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Antibacterial activity of Commiphora molmol (Myrrha) against the periodontal pathogen, Aggregatibacter actinomycetemcomitans

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Antibacterial Activity of *Commiphora molmol* (Myrrha) Against The Periodontal Pathogen, *Aggregatibacter actinomycetemcomitans*

By
Khalid Alharbi

A Master's Thesis Submitted to the Faculty of Richard L. Conolly College,
Long Island University
In Fulfillment of
The Requirements for the Degree of
Masters of Science
May 9, 2022

MAJOR DEPARTMENT

Biology

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A1. Characteristic of *Aggregatibacter actinomycetemcomitans*

Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*) is an exogenous pathogen responsible for causing a transmissible infection in people who are exposed to it. Young people who get periodontitis are more prone to infection by the bacteria. It is the causative agent in 90 percent of the localized aggressive periodontitis cases in young people but in only 30-40 percent of severe adult periodontitis cases. It has a capacity to produce a number of virulence factors responsible for its pathogenicity. In 1912, the bacterium was first reported by Klinger who discovered a previously unknown Gram-negative microorganism in actinomycotic lesions. Therefore, the word 'comitans' is common with Actinomyces (Klinger, 1912). *A. actinomycetemcomitans*, in addition to aggressive periodontitis, has also been associated with a number of systemic diseases like infectious endocarditis, brain abscesses and chest wall abscesses (Kaplan et al., 1989). Previously it was only considered to be the only cause of localized aggressive periodontitis (LAgP) (Zambon, 1985), but recent research showed that *A. actinomycetemcomitans* plays a significant role in a consortium of microorganisms related to the disease.

A. actinomycetemcomitans is related to human oral microbiota and considered to be highly host specific. Although the host shows a tendency to carry these strains starting from the teething phase till tooth loss at old age but the spread of the clones of bacteria is not a recurring event (Asikainen & Chen, 1999). *A. actinomycetemcomitans*, along with other bacterial strains, plays a significant role in suppressing the host defenses (Fine et al., 2019). *A. actinomycetemcomitans* falls in the category of low abundant oral pathobiont but as the disease progresses it specifically attacks the host mucosal immune system (Hornef, 2015).

A. actinomycetemcomitans belongs to the category of non-motile, rod-shaped Gram-negative bacteria. Its cell size ranges from 0.4–0.5 µm × 1.0–1.5 µm. The microscopic picture of it shows cocci shaped cells and they poorly grow in ambient air. They grow well in 5% CO_2 (Holm, 1954). When the cells are incubated for 24 hours in chocolate agar, the colonies appeared very small but after 48 hours of incubation, the size of the colony may grow to 1-2 mm (Henderson et al., 2010). The primary culture showed rough textured colonies that strongly adhere to the agar plates as shown in Figure 1.

Figure 1: Colonies of *A. actinomycetemcomitans* on solid media (Nørskov-Lauritsen et al., 2019). (A) shows rough textured colonies of *A. actinomycetemcomitans* strain HK1651 on chocolate agar. (**B**) Clinical isolate incubated on TSBV (tryptic soy-serum-bacitracin-vancomycin) agar for 4 days in 5% CO_2.

We can get the sample of *A. actinomycetemcomitans* from the periodontal pockets, mucosa of the oral cavity and saliva. The techniques involved in getting samples are the insertion of sterile paper in periodontal pockets, cotton swabs for the mucosal sample and a piece of paraffin is given to the patient to chew for the collection of stimulated saliva. Different media have been recommended for the transportation of these samples. The studies suggested that for the paper points transportation VMGA-III medium is used (Möller, 1966), and for cotton swab samples salt buffer or TE-buffer is required (Johansson et al., 2009). However, saliva samples have a short transportation time hence no additive is needed but if the samples have to be transported for a long time-duration they must be stored at room or freezing temperatures in Saliva DNA Preservation Buffer.

The common selective media used for *A. actinomycetemcomitans* culturing and isolation is TSBV (tryptic soy-serum-bacitracin-vancomycin) agar (Jorgen Slots, 1982). The information obtain after detection of *A. actinomycetemcomitans* in clinical samples is very limited for the disease management, prediction, and progression of periodontal disease. Therefore, it is absolutely necessary to study the population of a bacterium on the disease site. This constitutes ecological plaque hypothesis (Marsh, 1994) in which the bacterium is suspected when a formation of rough textured colonies that appear on selective agar after incubation of one or two days is observed. After initial detection, MALDI TOF Mass spectrometry technique is employed for identification of the bacterium (Couturier et al., 2011).

Several biological assays involving human cell lines have been performed to determine the leukotoxicity (the protein toxin secreted by bacterium to destroy the immune cells) of *A. actinomycetemcomitans* (J J Zambon et al., 1983) but a second method reported in previous studies is a semi-quantitative method based on hemolysis on agar blood plates (Haubek et al., 1997). It was reported earlier that the release of leukotoxin is from the surface of the bacterium, either in broth during the growth phase (Sampathkumar et al., 2017) or during the treatment of bacterial culture with hypertonic salt solution (Höglund Åberg et al., 2014). Still, it has to be validated that the amount of leukotoxin released from the bacterial cell surface is equal to the total amount of the leukotoxin produced.

