# TOTAL PHENOLIC CONTENT, CELLULAR ANTIOXIDANT ACTIVITY AND POTENTIAL HEPATOPROTECTIVE EFFECT OF FRUIT EXTRACTS

**Chunhabundit R [1]*, Kongkachuichai R [2], Norasing J[1], Visetpanich Y [3]**

[1] Graduate Program in Nutrition, Faculty of Medicine at Ramathibodi Hospital, Mahidol University, Thailand

[2] Institute of Nutrition, Mahidol University, Thailand

[3] Research Center, Faculty of Medicine at Ramathibodi Hospital, Mahidol University, Thailand

## ABSTRACT

Environmental pollutants are sources of several reactive oxygen species and other byproducts of oxidative stress. Such products involve the underlining processes of chronic diseases, either at the initial or progressive stages of the diseases. The antioxidant nutrients and related bioactive compounds common in vegetables and fruits have become a beneficial alternative to prevent oxidative stress in cells. The present study was done to investigate cellular antioxidant activity as well as total phenolic content of the extracts of selected fruits: strawberry, carambola, guava, longkong, pomelo, and tangerine. The protective effect of these fruits on $H_2O_2$ induced cytotoxicity was also evaluated in human liver carcinoma cell line (HepG2). Cellular antioxidant activity (CAA) assay was the measurement the ability of antioxidants in fruit extracts to prevent oxidation of cell membrane lipids and production of more radicals in cells. The cytoprotective potential was assessed by using MTT assay. The results showed that strawberry, carambola, and guava had higher phenolic contents than longkong, pomelo, and tangerine. The antioxidant efficacies of fruits in CAA values were consistent with their phenolic contents except longkong which exhibited the highest CAA even contained low level of phenolics. However, cell viability measured after co-treatment $H_2O_2$ with the fruit extracts showed the similar cytoprotective ability of all fruit extracts. The present study demonstrates that all selected fruit extracts have protective effect on $H_2O_2$ induced oxidative damage in human hepatocarcinoma cells and this effect is related to their cellular antioxidant properties.

**Keywords:** Phenolic content, Fruit extracts, Cellular antioxidant activity, Hepatoprotective effect

***Corresponding author:**

Dr. Rodjana Chunhabundit

Graduate Program in Nutrition, Faculty of Medicine at Ramathibodi Hospital,

Mahidol University, Bangkok 10400, Thailand

E-mail: rodjana.chu@mahidol.ac.th

# ปริมาณสารฟีนอลิครวม ฤทธิ์ต้านอนุมูลอิสระในเซลล์และความสามารถในการป้องกันเซลล์ตับของสารสกัดจากผลไม้

รจนา ชุณหบัณฑิต [1]*, รัชนี คงคาฉุยฉาย [2], จุฑามาศ นรสิงห์[1], ยุพิน วิเศษพานิช [3]

[1] กลุ่มสาขาวิชาโภชนศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพฯ 10400

