected for further study on their cytotoxic property, the MTT assay whose principle is based upon the quantitative colorimetric method for determining cell proliferation. Surviving cell numbers are determined indirectly by MTT dye reduction. The MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium-bromide) is a yellow water soluble tetrazolium dye that is reduced by mitochondrial dehydrogenase in live but not dead cells to a purple formazan product that is insoluble in aqueous solutions. The amount of MTT-formazan formation can be determined spectrophotometrically after being solubilized in a suitable solvent such as dimethylsulfoxide (DMSO). In the present study, MTT was carried out on two cell lines including TK6 human lymphoblasts (ATCC CRL-8015) and V79 Chinese hamster lung cells (ATCC CCL-93).

Subsequently, the active fractions were subjected to anti-oxidative DNA damage activity against ROS (H_2O_2) in TK6 cells by comet assay or Single Cell Gel Electrophoresis (SCGE). This assay is considered to be a very sensitive and can detect several types of DNA damage including single strand break (SSB), double strand break (DSB), alkali-labile site (ALS) of DNA and incomplete excision repair sites at an individual cell. The principle of comet assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field which exhibits the denatured DNA fragments migrating out of the cell nucleus.

In this study, TK6 cells were pre-treated with various concentrations of longkong active fractions and then, exposed to H_2O_2 for oxidative DNA damage induction. The treated cells were then mixed with low melting point (LMP) agarose and spread onto a microscope glass slide pre-coated with normal melting point (NMP) agarose gel. Following the lysis treatment of cells (whole glass slides) with detergent at high alkali salt concentration, DNA unwinding and electrophoresis were carried out at pH 13. When subjected to an electric field, the DNA migrated out of the nucleus of cells in the direction of the anode. The comet results were quantitatively analyzed in real time under a fluorescence microscope equipped with a camera couple device (CCD) and connected to a computer where Comet III software (Perceptive Instrument, UK) was established. The resultant images obtained look like a ‘‘comet” with a distinct head consisting of intact DNA, and a tail which contains damaged or broken pieces of DNA. The more DNA (single and double-strand) breaks produced, the greater in fluorescent intensity of the comet tail relative to the head occurred. The anti-oxidative DNA damage activity of active fractions against H_2O_2 was indicated by a significant reduction in DNA damage parameters either the tail length or tail moment (TM) values.

For anti-mutagenic activity, the best longkong active fraction was performed using cytokinesis-block micronucleus assay (CBMN) on TK6 cells following the protocol described by Fenech *et al*. (2003) with some modifications. The CBMN was developed to ensure that analyzed cells completed only one nuclear division by addition of the actin polymerisation inhibitor cytochalasin-B during targeted mitosis, resulting in the accumulation of dividing cells at the binucleated stage. Micronucleus frequencies are then evaluated and scored in only binucleated (BN) cells under light microscopy. Any samples inhibit this type of damage can be classified as anti-mutagenic agents. The standard mutagen, mitomycin C (MMC) was used as the positive control to promote micronucleus formation and the anti-mutagenic activity was measured following adding longkong sample fractions to inhibit mutagenesis induced by MMC.

Lastly, thin layer chromatography (TLC) technique was conducted to primarily identify some phytochemical substances of the longkong fractions that possess the highly active anti-oxidant, anti-oxidative DNA damage and anti-mutagenic activities. Then, the total phenolic content (TPC) of most active fractions was determined using Folin-Ciocalteu reagent and the total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method.

This study generated new and updated information on biological activity of longkong fruits that has not yet been published before. The fruitful results on non-cytotoxicity, free radical scavenging, anti-oxidative DNA damage, anti-mutagenic activities will promote and strengthen utilization of *L. domesticum* and may lead to a discovery of new alternative source of natural anti-oxidant and anti-mutagenic substances for disease treatment and health product industry.

CHAPTER II

MATERIALS AND METHODS

2.1 Sample preparation and extraction

Mature fruits of *L. domesticum* or longkong (Figure 2.1) used in this study were purchased from Talad-Thai market in Prathum Thani, Thailand.

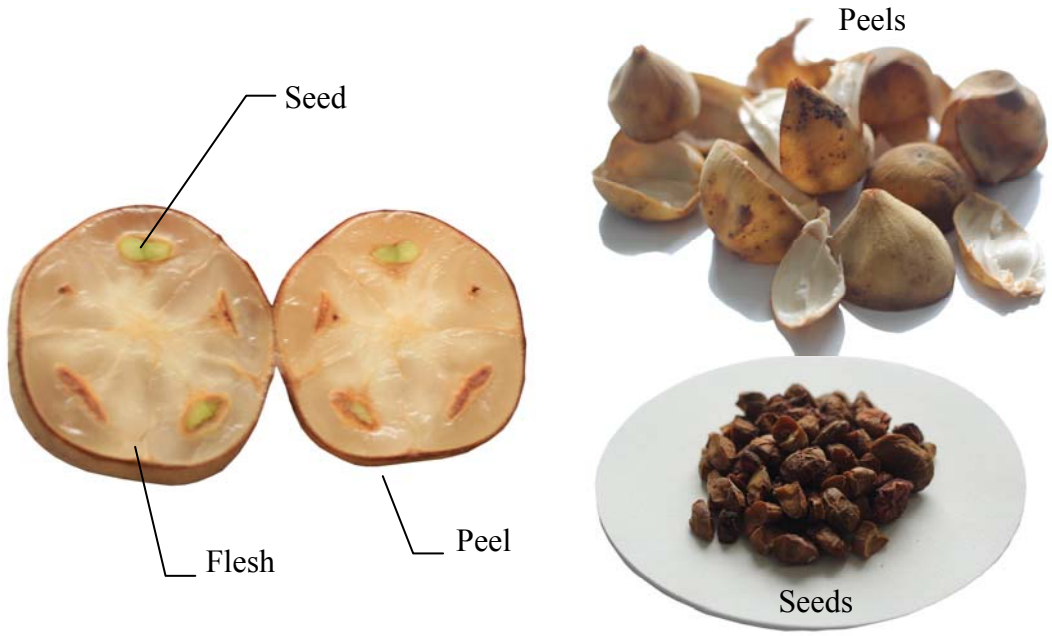


Figure 2.1 Illustration of flesh, peels and seeds of longkong fruits.

After washing, peels or skins (SK) and seeds (SD) of the fruits were separated and air-dried at 50°C in hot air oven for 1-2 days until their weights unchanged. Each dried sample was then, ground into very small pieces with an electrical grinder and stored at room temperature. The grounded samples were extracted with 50% or 95% (v/v) ethanol by maceration method. Firstly, 100 g. of either fine air-dried peels and seeds were mixed with 300 ml. of 50% or 95% (v/v) ethanol and left overnight at room temperature. Then, supernatant of each sample was kept and added with 50% or 95% (v/v) ethanol. This step was performed for 12 times to reach a completion of extraction. For each sample, all 12 extracts were pooled, then filtered using

Whattman No.1 filter paper before being evaporated to get rid of ethanol by rotary evaporator at 45ºC. The aqueous phase residues were further fractionated with 100 ml. ethyl acetate (EA) for 5 times as well as dichloromethane (DCM) of similar volume and times. All fractions were then concentrated by a rotary evaporator at 45ºC. The obtained twelve semisolid fractions were stored at 4ºC and protected from light until utilization. Table 2.1 shows the 12 fractions of longkong peels and seeds obtained.

Table 2.1 Twelve fractions prepared from peels and seeds of *L. domesticum* fruits.

Maceration	Fractionation	*Lansium domesticum* Corr.	
		Peels (SK)	Seeds (SD)
Ethanol 50% (v/v) concentration	Dichloromethane (DCM)	LDSK50-DCM	LDSD50-DCM
	Ethyl acetate (EA)	LDSK50-EA	LDSD50-EA
	Water (H_2O)	LDSK50-H_2O	LDSD50-H_2O
Ethanol 95% (v/v) concentration	Dichloromethane (DCM)	LDSK95-DCM	LDSD95-DCM
	Ethyl acetate (EA)	LDSK95-EA	LDSD95-EA
	Water (H_2O)	LDSK95-H_2O	LDSD95-H_2O

2.2 Determination of reactive oxygen species (ROS) radical scavenging capacity

2.2.1 Photochemiluminescence (PCL) assay

The anti-oxidant capacity of twelve above mentioned fractions of peels and seeds of longkong fruits was determined using PHOTOCHEM® (Analytik Jena, Germany) technique. Its principle is based upon measurement of photochemiluminescence (PCL). Briefly, superoxide anion radicals ($O_2^{-\bullet}$) were generated in the system by optical excitation or irradiation of luminol which was a photosensitizer substance. The anti-oxidant capacity of samples was measured by their inhibitory effect on luminescence generation, compared with the standard anti-

oxidant (constructed calibration curve). The results were presented in equivalent units (nmol) of ascorbic acid for anti-oxidative capacity of water soluble substances (ACW) system or trolox (synthetic vitamin E) units for anti-oxidative capacity of lipid soluble substances (ACL) system.

For the measurement, *L. domesticum* fractions were prepared by weighing 10 mg. of each sample fraction and dissolved in 1 ml. of dilution reagent (reagent 1) supplied with the ACL or ACW reagent kit (Analytik Jena, Germany). The solution was sonicated for 10 min. at room temperature to facilitate complete solubility. The supernatants were filtered through 0.45 µm syringe filter. The reaction was initiated by adding 10 µl of standard anti-oxidant compound (ascorbic acid and trolox) or test samples (longkong fractions) to the mixture of 2,300 µl of dilution reagent (reagent 1), 200 µl of reaction buffer (reagent 2) and 25 µl of protosensitizer (reagent 3). All samples were conducted and measured in triplicate.

2.2.2 Deoxyribose assay

Deoxyribose assay was performed to evaluate hydroxyl radicals ($OH^{\bullet}$) scavenging activity of the 12 fractions. The method was based on the determination of malondialdehyde (MDA) pink chromogen which was a degraded product of 2-deoxyribose (2-DR) damaged by $OH^{\bullet}$. Each sample fractions were prepared as prior mentioned in PCL assay except using distilled water as solvent. 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), 0.5 mM H_2O_2 and various concentrations of sample fractions in presence of 100 µM ascorbic acid (reducing agent) for starting the reaction and generated $OH^{\bullet}$. Reactions were carried out for 10 min. at room temperature and stopped by the addition of 0.5 ml. 2.8% trichloroacetic acid (TCA) followed by the addition of 0.5 mL thiobarbituric acid (TBA) solution. After boiling for 15 min, solutions were allowed to cool at room temperature. The absorbance of reaction mixture was measured to determine MDA pink chromogen at 532 nm in micro-plate reader system (GENios Plus, TECAN®, Australia). All samples were tested in triplicate.

2.3 Culturing and maintaining of the cell cultures

2.3.1 The TK6 Human lymphoblast cells

The stock TK6 human lymphoblast cell line (ATCC CRL-8015) was purchased from the American Type Culture Collection (ATCC), USA. The cells were grown as suspension and maintained as exponentially growth phase in RPMI 1640 medium (GIBCO®), supplemented with 10% (v/v) heat-inactivated horse serum (HS, GIBCO®) and 1% (v/v) penicillin-streptomycin (GIBCO®) in tissue culture flask (Corning®) and incubated at 37ºC in humidified atmosphere containing 5% CO_2. The cells were sub-cultured every 2-3 days and maintained by addition of flesh medium or replacement of flesh medium. The doubling time of TK6 cells is 12-14 h.

2.3.2 The V79 Chinese hamster lung cells

The V79 Chinese hamster lung cell line (ATCC CCL-93) was purchased from the American Type Culture Collection (ATCC), USA. The cells were grown as adherent in Dulbecco's Modified Eagle Medium (DMEM, GIBCO®) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO®) and 1% (v/v) penicillin-streptomycin (GIBCO®). The cells were propagated in tissue culture flask (Corning®) at 37ºC in humidified atmosphere incubator with 5% CO_2, sub-culturing every 2-3 days by trypsinization with 1 to 2 ml of 0.25 % trypsin-EDTA to allow detachment of cells and add flesh culture medium, aspirate and dispense into new culture flasks.

2.4 Determination of cytotoxicity by MTT assay

2.4.1 MTT assay in TK6 cells

After overnight cultivation, TK6 cells in 75 cm^2 tissue culture flask were routinely examined under inverted microscope. Then, the old medium was removed by centrifugation at 1,500 rpm. for 5 min. and the cells were washed with 1x of Hank's balanced salt solution (1x HBSS, GIBCO®) before being made to 4×10^5 cells/ml. for experimentation. Then, 0.5 ml of cell suspension was seeded into 24 well-plate. The cells were then treated with 0.5 ml. of sample fractions dissolved in RPMI (supplemented with 10% HS) at suitable final concentrations (LDSK50-EA 100, 200, 250, 300 , 400 µg./ml. and LDSK50-H_2O 1,000, 2,000, 3,000, 4,000, 5,000 µg./ml.).

Afterwards the cells were incubated at 37ºC. for 24 h in humidified atmosphere containing 5% CO_2. Concurrent vehicle control was included. After treatment, the chemical-containing medium was removed by centrifugation at 3,000 rpm. for 3 min. The treated cells were washed with 1x HBSS and re-suspended with 1 ml. of 0.625 mg./ml. of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, GIBCO®) in 1.5 ml. microcentrifuge tube. Then, the cells were incubated in darkness at 37ºC. in insoluble crystals fomazans were washed with 1x HBSS and dissolved in 200 µl of dimethyl sulfoxide (DMSO). The amount of formazan was evaluated to estimate the cell survival by measuring absorbance at 540 nm by micro-plate reader system.

2.4.2 MTT assay in V79 cells

The overnight culture of V79 cells were trypsinised using 0.25% trypsin-EDTA and the cells density was adjusted to 2×10^5 cells/ml with flesh DMEM. Then 200 µl of cell suspension was seeded in to 96 well-plate and incubated for 24 h at 37ºC in humidified atmosphere containing 5% CO_2. After incubation, 200 µl of complete culture media containing sample fractions at various final concentrations of LDSK50-EA and LDSK50-H_2O as mentioned above were added whereas concurrent vehicle control was included. After treatment, the cells were washed with 1x HBSS before adding 200 µl of 0.625 mg/ml MTT and incubated in dark at 37ºC in humidified incubator containing 5% CO_2 for 3 h. At the end of 3 h incubation time, MTT-containing media was discarded by pipetting. The crystals famazans formed in cells were washed once with 1x HBSS and then dissolved by adding 200 µl of DMSO. The absorbance value was measured at 540 nm by micro-plate reader system.

2.4.3 Cytotoxicity criteria

Values of the three independent experiments obtained from MTT assay were used to calculate the percentage viability of the cells using Equation (a). A graph of absorbance (y axis) plotted against sample concentration (x axis) was constructed. The cytotoxicity of the two active fractions (LDSK50-EA and LDSK50-H_2O) were

presented as 50% inhibitory concentration (IC_{50}), the concentration of test sample required to reduce the absorbance to half (50%) that of the control.

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \qquad \textbf{Equation (a)}$$

2.5 Determination of anti-oxidative DNA damage activity by comet assay

2.5.1 Cell culture and preparation

The TK6 human lymphoblast cells were also used in the comet assay. TK6 cells were cultured in RPMI medium as described previously (in the topic 3.3.1) and maintained as exponential growing phase prior to the experiment. Cell density at concentration of 2×10^5 cells/ml. was employed for each comet assay experiment. All treatments resulted in a minimum of 70% viable cells, a level sufficient for avoiding cytotoxicity artifacts in the comet assay.

2.5.2 Preparation of reagents

As a general rule, all buffers and reagents used in the assay should be given shelf-lives of no more than 1 month.

2.5.2.1 Agarose gel

Two agarose gels; 0.5% (w/v) LMP and 0.75% (w/v) NMP agarose gels were prepared by dissolving in phosphate buffered saline (PBS) and heated in microwave until fully dissolved. Melt LMP agarose was aliquoted into clean glass bottles approximately 3 ml./bottle and kept at 4°C. Prior to experimentation, LMP agarose aliquot was quickly melt in microwave and maintained at 37°C. in water bath.

2.5.2.2 Lysing solution

The solution was prepared by mixing of 2.5 M NaCl, 100 mM Na_2EDTA and 10 mM Trisma base in distilled water. Following completion of

solubility using magnetic stirring, the lysing solution was adjusted to pH 10.0 using NaOH pellets and stored at 4°C. Until being used, working lysing solution was fleshly prepared by adding Triton x-100 1% (v/v) and DMSO 10% (v/v) before refrigerated (4°C.) for at least 30 min., prior to slide addition.

2.5.2.3 Electrophoretic buffer

The stocks of electrophoresis buffers were separately prepared by dissolving of 10 N NaOH and 200 mM EDTA in distilled water using magnetic stirring, stored at 4°C. Prior to experimentation, working electrophoresis solution was fleshly prepared by mixing 30 ml. of 10 N NaOH and 5 ml. of 200 mM EDTA together and then adjusted the total volume to 1,000 ml. by distilled water. Thereafter, electrophoresis buffer was adjusted to pH 13 and kept chill at 4°C. until being used.

2.5.2.4 Neutralization solution

The neutralization buffer was prepared by dissolving 0.4 M Tris-base in distilled water using magnetic stirring. The buffer was adjusted to pH 7.5 with concentrated hydrochloric acid (conc. HCl) and stored at 4°C.

2.5.2.5 DNA staining dye

Ethidium bromide (EtBr) solution was employed for DNA staining. The EtBr was prepared at 20 µg./ml. by dilution of stock EtBr (1,000 µg./ml.) in PBS and stored at 4°C.

2.5.3 Cell treatment

After overnight cultivation, TK6 cells were centrifuged and the pellet were adjusted the density to 2×10^5 cell/ml. in flesh medium. One milliliter of cell suspension was added to 1 ml. volumes of complete medium contained 25, 50, 100 or 200 µg./ml. of LDSK50-EA or LDSK50-H_2O in a 12 well-plate and incubated at 37°C in 5% CO_2 incubator for 24 h.

2.5.4 Hydrogen peroxide treatment

After treatment as described in 3.5.3, the chemical-containing medium was removed by centrifugation at 3,500 rpm. for 3 min. Cells were washed twice with cold PBS before collected by centrifugation at 3,500 rpm. for 3 min. The cells were resuspended in 1 ml. of flesh medium containing 50 μM H_2O_2 and incubated at 4°C. for 5 min. to produce oxidative DNA damage to the cells. At the end of incubation period, the H_2O_2 treated cells were washed twice with cold PBS and resuspended in cold PBS for the comet assay.

2.5.5 Comet assay

The high alkaline (pH 13) comet assay was performed following the protocol described by Tice *et al*. (2000) with some modifications. The basic steps of the assay should be performed in a consistent manner to allow the reliable detection of genotoxic agents.

2.5.5.1 Comet slide preparation

Comet slides were prepared by pre-coating clean microscope slides with 0.75% (w/v) NMP agarose. Slides were allowed to dry for 1-2 h. at room temperature. The second or cell-containing layer was generally prepared from mixing 25 μl of treated cells with 75 μl of 0.5% (w/v) LMP agarose at 37°C. and the cell suspension was rapidly spread onto a pre-coated slide. The concentration of cells in agarose was the important parameters for ensuring a successful analysis. Thus, suitable cell concentration was required. At least two slides were made for each treatment. Each slide was covered with a 24×60 mm^2 coverslip and placed on flat surface ice box for 5 min. to solidify. The coverslip was gently removed and 95 μl of 0.5% (w/v) LMP agarose was then third-layered on the slide before covered with coverslips as prior described.

