Antibacterial Activity of *Ganoderma Lucidum* and *Psidium Gujava* against the Most Prevalent Endodontic Pathogens: A Preliminary Microbiological Study

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Abstract

Objective: The article aimed to define chemical constituents of *Ganoderma Lucidum* (GL) and *Psidium Gujava* (PG) and to compare the preliminary antibacterial activity of GL & PG with Modified Triple Antibiotic Paste (MTAP) and Calcium hydroxide Ca(OH)₂.

Material and Methods: Gas Chromatography Mass Spectroscopy (GCMS) was used to define the chemical constituents of *Ganoderma Lucidum* (GL) and *Psidium Gujava* (PG). The preliminary antimicrobial activity of hot aqueous and ethanolic extract of GL and PG was evaluated by Agar diffusion assay at 75, 50, 25, 10 and 5 µl/ml concentration and diameter of zone of inhibition was measured. The Minimum Inhibitory Concentration (MIC) was measured by serial dilution method at the highest concentration of 100 µg/ml (stock concentration) in
DMSO and serially diluted to working concentration ranging from 0.02 µg/ml. One hundred µl isolated strain of P intermedia, F nucleatum was added to each of the 10 serially diluted tubes so that the final volume per tube was 200 µl and incubated at 37°C for 48-72 h. After the incubation period, by visual inspection of the tubes, the MIC values were determined. Minimum Bactericidal Concentration (MBC) was performed by first 3 or 5 tubes which were sensitive to MIC were plated and colony count was taken. Time kill tests were performed at concentrations equal to MIC, twice the MIC and four times the MIC of the extracts, inoculums of size 1.0x10^5 CFU/ml were added and incubated at 37 degrees and colony count was taken (CFU/ml).

Results: Phytochemical extracts responsible for antibacterial activity of GL are flavanoids, flavanones and xanthose and for PG extract Flavanoids, flavanones, and Xanthose. Modified Triple antibiotic paste (MTAP) has the largest zone of inhibition and smallest MIC and MBC concentration. Correlating the results of disc diffusion assay, MIC and MBC both GL and Ca(OH)₂ have shown good antibacterial activity against P intermedia as compared to F nucleatum. However at the MIC values, PG is more sensitive to F nucleatum as compared to P intermedia. GL, PG and Ca(OH)₂ have shown 50% killing activity after 2 hours and MTAP 100% killing activity.

Conclusion: Within the limitation of the study GL and PG can be used as potential herbal intracanal medicaments in the treatment of root canal infections. Further research needs to be carried out to evaluate the antibacterial activity of these novel herbal intracanal medicaments on biofilm models.

Keywords
Biofilm; *Ganoderma Lucidum; Psidium Gujava;* P Intermedia; F Nucleatum; Modified Triple Antibiotic Paste; Calcium Hydroxide

Introduction
The infected root canal harbor polymicrobial microorganisms such as ae mode of growth [1]. Symbiosis among the bacteria associated with endodontic infections has led to coexistence of Prevotella intermedia and Fusobacterium nucleatum in biofilm mode of growth [2]. Complex structural anatomy of apical third of root canal make the disinfection task very tedious despite thorough cleaning and shaping [2]. Thus the use of intracanal medicaments.

Calcium hydroxide has been used as an intracanal medicament due to its antimicrobial properties due to its alkaline pH destroys it causes destruction of bacterial cell membrane and
protein structure. However recent findings suggest disadvantages associated with its long-term usage as well as the development of antimicrobial resistance. Gomes, et al. stated that calcium hydroxide is not equally effective against all microorganisms [3].

Triple Antibiotic Paste (TAP) is another most widely used intracanal medicament which is a mixture of Metronidazole, Ciprofloxacin, and Minocycline [4]. Several studies have proved the antibacterial activity of TAP. However the major disadvantage of TAP is tooth discoloration caused by minocycline. Thibodeau and Trope (2007) suggested substituting minocycline for cefaclor in the tri-antibiotic formula to avoid dentine discoloration [4]. Ruperal, et al., exposed triple and double antibiotic paste to SCAPs to assess their effect and found that both are detrimental.

Recently studies have shown that intracanal medicaments are toxic to stem cells of apical papilla and human periodontal ligament fibroblasts in concentrations currently used in endodontic regeneration [3]. The toxic side effects of these synthetic drugs have led the researchers to look for herbal alternatives with antimicrobial properties which can be used against endodontic pathogens. Since a long time, many herbal agents have been used to eradicate root canal microorganisms having antimicrobial and therapeutic effects.

Phytochemical extracts such as *Ganoderma lucidum* commonly known as red fungus used in traditional medicine in China. The *Psidium gujava* also known as Guava, the leaf, bark and fruit of this tree is used to treat many ailments in traditional Indian medicine. These herbal extracts consists of active ingredients such as Terpenoids, Steroids, Phenols, Nucleotides, Glycoproteins and their derivatives which have been reported to exert anti-inflammatory, anti-tumorogenic and immune-stimulating properties [5,6].

