"ANTIBACTERIAL EFFICACY OF *ACHYRANTHES ASPERA* AND *TRACHYSPERMUM AMMI* EXTRACTS WITH CHLORHEXIDINE AGAINST PERIODONTAL PATHOGENS: AN IN-VITRO STUDY"

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Karnataka

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Dr. ANIL. V. ANKOLA **M.D.S.**
Professor and Head,
Department of Public Health Dentistry,
KLE Vishwanath Katti
Institute of Dental Sciences,
Belagavi-590010.

Date : 26-12-2022
Place : Belagavi

Professor & Head
Department of Public Health Dentistry
KLE VK Institute of Dental Sciences
Nehru Nagar, Belagavi-10.

Dr. ALKA KALE **M. D.S.**
Principal,
KLE Vishwanath Katti
Institute of Dental Sciences,
Belagavi-590010

Date : 24/12/22
Place : Belagavi

PRINCIPAL
KLE V.K. Institute of Dental Sciences
Nehru Nagar, BELAGAVI-590010.

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I, **REGISTRATION NO: IL0220003**, post-Graduate student in the subject of Public Health Dentistry have completed research work on the topic **"ANTIBACTERIAL EFFICACY OF *ACHYRANTHES ASPERA* AND *TRACHYSPERMUM AMMI* EXTRACTS WITH CHLORHEXIDINE AGAINST PERIODONTAL PATHOGENS: AN IN-VITRO STUDY",** in the year 2022**.**

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KLE VK Institute of Dental Sciences

A Constituent Unit of KLE Academy of Higher Education and Research
(Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi - 590 010, Karnataka State

Accredited 'A' Grade by NAAC (2nd Cycle) **Placed in Category 'A' by MHRD (GoI)**

☎: 0831-2470362 **Web: http://www.kledental-bgm.edu.in**
FAX: 0831-2470640 **E-mail:principal@kledental-bgm.edu.in**

Date : 24.12.2022 **Serial No. :** 130

PLAGIARISM CHECK REPORT

Name of the Applicant : DR. RAM SURATH KUMAR.K
UG / PG / Ph.D / Staff : POSTGRADUATE STUDENT
Batch & Year : 2020-23
Department : PUBLIC HEALTH DENTISTRY

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KLEVK Institute of Dental Sciences
KAHER-Belagavi

Chairman
Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
KAHER - Belagavi

BIOSTATISTICIAN REPORT

KLE V.K. Institute of Dental Sciences

(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA

Re-Accredited 'A+' grade by NAAC (3rd Cycle) & Placed in Category 'A' by MHRD (GoI)

: 0831-2470362
FAX: 0831-2470640

Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation of **Dr. Ram Surath Kumar K,** Post Graduate Student, under the guidance of **Dr. Anil V. Ankola** M.D.S, Professor & Head, Department of Public Health Dentistry, KLE Vishwanath Katti Institute of Dental Sciences, entitled **"Antibacterial efficacy of *Achyranthes aspera* and *Trachyspermum ammi* extracts with chlorhexidine against periodontal pathogens: An in vitro study"** has been done under my guidance and considered satisfactory.

Dr. J. B. Prasad

Place: Belagavi
Date: 20/12/2022

Name & Signature of Biostatistician

LIST OF ABBREVIATIONS

CHX	:	Chlorhexidine
Ca2+	:	Calcium ion(2+)
K+	:	Potassium ion(K+)
Mg2+	:	Magnesium ion(2+)
A.aspera	:	Achyranthes aspera
T.ammi	:	Tachyspermum ammi
%	:	Percentage
Aa.comitans	:	Actinobacillus actinomyces comitans
P.gingivalis	:	Porphyromonas gingivalis
T.forsythia	:	Tannerella forsythia
MIC	:	Minimum Inhibitory Concentration
MBC	:	Minimum Bactericidal Concentration
Gms	:	Grams
i.e.	:	Id est. (Latin), Means that is
IRB	:	Institutional Review Board
ICMR-NITM	:	Institute of the Indian Council of Medical Research-National Institute of Traditional Medicine

RMRC	:	Regional Medical Research Center
°C	:	Degree celsius
Mg	:	Milligram
Min	:	Minute
ml	:	Millilitres
%	:	Percentage
BHI Broth	:	Brain Heart Infusion Broth
Pvt.Ltd..,	:	Private Limited
ATCC	:	American Type Culture Collection
BSRC	:	Basic Science Research Center
CFU	:	Colony Forming Unit
W/V	:	Weight/Volume
DMF	:	Dimethylformamide
rpm	:	Rotation per minute
sec	:	Second
SPSS	:	Statistical Package for Social Sciences
µl	:	Microlitre
+	:	Positive
-	:	Negative

GC	**:**	Growth control
SN	**:**	Serial number
WHO	**:**	World Health Organization
E.coli	***:***	*Escherichia coli*
P.aeruginosa	***:***	*Pseudomonas aeruginosa*
S.aureus	***:***	*Staphylococcus aureus*
B.subtilis	***:***	*Bacillus subtilis*
V/V%	**:**	Volume/Volume Percentage
S. mutans	***:***	*Streptococcus mutans*
P.Vulgaris	***:***	*Proteus Vulgaris*
K.Pneumonia	***:***	*Klebsiella Pneumonia*
B.megaterium	***:***	*Bacillus megaterium*

ABSTRACT

Aim: To assess and compare the antibacterial efficacy of *A. aspera* and *T. ammi* extracts with 0.12% CHX against periodontal microorganisms (*Aa. comitans*, *P. gingivalis* and *T. forsythia)*.

Methodology: Roots of *A. aspera* and Seeds of *T. ammi* were procured and were dried under shade and ground into a coarse powder. Ethanol extracts of A. aspera and T. ammi were prepared by the Soxhlet apparatus method. The freshly prepared crude extracts were qualitatively analyzed for the presence of flavonoids, alkaloids, tannins, steroids and volatile oils. The plant extracts (*A. aspera* and *T. ammi)* were assessed for Minimum Inhibitory Concentration (MIC), and Minimum Bacterial Concentration (MBC) against selected periodontal pathogens in comparison to 0.12% CHX. A Rezazurin microtitre assay was used to determine the MIC of plant extracts. MBC was determined by the spread plating method. The experiments were prepared in triplicates. Kruskal Wallis Test followed by the Bonferroni Post-Hoc test was employed wherein the statistical significance was set at $p \leq .05$.

Results: When compared with the plant extracts, 0.12% CHX was found to have a higher inhibitory effect (MIC - 0.02 to 0.04 mg/ml) and higher bactericidal effect (MBC - 0.08mg/ml) against tested periodontal pathogens ($p < .001$). The mean MIC and MBC of the plant extracts for *Aa. comitans, P. gingivalis, T. Forsythia* are 12.5 mg/ml and 25 mg/ml, respectively.

Conclusion: Both *A. aspera* and *T. ammi* possess comparable antibacterial activity against periodontal pathogens (*Aa. comitans*, *P. gingivalis* and *T. forsythia)*when compared to CHX. It could be a valuable tool against periodontal pathogens as it is widely available and cost-effective in addition to being efficacious. *A. aspera* and *T. ammi* can be regarded as

promising multipurpose therapeutic agents. Hence, further clinical trials should be conducted to prove its effectiveness.

Keywords: Achyranthes aspera, Trachyspermum ammi, Ajwain, Chlorhexidine, antibacterial, extract, periodontal pathogens

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INTRODUCTION

"Nature Itself is the Best Physician."

- ***Hippocrates***

Gingivitis is a commonly occurring oral disease, characterized by bleeding and gingival inflammation primarily caused by microorganisms and inadequate oral hygiene.[1] The main cause of gingivitis is plaque containing periodontal pathogens that form on the surface of teeth and gums. The onset and development of periodontal diseases are strongly associated with red complex pathogens. The red complex encompasses three species of bacteria named *Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola.*[2] *Aggregatibacter actinomycetemcomitans,* on the other hand, is also frequently related to periodontal diseases.[3]

Mechanical plaque control techniques are the mainstay in maintaining oral hygiene that are time-consuming and require skill and motivation to be effective.[4] As a result, antimicrobial agents have been widely employed as a supplement to mechanical plaque control techniques. Chlorhexidine gluconate (CHX) has been widely recommended and used as an antimicrobial agent.[5]

CHX has been the gold standard since the 1940s because of its antibacterial efficacy and its substantivity.[6] CHX is effective against both Gram-negative as well as Gram-positive bacteria.[7] Saliva itself demonstrates antibacterial activity for around five hours following a single CHX rinse, and salivary bacterial counts are suppressed for over 12 hours.[8] Periodic repetition and multiple rinses of CHX help reduce the number of aerobic and anaerobic organisms by 80% to 90%.[9] At lower concentrations, CHX

affects the permeability of bacterial membrane by displacing Ca2+, K+, and Mg2+ ions from the cell wall and producing a bacteriostatic effect. Higher concentrations of CHX (>0.1%) result in the leakage of cell organelles, resulting in a bactericidal effect.[10] Despite the varying advantages, chlorhexidine carries the disadvantage of being a chemical that can cause dysgeusia, staining of teeth and tongue, xerostomia, and precipitation of calcium and phosphate ions from the tooth surface when used for a longer period.[8,11] To overcome such adverse effects of CHX, extensive research is being conducted in the field of alternative medicine. Modern society is more focused on the use of natural derivatives for curing ailments. Traditional medicine has gained popularity, due to its affordability, therapeutic value, and reputation for having fewer side effects as compared to synthetic drugs.[12]

Achyranthes aspera Linn belongs to the family Amaranthaceae [Uttarani in Kannada; Aghara in Hindi], which is a bounteous indigenous herb of Asia, Africa, and South America. Apamarga taila and agnimukha are the names of the ayurvedic preparations made from this plant. Uses include the treatment of pneumonia, snake bites, skin rashes, and corneal opacities. The active ingredients of *A. aspera* are utilised as an antibacterial, antifungal, antiviral, antimalarial, antiarthritic, antileprotic, antispasmodic, oestrogenic, purgative, diuretic, oestrogenic, and cardiotonic agent.[13] In ancient times, fresh *A. aspera* root was used as a toothbrush. The phytochemical analysis of the *A. aspera* extracts indicated the presence of flavonoids, phenolic compounds, tannins, phenolic compounds, steroids, alkaloids, etc. that may be responsible for its antibacterial and antioxidant activity.[14]

Trachyspermum ammi Linn belongs to the family Apiaceae, known in India as Ajowan [Oma in Kannada; Ajwain in Hindi], which is widely dispersed throughout Iran and India. Ajwain seeds have therapeutic benefits in medicine, including antifungal, antinociceptive, cytotoxic, and digestive effects. *T. ammi* has been associated with a variety of biological activities, including aphrodisiac properties, analgesic, antibacterial, antiviral, antifungal, antioxidant, and anti-inflammatory activity.[15] *T. ammi* seeds encompass the major constituents are phenolic compounds (Thymol- 59.9–96.4%, p-cymene- 0.6–21.2%, γ-terpinene- 0.2–17.8%, and carvacrol- 0.4–2.8%).[16–18] The essential oils extracted from T. ammi seeds exhibited antibacterial action, suggesting that they have the ability to suppress the growth of pathogenic organisms.[19]

A thorough literature review revealed that the antibacterial efficacy of *A. aspera* and *T. ammi* against periodontal pathogens (*Aa. comitans, P. gingivalis and T. Forsythia.*) has not been evaluated so far. The present study aims to fill this lacuna in scientific literature.

AIM OF THE STUDY

To assess and compare the antibacterial efficacy of *A. aspera* and *T. ammi* extracts with 0.12% CHX against periodontal pathogens.

OBJECTIVES OF THE STUDY

1. To determine the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of ethanol extract of *A. aspera*, ethanol extract of *T. ammi* and 0.12% CHX against *Aa. comitans, P. gingivalis* and *T. Forsythia.*

2. To compare the MIC and MBC of crude extracts of *A. aspera* and *T. ammi* with 0.12% CHX against *Aa. comitans, P. gingivalis* and *T. Forsythia.*

NULL HYPOTHESIS:

There is no difference in the antibacterial effect of extracts of *A. aspera and T. ammi* in comparison to 0.12% CHX against *Aa. comitans, P. gingivalis* and *T. Forsythia*.

ALTERNATIVE HYPOTHESIS:

There is a difference in the antibacterial effect of extracts of *A. aspera and T. ammi* in comparison to 0.12% CHX against *Aa. comitans, P. gingivalis* and *T. Forsythia*.

REVIEW OF LITERATURE

1. Achyranthes aspera

Achyranthes aspera (A. aspera) grows as a weed on roadsides and along field boundaries across India.[20],[21] Tropical Asia, Baluchistan, Australia, Sri Lanka, Africa, and America are also indigenous to the plant.[22] It is commonly known as Uttarani in Kannada; Aghara in Hindi. A wide range of phytochemical compounds extracted from the plant exhibit anti-allergic, antiperiodic, purgative, diuretic, laxative, hepatoprotective, antiasthmatic, and other essential therapeutic characteristics. Extensive research has been conducted over the last few decades to demonstrate its biological activity and pharmacology. Many chemical components, including oleanolic acid, saponins, dihydroxy ketones, long-chain compounds, alkaloids, and others have been identified and isolated.

