



Original Article

บทวิทษฬฬกร

# *In vitro* study on the efficacy in assisting gutta-percha removal and cytotoxicity of essential oil from *Citrus maxima* (pomelo oil)

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## Abstract

**Objective** To evaluate the efficacy in assisting gutta-percha removal of an essential oil from *Citrus maxima* (pomelo oil) and its cytotoxicity on human gingival fibroblasts when compared to orange oil and xylene.

**Materials and methods** Forty human single-rooted teeth were instrumented and filled with gutta-percha at their apical one third. The gutta-percha filled in root canal was softened with each solvent, pomelo oil, orange oil, xylene or distilled water (n=10 for each group). Gutta-percha was firstly removed with #25 K-file to reach a working length and then completely removed with Hedström files. The complete removal was verified by a microscope and a digital radiography. Times required to reach the working length and to completely remove filled gutta-percha were recorded. For the toxicity test, the clinically used concentration of three solvents was tested on human gingival fibroblasts using the MTT assay. Cell viability was determined after exposure to each solvent for 1 s, 10 s and 30 s.

**Results** The results showed no statistically significant difference in time to reach the working length or time required to completely remove gutta-percha among three solvents ( $p > 0.05$ ). All three solvents were toxic to gingival fibroblasts but xylene was the most cytotoxic solvent in all exposure times. Pomelo oil was less cytotoxic than orange oil and xylene when the exposure time increased.

**Conclusion** This study demonstrated the usefulness of the pomelo oil in assisting the removal of gutta-percha and that it was less toxic to gingival fibroblasts than orange oil and xylene.

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**Key words:** cytotoxicity; gutta-percha solvent; orange oil; pomelo oil; xylene

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## Introduction

In non-surgical endodontic retreatment, old root canal filling must be removed to gain access to the entire root canal system to facilitate re-cleaning, re-shaping and re-filling.<sup>1</sup> Either with or without solvents, various physical techniques (heat, hand files, rotary instruments, and ultrasonic instruments) have been used to remove gutta-percha from the root canal.<sup>2</sup> Due to its softening effect, solvent has been clinically used to facilitate the removal of well-condensed gutta-percha and to reduce the risk of altering or mis-shaping the root canal morphology.<sup>3</sup> Some previous studies<sup>4-6</sup> have revealed several advantages in a less clinical working-time and an improvement in the root canal cleanliness by the usage of instruments combined with organic solvents.

Chloroform and xylene, as well as some other commercially available products, are common gutta-percha solvents. Despite its excellence in dissolving the gutta-percha,<sup>7-9</sup> chloroform possesses carcinogenic potentials.<sup>10-11</sup> Xylene is another available solvent for the clinical usages of removing the gutta-percha, but is toxic to living-tissues.<sup>12</sup> Frequent uses of xylene could increase a risk of some occupational exposures via inhalation and/or dermal absorption, both of which could cause local and systemic toxicological effects.<sup>12-15</sup>

Citrus plants are famous as sources of essential oils which are safe for the usage in flavoring foods, beverage, and pharmaceutical products.<sup>16</sup> Pomelo (*Citrus maxima*) is the largest citrus fruit native to Asia, and is best cultivated in China, southern Japan, Vietnam, Malaysia, Indonesia and Thailand.<sup>17</sup> Other terms for pomelo include pummelo, pommelo and Chinese grape-fruit. The essential oil can be obtained from the peels, which are abundant industrial waste. The major constituent in pomelo oil is *d*-limonene, a hydrocarbon classified as a terpene. Orange oil's *d*-limonene is able to soften the gutta-percha,<sup>18-19</sup> and has been

proposed as a gutta-percha solvent.<sup>19</sup> Pomelo oil contains as high limonene as orange oil, however, its softening effect on gutta-percha has not yet been evaluated.

To obtain an ideal solvent for root canal re-treatment, some assessments are needed to judge the balances among toxicity, clinical safety, and chemical solvent property.<sup>20</sup> The toxicity of pomelo oil compared to orange oil and xylene is completely unknown. Thus, this study was conducted to preliminary evaluate the efficacy in assisting gutta-percha removal of an essential oil from *Citrus maxima* (pomelo oil) and its cytotoxicity on human gingival fibroblasts when compared to orange oil and xylene.