Therefore the present study aims to evaluate the preliminary antimicrobial potential of two herbal extracts *Ganoderma Lucidum* (GL) and *Psidium Guajava* (PG), against most commonly found endodontic pathogens like *Prevotella Intermedia* (PI) and *Fusobacterium Nucleatum* (FN) by various microbiological assays.

**Subjects and Methods**

ATCC strains of P intermedia and F nucleatum were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.
Preparation of Guava Extract

The leaves were washed and dried at 37 °C for one week, when they became brittle and ground to a fine powder (Maceration). Ethanolic extract was obtained by mixing 100 g of powdered leaves with 250 ml of 80% ethanol and shaking vigorously five times a day for 12 days. The ethanolic extract was then concentrated under reduced pressure in the Soxhlet extractor. The dried extracts were stored in dark bottles at -20 °C until use.

Preparation of Ganoderma extract: The powdered *Ganoderma Lucidum* (25 grams) was mixed separately with 250 ml of ethanol the extract was then concentrated under reduced pressure in a Soxhlet extractor for 6 hours. The extractions were repeated twice.

Preparation of modified triple antibiotic paste: Entricoating of the tablets of Ciprofloxacin hydrochloride tablets 500 mg (Cipla Ltd, Tarpin block, Sikkim, India), Metronidazole tablet 400 mg (JB chemicals, India) and cefaclor tablets 250 mg (Icarus Health Care Pvt. Ltd) were removed and crushed in a sterile motar and pestle to a very fine powder. 300 mg USP grade antibiotic powder compounded of equal portions of Metronidazole, Ciprofloxacin, and Cefaclor was dissolved in 3 mL distilled water (33 mg of each antibiotic/mL) overnight. The mixtures were then centrifuged at 3000 rpm for 15 minutes to clarify the solutions and supernatant were taken.

Preparation of calcium hydroxide paste: A saturated solution of Ca(OH)₂ paste (Vishal Dental Care, Ahmadabad, India) was prepared by mixing 16 mg Ca(OH)₂ powder with distilled water on a clean sterile glass slab using a sterile spatula until a creamy paste is obtained.

**GC-MS Analysis**

GCMS analysis of the samples was carried out using Shimadzu Make QP-2010 with a non-polar 60 M RTX 5MS Column. Helium was used as the carrier gas and the temperature programming was set with the initial oven temperature at 400°C and held for 3 min and the final temperature of the oven was 4800°C with a rate at 100°C. A 2 μL sample was injected with split less mode. Mass spectra were recorded over 35-650 amu range with electron impact ionization energy 70 eV. The total running time for a sample is 45 min. The chemical components from the ethanolic extracts of plants were identified by comparing the retention times of chromatographic peaks using the Quadra pole detector with NIST Library to relative retention indices (Fig. 1 and 2).

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**Standardization of Concentration**

**Disc-Diffusion Assay**
Antibacterial susceptibility against ATCC strains of Prevotella intermedia and F nucleatum by disc diffusion assay, the bacterial culture was adjusted to 0.5 MacFarland standards (0.5x10^8 colony forming units in an optical density of 600 nm) a sterile swab was used to add this on blood agar. Wells (5 mm) were punched in the agar plate which was saturated with experimental medicaments Calcium hydroxide (Ca(OH)_2), *Ganoderma Lucidum* (GL) and *Psidium Gujava* (PG) and Modified Triple Antibiotic Paste (MTAP). After incubation at 37°C for 48-72 hours zones of inhibition were measured.

**MIC Procedure**

In this study a serial dilution technique was employed and the extract concentration was prepared to the highest concentration of 100 µg/ml (stock concentration) in DMSO and serially diluted to working concentration ranging from 0.02 µg /ml. Ten tubes, each having 100 µl thioglycollate broth was used for the MIC procedure. 100 µl extract solution was added to the first MIC tube containing 100 µl broth. After mixing, 100 µl solution from this tube was transferred to the second MIC tube. This process was continued till the 10th tube. From the 10th tube which was the last tube, 100 µl final solutions were discarded. The concentrations of the aqueous extract were achieved by a serial dilution method. 100 µl isolated strain of P intermedia, F nuclatum was added to each of the 10 serially diluted tubes so that the final volume per tube was 200 µl. The tubes were incubated at 37°C for 48-72 h. After the incubation period, by visual inspection of the tubes, the MIC values were determined.