Taxonomic classification

Kingdom	-	Plantae
Subkingdom	-	Tracheobinota
Super Division	-	Spermatophyta
Division	-	Mangoliophyta
Class	-	Magnoliopsida
Subclass	-	Caryophyllidae
Order	-	Caryophyllales
Family	-	Amaranthaceae
Genus	-	*Achyranthes*
Species	-	*Aspera*

Traditional Uses

The herb has traditionally been used to treat asthma, bronchitis and cough. It is a diuretic, pungent, antiperiodic, anti-phlegmatic, purgative, and laxative, and can be used to treat oedema, dropsy, piles, boils, and skin eruptions. In pneumonia, the crushed plant is cooked in water and used. In intestinal troubles, an infusion of the root part acts as a mild astringent. Ground blooming spikes or seeds mixed with water are used as an external therapy for venomous snake and reptile bites, as well as for night blindness and skin illnesses.[23] The ground root is administered together with water for snake bites until the victim pukes and regains consciousness. It is reported that inhaling the fumes of *A. aspera* combined with *Smilax ovalifolia* roots enhances appetite and heals several forms of gastrointestinal problems. It helps with haemorrhoids, and the seeds and leaves are emetic, hydrophobic, carminative, digestive, anti-inflammatory, and expel phlegm. Externally, the plant's ash is used to treat ulcers and warts. Crushed leaves are massaged on an aching back to relieve strain. [24] As a toothbrush, a fresh piece of root is utilised. Ophthalmia and corneal opacities are treated using a water-based root paste. Fresh leaf paste is used to relieve discomfort from wasp bites.[20] The herb can help with liver problems, rheumatism, scabies, and other skin issues.[21,25]

***A. aspera* as an antimicrobial agent**

According to Khan et al., the ethanol extract of the seeds showed an antibacterial effect against *Escherichia coli, Bacillus subtilis,* and *Pseudomonas aeruginosa.*[26] Saravanan et al. observed the antibacterial effect of leaf extracts against *E. coli, P. aeruginosa, Proteus vulgaris, S. aureus*, and *Klebsiella species.*[27] Sharma et al. investigated the antibacterial action against *S. aureus* in an alcoholic extract.[28] Manjula et al. investigated the antibacterial activity of *A. aspera* extracts against

various pathogenic organisms such as *E. coli, P. aeruginosa, Citrobacter species, B. subtilis,* and *Micrococcus species*.[29]

2. Trachyspermum ammi

Trachyspermum ammi (T. ammi) is an Egyptian native plant.[30] However, it is extensively spread and cultivated all around the world, including Iran, Iraq, Afghanistan, Pakistan, Afghanistan, India, and Europe.[31]

T. ammi is a medicinal plant of the Apiaceae family, which has 270 genera and species. In Hindi, it is known as Ajwain, in English as Bishop's weed, in Sanskrit as Yamini, in Kannada as Oma, and in Tamil as Omam.[32] The word Ajwain comes from the Sanskrit term ajomoda. Ajwain is a well-known and ancient Ayurvedic spice. It is a fragrant, tall annual plant with white blooms and little brownish berries. Ajwain seeds are tiny, grey, and have a bitter, peppery flavour when raw, but become milder when boiled. The seeds or fruit of the plant are the most widely utilised portion. It appears to be cumin seeds [15]. The antioxidant carotenoids, zeaxanthin, and lutein as well as other key nutrients, are concentrated in the dark green leaves.[33]

Ajwain seeds have been shown to have effects that are antiseptic, anaesthetic, stimulating, carminative, diuretic, antibacterial, antiviral, nematicidal, antiulcer, antihypertensive, bronchodilatory, antitussive, hepatoprotective, and antiplatelet, as well as antihyperlipidemic properties.

Taxonomic classification

Kingdom - Plantae

Division - Magnoliophyta

Order - Apiales

Class - Magnoliopsida

Family - Apiaceae

Genus - *Trachyspermum*

Species - *ammi* [34]

Traditional Uses

In the Indian medical system, ajwain is given for stomach problems, fruit paste is used externally to relieve colic pain, and fermented fruits are applied on the chest to treat asthma. Ajwan-ka-arak, an aqueous extract of ajwain, was utilised to treat diarrhoea.[35,36] Crushed ajwain leaves are capable of treating skin diseases. Ajwain powder was knotted in a thin handkerchief and regularly inhaled by Middle Easterners to lessen discomfort during the acute stage of a cold and/or migraine.[37] The tiny seeds have a fiery, spicy, bitter flavour and are an excellent appetiser. They have been used as a useful treatment for conditions including vomiting, heart illness, and oral diseases. It was thought to have diuretic and carminative properties and served as a successful treatment for illnesses including paralysis and limb weakness. Chest pain, liver illness, hiccups, and renal and spleen issues, were among the side effects of ajwain seeds. The ajwain seeds can be consumed with honey, protein, fat, minerals, fibre, carbs, iron, calcium, phosphorus, carotene, thiamine, riboflavin, and niacin. In India, Pakistan, and the southern part of Iran, ajwain seeds were used in vegetable curries, steaming cabbage, carrots, potatoes, and pumpkin.

In Indian cuisine, the seed was ground in a mortar and pestle and cooked with butter to prepare an aromatic butter sauce that was used to flavour vegetable dishes. One of its most well-known therapeutic benefits is its capacity to lessen flatulence. For the treatment of diarrhoea and colic, it can also be prepared into tea.

Additionally, it possesses significant germicidal and fungicidal effects. The roots of ajwain have a diuretic effect. Its seeds contain roughly 50% thymol, a well-known essential oil that has antibacterial properties, and thyme, which may be utilised to boost the immune system to fend against viral diseases like the flu and colds.

Ajwain seeds and the tamarind seed kernel may be combined to make a potent aphrodisiac by frying an equal amount of each in pure ghee. This is used before night as a virility enhancer. When making an ear drop for an earache, 30 ml of milk is heated with half a teaspoon of ajwain seeds until the scent of the seeds permeated the milk. Three grams of ajwain seeds and 40 grams of sesame oil were heated together to treat ear pain. After being strained and allowed to cool to body temperature, the ajwain seed oil was utilised as ear drops. Bad breath is avoided by making eating ajwain seeds a habit. Ajwain seeds are wrapped in cotton fabric, cooked in a frying pan, and then applied to the chest and neck for bronchial asthma. Ringworm, itching, menstruation, and postnatal diseases can all be treated with it.[38,39] It is the most popular spice in the kitchen and is simple to use in a regular diet to treat or ward off a variety of other illnesses.[40]

T. ammi as an antimicrobial agent

Mayaud et al. reported that except for *P. aeruginosa*, *T. ammi* seed oil showed antimicrobial activity against 55 bacterial strains at a MIC of 2% *(v/v)*.[41] Essential oil of *T. ammi* showed an antibacterial effect against three Gram-negative bacterial strains (*E. coli*, *P. vulgaris*, and *K. pneumonia*) and three Gram-positive bacterial strains (*B. subtilis, S. aureus,* and *B. megaterium)*.[42] *T. ammi* seed extracts in ethanol exhibited antibacterial activity against two Gram-negative food deterioration bacteria, *P. aeruginosa* and *E. coli.*[43]

3. Chlorhexidine

Chlorhexidine (CHX), a bisbiguanide base, is a cationic antiseptic with broad-spectrum antibacterial activity (against Gram-positive and Gram-negative bacteria and certain myocytes).[44]

The International Union for Pure and Applied Chemistry (IUPAC) defines CHX as 1,1'-hexamethyle ne bis (5-[4-chlorophenyl] biguanide), having the chemical formula 22Cl2N10H30. CHX is available in three formulations: digluconate, acetate (both water-soluble) and hydrochloride (poorly soluble in water).[45]

Pharmacodynamics

Antibacterial properties can be found in CHX. As it is a cationic molecule, It attaches to the negatively charged bacterial cell wall as a cationic molecule and has the following properties:

1. A bacteriostatic action, at smaller concentrations. CHX stimulates the release of light molecules by changing the osmotic equilibrium of the bacterium cell (potassium and phosphorus).[46]
2. A bactericidal action, at higher concentrations. CHX induces cytolysis, which kills cells by releasing the key intracellular components, from the bacterial cell membrane. This changes the protein structure of the cell and results in the precipitation or coagulation of cytoplasmic proteins.[46,47]
3. With strong and selective adsorption to phosphate-containing substances, the negatively charged cell surface of the bacterium is readily attracted by the cationic CHX molecule. As a result, the integrity of the bacterial cell membrane is compromised, and CHX is drawn to the inner cell membrane. In the inner

membrane, CHX binds to phospholipids, increasing its permeability and allowing low-molecular-weight substances, such as K+, to seep out. When excess CHX is eliminated by neutralizers during this bacteriostatic stage, the effects of CHX may be reversed and recovery of the bacterial cell can occur. Low-molecular-weight cytoplasmic components become less permeable as CHX concentration rises, which suggests that the synthesis of phosphated complexes such as nucleic acids and adenosine triphosphate is what causes the coagulation and precipitation of the cytoplasm.[48]

In the 1970s, the substantivity property of CHX was first explained. The concentration, pH, temperature, and amount of time the solution remains in touch with the oral surfaces all have an impact on its substantivity. The ability of CHX to maintain effective concentrations for a longer length of time was connected to this property, and this prolongation of its activity made it particularly suitable for the inhibition of plaque formation.[49–51] Saliva by itself indicates an antibacterial activity after a single rinse of CHX for up to five hours, even while persistence at oral surfaces has been demonstrated to suppress salivary bacterial counts for twelve hours.[52]

Uses of CHX in dentistry

The effectiveness of CHX for the management of certain pathologic disorders as well as for the antisepsis of the oral cavity has been investigated in several clinical investigations. For the main and secondary prevention of gingivitis, periodontitis, and tooth decay, mouthwashes with CHX have been recommended. Particularly in patients who are unable to adequately manage plaque using standard at-home mechanical oral hygiene techniques alone.[53,54]

Formulations for dental use

Various concentrations of CHX are available on the market ranging from 0.02-0.3%). Unlike other preparations, mouthwashes allow the active ingredient to act in a general way throughout the entire oral cavity. As mentioned earlier, the type of action is dose-dependent: bactericidal at higher concentrations (0.12-0.2%) and Bacteriostatic at lower concentrations (0.02% - 0.06%).[46,47]

Two recent literature reviews showed negligible differences between 0.12% and 0.2% concentrations of CHX in mouthwashes in terms of gingival inflammation and plaque indexes, which were significantly reduced in both cases.[55,56]

The 0.12% concentration had a similar effect to the 0.2% concentration, particularly when the volume of mouthwash was increased from 10 to 15 mL.[57,58]

CHX is regarded as the benchmark by which other anti-gingivitis and anti-plaque medications are evaluated.[48]

Local side effects

Several clinical trials have described the occurrence of side effects associated with the use of CHX, however, all of these are completely reversible once the treatment is suspended.[59]

The most prevalent local side effect of CHX is a marked but transient increase in the pigmentation of the dorsal surface of the tongue, denture bases, restorative materials, and teeth. The presence of untreated plaque and certain chromogenic nutrients (metal ions and polyphenols), which are largely present in coffee and tea, in

the diet seems to make the pigmentation of the tooth surface, which is of a dark brownish colour, more noticeable.[60]

The usage of CHX mouthwash has also been associated with other local negative effects that have been recorded.

1. Increase in the formation of supragingival calculus however, it is not clear whether the formation of subgingival calculus is affected. This is due to the ability of CHX to precipitate both inorganic salts and salivary proteins on tooth surfaces.[61]
2. Temporary dysgeusia.[62]
3. Temporary sensations of burning and dry oral mucosa.[63,64]

Occasionally, erythematous and desquamative lesions of the oral mucosa have been described.[65] Cases of facial paraesthesia, swelling of the parotid glands and sialoadenitis have also been reported.[66]

Systemic adverse effects and adverse reactions

Preclinical animal studies on rats and other mammals ruled out the presence of carcinogenic, mutagenic and fertility-altering effects and determined the maximum lethal dose.[45]

Hypersensitivity reactions

Some authors have reported rare hypersensitivity reactions to CHX following topical application on the mucosa. These included some cases of anaphylactic shock, even at very low doses.[67] The reviewed literature contains two cases of systemic adverse effects concerning the use of mouthwashes: Moghadam reported the

occurrence of fixed erythema,[68] whilst Sharma described a case of CHX-related urticarial.[69]

Determination of MIC by Resazurin Method

The smallest concentration of an extract or drug required to inhibit the growth of microorganisms is known as the minimum inhibitory concentration (MIC). The Rezasurin microtitre method is one of the antibacterial assays to determine the MIC of each extract. Resazurin, a redox-sensitive dye, is used to examine cell viability. Live cells cause the fluorescent red Resorufin to reduce from the non-fluorescent blue Resazurin, however, dead cells do not reduce the Resazurin. In this study, the MIC of ethanol-based extracts of *A. aspera* (roots) and ajwain (*T. ammi*) seeds against *Aa. comitans, P. gingivalis, and T. forsythia* were identified using this fluorescence and observable change in colour.