[2] กลุ่มวิชาอาหาร สถาบันโภชนาการ มหาวิทยาลัยมหิดล จ.นครปฐม73170

[3] สำนักงานวิจัย คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพฯ 10400

## บทคัดย่อ

มลพิษในสิ่งแวดล้อมเป็นแหล่งของสารอนุมูลอิสระของออกซิเจนและสารอื่นๆที่เกิดจากภาวะ oxidative stress ซึ่งเป็นสาเหตุของโรคเรื้อรังชนิดต่างๆหรือทำให้ความรุนแรงของโรคดังกล่าวเพิ่มขึ้น สารอาหารและสารออกฤทธิ์ทางชีวภาพที่พบมากในผักและผลไม้สามารถป้องกันและยับยั้งผลของอนุมูลอิสระในเซลล์ได้ ในการศึกษาครั้งนี้มีการตรวจสอบ ปริมาณสารฟีนอลิครวม ฤทธิ์ต้านอิสระในเซลล์ และความสามารถในการป้องกันการเกิดพิษต่อเซลล์ตับ (HepG2)ซึ่งเหนี่ยวนำโดยสารไฮโดรเจนเปอร์ออกไซด์ของสารสกัดจากสตรอเบอร์รี่ มะเฟือง ฝรั่ง ลองกอง ส้มโอ และส้มเขียวหวาน การวัดฤทธิ์ต้านอิสระในเซลล์เป็นการวัดความสามารถของสารสกัดในการป้องกันการเกิดออกซิเดชั่นของไขมันที่เยื่อหุ้มเซลล์และส่งผลให้มีการสร้างอนุมูลอิสระในเซลล์ ส่วนการป้องกันการเกิดพิษต่อเซลล์ใช้เทคนิค MTT assay ผลการศึกษาพบว่าปริมาณสารฟีนอลิครวมในสารสกัดจากสตรอเบอร์รี่ มะเฟือง และฝรั่งสูงกว่าในลองกอง ส้มโอ และส้มเขียวหวาน ประสิทธิภาพของการต้านอนุมูลอิสระในเซลล์ของสารสกัดจากผลไม้มีความสัมพันธ์กับปริมาณสารฟีนอลิครวม ยกเว้นลองกองซึ่งแสดงฤทธิ์ต้านอนุมูลอิสระในเซลล์ได้สูงสุดทั้งๆที่มีปริมาณสารฟีนอลิครวมต่ำ อย่างไรก็ตามจากการให้สารสกัดจากผลไม้ร่วมไปกับการให้สารไฮโดรเจนเปอร์ออกไซด์เพื่อเหนี่ยวนำให้เกิดพิษกับเซลล์ตับ พบว่าสารสกัดจากผลไม้ทุกชนิดมีประสิทธิภาพในการป้องกันหรือลดพิษจาก oxidative stress ไม่แตกต่างกัน การศึกษานี้แสดงให้เห็นว่าสารสกัดจากผลไม้ที่เลือกมาทุกชนิดสามารถป้องกันความเสียหายจากความเป็นพิษของสารไฮโดรเจนเปอร์ออกไซด์ในเซลล์ตับได้ ซึ่งผลดังกล่าวเนื่องมาจากความสามารถต้านอนุมูลอิสระในเซลล์ของสารสกัด

**คำสำคัญ:** สารฟีนอลิค, สารสกัดจากผลไม้, ฤทธิ์ต้านอนุมูลอิสระในเซลล์, ความสามารถในการป้องกันเซลล์ตับ

***Corresponding author:**

ดร. รจนา ชุณหบัณฑิต

กลุ่มสาขาวิชาโภชนศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพฯ 10400

E-mail: rodjana.chu@mahidol.ac.th

## INTRODUCTION

Oxidants and byproducts found in environmental pollutants have the capability to produce reactive radicals in biological systems and lead to oxidative stress, a state of the imbalance between the excessive formation of reactive oxygen species (ROS) and insufficient body antioxidant protection. The oxidative stress can initiate and implicate in several human chronic diseases such as cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders, and other chronic inflammatory diseases.[1, 2] Oxidative stress also plays an important role in the induction and progression of toxic liver diseases and other hepatic alterations.[3,4] Consumption of fruits and vegetables has been negatively associated with incidence and mortality rates caused by cancer, hypertension, cardio- and cerebrovascular diseases in several human studies. Dietary components in fruits and vegetables may act independently or in combination as natural antioxidants that have been shown to play a crucial role in prevention of such diseases.[5]The constituents of fruits and vegetables that act as antioxidants include vitamins such as vitamin C, vitamin E, β-carotene, and polyphenolic compounds.[6] Phenolic compounds possess antioxidant properties through free-radical scavenging activity resulted from their hydrogen- or electron-donating ability, their metal chelating properties, as well as the stability of the resulting antioxidant-derived radicals.[7, 8] It has been demonstrated that antioxidant activities of fruits show high correlations with levels of their total soluble phenolic contents.[9] However, chemical antioxidant activity does not account for bioavailability, uptake, and metabolism of the antioxidant compounds. Cell culture models can provide the tool for investigation of uptake, distribution, and metabolism. Therefore, a cell-based antioxidant activity assay was developed to evaluate foods, phytochemicals, and dietary supplements for potential biological activity.[10, 11]