## Materials and methods

### *Gutta-percha removal test*

Intact single-rooted teeth (n=40) were selected and used in this study. The teeth were horizontally cut at the cervical line using a diamond disc under water spray. Each root's length was adjusted to be 13 mm. A stainless steel K-file (size 15, Kerr, Romulus, MI, USA) was inserted into the root canal until it was visible at the apical foramen. Each root canal was instrumented 1 mm short of this length (working length = 12 mm). The coronal thirds of root canal was flared with Gate Glidden drills #3 and #4 (Dentsply-Maillefer, Ballaigues, Switzerland). The root canal was prepared in a step-back technique using K-files. The master apical file (size 60) was used and the subsequent five files were used at 1 mm shorter than the previous file. During instrumentation, the canal was irrigated several times with 2.5% sodium hypochlorite. The root canal was dried with absorbent paper points and filled by using the laterally condensed gutta-percha technique. A main gutta-percha cone (size 60, Hygenic Co., Akron, OH, USA) and accessory cones (medium-fine size, Hygenic Co.) were used without any root canal sealer. A root canal spreader (D11T) was used to

laterally condense until the distance of more than 7 mm could not be reached. Gutta-percha at both coronal and middle parts (9 mm) was removed with a heat instrument. The quality of root canal filling (3 mm at the apical 1/3) was examined using digital radiographs in bucco-lingual and mesio-distal directions as shown in figures 2a and 2b. The root canal fillings with a homogenous density, with a 1-mm distance between the end of the filling and the radiographic apex, and without space between filling and root canal wall were accepted as being good quality. Radiographs were digitized using a scanner (PSPIX, Sopro-Acteon, La Ciotat, France) and the digitized images were imported into an imaging software (VixWin 2000™, Gendex Corp., IL, USA). All specimens were stored at 37°C in 100% humidity for 1 week.

The solvents used in this study were distilled water (control), pomelo oil (*Citrus maxima* Merr.) (Punnapat Co., LTD, Bangkok, Thailand), orange oil (*Citrus aurantium* Var. *dulcis* & *Citrus aurantium* Var. *sinensis*) (Thai-China flavours and fragrances industry Co., LTD, Bangkok, Thailand) and xylene (Vidhayasom Co., LTD, Bangkok, Thailand). All specimens were then divided into four solvent groups, randomly and equally. Each solvent (4 µl) was pipetted into the root canal and left for 30 s. A K-file (ISO size 25) was inserted until it reached the working length, and then the time required (in minutes) was recorded. The complete removal of gutta-percha was performed using Hedström (H) files (size 25 to 60, Kerr, Romulus, MI, USA) in a reaming motion. An additional 2 µl solvent was added after four instruments were used. A total of 6 µl of solvent were used per one tooth. Gutta-percha was removed until no evidence of gutta-percha was found on the last file. A complete removal of the gutta-percha was evaluated under an operating microscope (OPMI pico, Carl Zeiss Meditec, Inc., Germany) at a magnification of 25X and the digital radiographic images in both bucco-lingual and mesio-distal directions. The total time (in minutes) required for the completion of

gutta-percha removal process was recorded with a stopwatch. If the remaining gutta-percha was found under the microscope or the digital radiographs, the procedure with H-files was continued until a complete removal was achieved. The additional time (the evaluation time excluded) was also recorded and incorporated in the total time required for complete removal of gutta-percha. During removal, an apical extrusion of debris was observed and reported as yes or no.

#### *Cellular viability test*

This study was approved by the Ethics Committees of Naresuan University. Human gingival fibroblasts from 3 patients were grown in a 35 x 15 mm tissue culture dish (Nunc, Rochester, NY, USA) in a Dulbecco Modified Eagle's Medium (DMEM) (Gibco®, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 3 µg/ml fungizone. The culture dishes were incubated at 37°C in a humidified atmosphere (95% air and 5% CO<sub>2</sub>). Cells exhibiting characteristic spindle-shaped morphology of fibroblasts, were passaged by washing with a phosphate-buffered saline (PBS) before being treated with 0.25% trypsin/1 mM EDTA (Gibco®) for 3 min. Cells from the third to the fifth passages were plated in a 60 x 15 mm glass plate at a density of 300,000 cells/plate, allowed to attach for 24 h, and changed to serum-free plus supplements for 4-6 h. Cells were prepared for 108 plates which were divided for four solvent groups and three exposure time groups equally (n=9 for each group). At this time, the medium was removed and tested solvent (1 ml) was added to each culture plate sealed with parafilm. The medium with supplements alone provided a negative control. The gingival fibroblasts were exposed to each solvent for 1 s, 10 s and 30 s.