Most of the leukotoxins produced belong to serotype b. In several studies it is noted that serotype b of *A. actinomycetemcomitans* is the most frequently found strain and is present in greater number in active periodontitis lesions, whereas the other strains such as serotype a and c are strongly associated with the oral health.

A2. Antibiotic resistance of *A. actinomycetemcomitans*

Amoxicllin is the most frequently used antibiotic for treating *A. actinomycetemcomitans*. It has been reported that amoxicillin has different rates of resistance development ranging from 0% to 84% as seen in Table 1, which also shows that amoxicillin resistance varies from country to country as it is negligible in Switzerland but extremely high in United Kingdom.

The molecular mechanisms of antibiotic resistance in *A. actinomycetemcomitans* are largely unknown and so, further studies in this area of research are warranted. In gram-negative bacteria, the most common cause of β-lactam resistance is the production of β-lactamase, but certain enzymes common in these bacteria have not yet been found in *A. actinomycetemcomitans* . The main reason for limited knowledge about antimicrobial resistance mechanisms is the challenges associated with studying such mechanisms due to fastidious nature of the *A. actinomycetemcomitans* . Currently no evidence has been found for the replacement therapy in case of oral amoxicillin when antibiotics are prescribed for the treatment of *A. actinomycetemcomitans*-associated periodontitis. These gram-negative microorganisms are the most susceptible towards the cephalosporins as compared to the penicillin class of β-lactams. Currently the disease-specific treatment options are accepted widely.

A3. Diseases caused by *A. actinomycetemcomitans*

The 10% of all cultivable *A. actinomycetemcomitans* is present periodontally in healthy children (Höglund Åberg et al., 2014). According to a study by Slots et al., (1980), prevalence of *A. actinomycetemcomitans* depends on age as well as whether a patient has periodontitis or not. The researchers found that the juveniles who did not have periodontitis had 20% *A. actinomycetemcomitans* carriage rate while for juvenile periodontitis patients, this number

increased to 96%. On the other hand, *A. actinomycetemcomitans* carriage rate in normal adults was higher than in normal juveniles at 36% but for adult periodontitis patients, it was significantly less than for juvenile periodontitis patients at 50%.

After colonization, *A. actinomycetemcomitans* remains detectable in periodontitis patients. The natural habitat of *A. actinomycetemcomitans* is the oral cavity but this species can also be isolated from a number of oral as well as non-oral infectious diseases like arthritis, bacteremia, endocarditis, osteomyelitis, skin infections, urinary tract infections and various types of abscesses (Arie J van Winkelhoff & Slots, 1999).

A. actinomycetemcomitans is a member of fastidious Gram-negative bacteria responsible for 1.4-3% of infective endocarditis (Brouqui & Raoult, 2001). This group, called the HACEK group, comprises of *Haemophilus* species, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella kingae* (Geraci & Wilson, 1982). Endocarditis is an infection of endocardium; and the lining that surrounds the interior of the heart. Usually, the valves of the heart are affected; the bacterial infection initially causes corrosion and accidental exposure of the endothelium tissues during the extensive valves' activity.

The development of periodontitis is mainly due to the unhealthy lifestyle and unhygienic conditions resulting in the development of this common chronic inflammatory disease (Henderson et al., 2010). The identification of specific disease-causing agent is delayed in the case of *A. actinomycetemcomitans* because of the complexity of the periodontal microbiome. Recent studies show a complex relationship between cultivable and non-cultivable bacteria in the oral cavity and their interplay with the host (Dewhirst et al., 2010; Ebbers et al., 2018; Henderson et al., 2010). The mechanism adopted by the *A. actinomycetemcomitans* is the suppression of the host immune system and the overgrowth of the partners associated with the disease (Fine et al., 2019).

Aggressive periodontitis was earlier classified as the rapid loss of the periodontal tissues based on the clinical cases presented (Armitage, 2000). Recently a study presented a new classification of chronic and aggressive forms of *A. actinomycetemcomitans* based on a multi-dimensional staging and grading system (Fine et al., 2018; Tonetti et al., 2018). The staging system assesses the severity of the disease and grading system tells us the progression of the risk associated with the disease and response towards the standard therapy (Tonetti et al., 2018).

A4. Treatment of infections caused by A. actinomycetemcomitans

The main step towards containing the spread of disease is to maximize the periodontal health (Teughels et al., 2014). The most effective therapy is the mechanical removal of the damaged tissues through biofilm but in this oral hygiene is very important. The persistence of periodontal lesions even 3-6 months after the initial surgery calls for a second therapy. Localized periodontitis that affects the adolescents has been found associated with the healing potential (Hamad et al., 2019). The stage III and IV of the disease periodontitis has been treated with regenerative techniques in surgery (Tonetti et al., 2018). Risk factors associated with the disease are non-compliance, smoking, increased bleeding index and uncontrolled production of plaques (Dopico et al., 2016).