In the present study, total phenolic contents and cellular antioxidant activities of the selected fruit extracts were determined. Furthermore, the antioxidative effect of the fruit extracts were confirmed by assessing their cytoprotective potential against hydrogen peroxide ($H_2O_2$) induced oxidative damage in human hepatic cell line (HepG2).

## MATERIALS AND METHODS

### Chemicals

2′,7′-Dichlorofluorescin diacetate (DCFH-DA), Folin Ciocalteu reagent, gallic acid and sodium carbonate were purchased from Sigma-Aldrich, Inc. (St. Louis, USA). 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA. Methanol and acetone were bought from Mallinckrodt

Baker, Inc. (Phillipsburg, USA). Dulbecco's Modified Eagle Medium (DMEM), Hanks' Balanced Salt Solution (HBSS) and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, USA). HepG2 human hepatocarcinoma cells were obtained from the American Type Culture Collection (ATCC) (Rockville, USA).

**Preparation of Fruit Extracts**

Strawberry, carambola, guava (Pan Sitong), longkong, pomelo (Thong Dee), and tangerine (Sai Nam Pung) were collected from three venders of local markets. Two hundred grams of edible portions of each kind of fruit from each vender were homogenized to make the pooled sample. The preparation of fruit extract was performed by modified method of Sun et al. (2002).[12] One hundred gram of each pooled fruit sample was extracted by stirring in chilled 80% acetone (1:2, w/v) at 5-8 °C for 6 hrs. After filtration through Whatman no. 1 paper, the solvent in extracts were evaporated under vacuum at room temperature until approximately 10% of original volumes remain. The concentrated extracts were reconstituted to 50 ml in 70% methanol and stored at -30°C. The methanol was evaporated under a stream of nitrogen, and the extracts were reconstituted to the same volume in water before use.

**Cell Culture**

HepG2 cells were grown in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in humidified incubator at 37ºC and 5% $CO_2$.

**Determination of Total Phenolic Content**

The total phenolic contents of the fruits were measured using a modified colorimetric Folin Ciocalteu method. [13] Briefly, fruit extracts were diluted with de-ionized water and introduced to a test tube. Then, 0.125 ml of Folin Ciocalteu reagent was added to the solution and allowed to react for 6 min. A 1.25 ml aliquot of 7% sodium carbonate solution was added into the test tubes. The mixture was then diluted to 3 ml with deionized water and allowed to stand for 90 min to develop color. Absorbance at 765 nm was measured spectrophotometrically. The measurement was compared to a standard curve of gallic acid concentrations and expressed as milligrams of gallic acid equivalents (GAE) per 100g of fresh weight.

**Cellular Antioxidant Activity (CAA) of Fruit Extracts**

The CAA assay was carried out according to the method of Wolfe et al. (2007).[10] Briefly, HepG2 cells were seeded at a density of $6 \times 10^4$ cells/ well on a 96-well microplate. The growth medium was

removed after 24 h, and the cells were washed with PBS. Cells were treated in triplicate for 1 h with treatment medium containing various concentrations of tested fruit extracts plus 25 μM DCFH-DA. Dye was removed and cells were washed with PBS. Then 600μM ABAP in HBSS was applied to the cells and the 96- well microplate was placed into a microplate reader (Wallac 1420, Finland) at 37 °C. Emission at 538 nm was measured after excitation at 485 nm every 5 min for 1 h.

