Original article:

Ethanolic Garlic Extract (Allium sativumL) Increased Viability and Proliferation of Human Gingival Fibroblast In Vitro

Bramanti I[1], Sudarso ISR[2], Wahyuningsih MSH[3], Wibawa T[4], Karina VM[5], Kusumawardani B[6]

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 $2x10^3$ cells, added with the fourteen serial concentration of garlic extract, and incubated in 37° C and 5% CO_2 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

Bangladesh Journal of Medical Science Vol. 17 No. 04 October'18. Page : 556-561
DOI: http://dx.doi.org/10.3329/bjms.v17i4.38315

Introduction

Dental caries is a major dental problem in deciduous teeth. The results of observations showed a extensive caries in deciduous teeth[1]. Extensive caries area can lead to pulp exposure and it lead to pulp necrotic.[2,3] Necrotic deciduous teeth should be treated to maintain teeth for mastication so that optimal nutrition for growth and development of children can be provided.[4] 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.[5,6] 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 cresophene, but it is necessary to study assessed both clinically and in vitro, as well as how they affect dental periapical tissues.[7]

1. I Bramanti, Students of Post Graduate Programme, Faculty of Dentistry UGM, Indonesia
2. ISR Sudarso, Lecturer of Pediatric Dentistry Department, Faculty of Dentistry UGM, Indonesia
3. MSH Wahyuningsih, Lecturer of Pharmacology and Therapy Department, Faculty of Medicine,UGM, Indonesia
4. T Wibawa, Lecturer of Microbiology Department, Faculty of Medicine,UGM, Indonesia
5. V. M. Karina, Student of Speciality Education Programme, Faculty of Dentistry,UGM, Indonesia
6. B. Kusumawardani, Lecturer of Biomedical department, Faculty of Dentistry, Jember University, Indonesia

Correspondence to: I Bramanti, Students of Post GraduateProgramme, Faculty of Dentistry UGM, Indonesia. Jl. Denta 1, Sekip Utara, Yogyakarta,55281, Indonesia, e-mail: bramantikg@gmail.com,

Recently, traditional medicine or "back to nature" tends to be preferred. It has developed because it does not produce any side effects.[8] One of medicinal plants are often used as a traditional medicine is a garlic-family.[9] Research on the effects of garlic extract in inhibiting the growth of bacteria has been studied, both against gram positive and gram negative bacteria.[10,11,12,13,14] Allicin is the active component in garlic as an antibacterial and anti-inflammatoryas well as Gurwitchray's that stimulates the growth of body cells.[15] Garlic also contains flavonoids which can regenerate tissues, by inhibiting lipooxigenase so that decrease prostaglandins production.[16]

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.[17]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 toot extracted for orthodontic treatment. Previously, patients had signed the informed consent. Toot hand 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 mm^3 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 37ºC,5% CO_2 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 deattached 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 $2x10^3$ cells /100 mL were seeded into 96 micro well plate and incubated for 24 hours at a temperature of 37ºC, 5% CO_2. Culture medium was removed and cells were washed with 100 mL PBS. Each well was added 100 mL culture media and garlic extract wiith concentration of 0.3125-2560 ug/ml in 0,25% DMSO, and incubated at 37ºCat 37ºC, 5% $CO_2$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% CO_2, 37ºC. 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