Physicians recommend an intravenous course for at least one month with third generation cephalosporins, or usually give a combination therapy including ampicillin and an aminoglycoside (Paturel et al., 2004). In a recent study the patients suffering with infective endocarditis were given an oral antimicrobial follow up regimen and showed positive results and are clinically stable as well (Iversen et al., 2018).

B. *Commiphora molmol* (Myrrha)

Commiphora molmol is a member of a family Burseraceae which includes the aromatic and resinous plants (De Rapper et al., 2012). It naturally occurs in Indian subcontinent, north-east Africa, Somalia, Kenya, Ethiopia and Saudi Arabia (Haffor, 2010). It is known as Myrrha in folklore medicine and is a commonly used herb in southern Arabian Peninsula. Myrrha is a resinous exudate obtained from multiple species of Burseraceae family possessing medicinal properties and is used as a traditional medicine for the treatment of hypertension, hyperlipidemia, various respiratory infections, mouth ulcers, colds, burn treatment and cancers (Haffor, 2010).

Myrrha has long been known for its antimicrobial activity and therefore, has been used to treat infections and inflammation either alone or in combination with other products obtained from different herbs. Dated back to 1100BC, the Sumerians used Myrrha against tooth infections and as an anti-worm medicine to treat intestinal worms. The oil extracted from Myrrha in older times has also been used against fungal infections caused by Candida albicans and Tinea pedis and also for treating wounds on skin, while the Egyptians used it for embalming (Haffor, 2010). The Britishers used it as a mouthwash as well as for treating ulcers. It has been approved by the European Commission to treat tropical inflammation of oral mucosa. Historically it has been considered as an important drug and has been used in Chinese Traditional Medicine system as a treatment for leprosy, rheumatism and syphilis. In Somalia and Ethiopia, Myrrha oil is used as a treatment against stomach ache (Haffor, 2010).

The studies on rats have been done to check the glucose tolerance of the Myrrha and resulted in effective decrease of blood glucose above the fasting concentration in both normal and diabetic rats and therefore they proved to be a beneficial therapeutic agent in treating non-insulin dependent diabetes mellitus (Al-Awadi & Gumaa, 1987). It also possesses strong antithrombotic activity. A

large number of studies have been done to support the traditional use of Myrrha in treating infectious diseases and other ailments (De Rapper et al., 2012; Haffor, 2010). The earliest records for the use of these oils in combination with other materials date back to 1500 BC. Ancient Egyptians have shown earliest evidence of using these oils as pharmaceuticals in combination therapy treating wounds and skin sores (Michie & Cooper, 1991). It is usually used in combination with frankincense but is also known to be added with other medicinal substances like opium and red ochre for treating pruritus and burn wounds. Various studies have been done to identify the beneficial effects of Myrrha but it was rejected to work as an antibiotic because of the presence of toxic inorganic elements (Ahamad et al., 2017). Along with the existence of various organic compounds, 62 inorganic compounds containing elements such as calcium, chromium, iron, phosphorous and some toxic inorganic elements like arsenic, mercury and lead were found to be present at lower toxic levels when compared to reference doses by US EPA (Ahamad et al., 2017).

A survey conducted in Iran has been portrayed in Iranian traditional books suggesting that the Myrrha has been used because of its activity against microbes, treating topical fungal infections, skin ulcers and increase in wounds healing (Bagatti, 1946; Mahboubi & Mohammad Taghizadeh Kashani, 2016). The use of Myrrha was common among midwives during labor in women to prevent infections (Walsh et al., 2010). The abscesses and wounds on the horse's skin were also treated with Myrrha as an ethnoveterinary medicine (Lans et al., 2006). The studies using different extracts of Myrrha (Abdallah & Khalid, 2012; Kim et al., 2011) such as crude extract (Rahman & Gibbons, 2007), Myrrha tincture (Romero et al., 2005), Myrrha oil (Mohammed & Samy, 2013) have revealed antibacterial activity against a broad spectrum of bacteria.

Multiple studies have been conducted till date to evaluate the biological active ingredient present in Myrrha resin along with its medicinal properties that were reported earlier. A large

amount of data has been published in support of its use as a traditional medicine highlighting the plant-based drug, however data regarding the inorganic constituents of myrrha resin has yet to be reported. Many studies support the historical and traditional use of Myrrha in combination with frankincense, but no supporting evidence has been found to evaluate the combination therapy to treat infectious diseases.

