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RADICAL-SCAVENGING AND CELLULAR DNA PROTECTIVE ACTIVITIES OF ACTIVE FRACTIONS FROM *LANSIUM DOMESTICUM* CORR. FRUIT

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KEYWORDS: *Lansium domesticum*, Long-kong, Superoxide ($O_2^{\cdot-}$), Hydroxyl ($OH^{\cdot}$), Hydrogen peroxide (H_2O_2), DNA damage, Comet test

INTRODUCTION

Lansium domesticum Corr belongs to the family Meliaceae. 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 (1). *L. domesticum* is known under a variety of common names in different countries and languages. In Thailand, the *L. domesticum* fruit, called "long-kong", 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 of *L. domesticum* seeds were investigated (1).

Most free radical reactions involve formation of ROS, including superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$) and hydrogen peroxide (H_2O_2) (2). These ROS can attack healthy cells and cause oxidative damage to DNA, lipid, protein and other bio-molecules. They can disrupt duplication of DNA, interfere with DNA maintenance, break open the molecule or alter the structure by reacting with the DNA bases. Lipids in cell membranes are quite prone to oxidative damage because free radicals tend to collect in cell membranes, known as lipid peroxidation. When a cell membrane is oxidized by an ROS, it becomes brittle and leaky. Eventually, the cell falls apart and dies (2-3). The objective of this study was to investigate the anti-oxidative activity against DNA damage of fraction extracted from the peels of long-kong fruits using single cell gel electrophoresis (SCGE) or comet assay in TK6 human lymphoblast cells.

MATERIALS AND METHODS

Preparation of crude extracts The fresh, mature fruits of *L. domesticum* Corr. purchased from Talad-Thai market were employed. After washing, skin (peel) and seeds of the fruits were manually separated, dried at 50°C in a hot air oven for 48 h and finally grounded into powder using a blender. One hundred g of dried powder of skin (SK) and seeds (SD) 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 yielded 12 fractions (namely; LDSK50-DCM, LDSK50-EA, LDSK50-H2O, LDSK95-DCM, LDSK95-EA, LDSK95-H2O, LDSD50-DCM, LDSD50-EA, LDSD50-H2O, LDSD95-DCM, LDSD95-EA and LDSD95-H2O). All twelve fractions were concentrated at 45°C, then kept at 4°C and protected from light until being used.

Evaluation of free-radicals scavenging activity of fractions Twelve long-kong fractions were subjected to photochemiluminescence (PCL) and deoxyribose assays to determine their anti-oxidant capacity on superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) scavenging activities.

Photochemiluminescence (PCL) assay In the PCL system, $O_2^{\cdot-}$ radicals were generated at about 1,000 times greater than in normal cells by optical excitation of photosensitizer substance (luminol). These $O_2^{\cdot-}$ radicals were partially eliminated by reaction with the anti-oxidants in the test samples. Then, the remaining $O_2^{\cdot-}$ caused the detector substance to emit luminescence. Therefore, the anti-oxidant capacities

of samples could be determined by their inhibitory effect on luminescence generation. Results of PCL assay indicated the $O_2^{\cdot-}$scavenging activity of 12 fractions of long-kong fruit extracts obtained from both the lipid- and water-soluble solvent systems to possess anti-oxidant capacities (4).

Deoxyribose assay 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 $OH^{\cdot}$ radical, formed in the reaction between iron (III)-EDTA and H_2O_2 in the presence of ascorbic acid (reducing agent), attacks 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 spectrophotometer at 532 nm. Thus, long-kong extract fractions that possessed $OH^{\cdot}$ scavenging activity could inhibit the deoxyribose degradation and yield less chromogen than the one without anti-hydroxyl radical activity (5)

Investigation on DNA protective activity in H_2O_2-treated TK6 cells by comet assay Subsequently, the active fractions were assayed for their anti-oxidative activity against DNA damage by hydrogen peroxide (H_2O_2) in TK6 human lymphoblast cells (ATCC CRL-8015) using the comet assay or single cell gel electrophoresis (SCGE) (6). The TK6 cells were pre-treated with various concentrations of long-kong active fractions and then exposed to H_2O_2 to induce DNA damage. The treated cells were then mixed with low melting point (LMP) agarose and spreaded 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. 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 with Comet III software. The anti-oxidative activity of active fractions was indicated by a significant reduction in DNA damage parameters: either the tail length (TL = distance of DNA migration from center of cell nucleus, μm) or 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, %) values (7, 11).

