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Antimicrobial Property of Lemon Grass Oil against Aggregatibacter Actinomycetemcomitans

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Abstract

Background: Lemon grass has been used in treatment of many diseases. Hence this study was aimed to determine antibacterial action of lemon grass oil against the Aggregatibacter Actinomycetemcomitans.

Materials and methods: Lemon grass was dried and hydrodistillation was performed to obtain the oil used in the study. GCMS analysis was done to know the compounds of lemon grass which effective against Dispersin B of Aggregatibacter Actinomycetemcomitans. Molecular docking technique was done to study these compounds obtained after GCMS analysis. Finally, minimum inhibitory concentration was done to know the antibacterial activity of lemon grass oil components against Aggregatibacter Actinomycetumcommitans.

Results: In the present study a total of 11 compounds were obtained after GCMS analysis but 3 compounds were selected as the compounds that were expected their presence in essential oil. The chemical compounds used for study were Citronellol, D-Limonene and Geraniol. Dlimonene however exhibited good binding with a score of -6.1. Citronellol exhibited a binding score of -4.9 whereas Geraniol exhibited a binding score of -5.5 for Aggregatibacter Actinomycetemcomitans. The minimum concentration of Cymbopogon citrates (lemon grass) causing inhibition was 150μ g/ml and 200μ g /ml respectively. The maximum effect was found using 200μ l and minimum effect was observed with 50μ l of lemon grass oil.

Conclusion: The active ingredients from lemongrass oil containing Citronellol, D-Limonene and Geraniol showed antibacterial activity against Aggregatibacter Actinomycetemcomitans with MIC of 15μ l/ml. Therefore, formulations encompassing these compounds can be used to treat plaque induced periodontal disease.

Keywords: Periodontitis; lemon grass; Aggregatibacter Actinomycetemcomitans; molecular docking.

Introduction

Periodontitis is a chronic, destructive, multifactorial inflammatory disease of the supporting tissues of the teeth with a high prevalence among the adult population. Several studies have shown that A. actinomycetemcomitans is significantly associated with both aggressive periodontitis and chronic periodontitis.^{1,2}

A variety of different drug classes have been evaluated as host modulation agents, including the non-steroidal antiinflammatory drugs (NSAIDs), bisphosphonates, tetracyclines and sub antimicrobial dose doxycycline (SDD).

Fibers (hollow and monolithic), strips and compacts, films, microparticles, gels and nanoparticles have been used as periodontal local delivery devices for the targeted delivery of antimicrobial agents.³

Undesirable side-effects such as vomiting, diarrhoea and tooth staining is evidenced to the commercially available chemical agents that can alter oral microbiota. ^{4,5} Hence, the search for alternative products continues and use of traditional medicine such as natural phytochemicals isolated from plants are considered as good alternatives to synthetic chemicals.⁶

Herbal remedies possess high benefit to low risk ratio and have an advantage over conventional antibiotic treatment which suffer the limitation of low benefit to high risk. ⁷ Therapeutic approaches with herbal medicine are often staggered due to lack of data on safety and efficacy and meticulous clinical trial evidence. It is recommended that more researches should be undertaken.⁸

Cymbopogon citrates commonly known as lemongrass and other Cymbopogon species is a tall, coarse grass with a strong lemon taste. Lemongrass is a perennial herb widely cultivated in the tropics and sub-tropics, designates two different species, East Indian Cymbopogn flexuosus stapf and West Indian, Cymbopogon citrates stapf. Antidepressant, antioxidant, antiseptic, astringent, bactericidal, fungicidal, nervine and sedative properties has been found by researchers in lemongrass. ⁹ Further, many workers had reported about the antibacterial activity of lemongrass oil against a diverse range of organisms comprising gram positive and gram-negative organism, yeast and fungi.⁹ Onawunmi et al. had observed that gram positive organisms were more subtle to the oil than gram negative organisms.¹⁰

Clinical trials for assessment of safety and efficacy of these herbal remedies are in its infant stage. In vivo studies using the herbal products cannot be used unless identification of molecular features of an herbal agent which are responsible for specific biological recognition and prediction of compound modifications that improve potency are done.¹⁰

Molecular docking studies are used to determine the interaction of two molecules and to find the best orientation of ligand which would form a complex with overall minimum energy. As predicted by the search algorithm, the small molecule, known as ligand usually fits within protein's cavity. These protein cavities become active when they come in contact with any external compounds and are thus called as active sites.¹¹

Minimum inhibitory concentrations (MICs) are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. The lowest concentration of an anti microbial that will inhibit the visible growth of a microorganism after overnight incubation is defined as minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) is the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media.¹² It reduces the wet lab time.

