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Original article:
Ethanolic Garlic Extract (Allium sativum L) Increased Viability and Proliferation of Human Gingival Fibroblast In Vitro

Bramanti I1, Sudarso ISR2, Wahyuningsih MSH3, Wibawa T4, Karina VM5, Kusumawardani B6

Abstract:
Introduction: Garlic is a natural herb which can be used to be a good alternative treatment because cheap and safe. Garlic contains allicin which may has act antibacterial and anti-inflammatory effect. Moreover, garlic extract has a good biocompatibility and can stimulate cell growth. Does garlic extract biocompatible and can stimulate cell growth that is seen from the proliferation of human gingival fibroblasts and how its work will be studied. Objective: The aim of this study was to analyze the biocompatibility of garlic extract by observing the viability and proliferation of human gingival fibroblasts in vitro. Methods: Biocompatibility test was conducted using serial concentration of garlic extract. Human gingival fibroblasts was seeded into 96 microwell plate with density of 2x104 cells, added with the fourteen serial concentration of garlic extract, and incubated in 37°C and 5% CO2 for 24, 48 and 72 hours. MTT assay was used to analyze the viability and proliferation of human gingival fibroblasts. Data were analyzed by the Kruskal Wallis and U Mann-Whitney test. Results: The result showed that in each time of observation, there is no significant difference in viability fibroblast (p>0.05), but there are significant difference between time of observation at 24, 48, and 72 hours (p <0.05). Data showed that all concentration of garlic extract increased the viability and proliferation of human gingival fibroblasts. Conclusions: The ethanolic garlic extract has a good biocompatibility to human gingival fibroblasts culture cell and can stimulate the proliferation of human gingival fibroblast.

Keywords: ethanolic garlic extract, human gingival fibroblast, in vitro, biocompatibility, proliferation

Introduction
Dental caries is a major dental problem in deciduous teeth. The results of observations showed a extensive caries in deciduous teeth. Extensive caries area can lead to pulp exposure and it lead to pulp necrotic. Necrotic deciduous teeth should be treated to maintain teeth for mastication so that optimal nutrition for growth and development of children can be provided. The endodontic or root canal treatment has been performed to bacterial elimination and reinfection protection. This treatment will restore teeth in the jaw for longtime, and maintain their function to support periodontal tissues health and free of pain. The endodontic or root canal treatment is performed with root canal irrigation, antibacterial agent, or root canals dressing. Some dressing materials are commonly used in pediatric dentistry is calcium hydroxide and cresphene, but it is necessary to study assessed both clinically and in vitro, as well as how they affect dental periapical tissues.

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Recently, traditional medicine or “back to nature” tends to be preferred. It has developed because it does not produce any side effects. One of medicinal plants are often used as a traditional medicine is a garlic-family. Research on the effects of garlic extract in inhibiting the growth of bacteria has been studied, both against gram positive and gram negative bacteria. Allin is the active component in garlic as an antibacterial and anti-inflammatory as well as Gurwitchray’s that stimulates the growth of body cells. Garlic also contains flavonoids which can regenerate tissues, by inhibiting lipoxygenase so that decrease prostaglandins production. It proposes that garlic extract may be a potential candidate dressing materials of root canal treatment for deciduous teeth, which has a good biocompatibility to dental periapical tissues. However garlic is a herbal, we have to make sure that’s garlic must not have a detrimental effect on the biological environment, both local and systemic. Therefore, our study aimed to analyze the biocompatibility of garlic extract by observing the viability and proliferation of human gingival fibroblasts in vitro.

Material and methods
This research is a purely experimental conducted in Laboratory of Pharmacology and Laboratory of Parasitology Faculty of Medicine, University of Gadjah Mada. The tools used are microplate96 wells, incubator, culture flask, and Elisa Reader. The materials used are gingival fibroblasts, garlic extract, MTT, DMEM high glucose, ethanol 75%, 95% and 100%, Fetal Bovine Serum (FBS) 10%, 2% penicillin-streptomycin, Fungizone 0.5%, aluminum foil, 10% povidone iodine, sterile PBS, trypsin 0.25%, NaCl and DMSO.

