

## The effect of ethyl acetate fraction of *Citrus limon* peel on mesenchymal cell proliferation and polybacterial growth

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### ABSTRACT

**Background:** Oral diseases remain to be global health problem. The common therapy involved the use of modern medicines with their various side effects. *Citrus limon* are potentials as anti-inflammatory, anti-fungal, anti-oxidant, anti-viral and anti-bacterial. **Purpose:** The purpose of this study was to determine the effect of ethyl acetate fraction of citrus limon peel extract on human gingival mesenchymal cell proliferation and palm commensal polybacterial growth. **Method:** This study was experimental study with post test only control group design. Citrus limon peel extracted and partitioned in order to obtain ethyl acetate fraction of 3.125%, 2.75%, 2.375%, 2%, and 1.5625%. Toxicity test was performed after 24 hours using the MTT Assays. Cell viability was measured by optical density formazonand read by ELISA reader 620 nm. **Results:** All treatment groups showed less than 60% cell viability. The highest cell was 19.36 (1.5625% concentration) and the lowest was 12.65 (3.125% concentration). The highest anti-bacterial inhibition value was 8.9125 mm (3.125% concentration) and the lowest was 6.0625 mm (1.5625% concentration). **Conclusion:** The higher concentration of ethyl acetate fraction Citrus limon peel extract, the higher toxicity and inhibitory properties against commensal palm polybacteria.

**Keywords:** Citrus limon; toxicity; gingival mesenchymal cell; palm polybacteria

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### INTRODUCTION

Oral diseases remain to be global health problem. The common therapy using modern medicines is preferrable in oral diseases treatment to give either immediate or non-immediate side effect, or accumulated side effect. Most of chemical medicines we consumed are international products which make their price high.<sup>1</sup>

Indonesia is a rich source of herbs to produce conventional herbal medicines. The addictive effect of using chemical medicines can be taken away by using herbal medicines. Herbal medicines are more effective than chemical medicines because they are affordable and are relatively safer than modern medicines. However, conventional medicines can also give negative effect if they are used in inaccurate way. The dose of conventional medicines is often empiric, without a clear prescription.

Some studies had been conducted to advance the use of conventional medicines, including: cellular level study (prominent potential test with toxicity test), guinea pig level study, human level study and human in a big scale level study (multicenter).

*Citrus limon*, also known as citron, is one of herbal medicine which is quite popular for its various merits. The most usable part of *Citrus limon* is its fruit. The fruit is good for health for increasing immune system, preventing bacteria and free radicals. Ascorbic acid in lemon functions as anti-inflammatory and accelerating the recovery process, whereas *Citrus limon* peel is not used.<sup>2</sup> The peel of *Citrus lemon* contains many chemical compounds polymethoxylated flavones, such as flavonoid glycosides, coumarins,  $\beta$  dan  $\gamma$ - sitosterol, terpenoid glycosides and volatile oils which have several significant bioactivities and rarely found in the other herbs. Polymethoxylated flavones

in *Citrus Limon* give many benefits for biological activities including anti-bacterial, anti-inflammatory, anti-fungal, anti-diabetic, anti-oxidant and anti-viral.<sup>2</sup>

The ingredients are required to be non-toxic, non-irritating and have biocompatibility, means that the produced ingredients must not harm either local or systemic biological environment.<sup>3</sup> Toxicity test must be absolutely conducted for conventional or herbal medicines before they are broadly circulated in the market to detect the toxic effect directly (in vitro) by culturing cell lines.<sup>4</sup> The observation on mesenchymal cell viability during culturing process can be used to indicate concentration effect and exposure time of a substance, including cytotoxic effect. Mesenchymal cell is multipotent cell which can be differentiated into several cells including fibroblast, due to its pureness and sensitivity when it is used as toxicity test.<sup>5</sup> Before and after contaminated by cytotoxin, mesenchymal cell viability which is shown by inactive cells percentage can be used as measured parameter, to find out cytotoxic effect of an ingredient.<sup>5-7</sup> If the ingredient is toxic on mesenchymal cell, it cannot be used as medicine; however, that ingredient can be used to control microbes population on the skin body through anti-bacterial potential and as extra oral antiseptic ingredient through anti-fungal. The purpose of antiseptic is to hinder the growth of bacteria. One of antiseptic is hand cleanser antiseptic or hand sanitizer. Hand sanitizer products are generally made from chemical substances consist of alcohol and triclosan. However, the use of alcohol as hand antiseptic has deficiencies, such as ineffectiveness use on wounded skin, inflammable, dryness and skin irritation on repetitive use.<sup>8</sup> This study was aimed to determine the ethyl acetate fraction of *Citrus limon* peel extract effect on human gingival mesenchymal cell proliferation and commensal palm polybacterial growth.