RESULTS AND DISCUSSION

Superoxide ($O_2^{\cdot-}$) radical –scavenging activity (PCL assay) The photochemiluminescence (PCL) assay measures the potential anti-oxidant property of *L. domesticum* fractions by two different protocols i.e. ACW and ACL that indicate the anti-oxidant capacity of the water and lipid soluble components, respectively (8-9). The anti-oxidant property is expressed in equivalent concentration units of ascorbic acid and Trolox for water and lipid soluble systems, respectively (10). All twelve *L. domesticum* fractions exhibited different degrees of the $O_2^{\cdot-}$ scavenging activity for both ACL (**Figure 1, left**) and ACW (**Figure 1, right**) measurement systems. Results of the ACL demonstrated the anti-oxidant capacity of these twelve fractions (at 10 μg/ml) range from 0.380 to 6.625 nmol of Trolox. Among these, LDSK50-EA possessed the highest anti-oxidant activity (equivalent to 6.625 nmol of Trolox). Interestingly, the anti-oxidant capacity of ACW system indicated that 50% ethanol extract of peels (LDSK50) still had high anti-oxidant capacity. The anti-oxidant capacity of all fractions was found to be in a wide range 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).

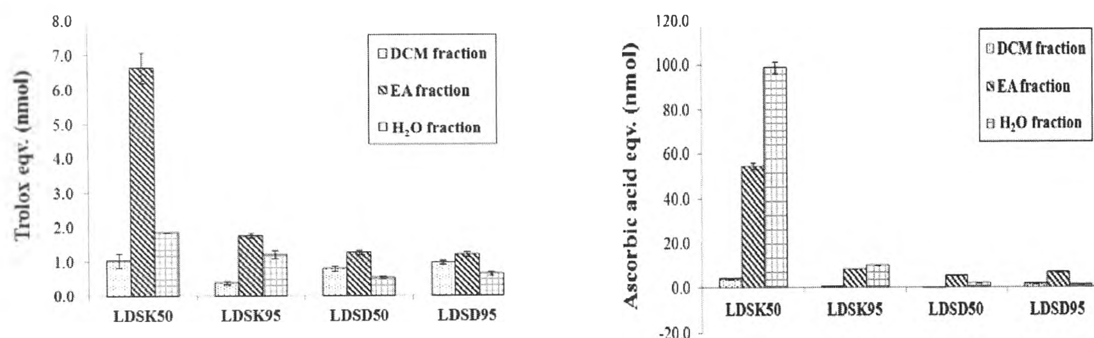

Figure 1 The anti-oxidant capacity in lipid (ACL, Left) and water phases (ACW, Right) of twelve *L. domesticum* fractions by PCL assay. Results were expressed as means ± SD (n=3). *Significant difference was detected from the lowest activity fraction of same part-extraction ($p \leq 0.05$). **Significant difference was detected from all fractions of same part-extraction ($p \leq 0.05$).

For the lipid-soluble (ACL) system, 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). For the water-soluble (ACW) system, the overall anti-oxidant capacity of these 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).

Hydroxyl ($OH^{\bullet}$) radical scavenging activity (deoxyribose assay) Inhibitory effect of *L. domesticum* fractions on 2-deoxyribose (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 activity was expressed as % inhibition for the test sample at the concentrations of 0.5, 1.0 and 2.0 mg/ml. **Figure 2** demonstrated a wide range of $OH^{\bullet}$ scavenging activity of these fractions from 0.50 ± 0.12 to 93.44 ± 0.84%. Fraction LDSK50-H_2O, at 2,000 µg/ml, displayed maximum inhibitory effect (%I) of up to 93.44 %, equal to that of tannic acid (reference anti-oxidant) at 80 ug/ml (data not shown).

DNA protective activity on H_2O_2-treated TK6 cells (comet assay) Fractions LDSK50-EA and LDSK50-H_2O, which were most potent against $O_2^{-\bullet}$ and $OH^{\bullet}$ radicals, were further studied using comet assay. TK6 cells were separately pre-treated with these fractions at 25, 50, 100 and 200 µg/ml for 24 h prior to H_2O_2 induction. The DNA protective effect of LDSK50-H_2O was indicated by a reduction in TL and TM damages compared to cells treated with H_2O_2 alone.

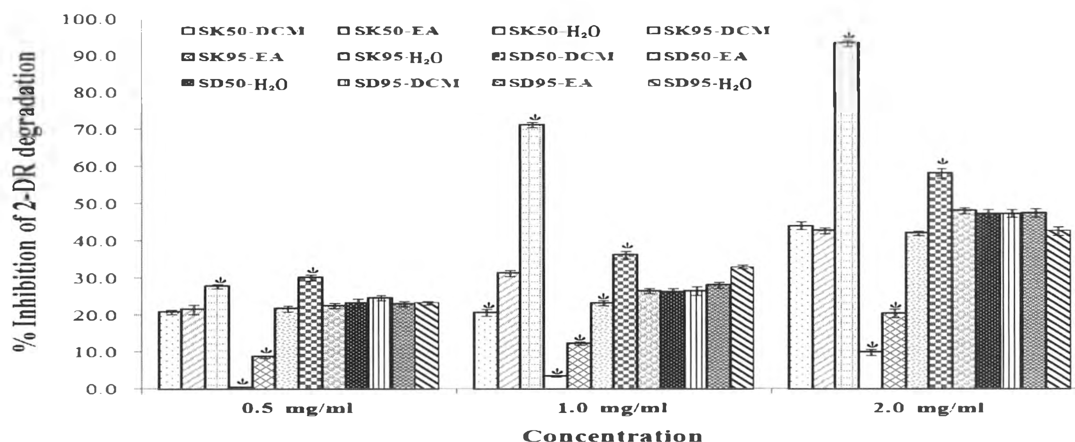

Figure 2 Inhibitory effect (%) of 2-deoxyribose degradation of twelve *L. domesticum* fractions by deoxyribose assay. Results were expressed as means ± SD (n=3). *Significant difference was detected from all fractions of same concentrations (0.5, 1.0 and 2.0 mg/ml) ($p \leq 0.05$).

