Inhibitory Effects of Date Fruit (*Pheonix dactylifera L.*) Extracts on Oral Cancer Cell Lines

Chaiyarit P Weerayutsil P** Wongraweewiwat R*** Kotchoom A**** Rattanathongkum A**

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Abstract

Date fruit (Phoenix dactylifera L.) has been known as a fruit with richness of nutrients, dietary fiber, essential minerals and vitamin contents. Previous studies reported that date fruit provided a variety of health benefits. Over the past five years, date palms have been cultivated throughout the north-eastern regions of Thailand, and date fruit becomes popular for Thai people. However, very limited data of date fruit in association with oral health benefits are available. The purpose of this study was to determine the effects of date fruit extracts on oral cancer cell lines. Date fruits derived from three stages including Kimri; Khalal; and Rutab were prepared for crude extraction by ethanol. Two oral squamous carcinoma cell lines (ORL-48T and ORL-136T) were used in this study. Human gingival epithelial cells were used as control. Cell cytotoxicity and cell viability were determined by MTT assay. DPPH assay was used to evaluate the antioxidant activity in date fruit extracts. According to analyzing chemical compositions, phenolic compounds and flavonoids were the essential components in date fruit extracts. According to MTT assays, date fruit extracts from three stages had inhibitory effects on two oral squamous carcinoma cell lines, as compared with human gingival epithelial cells ($p < 0.05$). The crude extracts from Khalal stage demonstrated the best inhibitory effect on reduction of oral squamous carcinoma cell growth. In conclusion, the present study provides new evidence of inhibitory effects of date fruit extracts on oral squamous carcinoma cell lines. These findings imply clinical use of date fruit extracts for oral health benefits.

Keywords: *Date fruit/ Human gingival epithelial cells/ Oral squamous carcinoma cell lines*

Introduction

Phoenix dactylifera L. (Date) is classified in a family of palm. Date has been cultivated for more than 6,000 years. Date palm can be found in various regions from north of Africa to Middle East countries, Middle and South America, and Southern Europe. Date palm can grow well in tropical climates[1]. Date fruit is the ideal food [2] known as a fruit with richness of nutrients and dietary fiber such as glucose, sucrose and fructose, lipids and proteins. Moreover, date fruit contains high levels of essential mineral and vitamin contents such as calcium, iron, magnesium, phosphate, zinc, and selenium [3, 4]. Date fruit has been ascribed to provide a variety of health benefits such as: boost energy, promote heart health, strengthen bones, decrease blood sugar level, and prevent peptic ulcer. Accumulated information indicates that date fruit has several biological functions such as anti-oxidant[5] and anti-microbial activities[6], and anti-cancer properties (Allogenic solid sarcoma-180)[7].

Over the past 5 years, date palm has been cultivated and becomes important for agricultural economy, especially in the north-eastern region of Thailand. However, limited studies of date fruit in the context of oral health benefits are available. Previous study reported that crude extracts of date fruit could induce apoptosis in human breast adenocarcinoma cell lines[8]. Taking these findings into account, we hypothesized

* Department of Oral Biomedical Sciences, Research Group of Chronic Inflammatory Oral Diseases and Systemic Diseases Associated with Oral Health, Faculty of Dentistry, Khon Kaen University, Amphur Muang, Khon Kaen.
** Dental Hospital, Faculty of Dentistry, Khon Kaen University, Amphur Muang, Khon Kaen.
*** Dental Department, Ban Phai Hospital, Amphur BnaPhai, Khon Kaen.
**** Dental Department, Waeng Noi Hospital, Amphur Waeng Noi, Khon Kaen.

that crude extracts of date fruit could have an inhibitory effect on oral cancer cells. The purpose of this study was to determine the inhibitory effect of date fruit extracts from 3 stages including: Kimri; Khalal; and Rutab on two oral squamous carcinoma cell lines including ORL-48T and ORL-136T.

Materials and methods

This study was approved by the institutional human ethics committee of Khon Kaen university number HE602169.

