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ORIGINAL ARTICLE

In vitro Antimicrobial Effect of Egyptian Propolis Pastes on Microorganisms in Necrotic Primary Molars: A Comparative Study

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ABSTRACT

Background: *Primary teeth are very important to be restored in children, so using natural antimicrobial products is nessecary to protect children from complications of chemical drugs.* ***Objectives:*** *The aim of this study was to evaluate the antimicrobial effect of three intracanal propolis containg pastes against polymicrobial cultures collected from thirty five primary molars of 4-8-years old children of both sexes having necrotic root canals.* ***Methodology:*** *Sterile paper point were introduced in the root canal of the necrotic teeth, placed in a transport medium containing brain heart infusion (BHI), followed by identification of the isolated organisms. Agar-well diffusion technique was used to determine the antimicrobial activity of the following pastes; Metapex mixed with propolis (paste 1), zinc oxide eugenol mixed with propolis (paste 2) and ethanol extract of propolis (EEP) paste (paste 3).* ***Results:*** *The predominant organisms isolated were Streptococcus mutans (51.4%), Enterococcus species (34.2%), followed by Porphyromonas gingivalis (28.5%), Fusobacterium nucleatum (25.6%) and Prevotella nigrescens (8.5%). There was a statistically significant difference between inhibition zone diameters of the different paste types (P-value <0.001). Pair-wise comparison between the paste types revealed that paste 2 showed statistically significantly highest mean inhibition zone diameter values. There was no statistically significant difference between paste 1and paste 3; both showed a statistically significant lowest mean inhibition zone diameter values.* ***Conclusion****: The addition of propolis to Metapex and zinc oxide eugenol could potentiate the antimicrobial effect of each other.*

INTRODUCTION

Primary teeth are important in the development of jaws, chewing, and food preparation in children so losing them might cause phonetic disturbances and harmful oral habits [1]. Treatment of pulp necrosis, especially in primary teeth, is not easy due to anatomical and physiological complexity[2]. Bacterial species such as *Enterococcus faecalis* is the most common organism affecting Turkish children [3].

Alternative medicine adds a lot to modern medical practice[4]. The role of natural products is very important in pediatric dentistry, as nearly all problems related to oro-dental region requires either indirect contact to the soft and hard tissue or direct contact of material and medicaments with oral mucosa. Thus, complications and side effects due to the use of man-made drugs have directed the use of natural products for pharmaco-therapeutic purposes [5].

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Apitherapy is the use of products made by honeybees in medical purposes. It has been used since thousands of years. This can involve the use of honey, propolis, royal jelly, pollen, and bee venom[6]. It is an old tradition that has been revitalized in recent research[7].

Propolis or "Bee glue" is a resinous material obtained from tree barks and buds. After propolis collection, bees mix it with wax flakes and their saliva in the hive. This mixture is used to cover the interior of the hive. Propolis form a part of the bees external immune defence system, which makes the bee hive one of the most sterile environments known in nature[8]. Propolis has many pharmacological properties such as anti-inflammatory, antimicrobial, healing, anesthetic properties. In the current research we are considering the use of propolis in dentistry to highlight its anti-inflammatory and antimicrobial activities particularly in oral surgery, periodontics, endodontics and pedodontics[9].

Different materials have been used as intracanal obturating agents in deciduous teeth. Among the most common materials are; unfortified zinc oxide eugenol, used either alone or applied with formocresol[10], iodoform and camphorated parachlorophenol pastes [11] as

well as iodoform and calcium hydroxide mixtures (such as Metapex) [12].

Zinc oxide eugenol has been the material of choice since 1930's, it has been found that Zinc oxide eugenol sets into hard material that resists resorption when extruded beyond the apices, so it can remain in the alveolar bone for months to years resulting in a mild foreign body reaction [13]. Therefore scientists are more interested in this area to improve the properties of zinc oxide eugenol [14]. Calcium is widely used in dentistry because it is able to stimulate mineralization and has an excellent antimicrobial action[15]. Metapex is a combination of calcium hydroxide and iodoform and other oily additives. It is marketed as a pre-mixed paste[16].