After blank subtraction, the area under the curve for fluorescence versus time was integrated to calculate the CAA value at each concentration of fruit as

CAA unit = 1 - (∫SA/∫CA)

∫SA is the integrated area under the sample fluorescence versus time curve

∫CA is the integrated area from the control curve

The median effect plot of log (fa/fu) versus log (dose) was done based on calculated CAA unit.

fa is the fraction affected (CAA unit) by the treatment.

fu is the fraction unaffected (1 - CAA unit) by the treatment.

The EC50 is then determined as concentration of extract at which fa/fu = 1 (i.e., CAA unit 50), as calculated from the linear regression of the median effect curve.

**Protective Effect on $H_2O_2$ Induced Oxidative Damage in HepG2 Cells**

HepG2 cells were seeded at $1x10^4$ cells/ well on a 96-well plate and incubated at 37 °C for 24 h. After removing medium, and washing cells with PBS, 200 μM $H_2O_2$ with or without different concentration of fruit extracts in 100 $\mu$l of HBSS were applied to the cells, then the plate was incubated at 37 °C for 24 h. After treatments, the cell monolayer was washed once with PBS, added with a 0.5 mg/ml MTT solution in DMEM and incubated at 37 °C for 2 h. Afterwards, the medium with unreacted MTT was removed, and then isopropanol/ HCL solution was added to dissolve the reduced dye. The absorbance was measured at 570 nm with the microplate reader (BioTek, USA). The viability of the treated groups was assessed as a percentage of non-treated control groups, which was assumed to be 100%.

**Statistical analysis**

All values were presented as mean± standard error of mean (SEM). Statistical analysis of the data for multiple comparisons was performed by one-way analysis of variance (ANOVA) followed by Multiple Comparison Test for pair wise comparison. A level of $P<0.05$ was accepted as statistical significant.

## RESULTS AND DISCUSSION

### Phenolic Content of the Fruit Extracts

The phenolic compounds have been established as the main contributors to the antioxidant activity of fruits and vegetables**.** They act as powerful antioxidants in a structure-dependent manner by scavenging reactive oxygen species (ROS), and chelating transition metals which play vital roles in the free radical reactions.[14] The redox properties can promote phenolic compounds to act as reducing agents, hydrogen donators and singlet oxygen quenchers.[5] Table 1 shows total phenolic contents (TPC) that categorized fruits into the high and low TPC groups. Strawberry, guava and carambola contained the high TPC of 272, 176, and 137 mgGAE/100g FW, respectively.

The low TPC values in longkong, pomelo and tangerine were in the range of 53-64 mgGAE/100g FW. These results are consistent with those observed in a previous study, in which TPC of guava and carambola extracts were 4-5 folds higher than TPC in longkong and tangerine extracts.[15] Isabelle et al. (2010) also investigated the TPC of fruits in Singapore and reported the TPC values of strawberry, guava, carambola, pomelo and tangerine that were similar to our findings.[16] This may result from most of the tropical fruits in Singapore are imported from South East Asian countries including Thailand.

**Table 1** Total phenolic content and cellular antioxidant activity of selected fruits expressed as EC50 values (mean ± SEM, n =3)

| Fruits | Total phenolic content (mg GAE /100 g FW) | EC50 (mg/mL) |
|---|---|---|
| Strawberry | 272.87± 3.56 | 5.75 |
| Guava | 176.48±.2.82 | 6.31 |
| Carambola | 137.82± 2.62 | 7.50 |
| Longkong | 64.21± 2.95 | 4.37 |
| Pomelo | 55.18± 5.48 | 55.95 |
| Tangerine | 53.10± 6.76 | 27.54 |