The effects of solvents on the viability of gingival fibroblast cells were evaluated with MTT (3-(4,5-dimethylthiazoyl)-2,5-diphenyl-SH-tetrazolium bromide) (USB corp., Cleveland, OH, USA) assay. The amount of

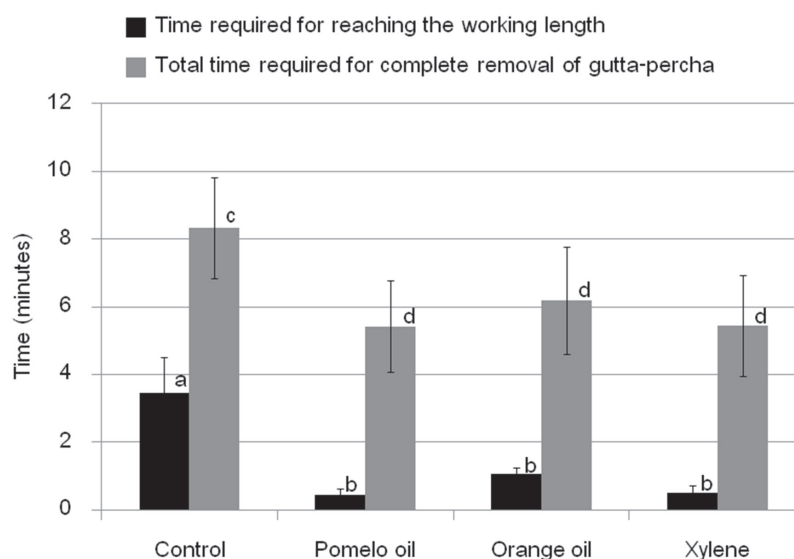
yellow MTT reduced to purple formazan was measured by a spectrophotometer at a 570 nm wavelength. This reduction took place only when mitochondrial reductase enzymes were active, thus the conversion was directly proportional to the number of viable cells in the culture. The production of purple formazan in cells treated with solvents was measured relative to the production in control cells. The culture medium was aspirated, replaced with a 0.5 mg/ml MTT solution, and incubated for 30 min in the CO<sub>2</sub> incubator. The solution was then aspirated and a 1,000 µl of DMSO (Sigma, St Louis, MO, USA) was added to dissolve the formazan crystals. The absorbance of the solution at the 570 nm wavelength was measured using a Genesis 10 UV-vis spectrophotometer (Thermo Spectronic, NY, USA). Viable cells were calculated from the standard curve of cell number by plotting a scattergram of the absorbance value against the known number of cells. The percentages of viable cells in each group were calculated and compared.