Garlic was obtained from the Institute for Traditional Medicine Medicinal Plants Tawangmangu, Central Java, Indonesia. After that, garlic was extracted by maceration method with 96% ethanol. Gingival tissue was obtained from tooth extracted for orthodontic treatment. Previously, patients had signed the informed consent. Tooth and gingival tissue were washed in PBS, added culture medium and immediately carried to laboratory for the next experiment. Tooth and the attached gingiva directly into the conical tube which filled with PBS and then washed by shaking the tube gently (3 times). The process of fibroblast cultures cells should be done as soon as possible, no more than 6 hours. Gingival tissue was minced into small pieces approximately 1 mm³ by sterile scissors, and washed in PBS. They were put into plates, added culture medium (DMEM) containing 10% FBS, 1% penicillin-streptomycin and 1% antifungal, and incubated at 37°C, 5% CO₂ for 4 days. Gingival tissue pieces were discarded and culture medium was replaced every three days. Cells were grown to 80% confluent. Fibroblasts were observed with inverted microscope. This study was taked up fibroblasts on 3rd passage. Subsequently, fibroblasts were detached with 0.25% trypsin 1 ml and washed in PBS. Fibroblasts were transferred into a new conical tube, added 2 ml DMEM and resuspended. The harvested cells were taken up into hemocytometer and counted under an inverted microscope.

MTT cytotoxicity assays Test Procedures
Cells with a concentration of 2x10⁴ cells /100 mL were seeded into 96 micro well plate and incubated for 24 hours at a temperature of 37°C, 5% CO₂. Culture medium was removed and cells were washed with 100 mL PBS. Each well was added 100 mL culture medium and garlic extract with concentration of 0.3125-2560 ug/ml in 0.25% DMSO, and incubated at 37°C for 24, 48 and 72 hours. At the end of incubation, the culture medium was removed and cells were washed with 100 mL PBS. Each well was added 100 mL culture medium and 10 mL MTT solution, and incubated for 4 hours at 5% CO₂, 37°C. The reaction was stopped by the MTT stopper reagent (10% SDS in 0.1N HCl). Plate was wrapped with aluminum foil and incubated overnight in room temperature. Then, the optical density (absorbance) was determined by an ELISA reader at λ=595 nm. Data were converted into the percentage of cell viability (CCRC, 2009).

\[
\text{Cell viability} = \frac{\text{Absorbance tests - Absorbance media}}{\text{Cells absorbance - Absorbance media}} \times 100\%
\]

Cell viability is a percentage of the cell’s life after the test. Absorbance test is Value Optical Density (OD) after the test. Absorbance Media is OD value on average every media control. Absorbance Cells is OD value on average of control cells.

The data was analized by Kruskal Wallisand U Mann Whitney test to determine the significance of differences within the group. The significance level was 95% (p<0.05).

Results
The time observations of this study were made at 24 hours, 48 hours, and 72 hours. Absorbance of fibroblast cells in each group is presented in Table 1. The pattern of influence of garlic extract concentration against absorbance of fibroblasts cells can be seen in Figure 1 below.
Table 1. The Mean and Standard Deviation of Fibroblast Cells Absorbance Based on groups and all observation periods

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean and Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>GF + GE 2560</td>
<td>0.334 ± 0.101</td>
</tr>
<tr>
<td>GF + GE 1280</td>
<td>0.308 ± 0.001</td>
</tr>
<tr>
<td>GF + GE 640</td>
<td>0.302 ± 0.001</td>
</tr>
<tr>
<td>GF + GE 320</td>
<td>0.328 ± 0.010</td>
</tr>
<tr>
<td>GF + GE 160</td>
<td>0.316 ± 0.006</td>
</tr>
<tr>
<td>GF + GE 80</td>
<td>0.307 ± 0.176</td>
</tr>
<tr>
<td>GF + GE 40</td>
<td>0.291 ± 0.12</td>
</tr>
<tr>
<td>GF + GE 20</td>
<td>0.281 ± 0.007</td>
</tr>
<tr>
<td>GF + GE 10</td>
<td>0.279 ± 0.009</td>
</tr>
<tr>
<td>GF + GE 5</td>
<td>0.297 ± 0.005</td>
</tr>
<tr>
<td>GF + GE 2.5</td>
<td>0.314 ± 0.020</td>
</tr>
<tr>
<td>GF + GE 1.25</td>
<td>0.313 ± 0.207</td>
</tr>
<tr>
<td>GF + GE 0.625</td>
<td>0.310 ± 0.215</td>
</tr>
<tr>
<td>GF + 0.3125</td>
<td>0.316 ± 0.007</td>
</tr>
<tr>
<td>GE</td>
<td>0.291 ± 0.005</td>
</tr>
</tbody>
</table>