Treatment with 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. **Figure 3** shows the DNA-protective activity of both fractions when cells were pre-treated at 25, 50, 100 and 200 µg/ml for 24 hr prior to exposure to H_2O_2. The effect was indicated by a reduction in TL and TM values in comparison to cells treated with H_2O_2 alone (**Figure 3**). Pre-treatment with LDSK50-EA could prevent DNA damage in a dose-dependent manner (**Figure 4**), with the highest effect found at 200 µg/ml concentration (% DNA damage inhibition = 53.47 ± 1.99). However, at dose greater than 200 µg/ml (up to 250 µg/ml), very little alteration in the effect was detected but with higher cytotoxicity (data not shown). In contrast, fraction LDSK50-H_2O exhibited only slight anti-oxidative activity at similar concentrations. Hence, LDSK50-EA possessed anti-oxidative DNA damage greater than LDSK50-H_2O fraction.

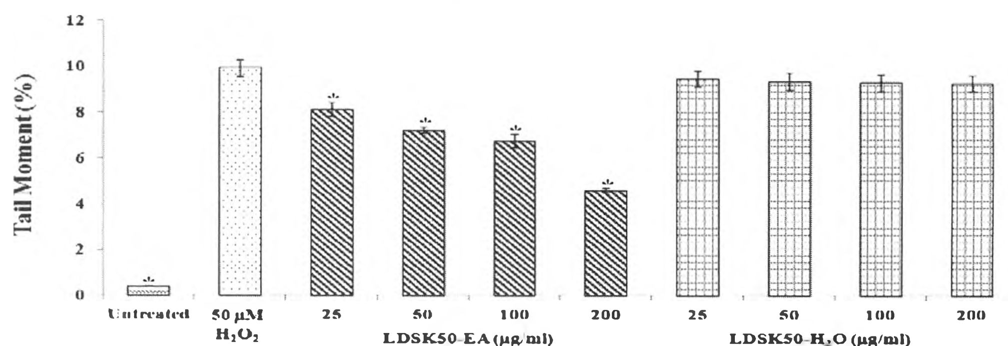

Figure 3 Tail moment (TM, %) values measured in pre-treated TK6 cells with LDSK50-EA and LDSK50-H_2O fractions followed by H_2O_2 damage induction by comet assay. Results were expressed as means ± SD (n = 3). *Significant difference was detected from 50 µM H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA).

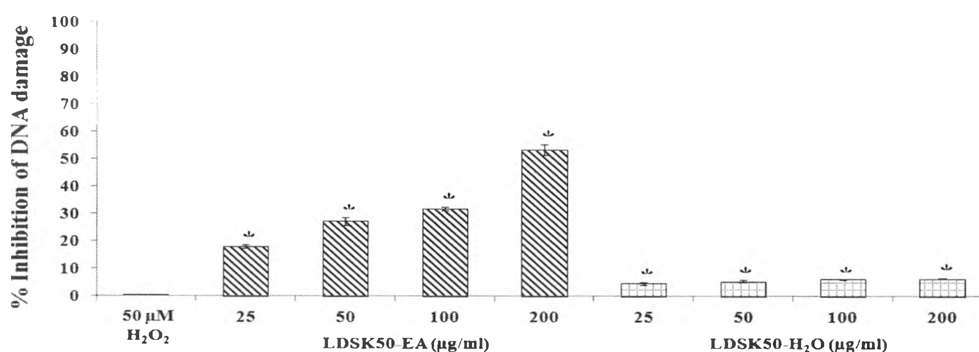

Figure 4 Inhibitory effect of LDSK50-EA and LDSK50-H_2O on H_2O_2-induced DNA damage in TK6 cells by comet assay. Results were expressed as means ± SD (n = 3). *Significant difference was detected from 50 µM H_2O_2 treatment groups at $p \leq 0.05$ (ANOVA)

CONCLUSION

This study generates new and updated information on biological activity of seeds and skins (peels) of long-kong (*L domesticum* Corr.) fruits that has not yet been studied before. The results on free radical ($O_2^{-\bullet}$ and $OH^{\bullet}$) scavenging activity and cellular DNA protective activity against H_2O_2 will promote and strengthen utilization of *L. domesticum*.

ACKNOWLEDGEMENTS

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