2.5.5.2 Cell lysis and DNA unwinding

After the agarose gel has been solidified, the coverslips were removed and then slides were immersed into fleshly prepared cold lysis solution in a seal box.

The box was placed in the 4°C. refrigerator for 2 h. After lysis, slides were exposed to flesh and chilled alkaline electrophoresis solution (pH 13) at 4°C. for 20 min. to allow DNA unwinding and expression of alkali labile sites as DNA break.

2.5.5.3 DNA Gel electrophoresis

After alkali unwinding, the single-stranded DNA in the gel was subjected to electrophoresis under alkaline conditions to produce comets. Firstly, the electrophoresis rig was set and placed in ice bath to keep temperature at 4°C. Following, the sufficient electrophoresis solution was added into the electrophoresis tank, the slides were placed on a horizontal gel electrophoresis platform. Electrophoresis conditions were carried out for 20 min. at a constant of 25 Volt and a current of 300 mA using PowerPac™ high-current power supply.

2.5.5.4 Neutralization and DNA straining

After electrophoresis, slides were gently washed for 5 min. three times in the neutralizing buffer to remove the alkali and detergents. Afterwards, each slide was stained with 35 μl of ethidium bromide solution (20 μg/ml.) and then covered with a 24×60 mm^2 coverslips.

2.5.5.5 Comet scoring

From each slide, fifty comet cells were randomly selected for comet analysis. The comet images were scored using the fluorescence microscope (at 200x magnification) coupled with CCD camera. The camera was linked to personal computer containing an automate comet image analysis software (Comet Assay III, Perceptive Instruments, UK). The two parameters selected as indicator of DNA damage was tail length (TL, the distance of DNA migration measured from the center of the nucleus towards the end of the tail, μm) and tail moment (TM, a measure of the distance between the center of the tail and the center of the head, multiplied by the percentage of DNA in the tail, %).

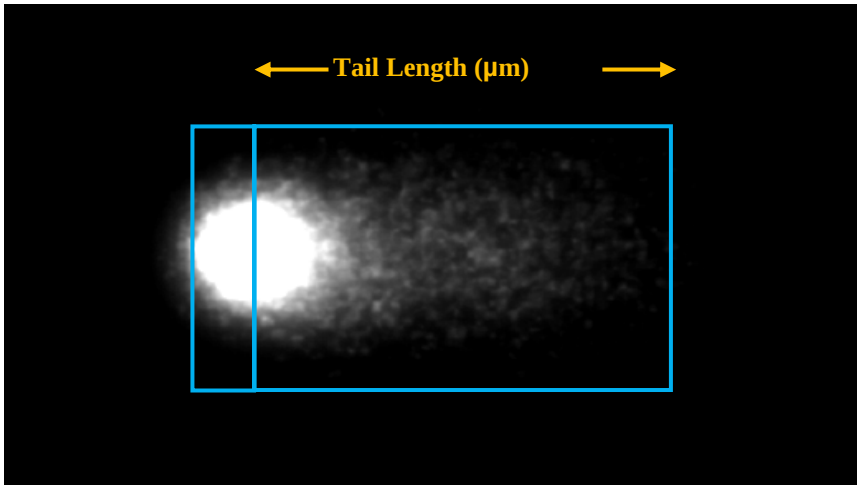


Figure 2.2 The comet cell illustrates DNA damage which is indicated by the tail length (TL) parameter.

2.5.6 Statistical analysis

According to the current recommendations for *in vitro* studies, the culture was used as the experimental unit. The mean values of 50 comet cells of all experiments were analyzed. All experiments were repeated on three separate occasions. The homogeneity of variance between concentration levels was determined using Levene's test. The statistical significance of the results was determined by means of one way analysis of variance (one-way ANOVA). When the results were significant, pair-wise comparisons of data from treated cultures with the controls were conducted using Tukey multiple comparisons. The result was considered statistically significant when p-value≤0.05. All analyses were performed using the SPSS statistics version 17.0, USA.

2.6 Determination of anti-mutagenicity activity by the *in vitro* cytokinesis-block micronucleus (CBMN) assay

Regarding results obtained from various assays (PCL, deoxyribose, MTT and comet test), the LDSK50-EA was obviously considered the most potent fraction. Therefore, the CBMN test was carried out only on LDSK50-EA to determine its anti-mutagenic property against known mutagen; mitomycin C (MMC) in human TK6 cells.

2.6.1 Chemical preparations

The standard mutagen, mitomycin C (MMC) was purchased from Kyowa (Japan) and used as the positive control to promote micronucleus formation. The stock solution of MMC (100 μg/ml) was prepared by dissolving MMC which was combined with 2.5 mg. NaCl in 1 ml. sterile distilled water. The concentration was expressed in term of pure MMC in microgram per microliter (μg/μl) of solution. For experiment, MMC was diluted with the culture medium (RPMI) and directly added into cultures to reach the final concentration of 0.8 μg/ml. Based on the percentage survival of the preliminary study, the top concentration of MMC producing high level of micronucleated cells (MNC) was selected.

The LDSK50-EA stock solution was diluted with complete RPMI medium prior to adding into the cultures at final concentrations of 25, 50, 100 and 150 μg/ml. These concentrations were selected depending on the preliminary experiment of cytotoxicity of LDSK50-EA when treated together with MMC, the minimal acceptance value not less than 70% cell viability. An inhibitor of action polymerization; cytochalasin-B (Cyt-B) was used in this study in order to stop cell membrane division of mitotic cells. Cyt-B was prepared as stock solution by dissolving in dimethyl sulfoxide (DMSO) at 1,000 μg/ml. Further dilutions in culture medium were fleshly performed before use. All working solutions were kept in dark container at -25°C.

2.6.2 Cell treatment

The anti-mutagenic experiment was carried out using CBMN test in TK6 cells. After overnight cultivations, TK6 cells were washed and collected by centrifugation at 1,200 rpm for 5 min. The viability of cells was determined using trypan blue exclusion (TBE) method. TK6 cells at 6×10^5 cells/ml in RPMI medium supplemented with 10% HS were seeded into 6-well plates and allowed to grow for 1-2 h. The cells were then treated with 1 ml. of LDSK50-EA fraction dissolved in RPMI (supplemented with 10% HS) at final concentrations of 25, 50, 100 and 150 μg/ml. in combination with 1 ml. MMC at final concentration 0.8 μg/ml. Cells were incubated for both short-term (4 h) and long-term periods (24 h) at 37°C. in 5% CO_2 incubator. The total

volume of each treatment was 3 ml. The positive (0.8 μg/ml. of MMC) and negative (only RPMI medium) controls were included in each experiment. At the end of short-term treatments, chemical-containing media were removed following centrifugation at 1,200 rpm for 5 min. The cells were collected and washed with 1x HBSS twice to remove remaining residues of test substances. The cells were then resuspended in 3 ml. of flesh RPMI medium containing 3 μg/ml. Cyt-B and further incubated for 24 h (from initiated culture) to allow binucleated (BN) cell accumulation. At the same time, Cyt-B was added into the long-term treatment plate to promote 3 μg/ml. concentration of Cyt-B in medium and further incubated for 24 h (from initiated culture) to allow BN cells accumulation as well.

2.6.3 *In vitro* Micronucleus (MN) analysis

After treatments as described in 3.6.2, the viabilities of the cells at all LDSK50-EA concentrations tested were immediately checked using the trypan blue exclusion (TBE) method. In order to distinguish the mutagenic property from cytotoxicity activity, the survival rate of treated cells should be greater than 70%. The treated cells were washed twice with cold 1x HBSS and resuspended in the optimization volume of RPMI medium without HS. Then, 80 µl of cell suspension was transferred to cytocentrifuge cups and prepared like a spot on glass slides using cytospin-3 equipment (SHANDON, United Kingdom) at speed 1500 rpm for 5 min. The slides were allowed to air dry at room temperature for 10-20 min. then fixed with cold methanol for 30 min. and stained with 10% (v/v) Giemsa in Gurr buffer for 20 min. After staining, the slides were air-dried and coverslips placed over the cells using Permount® SP15-500 mounting medium. At least 3 slides were prepared for each concentration treatment.

2.6.4 Slide assessment and micronuclei (MN) scoring

For micronucleus scoring, all slides were coded and then binucleated (BN) cells were scored blindly using light microscopy for the presence of micronuclei. MN frequency was expressed as the number of micronucleated cells (MNC) in 1,000 BN

cells scored. The criteria for the identification of micronuclei were those of Fenech *et al.* (2003).

The cell cycle alterations, including cytotoxic and cytostatic effects were concurrently determined by scoring 500 cells which were classified according to the number of nuclei. The number of cell cycle was expressed as cytokinesis block proliferation index (CBPI) which was calculated according to the formula in Appendix C. These measurements were made to assure that the treated cells had undergone mitosis during the MN assay.

2.6.5 Statistical analysis

All experiments were independently repeated three times and three slides were analysed for the present of MN frequency of each LDSK50-EA concentration as well as for positive and negative controls. The data were presented as the mean±standard error (SE) for all experiments. The MN data obtained were statistically analysed using one-way ANOVA with Tukey multiple comparisons to compare the MN frequencies of each treatment groups with the control. The *p*-value≤0.05 was considered significant difference. The Tukey multiple comparison was also used for the analysis of CBPI. All analyses were performed using the SPSS statistics version 17.0, USA.

2.7 Determination of phytochemical components

2.7.1 Thin layer chromatography (TLC)

Stock solution containing 100 mg./ml. of LDSK50-EA and 10 mg./ml. of each standard was prepared by dissolving in absolute ethanol. Then, approximately 10-20 μl of LDSK50-EA stock solution and standard phytochemicals of interest (e.g. rutin, chlorogenic acid, scopoletin) were spotted on silica gel F_{254} plates (Alufolien, Merck). The TLC plate was developed with various solvents to select the suitable system for separation and identification.

Solvent systems used were as the following:

System 1: toluene: ethyl acetate: formic acid (5:4:1).

System 2: ethyl acetate: formic acid: acetic acid: water (137:11:11:26).

Spray reagent: natural product (diphenylboryloxyethylamine)-polyethyleneglycol (PEG).

UV Detection: 366 nm.

Standard: scopoletin, rutin, chlorogenic acid.

The retention factor (Rf value) was used to characterize and compare components between LDSK50-EA fraction with any standard.

$$\text{Retention factor (Rf value)} = \frac{\text{Distance from origin to component spot}}{\text{Distance from origin to solvent front}}$$

2.7.2 Total phenolic content (TPC) determination

The contents of total phenolic were determined by using Folin-Ciocalteu method. The reaction mixture contained 100 µl of 2 mg./ml. LDSK50-EA in ethanol, 500 µl of the Folin-Ciocalteu reagent, and 1 ml. of 20% sodium carbonate. The final volume was made up to 10 ml. with pure water. After 1 h incubation, the absorbance at 760 nm was measured and used to calculate the phenolic contents using gallic acid as standard. Total polyphenol contents were expressed as mg. gallic acid equivalents (GAE) per mg. sample extract (mg. GAE/mg. extract). Triplicate reactions were conducted. Data were reported as mean ± standard deviation (SD).

2.7.3 Total flavonoid content (TFC) determination

The total flavonoid content was determined using the aluminum chloride colorimetric method with some modification. Briefly, 1 ml. of the LDSK50-EA (2 mg./ml.) or rutin standard solution was mixed with 5 ml. of distilled water in a test tube, followed by addition of 300 µl of a 5% (w/v) sodium nitrite solution. After 5 min., 300 µl of a 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for a further 1 min. before 2 ml. of 1 M NaOH was added. The mixture was made up to 10 ml. with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The mean (±SD) results of triplicate analyses were expressed as mg. of rutin equivalents (RE) per mg. sample extract (mg. RE/mg. extract).

CHAPTER III

RESULTS

3.1 Anti-oxidant capacity of twelve fractions of *L. domesticum* extractions

3.1.1 Photochemiluminescence (PCL) anti-oxidant assay

The principle of PCL assay is based on the photochemical generation of specific free radicals-superoxide anion radical ($O_2^{-\bullet}$) combined with the sensitive detection method by using chemiluminescence. This method differs from other procedures for anti-oxidant evaluation because it does not require oxidizing reagents for the production of the radical species. By this assay, the anti-oxidant capacity against $O_2^{-\bullet}$ of the twelve *L. domesticum* fractions could be determined.

As demonstrated in Figures 3.1 and 3.2, the anti-oxidant capacity of the fractions to counteract $O_2^{-\bullet}$ radicals, greatly varied within the parts of *L. domesticum* extracted and type of fractionation methods. The anti-oxidant capacity of each sample tested was expressed in nanomole (nmol) scale of trolox and ascorbic equivalent for the lipid-soluble (ACL) and water-soluble (ACW) substances system, respectively.

The results of the ACL are demonstrated in Figure 3.1 and Table 3.2. When all samples were tested at 10 µg/ml concentration, the overall anti-oxidant capacity range from 0.380 to 6.625 nmol of trolox. Among the twelve fractions, it was noticeable that the LDSK50-EA possessed the highest anti-oxidant activity (6.625 nmol of trolox) whereas the other fractions exhibited slightly different in anti-oxidant capacity. The degree of $O_2^{-\bullet}$ scavenging activity for all twelve fractions (from high to low) were as follows: LDSK50-EA (6.625 nmol) > LDSK50-H_2O (1.845 nmol) > LDSK95-EA (1.750 nmol) > LDSD50-EA (1.257 nmol) > LDSD95-EA (1.200 nmol) > LDSK95-H_2O (1.195 nmol) > LDSK50-DCM (1.028 nmol) > LDSD95-DCM (0.966 nmol) > LDSD50-DCM (0.795 nmol) > LDSD95-H_2O (0.635 nmol) > LDSD50-H_2O (0.525 nmol) > LDSK95-DCM (0.380 nmol).

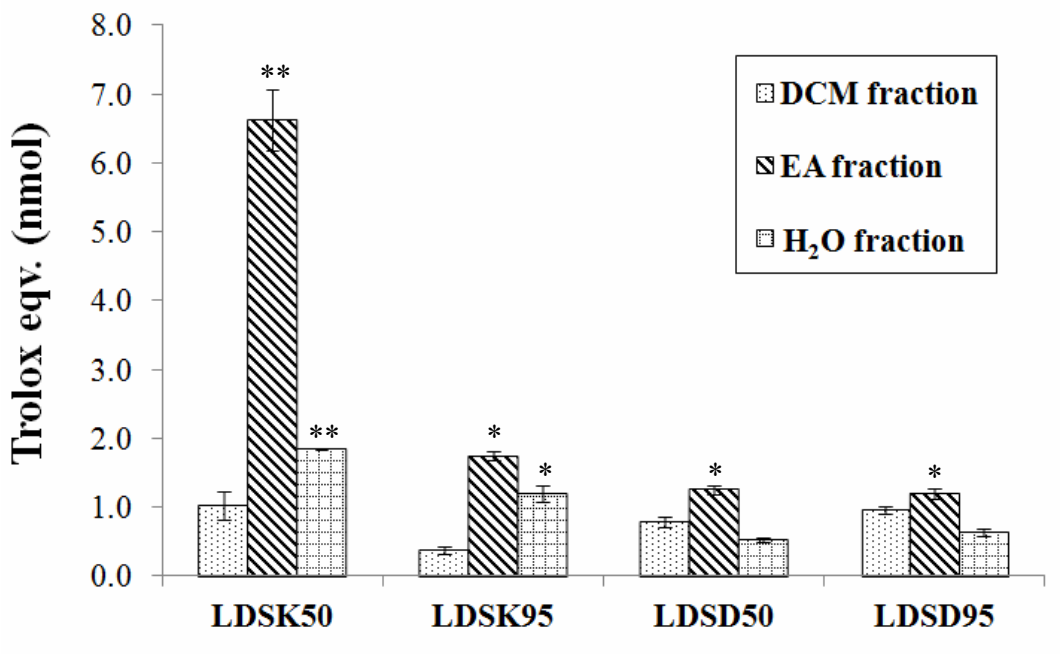


Figure 3.1 The anti-oxidant capacity in lipid phase (ACL) of the twelve *L. domesticum* fractions by PCL assay. Results were expressed as means±SD (*n*=3). *Significant difference was detected from the lowest activity fraction of same part-extraction (*p≤0.05*). **Significant difference was detected from all fractions of same part-extraction (*p≤0.05*).

The anti-oxidant capacitiy of the water-soluble system (ACW) of *L. domesticum* fractions were examined at 100 µg/ml concentration. The results were expressed as an ascorbic acid equivalent in nanomole (nmol) range and are shown in Figure 3.2 and Table 3.2. The wide range of anti-oxidant capacity of all fractions was found from -0.065 to 98.733 nmol of ascorbic acid. The highest anti-oxidant activity was in fraction of LDSK50-H_2O (98.733 nmol of ascorbic acid) followed by the LDSK50-EA (54.660 nmol of ascorbic acid). The overall capacity of all twelve *L. domesticum* fractions ranking from high to low are as follows; LDSK50-H_2O (98.733 nmol) > LDSK50-EA (54.660 nmol) > LDSK95-H_2O (9.910 nmol) > LDSK95-EA (8.350

nmol) > LDSD95-EA (6.880 nmol) > LDSD50-EA (5.410 nmol) > LDSK50-DCM (4.180 nmol) > LDSD50-H_2O (2.073 nmol) > LDSD95-DCM (1.513 nmol) > LDSD95-H_2O (1.105 nmol) > LDSK95-DCM (0.345 nmol) > LDSD50-DCM (-0.065 nmol).

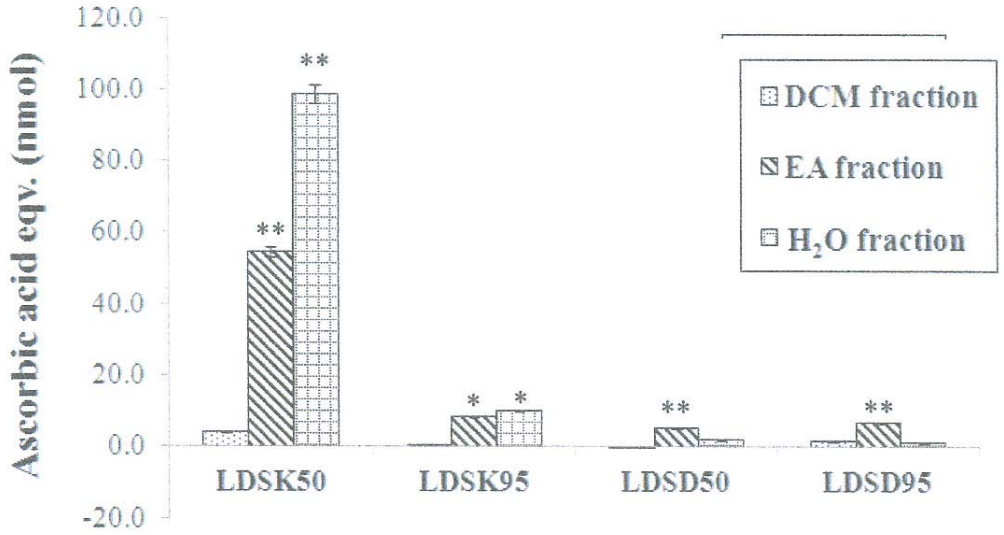


Figure 3.2 The anti-oxidant capacity in water phase (ACW) of twelve *L. domesticum* fractions by PCL assay. Results were expressed as means±SD (*n*=3). *Significant difference was detected from the lowest activity fraction of same part-extraction (*p≤0.05*). **Significant difference was detected from all fractions of same part-extraction (*p≤0.05*).

