



EVALUATION OF ANTIBIOFILM EFFICACY OF DIFFERENT NANO-HERBAL MEDICATIONS ON MULTISPECIES BACTERIAL INFECTED ROOT CANALS: AN INVITRO STUDY

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ABSTRACT

Background: The main objective of the present study was to evaluate antibiofilm efficacy of different nanoherbal medications on multispecies bacterial infected root canals.

Methods: Sixty freshly extracted permanent premolar with vertucci class 1 were collected. According to the type of the herbal applied the samples will be divided into three groups: Group (1) medicated with nano punica granatum herbal extract. Group (2) medicated with nano garlic herbal extract. Group (3) medicated with calcium hydroxide. Group (4) medicated without. The bacteria *e. fecalis* and staph epidermids were brought according to American tissue culture collection. The teeth samples were sectioned below the cementoenamel junction.. All teeth were then vertically sectioned into two halves. Application of nanoherbals and calcium hydroxide to the samples and incubated for one week. The samples stained and examined under confocal microscope.

Results: Negative control showed statistically significantly HIGHER % of live bacteria than the other three groups. Pomegranate showed statistically significantly HIGHER % of dead bacteria than other groups.

Conclusion: Pomegranate had a significant antibacterial effect on common oral bacteria. Garlic has great potential in the treatment of many microbial diseases. Nanoherbals showed better antibacterial effect than herbal extracts.

INTRODUCTION

Primary endodontic infection is caused by microorganisms colonizing the necrotic pulp tissue⁽¹⁾. Endodontic infections are polymicrobial in nature dominated by obligate anaerobic bacteria⁽²⁾. Donald and Costerton⁽³⁾ concluded that microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other, embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription are called biofilms. Biofilm helps in resisting the destruction

of the bacteria by making them thousand times more resistant to phagocytosis, antibodies and antimicrobial agents. This is attributed to the protective barrier provided by the extracellular matrix. ⁽⁴⁾The mature biofilm at the end of 6 weeks shows signs of mineralization ⁽⁵⁾.

In root canal infections bacteriological studies using various techniques have identified different multiple bacterial biofilms in the root canal after root canal treatment, this indicates the resilience and ability to survive under adverse conditions⁽⁶⁻⁹⁾. the choice of instrumentation, irrigating solutions and medications that permit bacterial neutralization

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and toxin inactivation without negative interference with the healing process is fundamental to the success of the treatment⁽¹⁰⁾. The constant increase in antibiotic resistant strains and side effects caused by synthetic drugs has also led to the search for herbal alternatives⁽¹¹⁾. Some products represents attention due to their beneficial effects confirmed by research and because of the current worldwide “back to nature” trend⁽¹²⁾.

Some of these natural products are herbals such as garlic and pomegranate. Allicin is one of the active ingredients of freshly crushed garlic homogenates, has various biological properties, including antibacterial, antifungal, antiparasitic, antiviral, anti-inflammatory and immunomodulatory activities^(13, 14). Bachrach et al.,⁽¹⁵⁾ tested the antimicrobial activity of garlic allicin on oral pathogens associated with dental caries and periodontitis. Allicin was found effective against all the tested bacteria. Pomegranates (*Punica granatum* L.) which have a long history of antibacterial use dating back to biblical times⁽¹⁶⁾. Egyptians used pomegranates to treat a number of different infections⁽¹⁶⁾. It was utilized as a traditional remedy for thousands of years under the Ayurvedic system of medicine, with extracts from the rind of the fruit and bark of the tree being effective against diarrhea and dysentery⁽¹⁷⁾. Abdollahzadeh et al.,⁽¹⁸⁾ evaluated the effect of methanolic extract of *Punica granatum* peel (MEPGP) against oral organisms. This study suggests that MEPGP might be used as an antibacterial agent in controlling oral infections. Carbonell-Barrachina A.⁽¹⁹⁾ reported different parts of the pomegranate fruit, especially the peel, may act as potential antimicrobial agents and thus might be proposed as a safe natural alternative to synthetic antimicrobial agents.