### Statistical analyses

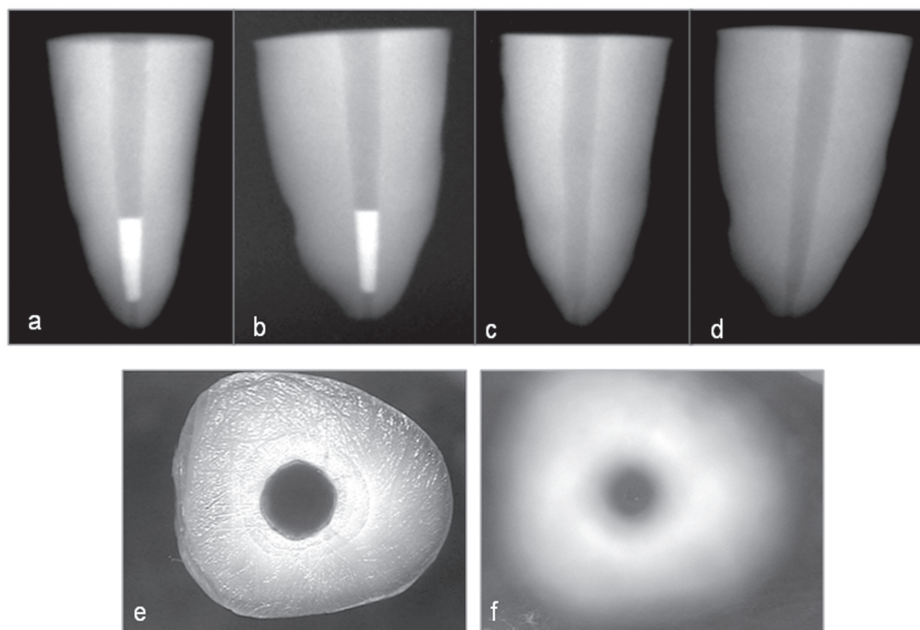
The time required for reaching the working length and for removing the gutta-percha and the percentage of cells viability were statistically analyzed by a one-way analysis of variance. Tukey multiple comparisons were used to specify the inter-group's differences test at the 95% level of confidence.

## Results

All specimens showed neither remaining gutta-percha under the microscope (Figs. 2e and 2f) nor radiopaque mass in the digital radiographic images in both bucco-lingual (Fig. 2c) and mesio-distal (Fig. 2d) views. In addition, no apical extrusion was observed in any of the groups. The means and the standard deviations of the time required for reaching the working length and for complete removal of gutta-percha for the groups are shown in Fig. 1.



**Fig. 1** Time required for reaching the working length and for complete removal of gutta-percha. All numerical data are shown in mean and standard deviation. The different letters (a/b/c/d) present the significantly different results ( $p < 0.05$ ) among groups.



**Fig. 2** Digital radiographic images showing the specimen with root canal filling in bucco-lingual (a) and mesio-distal (b) views, and the specimen after removal of the gutta-percha in bucco-lingual (c) and mesio-distal (d) views. Microscopic pictures of root canal after complete removal of gutta-percha showing no remaining debris in root canal focused on coronal 1/3 (e) and apical 1/3 (f).

#### *Time required for reaching the working length*

When compared to that with the control, the use of hand file with softening solvents required significantly less time. There was no statistically significant difference ( $p > 0.05$ ) among the three solvents used.

#### *Total time required for complete removal of gutta-percha*

The times (mean  $\pm$  standard deviation) for control, pomelo oil, orange oil and xylene groups respectively were  $8.33 \pm 1.49$ ,  $5.42 \pm 1.35$ ,  $6.19 \pm 1.58$ , and  $5.43 \pm 1.49$ . Total time required for complete removal of gutta-percha with softening solvents was less than that with the control. There was no statistically significant difference ( $p > 0.05$ ) among the three solvents used.

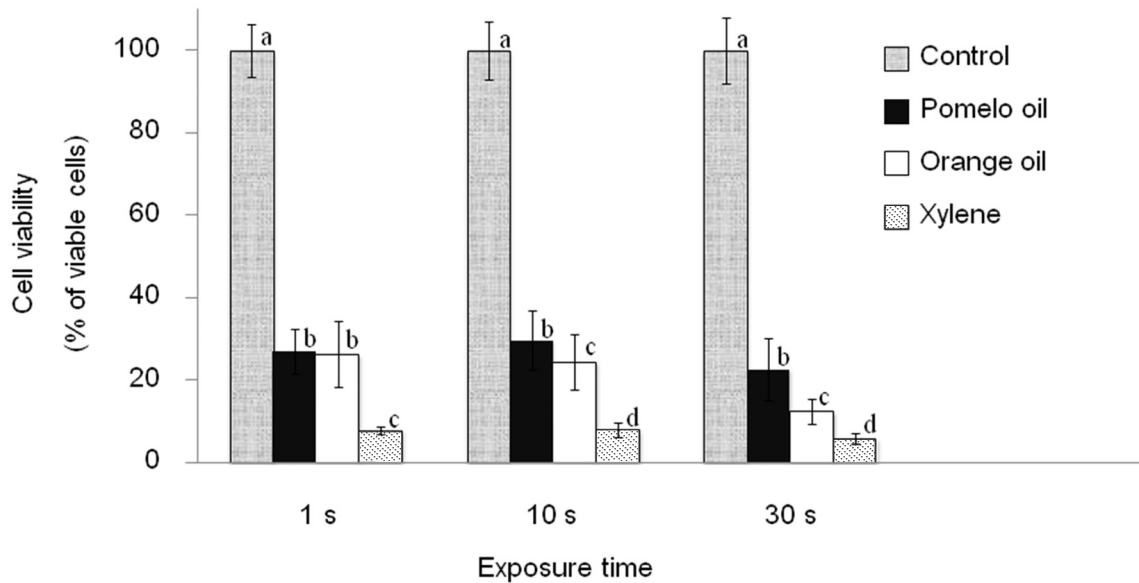
To consider the time of using H-files (total time required for complete removal of gutta-percha minus time required for reaching the working length), there was no statistically significant difference ( $p > 0.05$ ) between control, pomelo oil, orange oil and xylene.