B1. Antibacterial activity of *Commiphora molmol* (Myrrha)

Gradually with the passage of time humans recognized the medicinal values of the resinous extracts of the genus Commiphora (Barnett, 2004) and used it against several illnesses like wound treatment, pain, arthritis, fractures, obesity, infections of gastrointestinal diseases and infections caused by parasites (Abdul-Ghani et al., 2009).

Recent studies showed that *Commiphora myrrha* has antimicrobial activity against staphylococci, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* (Dolara et al., 2000). However, in most of these studies, the antibacterial effect of myrrh against organisms was found to be weak. This is because all these studies used traditional testing methods, which are designed to test for antibacterial activity against growing bacteria. A recent paper published in 2020 reported that Myrrha showed strong and unique antibiotic activity preferentially against nongrowing bacteria (Bhattacharjee & Alenezi, 2020). To detect this activity, the authors have developed a new method that is specially designed to test for activity against non-growing bacteria, which are also known as persisters or dormant bacteria. This preferential activity against non-growing bacteria is a property that differentiates myrrha from other commercially available antibiotics. Another important observation reported by the authors is that there was no detectable development of antibiotic resistance against myrrha.

More research is required to study the target of the antibiotic in Myrrha whereas it is considered that a biochemical process taking place in nongrowing cells can be a target. The possible sites can be membranes of bacteria or bacterial enzymes associated with membrane. Persistent infections are known to be treated by the disruption of the bacterial membrane proteins (Hurdle et al., 2011).

It can be concluded that Myrrha oil preferentially kill nongrowing bacteria and hence can be considered as a significant candidate for future antibiotic development. According to the literature in traditional medication, Myrrha plant causes low toxic effect however, formal toxicity studies are still required to corroborate this information. Therefore, future studies should focus on the identification of the active compound in Myrrha oil and its pharmacokinetic and pharmacodynamic properties.

II. MATERIALS AND METHODS

A. Microbial strains used

The microbial strain used were following: Y4Nal (stock # LIU1142), a periodontal pathogen *Aggregatibacter actinomycetemcomitans*, Y4Nal(pVJT128) (stock# LIU1239), where the plasmid pVJT128 is used for transposon mutagenesis and the *Escherichia coli* strain MV10Nal (stock # LIU4).

B. Preparation of the growth medium broth and plate

AAGM (*Aggregatibacter actinomycetemcomitans* growth medium) was made by dissolving 0.9 grams of yeast extract and 3.75 grams of tryptic soy broth in 180 ml of autoclaved water and adjusting the pH at 7.5 using 10M NaOH. Solutions were sterilized by the microwave method that has been described before (Bhattacharjee et al., 2009, Bhattacharjee and Delsol 2014). For making AAGM plates, a previously autoclaved solution of 4.5g agar in 150 ml of water was melted by heating in a microwave oven. In a separate bottle, 7.25 grams of tryptic soy broth and 1.8 grams of yeast extract were added to 180 ml of previously autoclaved water, pH was adjusted to 7.5 by adding 140 µl of 10M NaOH. This broth mixture was heated using microwave and added to the heated agar solution. The mixture (300 ml) was then cooled and poured to make 12 plates. Plates were then streaked with bacteria and grown in carbon dioxide environment as follows: plates were placed inside a plastic bag containing a beaker having 50 ml of 0.5 M HCl. Outside the beaker 2 grams of sodium bicarbonate containing paper pouch was placed. The plastic bag was heat sealed and the pouch containing bicarbonate was then placed inside the beaker. The reaction of acid and base produced carbon dioxide gas in the sealed bag, which was then incubated at 37°C.

C. Myrrha extract

Myrrha oil was isolated by the extraction method described before (Bhattacharjee and Alenezi 2020). The Myrrha resin, approximately 2.0 g, was finely ground and soaked three times in 4.3 ml of 95% ethanol. The combined ethanol extract was centrifuged, and the supernatant was collected. Rotary evaporator was then used to evaporate the ethanol after which a yield of 0.72 g of oil was obtained. Since the oil is insoluble in water, a 20% solution of oil in ethanol was used as a stock solution for all experiments described here.

D. **Construction of transposon insertion mutant library**

In bacteria the process of mutagenesis demands the efficient administration of a transposon suicide vector. An experiment was conducted to develop an IS903-based transposon mutagenesis system for a diverse group of gram-negative bacteria. IS903 consists of an insertion sequence of 1,057 bp containing 18 bp of inverted repeats at its end and a single gene for transposase (tnp). On insertion of the IS903 transposon, it generates 9-bp targeted duplication (Grindley & Joyce, 1981). This straightforward approach can be used to create insertion mutagenesis vectors that allow for stable insertions in host genomes. The IS903 based transposon with a cryptic kan gene was constructed. The transposon IS903φkan, is expressible only when there is an efficacious transposition resulting in the stable entry of the vector inside the host. IS*903φkan* was transferred into an IncQ plasmid vector having a transposase gene outside the transposon. This transposition from pVJT128 was done through IPTG induction of the *tac* promoter. IncQ plasmid vector of the IS903φkan containing plasmid pVJT128 is widely functional in many gram-negative bacteria such as *A. actinomycetemcomitans* (Thompson, et al., 1999) in isolating mutants from insertions. Map of pVJT128 is shown in (Figure 2).