Group	Mean and Standard Deviation		
	24 hours	48 hours	72 hours
GF + GE 2560	0.334 ± 0.101	0.364 ± 0.008	0.287 ± 0.007
GF + GE 1280	0.308 ± 0.001	0.367 ± 0.007	0.298 ± 0.127
GF + GE 640	0.302 ± 0.001	0.367 ± 0.229	0.318 ± 0.122
GF + GE 320	0.328 ± 0.010	0.423 ± 0.006	0.353 ± 0.008
GF + GE 160	0.316 ± 0.006	0.390 ± 0.213	0.308 ± 0.006
GF + GE 80	0.307 ± 0.176	0.357 ± 0.113	0.286 ± 0.209
GF + GE 40	0.291 ± 0.12	0.331 ± 0.151	0.260 ± 0.601
GF + GE 20	0.281 ± 0.007	0.398 ± 0.180	0.272 ± 0.106
GF + GE 10	0.279 ± 0.009	0.279 ± 0.009	0.421 ± 0.003
GF + GE 5	0.297 ± 0.005	0.417 ± 0.105	0.287 ± 0.189
GF + GE 2.5	0.314 ± 0.020	0.441 ± 0.144	0.288 ± 0.172
GF + GE 1.25	0.313 ± 0.207	0.442 ± 0.005	0.335 ± 0.217
GF + GE 0,625	0.310 ± 0.215	0.412 ± 0.162	0.255 ± 0.317
GF + 0.3125 GE	0.316 ± 0.007	0.390 ± 0.006	0.272 ± 0.418
GF + Media	0.291 ± 0.005	0.316 ± 0.28	0.345 ± 0.88

Description: GF = gingiva fibroblasts; GE = Garlic Extract

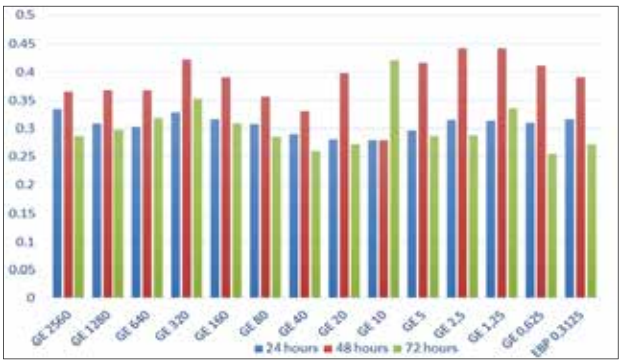

Figure 1. The pattern of Absorbance Fibroblasts cells at time of observation 24 hours, 48 hours and 72 hours

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

Group	Mean and Standard Deviation		
	24 hours (%)	48 hours (%)	72 hours (%)
GE 2560	115.94	112.127	94.52
GE 1280	103.04	113.4	99.54
GE 640	100	114	108.68
GE 320	113.043	137.23	124.66
GE 160	107.246	123.33	104.11
GE 80	102.415	109.14	94.06
GE 40	95.16	98	82.19
GE 20	89.85	126.59	87.67
GE 10	88.88	136.24	84.47
GE 5	97.58	134.68	94.52
GE 2.5	105.797	144.89	95.30
GE 1.25	105.797	**145.46**	116.75
GE 0,625	104.347	132.83	**79.90**
GE 0.3125	107.246	123.33	88.13

Description: GE = Garlic Extract

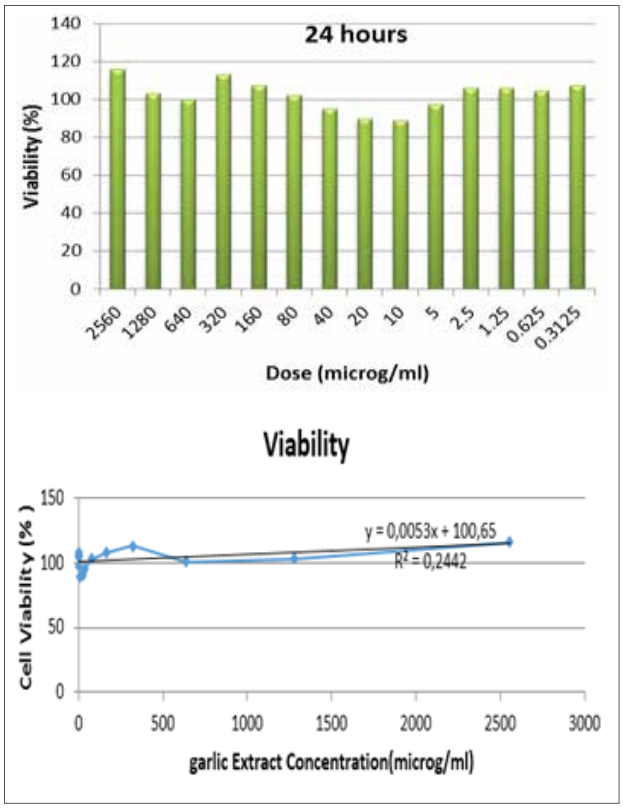

Figure 2.The effect of garlic extract to viability of human gingival fibroblasts at 24 hours