3.2.2 Deoxyribose anti-oxidant assay

The method is based on the determination of malondialdehyde (MDA) pink chromogen which is a degraded product of 2-deoxyribose (2-DR) damaged by hydroxyl radical ($OH^{\bullet}$). The inhibitory effect of *L. domesticum* fractions on 2-DR degradation was determined by measuring the competition between 2-DR 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-DR degradation for the test sample of 0.5, 1.0 and 2.0 mg/ml. As shown in Figure 3.3 and Table 3.1, 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 of 2-DR degradation.

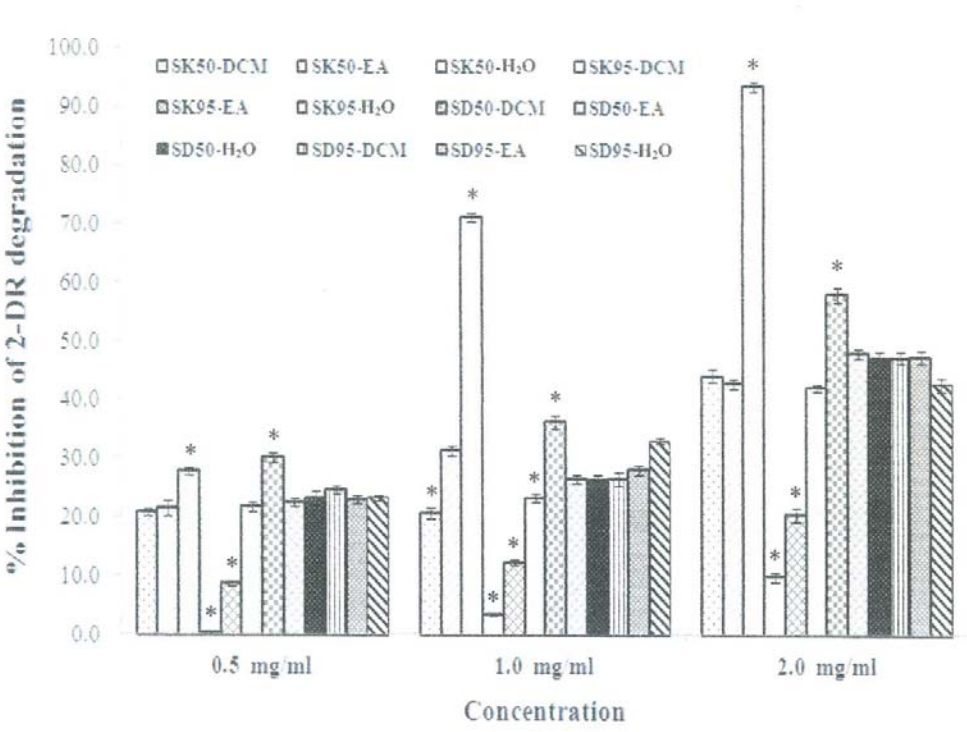


Figure 3.3 Inhibitory effect (%) of 2-deoxyribose degradation of twelve *L. domesticum* fractions by deoxyribose assay. Results were expressed as means±SD (*n*=3). *Significant difference was detected from all fractions of same concentrations (0.5, 1.0 and 2.0 mg/ml) (*p≤0.05*).

Table 3.1 Percent inhibition of 2-deoxyribose degradation of the twelve *L. domesticum* fractions by deoxyribose assay

Fractions		% Inhibition (Mean±SD)		
		0.5 mg/ml	**1.0 mg/ml**	**2.0 mg/ml**
LDSK50	DCM	20.79 ± 0.62	20.61 ± 0.87*	43.94 ± 1.03
	EA	21.49 ± 1.28	31.24 ± 0.86	42.70 ± 0.86
	H_2O	27.79 ± 0.54*	**71.21 ± 0.73***	**93.44 ± 0.84***
LDSK95	DCM	0.50 ± 0.12*	3.48 ± 0.28*	9.86 ± 0.89*
	EA	8.69 ± 0.29*	12.31 ± 0.44*	20.47 ± 1.14*
	H_2O	21.68 ± 0.91	23.30 ± 0.72*	42.03 ± 0.58
LDSD50	DCM	**30.10 ± 0.79***	36.11 ± 1.06*	58.03 ± 1.37*
	EA	22.55 ± 0.63	26.48 ± 0.64	48.00 ± 0.87
	H_2O	23.23 ± 1.08	26.24 ± 0.81	47.24 ± 1.12
LDSD95	DCM	24.59 ± 0.76	26.49 ± 1.14	47.21 ± 1.06
	EA	22.94 ± 0.65	28.02 ± 0.87	47.42 ± 1.09
	H_2O	23.30 ± 0.47	32.95 ± 0.49	42.53 ± 1.11

The results were expressed as mean±SD values (n=3). *Significant difference was detected in all fractions of same concentrations ($p \leq 0.05$). The bold characters demonstrated the maximum % inhibition of 2-DR degradation found for each concentration.

Table 3.2 The anti-oxidant capacity of twelve *L. domesticum* fractions determined by PCL and deoxyribose assays

Fractions		ACL	ACW	Deoxyribose
		Trolox eqv. (nmole) ± S.D	Ascorbic eqv. (nmole) ± S.D	Inhibition (%) ± S.D
LDSK50	DCM	1.030 ± 0.198	4.180 ± 0.211	43.94 ± 1.03
	EA	**6.625 ± 0.445**	54.660 ± 1.413	42.70 ± 0.86
	H_2O	1.845 ± 0.007	**98.733 ± 2.516**	**93.44 ± 0.84**
LDSK95	DCM	0.380 ± 0.057	0.345 ± 0.007	9.86 ± 0.89
	EA	1.750 ± 0.057	8.350 ± 0.072	20.47 ± 1.14
	H_2O	1.195 ± 0.120	9.910 ± 0.144	42.03 ± 0.58
LDSD50	DCM	0.795 ± 0.078	-0.065 ± 0.120	58.03 ± 1.37
	EA	1.257 ± 0.060	5.410 ± 0.240	48.00 ± 0.87
	H_2O	0.525 ± 0.035	2.073 ± 0.101	47.24 ± 1.12
LDSD95	DCM	0.965 ± 0.050	1.513 ± 0.050	47.21 ± 1.06
	EA	1.200 ± 0.085	6.880 ± 0.028	47.42 ± 1.09
	H_2O	0.635 ± 0.050	1.105 ± 0.007	42.53 ± 1.11

ACL (Anti-oxidant capacity in lipid phase) tested at 10 µg of sample.

ACW (Anti-oxidant capacity in water phase) tested at 100 µg of sample.

Deoxyribose assay tested at 2.0 mg/ml of sample.

The results were expressed as mean±SD (*n*=3).

The bold characters demonstrated the maximum value of each assay.

Regarding results demonstrated in Table 3.2, the *L. domesticum* fractions that exhibited the greatest anti-oxidant activity by PCL and deoxyribose assays were LDSK50-EA and LDSK50-H_2O. These two fractions were classified as active fractions and selected for study on their biological activity through the cell-based model in next step.

3.2 Cytotoxicity of the two active fractions: LDSK50-EA and LDSK50-H_2O by MTT assay in TK6 and V79 cell lines

The MTT assay is based on the reduction of the yellowish MTT (3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) to purple formazan by viable and metabolically active cells. Following treatment of cells with MTT, the amount of formazan was evaluated to estimate the cell viability by measuring absorbance at 540 nm using micro-plate reader system. The resulting absorbance values (correlation between the cell number and the amount of formazan product) were taken for cell viability calculation, details as described in Chapter II.

The cytotoxicity of the two *L. domesticum* active fractions e.g. LDSK50-EA and LDSK50-H_2O was assessed by MTT assay in two different cell lines including TK6 human lymphoblasts and V79 Chinese hamster lung cells. Both cell lines were pre-treated with different concentrations of LDSK50-EA and LDSK50-H_2O for 24 h and the viability of cells was determined according to its principle as described above.

3.2.1 Cytotoxic effect of LDSK50-EA

The anti-proliferative activity of LDSK50-EA was determined using MTT cell viability assay, where the IC50 value was used as a parameter of cytotoxicity. Treatments of LDSK50-EA (at 100, 200, 250, 300, 400 µg/ml) for 24 h, resulted in cytotoxic effect in TK6 and V79 cell lines in a dose-dependent manner as illustrated in Figure 3.4. Moreover, the result indicated that V79 cell line was more sensitive to LDSK50-EA than TK6 cell line. The IC_{50} (inhibitory concentration inhibited cell growth by 50%) value of LDSK50-EA in TK6 cells was 280 µg/ml whereas that of V79 cells was 231 µg/ml (Table 3.3).

3.2.2 Cytotoxic effect of LDSK50-H_2O

Unlike the LDSK50-EA, the cytotoxic effect of LDSK50-H_2O was able to obtain only at higher concentrations (1,000, 2,000, 3,000, 4,000, 5,000 µg/ml). As shown in Figure 3.5 and Table 3.3, LDSK50-H_2O was less toxic than LDSK50-EA in both TK6 and

V79 cells. This was evident by % cell viability greater than 80% at all concentrations tested in TK6 cells. The values of IC_{50} of LDSK50-H_2O were 4,309 µg/ml and greater than 5,000 µg/ml for V79 and TK6 cells, respectively (Table 3.3).

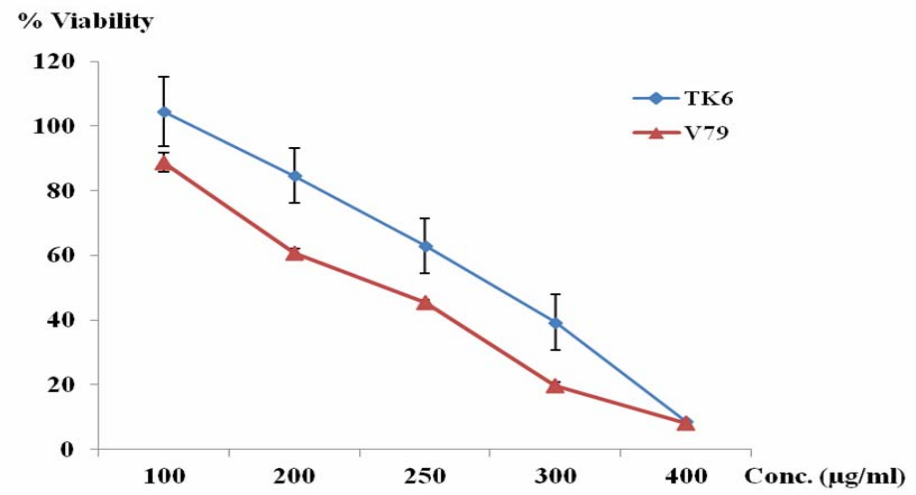


Figure 3.4 Concentration-viability curve of LDSK50-EA treated TK6 and V79 cell lines for 24 h exposure and determined by MTT assay.

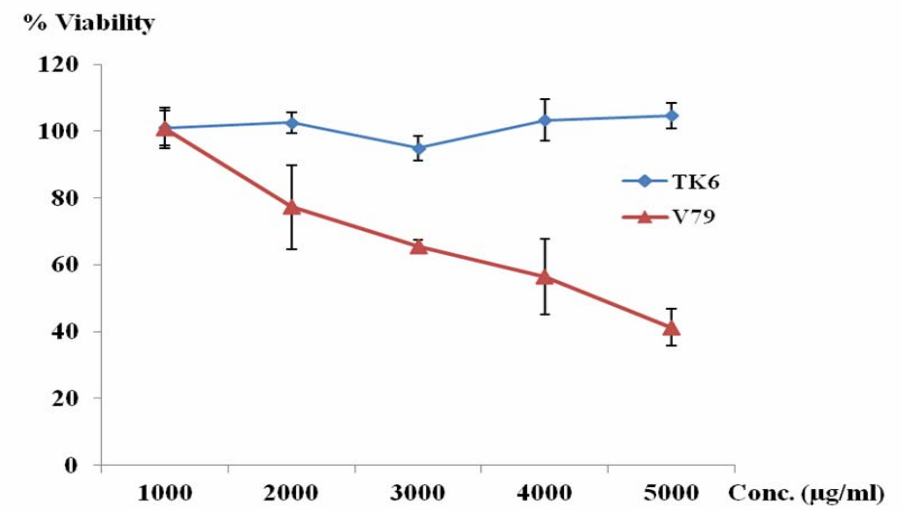


Figure 3.5 Concentration-viability curve of LDSK50-H_2O treated TK6 and V79 cell lines for 24 h exposure and determined by MTT assay.

Table 3.3 The growth inhibitory effects-IC50 values (μg/ml) of LDSK50-EA and LDSK50-H_2O on TK6 and V79 cell lines by MTT assay after 24 h exposure

Fractions	IC_{50} (μg/ml)	
	TK6 cells	V79 cells
LDSK50-EA	280	231
LDSK50-H_2O	> 5,000	4,309

3.3 Anti-oxidative activity of two active fractions: LDSK50-EA and LDSK50-H_2O on TK6 cells by comet assay

The comet assay was performed as previously described in Chapter II. With this assay, effects such as DNA single-strand breaks, incomplete excision repair sites, and alkali-labile sites can be detected by analyzing the amount of DNA that migrates out of immobilized cell nuclei that were subjected to electrophoresis. Two major DNA damage parameters defined as Tail length (TL = distance of DNA migration from center of cell nucleus, μm) and Tail moment (TM = distance between the center of the tail and the center of the head, multiplied by the percentage of DNA in the tail, %) were evaluated.

To investigate the anti-oxidative activity of LDSK50-EA and LDSK50-H_2O in protection of DNA damage, the TK6 cells were separately pre-treated with these two fractions at 25, 50, 100 and 200 μg/ml concentrations for 24 h prior to H_2O_2 induction. Treatments of TK6 cells with LDSK50-EA and LDSK50-H_2O at these assigned doses for 24 h did not exhibit inhibitory effect on cell growth rates. The results shown in Table 3.4 indicated the percentage of TK6 living cells prior to H_2O_2 exposure (pre-H_2O_2) and after H_2O_2 exposure (post-H_2O_2) with different concentrations of LDSK50-EA and LDSK50-H_2O fractions. In this study, any concentrations produced cell viability less than 70% were discarded in order to distinguish the oxidative effect from cytotoxic effect.

Table 3.4 Percentage of living cells of pre- and post-H_2O_2 induction following treatments of TK6 with LDSK50-EA and LDSK50-H_2O measured by the trypan blue exclusion method

LDSK50-EA			LDSK50-H_2O		
Concentration (µg/ml)	TK6 Viability (%)		Concentration (µg/ml)	TK6 Viability (%)	
	Pre-H_2O_2	Post-H_2O_2		Pre-H_2O_2	Post-H_2O_2
0	97.52±2.50	95.02±6.80	0	97.52±2.50	95.02±6.80
25	98.15±3.21	92.95±4.37	25	98.72±2.22	93.50±5.73
50	99.57±0.75	96.38±6.28	50	97.24±2.45	93.53±5.29
100	97.62±2.86	98.24±1.94	100	97.70±3.98	95.98±6.97
200	97.76±2.72	94.33±4.27	200	98.89±1.92	92.47±7.96

Results were expressed as means ± SD (*n*=3).

In present study, the results detected by comet or single cell gel electrophoresis (SCGE) assay revealed that treatment of 50µM H_2O_2 for 5 min. produced DNA damage (%TM) in TK6 cells at about 10-fold greater than untreated cells (Figure 3.7). Interestingly, this DNA damage could be prevented by pretreated the TK6 cells with LDSK50-EA at 25, 50, 100, 200 µg/ml. for 24 h. The effect was found to be in dose-dependent manner. The highest DNA preventive effect was found at 200 µg/ml. concentration (Figure 3.8 and Table 3.5).

In contrast, the LDSK50-H_2O fraction exhibited a slight inhibitory effect on oxidative DNA damage when tested at the similar concentrations range (Figure 3.8 and Table 3.5). The DNA protective effect against H_2O_2 of LDSK50-H_2O was indicated by a reduction in TL and TM damage parameters in comparison to cells treated with H_2O_2 alone.

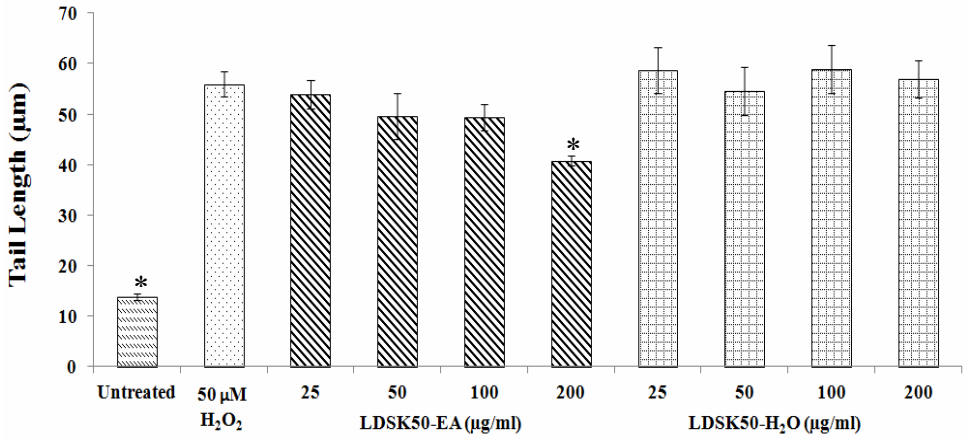


Figure 3.6 Tail lenght (TL, µm) values measured in pre-treated TK6 cells with LDSK50-EA and LDSK50-H_2O fractions followed by H_2O_2 damage induction by comet assay. The results were expressed as means±SD (*n*=3). *Significant difference was detected from 50 µM H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA).

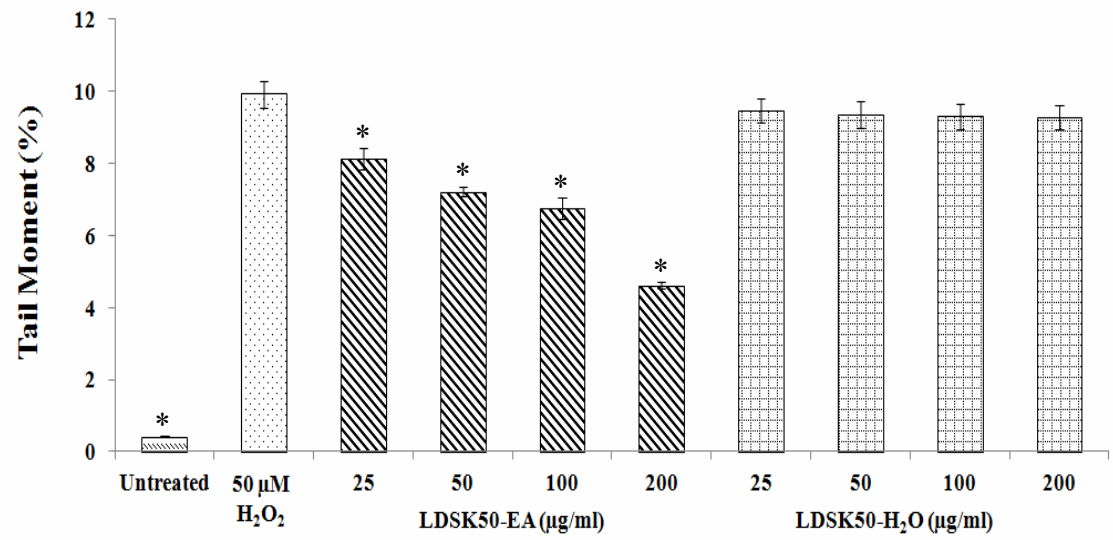


Figure 3.7 Tail moment (TM, %) values measured in pre-treated TK6 cells with LDSK50-EA and LDSK50-H_2O fractions followed by H_2O_2 damage induction by comet assay. The results were expressed as means±SD (*n*=3). *Significant difference was detected from 50 µM H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA).