**Procedure for MBC**

From the MIC dilution tubes, the first 3 or 5 tubes were plated (which was sensitive in MIC) and incubated for 24 hrs then the next day the colony count was taken. MBC was done to see whether there was bacteriostatic or bactericidal effect of the extract (Drug) against the organism, if there is no growth then - its bactericidal effect, if there is growth then it’s the bacteriostatic effect.

**Time Kill Curve Assay**

Concentrations equal to MIC, twice the MIC and four times the MIC of the extracts were prepared. An inoculum of size 1.0x10^5 CFU/ml was added and incubated at 37 degrees. The aliquots of 1.0 ml of the medium were taken at time intervals of 0, 5, 10, 30 minutes and 2 hours and inoculated aseptically into 20 ml in blood agar and incubated at 37 degree for 24
hours. A control test was performed for the organisms without the extract or reference antibiotic. The colony-forming unit of the organisms was determined five times.

**Results**

Findings obtained from the disc diffusion assay were subjected to descriptive statistics. Because of violation of normality assumption, nonparametric Kruskal Wallis and Man Whitney U-tests were employed to compare antibacterial activity against P. intermedia and F. nucleatum. The zones of inhibition of medicaments at five different concentrations were measured, followed by MIC, MBC and Time Kill Curve assay.

The Table 1, shows the mean diameter of the zone of inhibition of all the medicaments against P. intermedia, GL has shown a higher zone followed by Ca(OH)\(_2\) and PG. MTAP has shown the highest zone at all the dilutions. Table 2 shows the mean diameter of the zone of inhibition of all the medicaments against F. nucleatum, both GL and PG have shown almost similar inhibition zones as compared to Ca(OH)\(_2\). MTAP has shown the highest zone of inhibition.

Table 3 shows the MIC, MBC Values of four medicaments against P. intermedia for GL (6.24 mg/ml) and (12.50 mg/ml) for PG (6.25 mg/ml) and (14.06 mg/ml) for Ca(OH)\(_2\) (3.51 mg/ml) and (7.03 mg/ml) followed by for MTAP is (3.51 mg/ml) and (3.90 mg/ml) respectively. At MIC and MBC concentration PG, GL and Ca(OH)\(_2\) are bacteriostatic in nature whereas MTAP was bactericidal.

MIC and MBC concentration for F. nucleatum for GL (137.5 mg/ml) and (150 mg/ml) for PG (6.25 mg/ml) and (112mg/ml), Ca(OH)\(_2\) (31.25 mg/ml) and (50.46 mg/ml) followed by for MTAP is (2.73 mg/ml) and (4.68 mg/ml) respectively. At MIC and MBC concentration GL was more bacteriostatic in nature as compared to PG followed by Ca(OH)\(_2\). At MIC and MBC concentration PG and GL are bacteriostatic followed by Ca(OH)\(_2\) and MTAP is bactericidal.

Table 4 shows time-dependent killing action of the medicaments against P. intermedia, GL has shown better killing activity at all the time intervals as compared to PG followed by Ca(OH)\(_2\) and MTAP. MTAP has shown sustained anti-bacterial activity at all time intervals.

Table 5 shows time-dependent killing action of the medicaments against F. nucleatum, PG has shown better killing activity after 10 minutes than GL, after two hours both PG and GL were equally effective. Ca(OH)\(_2\) has shown the least killing activity among all the medicaments. MTAP has shown sustained anti-bacterial activity at all time intervals.
The table 1 shows the descriptive statistics for disc diffusion for P. intermedia. The table 2 displays the descriptive statistics for disc diffusion for F. nucleatum. The table 3 presents the descriptive statistics for MIC and MBC for P. intermedia and F. nucleatum. The table 4 represents the time kill assay for P. intermedia by Kruskal-Wallis test.
Table 5: Time kill assay for F nucleatum by Kruskal-Wallis test.

<table>
<thead>
<tr>
<th>Medicaments</th>
<th>0 hrs</th>
<th>5 min</th>
<th>10 min</th>
<th>30 min</th>
<th>2 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>46±3.03×10^5</td>
<td>36±3.74×10^5</td>
<td>24.8±4.15×10^5</td>
<td>12±5.55×10^5</td>
<td>0</td>
</tr>
<tr>
<td>PG</td>
<td>69±1.48×10^5</td>
<td>58±2.19×10^5</td>
<td>13±1.92×10^5</td>
<td>10±2.79×10^5</td>
<td>2±1.67×10^5</td>
</tr>
<tr>
<td>Ca(OH)_2</td>
<td>78±5.46×10^5</td>
<td>71±5.93×10^5</td>
<td>58±7.09×10^5</td>
<td>18±6.20×10^5</td>
<td>22±4.15×10^5</td>
</tr>
<tr>
<td>MTAP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Discussion

Plant extracts have essential components having antimicrobial properties such as Flavonoids Tannins and Alkaloids. Porphromonas gingivalis and Fusobacterium nucleatum are the most commonly isolated microorganisms from chronic apical periodontitis and abscess whereas P.intermedia is an important microorganism involved in periodontal diseases. Herbal extracts show antibacterial activity by various modes of action such as altering the interfacial surface tension of bacterial cell wall and mitochondria leading to bacterial death [7,8].