Calcium hydroxide is presently considered the intracanal medication of choice and has been used in treating perforations, resorption, weeping canals, conservative management of apical lesions, incompletely formed roots, root fracture etc⁽¹²⁾.

Nanotechnology is a rapidly growing interdisciplinary area of science and technology that integrates materials science and biology⁽²⁰⁾. There is an immense interest in biomedicine applications of nanoparticles owing to their size and structural similarity with biological molecules. So nanosize from *punica granatum* and garlic extracts was prepared then their antibacterial effect was investigated as a trial to utilize a novel antibiofilm agents.

SUBJECTS AND METHODS

95 extracted permanent lower premolar single rooted teeth with vertucci class 1 were collected from outpatient clinic of Oral Surgery Department, Faculty of Dental Medicine, Boys, Cairo, Al Azhar University and radiographically examined for selection. Teeth will be stored in distilled water until tested. After that, the samples were cleaned from superficial debris, calculus, tissue tags and stored in normal saline. According to the type of the herbal applied the samples will be divided into three groups: Group (1) medicated with nano *punica granatum* herbal extract medicament (gel/cream). Group (2) medicated with nano garlic herbal extract medicament (gel/cream). Group (3) medicated with calcium hydroxide as a control group. Group (4) medicated without as a negative control group. The bacteria *e. fecalis* and staph epidermids were brought according to American tissue culture collection from fungi center, Cairo, AL-Azhar University then tested for viability then stored in the refrigerator till testing.

Pomegranates were collected from the local market of Cairo then washed then peeled and shade dried and subsequently powdered. Two hundred grams of powder peel was soaked in three different solvents (Ethanol, ethyl acetate and methanol) at room temperature for 72 h in rotatory shaker (120 rpm). The powder and solvent were taken in the ratio of 1:3. The filtrates were further concentrated under reduced pressure at 40 °C and stored in a refrigerator at 2–8 °C for use in subsequent experiments.

Then the anti-bacterial effect of the nano herbals were compared to the original herbal extract and it was found that nano herbal extracts has stronger antibacterial effect than original herbal extract when its used with the same concentration of 50 micrograms per milliliter. The teeth samples were sectioned below the cemento-enamel junction with a diamond disc to obtain a standardized tooth length of 8 mm for uniform sample. The root canals were then cleaned and shaped using the crown down technique and protaperrotary instruments to an apical size of F3. Two milliliters of 5.25% NaOCl was used between each instrument during the cleaning and shaping procedures. All teeth were then vertically sectioned along the mid-sagittal plane into two halves. The concave tooth surface was minimally grounded to achieve flat surface. Then the samples were cut by the isomet saw. Confirmation of the formation of the bacterial biofilm by E.M. Application of nanoherbals and calcium hydroxide to the samples till its fully covered and incubated at the incubator for one week after one week the samples were removed from the gel and calcium hydroxide, washed by deionized water, and stained by viable stains iodid promide and left for 15 min then it examined under confocal microscope.

RESULTS

I. Comparison between all test groups regarding the absolute bacterial count:

1. Dead bacterial cell count:

When comparing between the dead bacterial cell count among all the groups, it was found that group (A) (pomegranate) showed the highest significant absolute mean score (7.32 ± 0.12) followed by group (B) (Garlic) (7.24 ± 0.08) with no significant difference between them, while group (D) (negative control) group showed the least significant absolute mean score (5.89 ± 0.19).

2. Alive bacterial cell count:

When comparing between alive bacterial cell count among all the groups, it was found that group (D) (negative control group) showed the highest significant absolute mean score (7.44 ± 0.12) while (A)(pomegranate) showed the least significant absolute mean score (7.00 ± 0.16) followed by group (B) (garlic) (7.03 ± 0.08) with no significant difference between them.

II. Comparison between all test groups regarding the percentage change of bacterial count:

1. Dead bacteria:

When comparing between the percentage change in dead bacterial cells among all groups it was found that group (A) (pomegranate) showed the highest significant percentage change score ($67.47\% \pm 5.10$) while group (D)(negative control group) showed the least significant percentage change score ($3.00\% \pm 0.85$). Moreover, it was found that there was a significant difference in percentage change of dead bacterial cells between group (B) (garlic) ($61.60\% \pm 5.80$) and group (C) (calcium hydroxide) ($29.80\% \pm 7.07$).