Figure 3.8 shows the inhibitory oxidative DNA damage effect of LDSK50-EA and LDSK50-H_2O in TK6 cells against H_2O_2 induction. The results were calculated in percentage inhibition of DNA damage. The concentration-dependent DNA protective effect was found in LDSK50-EA by 18.04±0.66%, 27.29±1.37%, 31.99±0.68% and 53.47±1.99% for 25, 50, 100 and 200 µg/ml., respectively. For the LDSK50-H_2O, a slight DNA protective activity was found and not significantly different compared among four concentrations tested. The % inhibition of DNA damage of LDSK50-H_2O exhibited from low to high by 4.66±0.36%, 5.59±0.36%, 6.28±0.06% and 6.54±0.25% for 25, 50, 100 and 200 µg/ml., respectively (Table 3.5).

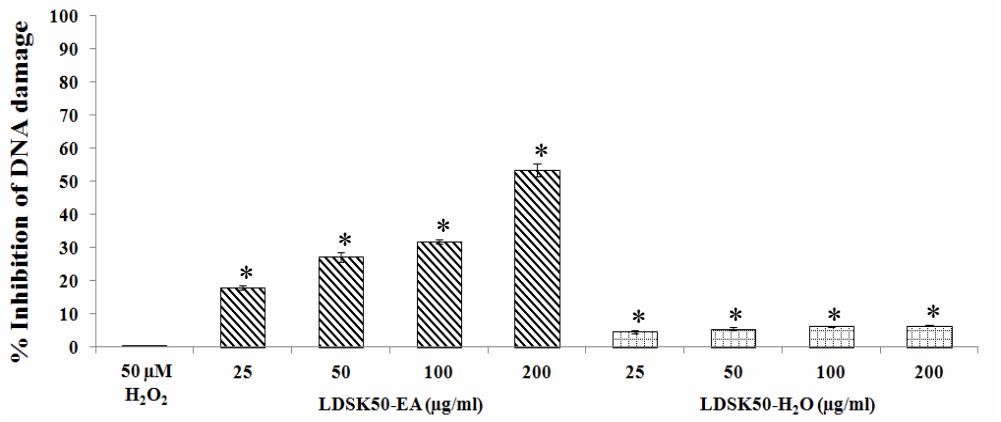


Figure 3.8 Inhibitory effect of LDSK50-EA and LDSK50-H_2O on H_2O_2-induced DNA damage in TK6 cells by comet assay. The results were expressed as means±SD (*n*=3). *Significant difference was detected from 50 µM H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA).

Table 3.5 DNA damage parameters including tail length (TL) and tail moment (TM) and % inhibitory effect on DNA damage of LDSK50-EA and LDSK50-H_2O in TK6 cells by comet assay

Treatment	Tail length (TL) (μm)	Tail moment (TM) (%)	DNA damage inhibition (%)
Untreated	13.80 ± 0.73*	0.42 ± 0.02*	NC
H_2O_2 50 μM	55.97 ± 2.56	9.93 ± 0.37	0.00 ± 0.00
LDSK50-EA 25 μg/ml	53.82 ± 2.82	8.14 ± 0.30*	18.04 ± 0.66*
LDSK50-EA 50 μg/ml	49.50 ± 4.56	7.21 ± 0.13*	27.29 ± 1.37*
LDSK50-EA 100 μg/ml	49.37 ± 2.67	6.75 ± 0.30*	31.99 ± 0.68*
LDSK50-EA 200 μg/ml	40.75 ± 1.01*	4.61 ± 0.10*	53.47 ± 1.99*
LDSK50-H_2O 25 μg/ml	58.72 ± 4.56	9.46 ± 0.33	4.66 ± 0.36*
LDSK50-H_2O 50 μg/ml	54.60 ± 4.58	9.37 ± 0.37	5.59 ± 0.36*
LDSK50-H_2O 100 μg/ml	58.94 ± 4.81	9.30 ± 0.35	6.28 ± 0.06*
LDSK50-H_2O 200 μg/ml	56.89 ± 3.72	9.28 ± 0.35	6.54 ± 0.25*

The results were expressed as mean±SD (n=3). *Significant difference was detected from 50 μM H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA). NC = not calculated

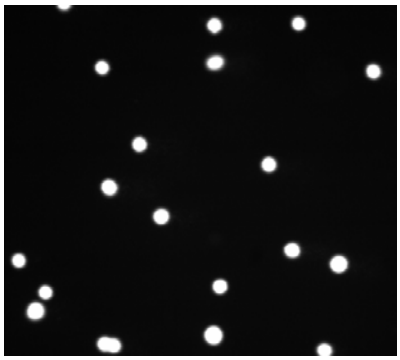
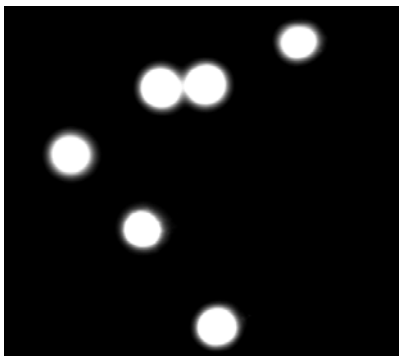
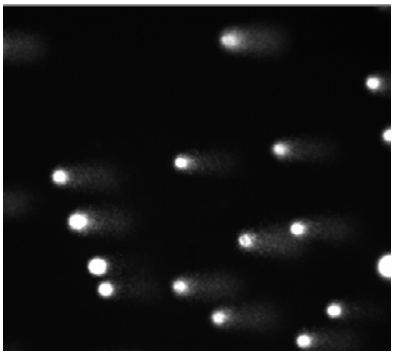
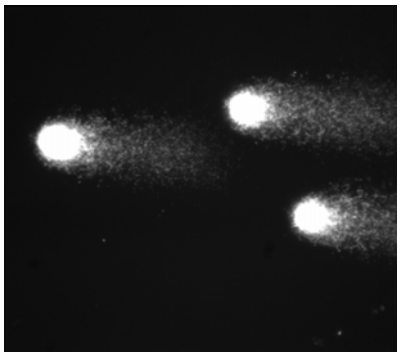
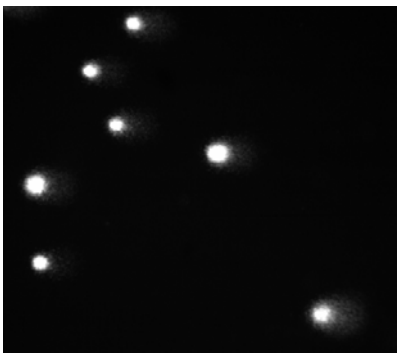
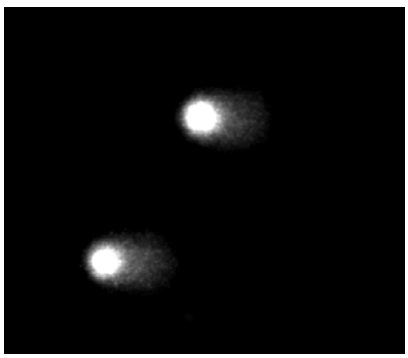
Treatment	TK6 Comet images	
	Medium magnification (200x)	High magnification (400x)
Untreated (Undamaged)		
Induced DNA damage by 50μM H_2O_2		
100 μg/ml Trolox		

Figure 3.9 The comet images of TK6 cells (from top to bottom) of following treatments: control or untreated (undamaged DNA), 50 μM H_2O_2, 100 μg/ml Trolox (positive control), 200 μg/ml LDSK50-EA and 200 μg/ml LDSK50-H_2O. Cells were stained with ethidium bromide and taken by fluorescence microscope at medium (200x) and high (400x) magnification.

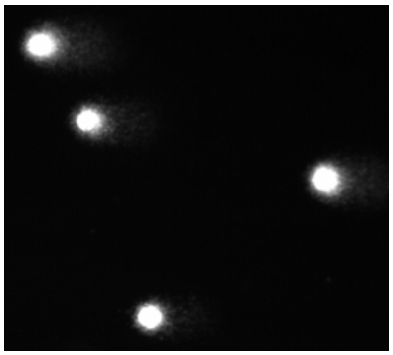
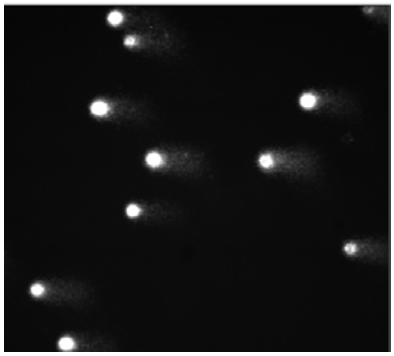
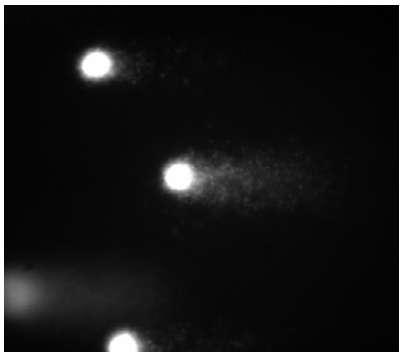
Treatment	TK6 Comet images	
	Medium magnification (200x)	High magnification (400x)
200 µg/ml LDSK50-EA		
200 µg/ml LDSK50-H_2O		

Figure 3.9 (continued)

3.4 Anti-mutagenic activity of the most active LDSK50-EA fraction on TK6 cells by the *in vitro* CBMN assay

According to the results obtained from comet assay, it was clearly evident that the LDSK50-EA fraction exhibited potent activity on anti-oxidative DNA damage induced by H_2O_2 in TK6 cells greater than LDSK50-H_2O. Therefore, only LDSK50-EA was chosen for further study on anti-mutagenic property on TK6 cells using the cytokinesis-block micronucleus (CBMN) test.

Briefly, the cells were simultaneously treated with known mutagen mitomycin C (MMC) at 0.8 μg/ml. and LDSK50-EA at concentrations of 25, 50, 100, 150 μg/ml. for 4 h at 37°C in humidified atmosphere containing 5% CO_2. The concentrations of LDSK50-EA were designed based on the results obtained from the cytotoxicity test as well as the concentration-dependent DNA protective effect doses of comet assay. However, the maximum concentration of LDSK50-EA (200 μg/ml) was excluded from CBMN test due to its cytotoxic effect to TK6 cells (% viability < 50). The results were expressed as number of micronucleated cells (MNC) per 1,000 binucleated (BN) cells in means±SE (*n*=3). The survival rates of TK6 cells of all treatments were monitored and focused on greater than 70% in order to discriminate cytotoxic effect of LDSK50-EA.

The results of CBMN are shown in Figure 3.10 and Table 3.6. The mean values of micronucleus (MN) formation (number of MNC per 1,000 BN cells scored) of untreated TK6 cells (receiving RPMI) were 11.33±1.86 and 13.00±1.53, respectively for 4 h and 24 h treatment times. 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 MMC at 0.8 μg/ml at $p \leq 0.05$ (ANOVA). Therefore, the reproducibility of the CBMN test was ascertained.

Interestingly, the MN formation in BN cell was suppressed in the presence of LDSK50-EA (Figure 3.10 and Table 3.6). At 24-h treatment time, the suppressive

effects of LDSK50-EA against MMC-induced MN formation at concentrations of 25, 50, 100, and 150 μg/ml were 69.33±7.51, 68.33±6.74, 65.67±6.94 and 59.67±4.33 MNC cells for 1,000 BN cells scored, respectively. However, the anti-mutagenic effect of LDSK50-EA was not observed in TK6 cells when tested for 4 h at all concentrations. This was indicated by no significant difference in MN frequency of LDSK50-EA treated groups in comparison to MMC positive control group ($p \leq 0.05$, ANOVA) (Figure 3.10 and Table 3.6).

The survival rates of TK6 cells after short-term (4 h) and long-term (24 h) treatments were greater than 70% in all tested LDSK50-EA concentrations but not the highest concentration (150 μg/ml) at 24 h where a very slightly lower survival rate (69.89%) than acceptance value (70%) was observed. This suggested a possible cytotoxic effect of the combined treatment of MMC and LDSK50-EA treatment at the higher dose.

The cytokinesis block proliferation index (CBPI) values were calculated and used as a parameter to investigate cell proliferation in order to assure that the treated TK6 cells had undergone one nuclear division (mitosis) during experiment. This parameter was determined in all experiments (calculated according to the formula shown in Appendix C). The lowest CBPI value possible was 1.0, which occurred if all of the viable cells failed to divide during the cytokinesis-block period and therefore all mononucleated. If all viable cells completed one nuclear division and therefore all binucleated, the CBPI value would be 2.0.

The results of CBPI are demonstrated in Table 3.6. The average CBPI values of TK6 cells of RPMI treatment (control) for 4 and 24 h treatments were 1.91 and 1.90, respectively. While the average CBPI values of MMC positive control for 4 and 24 h treatments were reduced to 1.31 and 1.29, respectively. The combination treatment of LDSK50-EA and MMC, exhibited similar CBPI value of MMC alone but was significantly lower than CBPI value of the control group ($p \leq 0.05$, ANOVA).

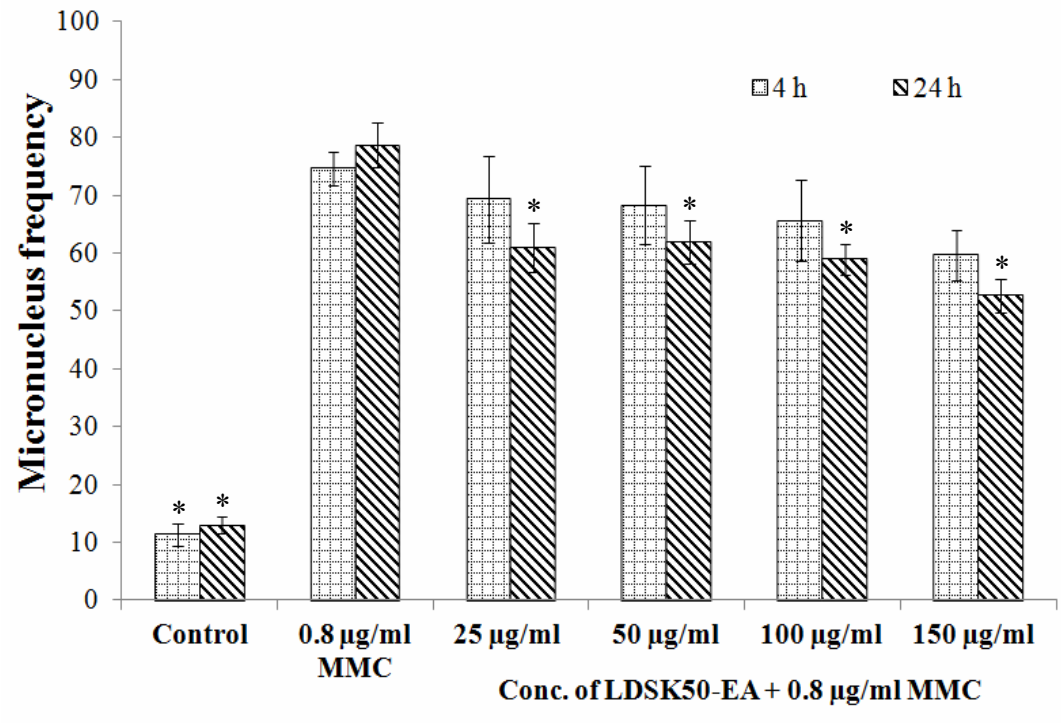


Figure 3.10 MN frequency (number of MNC per 1,000 BN cells scored) in TK6 cells after treatments with a combination of LDSK50-EA (25, 50, 100 and 150 µg/ml) and MMC (standard mutagen, 0.8 µg/ml) for 4 h and 24 h. The results were expressed as means±SE (*n*=3). *Significant difference was detected from each MMC treatment groups at $p \leq 0.05$ (ANOVA).

Table 3.6 The micronucleus (MN) frequencies, cell viability and cytokinesis block proliferation index (CBPI) of TK6 cells after treatment with MMC and a combination of LDSK50-EA and MMC by the CBMN assay

Treatment	MN frequency[a] (Mean ± SE)	Cell viability[b] (%)	CBPI[c]
4 h Treatment			
Control	11.33 ± 1.86*	95.71	1.91
MMC 0.8 μg/ml	74.67 ± 2.96	88.78	1.31*
LDSK50-EA 25 μg/ml	69.33 ± 7.51	89.67	1.32*
LDSK50-EA 50 μg/ml	68.33 ± 6.74	80.65	1.25*
LDSK50-EA 100 μg/ml	65.67 ± 6.94	85.13	1.26*
LDSK50-EA 150 μg/ml	59.67 ± 4.33	79.75	1.29*
24 h Treatment			
Control	13.00 ± 1.53*	94.36	1.90
MMC 0.8 μg/ml	78.67 ± 3.84	80.03	1.29*
LDSK50-EA 25 μg/ml	61.00 ± 4.16*	80.66	1.31*
LDSK50-EA 50 μg/ml	62.00 ± 3.79*	75.15	1.34*
LDSK50-EA 100 μg/ml	59.00 ± 2.65*	75.89	1.21*
LDSK50-EA 150 μg/ml	52.67 ± 2.85*	69.89	1.22*

[a]Number of MNC per 1,000 BN cells scored. The results were expressed as means±SE (n=3). *Significant difference was detected from MMC treatment groups at $p \leq 0.05$ (ANOVA).

[b]Cell viability. The results were expressed as mean values of three independent experiments.

[c]Cytokinesis block proliferation index (CBPI). The results were expressed as mean values of three separated experiments. *Significant difference was detected from control groups at $p \leq 0.05$ (ANOVA).