**Cellular Antioxidant Activity (CAA)**

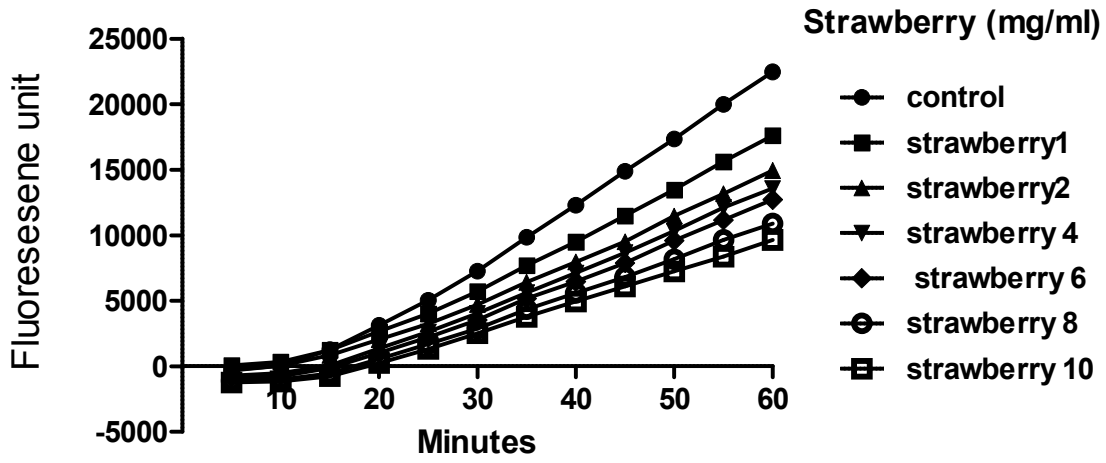
The antioxidant potential of selected fruits was evaluated by measuring their ability to prevent ABAP induced oxidation of non-fluorescent dichlorofluorescin diacetate (DCFH-DA) to fluorescent dichlorofluorescin (DCF) in HepG2 cells. The example of dose dependent inhibition of ABAP induced increase in fluorescence by strawberry and longkong extracts were illustrated in Figure 1A. and 2A. The median effect plot generated using the data of strawberry and longkong extracts were shown in Figure 1B. and 2B.

In this study, the cellular antioxidant activities of selected fruits were determined and expressed by the EC50 values that represented the concentration of fruit extracts which inhibited 50% of intracellular ROS generation. In general, the lower EC50 values indicated the higher anti-free radical efficacy (CAA) of the extracts.

The EC50 values shown in Table 1 demonstrate that longkong extract has the lowest EC50 value, followed by strawberry, guava, and carambola which have similar EC50 values. Tangerine and pomelo extracts show the much higher EC50 values.

Therefore, of the fruit tested, longkong, strawberry, guava, and carambola exhibited the much high antioxidative activities in HepG2 cells compared to tangerine and pomelo. Except longkong, all fruits with the high CAA showed the correlation between the higher TPC and the lower EC50 values. From this point of view, the phenolic compounds in strawberry, guava and carambola may directly contribute to their cellular antioxidant action. In contrast, longkong which was the fruit had the highest CAA as well as tangerine or pomelo which presented the low CAA, contained the similar low level of phenolic contents. Tangerine possessed higher CAA than pomelo in our study had been reported to contain 7- and 10 fold, respectively, higher levels of beta-carotene and lycopene than pomelo. However, longkong which showed the highest CAA but low in TPC in this study did not contain beta-carotene and lycopene in the detected level.[17] These results suggested that the CAA of later fruit group may be contributed from other non-phenolic antioxidants. Moreover, the bioavailability and potency of antioxidants presented in each fruit are important factors influencing antioxidative potential in cells.