The minimum inhibitory concentration method allows comparisons between the microorganisms exposed to the same chemical agents, but does not allow analog comparisons between the activities of different chemical agents. A. actinomycetemcomitans produces a soluble glycoside hydrolase named Dispersin B.¹³ The bacteria secrete Dispersin B to release adherent cells from a mature biofilm colony by disrupting biofilm formation.

In this study molecular docking was conducted to know the accurate structural modeling of lemon grass and correct prediction of its activity by the identification of its molecular features and it was tested against bacterial proteins. Hence this study was aimed to determine antibacterial action of lemon grass oil against the Aggregatibacter actinomycetemcomitans.

Materials And Methods

About 4-5 kilos of fresh lemon grass was used in this study which was purchased from Indian Institute Of Horticulture Hesaraghatta, Bangalore. The procedures and processing were conducted in the Dextrose Technologies Pvt Ltd. The procedures performed are given below.

Objectives

- 1. Clinical isolate of Aggregatibacter actinomycetemcomitans was used as test organism.
- 2. Lemon grass extract was used as the antimicrobial agent.

Hydrodistillation

For this method, about 500 g sample of fresh lemongrass was weighed in a 500 ml flask and was subjected to hydrodistillation for 6 hours.(Figure 1) The distillate was saturated with sodium chloride and n-hexane was added to this mix. Then, the ether layer and hydro layer were separated using separation funnel. After being dehydrated by anhydrous sodium sulphate, the n-hexane layer was further evaporated at 40 °C to concentrate oil to 5 ml and subjected to Gas Chromatography Mass Spectrometry Analysis (Guan et al.).¹⁴

Gas Chromatography Mass Spectrometry Analysis Lemon grass sample was concentrated using a rotary evaporator to 1 mL in GC vial before subjected to Gas chromatography mass spectrometry analysis (GCMS). Analyses of the samples were performed on a WATER's Gas Chromatograph-MS* using a fused silica capillary column DB-5 (20 m×0.188 mm internal diameter, 0.4 μ m film thickness). The program started at 100°C for 1 min. Then, the temperature was increased to 120°C at rate of 1°C/min. Injector and detector temperatures were kept constant at 260 °C and

volume injected was 2 μ L and injections were done in triplicates. Finally, compounds detected by GCMS were referred to Flavour 2L and NIST library. (Graph 1)

Molecular Docking of selected active compound(s) against bacterial receptor proteins

In order to assess the effectiveness and the mechanism of action, molecular docking studies were intensively performed using Citronellol, D-Limonene and Geraniol, the main active ingredients in lemon grass oil as ligands. Evaluation of Antimicrobial activity (Figures 1 and 2)

- 1. Well diffusion method was carried out in trypticase soy agar plates with 50, 100, 150, 200 μ l of lemon grass oil. It was incubated at anaerobic condition at 37 for 72hrs, with 10% CO₂, 5% hydrogen and 85% nitrogen.
- 50μl of Aggregatibacter actinomycetemcomitans in trypticase soy broth (10ml) with 50μl,100μl, 150μl and 200 μl of lemon grass oil was inoculated and incubated at 37°C for 24hrs, with 10% CO₂, 5% hydrogen and 85% nitrogen. .(Figure 2)
- After 24 hrs of incubation, the 50µl of sample of each concentration of the lemongrass oil was again plated

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on trypticase soy agar medium for MIC and incubated at 37 for 72hrs, with 10% CO_{2} , 5% hydrogen and 85% nitrogen.

4. After 72 hrs the number of colony forming units were noted down using microbial counter.

Aggregatibacter Actinomycetemcomitans produces a soluble glycoside hydrolase named Dispersin B. The main focus of the study was to explore protein - ligand interactions using molecular docking software.¹⁵ The interaction of enzyme Dispersin B from Aggregatibacter Actinomycetemcomitans with chemical compounds from lemon grass was studied. The chemical compounds used for study were Citronellol, D-Limonene and Geraniol. The structure of enzyme Dispersin crystal B from Aggregatibacter Actinomycetemcomitans was retrieved protein from databank

http://www.rcsb.org/pdb/home/home.do.¹⁶ the proteins and ligands were prepared using GOLD default settings. The docking procedure used for a rigid protein and a flexible ligand was as per the rule. In order to calculate interaction energies between the compounds and the proteins, a grid was created using Autogrid 4.¹⁶ Molecular docking was carried out three times with Dispersin B domain. ADT 1.5.6 was used to view and extract lowest binding energy complexes of ligands with Dispersin B. Discovery studio 4.1 client and Protein Plus Server Centre for Bioinformatics online data were used to determine binding mode and intermolecular interactions between proteins and ligands. Docking studies was carried out three times with Dispersin B domain.¹⁶