Therefore associating calcium hydroxide with propolis to add all their beneficial biological properties as anti-inflammatory, immunomodulation, antibacterial, antifungal and antiviral properties in addition to it's low-speed dissociation and diffusion in an endodontic paste for primary teeth to those of calcium hydroxide [7]. This study aimed to evaluate and compare the antimicrobial effect of propolis paste and pastes of Metapex and zinc oxide eugenol mixed with Egyptian propolis against microorganisms found in necrotic primary teeth. The tested hypothesis was that the addition of propolis to calcium hydroxide and zinc oxide eugenol would improve the antimicrobial properties of both medications.

METHODOLOGY

1- Samples:

Thirty five patients aged from 4 to 8years of both sexes with necrotic primary molars were selected from the Outpatient of the Pediatric Dentistry Clinic, Faculty of Dentistry, Ain Shams University. Ethical committee of Faculty of Dentistry Ain Shams University approved this study. An informed consent was obtained from each of the participants before recruitment in the study and signed by one of their parents. Thirty five necrotic primary posterior teeth either D or E (one from each patient) were included in the study according to inclusion criteria which were :Apparently healthy children with necrotic primary molars, Child did not take any antibiotic for at least one month before sample collection , Primary molars showing root resorption not more than half the roots, Primary molars should be restorable while the exclusion criteria were :Child known to have a systemic illness and child under antibiotic therapy [17].

A rubber dam was used to isolate the root canals involved in the study, without using antiseptic solutions in the root canal. We used sterile paper points which were introduced into the root canals and left for about 1 minute. Each paper point was placed in a transport medium containing brain heart infusion (BHI) supplied by (OXOID LTD., BASINGSTOKE, HAMOSHIRE, ENGLAND) in a reduced oxygen atmosphere.

2- Test materials:

Three pastes were evaluated: Paste1: mixture of propolis (EEP) and metapex prepared with paste consistency prepared by mixing 1 gm of metapex and 2 ml of propolis with alcohol. Paste 2: mixture of propolis (EEP) and zinc oxide eugenol prepared with paste consistency (2gm zinc oxide powder: 1gm propolis: 1ml eugenol).Paste 3: Ethanol extract of propolis paste (EEP) prepared by mixing 10 g of propolis to 100 ml of solvent (ethanol 80%v/v).

3- Laboratory procedure:

Anaerobic bacterial were directly identified by preparing smears on a sterile glass slide from each specimen collected and subjected to gram staining and were observed under oil immersion lens to identify the most predominant organism in each sample ,followed by culture on nutrient agar, blood agar and MacConkey agar under aneorobic conditions. For Gram positive organisms: catalase, coagulase, litmus milk reduction tests were done. Most prominant Gram negative organisms were isolated and resuspended in 100 µL of sterile water. Centrifugation was done and the supernatant was stored at -80°C.PCR assays were performed in a thermal cycler (BioRad, USA) with specific primers for *Fusobacterium nucleatum,*, *Porphyromonas gingivalis* and *Prevotella nigrescens* (table 1) with final volume containing 25µL containing 1X PCR buffer, 0.2 mM of dNTP mixture, 0.4 µM of each primer, 50 mM MgCl2, 0.5 U Platinum Taq DNA polymerase, and 1 ng DNA. PCR products were separated by gel electrophoresis on 2% agarose gel containing 0.5 µg/ml ethidium bromide. In all PCR reactions, sterile water was used as negative control instead of DNA.

Table 1: Primers used for PCR ampilification

Microorganism	*Sequence 5'-------3'*	*Amplicon*	*References*
Fusobacterium nucleatum	CAA ATG CTT GTG TCA ATA ATA CT TTT AGA AGA AAT GGT AGA ATA AT	500	18
Porphyromonas gingivalis	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404	19
Prevotella nigrescens	ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	804	19

4- Antimicrobial activity test:

Antimicrobial activity was done by using the agar-well diffusion technique. The paper points was prepared with an overnight culture of the root canal samples in an anaerobic medium at 37 ºC inside the incubator. In the second day, after bacterial growth was confirmed by the precipitation of the bacterial deposit or turbidity in the tube and adjusted to 0.5 McFerland standard turbidity, the inoculum was mixed all together by the use of vortex (MAXI MIX plus TM) to ensure even distribution of the bacteria throughout the inoculum. The Mueller Hinton agar was melted and left to cool to 44 ºC. ½ ml of the inoculum was poured inside each sterile plastic petri dish. Thirty ml of melted Mueller Hinton agar was mixed with the inoculum and left to solidify in laminar flow. Equidistant wells 5 mm in diameter and 4 mm deep using sterile pipette with same diameter were done in each plate. Each plate was divided into portions according to the previously mentioned paste groups. The wells were completely filled with the test materials using sterile syringes. Plates were incubated in an anaerobic jar using the gas pack system supplied by (Oxoid Ltd by Mitsubishi Gas Chemical Company Inc.-Japan) at 37^0c for 48hours Antimicrobial activity was determined by measuring the diameters of polymicrobial growth inhibition zones around each one of the three pastes in millimeters [20] as shown in figure 1. The collected data was tabulated and presented for statistical analysis.

Fig. 1: Antimicrobial activity was determined by measuring the diameters of polymicrobial growth inhibition zones around each well in millimeters

5- Statistical Analysis

Quantitative data were presented as mean, median, standard deviation (SD), range (Minimum – Maximum). Data were explored for normality by checking the data and using Kolmogorov-Smirnov and Shapiro-Wilk tests. Data showed non-parametric distribution. Kruskal-Wallis test was used to compare between the three paste types. Mann-Whitney U test with Bonferroni's adjustment was used for pair-wise comparisons when Kruskal-Wallis test is significant. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM SPSS Statistics Version 20 for Windows.

RESULTS

All samples (35patients) (100%) showed growth of different bacterial growth. The presence of bacteria in the samples showed statistical significant difference.

Table 2: Frequency and percentage (%) of the isolated microorganisms

		Bacterial isolates						***p-value***
		Gr.+ve Cocci		**Gr.+ve Bacilli**		**Gr. –ve Bacilli**		
		N	**%**	**N**	**%**	**N**	**%**	
Presence of bacteria	-ve	5	14.3%	32	91.4%	10	28.5%	≤0.001*
	+ve	30	85.7%	3	8.6%	25	71.4%	

Out of the Thirty five samples, thirty samples showed Gram positive cocci, these isolates were subjected to biochemical tests. *Streptococcus mutans* presented (51.4%),and *Enterococcus species* presented (34.2%). Twenty five samples showed Gram negative bacilli, these isolates were subjected to PCR assay. *Porphyromonas gingivalis* presented (28.5%), *Fusobacterium nucleatum* (25.6%) and *Prevotella nigrescens* (8.5%) was observed. While 3 Gram negative organisms were not identified by PCR assay used in our study.

There was a statistically significant difference between inhibition zone diameters of the different paste types (*P*-value <0.001). Paste 2 showed the statistically significantly highest mean inhibition zone diameter of 17.5 mm, Paste 1 showed mean inhibition zone of 13.4 mm. Paste 3 showed mean inhibition zone of 13.2 mm, there was none statistically significant difference between Paste 1 and Paste 3. Both showed the lowest mean inhibition zone diameter values. The results are shown in table 3and figure 2

Table 3: Descriptive statistics of inhibition zone diameter (in millimeters) values for the three paste types

Paste 1		***Paste 2***		***Paste 3***		**P-*value***
Mean	SD	Mean	SD	Mean	SD	
13.4	2.7	17.5	3.0	13.2	3.6	<0.001*