Extraction of date fruit Date fruit crude extracts (DFE) were obtained from the local cultivar named KL-1 Mae-Jo-36, developed from *Barhi* or *Barhee* and *Deglet Noor* varietals. One kilogram of Kimri stage, two kilograms of Khalal stage and one kilogram of Rutab stage were shred and splitted the seeds from fruit. Pulp of date fruit was blended with ethanol 1:3 ratio, incubated for 24 hours, and filtered with Whatman paper number 1 (N-1000,Tokyo Rikakikai Co.Ltd., Japan). DFE was dried with rotary evaporator (Eyela rotary N-1000, Tokyo Rikakikai Co.Ltd., Japan) at 60°C, 150 rpm, 3-4 hours/1000 mL and lyophilized (Alpha 2-4 LD plus, Martin Christ Co Ltd., Germany) at -80°C, 0.001 mbar, 48 hours. DFE was stored in the freezer at -80°C until further use.[8]

Determination of total phenolic content (TPC) All crude extracts were dissolved in distilled water and the final concentration was adjusted to 20 mg/mL. Twenty five microliters of the crude extract was mixed with 50 µL of 0.2 N Folin-Ciocalteau reagent and incubated in the dark for 30 min. Then Na_2SO_4 was added. After standing in the dark for 5 min, the absorbance at 760 nm was recorded by using a spectrometer. Gallic acid with concentrations ranging 6.25-100 µg/mL was used for preparation of a calibration curve. The concentration of total phenolic was expressed as milligram of gallic acid equivalent per gram dry weight (mg GAE/g dry wt.)[9]. The experiment was carried out in triplicate.

Determination of total flavonoid content (TFC) Five hundred microliters of crude extracts were mixed with 1.5 mL of 95% ethanol. After standing for 5 min, 100 µL of 10% $AlCl_3 \bullet 6H_2O$ was added into the mixture and left for another 6 min. Five hundred µL of 1 M KCH_3COO was added and total volume was adjusted to 5 mL with distilled water and incubated at room temperature for 30 min. The absorbance at 415 nm was read. A blank and standard compound were distilled water and quercetin (concentrations ranging 12.5-100 µg/mL), respectively. Total flavonoid content was reported as milligram of quercetin equivalent per 1 g dry weight (mg QE/g dry wt.) [10]. The experiment was carried out in triplicate.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay One hundred and fifty microliters of 625 mg/mL of crude extracts and 800 µg/mL DPPH reagent were mixed in each well. The mixture was left for 30 min at room temperature. The absorbance was measured at 517 nm by using a microplate reader (Tecan, France)[11]. All measurements were carried out in triplicate. The percentage of an inhibition activity was calculated as follows:

$$\% \text{ DPPH inhibition} = [(A_0 - A_1)/A_0] \times 100$$

A_0 = the absorbance of DPPH reagent with 120 µL of distilled water

A_1 = the absorbance of DPPH reagent with 120 µL of standard gallic acid or the extract

Cells culture condition The cells used in this study were human gingival epithelial cells (HGEP) (CELLnTec Co.Ltd., Switzerland) as a control, and 2 oral squamous carcinoma cell lines including ORL-48T and ORL-136T (kindly provided by Professor Sok Ching Cheong from the Cancer Research Initiatives Foundation (CARIF, Malaysia). HGEP were cultured in Keratinocyte-SFM (1X), and two oral cancer cell lines were cultured of DMEM/F-12.

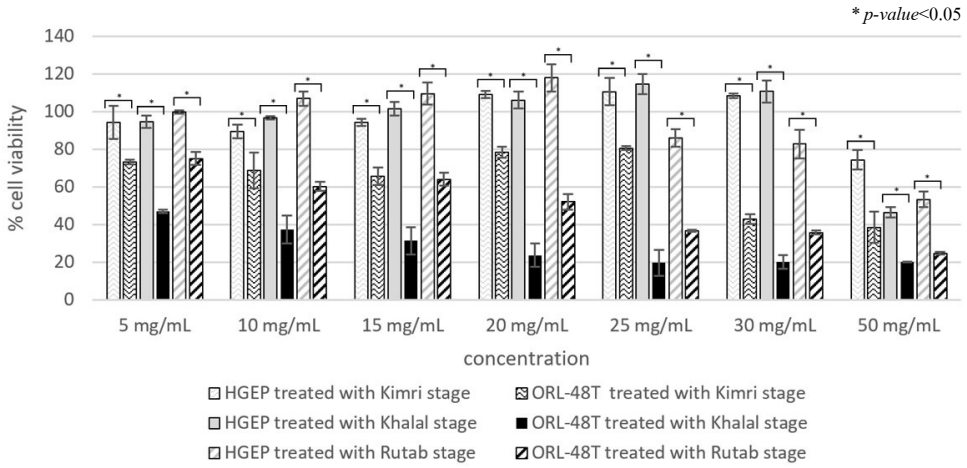

Figure 1 Cell Viability of ORL-48T and HGEP treated with DPF was examined by the MTT assay at 24 hours