Studies on *A. aspera* and *T. ammi*

An in-vitro study conducted by **Dadpe et al., 2018**[70] concluded that ajwain oil has a comparable antibacterial effect against oral microorganisms to CHX and has the potential to be a safe, natural, and affordable therapeutic antiplaque agent. Compared to CHX at the same dose, it exhibits much greater bacteriostatic and bactericidal activity. However, the findings were rather intriguing. *T. ammi* oil is widely and organically available. It does not irritate or harm people in any way. Compared to CHX made artificially, it is less expensive. The study suggests that *T. ammi* oil may one day be employed as a powerful antiplaque agent.

A study conducted by **Boyapati et al., 2017**[71] reported that *A. aspera* gel, used as a nonsurgical local drug delivery system in periodontitis patients after a complete Scaling and root planing, has demonstrated satisfactory outcomes with a favourable prognosis and minimal adverse effects. Local delivery of *A. aspera* gel combined with SRP demonstrated a positive outcome. As a non-surgical local drug delivery system technique, *A. aspera* gel has been shown to be completely safe for the treatment of periodontitis. *A. aspera* gel possesses a wide range of pharmacological activities, including potent anti-inflammatory and antioxidant actions.

An in vitro study conducted by **Yadav et al., 2016**[72] concluded that The prevention of dental caries and periodontal disease is greatly aided by *A. aspera,* which exhibited strong antibacterial activity against oral microorganisms, particularly those that contribute to the formation of sub and supra-gingival biofilms.

A randomized controlled trial conducted by **Petramfar et al., 2016**[73] concluded that a 10% topical cream containing ajwain essential oil can help neuropathy patients' burning feet and be used as a solution to treat neuropathic pain.

A randomized controlled trial conducted by **Bansal et al., 2015**[74] concluded that the effectiveness of CHX, *A. aspera,* and *Punica granatum* against *Streptococcus mutans* was statistically significant, with CHX being just slightly superior to the other two. The findings of this 7-day trial show that even a brief period of mouthwash usage significantly lowers *S. mutans* count and plaque index scores. Natural substances like *P. granatum* and *A. aspera* may have advantageous effects.

According to **Khan et al., 2010**[75] in vitro investigation, the cariogenic potential of *S. mutans* was tested against the activity of T. ammi seeds. It substantially

decreased its adhesion, biofilm generation, hydrophobicity, and GTF's ability to synthesise insoluble glucans. These findings suggest that this substance has strong anti-cariogenic potential.

Kumar V et al., 2009[76] in vivo investigation on Wistar albino rats concluded that the investigation into the potentially beneficial anti-inflammatory properties of *A. aspera* roots had been scientifically proved.

An in-vivo study conducted by **Edwin et al., 2008**[77] found that ethanol-based extract of *A. aspera* has substantial antioxidant and wound healing activity in Wistar rats. The findings of this study appear to support the long-standing practice of using *A. aspera* to treat wounds. It is possible to create phytomedicines for the treatment of septic wounds using the extracts of the plant.

MATERIAL AND METHODS

The current study was designed to assess and compare the antibacterial efficacy of *A. aspera* and *T. ammi* extracts with 0.12% CHX against periodontal microorganisms. It was an in vitro study designed and conducted from March to July 2021 in the Department of Public Health Dentistry, KLE VK Institute of Dental Sciences, KLE Academy of Higher Education and Research, Belagavi, India.

Ethical Considerations

The ethical clearance was obtained from the Institutional Research and Ethics Committee with reference number: 1454 (Annexure-I).

Steps In Evaluation Of Antibacterial Efficacy Of *A. Aspera* And *T. Ammi*

1. Procurement of materials
2. Preparation of plant extracts
3. Phytochemical screening
4. Instruments and materials
5. Determination of minimum inhibitory concentration
6. Determination of minimum bactericidal concentration

1. PROCUREMENT OF MATERIALS:

Roots of *A. aspera* were obtained from the Xavier Research Foundation, St. Xavier's College, Tirunelveli, Tamil Nadu (Annexure-II). Seeds of *T. ammi* were obtained from the KLE Ayurveda Pharmacy of KAHER's Ayurveda College, Belagavi, Karnataka.

2. PREPARATION OF EXTRACTS

Plant Materials Collection and Authentication

A. aspera plant was authenticated by the taxonomist in the ICMR-National Institute of Traditional Medicine (ICMR-NITM), Belagavi and the herbarium specimen of the same has been deposited in the herbaria for future use with accession number RMRC-1618 (Annexure-III).

Extract Preparation of A. aspera and T. ammi

Fresh roots of *A. aspera* and seeds of *T. ammi* were dried under shade and ground into a coarse powder. Ethanol extracts of *A. aspera* and *T. ammi* were prepared by the Soxhlet apparatus method in 600ml of solvent under 50ºC *A. aspera* and *T. ammi* extracts were prepared after the cyclic procedure with the duration of 8 hours and 5.5 hours, respectively. The cycles were repeated until the solvent transformed from a coloured to a colourless one. A total of 150 gms of coarse powders (*A. aspera* and *T. ammi*) were utilized in 600 ml of solvent each in a 1:4 ratio to produce an extraction yield of 13.9 gms of *A. aspera* (9.3%) and 30.2 gms (20.1%) of *T. ammi* crude extracts. The extracts were removed from the apparatus and dried in the rotary evaporator. The sterile crude extracts were stored at −20 °C for further use.

3. PHYTOCHEMICAL SCREENING

Freshly prepared crude extracts of *A. aspera* and *T. ammi* were tested qualitatively for the presence of phytochemical constituents that are responsible for antibacterial activity. Preliminary phytochemical screening was conducted using the standard procedures as suggested by Evans et al.[78] and Sofowora et al.[79]. The extract of *A. aspera* was qualitatively analyzed for the presence of flavonoids, alkaloids, tannins, phenolic compounds, and steroids. Phytochemical screening was performed

using different chemical tests as follows: (i) for *Flavonoids* - Sulphuric acid test and Lead acetate tests; (ii) for *Alkaloids* - Dragendorff's test and Mayer's test; (iii) for *Tannins and Phenolic compounds* – Ferric chloride test and Lead Acetate test; (iv) for *Steroids* - Salkowski's reaction and Liebermann's test. (Annexure-IV). Similarly, the extract of *T. ammi* was also qualitatively analyzed by using the above-mentioned methods for the presence of flavonoids, alkaloids, tannins, phenolic compounds, steroids, and volatile oils- Solubility test in 90 % Alcohol (Annexure-V).

4. INSTRUMENTS AND MATERIALS

1. Materials required for preparation of extracts
    - Coarse powder of *A. aspera*
    - Coarse powder of *T. ammi*
2. Armamentarium required for the microbiological investigation:
    - Test tubes
    - Flasks
    - Muslin cloth
    - Culture plates
    - Inoculating loops
    - Micropipette
    - Brain–heart infusion (BHI) broth and agar, HiMedia Laboratories Pvt. Ltd., Mumbai, India.
    - Resazurin dye
    - Distilled water
    - Chlorhexidine hydrochloride salt (CHX) BP grade, ICPA Health Products Ltd., Mumbai, India.

3. Standard bacterial strains:

   - *Aggregatibacter actinomycetemcomitans*,
   - *Porphyromonas gingivalis*,
   - *Tannerella forsythia*.

5. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

Source of microorganisms: The standard strains of *Aa. comitans* (ATCC 29523), *P. gingivalis* (ATCC 33277) and *T. forsythia* (ATCC 43037) were procured from KLE's Dr. Prabhakar Kore Basic Science Research Centre (BSRC).

Preparation of microbial inocula/bacterial suspension: A direct colony suspension of each bacterial isolate was prepared in Brain–Heart Infusion (BHI) broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The turbidity was adjusted to 0.5 McFarland Standard ($1X10^8$ CFU/ml).

Preparation of BHI broth: About 3.7g of the brain–heart infusion broth was added and mixed in 100ml of distilled water to prepare the BHI broth and kept in a refrigerator.

Preparation of stock solutions [A. aspera (w/v) and T. ammi (w/v)]: About 50 mg of sterile extracts [*A. aspera (w/v)* and *T. ammi (w/v)*] which is in semi-solid form were reconstituted in 1000 µl of 10% Dimethylformamide (DMF) *(w/v)*. The mixture was shaken vigorously by using a MixMate® Vortex agitator (Eppendorf, Sydney, Australia) for 3 minutes at 1000 rpm (maximum setting) and then bath sonicated using

Branson bath® sonicator 1800 (Branson Ultrasonics, Danbury, CT) for 15 minutes giving a final concentration of extract at 50 mg/ml.

Preparation of 0.12% chlorhexidine solution (positive control): Twelve mgs of CHX salt was mixed and diluted in 10 ml of distilled water (1.2 mg/ml). The mixture was shaken vigorously by using a MixMate® Vortex agitator (Eppendorf, Sydney, Australia) for 3 minutes at 1000 rpm (maximum setting) and then bath sonicated using Branson bath sonicator 1800 (Branson Ultrasonics, Danbury, CT) for 15 minutes to obtain a final concentration of 10 ml of 0.12% of CHX solution.

Preparation of negative control and growth control: Uninoculated tubes containing BHI supplemented with various plant extracts were used as a negative control. In the preparation of growth control, about 100 µl of bacterial strain were inoculated into tubes containing BHI without the plant extract

Determination of Minimum inhibitory concentration (MIC) by Resazurin Method:

A Resazurin microtitre assay was used to determine the MIC of plant extracts (*A. aspera* and *T. ammi)* and 0.12% CHX (positive control) against *Aa. comitans, P. gingivalis, T. Forsythia*.

A volume of 100 µl of the test material was added in the first column of the 96-well plate (*A. aspera* – 1A to 1C, *T. ammi* – 1D to 1F, Positive control in 1H). To all the wells, 100 µl of sterile BHI broth was added. The different concentrations (50 to 0.2 mg/mL) of extracts were prepared by a two-fold serial dilution method. Finally, 10 µl of standardized bacterial suspension ($1x10^8$ CFU/ml) was added to the respective wells containing the serially diluted plant extracts.

In addition to these, well labelled as 12F was filled with 200 µl of BHI broth to ensure the sterility of the broth (negative control), confirming no contamination occurred while preparing the plate. About 200 µl of BHI broth was added along with 10 µl of bacterial suspension to another well marked 12A, which served as growth control to ensure bacterial growth. The BHI broth of 200 µl was added along with 10 µl of bacterial suspension to another well labelled as 12A served as a growth control to ensure bacterial growth. The microtitre plates were prepared in triplicate. The plates were sealed and incubated anaerobically in an anaerobic jar using the microaerophilic atmosphere generation system at 37 °C for 30 hours. After the period of incubation, 10 µl of Resazurin solution (0.5mg/ml) was added to each well and further incubated for 4 hrs. The resulting change in colour to pink/red was inferred as an indication of bacterial growth and no change in Resazurin blue/purple was inferred as an indication of inhibition of bacterial growth. The MIC was the lowest concentration with no change of resazurin colour (inhibition of bacterial growth). The range of extract concentration in the wells was 50–0.19 mg/ml (Figures.1 and 2).

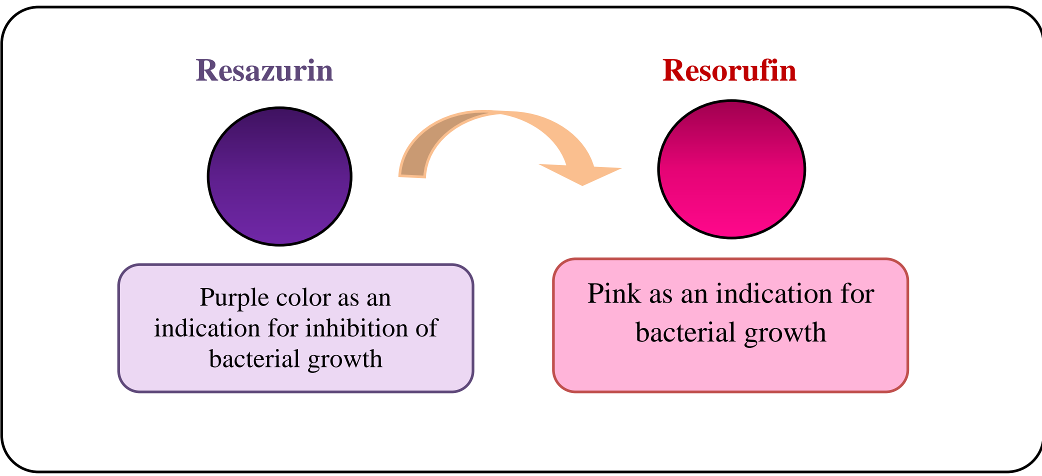


Figure 1. Mechanism of Action

Preparation of a microtitre plate in aseptic conditions

↓

Preparation of a stock solution of test samples (w/v)

↓

Different concentrations (50–0.19 mg/ml) of the test material was transferred to the wells to attain serial dilutions

↓

100 µl of sterile BHI broth was filled into the wells

↓

Finally 10 µl of bacterial suspension (1x108 CFU/ml) was added to each well

↓

Plates were placed in an incubator set at 37 °C for 30 hrs

↓

To each well, 10 µL resazurin dye (0.5mg/ml) was added

↓

Colour change from purple to pink indicated the presence of living bacteria (Resazurin reduced to Resorufin)

↓

Lowest concentration at which the color change observed was taken as MIC values for the test material.