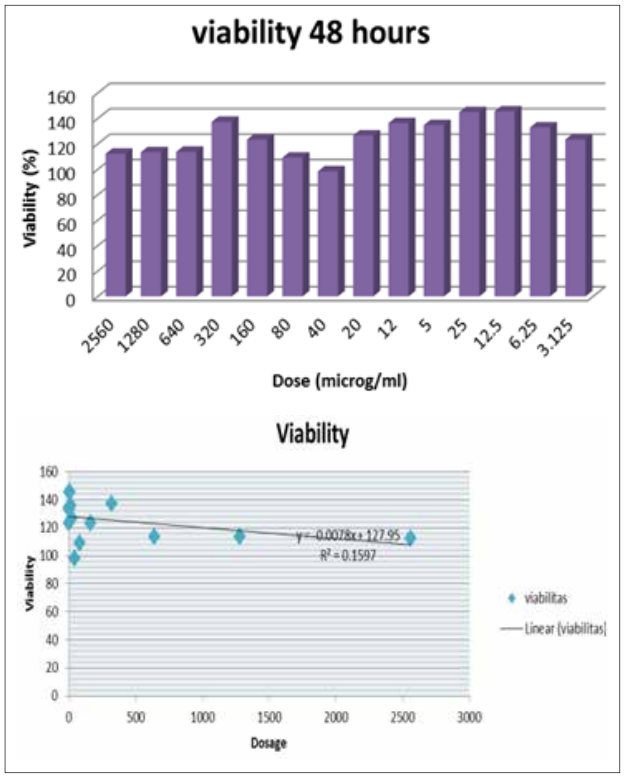

Figure 3.The effect of garlic extract to viability of human gingival fibroblasts at 48 hours

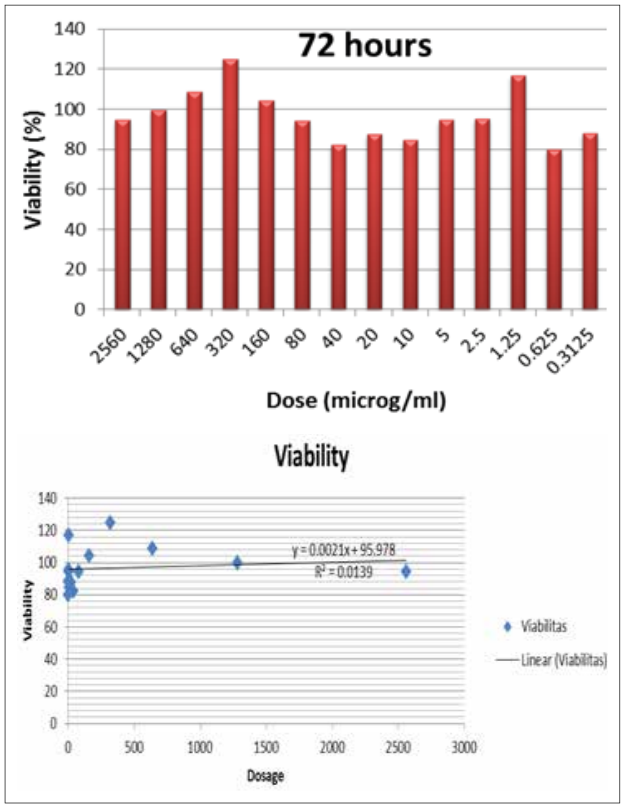

Figure 4.The effect of garlic extract to viability of human gingival fibroblasts at 72 hours

