The Thai Journal of Pharmaceutical Sciences

Volume 36
Issue 0 *supplement issue*

Article 22

1-1-2012

EVALUATION ON ANTI-OXIDANT, ANTI-MUTAGENIC AND CYTOTOXIC PROPERTIES OF ACTIVE FRACTIONS OF THAI "LONG-KONG" (LANSIUM DOMESTICUM CORR.) FRUITS

Prapaipat Klungsupya

Nava Suthepakul

Sarunya Laovitthayanggoon

Jeerayu Thongdon-A

Srisak Trangwacharakul

See next page for additional authors

EVALUATION ON ANTI-OXIDANT, ANTI-MUTAGENIC AND CYTOTOXIC PROPERTIES OF ACTIVE FRACTIONS OF THAI “LONG-KONG” (LANSIUM DOMESTICUM CORR.) FRUITS

Authors

Prapaipat Klungsupya, Nava Suthepakul, Sarunya Laovitthayanggoon, Jeerayu Thongdon-A, Srisak Trangwacharakul, and Srichan Phomchirasilp

BP-12

EVALUATION ON ANTI-OXIDANT, ANTI-MUTAGENIC AND CYTOTOXIC PROPERTIES OF ACTIVE FRACTIONS OF THAI "LONG-KONG" (*LANSIUM DOMESTICUM* CORR.) FRUITS

Prapaipat Klungsupya[1], Nava Suthepakul[3], Sarunya Laovitthayanggoon[1], Jeerayu Thongdon-A[1], Srisak Trangwacharakul[2] and Srichan Phornchirasilp[3].

[1]*Department of Pharmaceuticals and Natural Products (PNPD),* [2]*Department of Food Technology, Thailand Institute of Scientific and Technological Research (TISTR), 35Moo 3 Techno Polis, Klong Luang, Pathum Thani 12120, Thailand*
[3]*Department of Pharmacology, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand*

KEYWORDS: *Lansium domesticum*, long-kong, antioxidant, mutagenic, cytotoxic

INTRODUCTION

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 (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. 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 fruits of *L. domesticum* Corr. at the mature stage purchased from the Talad-Thai market were employed. 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 100 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 resulted in a yield of 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 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 obtained by partition step 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) systems.

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

The hydroxyl radical ($OH^{\bullet}$) scavenging activity of twelve *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) = $(Abs_{control} - Abs_{sample})/Abs_{control} \times 100$

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 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). Briefly, TK6 cells ($2x10^5$cells/ml) were treated with *L. domesticum* fractions at final concentrations of 500, 700, 800, 900 and 1,000μg/ml for 4 hr at 37°C. Cells were 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.

Anti-mutagenic 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

All twelve *L. domesticum* fractions (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 and ACW 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. Interestingly, the anti-oxidant capacity of ACW system 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).

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

MTT assay

The MTT (methylthiazol tetrazolium) assay was chosen for determining cytotoxic-effect of the two active fractions; LDSK50-H_2O and LDSK50-EA. These two fractions exhibited the greatest antioxidant activity, determined by PCL assay. 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. This observation was supported by the IC_{50} (inhibitory concentration inhibited cell growth by 50%) value of LDSK50-EA in TK6 cells which was 280 µg/ml whereas in V79 cells it was 231 µg/ml. No cell proliferation inhibition activity was found for LDSK50-H_2O. This was evident by % cell viability greater than 80% at concentrations up to 5,000 µg/ml. The values of IC_{50} of LDSK50-H_2O were 4,309 µg/ml inV79 cells and were greater than 5,000 µg/ml in TK6 cells.

Micronucleus assay

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

DISCUSSION

Regarding PCL results, 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 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. 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 was conducted on TK6 and V79 cell lines due to their high sensitivity in cytotoxicity and genotoxicity testings (Guillamet *et al.*, 2008).

Under the condition used in our study, it was noticed that V79 cells were more sensitive to LDSK50-EA than TK6 cells. 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 231μg/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. No cell proliferation inhibition activity was found for LDSK50-H_2O. This was evident by % cell viability greater than 80% at concentrations up to 5,000 μg/ml. The values of IC_{50} of LDSK50-H_2O were 4,309 μg/ml 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; OECD, 2009; Tomasz & Palom, 1997). Thus, we could call it a substance that is able to produce MN from a lagging acentric chromosome fragment as a clastogen (Kayani and Parry, 2010)

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

This project was carried out during 2009-2012 and funded by the Ministry of Science and Technology for the Thailand Institute of Scientific and Technological Research (TISTR).

REFERENCES

1. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, and Zeiger E, (2008). Human project: detailed description of the scoring criteria for the cytokinesis block micronucleus assay using isolated lymphocyte cultures. Mutat. Res. 534: 65-75.
2. Gad Shayne C, (1999). Alternatives to *in vivo* studies in toxicology, in: B Ballantyne, TC Marrs and TLM Syversen (editors), General and applied toxicolog, London; New York, NY: Macmillan, Vol.1, pp. 178.
3. Guillamet E, Creus A, Farina M, Sabbioni E, Fortaner S, and Marcos R, (2008). DNA-damage induction by eight metal compounds in TK6 human lymphoblastoid cells: Results obtained with the alkaline Comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 654, 22-28.
4. Kayani M A, and Parry J M, (2010). The *in vitro* genotoxicity of ethanol and acetaldehyde. Toxicology in vitro : an international journal published in association with BIBRA, 24: 56-60.
5. Genaro-Mattos TC, Dalvi LT, Oliveira RG, Ginani JS, and Hermes-Lima M, 2009. Reevaluation of the 2-deoxyribose assay for determination of free radical formation. Biochim Biophys Acta.1790: 1636-1642.
6. Oka M, Maeda S, Koga N, Kato K, and Saito T, (1992). A modified colorimetric MTT assay adapted for primary cultured hepatocytes: Application to proliferation and cytotoxic assay. Biosci Biotechnol Biochem, 56:1472-1473
7. Popov I, and Lewin G, (1999). Antioxidative homeostasis: characterization by means of chemiluminescent technique. Methods Enzymol, 300: 437-456.
8. Tilaar M, Wong LW, Ranti AS, Wasitaatmadja SM, and Junardy FD, (2008). Review of *Lansium domesticum* Corrêa and its use in cosmetics. Bol. Latinoam. Caribe Plant. Med. Aromaticas, 7: 183-189.