Description: GF = gingiva fibroblasts; GE = Garlic Extract

Figure 1. The pattern of Absorbance Fibroblasts cells at time of observation 24 hours, 48 hours and 72 hours

After getting the data of absorbance fibroblasts cells, then the data is processed to obtain data fibroblast cell viability. This study resulted that the viability of fibroblasts were the lowest in the group treated with garlic extract of 0.625 μg/ml at 72 hours (79.9%). However, the highest cell viability was in the group treated with the garlic extract concentration of 1.25 μg/ml at 48 hours (145.46%). Data showed that all concentration of garlic extract increased the viability and proliferation of human gingival fibroblasts at 24, 48, and 72 hours. Data can be seen in Table 2. The pattern of increase and decrease fibroblast cell viability as shown in Figure 2,3,4 below.

Table 2. The effect of garlic extract to viability of human gingival fibroblasts based on groups and all observation periods

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean and Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours (%)</td>
</tr>
<tr>
<td>GE 2560</td>
<td>115.94</td>
</tr>
<tr>
<td>GE 1280</td>
<td>103.04</td>
</tr>
<tr>
<td>GE 640</td>
<td>100</td>
</tr>
<tr>
<td>GE 320</td>
<td>113.043</td>
</tr>
<tr>
<td>GE 160</td>
<td>107.246</td>
</tr>
<tr>
<td>GE 80</td>
<td>102.415</td>
</tr>
<tr>
<td>GE 40</td>
<td>95.16</td>
</tr>
<tr>
<td>GE 20</td>
<td>89.85</td>
</tr>
<tr>
<td>GE 10</td>
<td>88.88</td>
</tr>
<tr>
<td>GE 5</td>
<td>97.58</td>
</tr>
<tr>
<td>GE 2.5</td>
<td>105.797</td>
</tr>
<tr>
<td>GE 1.25</td>
<td>105.797</td>
</tr>
<tr>
<td>GE 0.625</td>
<td>104.347</td>
</tr>
<tr>
<td>GE 0.3125</td>
<td>107.246</td>
</tr>
</tbody>
</table>

Description: GE = Garlic Extract

Figure 2. The effect of garlic extract to viability of human gingival fibroblasts at 24 hours
The results of Kruskal Wallis test show the value of $p < 0.005$ ($p = 0.000$). It shows there are statistically significant differences between groups. Post Hoc test by U Mann Whitney test is required to find the significance between the viability of each group and time of observation (Table 3).

**Table 3.** The Summary of U Mann-Whitney Test of Fibroblasts Viability based on groups and all observation periods

<table>
<thead>
<tr>
<th>Group</th>
<th>24-48 hours</th>
<th>24-72 hours</th>
<th>48-72 hours</th>
</tr>
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<tbody>
<tr>
<td>GE 2560</td>
<td>0.47</td>
<td>0.001*</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 1280</td>
<td>0.000*</td>
<td>0.475</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 640</td>
<td>0.000*</td>
<td>0.270</td>
<td>0.001*</td>
</tr>
<tr>
<td>GE 320</td>
<td>0.000*</td>
<td>0.090</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 160</td>
<td>0.000*</td>
<td>0.564</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 80</td>
<td>0.001*</td>
<td>0.142</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 40</td>
<td>0.007*</td>
<td>0.32</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 20</td>
<td>0.000*</td>
<td>0.549</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 10</td>
<td>0.000*</td>
<td>0.322</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 5</td>
<td>0.000*</td>
<td>0.504</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 2.5</td>
<td>0.000*</td>
<td>0.78</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 1.25</td>
<td>0.000*</td>
<td>0.130</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 0.625</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>GE 0.3125</td>
<td>0.000*</td>
<td>0.003*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Description: GE = extract of garlic
(*) = significant ($p<0.050$)

The summary of U Mann-Whitney test (Table 3) resulted that there are significant difference between time of observation at 24, 48, and 72 hours ($p < 0.05$). The most of group that had a significant difference in 24 hours time of observation compared to 48 hours and 48 hours compared to 72 hours. The comparison between 24 hours and 72 hours mostly showed no significant difference.