**A**




**B**

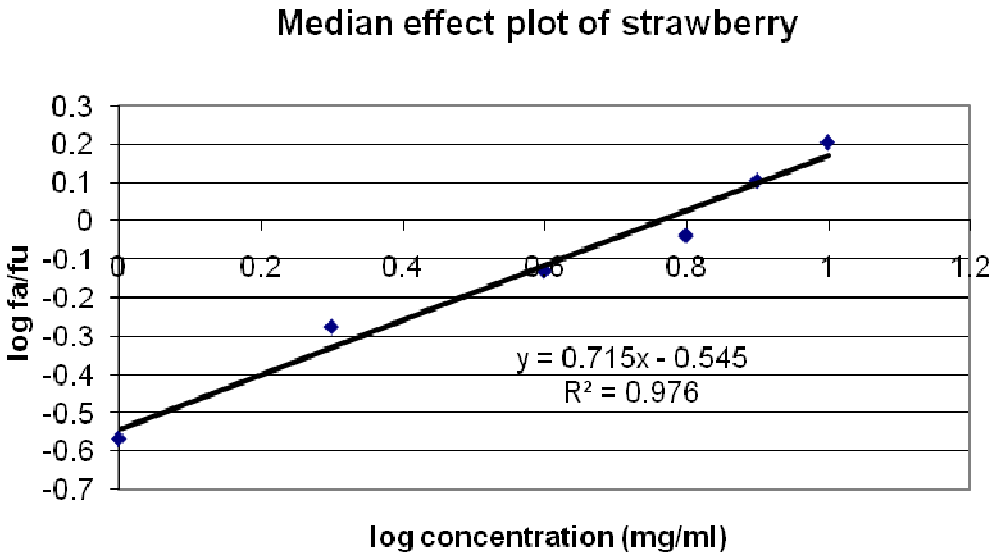
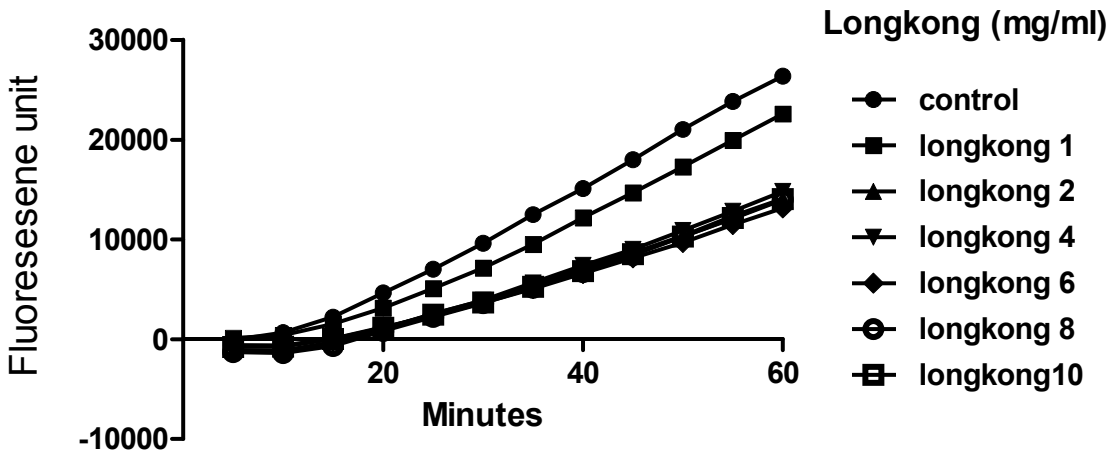



**Figure 1** Cellular antioxidant activity assay results. (A) Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by strawberry (B) Median effect plots for inhibition of peroxyl radical-induced DCFH oxidation by strawberry (mean ± SEM, n =3)