2. Alive bacteria:

When comparing between the percentage change of alive bacterial cells among all groups, it was found that group (D) (negative control group) showing the highest significant percentage change score among all the groups ($97.00\% \pm 0.85$) while group (A) (pomegranate) showed the least percentage change ($32.53\% \pm 5.10$) among all the groups followed by group (B) (garlic) ($38.40\% \pm 5.80$) then group (C) (calcium hydroxide) ($70.20\% \pm 7.07$) with significant difference between all of them.

TABLE (1): Comparison between all test groups regarding the percentage change and absolute bacterial count:

Variables	Bacterial percentage			Absolute bacterial count		
	Dead bacteria	Alive bacteria	p-value	Dead bacteria	Alive bacteria	p-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Negative control	3.00 ± 0.85	97.00±0.85	≤0.001*	5.89±0.19	7.44±0.12	≤0.001*
Calcium hydroxide	29.80 ± 7.07	70.20±7.07	≤0.001*	6.83±0.23	7.22±0.11	≤0.001*
Garlic	61.60± 5.80	38.40±5.80	≤0.001*	7.24±0.08	7.03±0.08	≤0.001*
Pomegranate	67.47± 5.10	32.53±5.10	≤0.001*	7.32±0.12	7.00±0.16	≤0.001*
p-value	≤0.001*	≤0.001*		≤0.001*	≤0.001*	

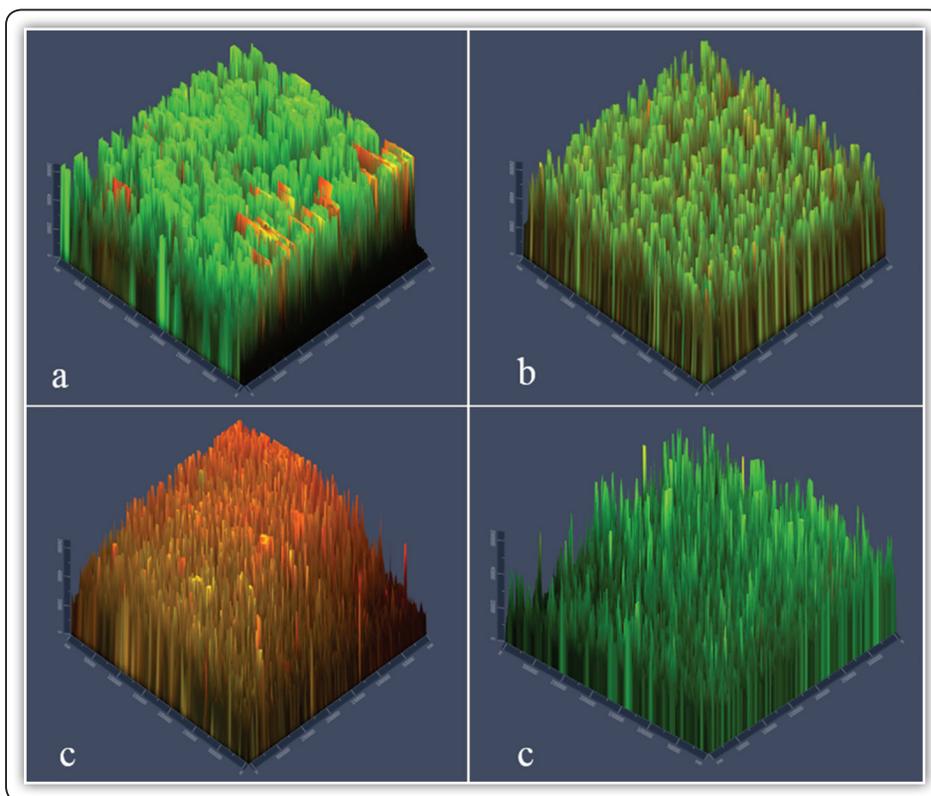


FIG (1) 3D confocal microscope showing Antibacterial effect of calcium hydroxide, garlic, nano pomegranate, and negative control group on multispecies bacterial biofilm

DISCUSSION

Microorganisms play a major role in infecting root canal system. Endodontic infections occur and progress when the root canal system gets exposed to the oral environment by one reason or the other and simultaneously when there is fall in the body's immune response⁽²¹⁾.