↓

The experiment was performed in triplicate.

Figure 2. Determination of Minimum Inhibitory Concentration

6. DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATION (MBC)

MBC was determined by the spread plating method. About 20 µl of the bacterial suspension from the wells with concentrations higher than the MIC value was subjected to inoculation on plates containing BHI agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) and were incubated at 37 °C for 24 hours. The concentration at which the bacteria were completely killed was taken as MBC. The experiments were prepared in triplicate.

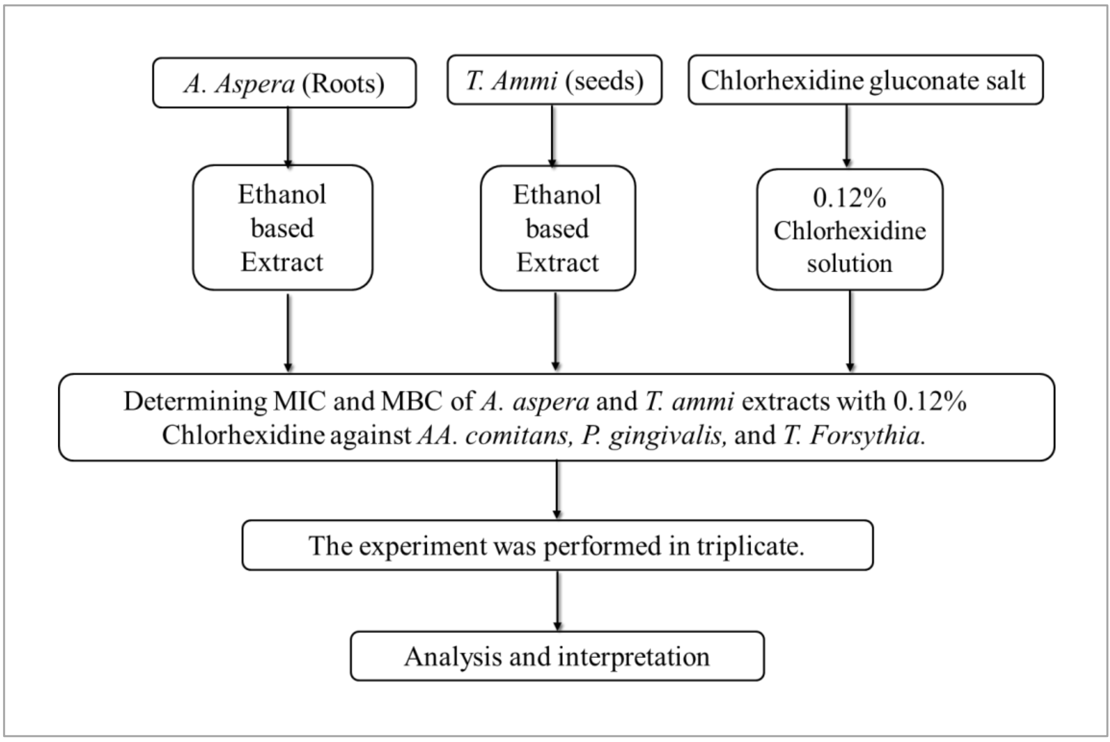


Figure 3. Methodology in flow diagram

STATISTICAL ANALYSIS

"Data obtained were entered in Microsoft Excel (2020) and analyzed using the SPSS® (IBM Corp. Released 2012 IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). The descriptive statistics were presented as mean ± standard deviation for continuous variables."

Kruskal Wallis Test followed by the Bonferroni Post-Hoc test was employed to compare the differences in the antibacterial activity of individual extracts and 0.12% CHX against *Aa. comitans, P. gingivalis, T. Forsythia*. Statistical significance was set at $p \leq 0.05$.

PHOTOGRAPHS

Photograph 1a) ***Achyranthes aspera*** **plant**

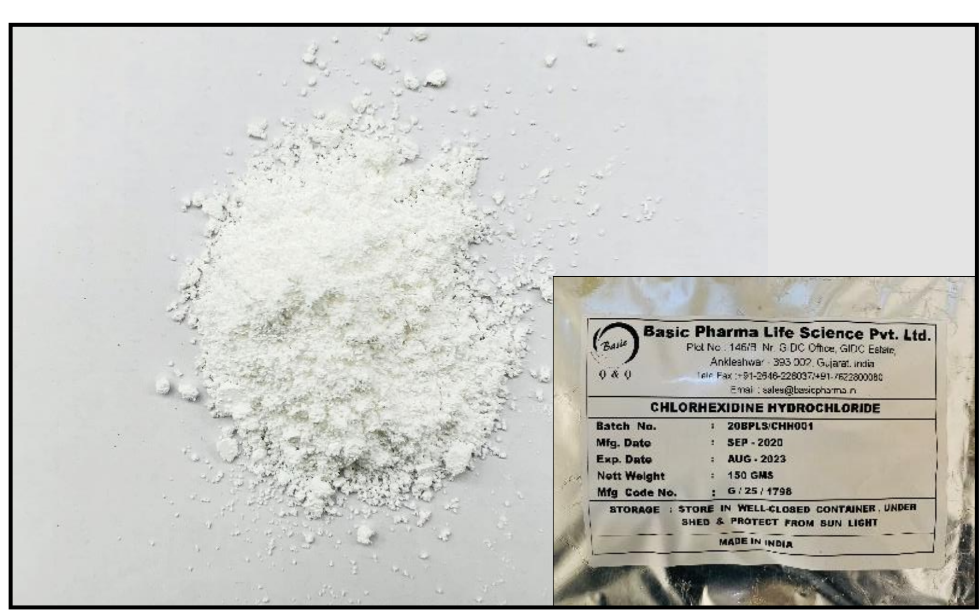
Photograph 1b) ***Trachyspermum ammi*** **plant**

Photograph 1c) ***Achyranthes aspera*** **(Roots)**

Photograph 1d) ***Trachyspermum ammi*** **(Seeds)**

a) *Achyranthes aspera* b) *Trachyspermum ammi*

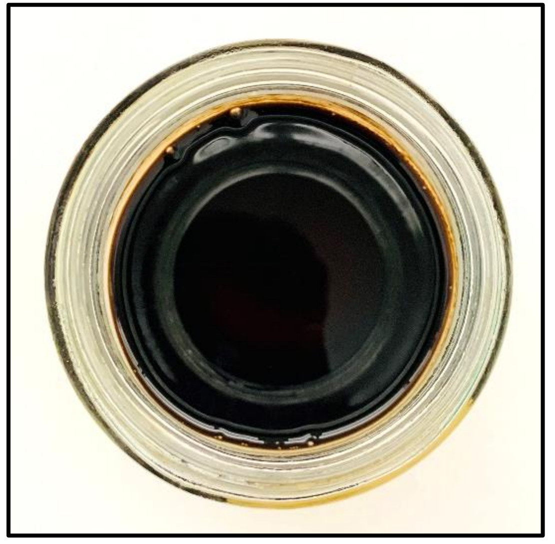
Photograph 2. Coarse powder



Photograph 3. Chlorhexidine Hydrochloride salt

Photograph 4. Soxhlet Apparatus for preparation of *Achyranthes aspera* and *Trachyspermum ammi* crude extracts

Photograph 5. IKA Rotary evaporator to extract solvent from crude extracts



a) **Ethanol based *Achyranthes aspera* extract**

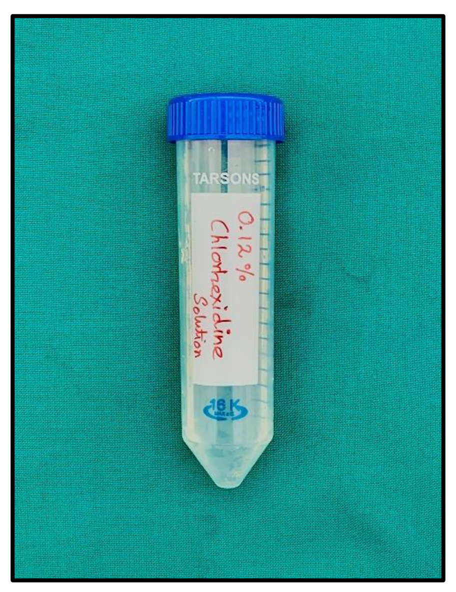
b) **Ethanol based *Trachyspermum ammi* extract**

Photograph 6. Crude Extracts

a) Vortex b) Tip Sonicator c) Bath Sonicator

Photograph 7. Preparation of the stock solutions

a) *Achyranthes aspera* and *Trachyspermum ammi* (50 mg/ml)



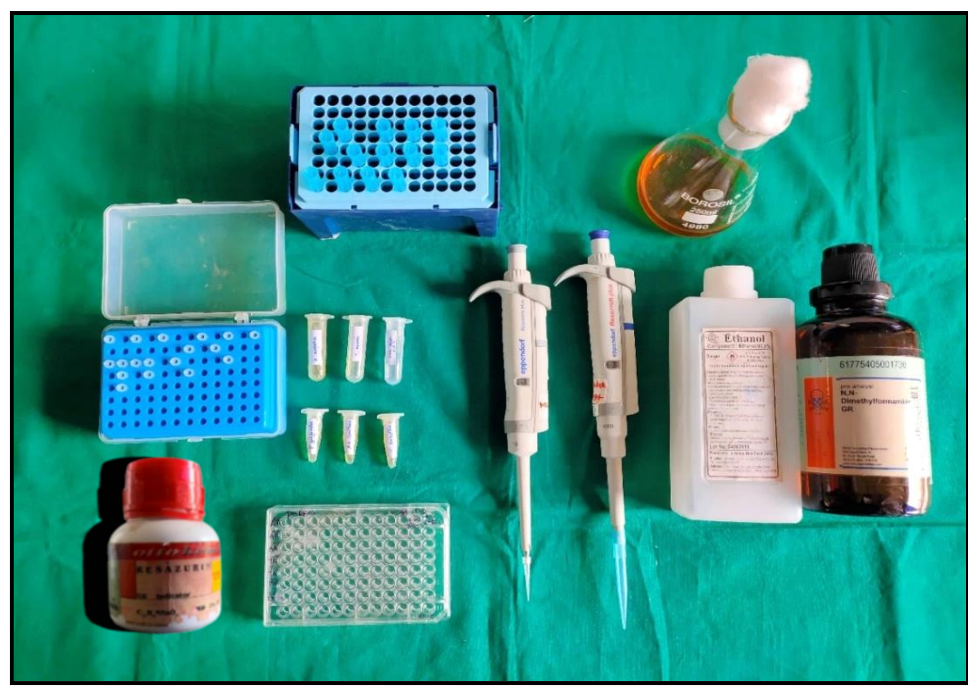
b) 0.12% Chlorhexidine solution (1.2 mg/ml)

Photograph 8. Stock solutions

Photograph 9. Preparation of working Solutions

a) *Achyranthes aspera extract* (50 mg/ml), b) *Trachyspermum ammi* (50 mg/ml) and c) 0.12% Chlorhexidine solution (1.2 mg/ml)

Photograph 10. Preparation of microbial inocula/bacterial suspension
The standard strains of *Aa. comitans* (ATCC 29523), *P. gingivalis* (ATCC 33277) and *T. forsythia* (ATCC 43037)



Photograph 11. Armamentarium used for microbiological investigation

Photograph 12. Microbiological investigation

RESULTS

Qualitative phytochemical screening

The preliminary qualitative phytochemical screening of the *A. aspera* determined various bioactive components of which flavonoids, alkaloids, tannins, phenolic compounds and steroids were identified. Similarly, phytochemical screening of the *T. ammi* extract determined bioactive components like flavonoids, alkaloids, tannins, phenolic compounds, steroids, and volatile oils. The results of the phytochemical screening of *A. aspera* and *T. ammi* extracts are outlined in Tables 1 and 2.

Antibacterial activity

Tables 2 and 3 summarize the mean MIC and MBC of the plant extracts (*A. aspera, T. ammi*) and 0.12% CHX against *Aa. comitans, P. gingivalis, T. Forsythia*. When compared with the plant extracts, 0.12% CHX was found to have a higher inhibitory effect (MIC - 0.02 to 0.04 mg/ml) and higher bactericidal effect (MBC - 0.08mg/ml) against *Aa. comitans, P. gingivalis, T. Forsythia*. Kruskal Wallis test indicated a statistically significant difference between the plant extracts and CHX, *p*-value = 0.018 (Figures 4 and 5).