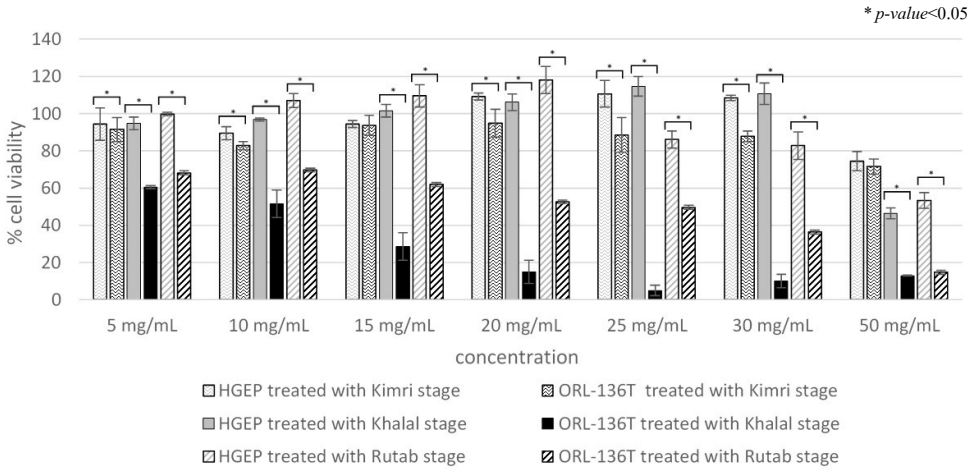

Figure 2 Cell Viability of ORL-136T and HGEP treated with DPF was examined by the MTT assay at 24 hours

Cell viability assay HGEP as a control, and 2 oral squamous carcinoma cell lines (ORL-48T and ORL-136T) were seeded ($2x10^5$ cells/mL) 100 µL in a 96 well plate (Corning, Costar, USA.) and incubated at 37°C in 5% CO_2 and 95% relative humidity for 24 hours. DFE was diluted with culture medium to make final concentrations of 1, 5, 10, 15, 20, 25, 30, 50, 80 and 100 mg/mL. Cells were treated with DFE in which each sample was tested in triplicate and control group was treated with culture medium 100 µL/well, and incubated at 37°C in 5% CO_2 and 95% relative humidity for 24 hours. After incubation, MTT reagent (3,[4, 5-dimethylthiazol- 2-yl]- 2, 5-diphenyltetrazolium bromide, MTT: Sigma Aldrich, St. Louis, Missouri, USA) was added to each well (50 µL/well), and incubated for 4 hours to form the formazan crystals. After that, Dimethyl Sulfoxide (DMSO) (100 µL/well) was added. The plate was mixed vigorously on a plate shaker. When the crystals were dissolved, absorbance was measured at 570 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific Inc., Massachusetts, USA). Blank controls were the well containing only DFE[12]. Percent cell viability was calculated by a formula below.

% Cell viability = [(A sample – A control)/ A of cell without treatment] x 100

A control = absorbance at 570 nm of well with 100 µL 1% DMSO,

A sample = absorbance at 570 nm of well with crude extract

Statistical analysis The results were expressed as mean ± standard deviation (SD). Cell viability was analyzed with independent-samples T-test using SPSS windows version 20.0. *P-value*<0.05 was considered as statistically significant.

Results

Percent yield of Date fruit crude extracts DFE from Rutab stage had the highest percent yield followed by those from Khalal and Kimri stages (Table 1).

Total phenolic and total flavonoid contents and antioxidant activity of date fruit crude extracts The highest contents of TPC and TFC were found in DFE from Kimri stage. Among all extracts, DFE from Kimri stage had a higher reducing power (136.74 ppm) than Khalal stage (Table 2).

Table 1 Percent yield of date fruit crude extracts (DFE) from three stages

Stages of DFE	%Yield
Kimri	6.522
Khalal	17.042
Rutab	17.978

Cytotoxic effect of Date fruit crude extracts on human epithelial cells and oral squamous carcinoma cell lines DFE from three stages was non-cytotoxic to HGEP. In contrast, DFE from all three stages could inhibit oral squamous carcinoma cell lines (ORL-48T and ORL-136T), except that DFE from Kimri stage could not inhibit ORL-136T. DFE from Khalal stage had the lowest CC_{50} for both oral squamous carcinoma cell lines (Table 3).