Many intracanal medicaments were introduced to be used to disinfect root canal such as calcium hydroxide. The calcium hydroxide is the most widely used for disinfection of the root canal as it exerts antibacterial effects in the root canal system as long as a high pH is maintained.

Many herbals have been used as antibacterial agents such as pomegranate and garlic. Pomegranate was used as medicament as its high in hydrolyzable tannins (punicalins and punicalagins), ellagic acid, a component of ellagitannins, and gallic acid, a component of gallotannins. Mass spectrometry data shows that pomegranate contains oligomeric ellagitannin with a degree of polymerization of up to 5 core glucose units. These molecules may be the most potent antibacterial compounds in pomegranate. However, other compounds also have activity and may contribute synergistically as mixtures to bring about the effects, including anthocyanins (pelargonidin-3-galactose and cyanidin-3-glucose) and flavonols (quercetin and myricetin)⁽²²⁾.

Garlic (*Allium sativum*) also used as medicament as the antimicrobial activity derived from alliums was identified nearly 70 years ago and subsequently the chemical structure of allicin (2-propenylthiosulphinate), and its properties was evaluated over a series of papers by researchers at The Winthrop Chemical Company. Nanotechnology has become one of the most important and exciting fields in science. It shows great promise for providing us in the near future with many breakthroughs that will change the direction of technological advances in a wide range of applications. Research and development of this futuristic field is directed towards creating improved material, devices and systems that expressed the new properties⁽²³⁾.

So this study was focused on the use of nanotechnology for "*phytotherapy*" or treatment of various diseases by herbal medicines/drugs, where current and emerging nanotechnologies could enable entirely novel classes of therapeutics. The teeth included in the study were selected as single rooted, mature apices with degree of curvature from 0 to 10 degrees and were sectioned below the cement enamel junction to obtain a standardized root length of 14 mm for standardization purposes. .. Also isomet saw was used for samples sectioning and cutting other than disk and low speed hand piece

to obtain more precise cutting without destruction of the samples and to facilitate preparation of the teeth.. 6% citric acid was used to remove smear layer and to keep dentinal tubules patent to receive the bacterial biofilm. The choosing of ethyl acetate herbal extract was done after making extraction with three different solvents (ethyl acetate, methanol and ethanol) to obtain maximum antibacterial effect.

Multispecies biofilm was prepared to resemble in vivo intra canal bacterial biofilm. Antagonism test was done to assure that each bacterial has no effect on the other. Sterile air laminar flow cabinet was used to assure performing all procedures in sterile condition. Many methods have been used for preparation of nanoparticles, in our study nanoherbals were prepared by nanoemulsification using sonication⁽²⁴⁾ as it doesn't require drug delivery system as the nanoherbal is going to be applied directly on the bacterial biofilm and ability to control the nanoparticle size by controlling the intensity of the ultrasonic waves in addition to its availability⁽²⁵⁾.

Methylcellulose base gel was used as base gel for the nanoherbal as it doesn't have any bacterial effect on and its availability⁽²⁶⁾. Many methods of evaluation were used to detect bacterial biofilm and to evaluate the effect of different antimicrobial agents as Scanning electron microscope (SEM), transmission (SEM) and recently confocal microscope was introduced in such field⁽²⁷⁾.

Confocal microscopy is a powerful tool that creates sharp images of a sample that would otherwise appear blurred when viewed under a conventional microscope, as well as the images obtained by confocal microscope have less haze and better contrast than that of a conventional microscope and represents a thin cross-section of the sample⁽²⁸⁾. Finally it was reported that confocal microscope is capable of differentiation between dead and alive bacterial cells⁽²⁹⁾.

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