The mean MIC and MBC of the plant extracts for *Aa. comitans, P. gingivalis, T. Forsythia* are 12.5 mg/ml and 25 mg/ml, respectively. Figures 6-8 depict the MIC and Figures 9-11 depict the MBC of the plant extracts and CHX against *Aa. comitans, P. gingivalis, T. Forsythia*.

TABLES

Table 1. Phytochemical Screening of the *Achyranthes aspera extract*

Test done	Observation	Inference
	Flavonoids	
Sulphuric acid test	A deep yellow solution was observed	+
Lead acetate tests	A yellow precipitate was observed	+
	Alkaloids	
Dragendoff's test	An orange-brown precipitate was observed	+
Meyer's test	Precipitate was observed	+
	Tannins and Phenolic compounds	
Ferric chloride test	A deep blue-black colour was observed	+
Lead acetate	A white precipitate was observed	+
	Steroid	
Salkowski's reaction	An appearance of red chloroform layer or greenish-yellow fluorescence in the acid layer	+
Liebermann's test	No appearance of blue colour	-

Positive (+): Present; Negative (-): Absent

Table 2. Phytochemical Screening of the *Trachyspermum ammi extract*

Test done	Observation	Inference
Flavonoids		
Lead acetate tests	A yellow precipitate was observed	+
Alkaloids		
Meyer's test	Precipitate was observed	+
Tannins and Phenolic compounds		
5 % Ferric chloride	A deep blue-black colour was observed	+
Steroid		
Salkowski's reaction	An appearance of red chloroform layer or greenish-yellow fluorescence in the acid layer	+
Volatile oil		
Solubility test in 90 % Alcohol	Soluble in Chloroform	+

Positive (+): Present; Negative (-): Absent

Table 3. Determination of MIC of *Achyranthes aspera* extract, *Trachyspermum ammi* extract, and 0.12% Chlorhexidine hydrochloride against tested periodontal pathogens

Test organism	MIC (mg/ml)			Statistics	
	0.12% CHX	***Achyranthes aspera***	***Trachyspermum ammi***	***Z*-value**	***p*-value**
Aggregatibacter actinomycetemcomitans	$0.04^{\alpha\beta}$	12.5^{α}	12.5^{β}	8.000	0.018*
Porphyromonas gingivalis	$0.04^{\alpha\beta}$	12.5^{α}	12.5^{β}	8.000	0.018*
Tannerella forsythia	$0.02^{\alpha\beta}$	12.5^{α}	12.5^{β}	8.000	0.018*

MIC: Minimum inhibitory concentration; CHX: Chlorhexidine hydrochloride salt.
The results are shown as average values of triplicate.
Test Applied: Kruskal Wallis test; Post-Hoc test Applied: Bonferroni Post-Hoc test, superscript with similar Greek symbols indicate a significant difference between the CHX and extracts (in the row); level of significance: * $p \leq 0.05$ is considered statistically significant.

Table 4. Determination of MBC of *Achyranthes aspera* extract, *Trachyspermum ammi* extract, and 0.12% Chlorhexidine hydrochloride against tested periodontal pathogens

Test organism	MBC (mg/ml)			Statistics	
	0.12% CHX	***Achyranthes aspera***	***Trachyspermum ammi***	***Z*-value**	***p*-value**
Aggregatibacter actinomycetemcomitans	$0.08^{\alpha\beta}$	25^{α}	25^{β}	8.000	0.018*
Porphyromonas gingivalis	$0.08^{\alpha\beta}$	25^{α}	25^{β}	8.000	0.018*
Tannerella forsythia	$0.08^{\alpha\beta}$	25^{α}	25^{β}	8.000	0.018*

MBC: Minimum bactericidal concentration; CHX: Chlorhexidine hydrochloride salt.
The results are shown as average values of triplicate.
Test Applied: Kruskal Wallis test; Post-Hoc test Applied: Bonferroni Post-Hoc test, superscript with similar Greek symbols indicate a significant difference between the CHX and extracts (in the row); level of significance: * $p \leq 0.05$ is considered statistically significant.

FIGURES

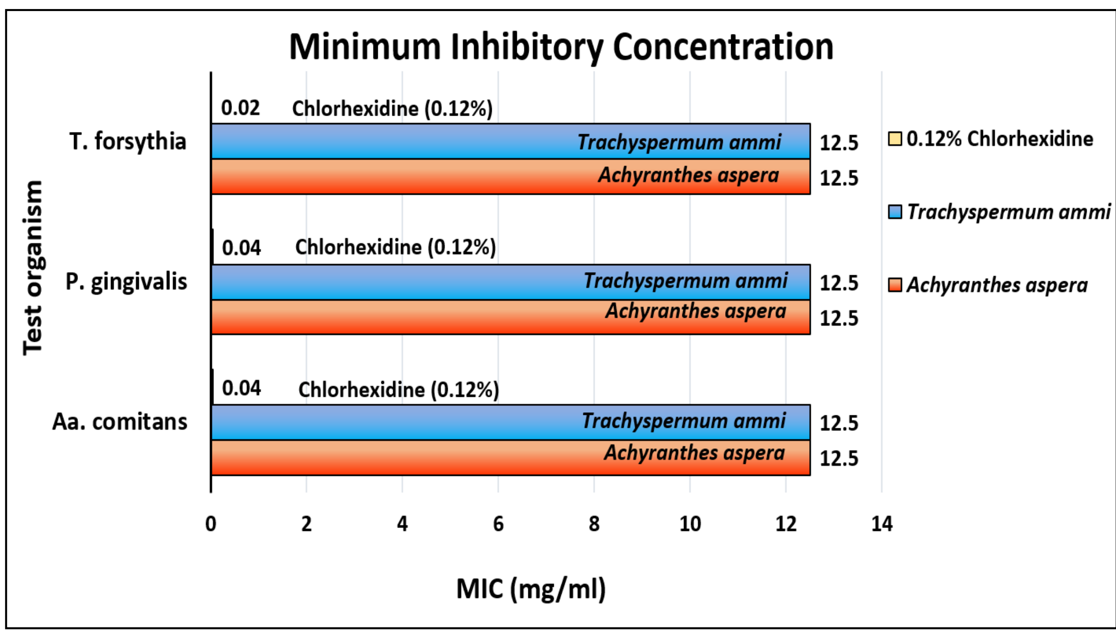


Figure 4. MIC of *Achyranthes aspera* extract, *Trachyspermum ammi* extract, and 0.12% Chlorhexidine hydrochloride against tested periodontal pathogens

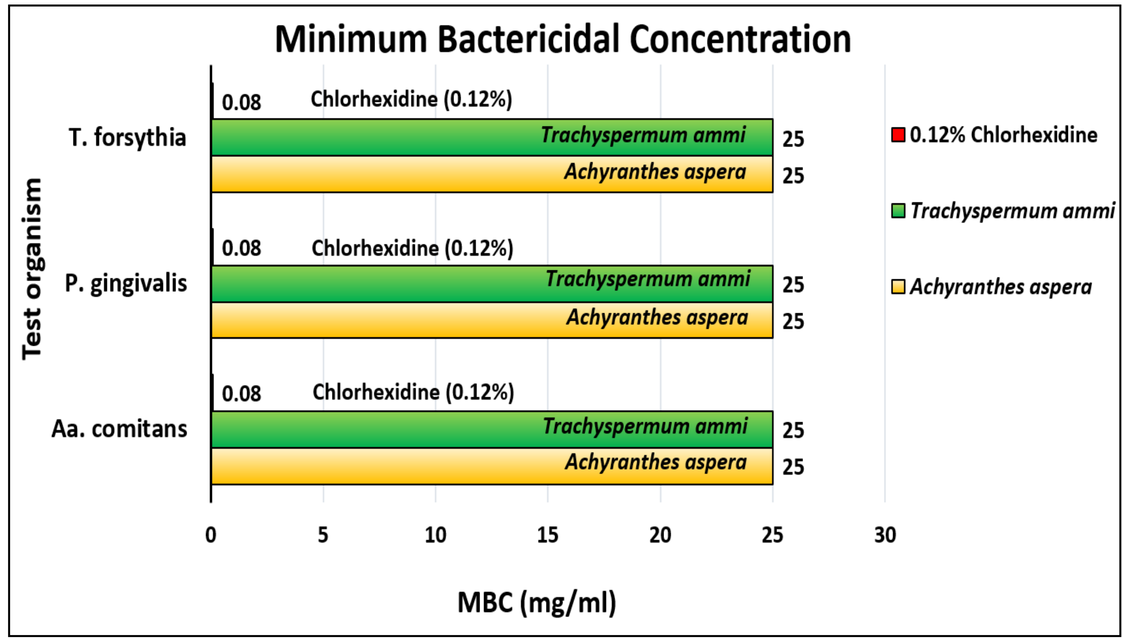
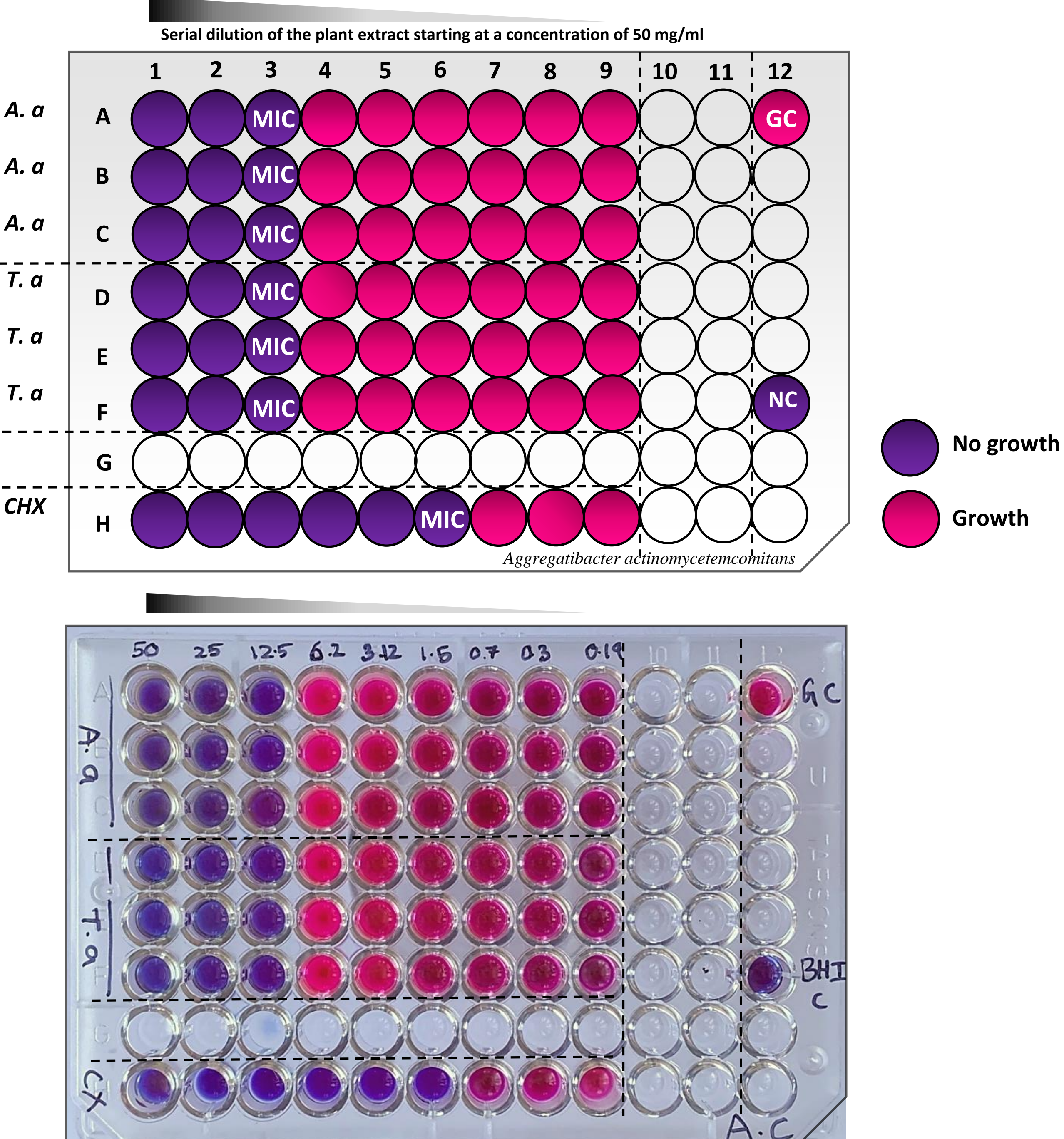
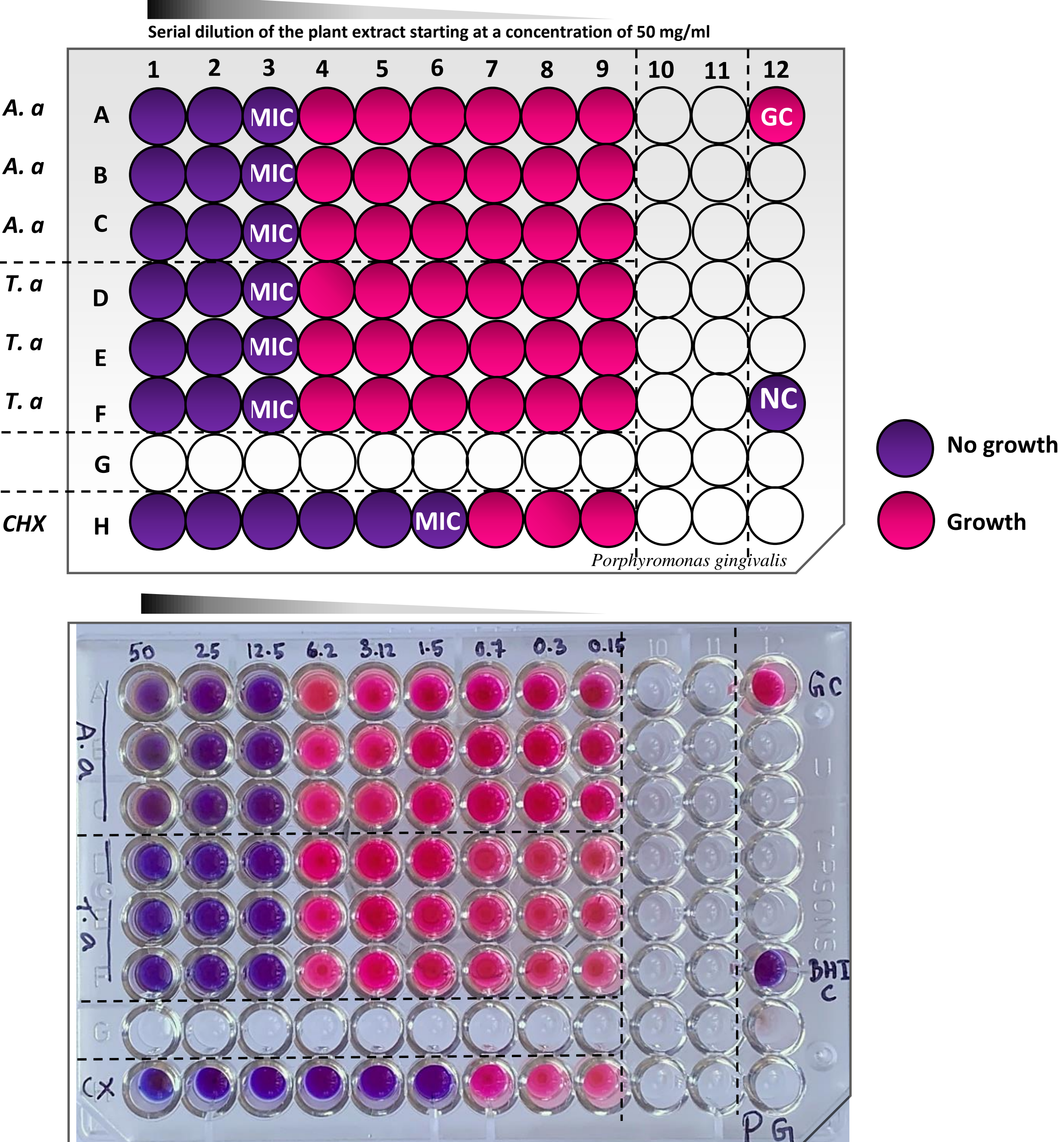