**A**




**B**

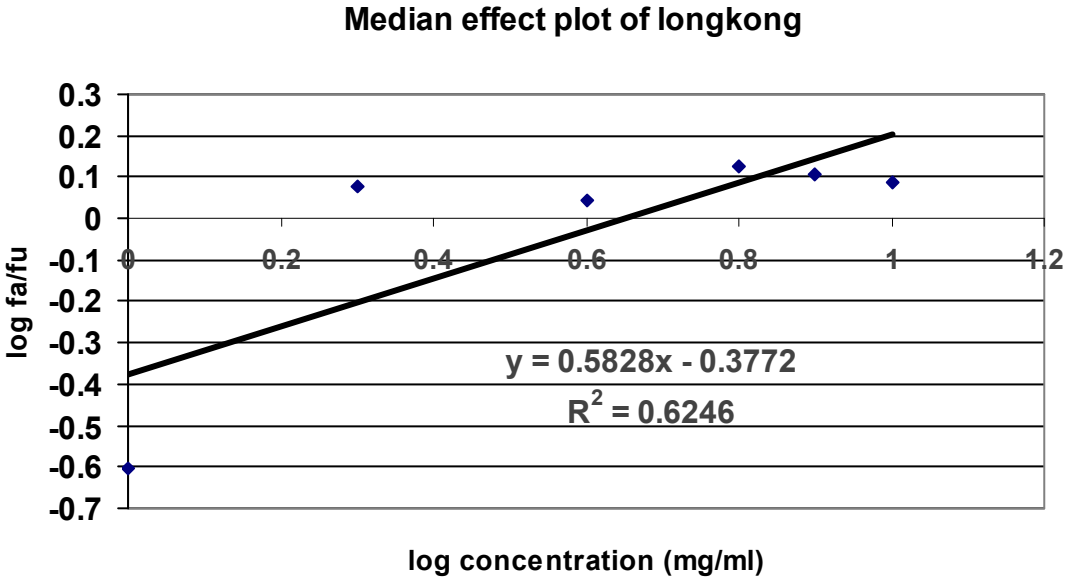



**Figure 2** Cellular antioxidant activity assay results. (A) Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by longkong (B) Median effect plots for inhibition of peroxyl radical-induced DCFH oxidation by longkong (mean ± SEM, n =3)