E. Minimum inhibitory concentration (MIC) for Myrrha on plates

Plates containing various concentrations of the Myrrha oil were made (0, 0.05, 0.1, 0.2, 0.4 and 0.8%). Due to the insolubility of the oil in water, the pure oil was not directly added to the molten agar. Instead, a 20% solution of the oil in ethanol was used. Serial dilutions of bacterial cells (10^{-7}, 10^{-5}, and 10^{-3}) were spread on the plates containing various amounts of the Myrrha oil. The plates were then incubated at 37ºC for 48 hours. The number of colonies that grew in each was counted.

F. Rate of killing by Myrrha oil extract in AAGM and phosphate buffer

Aggregatibacter Actinomycetemcomitans LIU1239 and LIU1380 were grown overnight. The A_{600} was measured and the cells were distributed into two microfuge tubes, 100 µl in each. In the first tube 1.5 µl of ethanol was added as a control while 1.5 µl of 20% Myrrha oil was added to the second tube (final concentration 0.30%). The tubes were incubated at 37ºC for various times from 0 to 20 hours. Different serial dilutions from 10^{-7} to 10^{-3} were spread on AAGM plates which were then incubated for 48 hours at 37 ºC. The number of colonies that grew in each plate was counted. For evaluating the rate of killing by Myrrha oil in phosphate buffer, LIU1239 and LIU1380 were grown overnight. The cells were centrifuged and then resuspended in 50 µl of 0.02M phosphate buffer pH 7.0 and distributed into two microfuge tubes, 100 µl in each. The remaining procedure was same as described above for rate of killing in AAGM.

III. RESULTS

A. Transposon mutagenesis

The *Aggregatibacter Actinomycetemcomitans* strain (LIU 1239) Y4Nal (pVJT128) was used to make a library of mutants containing transposon insertions. The plasmid in the cells contains a gene for transposase enzyme, which was cloned under the control of the *tac* promoter. Cells were grown for 24 hours on plates containing 1 mM IPTG, which induces expression of the transposase gene and initiates the transposition process (Table 2). Colonies from the IPTG plate were then resuspended in AAGM and then were spread on kanamycin-containing plates to select for a library of mutants with transposon insertions. The transposon contains a promoterless kanamycin resistance gene which can be expressed only if the transposon inserts in an active gene. In this experiment, about 20 colonies from IPTG plate were resuspended in 1.2 ml of growth medium and 200 μl of cells were added to 0.8 ml AAGM for zero time without antibiotic the remaining 1 ml of cells were added to 4 ml AAGM with 200 μl ampicillin (100 mg/ml). The final concentration of ampicillin was 3.8 mg/ml. The tube was incubated at 37 ^{o}C and at the indicated times, 1.0 ml of cells were removed, centrifuged and resuspended in 100 μl of AAGM and then spread on kanamycin (50 μg/ml) plates. The results in Table 2 show the number of viable cells surviving exposure to ampicillin. From the plate corresponding to 6 h time point, five smallest colonies were selected and grown in broth and saved as LIU1378, LIU1379, LIU1380, LIU1381 and LIU1382. Of these, the mutant strain LIU 1380 was selected for further studies. It was confirmed to be a slow growing mutant by sequential streaking on AAGM plates three times and comparing colony sizes with that of wild type bacteria (Figure 2). The reason for using ampicillin is that it can kill only growing bacteria. Since ampicillin is bactericidal, at the high concentration used in this

experiment, majority of the bacterial cells were killed. Only the non-growing or slow-growing cells, are expected to survive the antibiotic treatment, which targets the processes of growth of the cells.

B. Viability of cells in the dense and the isolated region of the plate

In this study, we determined the cell viability of the clinical isolates of *A. actinomycetemcomitans* in the dense and isolated regions of plates. For both experiments, the plates were incubated at 37ºC 3-5 days for taking colonies from the dense region and 3-9 days for taking colonies from the isolated regions. Colonies were resuspended in 1 ml AAGM and the A_{600} was measured immediately. Serial dilutions (10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}) were spread on AAGM plates, which were then incubated overnight at 37ºC. Colonies that grew in each plate were counted, and the results were used to calculate cell viability as number of cells/ml/A600. The results in Table 3 for dense regions of both strains LIU 1239 and LIU 1380 show that viability decreased after 3 days of growth but the decrease was much less for LIU1380, which is a slow growing mutant. The results in Table 4 show that the viability of cells in isolated colonies of LIU1239, the wild type strain, decreased from the 3rd day to the 9th day by 66% while the viability of the slow growing mutant strain actually increased by 123% during the same period. This confirms our hypothesis that the slow growing mutants secrete less acid and so, are more viable for longer periods of time.