## MATERIALS AND METHODS

*Citrus limon* peel polar compounds (ethyl acetate fraction) and the screening of phytochemical *Citrus limon* peel active compounds (ethyl acetate fraction) were made in the Laboratory of Pharmacology and Phytochemical, Faculty of Pharmacy, Universitas Widya Mandala Surabaya. Extract experimental test with MTT assay by using human mesenchymal cell culture was conducted in Institute of Tropical Disease, Universitas Airlangga Surabaya. Resistance capacity experimental test was conducted in the Laboratory of Microbiology, Faculty of Dentistry, Universitas Airlangga Surabaya.

Ethyl acetate fraction toxicity of *Citrus limon* peel extract on human gingival mesenchymal cell was using MTT method (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Mesenchymal cell was cultured into line cell. In splinting line cell, Dulbeco Modification of Eagle's (DMEM) medium and fetal bovine serum (FBS) was required. Compound contents of flavonoid class that often researched was flavonoid glycosides. Compounds

generated from flavonoid glycosides include hesperidin, luteolin, and quercetin<sup>2</sup> The compounds contained in ethyl acetate fraction in this research material were then analyzed in Badan Penelitian dan Konsultasi Industri (BPKI), which contained: hesperidin 3.61%, luteolin 1.82%, quercetin 2.16%, volatile oil 1.88%, lemonene 1.36%, and citric acid 0.51%. In this research, 5 treatments were given with concentration of ethyl acetate fraction for each was 1.5625%, 2%, 2.375%, 2.75% and 3.125%. MTT method test (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is an enzymatic test to measure the active cells ability based on the mitochondrial activities from cultured cells. The measurement was performed by using spectrophotometer at the wave length of 620 nm, that resulted in numerical data optical density. The scale of optical density was the value of colour concentration from the cultured cells. The more concentrated the colour resulted, the higher the value of optical density. The percentage number of human gingival mesenchymal cells that still active after the sprinkling of *Citrus Limon* peel extract was counted by active cells percentage formula:<sup>3</sup>

$$\% \text{ active cells} = \frac{\text{OD treatment} - \text{OD media}}{\text{OD cells control} - \text{OD media}} \times 100 \%$$

Notes :

- % Active cells : percentage of total live cell after test;
- OD treatment : optical density value of mesenchymal cells for each sample after the reading result of ELISA reader test;
- OD media : optical density value of mesenchymal cells on media control;
- OD cells control : optical density value of mesenchymal cells on control cells.

It is toxic if the number of the active mesenchymal cells after the test is less than 60% or the inactive mesenchymal cells after the test is greater than 50%.<sup>3</sup> Anti-microbial effects on palm can be examined through the positive polybacteria inhibitory if the inhibition zone is in the form of clear zone surround the disc paper (Figure 1). Positive control employed in this research was phenol compounds 5%. The population of bacteria were taken from the palm where each palm must be brushed together before checked so that the bacterial content in the palms will be homogeneous, and then the palms were swabbed using

**Table 1.** The classification of inhibitory responses to the microbila growth

Diameter of the inhibition zone	Inhibitory growth responses
< 5 mm	Weak
5 – 10 mm	Moderate
10 – 20 mm	Strong
> 20 mm	Very strong

the sterile cotton soaked by 0.9% NaCL, stroked in the opposite direction with the palm line, and then the cotton was caressed and reproduced using brain heart infusions (BHI) media. The ability of the researched substance in inhibiting the growth of bacteria was determined by the scale of inhibition zone that was the area around the disc paper where the growth of polybacteria was not found. The classification of inhibitory responses to the microbial growth on anti-microbial compounds can be seen in the Table 1.

## RESULTS

The data were analyzed using active cells percentage formula<sup>3</sup> and the obtained results can be seen in Table 1. The result from the cells viability calculation (active cells percentage). Each concentration from ethyl acetate fraction within *Citrus limon* peel extract inhibit mesenchymal cells proliferation with the value of cells viability less than 60%, which means toxic. Statistically, it can be concluded that the higher the concentration of ethyl acetate fraction of *Citrus limon* peel extract, the higher the toxicity.