Results

In the present study a total of 11 compounds were obtained after GCMS analysis but 3 compounds were selected as the compounds that were expected their presence in essential oil. The chemical compounds used for study were Citronellol, D-Limonene and Geraniol. The crystal structure of enzyme Dispersin B from Aggregatibacter Actinomycetemcomitans was retrieved and molecular docking was performed.

In this particular study Dispersin B from Aggregatibacter Actinomycetemcomitans was used as ligand. The binding В site on Dispersin from Aggregatibacter Actinomycetemcomitans were explored computationally using Autodock 4. The outcomes of the docking trials in terms of binding affinity are presented in Table 1. Of the 3 active ingredients from lemon grass tested for their binding affinity with the target protein, Citronellol showed highest binding affinity followed by Geraniol and D-Limomene which are the other two active ingredients. Geraniol showed potential levels of binding affinity to the target proteins. D-Limonene however didn't show any good binding affinity (Table 1).

The docking study has revealed different types of molecular interactions leading to non-covalent interactions and bonds of various types between the ligands and the protein. Table 1 also illustrates the number of bonds formed by individual ligands with the proteins. Hydrogen bonds, Alkyl bonds, Pi-sigma bonds and Pi-alkyl bonds are the types of bonds developed between various ligands and the proteins.

Probable active sites that took part in the interaction between enzyme of Aggregatibacter actinomycetum commitans and ligands were GLU (A:332), VAL (A:280), TRP (A:330), ARG (A:27), TRP (A:237), TYR (A:278), GLU (A:184), HIS (A:120), ASP (A:183), TRP (A:216). Citronellol, D-Limonene and Geraniol when docked with Dispersin B of Aggregatibacter Actinomycetemcomitans, exhibited 3 different kinds of interactions.

Citronellol vs Dispersin B of Aggregatibacter actinomycetum commitans. Few amino acids of the enzyme formed hydrogen bonds with the ligand and some other amino acids near the vicinity of the ligand developed

Vander waal's interactions with it. The other type of interaction observed were is pi –sigma, pi-alkyl and alkyl bonds between few amino acids of the protein and the ligand.

D-Limonene vs Dispersin B of Aggregatibacter actinomycetum commitans. Few amino acids near the vicinity of the ligand developed Vander waal's interactions with it. The other type of interaction observed is pi –sigma bonds between few amino acids of the protein and the ligand.

Geraniol vs Dispersin B of Aggregatibacter actinomycetum commitans. Few amino acids of the enzyme formed hydrogen bonds with the ligand and some other amino acids near the vicinity of the ligand developed Vander waal's interactions with it. The third type of interaction observed is pi –sigma and pi-alkyl bonds between few amino acids of the protein and the ligand.

D-limonene however exhibited good binding with a score of -6.1. Citronellol exhibited a binding score of -4.9 whereas Geraniol exhibited a binding score of -5.5 for Aggregatibacter Actinomycetemcomitans. (Table 1)

The result showed that the minimum concentration of Cymbopogon citratus causing inhibition was 150μ g/ml and 200 μ g /ml respectively. After MIC was carried out the number of colony forming units were calculated. 150μ l of extract caused 54.2% reduction in number of colonies of Aggregatibacter actinomycetum commitans. MIC was further carried out using 200 μ l of lemon grass oil and 59.2% reduction in number of colonies of Aggregatibacter actinomycetum commitans was observed (Table 2).

Antibacterial effect of lemon grass was tested using its oil. The results revealed that the bacteria were sensitive, and a a clear zone of growth inhibition was evident. The antibacterial activity was found progressively increasing with the increase in the quantity of lemon grass oil. The maximum effect was found using 200 μ l and minimum effect was observed when 50 μ l of lemon grass oil was used. (Table 2)

Discussion

The primary focus of this study has been on interaction of enzyme Dispersin B from Aggregatibacter Actinomycetemcomitans that inhabit oral cavity with Citronellol, D-Limonene and Geraniol of lemon grass.