Figure 5. MBC of *Achyranthes aspera* extract, *Trachyspermum ammi* extract, and 0.12% Chlorhexidine hydrochloride against tested periodontal pathogens



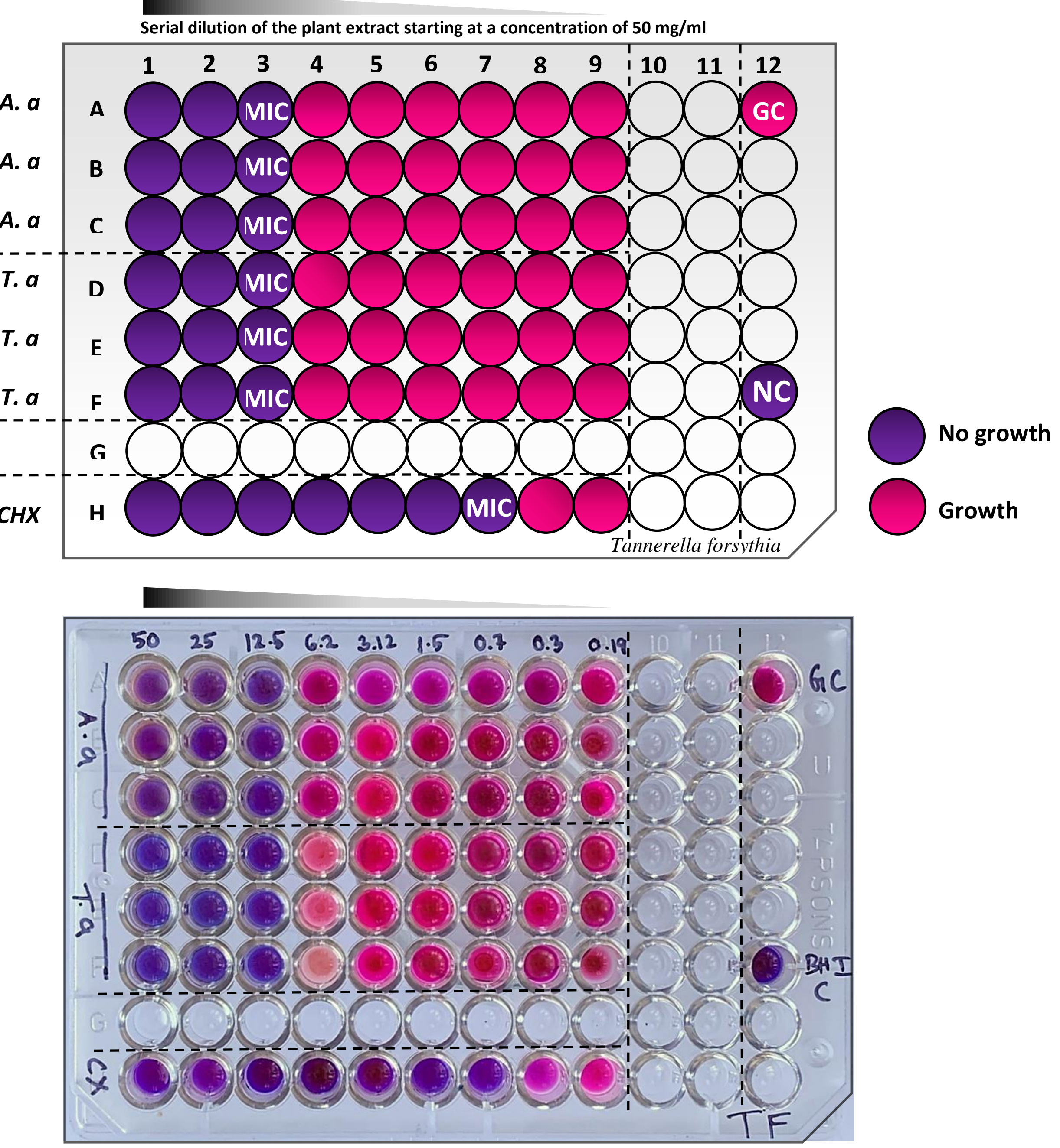
A.a, Achyranthes aspera; T.a, Trachyspermum ammi; CHX, 0.12% Chlorhexidine Hydrochloride; GC, Growth control; BHI C, Negative/Sterility control

Figure 6. Resazurin microtitre assay for determining MIC of *Achyranthes aspera, Trachyspermum ammi,* and 0.12% Chlorhexidine Hydrochloride (positive control) against *Aggregatibacter actinomycetemcomitans*. The highest concentration incorporated into the plate is 50 mg/ml and the lowest achieved through double serial dilution is 0.19 mg/ml. Column 3 shows no changes in colour from blue to pink, therefore the concentration of *A. aspera* and *T. ammi* extracts in that column was taken as the MIC value. The range of extract concentration in the wells was 50–0.19 mg/ml.



A.a, Achyranthes aspera; T.a, Trachyspermum ammi; CHX, 0.12% Chlorhexidine Hydrochloride; GC, Growth control; BHI C, Sterility control

Figure 7. Resazurin microtitre assay for determining MIC of *Achyranthes aspera, Trachyspermum ammi,* and 0.12% Chlorhexidine Hydrochloride (positive control) against *Porphyromonas gingivalis*. The highest concentration incorporated into the plate is 50 mg/ml and the lowest achieved through double serial dilution is 0.19 mg/ml. Column 3 shows no colour changes therefore the concentration of *A. aspera* and *T. ammi* extracts in that column was taken as the MIC value. The range of extract concentration in the wells was 50–0.19 mg/ml.



A.a, Achyranthes aspera; T.a, Trachyspermum ammi; CHX, 0.12% Chlorhexidine Hydrochloride; GC, Growth control; BHI C, Sterility control

Figure 8. Resazurin microtitre assay for determining MIC of *Achyranthes aspera, Trachyspermum ammi,* and 0.12% Chlorhexidine Hydrochloride (positive control) against *Tannerella forsythia*. The highest concentration incorporated into the plate is 50 mg/ml and the lowest achieved through double serial dilution is 0.19 mg/ml. Column 3 shows no colour changes therefore the concentration of *A. aspera* and *T. ammi* extracts in that column was taken as the MIC value. The range of extract concentration in the wells was 50–0.19 mg/ml.

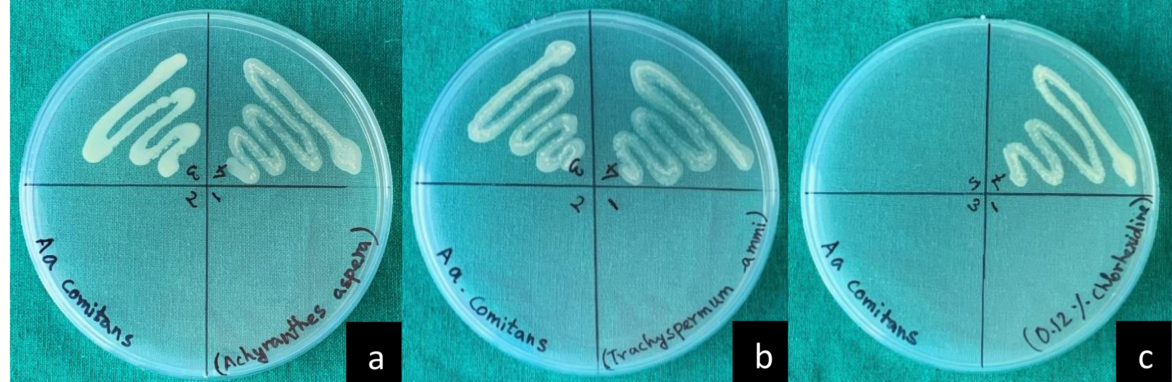


Figure 9. Photographs showing MBC of *A. aspera* and *T. ammi* extracts, and 0.12% Chlorhexidine hydrochloride (CHX) against *Aa. comitans*. The serial numbers on each BHI agar plate corresponded to the columns of the 96-well plate for each panel. The concentration of *A. aspera* and *T. ammi* extracts in the serial number (SN) 1 was 50 mg/ml, SN 2 was 25 mg/ml, SN 3 was 12.5 mg/ml, and SN 4 was 6.3 mg/ml. The concentration of CHX in the SN 1 was 1.2 mg/ml, SN 3 was 0.3 mg/ml, SN 5 was 0.08 mg/ml, and SN 7 was 0.02 mg/ml. SN 2 shows no growth, therefore the concentration of *A. aspera* and *T. ammi* extracts in that SN was taken as the MBC value.

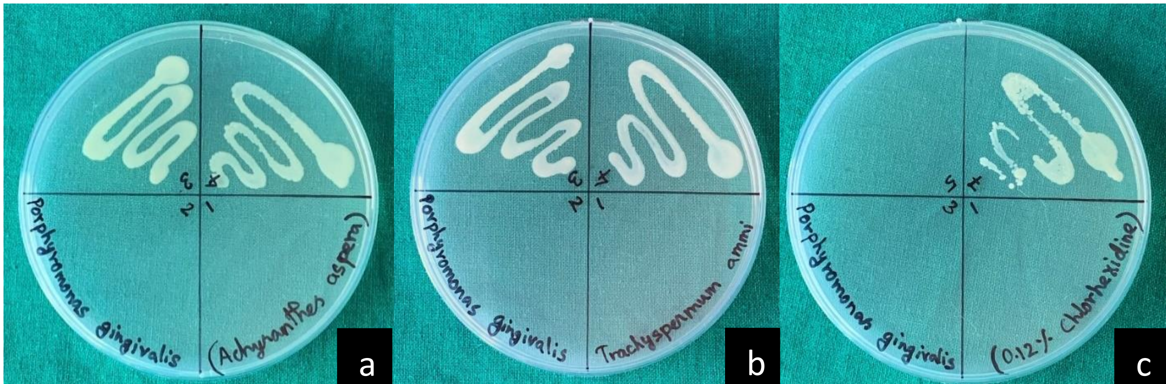


Figure 10. Photographs showing MBC of *A. aspera* and *T. ammi* extracts, and 0.12% Chlorhexidine hydrochloride (CHX) against *P. gingivalis*. The serial numbers on each BHI agar plate corresponded to the columns of the 96-well plate for each panel. The concentration of *A. aspera* and *T. ammi* extracts in the serial number (SN) 1 was 50 mg/ml, SN 2 was 25 mg/ml, SN 3 was 12.5 mg/ml, and SN 4 was 6.3 mg/ml. The concentration of CHX in the SN 1 was 1.2 mg/ml, SN 3 was 0.3 mg/ml, SN 5 was 0.08 mg/ml, and SN 7 was 0.02 mg/ml. SN 2 shows no growth, therefore the concentration of *A. aspera* and *T. ammi* extracts in that SN was taken as the MBC value.