Table 2 Total phenolic Contents, total flavonoid content and antioxidant activity of date fruit crude extracts (DFE)

Stages of DFE	TPC (mg GAE/g dry wt)	TFC (mg QE/g dry wt)	IC_{50} value of antioxidant activity by DPPH assay (ppm)
Kimri	15.05±1.66	1.64±0.95	136.74
Khalal	9.67±1.54	1.45±0.89	397.45
Rutab	7.23±1.58	1.41±0.92	No detectable

TPC, TFC, and IC_{50} value of antioxidant activity by DPPH assay are expressed as mean ± SD, (n = 3)

Table 3 Cytotoxicity of date fruit crude extracts (DFE) on human gingival epithelial cells (HGEP) and oral squamous carcinoma cell lines (ORL-48T and ORL-136T)

Stages of DFE	CC_{50} value (mg/mL)		
	HGEP	ORL-48T	ORL-136T
Kimri	87.782	35.058	73.615
Khalal	55.032	1.110	8.199
Rutab	51.309	22.001	24.289

Cell viability of HGEP, ORL-48T, and ORL-136T treated with DFE from Kimri, Khalal and Rutab stages was shown in Figures 1 and 2. When DFE from three stages were used to treat these cell lines, there were statistically significant differences in cell viability between HGEP and oral squamous cancer cell lines (p-value<0.05 for ORL-48T; and p-value<0.05 for ORL-136T).

Discussion

First, we demonstrated that major chemical components in date fruit were phenolic compounds and flavonoids. According to our study, DFE from Kimri stage had the highest reducing power (136.74 ppm). This finding was in agreement with the result showing that contents of TPC and TFC were found the most in DFE from Kimri stage. It was reported that phenolic compounds and flavonoids had an anti-cancer activity by suppressing the oncogenic proteins and inducing the expression of tumor suppressors P53, P21 and P27[15]. Thus, it is reasonable to speculate that DFE from Kimri stage which had phenolic compounds and flavonoids in date fruit extracts may play a role in the inhibition of oral cancer cells. Therefore, it would be of importance for future studies to determine whether phenolic compounds and flavonoids could inhibit oral cancer cells.

Second, according to our observations, crude extracts from date fruit inhibited cell viability of oral squamous carcinoma cell lines, but did not inhibit cell viability of human gingival epithelial cells. Our findings are in agreement with previous studies demonstrating that crude extracts of date fruit could inhibit cancer cells such as breast cancer[8] and GI tract cancer[13]. In the present study, inhibitory effects of date fruit on oral squamous carcinoma cell lines are different between ORL-136T and ORL-48T. The possible explanation may be due to different molecular characteristics between ORL-48T and ORL-136T. It was reported that ORL-48T demonstrated an increased expression of mouse double minute 2 homolog (MDM2), whereas ORL-136T revealed an increased expression of epidermal growth factor receptor (EGFR)[14]. It would be of interest to investigate whether date fruit extracts have inhibitory effects on other types of oral cancer cells. Furthermore, from our finding, although DFE from Khalal stage had TPC and TFC lower than DFE from Kimri stage, it showed the most inhibition on cell viability of oral squamous carcinoma cell lines. Thus, It is possible to speculate that DFE would have other compounds that may play a role in the inhibition of oral squamous carcinoma cell line.

In conclusion, Our study demonstrated that crude extracts from date fruit specifically inhibited oral squamous carcinoma cell line viability but did not have toxic effect on human gingival epithelial cells at concentration between 5-100 mg/mL for 24 hours. Our findings might be implicated in the future research to understand molecular mechanisms of date fruit in association with oral cancer.

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Corresponding author

Ariya Rattanathongkum
Department of Oral Biomedical Sciences,
Faculty of Dentistry, Khon Kaen University,
Amphur Muang, Khon Kaen.
Tel.: +66 4320 2405 #45281
Fax: +66 4320 2862
E-mail: ariya@kku.ac.th

ผลการยับยั้งของสารสกัดหยาบจากผลอินทผลัมต่อเซลล์มะเร็งช่องปาก

พลธรรม ไชยฤทธิ์ พีรดา วีระยุทธศิลป์** รมิดา วงศ์รวีวิวัฒน์*** อริสา โคตรชุม**** อาริยา รัตนทองคำ**