De Oliveria et al. conducted a study in which he found that Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis are the two bacteria likely to cause aggressive periodontal disease. The above organisms along with multiple deep pockets in the gum, are associated with resistance to standard treatments for gum disease.¹⁷

A. actinomycetemcomitans produces a soluble glycoside hydrolase named Dispersin B.¹⁸ The bacteria secrete Dispersin B to release adherent cells from a mature biofilm colony by disrupting biofilm formation. The enzyme catalyzes the hydrolysis of linear polymers of Nacetyl-D-glucosamines found in the biofilm matrices. In the structural integrity of the biofilms of various Grampositive bacteria and Gram-negative bacteria, Poly-acetyl glucosamines are integral and are referred to as PIA in Staphylococcus species (PNAG.PS/A) and PGA in Escherichia coli.^{13,19} By degrading the biofilm matrix, Dispersin B allows for the release of bacterial cells that can adhere to new surfaces close by and extend the biofilm or start new colonies.^{13,19}

The essential oil selected for this study was because of its folk use for treatment of different diseases. Cymbopogon citratus, from plant family of Poaceae, is mainly known for its insecticidal and antiseptic properties in Moroco.²⁰ Its antimicrobial properties were demonstrated by several studies, but there is less evidence of its effect in oral infectious diseases.⁴¹ Cymbopogon citratus contains also,

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another minor component; limonene, which was found to possess an efficient antibacterial property²⁰ and that may contribute to improve the observed antimicrobial activity. Li Z-kh et al. study results showed that Cymbopogon citratus was the most active oil against Aa. The chemical analyses of this oil in the above study revealed that the main constituents were Geraniol, Citronellol and Citronellal.²¹ In the present study Citronellol and Geraniol extracted from lemon grass were found to be more effective against A. actinomycetemcomitans as depicted through their lowest binding affinity scores (table 1 for binding affinity scores). The lower the binding affinity score, the more effective it is in attacking the target protein.

Carbajal et al.²¹ reported the activity of the essential oil from Cymbopogon citratus (lemon grass) in reducing blood pressure in animal models after venous application and antiinflammatory activity after oral application. Furthermore, high doses of the essential oil from Cymbopogon citratus showed no adverse effects on either the intestinal or central nervous systems in animal studies in a study conducted by Carini et al.²²

In the present study, it was found that the essential oils from medicinal plants offer a new choice for combination therapy against periodontal pathogens, especially the essential oil from lemon grass (Cymbopogon citratus). From the study conducted by Sakornrat et al.²³, it was concluded that the essential oil from Cymbopogon citratus (DC.) Stapf. (lemon grass) showed antimicrobial activity against periodontal pathogens, especially the reference strains A. naeslundii and P. gingivalis. This might be due against to the antimicrobial activity A. Actinomycetemcomitans ATCC43718, Strep. mutans ATCC 25175 and P. gingivalis WP50 which are odourigenic micro-organisms. Taweechaisupopong et al. found that lemongrass oil can be an effective anti-plaque agent by reducing the adherence ability of the cells and having inhibitory effect on biofilm formation.^{24,25} These can be attributed to the presence of various chemical components in the oil such as citral and geraniol²⁶ which lead to a decrease in the bacterial load and inhibit plaque biofilm formation. Antiplaque activity of lemongrass mouthwash was also confirmed in the study conducted by Kukkamalla et al.²⁷

The binding of ligands to receptors are governed by the **concept** of chemical bonding. Intra and intermolecular forces of attraction play a big role in understanding the binding chemistry between the ligands and the receptors. In understanding the binding chemistry between the ligands and the receptors intra and intermolecular forces of attraction play an important role. Covalent bonding. ionic bonding. and dipoledipole interactions are included in these interactions. The bond formed is very strong that detachment of the ligand is very hard and non-spontaneous.

Hydrogen bonds, Alkyl bonds, Pi-sigma bonds and Pialkyl bonds are the types of bonds developed between various ligands and the proteins. Within drugs and their targets, the majority of the bonds will be covalent. Covalent bonds are strong and hence drugs forming them will usually be permanently bound to their target. These include the alkyl, pi-sigma and pi-alkyl bonds which have been observed in our study. Hydrogen bonds play an important role in the interaction of ligand with protein. Hydrogen bonds are important in holding together the structure of proteins and DNA other than in drug-target interactions.