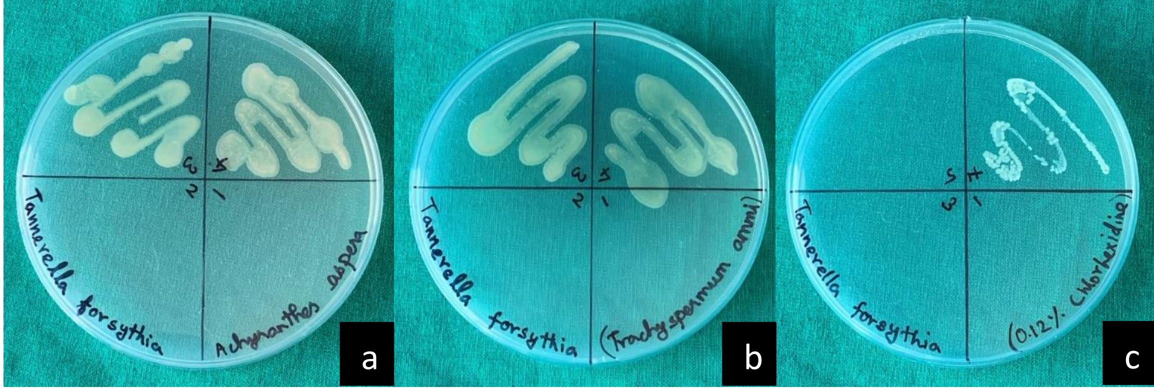


Figure 11. Photographs showing MBC of *A. aspera* and *T. ammi* extracts, and 0.12% Chlorhexidine hydrochloride (CHX) against *T. forsythia*. The serial numbers on each BHI agar plate corresponded to the columns of the 96-well plate for each panel. The concentration of *A. aspera* and *T. ammi* extracts in the serial number (SN) 1 was 50 mg/ml, SN 2 was 25 mg/ml, SN 3 was 12.5 mg/ml, and SN 4 was 6.3 mg/ml. The concentration of CHX in the SN 1 was 1.2 mg/ml, SN 3 was 0.3 mg/ml, SN 5 was 0.08 mg/ml, and SN 7 was 0.02 mg/ml. SN 2 shows no growth, therefore the concentration of *A. aspera* and *T. ammi* extracts in that SN was taken as the MBC value.

DISCUSSION

Role of microorganisms in periodontal disease

Periodontal disease is an inflammatory condition that affects both the soft and hard tissues of the teeth that results from bacterial growth and accumulation of dental plaque. Gingivitis arises from local risk factors, periodontal pathogens, microbial resistance, and plaque pathogenicity.[80] More than 700 bacterial species have been documented in the human oral cavity, although only a small number of them have been classified as prominent periodontal pathogens.[81]

The red complex which includes *P. gingivalis, T. denticola,* and *T. forsythia*, is considered to be the most pathogenic of the subgingival microbial complexes. There is a strong positive correlation between the red complex and the bleeding on probing/depth of the periodontal pocket.[82] Different combinations of bacterial species from other complexes, in addition to the red complex, have shown to be essential for periodontal diseases. According to the Consensus Report of the American Academy of Periodontology 1996, *Aa. comitans*, *P. gingivalis*, and *T. forsythia* are the principal microorganisms that have been first identified and classified as the primary causative agents of periodontal diseases.[83] Therefore, in the present study, the antibacterial efficacy of *A. aspera* and *T. ammi* extracts with chlorhexidine was assessed against the three periodontal microorganisms mentioned above.

Importance of herbal drugs in oral diseases

Herbal knowledge has been passed down from generation to generation for thousands of years.[84] Medicinal plants or herbs were utilized as preventative and curative therapy for a variety of human ailments in ancient medicine because they

match the immediate human need, and are readily accessible and affordable.[85] The same practice is still being used as the main therapeutic approach in rural areas of developing countries. Herbal medications have a deep traditional foundation and are as effective and safe as chemotherapeutic agents in treating various oral diseases.[86]

According to the WHO, traditional herbal remedies are employed in primary health care by more than 80% of the global population.[87] There is tremendous scope for the identification and use of new bioactive components, as fewer than 1% of the 5,00,000 plant species present globally have been phytochemically investigated.[88] Due to the substantial cumulative and irreversible effects of modern medicines, there has been a huge turnaround toward herbal remedies in recent times.[89] Herbal medicines are frequently employed owing to various benefits such as improved patient tolerance, acceptance, simplicity of cultivation and processing, and eco-friendliness. The ease with which herbal plants may be obtained has resulted in extensive research being performed in India and other regions of the world to supply a diversity of medications to modern medicine.[89]

Chlorhexidine as a chemical plaque control agent

In the present study, the antibacterial activity of CHX is 0.02-0.08 mg/ml, which is superior to the plant extracts (*A. aspera* and *T. ammi)*. According to the published systematic review of Manipal et al., few studies favour the use of herbal mouthwashes whereas, the majority of the studies favour the use of CHX.[90] According to a recent systematic review and meta-analysis (2022), 60% of the included studies demonstrated that CHX was as effective as herbal-based mouthwashes. On the other hand, 40% of the included studies proved that CHX mouthwash is superior to herbal mouthwashes.[91] The widely used supplementary chemotherapeutic medications for

gingivitis are CHX, cetylpyridinium chloride and Listerine.[92] However, some documented drawbacks associated with its use, including staining of tooth and restoration, burning sensation, parotid swelling, change in taste, and increased formation of supragingival calculus, have made it necessary to look for a more effective substitute.[92]

A. aspera and *T. ammi* as antibacterial agents

A. aspera grows as a weed on roadsides and along field boundaries across India.[20],[21] Baluchistan, Africa, Sri Lanka, Australia Tropical Asia, , and America are also indigenous to the plant.[22] According to Khan et al., the ethanol extract of the seeds showed an antibacterial effect against *Escherichia coli, Bacillus subtilis,* and *Pseudomonas aeruginosa.*[26] Saravanan et al. observed the antibacterial effect of leaf extracts against *E. coli, Proteus vulgaris, S. aureus*, *P. aeruginosa,* and *Klebsiella species.*[27] Sharma et al. investigated the antibacterial action against *S. aureus* in an alcoholic extract. [28] Manjula et al. investigated the antibacterial activity of *A. aspera* extracts against various pathogenic organisms such as *P. aeruginosa, E. coli, Citrobacter species, B. subtilis,* and *Micrococcus species*.[29] Gokhale et al. reported that *A. aspera* was shown to have anti-inflammatory properties in inbred Wistar rats and Swiss albino mice.[93]

T. ammi is an Egyptian native plant.[30] However, it is extensively spread and cultivated all around the world, including Iran, Iraq, Afghanistan, Pakistan, Afghanistan, India, and Europe.[31] Mayaud et al. reported that except for *P. aeruginosa*, *T. ammi* seed oil showed antimicrobial activity against 55 bacterial strains at a MIC of 2% *(v/v)*.[41] Essential oil of *T. ammi* showed antibacterial activity against three Gram-negative bacteria (*E. coli*, *P. vulgaris*, and *K. pneumonia*) and three Gram-

positive bacterial strains (*B. subtilis, S. aureus,* and *B. megaterium).*[42] *T. ammi* seed extracts in ethanol exhibited antibacterial activity against two Gram-negative food deterioration bacteria, *P. aeruginosa* and *E. coli.*[43]

Antibacterial activity of *A. aspera* and *T. ammi* against oral pathogens

The MIC and MBC are viewed as the gold standard for evaluating the antibacterial activity of organisms against various antimicrobials.[94] They are utilized for quantitative determination and can distinguish between bactericidal and bacteriostatic effects. In the present study, the mean MIC and MBC of the plant extracts of *A. aspera* and *T. ammi* for *Aa. comitans, P. gingivalis,* and *T. forsythia* were 12.5 mg/ml and 25 mg/ml, respectively. Study conducted by Ambulkar et al. showed *A. aspera* to exhibit significant antibacterial activity against bacteria majorly associated with dental caries *(Streptococcus mutans, Enterococcus faecalis, Streptococcus anginosus* and *Rothia dentocariosa).*[95] On the other hand, Lakshmi et al. reported that *A. aspera* (bark extract) exhibited antibacterial activity against *Lactobacillus acidophilus*; however, no activity against *S. mutans*.[96] In vivo study conducted by Bansal et al. reported a reduction in the salivary *S. mutans* count in children after one-week usage of *A. aspera* mouthwash.[97] An exploration of the literature revealed that there were no studies evaluating the antibacterial effects of *A. aspera* on periodontal pathogens.

Dadpe et al. reported that *T. ammi* oil exhibits antibacterial and antifungal properties against *S. mutans*, *Streptococcus oralis*, *L. acidophilus*, *Lactobacillus fermentum*, and *Candida albicans.*[98] Khan et al proved that a naphthalene derivative isolated from seeds of *T. ammi* exhibits antibiofilm activity against the cariogenic properties of *S. mutans* by significantly reducing its adherence and biofilm

formation.[99] Beegam et al. observed the antibacterial effect of *T. ammi* oil against *E. faecalis.*[100] There were no studies evaluating the antibacterial effects of *T. ammi* on periodontal pathogens except for a study conducted by Yavagal et al., 2022 who reported antibacterial activity of commercially available 100% *T. ammi* oil against (*Aa. comitans, P. gingivalis,* and *Fusobacterium nucleatum*) in comparison to CHX.[101]

Soxhlet extraction

The pharmacologically active elements can be extracted using several methods, such as cold or warm maceration, percolation, and continuous extraction like Soxhlet extraction. In the present study, the Soxhlet extraction technique was employed to prepare extracts, as it is the most commonly used, simple, cost-effective and has been proven to be more efficient because the crude plant extract component is repeatedly exposed to fresh solvent.[102] The active compound of any natural product must be extracted using a solvent that dissolves the chemicals of interest. Ethanol, methanol, petroleum ether, and water are the most commonly employed solvents for extracting components for the determination of antibacterial activity. Ethanol can extract polyphenolic compounds more effectively because it dissolves both non-polar and polar compounds, including a range of plant-derived chemicals and has been shown to be safe for humans. Hence, ethanol was chosen as the solvent in the present study.[103]

Qualitative phytochemical screening

In the present study, Preliminary phytochemical analysis of *A. aspera* extract roots indicated the presence of bioactive substances such as flavonoids, alkaloids, tannins, phenolic compounds and steroids. In the same way, the phytochemical screening of *T. ammi* seed extract also revealed the presence of flavonoids, tannins,

alkaloids, phenolic compounds, and steroids with the addition of volatile oils. Similar phytoconstituents have been reported in various studies. [104–106] All of the above phytochemicals exhibit significant antioxidant activity and have been proven to possess antibacterial, anti-inflammatory, and anticancer properties, as well as serving as an alternative to synthetic drugs.

In the current study, the specific mechanism of action and biochemical components responsible for the antibacterial activity against periodontal pathogens are unclear in *A. aspera* and *T. ammi*. In general, the antimicrobial mechanism of flavonoids can be categorized as inhibiting cytoplasmic membrane function, synthesis of nucleic acid, and energy metabolism.[107] Pandey et al. reported that flavonoids present in *A. aspera* are responsible for antioxidant and antibacterial activity.[108] Various studies revealed that the major constituents were phenolic compounds (Thymol- 59.9–96.4%, p-cymene- 0.6–21.2%, γ-terpinene- 0.2–17.8%, and carvacrol- 0.4–2.8%) present in *T. ammi* seeds exhibit strong antioxidant and antibacterial activity against various microorganisms.[18,109]

A. aspera and *T. ammi* have been reported to be safe plants as assessed by the cytotoxicity study.[110] Furthermore, the concentration of crude extracts of both *A. aspera* and *T. ammi* was low in the present study. This addresses the safety concern of using these extracts in humans.

Limitations

Periodontal disease is a multifactorial disease, due to the interaction of agent, host, and environmental factors. It is crucial to remember that laboratory experiments do not reflect the real condition that exists in the gingival sulcus. The aforementioned elements are not taken into account. The specific interaction cannot be determined by laboratory investigations, since the antibacterial activity is evaluated against selective periodontal pathogens.