Based on Table 2, it can be stated that the highest mean value of optical density formazon of ethyl acetate fraction of *Citrus limon* peel extract is in concentration 1.5625%, that is 19.36109. The greatest standard deviation is in concentration 3.125%, that is 5.238175. This concludes that the higher the ethyl acetate fraction concentration of *Citrus limon* peel extract, the smaller the value of optical density formazon which means the more mesenchymal cells will die.

Table 3 shows the inhibitory value of ethyl acetate fraction of *Citrus limon* peel extract on commensal palm polybacteria, in the group 3.125% has the highest value (8.9125). Visually, it can be stated that the higher concentration, the greater the inhibitory value of commensal palm polybacteria; however, it needs to be proved statistically through normality test, homogeneity test, and comparison test intra inhibitory groups on commensal palm polybacteria towards ethyl acetate fraction of *Citrus limon* peel extract. Statistically it can be concluded that the higher the concentration of ethyl acetate fraction of *Citrus limon* peel extract, the higher the toxicity and the inhibitory of commensal palm polybacteria, which is in accordance with the hypothesis.

## DISCUSSION

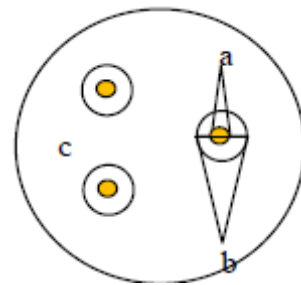
The compounds of ethyl acetate fraction of *Citrus limon* peel extract have anti-fungal ability with minimum inhibitory concentration (MIC) value is 1.5625% and minimum fungal concentration (MFC) value is 3.125%.<sup>10</sup> Those materials can be used as mouthwash ingredients. Conditions where a material can be used as medicine are non-toxic, non-irritating, and biocompatible, means the

**Table 2.** The mean value of optical density formazon and the standard deviation of ethyl acetate fraction of *Citrus limon* peel extract

Concentration of ethyl acetate fraction (%)	Mean value (%) optical density formazon ethyl acetate fraction $\pm$ Standard Deviation
1.5625%	19.36 $\pm$ 3.7
2%	17.26 $\pm$ 5.1
2.375%	14.46 $\pm$ 4.7
2.75%	12.64 $\pm$ 5.4
3.125%	17.12 $\pm$ 5.2

**Table 3.** The mean value of inhibitory and standard deviation of commensal palm polybacteria on ethyl acetate fraction of *Citrus limon* peel extract

Ethyl acetate fraction concentration	The mean of polybacteria inhibitory (mm) $\pm$ Standard Deviation
1.5625%	6.06 $\pm$ 0.0
2%	6.53 $\pm$ 0.0
2.375%	7.03 $\pm$ 0.0
2.75%	7.55 $\pm$ 0.0
3.125%	8.91 $\pm$ 0.0
Control +	8.02 $\pm$ 0.0



**Figure 1.** The diameter measurement of anti-bacterial inhibitory zone.<sup>6</sup>

Notes: a = Diameter of paper disc (6 mm); b = Diameter of the formed inhibitory zone (mm); c = The area of bacterial growth.

produced materials do not harm biological environment, either locally or systemically.<sup>1</sup> It underlies this research to find out the toxicity of ethyl acetate fraction of *Citrus Limon* peel extract on human gingival mesenchymal cells by using MTT method (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.

The national toxicity program (NTP) is stated that the recommended dose of *limonene* on rats is no more than 1650 mg/kg body weight per day, and if it is used more than the recommended dose will likely result in the increase of *renal tubuli tumor* as the activation of pro-oxidant that cause nephropathy.<sup>10</sup> *Limonene* within ethyl acetate extract in this research contains 1.36%

concentration which equals to 12240 mg/kg, exceeding the recommended dose of *limonene*. In several studies comparing cancer cells to normal cells, oxidative stress related to oncogenic transformation, metabolic activity, and reactive oxygen species (ROS) is increasing. The increased ROS in cancer cells will stimulate cell proliferation, gene mutation, and cellular sensitivity on anti-cancer agents. ROS is a reactive molecule that chemically contains oxygen, namely superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydrogen radical (OH<sup>-</sup>). ROS plays important role in pathogenesis including cancers, ageing, and the other degenerative diseases. Mitochondrial DNA codes protein which is the important component of energy formation pathway within mitochondria, namely oxidative phosphorylation (OXPHOS) that produces ROS to create adenosine-5'-triphosphate (ATP) product through mitochondrial respiratory chain and initiate the cell death.<sup>12</sup> DNA cell damage caused by toxic compounds can induce cells to initiate apoptosis process. For instance, the genome damage within nucleus, there is PARP-1 enzyme triggered the apoptosis. This enzyme has important role to keep genome integrity, even though the excessive activation can waste ATP, that can change the process of cell death into necrosis (unprogrammed cell death).<sup>13</sup>