**Discussion**

This study analized the effect of garlic extract to viability of human gingival fibroblasts *in vitro*, and resulted that human gingival fibroblasts have survived after treated with garlic extracts (Table 1). The highest viability of human gingival fibroblasts was determined in group treated with garlic extract 1.25 μg/ml at 48 hours (145.15%), and the lowest viability of human gingival fibroblasts was determined in group treated with garlic extract 0.625 μg/ml at 72 hours (79.90%). The concentration was
highest viability of human gingival fibroblast, may be considerable as a root canal dressing materials. Decreased viability of human gingival fibroblasts for 72 hours incubation may be caused by caused by the growth of cells entering the stationary phase. Nevertheless, Emilda et al. (2014) suggested that garlic extract which exposed in a longer time (120 hours) did not affect cell viability as the shorter exposures.¹⁸ These garlic extract did not interfere viability of human gingival fibroblasts because cell viability of all groups gained more than 50%. It suggested that the ethanolic garlic extract has a good biocompatibility to human gingival fibroblasts. This was accordance with previous study that garlic extract with concentration 50% to 100% did not have cytotoxic effect to fibroblast cell culture BHK-21. However, Ozan et al. (2013) suggested that garlic had mild cytotoxic effect to fibroblasts in vitro, but when compared with chlorhexidine as a positive control, chlorhexidine still more toxic.¹⁷ The garlic extract has no toxic effects on the proliferation of fibroblasts both the lowest and highest concentration. The results of the regression analysis on the entire time observations have shown no correlation between the magnitude of the high concentration of cell viability, even though dilution with the lowest concentration of 0.3125 microg / ml still showed a high viability (above 100%). It was showed that the magnitude of cell viability was not affected by the amount of concentration. It is thought to be influenced by the active compounds of garlic, such as diallyl disulfide, flavonoid, and allicin. Diallyl disulfide is able to break down proteins in the damaged cells so the protein is easily digested by the body. Diallyl disulfide also can increase phagocytic activity and stimulate the activity of cells involved in the immune response.¹⁹ Histological study showed that topical allicin on second-intention wound healing can decrease density of inflammatory cells but increase density of fibroblasts and fibrosit at the 7th day after treatment the dog back skin.²⁰ Allicin has bioactivity to penetrate the membrane phospholipids.²¹ Besides, the ability of allicin as an anti-inflammatory agent evidenced by a negative feedback of allicin to reduce and suppress spontaneously TNF-α production, which stimulates the secretion of IL-1, IL-6, IL-8 and leukotriene with decrease levels of mRNA and inhibits activation of NFk-B.²² Allicin is also capable to stimulate the production of IL-10 in which will suppress the production of TNF-α and IL-2, IL-6, IL-12 by T cells and ICAM. ICAM play a role in the regulation of inflammatory cell responses and then suppress the inflammatory process in the repair phase marked by increasing fibroblasts proliferation.²³ At this point, the activity of allicin to be as an anti-inflammatory and immunomodulatory agents.²⁴ In addition, flavonoids in garlic has properties to regenerate tissues and inhibit inflammation response by inhibiting lipoxygenase cycle that produce prostaglandin.²⁵ Flavonoids also have anti-inflammatory and antioxidant properties.²⁶ Flavonoids are able to regulate cell function by stimulating TGF-β (transforming growth factor-β) production which enhance the migration and proliferation of fibroblasts in the wound area, and induces VEGF (Vascular Endothelial Growth Factor) that plays a role in new blood vessels formation.²⁶,²⁷ The increasing of fibroblasts proliferation caused by the ability of garlic extract in increasing the cellular activity of cells through the induction of various growth factors that’s contained in garlic. It has been proven that garlic have many potential properties such as antibacterial, anti-inflammatory, and stimulates the cell growth. Therefore, the application of garlic extract in the oral cavity can be considered in Dentistry.

**Conclusion**

The conclusion of this research was the ethanolic garlic extract has a good biocompatibility to human gingival fibroblasts culture cell and can stimulate cell growth that is seen from the proliferation of human gingival fibroblasts cell.

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Reference