CONCLUSION

Both *A. aspera* and *T. ammi* possess comparable antibacterial activity against periodontal pathogens (*Aa. comitans*, *P. gingivalis* and *T. forsythia)*when compared to CHX. In terms of public health aspect, it could be a valuable tool against periodontal pathogens as it is widely available and cost-effective in addition to being efficacious. *A. aspera* and *T. ammi* can be regarded as promising multipurpose therapeutic agents. Hence, further clinical trials should be conducted to prove its effectiveness.

RECOMMENDATIONS

1. Studies should be conducted to identify key chemical compounds and their specific mechanism of action that is responsible for the antibacterial activity and to understand the synergistic effect of *A. aspera* with *T. ammi* extracts.
2. The bioactive molecules that are involved in biological processes should be isolated. Furthermore, these extracts can be used to assess nano-formulations in nano-biotechnology.
3. *In vivo* studies should be carried out to demonstrate its antibacterial effectiveness against periodontal pathogens without showing substantial local or systemic adverse effects.
4. To evaluate the long-term efficacy of *A. aspera* and *T. ammi* in combination as various medicinal agents such as mouthwash, intracanal medicament and root canal irrigant, and local drug delivery system using in larger sample size on different age groups in real-life conditions.
5. The production of novel pharmaceuticals for the treatment of oral infections may take advantage of the commercialization of natural products with added value.

SUMMARY

The current *in vitro* study was designed to assess and compare the antibacterial efficacy of *A. aspera* and *T. ammi* extracts with 0.12% CHX against periodontal microorganisms (*Aa. comitans*, *P. gingivalis* and *T. forsythia)*. Ethical clearance was obtained from the Institutional review board.

Roots of *A. aspera* and Seeds of *T. ammi* were procured. *A. aspera* plant was authenticated by the taxonomist in the ICMR-NITM. Fresh roots of *A. aspera* and seeds of *T. ammi* were dried under shade and ground into a coarse powder. Ethanol extracts of A. aspera and T. ammi were prepared by the Soxhlet apparatus method. The freshly prepared crude extracts were qualitatively analyzed for the presence of flavonoids, alkaloids, tannins, phenolic compounds, steroids and volatile oils.

The plant extracts (*A. aspera* and *T. ammi)* were assessed for Minimum Inhibitory Concentration (MIC), and Minimum Bacterial Concentration (MBC) against three standard strains of periodontal pathogens (*Aa. comitans*, *P. gingivalis* and *T. forsythia)* in comparison to 0.12% CHX. A Resazurin microtitre assay was used to determine the MIC of plant extracts. The resulting change in colour to pink was inferred as an indication of bacterial growth and no change in Resazurin blue was inferred as an indication of inhibition of bacterial growth. The MIC was the lowest concentration with no change of Resazurin colour (inhibition of bacterial growth). MBC was determined by the spread plating method. The experiments were conducted in triplicates. Kruskal Wallis Test followed by the Bonferroni Post-Hoc test was employed to compare the differences in the antibacterial activity of individual extracts

and 0.12% CHX against periodontal pathogens wherein the statistical significance was set at $p \leq .05$.

When compared with the plant extracts, 0.12% CHX was found to have a greater inhibitory effect (MIC - 0.02 to 0.04 mg/ml) and greater bactericidal effect (MBC - 0.08mg/ml) against tested periodontal pathogens ($p < .001$). The mean MIC and MBC of the plant extracts for *Aa. comitans, P. gingivalis, T. Forsythia* are 12.5 mg/ml and 25 mg/ml, respectively.

Both *A. aspera* and *T. ammi* possess comparable antibacterial activity against periodontal pathogens (*Aa. comitans*, *P. gingivalis* and *T. forsythia)*when compared to CHX. In terms of public health aspect, it could be a valuable tool against periodontal pathogens as it is widely available and cost-effective in addition to being efficacious. *A. aspera* and *T. ammi* can be regarded as promising multipurpose therapeutic agents. Hence, further clinical trials should be conducted to prove its effectiveness.

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ANNEXURES

ANNEXURE I

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC   Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362   Web: http://www.kledental-bgm.edu.in
FAX: 0831-2470640   E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1454

CERTIFICATE

This is to Certify that the synopsis titled Comparative evaluation of antibacterial efficacy of Achyranthes aspes and Trachyspermum ammi extracts with chlorexhidine against Periodontal pathogens - An in vitro study *Submitted by*

Dr. REG NO: IL0220003 *P. G. Student /*

Staff, Guided by ______ *from Department of* Public Health dentistry *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE II

PROCUREMENT AND AUTHENTICATION CERTIFICATE

XAVIER RESEARCH FOUNDATION
(XRF)
ST. XAVIER'S COLLEGE (AUTONOMOUS)
Palayamkottai – 627002, Tamil Nadu, India
Website: www.stxavierstn.edu.in Email: xrfsxc@gmail.com

Date: 01/11/2021

Authentication Certificate

Based on the Taxonomic features and personal observations the plant material/s submitted by -- **REG NO: IL0220003** -- is botanically identified and confirmed as here under with the Register No: XCH - 40399

Botanical Name: Achyranthes aspera L.

Syn. Name: /

Family: Amaranthaceae

Part: Root.

Reference: Flora of Tamilnadu Vol: II Page: 189, 1987
Botanical Survey of India, Coimbatore.

Place: Tirunelveli - 2.

Signature

Dr. S. MUTHEESWARAN, M.Sc., M.Phil., Ph.D.
Scientist
Xavier Research Foundation
St. Xavier's College
Palayamkottai - 627 002.
Tamil Nadu, India.

ANNEXURE III

AUTHENTICATION CERTIFICATE

राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR-NATIONAL INSTITUTE OF TRADITIONAL MEDICINE
(भूतपूर्वक्षेत्रीयआयुर्विज्ञानअनुसंधानकेन्द्रFormerly Regional Medical Research Centre)
Nehru Nagar, Belagavi-590 090

भारतीयआयुर्विज्ञानअनुसंधानपरिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
स्वास्थ्यअनुसंधानविभाग, स्वास्थ्यऔरपरिवारकल्याणमंत्रालय, भारतसरकार
Department of Health Research,
Ministry of Health & Family Welfare, Govt. of India

Dr. Harsha Hegde
Scientist-E
harshah@icmr.gov.in

Date: 05-02-2021

AUTHENTICATION

This is to authenticate that the plant material brought by **REG NO: IL0220003** I year MD, KLE VK Institute of Dental Sciences, Belagavi, is identified as ***Achyranthes aspera* L.** belonging to family Amaranthaceae. The herbarium specimen of the same has been deposited in our herbaria with accession number RMRC-1618.

Harsha Hegde
Scientist-E

ANNEXURE IV

PHYTOCHEMICAL SCREENING REPORT

A) *Achyranthes aspera*

SHRI B M KANKANAWADI AYURVED MAHAVIDYALAYA
A Constituent Unit of KLE ACADEMY OF HIGHER EDUCATION & RESEARCH (DEEMED-TO-BE-UNIVERSITY)
(Re-Accredited 'A' Grade by NAAC (2nd Cycle) || Placed under Category 'A' by MHRD GoI)
CENTRAL RESEARCH FACILITY
(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Outward No:-BMK/CRF/ /2021-22

Reference No:
Submitted by: **REG NO: IL0220003**
Sample : *Achyranthes aspera*
Product : Plant
(* N/A - Not Available)

Batch No. : NA
Sample Qty : 10gm

Registration Dt:-02/01/2021
Requisition no :----
Part/Form:Powder
Report Date : 16/01/2021

TEST REPORT
Form-50 [See Rule 160-D (f)]
(The Drugs & Cosmetic Act 1940 and the rules there under)

Preliminary Phytochemical Screening:

TESTS	
Test for Flavonoids	Positive
Test for Alkaloids	Positive
Test for Tannins	Positive
Test for Steroids	Positive

ANALYST

AUTHORISED SIGNATORY

PHYTOCHEMICAL SCREENING REPORT

B) ***CHLORHEXIDINE HYDROCHLORIDE***

Basic Pharma Life Science Pvt. Ltd.

Manufacturers of Bulk Drugs, Drug Intermediates and Fine Chemicals

Plot No. 146/B, Opp. New Fire Station, GIDC Estate, Ankleshwar - 393 002. Gujarat. India

NAME OF PRODUCT	CHLORHEXIDINE HYDROCHLORIDE BP / CHLORHEXIDINE DIHYDROCHLORIDE EP		
BATCH NO	: 20BPLS/CHH001	A.R. NO.	: 20FP514
MANUFACTURING DATE	: SEP - 2020	BATCH SIZE	: 305.0 kg
EXPIRY DATE	: AUG - 2023	QTY. SAMPLED	: 150 g
ALL TEST AS PER	: BP/EP	H.S. CODE:	: 29251900

CERTIFICATE OF ANALYSIS

TEST	REQUIREMENTS	RESULTS
Appearance	A white or almost white, crystalline powder.	An almost white, crystalline powder.
Solubility	Very slightly soluble in water, slightly soluble in propylene glycol, very slightly soluble in ethanol (96 percent).	Complies
Identification A. IR B. Chemical Test C.Melting Point D. Reaction of chloride	 A. Comparison with chlorhexidine dihydrochloride CRS. B. Dark red colour produced C. Between 132 °C to 136 °C D. A curdy white precipitate is formed which dissolves easily in ammonia solution.	 Complies Complies 131.2°C to 132.6 °C Complies
Impurity P (chloroaniline)	Maximum 300 ppm	50 ppm
Related substances Imp. N Imp. B Imp. A Imp. H Imp. I+O Imp. K Maximum unspecified Imp. Total impurities	 Not more than 0.15 % Not more than 0.10 % Not more than 0.15 % Not more than 0.5 % Not more than 0.4 % Not more than 0.4 % Not more than 0.10 % Not more than 1.0 %	 Below disregard Limit Below disregard Limit Not detected 0.21 % Below disregard limit Below disregard Limit 0.05% 0.26 %
Loss on Drying	Maximum 1.0 % w/w	0.61 % w/w
Sulphated Ash	Maximum 0.1 % w/w	0.047 % w/w
Assay	98.0 % to 101.0 % w/w (dried substance)	99.48 % w/w

REMARKS: The material complies with respect to the above specifications.

Prepared by	: [signature]	Checked by	: [signature]	Approved by	: [signature]
Date	: 18/09/2020	Date	: 18/09/2020	Date	: 18/09/2020

AN ISO 9001:2015 COMPANY

Quantity is Must but Quality is First

Ph. : +91-2646-226037 / +91-7622800080 • Telefax: +91-2646-226037

Email : sales@basicpharma.in, info@basicpharma.in

Skype ID : bplspl, Web : http://www.basicpharma.in

CIN : U24230GJ2005PTC046278

ANNEXURE V

PHYTOCHEMICAL SCREENING USING VARIOUS CHEMICAL TESTS

TEST FOR FLAVONOIDS

Flavonoids contain conjugated aromatic systems and therefore show prominent/ intense absorption bands in visible and UV regions of the spectrum. e.g. 470 nm - 560 nm (principal maxima) and 275 nm (subsidiary maxima) indicate the presence of anthocyanin.

The Colour properties of Flavonoids in visible and UV light also help in their identification. e.g. most flavonol glycosides have a very pale yellow colour in visible light while in UV light they show dark brown colour (alone) and with ammonia light yellow / yellow-brown colouration.

a) ***Sulphuric Acid Test:*** On addition of sulphuric acid (66% or 80 %), flavones and flavonols dissolve into it and give a deep yellow solution. Chalcones and aurones give red or red-bluish solutions. Flavanes give an orange to red colours.
b) **Lead acetate test:** To a small quantity of residue, add lead acetate solution. The yellow-coloured precipitate is formed.

TEST FOR ALKALOIDS

Evaporate the aqueous, alcoholic and chloroform extracts separately. To the residue, add dilute HCL. Shake well and filter. With filtrate, perform the following tests:

a) ***Dragendorff's test*** : To 2-3 ml filtrate, add few drops Dragendorff's reagent. Orange-brown ppt. is formed.

b) ***Mayer's test:*** 2-3 mi filtrate with a few drops of Mayer's reagent gives ppt.

TESTS FOR TANNINS AND PHENOLIC COMPOUNDS

To 2-3 ml of aqueous or alcoholic extract, add a few drops of the following reagents:

a) ***5% FeCIs solution***: deep blue-black colour.

b) ***Lead acetate solution***: white ppt.

TESTS FOR STEROID

a) ***Salkowski reaction:*** To 2 ml of extract, add 2 ml chloroform and 2 ml conc. H2SO4. Shake well. The chloroform layer appears red and the acid layer shows greenish-yellow fluorescence.

b) ***Liebermann's reaction:*** Mix 3 ml extract with 3 ml acetic anhydride. Heat and cool. Add a few drops of conc. 125O. The blue colour appears.

TESTS FOR VOLATILE OILS

Hydrodistillate material. Separate volatile oil from distillate and perform the following tests:

a) Volatile oils have a characteristic odour.

b) Filter paper is not permanently stained with volatile oil.

c) ***Solubility test:*** Volatile oils are soluble in 90% alcohol or chloroform.