#### *Cellular viability*

The effects of tested solvents on human gingival fibroblast viability are shown in Fig. 3. All three solvents were significantly toxic to gingival fibroblasts when compared to control ( $p < 0.05$ ). Xylene was the most toxic solvent in all exposure times (1 s, 10 s and 30 s). After 30 s of exposure, pomelo oil was less cytotoxic than orange oil and xylene ( $p < 0.05$ ).

## **Discussion**

Several investigations described the efficacy of solvents on dissolving or softening gutta-percha by the measurement of either applied forces or the consuming-time of instrument to penetrate into the gutta-percha,<sup>19-21</sup> the dissolution of gutta-percha cone<sup>22</sup> or condensed gutta-percha in a stainless steel mould,<sup>23</sup> or the removal time.<sup>18</sup> To simulate the clinical environment, the amount of the time required to reach the working length and to completely remove the gutta-percha in



**Fig. 3** Effects of pomelo oil, orange oil and xylene on human gingival fibroblasts. Results of viable cells are expressed in mean percentage and standard deviation (mean  $\pm$  SD); different letters (a/b/c/d) present the significantly different results ( $p < 0.05$ ) among groups after a 1, 10 or 30-second exposure.

human teeth was measured in this study. The crowns were removed and the root length was also standardized at 13 mm to allow a better visualization of the root canal morphology and to eliminate any coronal interferences during root canal preparation, root canal filling, and gutta-percha removal.<sup>24</sup>

The usage of softening solvents with hand files in this study facilitated complete removal of gutta-percha from the root canals and consumed less time to reach the working length and to completely remove gutta-percha, when compared with that of the distilled water. An initial penetration with a K-file in solvent groups was observed to be easier and faster to reach the working length than that in the control because the softened gutta-percha was less resistant.<sup>25</sup> Among three solvents including pomelo oil, orange oil and xylene, there was no statistically significant difference in both times required to reach the working length and to completely remove gutta-percha.

On the other hand, using softening solvents did not reduce the H-files usage time, because of no significant difference in such usage time between

control, pomelo oil, orange oil and xylene. This finding suggests that an addition of the 2- $\mu$ l solvent after reaching the working length of instrument may be unnecessary.

Pomelo oil and orange oil possess similar capacities to assist in gutta-percha removal. Because of the fact that *d*-limonene, the main component<sup>18</sup> which is able to soften gutta-percha, in both essential oils is as high as 90%, thus their gutta-percha softening abilities were not significantly different.

According to a previous investigation with the mould of gutta-percha or sealer in different solvents, the solvents' dissolving effect on sealer may be influenced by the presence of gutta-percha,<sup>21</sup> resulting in the indistinguishable overall results. Hence, the filling of root canal in this study was performed without a sealer to reduce the confounding factors. The dissolving effects of pomelo oil, orange oil, and xylene on various sealers still need further investigations.