C. MIC of Myrrha oil against *A. actinomycetemcomitans*

In this study, all the minimum inhibitory concentration (MIC) experiments were done on AAGM plates containing various concentrations of the Myrrha oil. First, *A.*

actinomycetemcomitans LIU 1142 cells were grown overnight and approximately 10^7 cells were spread on each plate containing varying concentrations of the Myrrha oil (0, 0.05, 0.1, 0.2, 0.4 and 0.8%). The plates were incubated overnight and the colonies that grew were counted. The results shown in Table 5 demonstrate that LIU 1142 (wild type) cells were inhibited with increasing concentration of oil and were completely inhibited at 0.2% myrrha oil. The same experiment was repeated using LIU 1239 (wild type with plasmid) and LIU 1380 (small colony mutant with plasmid) cells, which were spread on plates containing various concentrations of Myrrha oil (0, 0.05, 0.1, 0.2 and 0.3%) and the results are shown in Table 6. On the basis of these results, it can be concluded that Myrrha oil killed all *A. actinomycetemcomitans* cells at 0.3% concentration. At lower concentrations, the small colony mutant is more susceptible to the antibiotic in Myrrh than the wild type cells. For example, at 0.2 % myrrha oil concentration, the small colony mutant is 35-fold more susceptible than the wild type cells.

D. The antimicrobial activity of oil extract in AAGM broth and in phosphate buffer.

This experiment was designed to show the antimicrobial activity of Myrrha oil in AAGM and phosphate buffer. To measure the antimicrobial activity of Myrrha oil in AAGM, LIU1239 and LIU1380 were grown overnight to give A_{600} readings of 0.129 and 0.098 respectively. Myrrha extract was added to bacteria in 100 µl of AAGM in two microfuge tubes. The first tube contained 1.5 µl of 20% Myrrha oil (final concentration 0.30%) and the second tube contained 1.5 µl of ethanol as control. Both tubes were incubated at 37 ºC for various times up to 20 hours. Serial dilutions (10^{-7},10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}) were spread on AAGM plates, which were then incubated for 48 hours at 37 ºC. The number of colonies that grew in each plate was counted. For the antimicrobial activity of Myrrha oil in phosphate buffer, LIU1239 and LIU1380 were grown

overnight to give A_{600} readings of 0.249 and 0.096. Aliquots (1 ml) of each were centrifuged and the pellets were resuspended in equal volume of phosphate buffer. Then, the same procedure was performed in phosphate buffer instead of AAGM as described above. Myrrha oil was shown to have antimicrobial activity against LIU 1239 and LIU 1380 bacteria as shown in Table 7 and 8. In AAGM broth, it took twenty hours of exposure to Myrrha oil to kill most cells of both strains and at 5 hours, both strains were killed to the same extent with about 9% viable cells remaining for each (Table 7). However, in nutrient-free phosphate buffer, myrrha killed the small colony mutant much faster than the wild type cells. For example, at 3 hours and 5 hours, the viability of the small colony mutant was five-fold less than that of the wild type strain (Table 8). When cells are transferred from regular to nutrient-free medium, the small colony mutant probably switches to non-growing or slow-growing phenotype earlier than the wild type cells and thus are killed by myrrha, which has been shown to preferentially kill non-growing bacteria (Bhattacharjee and Alenezi, 2020).

IV. DISCUSSION

Periodontitis is a bacterial infection of the subgingival region caused by an anaerobic and facultative anaerobic biofilm of oral bacteria. Among these bacteria, *A. actinomycetemcomitans* is one of the clinically most important. Periodontitis is treated with the mechanical removal of subgingival and supragingival areas, which reduces the total periodontal bacterial burden. Bacterial reserves in the depths of the pockets, on the other hand, are notoriously hard to eliminate and can be blamed for poor treatment results. As a result, antibiotic treatment may be recommended for specific patient groups (Veloo et al., 2012). Antibiotics are major drugs that treat a wide range of infectious diseases. These drugs act by killing or reducing the growth of bacteria (Nascimento et al., 2000). As there are more than 700 bacterial species present inside the mouth, it can be difficult to choose the suitable antimicrobial therapy (Pretzl et al., 2019). Moreover, it is important to note that the increasing usage of antibiotics is increasing resistancedevelopment against them and many bacteria that cause major diseases have shown resistance to all existing antibiotics. This is also true for *A. actinomycetemcomitans* as evidenced by the increase in antibiotic resistance by *A. actinomycetemcomitans* in countries where antibiotic usage against these pathogens is high (Ardila et al., 2010; A J Van Winkelhoff et al., 2005). Because of the increasing antibiotic resistance trend, searching alternative antimicrobial therapies is urgently warranted (Czaplewski et al., 2016).