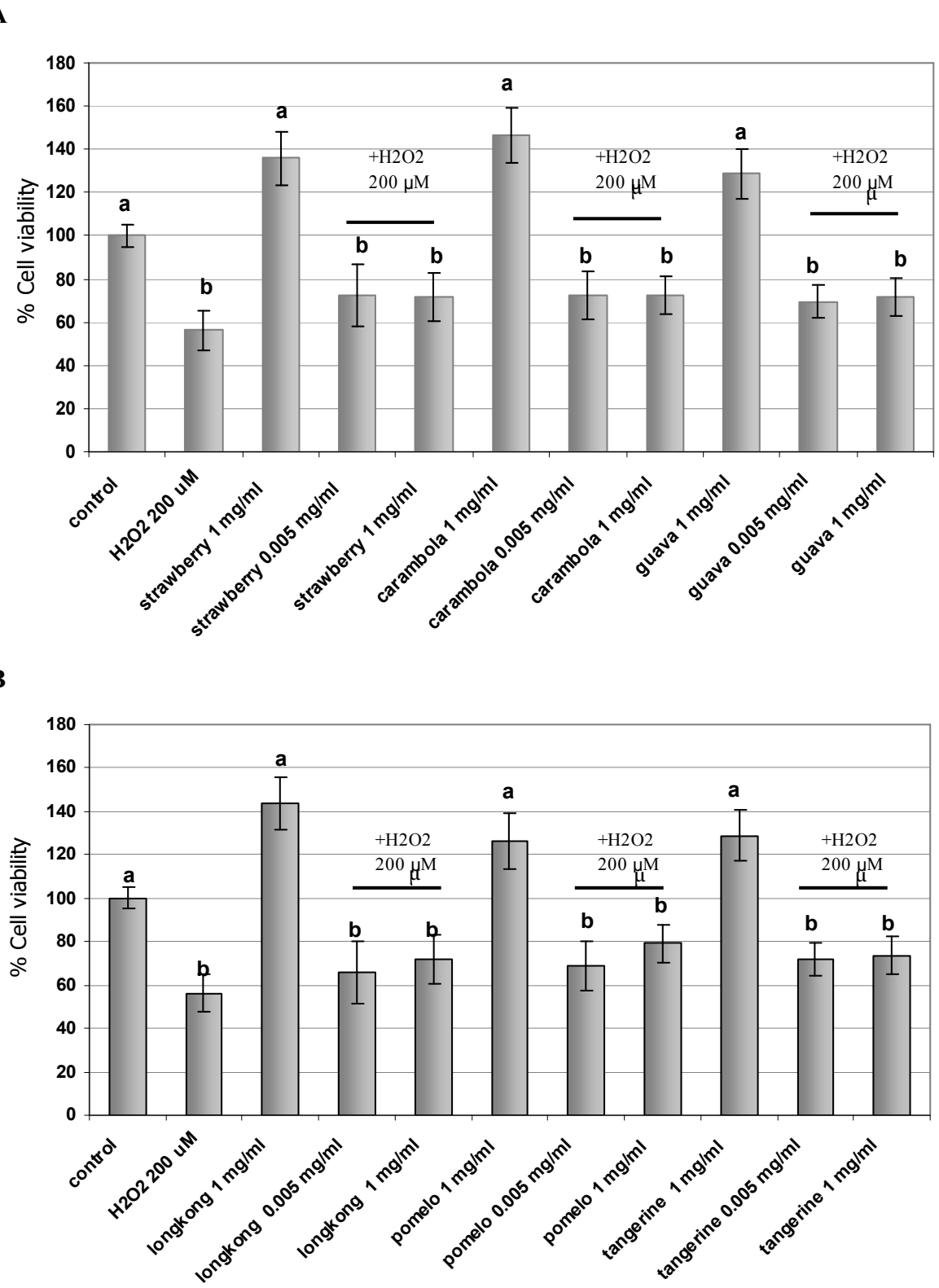



**Figure 3** Effect of fruit extracts on $H_2O_2$-induced oxidative damage in HepG2 cells. (A) Cells incubated in 200 µM $H_2O_2$ with or without 0.005, 1 mg/mL of strawberry, carambola, and guava extracts for 24 h. (B) Cells incubated in 200 µM $H_2O_2$ with or without 0.005, 1 mg/mL of longkong, pomelo, and tangerine extracts for 24 h. (mean ± SEM, n =6**)**

**Protective effect on oxidative damage in HepG2 cells**

$H_2O_2$ generated from sources of oxidative stress and oxygen radicals can form highly reactive radicals such as hydroxyl radicals in the presence of transition metal ions or by various other mechanisms. The formation of hydroxyl radicals and other ROS initiates oxidation of major cellular constituents such as lipid, DNA and protein and induces oxidative stressed cell damage.[18] Therefore, protective effect of strawberry, guava, carambola, longkong, pomelo, and tangerine extracts against $H_2O_2$-induced cell death was investigated in this study. As shown in Figure 3A and 3B, all fruit extracts at the maximum concentration used 1mg/ml increased the cell viability up to about 120-140% as compared to control. It was indicated that the fruit extracts at the level up to 1mg/ml was not toxic to HepG2 cells. The addition of 0.005 and 1 mg/ml of fruit extracts simultaneously with $H_2O_2$ increase the cell viability to 65-80% as compared to 56% in $H_2O_2$-treated cells. These results suggest that co-administration of all fruit extracts with $H_2O_2$ tends to improve cell survival rate although not to a level that is statistically significant. It was reported that strawberry, carambola and guava which presented the high CAA in this study also had the high antioxidant activities evaluated by oxygen radical scavenging activity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH). [9, 15, 19] However, the protective ability against $H_2O_2$-induced oxidative cell damage of all six selected fruits was not different. This result is probably due to by co-administration of $H_2O_2$ with the fruit extracts, the antioxidants in extracts can react directly with induced free radical before entry the cells. In addition, the doses of fruit extracts used may release the mixed antioxidants which can neutralize and reduce free radicals to the similar levels.

## CONCLUSION

Overall, the data from this study has demonstrated the cellular antioxidant activity of fruits which account for total antioxidant contents in fruits and cellular mechanism. Screening approaches by using CAA assay may obtain the more databases associate with potential biological activity that is not found from the chemistry antioxidant activity assays. Consumption of all tested fruit varieties may deliver healthful benefits by supplying natural antioxidants that are protective against oxidative hepatic cellular damage.

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