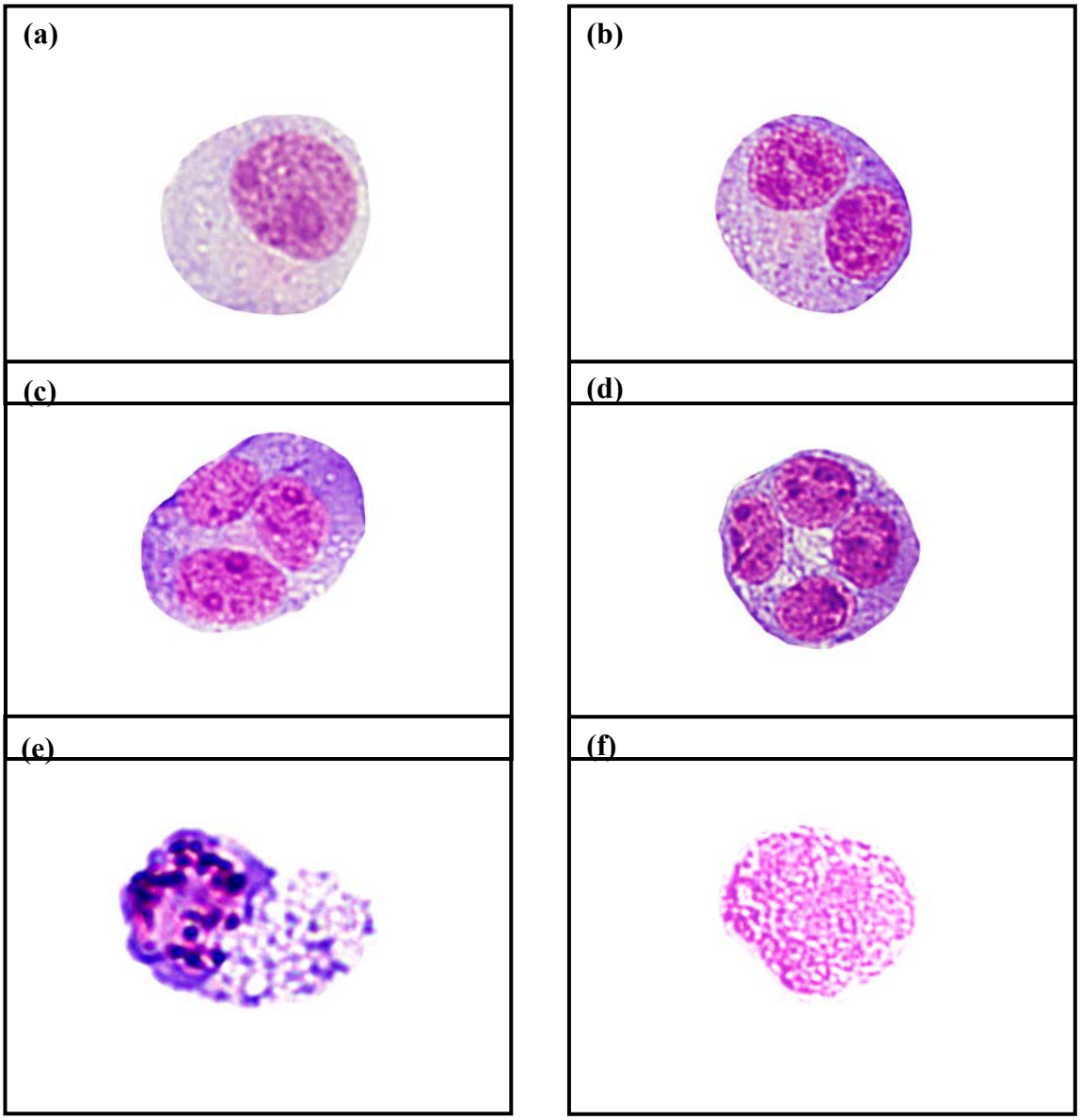


Figure 3.11 Photomicrographs illustrated the characteristic of TK6 cells stained with 10% Giemsa solution found in the CBMN assay : (a) mononucleated cell; (b) binucleated cell; (c) - (d) multinucleated cells; (e) apoptotic cell and (f) necrotic cell.

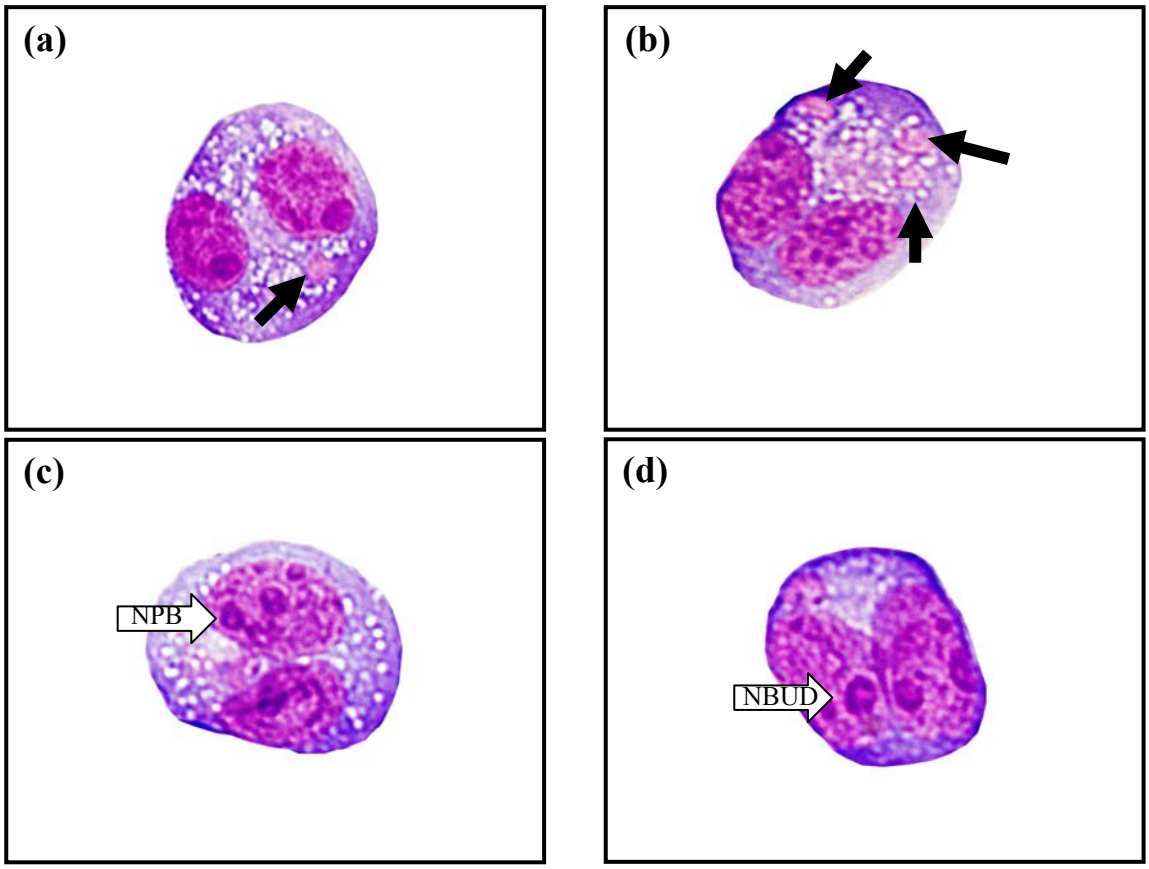


Figure 3.12 Photomicrographs of binucleated (BN) cells with micronucleus (arrow point). (a) BN cell with one micronucleus; (b) BN cell with three micronuclei. (c) BN cell containing an nucleoplasmic bridge (NPB) between nuclei; (e) BN cell containing nuclear bud (NBUD, on the right nucleus) that resemble micronucleus but should not be classified as micronucleus.

3.5 Determination of phytochemical components in LDSK50-EA

3.5.1 Thin layer chromatography (TLC)

LDSK50-EA was dissolved in absolute ethanol at concentration of 100 mg./ml., and spotted in 10-20 µl aliquots onto a silica gel F_{254} plates. The developing solvents were System 1: toluene: ethyl acetate: formic acid (5:4:1) and System 2: ethyl acetate: formic acid: acetic acid: water (137:11:11:26). After development, the plates were dried and sprayed with natural product (diphenylboryloxyethanolamine)-polyethyleneglycol (PEG) reagent. The bands were visualized under UV detector at 366 nm and their Rf values were recorded and compared with three standard phytochemicals including scopoletin, rutin and chlorogenic acid. TLC analysis of LDSK50-EA is shown in Figure 3.13. Under the detecting conditions used in this study, the results clearly revealed a presence of scopoletin (R_f 0.44), rutin (R_f 0.34) and chlorogenic acid (R_f 0.49) in LDSK50-EA.

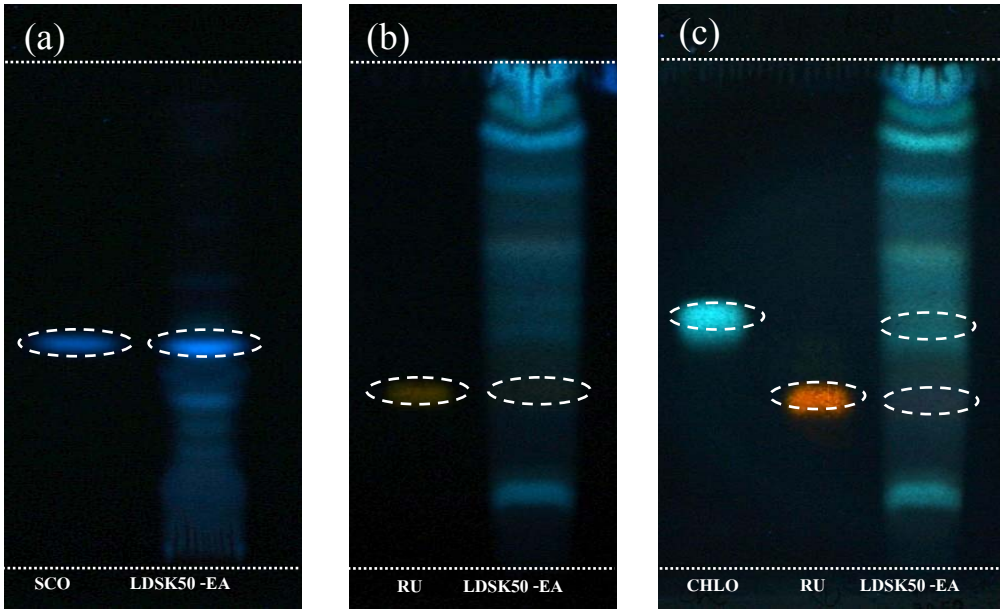


Figure 3.13 TLC analysis of LDSK50-EA fraction detected with NP/PEG spray reagent (366 nm) and against phytochemical standards including scopoletin (SCO), rutin (RU) and chlorogenic acid (CHLO).
(a) toluene: ethyl acetate: formic acid (5:4:1) system; (b), (c) ethyl acetate: formic acid: acetic acid: water (137:11:11:26) system.

3.5.2 Total phenolic content (TPC)

Content of phenolic compounds was determined following the Folin-Ciocalteu method in comparison with standard gallic acid. The results expressed in terms of mg. gallic acid equivalent (GAE)/mg. sample extract. From this study, the TPC value for LDSK50-EA was 0.198±0.001 mg GAE/mg extract (Table 4.7).

3.5.3 Total flavonoid content (TFC)

Content of flavoniod compounds was determined using the aluminum chloride colorimetric method in comparison with standard rutin and the results expressed in terms of mg. rutin equivalent (RE)/mg. sample extract. This study showed that TFC value of LDSK50-EA was 0.415±0.005 mg RE/mg extract (Table 3.7).

Table 3.7 Determination of total phenolic and total flavonoid contents in LDSK50-EA fraction

Fraction	Total phenolic content (TPC) (mg. GAE/mg. extract)	Total flavonoid content (TFC) (mg. RE/mg. extract)
LDSK50-EA	0.198 ± 0.001	0.415 ± 0.005

The results were expressed as mean±SD (n = 3).

CHAPTER IV

DISCUSSION AND CONCLUSION

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 degenerative diseases. These substances possess a high anti-oxidative potential and are counterparts to oxidative stress (Comhair and Erzurum 2010). Consequently, fruit is considered to be an important source of natural anti-oxidants, especially, peels (skins) and seeds which become wastes unless recycled or applied to use (Okonogi *et al.* 2007).

In such conditions, dietary intakes of anti-oxidant compounds are necessary for the body to neutralize the free radicals and remove the harmful effects of oxidative stress. Fruits, vegetables, grains and medicinal plants are known to contain a number of phenolic compounds with strong anti-oxidant activity. These compounds are found to be well correlated with anti-oxidant potential.

Nowadays, with safety concern, there is an increasing trend to replace synthetic anti-oxidants, with the natural anti-oxidants available from plant extracts or isolated products of plant origin. Even through Thailand has a variety of fruits, only some of them are widely consumed. Among these, fruits of longkong (*Lansium domesticum* Corr.) have been a very popular one in Thailand and some other countries in Southeast Asia. Thus, this study was undertaken on longkong to investigate the biological activity as well as the certain anti-oxidant mechanisms using both cell-based (anti-oxidative DNA damage activity) and non-cell based systems (ROS scavenging property). In addition, the toxicity of active fractions of longkong fruit extracts was also evaluated.

4.1 PCL and deoxyribose assays of twelve fractions of *L. domesticum* extractions for anti-oxidant capacity against $O_2^{-\bullet}$ and $OH^{\bullet}$ radicals.

It is well accepted that reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are highly reactive chemical species and can cause the oxidation of various biological molecules such as lipid, polypeptides, protein and DNA. Excess production and accumulation of ROS lead to oxidative stress, which can cause number of diseases. In comparison with many other radicals, $O_2^{-\bullet}$ is unreactive, but it can be converted into highly reactive species such as $OH^{\bullet}$, 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 (Ďuračková and Gvozdjáková 2008). In such conditions, dietary intakes of anti-oxidant compounds are needed in assisting the body to neutralize the free radicals and remove the harmful effects of oxidative stress. Therefore, this study is aimed to evaluate the free radical scavenging activity of longkong *L. domesticum* extracts.

The photochemiluminescence (PCL) measures the potential anti-oxidant property of *L. domesticum* fractions by two different protocols e.g. ACW and ACL that consent to measure the anti-oxidant capacities of the water and lipid soluble components, respectively (Popov and 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.

As illustrated in Table 3.2, all twelve *L. domesticum* fractions exhibited the $O_2^{-\bullet}$ scavenging activity at different degree of activity for both ACL and ACW measurement systems. The results of the ACL demonstrated the overall anti-oxidant capacities 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, LDSK50-EA possessed the highest anti-oxidant activity with an equivalent to 6.625 nmol of trolox whereas other fractions exhibited slightly different in anti-oxidant capacity. Interestingly, the

anti-oxidant capacity of ACW system indicated that 50% ethanol extract of peels (LDSK50) still had high anti-oxidant capacity. The wide range of anti-oxidant capacities of all fractions were found from -0.065 to 98.733 nmol of ascorbic acid. The highest anti-oxidant activity was found in fraction of LDSK50-H_2O (98.733 nmol of ascorbic acid) followed by the LDSK50-EA (54.660 nmol of ascorbic acid).

Regarding the PCL results, it indicated that peels of *L. domesticum* fruits possessed higher $O_2^{-\bullet}$ scavenging activity than seeds, particularly when extracted with 50% aqueous ethanol and partitioned with ethyl acetate (LDSK50-EA) which had high potential of both hydrophilic and lipophilic anti-oxidants. The results of ACL and ACW suggested that the $O_2^{-\bullet}$ scavenger in LDSK50-EA fractions was of both polar and non-polar phytochemical groups.

Furthermore, the hydroxyl ($OH^{\bullet}$) radical scavenging activity of *L. domesticum* was also determined by the deoxyribose assay, another cell-free radical generating system. This assay monitored an inhibitory effect of *L. domesticum* fractions on 2-deoxyribose (2-DR) degradation by measuring the competition between 2-DR and sample fractions for the $OH^{\bullet}$ generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. $OH^{\bullet}$ radicals formed in solution were detected by their ability to degrade 2-DR into fragments that on heating with TBA at low pH formed a pink chromogen. The absorbance read at the end of the experiment was used for the calculation of the percentage inhibition of 2-DR degradation by the test samples.

When *L. domesticum* fractions were added to the reaction mixture, they removed $OH^{\bullet}$ from the sugar and prevented their degradation. The scavenging effect of *L. domesticum* fractions on $OH^{\bullet}$ was determined by monitoring the reduction of deoxyribose degradation. The results were expressed as % inhibition of 2-DR degradation. As shown in Figure 3.3 and Table 3.1, in the presence of *L. domesticum* fractions (0.5, 1.0 and 2.0 mg./ml. concentration), a wide range of $OH^{\bullet}$ scavenging activity found from 0.50±0.12 to 93.44±0.84. The LDSK50-H_2O fraction has clearly presented to be the most effective inhibitor of the $OH^{\bullet}$ by exhibiting 93.44±0.84%

inhibition on 2-DR degradation. However, the wide range of % inhibition values among various *L. domesticum* fractions was possibly caused by their solubility character in the water which was the solvent mainly used in the deoxyribose assay.

4.2 Cytotoxicity of LDSK50-EA and LDSK50-H_2O determined by MTT assay in TK6 and V79 cell lines

Regarding the results of overall anti-oxidant capacity of the *L. domesticum* fractions evaluated by ACL, ACW and deoxyribose assays, the LDSK50-EA and LDSK50-H_2O exhibited the greatest $O_2^{-\bullet}$ and $OH^{\bullet}$ scavenging activities. Thus, these two fractions were classified as active fractions and selected for study on their cytotoxicity as well as biological activity through the cell-based model in further experiments.

While the active fractions possess high anti-oxidant properties, their safety is more important. Hence, the cytotoxic property of the active fractions of *L. domesticum*, was therefore conducted. The MTT colorimetric assay based on the reduction of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide) by mitochondrial dehydrogenase to a purple formazan product, was used to assess the cytotoxic effect of the two active fractions, LDSK50-EA and LDSK50-H_2O, on both human (TK6) and animal (V79) cells. Due to their high sensitivity, the TK6 and V79 cell lines have widely been employed in cytotoxicity and genotoxicity testings.

In the present study, the MTT assay was carried out by incubating small amounts of cells in the presence of LDSK50-EA or 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 conditions used in this study, V79 cells seemed to be more sensitive to LDSK50-EA than TK6 cells.

This observation was supported by the IC_{50} values (the concentration that inhibited cell growth by 50%). While IC_{50} value of LDSK50-EA in TK6 cells was 280 µg/ml., the value in V79 cells was 231 µg/ml. (Table 3.3). According to the classification of the cytotoxicity for natural ingredients (Gad Shayne 1999) (Table 4.1), the LDSK50-EA could be categorized as potentially harmful substance. Its degree of cytotoxicity suggested that LDSK50-EA might be useful for cancer treatment. However, prior to the implementation, the cytotoxicity of this substance on cancer cell lines must be thoroughly established.

In contrast to LDSK50-EA, LDSK50-H_2O was classified as potentially non-toxic in both TK6 and V79 cells. No inhibition of cell proliferation was found in LDSK50-H_2O. This was apparent 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. and greater than 5,000 µg/ml. for V79 and TK6 cells, respectively.

Table 4.1 Classification of the cytotoxicity for natural ingredients (Gad Shayne 1999)

Category	**IC_{50}**
Potentially very toxic	IC_{50} < 10 µg/ml.
Potentially toxic	10 µg/ml. < IC_{50} < 100 µg/ml.
Potentially harmful	100 µg/ml. < IC_{50} < 1,000 µg/ml.
Potentially non toxic	IC_{50} > 1,000 µg/ml.

4.3 Anti-oxidative DNA damage activity of two active fractions: LDSK50-EA and LDSK50-H_2O on TK6 cells by comet assay

In the last two decades, the comet assay or single cell gel electrophoresis (SCGE) has swiftly become one of the most popular methods in genetic toxicology. Its advantage is based upon relatively fast, simple, and sensitive technique for the analysis of single strand break (SSB), double strand break (DSB), alkali-labile site (ALS) of DNA and incomplete excision repair sites in eukaryotic individual cell. Moreover, the comet assay has been extensively used for the investigation of the effects of anti-oxidants. Among underlying principles, the alkaline (pH>13) version of comet assay is superior for evaluating a broad spectrum of DNA lesions, and maximizes sensitivity for detection of low levels of damage. Thus, it has been chosen as a useful general tool for monitoring DNA damage (Tice *et al*. 2000).

In this study, comet assay on TK6 cells was performed with the aim to evaluate anti-oxidative DNA damage mechanism of the LDSK50-EA against H_2O_2 induction. H_2O_2 is a direct non-radical reactive oxygen species (ROS). Though, H_2O_2 itself is incapable of damaging DNA directly, it is the main source of $OH^{\bullet}$ through Haber-Weiss and Fenton reactions. The analysis of the results obtained from comet assays was based on two major DNA damage parameters e.g. the tail length (TL, in μm) and tail moment (TM, in %). However, there are comments concerning the uses of these parameters since TL would reach the plateau value after migrating to the certain distance but grow in intensity. Therefore, TM is generally considered as the main representation of DNA damage.