Citric acid can cause hypercalcemia, hypotension and tachycardia if exceeding the dose of 530g/L. Citric acid functions as anti-oxidant to inhibit the oxidative stress.<sup>14</sup> Oxidative stress is caused by the balance disruption between ROS as metabolism product within normal cells which is pro-oxidant on enzymes and anti-oxidative co-factor. This imbalance condition is caused either by the excessive production of ROS H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub> and OH, or the shortage of ROS release due to oxidant defense mechanism.<sup>14</sup> The function of the anti-oxidant may increase the mesenchymal cell proliferation, as the dose of the extract in this research was 0.51% which equals to 4.131g/L.

The use of quercetyne over the recommended dose 135mg/kg can cause toxicity, while for the fraction in this study the concentration used was 19440 mg/kg, the toxic effect was as pro-oxidant that caused the chain formation of ROS seperti superoxide and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), the pro-oxidant activity will damage the fat, protein and DNA.<sup>16</sup> That condition will also damage the local organ cells and this necrosis networking process can even inhibit the mesenchymal cells proliferation<sup>13</sup>.

Hesperidin functions to inhibit the enzyme phospholipase and lipoxigenase, the histamine release from the mast cells, and lipid peroxidase forming free-radicals;<sup>17</sup> however, a bioactive material compound can cause toxicity if it is used in a high dose,<sup>18</sup> the recommended dose of hesperidin is 2 g/kg<sup>19</sup> in this extract 3.61% equals to 32.49 g/kg, therefore it is assumed to be the cause of cell death and inhibit the proliferation of mesenchymal cells.

Luteolin functions to inhibit the Nf- $\kappa$ B activity, so that pro-inflammatory cytokines can be blocked<sup>20</sup> and inhibit the inflammatory medium such as nitride oxide resulted by liposacharide (LPS), and inhibit IL-5 activity, where IL-5

is chemotactic factor that stimulates eosinophils which plays role in inflammatory condition due to allergy.<sup>21</sup> The recommended dose of luteolin is not more than 1-2 mg/kg.<sup>21</sup> In this extract the concentration was 1.82% that equals to 16.38 or 16380 mg/kg, while according to the other studies, it is stated that the toxic dose of luteolin is over 411 mg/kg i.p and 592 mg/kg i.m.<sup>21</sup> Toxic dose of luteolin emerges may be because of the concentration change of alpha-tocopherol that affects the absorption of beta-carotene that will disrupt the functions of liver, eyesight, blood vessels regeneration process in eyes, and also increase the cancer risk.<sup>22</sup>

The compounds of ethyl acetate fraction in this research, limonene, quercetin, hesperidin and luteolin inhibit the proliferation of mesenchymal cells, only citric acid can increase the proliferation of mesenchymal cells, so that the most of ethyl acetate fraction in this research is inhibiting the proliferation of mesenchymal cells rather than increasing.

Ethyl acetate fraction of *Citrus Limon* peel extract in this research was toxic even in the lowest concentration, and the higher the concentration, the higher the toxicity. Based on the evidence found in this study, ethyl acetate fraction of *Citrus limon* peel is toxic to be used as intra-oral medicines. To take the benefit of it, the further research was conducted, that is as herbal hand antiseptic materials.

The LSD results showed that significance value is (p)<0.05, it states that there was a significant difference between each inhibitory group on commensal palm polybacteria. The significance was caused by the activity of phenols compound, volatile liquid, and citric acid that inhibit the cell wall biosynthesis and increase the cytoplasmic membranes permeability and disrupt the bacterial protein synthesis if the concentration of ethyl acetate fraction of the lime peel extract is higher. Concentra of 3.125% is the maximum concentration that can kill greater number of bacteria than positive control. Phenols are used as positive control for it was the basic compound in the desinfectan test and has a larger work mechanism. Phenols can harm cell walls and cell membranes, coagulates proteins, damage ATP use damage sulphohydril from protein, and damage DNA so that it is effective to kill bacteria.<sup>23</sup>