As with any bacteria, When *A. actinomycetemcomitans* are streaked on plates, dense smears and isolated colonies are formed. Cells in colonies taken from the dense smears show 10^6 to 10^7 fold lower viability than cells in the isolated colonies because *A. actinomycetemcomitans* cells are known to be extremely sensitive to metabolic acids secreted during growth (Bhattacharjee

et al., 2011). Similarly, there is an inverse relationship between the glucose concentration in the growth medium and the viability of bacterium. Higher glucose concentration results in faster growth and thus secretion of more acids. Even a mildly acidic pH of 6 will result in rapid loss of cell viability. The decrease in pH is observed when the zones of diffusion of two neighboring colonies starts to merge together. In this study, the results in Table 3 and 4 shows the cells for in isolated region more viable than in dense region for select which the strain has more viable. Because of that, the result indicates LIU 1380 is a slow growing mutant strain and supports the idea that a slow growing mutant strain is more viable for a longer time.

Herbal compounds have been widely introduced into the oral care products in recent years, many of which have intriguing medicinal and physicochemical properties (Moghadam et al., 2020). Myrrha from trees in the genus *Commiphora* has been traditionally used for oral hygiene in many cultures around the world. Moreover, the scientific literature shows that Myrrha has antimicrobial activities and thus can be employed for therapeutic purposes. However, limited scientific studies and unknown mechanism of action of this herb impede its wide usage (Adnan, 2021). Phytochemical screening of Myrrha has discovered the presence of primary and secondary compounds in the extract. It has been reported that among the secondary compounds in the plants, phenols have broad range antibacterial activities (Özçelik et al., 2011). In this study, oil extracted from Myrrha was checked for antimicrobial activity against *A. actinomycetemcomitans*. To the best of our knowledge, this is the first study in which strong antimicrobial effects of Myrrha against *A. actinomycetemcomitans* was achieved by doing the test in nutrient-free medium. The results show that Myrrha oil had significant activity against *A. actinomycetemcomitans* and both growing and non-growing bacteria can be killed. The results also show that the antimicrobial activity of Myrrha oil increased with increasing concentration until reaching maximum at 0.3 % against

LIU1142, LIU 1239 and 1380 mutant (Table 5 and 6). To corroborate these findings, the effect of Myrrha oil in phosphate buffer outcompeted that in AAGM. The results in Table 7 and 8 support the idea that myrrh extract preferentially kills non-growing or slow-growing bacteria. This is especially significant because oral bacteria usually are slow-growing. Many oral bacteria, including *A. actinomycetemcomitans,* form biofilms in which growth of bacteria is very slow.

Despite a dearth of data on usage of Myrrha for oral and dental care, few in vitro studies and clinical trials conducted over the years have provided promising results. For example, in a study by Al-Madi *et al.*, (2019), 0.03 mg/μL Myrrha extract significantly reduced growth of *Enterococcus faecalis* and *Fusobacterium nucleatum* ($p < 0.0001$ for both) in premolar teeth infected with both oral pathogens. Saeedi et al., (2003) performed a clinical trial for assessing the oral mucosal effects of myrrh. For everyday use, test participants were given either only myrrh containing toothpaste or with chamomile toothpaste, while control subjects got only placebo toothpaste. The researcher discovered that people who took toothpaste containing myrrh had significantly less gingival bleeding than those who did not, implying that myrrh-containing toothpastes could be beneficial for the management of gingival lesions. A randomized clinical trial was conducted by Eid, (2021) to evaluate Myrrha as a mouth rinse following tooth extractions. A total of 40 people were divided into two groups of equal size. The participants included in the test group were given 1.25 grams of ground myrrh, which was mixed with 250 milliliters of mild warm water and rinsed twice daily for seven days. Saline mouthwash (0.90 percent w/v NaCl) was used in the control group. Both of these mixtures were studied for treating clinical symptoms like redness, swelling, discomfort, bad odor, and discharge etc. After the end of clinical trial, most patients reported a decrease in clinical symptoms with the regular use of Myrrh based mouth rinse.

The current research as well as research done over the years support use of Myrrh as an antibacterial agent that can be used for oral conditions like periodontitis. In the current study, we have provided evidence for the antimicrobial activity of Myrrha oil against an important oral pathogen (*A. actinomycetemcomitans*) which is becoming highly resistant against antibiotics due to the overuse of latter (Ardila et al., 2010). The extracted oil was evaluated on the basis of activity against wild type and small colony mutant of *A. actinomycetemcomitans*. However, more research is needed before it may be used in root canals to determine its biocompatibility in the oral environment. Research endeavors are necessary to compare Myrrh with other antimicrobials to make a broad range comparison for the discovery of effective alternatives of antibiotics for periodontitis (Adnan, 2021; Al-Madi et al., 2019)

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V. TABLES AND FIGURES

Table 1: Amoxicillin resistance of *A. Actinomycetemcomitans*.

S.No.	Country	Percent (%)	Reference
1.	Switzerland	0%	45
2.	Spain	33%	46
3.	United Kingdom	84%	47

Table 2: Number of viable cells of *A. actinomycetemcomitans* mutant library that survive exposure to Ap (100 μg/ml) for indicated times.