This could be the force that probably is responsible for inactivating the protein thus preventing disease progression in some cases and in other cases complete suppression. Citronellol and Geraniol, compounds from lemon grass interacted with the enzyme Dispersin B from

Aggregatibacter Actinomycetemcomitans effectively. Thus, we can effectively use lemon grass as it could have potential use in the inhibition of the Aggregatibacter Actinomycetemcomitans and act as potential natural products against periodontal diseases.

Molecular docking technique is very helpful as it is possible to study these chemicals and their interactions with the target protein at structural and functional levels. There is a possibility that the other herbs which can produce such chemicals that may exhibit similar effect on the bacteria and help in reducing their number. Therefore, molecular docking technique can be used as an effective method for screening natural compounds against pathogens as a preliminary step in drug discovery process for treating various diseases. Also, this will be helpful in saving years of wet lab research. It can save research and development resources and time to a great extent in drug discovery process.

Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identfied and quantified. This makes it supreme for the examination of the hundreds of relatively low molecular weight compounds found in environmental materials. In the present study a total of 11 compounds were obtained after GCMS analysis but 3 compounds were selected as the compounds that were expected their presence in essential oil. It is well-known that grass plants produce terpenoidal hydrocarbons and ethylene oxides that can be grouped as medicinal, industrial, and perfumery, depending on their chemical composition. The major components were geraniol, citronellol and D-limonene. The quality of lemon grass is generally determined by its citral content.²⁸

The MIC is the lowest concentration of the extract that did not permit any visible growth of the organism. Onawunmi et al. had also reported the similar antibiotic susceptibility pattern and had suggested that the test organisms particularly gram negative were found to be more susceptible to lemongrass than standard antibiotics.²⁹ In a 13-oil studies, lemongrass oil was found to be among the most active against human dermatophyte strains inhibiting 80% of strains were reported and this is confirmed by the antifungal activity of Cymbopogon Citratus against strains of fungi species used as test organism and also confirm reports by traditional users of lemongrass against ring worm infections.^{30,31} The isolation of volatile oils in Cymbopogon citratus confirms the activity showed against the test organisms by this plant and also in part confirms the report of the oils isolated from same plant by distillation to exhibit great antibacterial activity and also confirms the potency of this particular plant against skin cancer prevention was reported.³² The limitation of the present study is the use of lemon grass in vivo(oral cavity) and conducting clinical trials to collect samples from oral cavity to analyse the reduction in microbial counts.

Conclusion

In present era of emerging multidrug resistance among gram positive and gram-negative organisms, lemongrass oil will be helpful in treating such diseases. GCMS analysis and minimum inhibitory tests helped us to know the antibacterial activity of lemon grass oil components against Aggregatibacter actinomycetum commitans. From the results of the present study, it can be concluded that the active ingredients from lemongrass oil containing **D**-Limonene Geraniol Citronellol. and showed antibacterial activity against Aggregatibacter actinomycetemcomitans with MIC of 15µl/ml. Therefore, formulations encompassing these compounds can be used to treat plaque induced periodontal disease.

*Model HP 5971 MSD, Hewlett Packard, USA.

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Tables and figures

Table 1: Docking studies conducted using components oflemon grass and Dispersin B

Protein ID	Active compounds (ligands)	Target bacterial protein	Binding affinity	Hydrogen bonds
1YHT	Citronellol		-4.9	1
	D-	Dispersin	-6.1	0
	Limonene	В	0.1	0
	Geraniol		-5.5	1

Table 2: Microbial inhibitory concentration(colony count CFU's 10^6)-Aggregatibacter actinomycetemcomitans vs Lemongrass **extract**

	+ve	50µ1	100µ1	150µ1	200µ1
	control				
Trial 1	437	203	253	189	169
Trial 2		287	321	210	186
Avg	437	245	287	200	178
%		43.9%	34.3%	54.2%	59.2%
Reduction					

Graph 1: Gas Chromatography Mass Spectrometry analysis



Graph 2: Percentage reduction in number of colonies of Aggregatibacter actinomycetum commitans



Figure 1: Molecular Structure of Dispersin-B



Figure 2: Hydrodistillation



Page 🖌

Figure 3: Filteration



Figure 4: Antimicrobial Property Of Lemon Grass Oil Against Aggregatibacter Actinomycetemcomitans By Well Diffusion Method



Figure 5: Antimicrobial Property Of Lemongrass Oil Aggregatibacter Actinomycetemcomitans In The Trypticase Soy Agar Plates (Mic)