บทคัดย่อ

อินทผลัมเป็นผลไม้โบราณ มีรสชาติหวาน มีคุณค่าทางอาหารสูง และอุดมไปด้วยวิตามินต่าง ๆ เป็นผลไม้ที่ดีต่อสุขภาพ เนื่องจากมีฤทธิ์ทางเภสัชวิทยาที่หลากหลาย ในช่วง 5 ปีที่ผ่านมา อินทผลัมกลายเป็นผลไม้ได้รับความนิยมในประเทศไทยมากขึ้น โดยปลูกมากที่ภาคตะวันออกเฉียงเหนือของประเทศไทย อย่างไรก็ตามยังไม่เคยมีการศึกษาผลของสารสกัดหยาบจากผลอินทผลัมกับเซลล์ในช่องปากมาก่อน การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดหยาบจากผลอินทผลัมในระยะผลดิบ ผลสุก และสุกงอมต่อเซลล์ในช่องปาก ซึ่งเซลล์ที่ใช้ศึกษาประกอบด้วยเซลล์เยื่อบุผิวเหงือกชนิดปฐมภูมิ และเซลล์มะเร็งช่องปากชนิดสแควมัสเซลล์ 2 ชนิด ได้แก่ ORL-48T และ ORL-136T โดยการสกัดผลอินทผลัมด้วยตัวทำละลายเอทานอล ทำการทดสอบความเป็นพิษของสารสกัดหยาบจากผลอินทผลัมต่อเซลล์ในช่องปากด้วยวิธีเอ็มทีทีและวิเคราะห์ฤทธิ์การต้านอนุมูลอิสระของสารสกัดหยาบจากผลอินทผลัมด้วยวิธีดีพีพีเอช จากวิเคราะห์องค์ประกอบทางเคมีของสารสกัดหยาบจากผลอินทผลัมพบว่า สารประกอบฟีนอลิกและฟลาโวนอยด์เป็นสารประกอบสำคัญของสารสกัดหยาบจากผลอินทผลัม ผลการทดสอบความเป็นพิษของสารสกัดหยาบจากผลอินทผลัมต่อเซลล์ในช่องปากด้วยวิธีเอ็มทีทีพบว่า สารสกัดหยาบจากผลอินทผลัมในระยะผลดิบ ผลสุก และสุกงอม มีความเป็นพิษต่อเซลล์มะเร็งช่องปากทั้ง 2 ชนิด แต่ไม่มีความเป็นพิษต่อเซลล์เยื่อบุผิวเหงือกชนิดปฐมภูมิ ($p < 0.05$) โดยสารสกัดหยาบจากผลอินทผลัมในระยะผลสุกมีความเป็นพิษต่อเซลล์มะเร็งช่องปากทั้ง 2 ชนิดมากที่สุดเมื่อเปรียบเทียบกับสารสกัดหยาบจากผลอินทผลัมในระยะผลดิบและผลสุกงอม การวิจัยในครั้งนี้เป็นการศึกษาเบื้องต้นของสารสกัดหยาบจากอินทผลัมในระยะผลดิบ ผลสุก และผลสุกงอมในระดับเซลล์ จึงควรมีการศึกษาต่อไปในระดับโมเลกุลเพื่อให้ได้ทราบถึงกลไกการออกฤทธิ์ของสารสกัดหยาบจากผลอินทผลัมที่มีต่อเซลล์ดังกล่าวและสามารถนำไปต่อยอดความรู้ในการดูแลสุขภาพช่องปาก

คำไขรหัส : *ผลอินทผลัม/ เซลล์เยื่อบุผิวเหงือกชนิดปฐมภูมิ/ เซลล์มะเร็งช่องปากชนิดสแควมัสเซลล์*

ผู้รับผิดชอบบทความ

อาริยา รัตนทองคำ
สาขาวิชาชีววิทยาช่องปาก
คณะทันตแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
อำเภอเมือง จังหวัดขอนแก่น
โทรศัพท์: 0 4320 2405 #45281
โทรสาร: 4320 2862
จดหมายอิเล็กทรอนิกส์: ariya@kku.ac.th

** สาขาวิชาชีววิทยาช่องปากและกลุ่มวิจัยโรคอักเสบเรื้อรังช่องปากฯ คณะทันตแพทยศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น*
*** โรงพยาบาลทันตกรรม คณะทันตแพทยศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น*
**** ฝ่ายทันตกรรม โรงพยาบาลบ้านไผ่ อำเภอบ้านไผ่ จังหวัดขอนแก่น*
***** ฝ่ายทันตกรรม โรงพยาบาลแวงน้อย อำเภอแวงน้อย จังหวัดขอนแก่น*