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

	Time observation		
Group	24-48 hours	24-72 hours	48-72 hours
GE 2560	0.47	**0,001***	**0,000***
GE 1280	**0,000***	0.475	**0,000***
GE 640	**0,000***	0,270	**0,001***
GE 320	**0,000***	.090	**0,000***
GE 160	**0,000***	0,564	**0,000***
GE 80	**0,001***	0,142	**0,000***
GE 40	**0.007***	0.32	**0,000***
GE 20	**0,000***	.549	**0,000***
GE 10	**0,000***	0.322	**0,000***
GE 5	**0,000***	0.504	**0,000***
GE 2.5	**0,000***	0.78	**0,000***
GE 1.25	**0,000***	.130	**0,000***
GE 0,625	**0,000***	**0,000***	**0,000***
GE 0.3125	**0,000***	**0,003***	**0,000***

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 concentrationin the

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 the 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.[18]However, Ozan *et al.* (2013) suggested that garlic extract 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 fibroblats 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 lipooxygenase cycle that produce prostaglandin.[18] Flavonoids also have anti-inflammatory and antioxidant properties.[25] 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.

Acknowledgements

The author would like to acknowledge Faculty of Dentistry UGM on the research funded and also many thanks to all those who have helped the process of this study were couldn't mention one by one.