The current research was performed to investigate the preliminary antibacterial potential of Ganoderma Lucidum (GL) and Psidium Gujava (PG) used as an intracanal medicament against microorganisms causing apical periodontitis. The chemical components of both the extracts were evaluated using GC-MS to avoid lack of standardization and for a better understanding of bioactivity. As per the results of the tests, the phytochemical molecules responsible for the antibacterial activity of GL were flavanoids, flavanones and xanthose. For PG extract Flavanoids, Flavanones and Xanthose are responsible for their antibacterial activity. A minimum.

The second part of the study included a comparison of antibacterial activity GL and PG against P intermedia and F nucleatum with the most commonly used intracanal dressing material Ca(OH)_2 and MTAP. The main idea of this study was obtained from the preliminary investigation done by the various authors on antibacterial potential of GL and PG. The results are partially confirmatory and in line with the study done by Nayak, et al., and Yoon, et al., Nayak, et al., stated spore powder of GL can be used as a topical agent on the infected tissues which can act as an efficient drug delivery system for P intermedia associated periodontitis [9]. Yoon, et al. studied antibacterial activity of GL on gram-positive and gram-negative bacteria, strongest.

Correlating the results of disc diffusion assay, MIC 0.8 mg/ml and MBC (6.25 mg/ml), GL has shown good antibacterial activity against P intermedia as compared to F nucleatum. The killing
activity of GL is time-dependent which increases as the contact time is increased as this may be correlated to the slow diffusion of certain molecules in agar.

As per preliminary antibacterial tests at MIC (1.6 mg/ml) and MBC (6.25 mg/ml) values, PG is more sensitive to F nucleatum as compared to P intermedia. But at the minimum bactericidal concentration, the extract is more sensitive to P intermedia as compared to F nucleatum. Therefore it can be said that guava extract is bacteriostatic in nature at the MIC concentration for P intermedia as compared to F nucleatum. The killing activity of Guava extract was time-dependent as the time advanced the killing activity was better and fewer colonies were found. Yadav, et al., studied the effect of guava leaf extract on the biofilm of pseudomonas aeruginosa and found that biofilm got inhibited in presence of guava leaf extract [11]. As per the study done by Massunari, et al., extracts from Psidium Cattleianum Hydroethanolic (PCHE) leaves were biocompatible and presented better antimicrobial effect against important pathogens associated with persistent endodontic infections [12].

For calcium Hydroxide at MIC (0.4 g/ml) and MBC values (3.12 mg/ml) the medicament has maximum antibacterial activity against P intermedia as compared to F nucleatum. At minimum bactericidal concentration the calcium hydroxide is less effective against F nucleatum. At the initial contact the Ca(OH)_2 has not shown killing activity against P intermedia whereas for F nucleatum the killing activity is sustained as the time is increased. The variation in the results of MIC, MBC and time-kill assay may dependent on the size of the molecule of the medicament, type of vehicle used to prepare the medicament, and ability to diffuse in to the agar.

Modified Triple Antibiotic Paste (MTAP) at MIC (0.4 mg/ml) and MBC (0.4 mg/ml) has shown a best antibacterial activity against F nucleatum as compared to P intermedia. The antibacterial begins from the time of contact of medicament till 2 hours. The antibacterial activity is sustained as the time advanced there is decrease in the colony counts. The antibacterial activity of MTAP is more bactericidal, among all the experimental groups MTAP is found to have good antibacterial activity against both microorganisms. According to Abbaszadegan, et al., extending the contact time of CA(OH)_2 and TAP resulted in enhanced antimicrobial activity but it is usually associated with dentin discoloration and weakening [13]. The present study aims to highlight the usage of phytochemicals having antimicrobial properties not only to eliminate remaining microorganisms from the root canal but also to increase the favorable outcome of root canal treatment. Although the results of the present study support GL and PG are rich in phytochemicals and many more essential compounds. It is noteworthy that the chemical, physical and pharmacological interaction properties of these herbal extracts with the endodontic filling material are still unclear. Conclusive remarks on the usage of these medicaments in root canal treatment cannot be made until further evaluation is performed on animal and human models.
Conclusion

Under the conditions of this study, GL and PG exhibited good antibacterial activity against both tested microorganisms however there is no statistically significant difference in antibacterial activity of Ca (OH)$_2$ and other herbal medicaments. MTAP has shown the best antibacterial activity among all the tested medicaments.

Conflict of Interest

It is stated that there are no conflicts of interest between the proponents and participants in the present work.

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References


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