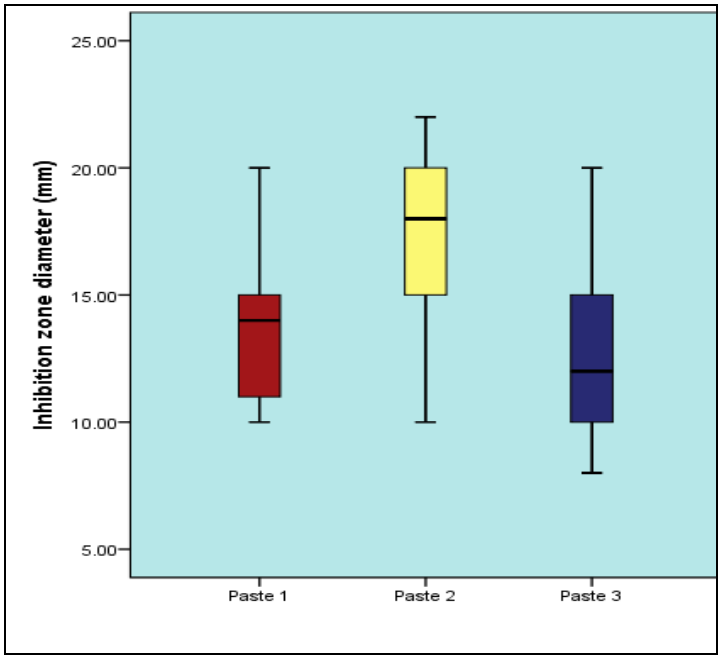

Fig. 2: Box plot representing median and quartile values of inhibition zone diameters of the three paste types.

DISCUSSION

Pulpectomy is an accepted line of treatment for saving infected primary teeth. In order to achieve a successful pulpectomy; good instrumentation, irrigation, intracanal medication and the use of an antimicrobial filling material are important to enhance the disinfection of the root canals in the infected cases [21]. Therefore there is a great trend towards using natural products in pediatric dental field because of its desirable effects and antimicrobial property. Among these natural products is "propolis" which is a natural resinous substance used in dentistry regarding its antimicrobial, anti-inflammatory and biocompatible properties [8].

In our study the most predominant isolated microorganisms in most samples were gram positive cocci (85.7%), gram negative bacilli (71.4%), then gram positive bacilli(8.6%). These results are comparable to the results of Antônio et al [22]who observed that different bacterial morphotypes in necrotic pulp and fistula samples were predominated by gram-positive cocci (81.8%), followed by gram negative coccobacilli (49%) and gram-positive bacilli (15.5%) .

In our study *Streptococcus mutans* were in (51.4%), *Enterococcus species* (34.2%), followed by *Porphyromonas gingivalis* (28.5%), *Fusobacterium nucleatum* (25.6%) and *Prevotella nigrescens* (8.5%). On the other hand Antônio et al [22] isolated *Enterococcus spp*. from (50%), *Porphyromonas gingivalis* from (49%), *Fusobacterium nucleatum* from (25%) and *Prevotella nigrescens* from (11.4%) of cases as he used wider range of primers in the PCR assay in his study.

Sundeep et al [23]found that black-pigmented bacilli were present in 53.33% of the cases while, anaerobic streptococci were found in 13.33% of the cases. Also Toyoshima et al [24] isolated black pigmented bacilli in 44.4% of retreatment cases, while another study reported their presence in 30% of the cases [25.] These differences between bacterial percentages might be due to individual variations during samples collection.

Antimicrobial activity was detected by the agar-well diffusion technique. It has been concluded that this is the best and most commonly used method for the determination of the antibacterial activity of natural products especially in endodontics [26].

It directly allows comparisons between the filling materials against the root canal bacteria, determining which material has the ability to eliminate intracanal bacteria. Results of the present study showed that Paste 2 (zinc oxide eugenol + propolis) has the statistically significantly highest mean inhibition zone diameter. The high antibacterial effect of paste 2 might be attributed to

the eugenol content as zinc oxide alone had no antibacterial activity against the microorganisms as mentioned by Sidney et al [27] or it may be due to propolis. However no more studies were found that studied the antibacterial effect of zinc oxide eugenol /propolis paste mixture .Paste 1 (Metapex mixed with propolis) showed higher mean inhibition zone diameter value than paste 3 (ethanol extract of propolis paste) but there was no statistically significant difference between them and both showed the statistically significantly lowest mean inhibition zone diameter values. These results are comparable to the results of DeRezende et al [28] who found that ethanol extract of propolis paste with calcium hydroxide paste showed larger mean inhibition zone than ethanol extract of propolis paste but with no statistical significant. The absence of significant statistical difference between paste 1and 3 in our study suggest that the antibacterial effect of paste 1 is attributed to propolis alone which was supported by Subramaniam &Gilhotra [29] and Ramakrishna & Reddy[30] who found that Vitapex and Metapex are non-inhibitory.

CONCLUSION

The present study suggested that the association of propolis with metapex and zinc oxide eugenol could be beneficial. However, further studies on these pastes mixtures' physicochemical properties and other pharmacological and microbiological aspects are necessary in order to support the use of these products.

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