*Citrus limon* peel contains volatile liquid that can inhibit the aero-bacterial growth, that is the anti-bacterial compounds limonene, linalool and mirsen that work by damaging the bacterial membrane cells. Anti-microbial activities may be occurred as their ability in affecting extracellular proteins and forming cell walls of the permeability of bacterial cell wall membranes.<sup>24</sup> *Limonene* is a hydrocarbon compound containing terpene cluster, a pale coloured liquid, and has a strong lime smell. *Limonene* content has anti-microbial ability by harming bacterial membranes. It damages the integrity of cytoplasmic membranes that act as selective permeability barriers, bring active transportation, and control internal cells composition.<sup>25</sup>

The damaged cytoplasmic membranes can cause cell membranes permeability lessen and the substance transportation into and out of cells becomes uncontrollable.

The substances within cells, such as enzyme organic ions, amino acids, and nutrients can come out of cells. If the enzymes come out of cells along with substances like water and nutrition, they can cause the inhibition of metabolism in which lead to the reduced ATP that required for cellular growth and reproduction, and then the bacterial cell growth become inhibited and died. Flavonoid compound can damage the cytoplasmic membranes and leak the important metabolites and activate bacterial enzyme systems. This damage may leak nucleotides and amino acids preventing the active substances to enter the cells, this causes the membrane of bacterial cell walls undergo lysis. On the damaged cytoplasmic membranes, H<sup>+</sup> ions from phenols compound and its derivative (flavonoid) will attack the polar cluster (phosphate cluster) so that the phospholipid will strand into glycerols, carboxylic acids and phosphoric acids. This causes phospholipides cannot maintain the cytoplasmic membrane form resulting in the leakage of the cytoplasmic membrane that the bacterial growth will be inhibited and eventually died.<sup>26</sup>

Volatile liquid is chemically composed of the blend of steroid compound and the other compound that acts as anti-bacterial by disrupting the formation process of membranes or cell walls that they are imperfectly formed.<sup>2</sup> The mechanism may be occurred by disrupting the constituent component of peptidoglycan within bacterial cells that causing the layer of the cell wall is not fully formed. The disruption of peptidoglycan synthesis resulted in the imperfect cell formation due to the absence of peptidoglycan and the cell wall is only involving the cell membranes. The base of bacterial cell wall is peptidoglycan layers. Peptidoglycan is constituted by N-acetyl glucosamine and N-acetyl muramic acid, bonded by 1,4-glycoside. In N-acetyl muramic acid, there are short amino acid chains: alanine, glutamic, diaminopimelic, lysine and alanine, bonded by peptide chain. The role of peptide chain is to link one chain to another.

The mechanism of bacterial wall damage occurs because the assembling process of bacterial cell wall initiated by the formation of peptide chains that form crossed bridge of peptides which integrate glycan chains from peptidoglycan to the other chains to form the perfect assembling of the cell wall. This causes the bacterial cells will easily undergo lysis, either physically or osmotically and causes the death of cell.<sup>26</sup>

Flavonoid is the largest phenols compound in the universe.<sup>9</sup> Phenols compound interacts with the bacterial cells through absorption process involving hydrogen bonds. On the low level, phenol protein complex formed in a frail bond and will immediately be apart, followed by phenols penetration into cells resulting in precipitation and protein denaturation. The protein denaturation of bacterial cell wall will cause the fragility that the cell wall is easily penetrated by the other active bacterostatics substances. If the denaturated protein is enzyme protein, the enzyme will not work causing the metabolism and nutrition absorption

process disrupted. On the high level, phenols cause protein coagulation and the lysis of membrane cells.<sup>8</sup> H<sup>+</sup> ions from phenols compound and its derivative (flavonoid) will attack the polar cluster (phosphate cluster) so that the phospholipid will break down into glycerols, carboxylic acids and phosphoric acids. This causes phospholipides cannot maintain the cytoplasmic membrane form resulting in the leakage of the cytoplasmic membrane that the bacterial growth will be retarded and eventually died.<sup>26</sup>

It can be concluded that ethyl acetate fraction within *Citrus limon* peel at concentration of 1.5625%, 2%, 2.375%, 2.75%, and 3.125% is toxic on human gingival mesenchymal cells, so it is not recommended to be developed into intra oral medicines; however, it has a very effective inhibitory power on commensal palm polybacteria, which had proven that in the lowest concentration it can inhibit the bacterial growth. The higher the concentration, the higher the inhibitory power resulted.

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