Reference

1. Warren, J.J., Levy, S.M., and Kanellis, M.J., Dental Caries in the Primary Dentition: Assessing Prevalence of Cavitated and Noncavitated Lesions, *Journal of Public Health Dentistry*. Vol. **62,** No. 2, Spring 2002
2. Orhan, Al., Oz FT., Ozcelik, B., and Orhan, K.. A Clinical and microbiological comparative study of deep carious lesion treatment in deciduous and young permanent molars. *Clin Oral Investig*. 2008;**12**(4) :369-78.
3. Whitworth, J.M. and Nunn, J.H.Paediatric endodontic in Paediatric Dentistry, Richard R. Welbury. Oxford: Oxford University Press;1997.141-144.
4. Kennedy, D.B., Konservasi Gigi Anak (Paediatric Operative Dentistry). Ed.3. Jakarta: EGC; 1992. 152-165.
5. Grossman, L.I., Olliet, S., and Del Rio, C.E., Endodontic Practice, 11thed,. Philadelphia: Lea and Febiger;1998. 242-270.
6. Harty, F.J., Endodonti Klinis (Harty's Endodontics in Clinical Practice.), ed.3, KOTA:Hipokrates; 1993. 159-183.
7. Kalchinov, V., Dimitrov, S.I., and Belsheva, M., In Vitro Study of Bactericidal Effect of Antimicrobial Agents Used in Modern Endodontics. *JIMAB.* 2009; **15:** 79-83.
8. Benzie IFF, and Wachtel-Galor S., Herbal Medicine: Biomolecular and Clinical Aspects. 2nd edition. CRC Press/Taylor & Francis; 2011.
9. Bongiorno, P.B., Fratellone, P.M., LoGiudice, P. Potential Health Benefits of Garlic (Allium Sativum): A Narrative Review. *Journal of Complementary and Integrative Medicine*, Vol. **5** [2008], Iss. 1, Art. 1
10. Nejad ASM, Shabani S, Bayat M, Hosseini SE. Antibacterial Effect of Garlic Aqueous Extract on Staphylococcus aureus in Hamburger. *Jundishapur Journal of Microbiology* 2014;**7.** doi:10.5812/jjm.13134.
11. Dhinahar, S. and Lakshmi, T., 2011, Role of Botanical of Antimicrobial Agent in Management of dental Infections- A Review, *International Journal of Pharma and Bio Sciences.***2** (4) :B690-B704
12. Velliyagounder, K., Ganeshnarayan,K., Velusamy, S.K., and Fine, D.H., In Vitro Efficacy of Diallyl Sulfides against the Periodonto pathogen *Aggregatibacter actinomycetemcomitans*. *Journals, ASM.org.* 2012.p. 2397–2407
13. Kemper, K.J.,*Garlic (Allium sativum)*, The Longwood Herbal Task Force (http://www.mcp.edu/herbal/default.htm) and The Center for Holistic Pediatric Education and Research (http://www.childrenshospital.org/holistic/),2000, p.1-48
14. Palombo, E.A.,Traditional Medicinal Plant Extracts and Natural Products with Activity against Oral Bacteria: Potential Application in the Prevention and Treatment of Oral Diseases (*Review Article*), *Evidence-Based Complementary and Alternative Medicine*. Hindawi Publishing Corporation,Vol. 2011, Article ID 680354, p.1-15.doi:10.1093/ecam/nep067
15. Zaenal, As'ad,S., Hadju, V., Bukhari, A., Role of Topical Cream Extract Garlic (Allium sativum) in Wound Healing Fibroblasts Judging from the Strain Wistar Rats with Acute Injury Models, *International Journal of Sciences: Basic and Applied Research (IJSBAR*), 2016, Vol. **26** (3) : 35-47
16. Khayyal MT, el-Ghazaly MA, el-Khatib AS. Mechanism involved in the antiinflamatory effects of propolis extract. *Drugs ExpClin Res* 1993; **19**(5): 197-203.
17. Ozan, F. Ozdem,M., Ozan,U., Sencimen, M., danPolat, Z.A.Evaluation of Cytotoxic effect of Garlic on human gingival fibroblast : A preliminary study. *Gulhane Med J.* 2013**; 55:** 276-280.
18. Emilda, Y., Budipramana, E., and Kuntari, S., Garlic *Allium Sativum* extract toxicity test on fibroblast cell culture. 2014. *Dental Journal.*Vol.**47** (4): 215-219
19. Arreola, R., Quintero-Fabián,S. López-Roa,R.I., Flores-Gutiérrez,E.O.,Reyes-Grajeda,J.P.,Carrera-Quintanar,L. and Ortuño-Sahagún, D., Immunomodulation and Anti-Inflammatory Effects of Garlic Compounds,*Journal of Immunology Research*, 2015, p.1-13
20. Sardari, K., Mirshasi, A., Maleki, M., Aslani, M.R., and Najar Barjasteh, M., Effect of topical allicin on second-intention wound healing in dogs (Histological Aspects), *Comp.Clin.Pathol.*2006.Vol.**15:**98-102
21. Mirron, T., Robinkov, A., Wilchek, M., Mirelman, D., and Weiner, L., The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity, 2000. *Biochim. Biophys.Acta*. 1463 : 20-30
22. Ok Ban, J., Oh, J.H., Kim, T.M., Kim, D.J., Jeong, H.S., Han, S.B., and Hong, J.T., Anti-inflammatory and arthritic effects of Thiacremonone, a novel sulfurcompound isolated from garlic via inhibition of NF-$_k$B. *Arthritis Res Ther*. 2009.**11**: R 145 : 1,2,11
23. Jiang, X.W., Hu, J., and Mian, F.I., A new therapeutic candidate for oral apthousulcer :*Allicin*. *Medical Hypotheses.* 2008. 71: 897-899
24. Aukhil, I., Biology of wound healing. 2000. *Periodontology 2000.***22** : 44-50
25. Intekhab, J. and Aslam, M., Isolation of A Flavonoid From The Roots of Citrus Sinensis, 2009,*Malaysian Journal of Pharmaceutical Sciences*, **7**(1): 1-8.
26. Sabir, A., Tabbu, C. R., Agustiono, P., Sosroseno, W., Histological Analysis of Rat Dental Pulp Tissue Capped with Propolis, 2005.*Journal of Oral Science*, **47**(3): 135-138
27. Kumar, V., Abbas, A.K., Fausto, N., *Pathologic Basic of Disease*. 2005. Elsevier Saunders. Philadelphia. p. 49-59, 95-113