The results of comet assay from this study revealed that treatment of H_2O_2 at 50 μM for 5 min. produced DNA damage (%TM) in TK6 cells at about 10-fold greater than in untreated cells. This indicated that H_2O_2 clearly played the important role of oxidative DNA damage in TK6 cells. The geno-protective activity of LDSK50-EA and LDSK50-H_2O in TK6 cells was found when cells were pre-treated with one of these two active fractions (25, 50, 100 and 200 μg/ml) for 24 hr prior to an exposure to H_2O_2. The DNA protective effect against H_2O_2 of LDSK50-EA and LDSK50-H_2O was

indicated by a reduction in TL and TM values in comparison to cells treated with H_2O_2 alone (Figures 3.6, 3.7 and Table 3.5).

Interestingly, the H_2O_2 induced DNA damage in TK6 cells was prevented by LDSK50-EA pre-treatment at 25, 50,100,200 µg/ml. and in dose-dependent manner. The highest DNA preventive effect was found at 200 µg/ml. concentration with % DNA damage inhibition of 53.47±1.99 (Figures 3.8 and Table 3.5). However, treatment of LDSK50-EA at dose greater than 200 µg/ml. (up to 250 µg/ml.) caused a very little alteration in % inhibitory effect but induced high cytotoxicity (data not shown). In contrast, the LDSK50-H_2O fraction exhibited a slight inhibitory oxidative DNA damage activity when tested at the similar concentrations of LDSK50-EA (Figures 3.8 and Table 3.5). Nevertheless, pre-treatment of cells with highest dose of LDSK50-EA (more than 1,000 µg/ml.) did not induce higher % inhibition effect (data not shown).

4.4 Anti-mutagenic activity of the most active LDSK50-EA fraction on TK6 cells by the *in vitro* CBMN assay

Since it was clearly evident by comet assay that the LDSK50-EA fraction exhibited more potent anti-oxidative DNA damage activity than that of LDSK50-H_2O, therefore only LDSK50-EA was chosen for further study on anti-mutagenic property. The micronucleus assay in TK6 cells using the cytokinesis-block micronucleus (CBMN) test (Fenech *et al.* 2003) was selected to explore this characteristic of LDSK50-EA. In the recent years, the CBMN has become an attractive tool for genotoxicity testing because of its simplicity of scoring and wide applicability in different cell types. The micronuclei (MN) is frequently used as simple biomarkers for cytogenetic damage in genotoxicity testing and is relevant for the increase of mutation rate leading to carcinogenesis event (Bonassi *et al*. 2011).

The assessment of anti-mutagenic activity using CBMN was performed only on LDSK50-EA which was considered the most active fraction. To ensure that the analyzed cells had completed only one nuclear division, addition of the actin polymerization inhibitor cytochalasin-B (Cyt-B) during targeted mitosis was employed.

Adding Cyt-B to the incubation system would result in an accumulation of dividing cells at the binucleated stage. Anti-mutagenic potential of LDSK50-EA was then analyzed in only binucleated (BN) cells in term of a reduction in micronucleus frequencies following MMC induction. Any samples inhibit this type of damage can be classified as anti-mutagenic agents.

In this study, the well-known mutagen, mitomycin C (MMC) was used as the positive control to promote micronucleus formation (OECD 2009). MMC is an alkylating and DNA cross-linking agent. It is the direct-acting mutagen and does not require any molecular activation. The mechanism of mutagenesis of MMC, similar to other alkylating agents, involves the transfer of a methyl or ethyl group to the nitrogenous bases of DNA, resulting in an alteration of base pairing. Alkylation activates error-free DNA repair processes which introduce transitions, transversions and changes in reading frames. Moreover, the DNA damage inducing agents MMC also has the capacity to produce intra- and inter-strand cross-links in DNA, induces chromosomal breaks which can result in various chromosomal anomalies including MN. The anti-mutagenic activity was measured following the addition of LDSK50-EA fractions to inhibit mutagenesis induced by MMC.

The incorporation of Cyt-B in the test system was not only to ensure the measurements of cell proliferation that underwent mitosis during the assay but also to confirm that the treatments were conducted at appropriate levels of cytotoxicity. The cytostasis/cytotoxicity ratio could be quantified from the Cytokinesis Block Proliferation Index (CBPI) from at least 500 cells per culture (OECD 2009). The lowest CBPI value possible was 1.0, reflected the failure of all of the viable cells to divide during the cytokinesis-block period and was therefore all mononucleated. But, if all viable cells completed one nuclear division and were therefore all binucleated, the CBPI value was 2.0.

The results obtained from this study demonstrated that when TK6 cells were treated with LDSK50-EA (25, 50, 100, 150 µg/ml.) and MMC (0.8 µg/ml.) for 24 h, a

clear anti-mutagenic effect occurred in a great extent than at 4 h treatment time. The mean values of micronucleus (MN) formation (number of MNC per 1,000 BN cells scored) of untreated TK6 cells (receiving RPMI) were 11.33±1.86 and 13.00±1.53, for 4 h and 24 hr treatment times, respectively. The MN formation was 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 $p \leq 0.05$ (ANOVA). This suggested the mutagenic mechanism of MMC is through DNA cross-link formation which led to chromosome breakage and initiated MN formation. Thus, we can call substance being able to produce MN from a lagging acentric chromosome fragment as clastogen.

As illustrated in Figure 3.10 and Table 3.6, the MN formation in BN cell was suppressed in the presence of LDSK50-EA. At 24-h treatment time (long-term treatment), the suppressive effect of LDSK50-EA against MMC-induced MN formation was found in dose-dependent manner. The highest anti-mutagenic effect of LDSK50-EA (59.67±4.33 MNC cells per 1,000 BN cells scored) was found when concentration of 150 μg/ml. was used. On the contrary, the anti-mutagenic effect of LDSK50-EA was not observed in TK6 cells when cells were treated for 4 h (short-term treatment) at all concentrations. This was indicated by no significant difference in MN frequency of all concentration of LDSK50-EA treated groups in comparison to MMC positive control group ($p \leq 0.05$, ANOVA).

Consideration on cytotoxic effect in CBMN test, it was found that the survival rates of TK6 cells after short-term (4 h) or long-term (24 h) treatments were greater than those in 70% in any LDSK50-EA concentrations, except when cells were incubated in the highest concentration (150 μg/ml.) at 24 h where a very slightly lower survival rate (69.89%) than acceptable value (70%) was observed. Though, it was not statistically significant different, it suggested a possible cytotoxic effect of MMC treatment in combination with higher doses of LDSK50-EA. While the observation of CBPI, the results illustrated the average CBPI values of TK6 cells of RPMI treatment (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, treatment with the combination of LDSK50-EA plus MMC exhibited CBPI value similar to MMC alone which was significantly lower than that of the control group ($p \leq 0.05$, ANOVA). This phenomenon suggested that MMC interfered cell proliferation.

When CBPI value was 1.30, the replication index (RI) and % cytostasis could be calculated from the formula presented in Appendix C which estimated equal to 34% and 66%, respectively. This implied that only 34% of treated cell completed one nuclear division while 66% of treated cell remained cytostasis or was restricted the cell division. The occurrence of cytostasis phenomenon was not only contributed by inhibitors of cell division but may also be the consequence of many cytotoxicity pathways leading to a delayed cell cycle. This confirmed that the clastogenic mechanism of MMC possessed the severe damage to the cell and expressed as the reduction of BN cells or cell division.

Based on the results obtained, it was reasonable to propose that the anti-mutagenic activity of LDSK50-EA was the result of its capability to block MN formation induced by MMC. However, more assays could be required to explore the mechanism of LDSK50-EA action such as chromosome aberration test (using metaphase analysis), cell cycle arrest assay and DNA repair assay. The results of these assays would support or confirm the anti-mutagenic activity of LDSK50-EA not only against MMC but also other mutagenic and carcinogenic substances.

4.5 Determination of phytochemical components in LDSK50-EA

Thin layer chromatography (TLC) is a separation technique that has been generally used in chemistry to separate compounds in the mixture. It is generally agreed that TLC is the most effective for the low-cost analysis of samples requiring minimal sample clean-up, or where TLC allows a reduction in the number of sample preparation steps. In this study, the TLC technique was used to detect the presence of phytochemicals in the LDSK50-EA active fraction. Following chromatogram development, the TLC plates were sprayed with various reagents such as natural

product (diphenylboryloxyethanolamine)-polyethyleneglycol (PEG) reagent to detect the phenolic compounds.

Phenolic compounds are characteristic of plants and as a group they are usually found as esters or glycosides rather than as free compounds. Current classification divides the broad category of phenolics into polyphenols and simple phenols, based solely on the number of phenol subunits presented. Polyphenols possess at least two phenol subunits that include the flavonoids and those compounds with three or more phenol subunits, referred as the tannins (hydrolyzable and non-hydrolyzable).

TLC analysis of LDSK50-EA is shown in Figure 3.1. Under the natural product-PEG detecting conditions, the results clearly revealed the presence of scopoletin (R_f 0.44), rutin (R_f 0.34) and chlorogenic acid (R_f 0.49) in LDSK50-EA. Subsequently, the total phenolic content (TPC) of LDSK50-EA was determined using Folin-Ciocalteu reagent to quantify the amount of phenolic compounds. The results expressed in terms of mg. gallic acid equivalents (GAE)/mg. sample extract. From this study, the TPC value for LDSK50-EA was 0.198±0.001 mg. GAE/mg. extract. At the same time, the total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method in comparison with standard rutin and the results expressed in terms of mg. rutin equivalents (RE)/mg. sample extract (Amensour *et al*. 2010 and Chang *et al.* 2002). The results illustrated the TFC value of LDSK50-EA to be 0.415±0.005 mg. RE/mg. extract.

The overall results of determination of phytochemical composition in peels extract of *L. domesticum* fruits **(**LDSK50-EA) have shown that it was the major source of phenolic and flavonoid compounds. This finding was consistent with the earlier studies (Huang *et al.* 2010; Okonogi *et al*. 2007). Due to the limitation of time and material availability (phenolic compound standards), only 3 phenolic compounds including scopoletin, rutin and chlorogenic acid were identified.

However, data of this study warrant good biological activities of LDSK50-EA including anti-oxidant, anti-oxidative DNA damage and anti-mutagenic activity. Its potent biological activities may be related to the occurrence of high potential phenolic and flavonoid substances. As many studies have demonstrated the anti-oxidant action of phenolic compounds, acting as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation.

A number of *in vitro* experiments have found that flavonoids exert a significant anti-oxidative ability, due to the presence of the hydroxyl groups in the B ring. It donates hydrogen atoms to radical reactions. The double bond at position 2, 3 in conjugation with the 4-oxo-group in the C ring and the hydroxyl groups are capable to bind transition metal ions such as iron and copper. Hence, these contribute to the chelating ability of flavonoids. On the organism, the positive effect of flavonoids is exerted via several pathways. In addition to the anti-oxidative effect mentioned above, flavonoids also possess other anti-oxidative ability, e.g. through stimulation of anti-oxidative enzymes, have vasodilating, anti-thrombotic, anti-inflammatory and anti-apoptic effects. Moreover, flavonoids also exhibit anti-mutagenic ability and can inhibit the bond of cancerogenic compounds to DNA (Ďuračková and Gvozdjáková 2008).

In summary, LDSK50-EA possesses anti-oxidant, anti-oxidative DNA damage and anti-mutagenic properties. However, further biochemical studies are needed to better characterize its interaction with DNA damage inducing agents for elucidation of its anti-mutagenic mechanism. In addition, more information of the potential toxic effects linked with LDSK50-EA in animal model is also necessary to ensure its safety use.

CHAPTER V

PRIMARY MARKETING ANALYSIS AND PROJECT IMPACT

Primary marketing analysis :

It was found from the data of global market research that the current trend of cosmetics was looking for natural ingredients for cosmetic industry. Since innovation in the cosmetics industry is intrinsically linked to product formulations, the search for new ingredients is intensifying. The research principle of this project of TISTR is based upon using the natural sources (plants/herbals) as a novel green ingredients for health products and cosmetics, particularly. Although a growing number of companies are looking to the seas and oceans for novel ingredients, the development of such raw materials raises many ethical and ecological questions.

This project had been carried out for 4 years during 2009-2012. The outcome of the project will be beneficial to cosmetic industry as follows:

1. Support small business exemptions for cosmetic companies so that they may create local jobs and contribute to local economies.
2. Reduce regulatory paperwork that has little to no impact on cosmetic safety but drives up the cost of doing business following the research conduction.
3. Support financial aid as a way to foster and grow small businesses (SME) in the country.
4. The development of products was through scientific process by controlling the amount extracted from the pharmacological properties and safety assessment. To the information and benefits to consumers as well as product registration.

The project impact :

(I) The economic and social impact :

Data at the global level indicates that among many cosmetics, the personal care products are receiving high popularity. Among these, the anti-aging products of natural ingredients and innovative solutions for hair protection are on top. Beauty manufacturers will also further explore simple formulas such as infusions and fluids but formulate them with a new generation of phytochemicals and natural process.

Our study generated new and updated information on biological activity of long-kong fruits that has not yet been published before. The fruitful results on non-cytotoxicity, free radical scavenging, anti-oxidative DNA damage and anti-mutagenic activities will promote and strengthen utilization of *L. domesticum*. Also, it may lead to a discovery of new alternative source of natural anti-oxidant. This can fulfill the requirement on new source of natural ingredients for cosmetic industry.

(II) The environmental impact :

Food waste (measures the decrease in edible food mass (excluding inedible parts and seed) or food loss (such as fuel or animal feed) is food that is discarded or lost uneaten. As of 2011, 1.3 billion tons of food, about one-third of the global food production, was lost or wasted annually. Loss and wastage occurs on all steps in the food supply chain. In low-income countries, most loss occurs during production while in developed countries much food –about 100 kilograms per person and year – is wasted at the consumption stage.

We had performed this research project on “Long-Kong” *Lansium domesticum* Corr. (Meliaceae) which is an economic plant and very popular in Thailand. This study entirely used the *L. domesticum* fruits in particular, the skins and seeds which are considered as waste. Therefore, this can be a fruitful way or tool to help getting rid of food/fruit waste in environment by developing health product for human.

Project product development:

The data obtained from present study show that 12 fractions isolated from peels and seeds of *L. domesticum* fruits exhibited the wide range of the anti-oxidant capacity against $O_2^{-\bullet}$ and $OH^{\bullet}$ radicals. Peels of *L. domesticum* fruits possessed higher $O_2^{-\bullet}$ and $OH^{\bullet}$ scavenging activity than seeds, particularly when extracted with 50% aqueous ethanol and partitioned with ethyl acetate (LDSK50-EA). This fraction had high potential of both hydrophilic and lipophilic anti-oxidants. Moreover, the LDSK50-EA had the geno-protective effect by reduction of the DNA and chromosome damage induced by H_2O_2 radicals and mitomycin C (MMC) mutagen. These findings were illustrated in results of the comet assay and the micronucleus test in TK6 cells.

This study generated new and updated information on biological activity of long kong fruits that has not yet been published before. The fruitful results on non-cytotoxicity, free radical scavenging, anti-oxidative DNA damage and anti-mutagenic activities will promote and strengthen utilization of *L. domesticum*.

Regarding our fruitful discovery in pharmacological property of LDSK50-EA sub-fraction of *L. domesticum*, we had used it as an active ingredient in development of two cosmetic products including the facial toner (Figure 5.1) and facial mask (Figure 5.2). Details of development of these two products are disclosed due to the matter of a patent arrangement.

Figure 5.1 the “Facial Toner” formulated using the LDSK50-EA of *L. domesticum* as an active ingredient.

Figure 5.2 the “Facial Mask” formulated using the LDSK50-EA of *L. domesticum* as an active ingredient.

CHAPTER VI

PROJECT SUGGESTION

- This study generated new and updated information on biological activity of long kong fruits that has not yet been published before. The fruitful results on non-cytotoxicity, free radical scavenging, anti-oxidative DNA damage, anti-mutagenic activities will promote and strengthen utilization of *Lansium domesticum* Corr.

- The results of this project research have spurred innovation and technology development of new cosmetic products derived from active fraction of *L domesticum* or "long-kong" extracts. Through the technology transfer process, these products will be industrialized manufactured and make significant impacts in the marketplace especially the cosmetic market from natural sources that hits the big wave of consumer's interest.

- Since the project will be completely ended by September 2012, the newly developed products have not yet been introduced to the business sectors. Therefore, process of technology transfer from the laboratory to a commercial organization is pretty straightforward. Companies that are interested in commercializing this technology from TISTR will be involved after releasing these products to a public in the near future after the end of this fiscal year (2012).

- However, during 27-30 August 2012, TISTR had arranged the free-transfer technology of cosmetic products derived from Long-Kong extracts to the muslim community in Narathiwat province (as shown in pictures). This arrangement was carried for local muslim people who are suffering from unpeaceful situation in the area. It's also served as Corporate Social Responsibility (CSR) that is the Government's policy.


วิทยาลัยสารพัดช่างนราธิวาส
ยินดีต้อนรับ
ผู้เข้าร่วมพิธีลงนามความร่วมมือ (MOU)
และผู้เข้าร่วมอบรมถ่ายทอดนวัตกรรมและ
ผลิตภัณฑ์แปรรูปผลผลิตทางการเกษตร "ลองกอง"
ห้องประชุมพฉาสนท์ อาคาร 3 ชั้น 3
ด้วยความยินดียิ่ง


ยินดีต้อนรับ


สารสกัดที่มีคุณประโยชน์จากลองกองและการนำสารสกัดมาใช้ในการผลิตเค
การเตรียมสารสกัดลองกองในห้องปฏิบัติการ
สรรพคุณของสารสกัดลองกอง
การนำสารสกัดมา
ในสูตรเครื่อ
สารสกัดจากแยมลองกอง
สารสกัดจากเปลือกลองกอง
6.แผ่นหลัง
7.สะโพก
8.ขา
9.ข้อเท้า


สารสกัดที่มีคุณประโยชน์จากลองกองและการนำสารสกัดมาใช้ในการ
การเตรียมสารสกัดลองกองในห้องปฏิบัติการ
สรรพคุณของสารสกัดลองกอง
สารสกัดจากแยมลองกอง
สารสกัดจากเปลือกลองกอง
6.แผ่นหลัง
7.สะโพก

CHAPTER VII

REFERENCES

Bonassi, S., El-Zein, R., Bolognesi, C., and Fenech, M., 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis,* **26**(1), pp. 93-100.

Comhair, S. A., and Erzurum, S. C., 2010. Redox control of asthma: molecular mechanisms and therapeutic opportunities. *Antioxidants & Redox Signaling,* **12** (1), pp. 93-124.

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

Fenech, M., Chang, W. P., Kirsch-Volders, M., Holland, N., Bonassi, S., and Zeiger, E., 2003. HUMAN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research,* **534**(1-2), pp. 65-75.

Gad Shayne, C., 1999. Alternatives to in vivo studies in toxicology. *In :* B. Ballantyne, T. C. Marrs & T. L. M. Syversen (Eds.), *General and applied toxicology* (Vol. 1, p. 178). London; New York, NY: Macmillan; Grove's Dictionaries.

Huang, W.-Y., Cai, Y.-Z., Corke, H., and Sun, M. 2010. Survey of antioxidant capacity and nutritional quality of selected edible and medicinal fruit plants in Hong Kong. *Journal of Food Composition and Analysis,* **23**(6), pp. 510-517.

Leaman, D. J., Arnason, J. T., Yusuf, R., Sangat-Roemantyo, H., Soedjito, H., Angerhofer, C. K., *et al.*, 1995. Malaria remedies of the Kenyah of the Apo Kayan, East Kalimantan, Indonesian Borneo: A quantitative assessment of local consensus as an indicator of biological efficacy. *Journal of Ethnopharmacology,* **49**(1), pp. 1-16.