Clinically, in patients who need retreatment, insertion of the instrument to reach the working length enables the operators to get a better control because

most of the remaining filling materials will be removed during the following preparation procedures, once the apex is reached.<sup>26</sup> These results coincided well with the previous report on the NiTi rotary systems with different solvents.<sup>4</sup> The study revealed less time consumption in all groups with solvent. On the other hand, some studies<sup>27-28</sup> found the root canal filling was removed faster using instruments with no solvent. The usage of solvents was claimed to result in a thin film of material over the root canal wall that was more difficult to remove. The solvent's volume in this study was then restricted to obtain only the softening effect, but not the dissolving effect on gutta-percha. To prevent an apical extrusion and a thin smear film on the root canal wall, the restriction of solvent's volume was particularly applied at the area adjacent to apical terminus.<sup>29</sup> Owing to the mentioned methods, this study succeeded in softening and mechanically removing the gutta-percha, without any apical extrusion when observed by naked eyes.

In this study, the gutta-percha in the coronal 1/3 and the middle 1/3 areas were removed with a heat instrument. Consequently, some spaces serving as reservoirs for the solvent were obtained. The root canal filling materials in such mentioned areas could be removed completely by various techniques including those with H-files.<sup>24</sup> The debris was previously reported to be detectable only in the apical 1/3 area,<sup>24,30</sup> which was thus evaluated in this work. Compared with those in previous reports,<sup>24,30</sup> a complete removal of gutta-percha at the apical 1/3 was more easily performed. This may be contributed to the selection of specimens with straight canals, the utilization of similar ISO hand files during preparing root canals and during removing gutta-percha, and no usage of root canal sealer.

In the studies on gutta-percha removal, evaluations of the remaining gutta-percha were achieved by radiography,<sup>6</sup> or by longitudinally splitting the teeth<sup>4-5,24</sup> followed by photography, magnification, and tracing.

However, the filling debris displaced during sectioning was claimed in the latter.<sup>31</sup> In the clinical situation, remnants of the old root canal fillings are routinely evaluated by radiography. To obtain more details, the complete removal of gutta-percha in this study was evaluated with the aid of the operating microscope and the digital radiography.

Regarding the cytotoxicity test, immediate exposure of the three solvents on gingival fibroblasts showed significant toxicity ( $p < 0.05$ ). After longer exposure (30 s), the toxicity of three solvents increased. Pomelo oil possessed the lowest toxicity compared to orange oil and xylene. This exposure method was different from previous study.<sup>32</sup> The tests were performed under a sealed condition to control some changes in the exposure concentration, due to chemical evaporation.<sup>33</sup> For the risk assessment in culture cell tests, two factors have been suggested to be overcome. First, the change of concentration over time should be controlled. Second, the exposure concentration should be expressed as the concentration in the tissue.<sup>33</sup> However we could not estimate the suitable concentration expressed as the concentration in the tissue. Hence, the solvents' concentrations usually employed clinically were evaluated in this study.

Taken together the balance between the chemical capacity and the level of toxicity, the facilitated gutta-percha removal of pomelo oil was similar to orange oil and xylene but pomelo oil showed less toxicity to gingival fibroblast than orange oil and xylene after 30-s exposure. Due to the toxicity of xylene to living-tissues<sup>12</sup> and its increased risk of some occupational exposures,<sup>12-14</sup> a replacement of xylene by pomelo oil should be conducted to obtain a safer, but similar, softening property.

Orange oil presented the same capacity of gutta-percha removal as pomelo oil and xylene, but its toxicity after 30-s exposure was higher than pomelo

oil but lower than xylene. Compared to chloroform and eucalyptol, orange oil in a previous study has been revealed to possess less cytotoxicity to Swiss mice's peritoneal macrophages.<sup>34</sup> Therefore, orange oil has been proposed as an alternative solvent for gutta-percha.<sup>19</sup> The different level of toxicities in this study might be resulted from the different organic components of essential oils varied with respect to ecological and geographical conditions, age of plant, and time of harvesting.<sup>35</sup>

The present results suggest that all tested solvents would be potentially toxic when they have reached the periapical tissues. Using solvents at the root canal's apical areas should be cautiously done to avoid the action of inadvertently pushing the solvent into such tissues.