TIME	0h	2h	4h	6h	8h
Number of viable cells	498	528	246	68	0

Table 3: Viability of cells per ml in dense colonies

Growth condition	Time	A_{600}	Number of viable cells (10^6/ml)	Viability (10^9cells/ml/A_{600})
LIU1239	3 days	0.088*5 = 0.440	1394	3.168
	5 days	0.028*5= 0.140	0.29	0.00207
LIU1380	3 days	0.035*5= 0.175	928	5.302
	5 days	0.030*5= 0.150	3.2	0.0213

Table 4: Viability of cells per ml in isolated colonies

Strain	Time	A_{600}	Number of viable cells (10^6/ml)	Viability (10^9cells/ml/A_{600})
LIU1239	3 days	0.012*5= 0.06	611	10.183
	9 days	0.020*5= 0.1	343	3.430
LIU1380	3 days	0.015*5 = 0.075	170	2.260
	9 days	0.012*5 = 0.06	302	5.033

Table 5: Number of colonies of LIU 1142 under different Myrrha oil concentrations in the plate

Oil concentration %	Number of viable cells (10^6/ml)	viability %
0	2540	100
0.05	1361	53.5
0.1	1671	65.8
0.2	202	8.0
0.4	0	0
0.8	0	0

Table 6: Minimum inhibitory concentration of viable cells against different concentrations of Myrrha oil.

Oil concentration %	Number of viable cells (10^6/ml)			
	LIU 1239	viability %	LIU 1380	viability %
0	850	100	5730	100
0.05	1186	60.5	1278	22.3
0.1	749	88.1	781	13.7
0.2	177	20.8	34.2	0.6
0.3	0	0	0	0

Table 7: Antimicrobial activity of Myrrha oil extract in AAGM

Time	Number of viable cells (10^6/ml)							
	LIU 1239 (wild-type)				**LIU 1380**			
	Myrrha oil	**viability%**	**Ethanol**	**viability%**	**Myrrha oil**	**viability%**	**Ethanol**	**viability%**
0 h	840	100	720	100	920	100	700	100
1 h	686	81.7	750	95.8	655	71.2	728	96
3 h	391	46.5	490	68.1	415	45.1	516	73.7
5 h	78.0	9.3	411	57.1	84.2	9.2	450	64.3
20 h	0	0	0	0	0	0	0	0

Table 8: Antimicrobial activity of Myrrha oil extract in Phosphate buffer

Time	Number of viable cells (10^6/ml)							
	LIU 1239 (wild-type)				**LIU 1380**			
	Myrrha oil	**viability%**	**Ethanol**	**viability%**	**Myrrha oil**	**viability%**	**Ethanol**	**viability%**
0 h	1180	100	1550	100	930	100	970	100
1 h	670	56.8	1410	91	410	44.1	830	85.6
3 h	169	14.3	1270	82	27	3	710	73.2
5 h	87.6	7.4	920	59.8	14.4	1.5	682	70.3
20 h	0	0	0	0	0	0	0	0

Figure 2: shows the map of pVJT128 plasmid which contains the transposase gene (tnp) which is found in the *cis* position to the transposon which contains 18bp inverted repeats indicated by "IR." The plasmid also contains the gene for kanamycin resistance which is represented by 'kan.' Oriv is the start site for circular DNA replication. Rep genes code for proteins that play a role in DNA replication. *OriT* is the origin of transfer (Thompson *et al.* 1999).

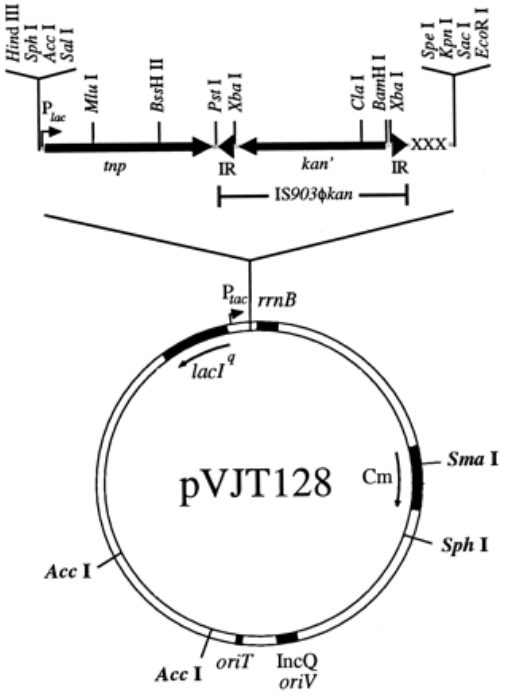

Figure 3å: The pictures show comparison of colony size between LIU 1239 (wild type with plasmid) with LIU 1378, LIU 1379 and LIU 1380 (small colony mutant with plasmid)