Mayanti, T., Tjokronegoro, R., Supratman, U., Mukhtar, M. R., Awang, K., and Hadi, A. H., 2011. Antifeedant triterpenoids from the seeds and bark of *Lansium domesticum* cv Kokossan (Meliaceae). *Molecules,* **16**(4), pp. 2785-2795.

Monzon, R. B., Alvior, J. P., Luczon, L. L., Morales, A. S., and Mutuc, F. E., 1994. Larvicidal potential of five Philippine plants against *Aedes aegypti* (Linnaeus) and *Culex quinquefasciatus* (Say). *The Southeast Asian Journal of Tropical Medicine and Public Health,* **25**(4), pp. 755-759.

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

Okonogi, S., Duangrat, C., Anuchpreeda, S., Tachakittirungrod, S., and Chowwanapoonpohn, S., 2007. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. *Food Chemistry,* **103**(3), pp. 839-846.

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

Saewan, N., Sutherland, J. D., and Chantrapromma, K., 2006. Antimalarial tetranortriterpenoids from the seeds of *Lansium domesticum* Corr. *Phytochemistry,* **67**(20), pp. 2288-2293.

Tanaka, T., Ishibashi, M., Fujimoto, H., Okuyama, E., Koyano, T., Kowithayakorn, T., *et al.*, 2002. New onoceranoid triterpene constituents from *Lansium domesticum*. *Journal of Natural Products,* **65**(11), pp. 1709-1711.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., *et al.*, 2000. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* **35**(3), pp. 206-221.

Tilaar, M., Wih, W. L., Ranti, A. S., Wasitaatmadja, S. M., Suryaningsih, Junardy, F. D., *et al*., 2008. Review of Lansium domesticum Corrêa and its use in cosmetics. *Boletin Latinoamericano y del Caribe de Plantas Medicinales y Aromaticas,* **7**(4), pp. 183-189.

APPENDICES

APPENDIX A
SAMPLE PREPARATION

1. Sample preparation for PLC and deoxyribose assay

he sample fractions were freshly prepared by weighing 10 mg of each *L. domesticum* fractions and dissolved in 1 ml of dilution reagent supplied with the ACL and ACW reagent kit. While used distilled water as solvent for deoxyribose assay. The solution was sonicated for 10 min to facilitate complete solubility. The supernatants were filtered through 0.45 mm syringe filter.

2. Sample preparation for MTT assay, Comet assay and CBMN assay

The stock solution containing 100 mg/ml of sample fractions were prepared by dissolving *L. domesticum* fractions 100 mg in 1 ml of DMSO, mixed well using sonication. These solutions were then aliquoted into micro-centrifuged tube and stored at freezer (-25°C), protect from light until being used.

3. Sample preparation for TLC

The stock solution containing 100 mg/ml of sample fraction was prepared by dissolving *L. domesticum* fractions 100 mg in 1 ml of absolute ethanol. The solution was sonicated for 10 min to facilitate complete solubility and stored at freezer (-25°C), protect from light.

4. Sample preparation for determination of TPC and TFC

The solution containing 2 mg/ml of sample fraction was freshly prepared by dissolving *L. domesticum* fractions 2 mg in 1 ml of absolute ethanol. The solution was sonicated for 10 min to facilitate complete solubility.

APPENDIX B

CHEMICALS AND REAGENTS PREPARATION

1. Chemicals and reagents preparation for comet assay

As a general rule, all buffers and reagents used in the assay should be given shelf-lives of no more than 1 month (Hartmann et al. 2003).

A. Agarose

0.50% LMP: dissolving 0.50 g LMP in 100 ml PBS.

0.75% NMP: dissolving 0.75 g NMP in 100 ml PBS.

Warmed in microwave until completely dissolve. LMP agarose was aliquoted into cleaned glass bottle 5 ml and stored at 4°C refrigerated. When used, quickly melt agarose in microwave and hold at 37°C in water bath until needed.

B. Lysing solution

Stock solution

NaCl	146.1	g
$Na_2EDTA{\cdot}2H_2O$	37.2	g
Trizmabase	1.2	g

Added each ingredient to about 700 ml distilled water and begin stirring. Adjusted pH to 10.0 using NaOH pellets and q.s. (quantity sufficient) to 1000 ml with distilled water, stored at refrigerator (4°C). The final concentration of stock solution compose of 2.5 M NaCl, 100 mM $Na_2EDTA{\cdot}2H_2O$ and 10 mM Trizmabase.

Working solution (freshly prepare)

Triton X-100	1	ml
DMSO	10	ml

Quantity sufficient to 100 ml with lysing stock solution and then refrigerated (4°C) before use.

C. Electrophoresis Buffer

Stock solution

10N NaOH**:** dissolving NaOH 200 g in 500 ml distilled water

200 mM EDTA**:** dissolving EDTA 14.89 g in 200 ml distilled water

Store both solutions at refrigerator (4°C) until being used.

Working solution (freshly prepare)

10N NaOH	30	ml
200 mM EDTA	5	ml

Quantity sufficient to 1000 ml with distilled water, mix well. Prior to use, measure the pH of the buffer to ensure pH > 13, chill on 4°C at least 1 h before used.

D. Neutralizing buffer

0.4 M Tris-base 48.5 g

Dissolved in 800 ml distilled water using stirring, adjusted pH to 7.5 with concentrated HCl, q.s. to 1000 ml with distilled water, and stored at refrigerator (4°C).

E. Staining solution

20 µg/ml ethidium bromide was prepared by dilution of 10 mg/ml ethidium bromide in PBS, then aliquoted into micro-centrifuged tube, and stored at freezer (-25°C), protect from light until being used.

2. Chemicals and reagents preparation of micronucleus test

A. Mitomycin C (MMC) solution

The stock solution of MMC (100 µg/ml) was prepared by dissolving MMC, which was combined with NaCl, 25 mg in water 10 ml, mixed well and filtered through a 0.2 µm filter to obtain sterility. The concentration was expressed in term of pure MMC in microgram per microliter (µg/ml) of solution. Finally, the stock solution of MMC was aliquoted into micro-centrifuged tube and stored at freezer (-25°C).

B. Cytochalasin-B (Cyt-B) solution

Cyt-B stock solution (1000 μg/ml) was prepared by dissolving Cyt-B 5 mg in dimethyl sulfoxide (5 ml), mixed well. Then sterilized and kept them similar to MMC as described above.

C. Gurr buffer solution

Gurr buffer solution was prepared by dissolving 1 Gurr tablet (Gibco Invitrogen Cooperation, USA) in 1000 ml distilled water using stirring at room temperature to facilitate dissolve. These solutions were kept at refrigerator (4°C).

D. Giemsa stain solution

Giemsa solution 10% (v/v) was prepared by dissolving 10 ml of Giemsa solution in Gurr buffer solution (adjusted to 100 ml) then filtered through a 0.5 μm filter.

APPENDIX C

FORMULATION AND CALCULATIONS

1. Cytotoxicity assessment of micronucleus assay

When Cyt-B was applied in the experiment, evaluation of cytotoxicity should be based on the CBPI (Cytokinesis Block Proliferation Index) or RI (Replication Index). The CBPI indicates the average number of cell cycles per cell during the period of exposure to Cyt-B, and may be used to calculate cell proliferation. The RI indicates the relative number of nuclei in treated cultures compared to control cultures and can be used to calculate the % cytostasis (cytostatic effects).

$$\textbf{CBPI} = M_1 + 2M_2 + 3M_3 + 4M_4 / N$$

$$\textbf{RI} = \frac{[M_2 + 2(M_3 + M_4)] / N_T}{[M_2 + 2(M_3 + M_4)] / N_C} \times 100$$

$$\textbf{\% Cytostasis} = 100 - 100[(CBPI_T - 1) / (CBPI_C - 1)] \quad \text{or}$$

$$= 100 - RI$$

Where M_1-M_4 represent the number of cells with 1-4 nuclei, N is the total number of viable cells scored (excluding necrotic and apoptotic cells), T abbreviate from treated culture and C abbreviate from control culture.

In this study, cytotoxicity was estimated by comparing CBPI values of all treatments in each experiment (4 and 24 h) and the result was analyzed by the Tukey multiple comparisons at statistical significant $p \leq 0.05$ (ANOVA).

APPENDIX D

INTERNATIONAL PUBLICATION

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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 50°C 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 45°C. Then, all fractions were kept at 4°C 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 37°C 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 37°C 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).

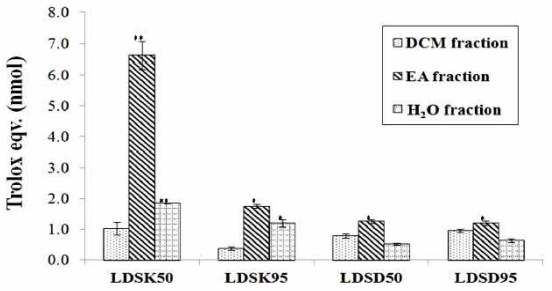

Fig. 1: Lipid-soluble antioxidant capacity (ACL) of twelve *L. domesticum* fractions by PCL assay. Results were expressed as means ± SD (n=3). *Significant difference was detected from the lowest activity fraction of same part-extraction ($p\leq0.05$). **Significant difference was detected from all fractions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

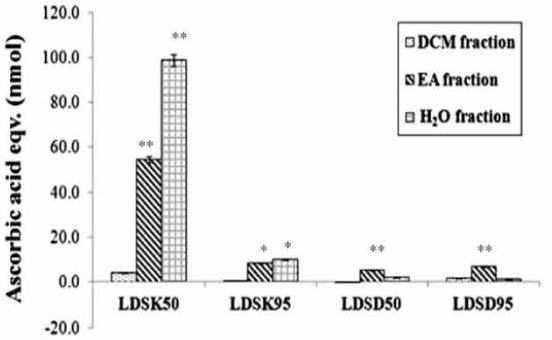

Fig. 2: Water-soluble antioxidant capacity (ACW) of twelve *L. domesticum* fractions by PCL assay. Results were expressed as means ± SD (n=3). *Significant difference was detected from the lowest activity fraction of same part-extraction ($p\leq0.05$). **Significant difference was detected from all fractions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

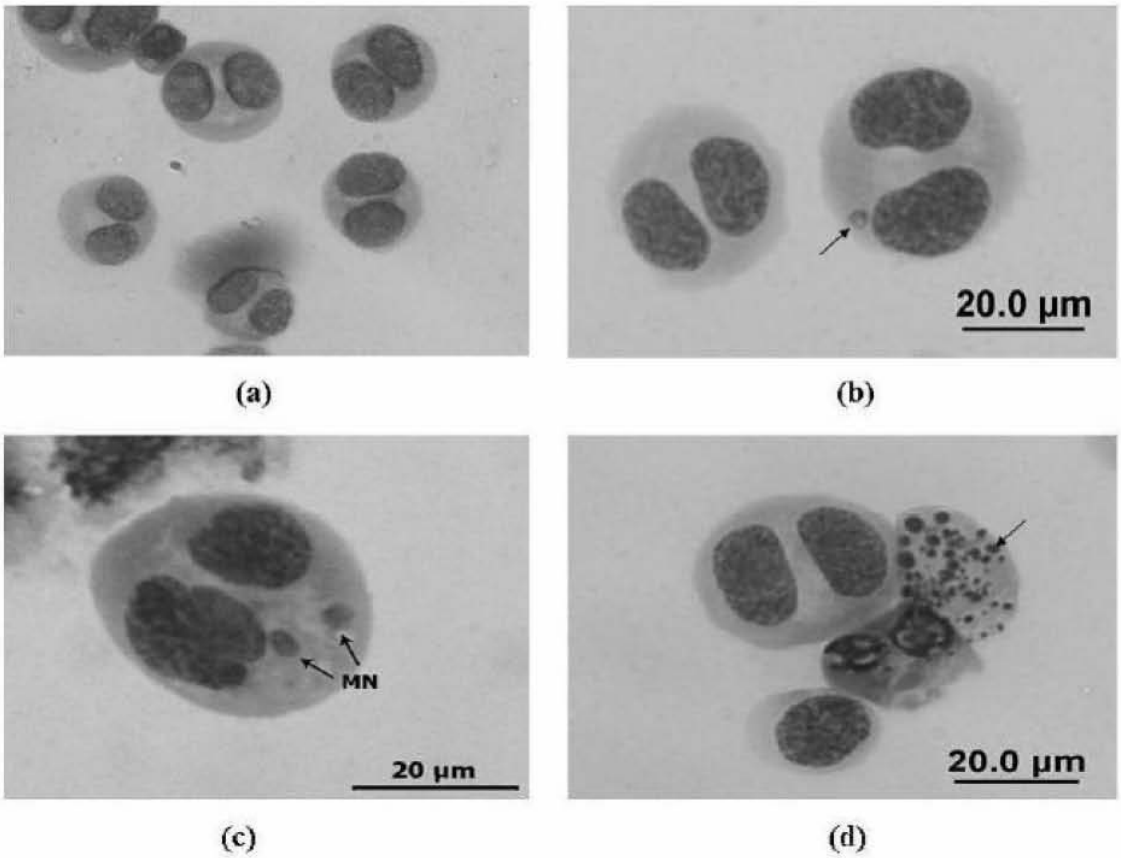

Fig. 6: Micronucleated (MNC) cells of TK6 in the CBMN assay: (**a**) an accumulation of binucleated cells (BNC) following cytochalasin B treatment, (**b**) BNC with one micronuleus (arrow), (**c**) BNC with two micronuclei (arrows), (**d**) apoptotic cell (arrow, not included in micronucleus scoring).

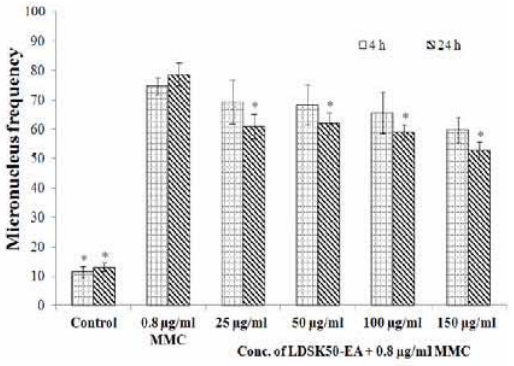

Fig. 7: Micronuclei frequency (number of MNC per 1,000 BNC scored) in TK6 cells after combination treatments of LDSK50-EA (at 25, 50, 100 and 150 µg/ml) and MMC (standard mutagen, 0.8 µg/ml) for 4 and 24 h. Results were expressed as means ± SE values of three independent experiments. *Significant difference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

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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.

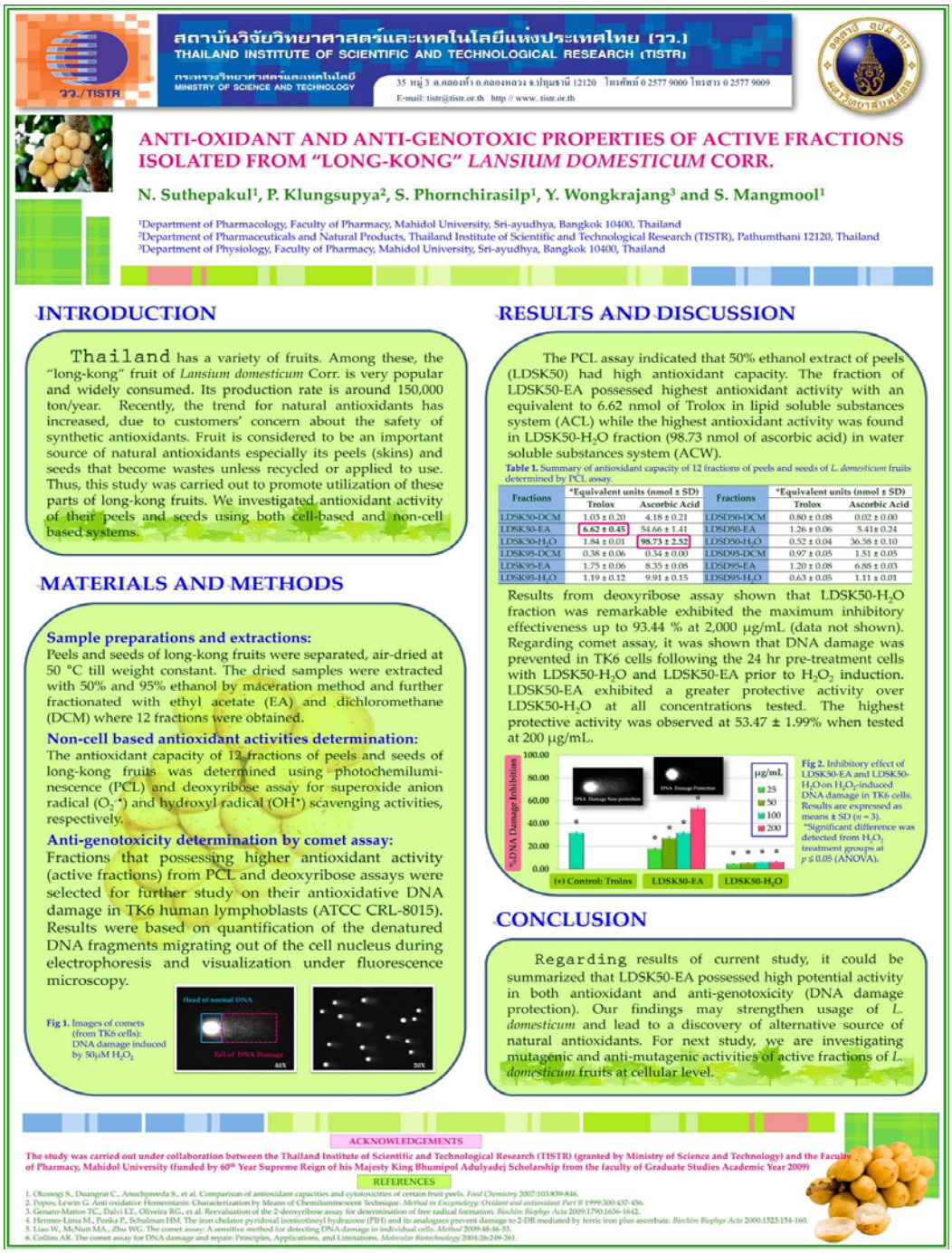
APPENDIX E

INTERNATIONAL POSTER PRESENTATION

2012 Klungsupya P, Suthepakul N, Laovitthayanggoon S, Thongdon-A J,Trangwacharakul S and Phornchirasilp S. **Biological activity evaluation and development of health products from natural antioxidants of Thai "Long-Kong" Lansium domesticum Corr. fruits**. The 13th international Congress of the Society for Ethnopharmacology (ISE13) September 2nd-6th, 2012 University of Graz, Graz, Austria.

2010 <u>Suthepakul N</u>, Klungsupya P, Rerk-am U, Trangvacharakul S, Arunpairoj V, and Phornchirasilp S, 2010. **Antioxidant capability of *Lansium domesticum* Corr. fruits by photochemiluminescence assay.** The 9th NRCT-JSPS Joint Seminar 2010: Natural Medicine Research for the Next Decade: New Challenges and Future Collaboration. December 8-9th, 2010, Bangkok,Thailand.

2011 Suthepakul N, Klungsupya P, Phornchirasilp S, Wongkrajang Y and Mangmool S, 2011. **Anti-oxidant and anti-genotoxic properties of active fractions isolated from "Long-Kong" *Lansium domesticum* Corr.** The 7th Indochina Conference on Pharmaceutical Sciences (Pharma Indochina VII): Advancing Pharmacy for ASEAN Community. December 14-16th, 2011, Bangkok, Thailand.



สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย (วว.)
THAILAND INSTITUTE OF SCIENTIFIC AND TECHNOLOGICAL RESEARCH (TISTR)

ANTI-OXIDANT AND ANTI-GENOTOXIC PROPERTIES OF ACTIVE FRACTIONS ISOLATED FROM "LONG-KONG" *LANSIUM DOMESTICUM* CORR.

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INTRODUCTION

Thailand has a variety of fruits. Among these, the "long-kong" fruit of *Lansium domesticum* Corr. is very popular and widely consumed. Its production rate is around 150,000 ton/year. Recently, the trend for natural antioxidants has increased, due to customers' concern about the safety of synthetic antioxidants. Fruit is considered to be an important source of natural antioxidants especially its peels (skins) and seeds that become wastes unless recycled or applied to use. Thus, this study was carried out to promote utilization of these parts of long-kong fruits. We investigated antioxidant activity of their peels and seeds using both cell-based and non-cell based systems.

MATERIALS AND METHODS

Sample preparations and extractions:
Peels and seeds of long-kong fruits were separated, air-dried at 50 °C till weight constant. The dried samples were extracted with 50% and 95% ethanol by maceration method and further fractionated with ethyl acetate (EA) and dichloromethane (DCM) where 12 fractions were obtained.

Non-cell based antioxidant activities determination:
The antioxidant capacity of 12 fractions of peels and seeds of long-kong fruits was determined using photochemiluminescence (PCL) and deoxyribose assay for superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$) scavenging activities, respectively.

Anti-genotoxicity determination by comet assay:
Fractions that possessing higher antioxidant activity (active fractions) from PCL and deoxyribose assays were selected for further study on their antioxidative DNA damage in TK6 human lymphoblasts (ATCC CRL-8015). Results were based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis and visualization under fluorescence microscopy.

Fig 1. Images of comets (from TK6 cells): DNA damage induced by 50μM H_2O_2

RESULTS AND DISCUSSION

The PCL assay indicated that 50% ethanol extract of peels (LDSK50) had high antioxidant capacity. The fraction of LDSK50-EA possessed highest antioxidant activity with an equivalent to 6.62 nmol of Trolox in lipid soluble substances system (ACL) while the highest antioxidant activity was found in LDSK50-H_2O fraction (98.73 nmol of ascorbic acid) in water soluble substances system (ACW).

Table 1. Summary of antioxidant capacity of 12 fractions of peels and seeds of *L. domesticum* fruits determined by PCL assay.

Fractions	*Equivalent units (nmol ± SD)		Fractions	*Equivalent units (nmol ± SD)	
	Trolox	Ascorbic Acid		Trolox	Ascorbic Acid
LDSK50-DCM	1.03 ± 0.20	4.18 ± 0.21	LDSD50-DCM	0.80 ± 0.08	0.02 ± 0.00
LDSK50-EA	**6.62 ± 0.45**	54.66 ± 1.41	LDSD50-EA	1.26 ± 0.06	5.41± 0.24
LDSK50-H_2O	1.84 ± 0.01	**98.73 ± 2.52**	LDSD50-H_2O	0.52 ± 0.04	36.58 ± 0.10
LDSK95-DCM	0.38 ± 0.06	0.34 ± 0.00	LDSD95-DCM	0.97 ± 0.05	1.51 ± 0.05
LDSK95-EA	1.75 ± 0.06	8.35 ± 0.08	LDSD95-EA	1.20 ± 0.08	6.88 ± 0.03
LDSK95-H_2O	1.19 ± 0.12	9.91 ± 0.15	LDSD95-H_2O	0.63 ± 0.05	1.11 ± 0.01

Results from deoxyribose assay shown that LDSK50-H_2O fraction was remarkable exhibited the maximum inhibitory effectiveness up to 93.44 % at 2,000 μg/mL (data not shown). Regarding comet assay, it was shown that DNA damage was prevented in TK6 cells following the 24 hr pre-treatment cells with LDSK50-H_2O and LDSK50-EA prior to H_2O_2 induction. LDSK50-EA exhibited a greater protective activity over LDSK50-H_2O at all concentrations tested. The highest protective activity was observed at 53.47 ± 1.99% when tested at 200 μg/mL.

Fig 2. Inhibitory effect of LDSK50-EA and LDSK50-H_2O on H_2O_2-induced DNA damage in TK6 cells. Results are expressed as means ± SD ($n = 3$). *Significant difference was detected from H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA).

CONCLUSION

Regarding results of current study, it could be summarized that LDSK50-EA possessed high potential activity in both antioxidant and anti-genotoxicity (DNA damage protection). Our findings may strengthen usage of *L. domesticum* and lead to a discovery of alternative source of natural antioxidants. For next study, we are investigating mutagenic and anti-mutagenic activities of active fractions of *L. domesticum* fruits at cellular level.

ACKNOWLEDGEMENTS
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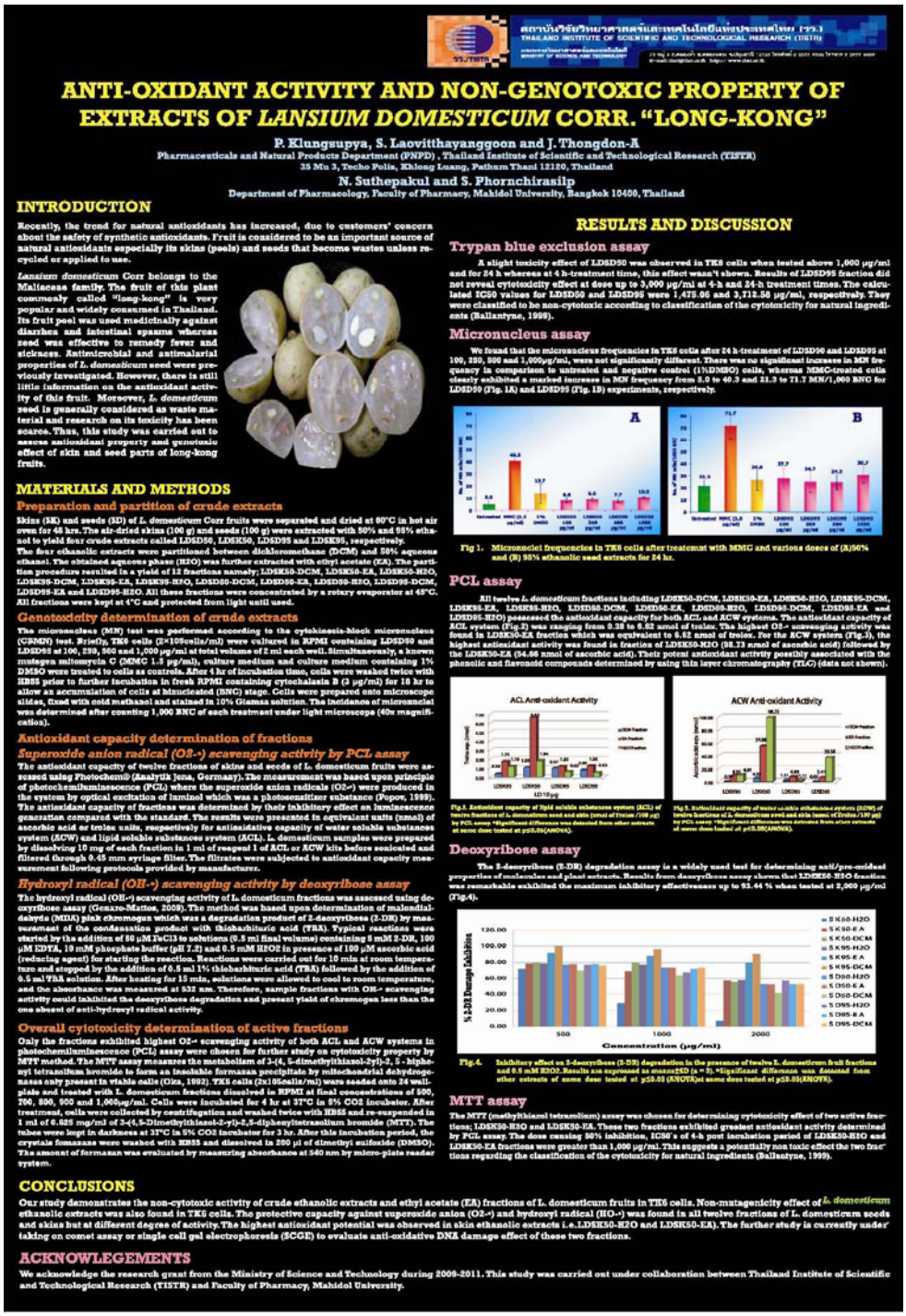
REFERENCES

1. Okonogi S., Duangrat C., Anuchpreeda S., et al. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. *Food Chemistry* 2007;103:839-846.
2. Popov, Lewin G. Anti oxidative Homeostasis: Characterization by Means of Chemiluminescent Technique. *Method in Enzymology: Oxidant and antioxidant Part B* 1999:300-437-456.
3. Genaro-Mattos TC., Dalvi LT., Oliveira RG., et al. Reevaluation of the 2-deoxyribose assay for determination of free radical formation. *Biochim Biophys Acta* 2009:1790:1636-1642.
4. Hermes-Lima M., Ponka P., Schulman HM. The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and its analogues prevent damage to 2-DR mediated by ferric iron plus ascorbate. *Biochim Biophys Acta* 2000:1523:154-160.
5. Liao W., McNutt MA., Zhu WG. The comet assay: A sensitive method for detecting DNA damage in individual cells. *Method* 2009-48-46-53.
6. Collins AR. The comet assay for DNA damage and repair: Principles, Applications, and Limitations. *Molecular Biotechnology* 2004:26:249-261.

2011 Klungsupya P, Suthepakul N, Laovitthayanggoon S, Thongdon-A J and Phornchirasilp S, 2011. **Anti-oxidant activity and non-genotoxic property of extracts of *Lansium domesticum* Corr. "Long-Kong" fruits.** The Royal Flora Ratchaphruek 2011: The International Symposium on Medicinal and Aromatic Plants. December 14-18th, 2011, Chiang Mai, Thailand.



ANTI-OXIDANT ACTIVITY AND NON-GENOTOXIC PROPERTY OF EXTRACTS OF *LANSIUM DOMESTICUM* CORR. "LONG-KONG"

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INTRODUCTION

Recently, the trend for natural antioxidants has increased, due to customers' concern about the safety of synthetic antioxidants. Fruit is considered to be an important source of natural antioxidants especially its skins (peels) and seeds that become wastes unless recycled or applied to use.

Lansium domesticum Corr belongs to the Maliaceae family. The fruit of this plant commonly called "long-kong" is very popular and widely consumed in Thailand. Its fruit peel was used medicinally against diarrhea and intestinal spasms whereas seed was effective to remedy fever and sickness. Antimicrobial and antimalarial properties of *L. domesticum* seed were previously investigated. However, there is still little information on the antioxidant activity of this fruit. Moreover, *L. domesticum* seed is generally considered as waste material and research on its toxicity has been scarce. Thus, this study was carried out to assess antioxidant property and genotoxic effect of skin and seed parts of long-kong fruits.

MATERIALS AND METHODS

Preparation and partition of crude extracts

Skins (SK) and seeds (SD) of *L. domesticum* Corr fruits were separated and dried at 60°C in hot air oven for 48 hrs. The air-dried skins (100 g) and seeds (100 g) were extracted with 50% and 95% ethanol to yield four crude extracts called LDSD50, LDSK50, LDSD95 and LDSK95, respectively. The four ethanolic extracts were partitioned between dichloromethane (DCM) and 50% aqueous ethanol. The obtained aqueous phase (H2O) was further extracted with ethyl acetate (EA). The partition procedure resulted in a yield of 12 fractions namely; LDSK50-DCM, LDSK50-EA, LDSK50-H2O, LDSK50-DCM, LDSK50-EA, LDSK95-H2O, LDSD50-DCM, LDSD50-EA, LDSD50-H2O, LDSD95-DCM, LDSD95-EA and LDSD95-H2O. All these fractions were concentrated by a rotary evaporator at 45°C. All fractions were kept at 4°C and protected from light until used.

Genotoxicity determination of crude extracts

The micronucleus (MN) test was performed according to the cytokinesis-block micronucleus (CBMN) test. Briefly, TK6 cells (2×10⁵cells/ml) were cultured in RPMI containing LDSD50 and LDSD95 at 100, 250, 500 and 1,000 µg/ml at total volume of 2 ml each well. Simultaneously, a known mutagen mitomycin C (MMC 1.5 µg/ml), culture medium and culture medium containing 1% DMSO were treated to cells as controls. After 1 hr of incubation time, cells were washed twice with HBSS prior to further incubation 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 light microscope (40x magnification).

Antioxidant capacity determination of fractions

Superoxide anion radical (O2·-) 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 principle of photochemiluminescence (PCL) where the superoxide anion radicals ($O_2^{\cdot-}$) were produced in the system by optical excitation of luminol which was a photosensitizer substance (Popov, 1999). The antioxidant capacity of fractions was determined by their inhibitory effect on luminescence generation compared with the standard. The results were presented in equivalent units (nmol) of ascorbic acid or trolox units, respectively for antioxidative capacity of water soluble substances system (ACW) and lipid soluble substances system (ACL). L. domesticum samples were prepared by dissolving 10 mg of each fraction in 1 ml of reagent 1 of ACL or ACW kits before sonicated and filtered through 0.45 mm syringe filter. The filtrates were subjected to antioxidant capacity measurement following protocols provided by manufacturer.

Hydroxyl radical (OH-·) scavenging activity by deoxyribose assay

The hydroxyl radical (OH-·) scavenging activity of L. domesticum fractions was assessed using 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) by mainstreat of the condensation product with thiobarbituric acid (TBA). Typical reactions were started by the addition of 50 µM FeCl3 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 H2O2 in presence of 100 µM ascorbic acid (reducing agent) for starting the reaction. Reactions were carried out for 10 min at room temperature and stopped by the addition of 0.5 ml 1% thiobarbituric acid (TBA) followed by the addition of 0.5 ml TBA solution. After heating for 15 min, solutions were allowed to cool to room temperature, and the absorbance was measured at 532 nm. Therefore, sample fractions with OH-· scavenging activity could inhibited the deoxyribose degradation and present yield of chromogen less than the one absent of anti-hydroxyl radical activity.

Overall cytotoxicity determination of active fractions

Only the fractions exhibited highest $O_2^{\cdot-}$ scavenging activity of both ACL and ACW systems in photochemiluminescence (PCL) assay were chosen for further study on cytotoxicity property by 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 dehydrogenase only present in viable cells (Oita, 1992). TK6 cells (2x10⁵cells/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 37°C in 5% CO2 incubator. After treatment, cells were collected by centrifugation and washed twice with HBSS and re-suspended in 1 ml of 0.625 mg/ml of 2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The tubes were kept in darkness at 37°C in 5% CO2 incubator for 3 hr. After this incubation period, the crystals formazans 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 micro-plate reader system.

RESULTS AND DISCUSSION

Trypan blue exclusion assay

A slight toxicity effect of LDSD50 was observed in TK6 cells when tested above 1,000 µg/ml and for 24 h whereas at 4 h-treatment time, this effect wasn't shown. Results of LDSD95 fraction did not reveal cytotoxicity effect at dose up to 3,000 µg/ml at 4-h and 24-h treatment times. The calculated IC50 values for LDSD50 and LDSD95 were 1,475.06 and 3,718.58 µg/ml, respectively. They were classified to be non-cytotoxic according to classification of the cytotoxicity for natural ingredients (Ballantyne, 1999).

Micronucleus assay

We found that the micronucleus frequencies in TK6 cells after 24 h-treatment of LDSD50 and LDSD95 at 100, 250, 500 and 1,000µg/ml, were not significantly different. There was no significant increase in MN frequency in comparison to untreated and negative control (1%DMSO) cells, whereas MMC-treated cells clearly exhibited a marked increase in MN frequency from 3.0 to 40.3 and 33.3 to 71.7 MN/1,000 BNC for LDSD50 (Fig. 1A) and LDSD95 (Fig. 1B) experiments, respectively.

Fig 1. Micronuclei frequencies in TK6 cells after treatemnt with MMC and various doses of (A)50% and (B) 95% ethanolic seed extracts for 24 hr.

PCL assay

All twelve *L. domesticum* fractions including LDSK50-DCM, LDSK50-EA, LDSK50-H2O, LDSK95-DCM, LDSK95-EA, LDSK95-H2O, LDSD50-DCM, LDSD50-EA, LDSD50-H2O, LDSD50-DCM, LDSD95-EA and LDSD95-H2O) possessed the antioxidant capacity for both ACL and ACW systems. The antioxidant capacity of ACL system (Fig.2) was ranging from 0.38 to 6.82 nmol of trolox. The highest $O_2^{\cdot-}$ scavenging activity was found in LDSK50-EA fraction which was equivalent to 6.82 nmol of trolox. For the ACW system (Fig.3), the highest antioxidant activity was found in fraction of LDSK50-H2O (98.73 nmol of ascorbic acid) followed by the LDSK50-EA (54.66 nmol of ascorbic acid). Their potent antioxidant activity possibly associated with the phenolic and flavonoid compounds determined by using thin layer chromatography (TLC) (data not shown).

Fig 2. Antioxidant capacity of lipid soluble substances system (ACL) of twelve fractions of L. domesticum seed and skin (nmol of Trolox / 100 µg) by PCL assay. *Significant difference was detected from other extracts at same dose tested at p≤0.05(ANOVA).

Fig 3. Antioxidant capacity of water soluble substances system (ACW) of twelve fractions of L. domesticum seed and skin (nmol of Trolox /100 µg) by PCL assay. *Significant difference was detected from other extracts at same dose tested at p≤0.05(ANOVA).

Deoxyribose assay

The 2-deoxyribose (2-DR) degradation assay is a widely used test for determining anti/pro-oxidant properties of molecules and plant extracts. Results from deoxyribose assay shown that LDSD50 H2O fraction was remarkable exhibited the maximum inhibitory effectiveness up to 93.44 % when tested at 2,000 µg/ml (Fig.4).

Fig.4. Inhibitory effect on 2-deoxyribose (2-DR) degradation in the presence of twelve L. domesticum fruit fractions and 0.5 mM H2O2. Results are expressed as means±SD (n = 3). *Significant difference was detected from other extracts of same dose tested at p≤0.05 (ANOVA)at same dose tested at p≤0.05(ANOVA).

MTT assay

The MTT (methylthiazol tetrazolium) assay was chosen for determining cytotoxicity effect of two active fractions; LDSK50-H2O and LDSK50-EA. These two fractions exhibited greatest antioxidant activity determined by PCL assay. The dose causing 50% inhibition, IC50's of 4-h post incubation period of LDSK50-H2O and LDSK50-EA fractions were greater than 1,000 µg/ml. This suggests a potentially non toxic effect the two fractions regarding the classification of the cytotoxicity for natural ingredients (Ballantyne, 1999).

CONCLUSIONS

Our study demonstrates the non-cytotoxic activity of crude ethanolic extracts and ethyl acetate (EA) fractions of L. domesticum fruits in TK6 cells. Non-mutagenicity effect of *L. domesticum* ethanolic extracts was also found in TK6 cells. The protective capacity against superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$) was found in all twelve fractions of L. domesticum seeds and skins but at different degree of activity. The highest antioxidant potential was observed in skin ethanolic extracts i. e. LDSK50-H2O and LDSK50-EA). The further study is currently under taking on comet assay or single cell gel electrophoresis (SCGE) to evaluate anti-oxidative DNA damage effect of these two fractions.

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