### Conclusions

Based on the *in vitro* methods and procedures used in this study, the efficacy of pomelo oil in assisting gutta-percha removal was similar to that of orange oil and xylene. Regarding their toxicities to human gingival fibroblast cells, pomelo oil has the lowest toxicity, followed by orange oil and xylene, respectively. This study demonstrated the usefulness of pomelo oil as an alternative gutta-percha solvent instead of toxic xylene.

### Acknowledgements

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# การศึกษาประสิทธิภาพการเสริมการรื้อ กัตทาเพอร์ชาและความเป็นพิษต่อเซลล์ของ น้ำมันหอมระเหยส้มโอในห้องปฏิบัติการ

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<sup>3</sup>ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยนครสวรรค์

## บทคัดย่อ

**วัตถุประสงค์** เพื่อประเมินประสิทธิภาพในการเสริมการรื้อกัตทาเพอร์ชาของน้ำมันหอมระเหยส้มโอและความเป็นพิษต่อเซลล์เหงือกมนุษย์เมื่อเปรียบเทียบกับน้ำมันส้มและไชลิน

**วัสดุและวิธีการ** นำฟันมนุษย์ที่มีรากเดียวจำนวน 40 ซี่ มาทำการขยาย และอุดคลองรากฟันในส่วน 1/3 ปลายราก ใช้ น้ำมันส้มโอ น้ำมันส้ม ไชลิน และน้ำกลั่น มาทำกัตทาเพอร์ชาที่อุดในคลองรากฟันอ่อนตัว (กลุ่มละ 10 ซี่) เริ่มต้นการรื้อด้วย เคไฟล์ ขนาด #25 ใส่ลงไปให้ถึงความยาวทำงาน แล้วจับเวลา หลังจากนั้น ทำการรื้อกัตทาเพอร์ชาให้สมบูรณ์ด้วยเฮดสโตรมไฟล์ จับเวลาและตรวจสอบการรื้อกัตทาเพอร์ชาโดยใช้กล้องไมโครสโคป และการถ่ายภาพรังสีแบบดิจิตอล บันทึกเวลาที่เครื่องมือแทรกผ่านถึงความยาวทำงาน และเวลาทั้งหมดที่ใช้รื้อวัสดุอุดกัตทาเพอร์ชาออกอย่างสมบูรณ์ ทดสอบความเป็นพิษของน้ำมันส้มโอ น้ำมันส้ม และไชลิน ณ ความเข้มข้นที่ใช้ในคลินิกต่อเซลล์เหงือกของมนุษย์โดยใช้วิธีวิเคราะห์ด้วยสารเอ็มทีที วัดความมีชีวิตของเซลล์ภายหลังจากการสัมผัสตัวทำลายแต่ละชนิดภายในเวลา 1 วินาที 10 วินาที และ 30 วินาที

**ผลการศึกษา** ผลการศึกษาแสดงให้เห็นว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติในเวลาที่ใช้เครื่องมือแทรกผ่านกัตทาเพอร์ชาถึงความยาวทำงาน หรือเวลาที่ใช้ในการรื้อกัตทาเพอร์ชาออกให้สมบูรณ์ท่ามกลางตัวทำลายทั้ง 3 ชนิด ( $p > 0.05$ ) ความเป็นพิษต่อเซลล์เหงือกพบว่าตัวทำลายทั้ง 3 ชนิดเป็นพิษต่อเซลล์ แต่ไชลินเป็นพิษมากที่สุดในทุกช่วงเวลาทดสอบ น้ำมันส้มโอเป็นพิษน้อยกว่าน้ำมันส้ม และไชลินเมื่อระยะเวลาการสัมผัสเพิ่มขึ้น

**สรุป** การศึกษานี้แสดงให้เห็นประโยชน์ของน้ำมันส้มโอในการเสริมการรื้อกัตทาเพอร์ชา และมีความเป็นพิษต่อเซลล์เหงือกน้อยกว่าน้ำมันส้มและไชลิน

(จ.ทันต. จุฬาฯ 2557;37:289-98)

**คำสำคัญ:** ความเป็นพิษต่อเซลล์; ไชลิน; ตัวทำลายกัตทาเพอร์ชา; น้ำมันส้ม; น้ำมันส้มโอ

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