

**“ANTIMICROBIAL EFFICACY OF ESSENTIAL OILS AND
THEIR COMBINATION ON MICROORGANISMS
ASSOCIATED WITH POST-RADIATION THERAPY IN
HEAD AND NECK CANCER PATIENTS:
AN IN-VITRO STUDY”**

By

REGISTRATION NO: IL0220001

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Submitted to

**KLE Academy of Higher Education and Research
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**In partial fulfillment
Of the requirements for the degree of**

MASTER OF DENTAL SURGERY

IN

PUBLIC HEALTH DENTISTRY

(BRANCH - VII)

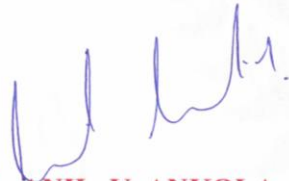
**DEPARTMENT OF PUBLIC HEALTH DENTISTRY
KLE VISHWANATH KATTI INSTITUTE OF DENTAL SCIENCES
KAHER, BELAGAVI, KARNATAKA**

2020 - 2023

**KLE Academy of Higher Education & Research, Belagavi
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LIST OF ABBREVIATION

EOs	:	Essential Oils
CHX	:	Chlorhexidine
MSSA	:	Methicillin Susceptible <i>Staphylococcus aureus</i>
MRSA	:	Methicillin Resistant <i>Staphylococcus aureus</i>
MIC	:	Minimum Inhibitory Concentration
MBC	:	Minimum Bactericidal Concentration
COA	:	Certificate Of Analysis
BHI	:	Brain Heart Infusion
DMSO	:	Dimethyl Sulphoxide
BSRC	:	Basic Science Research Centre
MTCC	:	Microbial Type Culture Collection
ATCC	:	American Type Culture Collection
mg	:	milligram
ml	:	milliliter
Min	:	Minute
CLSI	:	Clinical and Laboratory Standard Institute
µl	:	Microlitre
UV	:	Ultraviolet
SPSS	:	Statistical Package for Social Sciences
ISO	:	International Organization for Standardization
SD	:	Standard Deviation
WN	:	Well Number
GC	:	Gas Chromatography
etc	:	Etcetera

Spp.	:	Species
<i>S.aureus</i>	:	<i>Staphylococcus aureus</i>
<i>E.faecalis</i>	:	<i>Enterococcus faecalis</i>
<i>C.albicans</i>	:	<i>Candida albicans</i>
CO ₂	:	Carbon dioxide
H ₂	:	Hydrogen
N ₂	:	Nitrogen
i.e.	:	That is
BP	:	British Pharmacopoeia
ICPA	:	Independent Community Pharmacy Association
Ltd	:	Limited
IBM	:	International Business machines
Corp	:	Corporation
w/v	:	Weight/volume
w/w	:	Weight/Weight
P-value	:	Probability Value
°C	:	Degree Celsius
%	:	Percentage
et.al.	:	Et alia
vs	:	Versus
<i>C. Zeylanicum</i>	:	<i>Cinnamomum Zeylanicum</i>

ABSTRACT

Background:

The sixth most common cancer in the world is head and neck cancer. Despite the fact that there are many effective treatment options for oral cancer, including surgery, radiation, chemotherapy, or a combination of these, patients' local and systemic immunity declines after treatment. When exposed to the outside environment, the intraoral bacterial population swiftly changes. Immune cells are encouraged to invade the mouth mucosa by inflammatory exposure. It is widely acknowledged that the hallmarks of malignant transformation include inflammation and immune-related mediators.

The most common and abundant phyla linked to oral neoplasms include *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*. *Staphylococcus* species were found highly prevalent in patients of oral cancer before radiotherapy, while *Candida albicans*, *Klebsiella species*, and *Pediococcus species* were the significant pathogens isolated in postradiotherapy cancer patients. Reduced saliva production brought on by post-radiation therapy upsets the delicate balance between bacterial load and a weakened immune system. In patients who have undergone radiotherapy or who have had their oral malignancies removed, oral hygiene is commonly neglected. Numerous studies have demonstrated that bacteria can influence the chance of acquiring cancer, dental problems, and chronic systemic inflammation. Chlorhexidine (CHX), at low concentrations (0.02%-0.06%) has bacteriostatic activity, whereas at higher concentrations (>0.12%) acts bactericidal. Because of its diverse activity, Chlorhexidine has been the gold standard antiseptic and disinfectant for ages. Despite of this, it carries numerous disadvantages such as alteration of taste,

staining of teeth, dry mouth, mucositis due to radiation therapy etc.

An extensive review of the literature demonstrates the antibacterial properties of essential oils derived from plant materials, which may be able to prevent the development of such opportunistic microorganisms in the oral cavity. Cinnamon bark EO and Cajeput EO are known to show antimicrobial efficacy against varied microorganisms which include *S. aureus*, *E. coli*, *A. baumannii*, *E. faecalis*, *C. albicans* etc. and *P. aeruginosa* *Micrococcus luteus*, *Staphylococcus aureus*, *S. capitis*, *S. epidermidis*, *S. faecalis*, *Klebsiella spp.* *Staphylococcus aureus* etc. respectively.

Aim:

To assess the antimicrobial efficacy of Cinnamon bark oil (*Cinnamomum verum*), Cajeput oil (*Melaleuca leucadendron*), their combination and Chlorhexidine gluconate against the standard strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*.

Objectives:

To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Cinnamon oil, Cajeput oil and their combination against standard strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*.

To evaluate and compare the antimicrobial efficacy of Cinnamon oil, Cajeput oil and their combination with chlorhexidine against standard strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*.

Methodology:

The cinnamon bark essential oil and Cajeput essential oil were procured and checked for their solubility in DMSO. The final ratio at which the oils were found to be soluble was at 1:1 ratio. MIC of Cinnamon bark oil (*Cinnamomum verum*) and Cajeput oil (*Melaleuca leucadendron*) against *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans* was determined by serial dilution method using **Resazurin dye**. Plates were prepared in triplicates for *S. aureus*, *E. faecalis* and *C. albicans*. The 96-well microtiter plates were sealed and were placed in the incubator set at 37° C for 18-24 hours. After ~24 hours, 10 µl of freshly prepared Resazurin dye (0.015%) was aseptically added to each well under the UV light laminar air flow. . Lowest concentration at which the colour change observed was taken as MIC value of the respective essential oil and their combination against *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*.

Results:

The highest concentration of the positive control (0.12% CHX solution) was required against *E. faecalis* (15.63 ± 0.02 mg/ml), whereas the lowest concentration was required against *S. aureus* (6.51 ± 2.26 mg/ml). The cinnamon bark EO was most effective against *S. aureus* and least against *C. albicans*. Similarly, Cajeput EO was most efficacious against *Enterococcus faecalis* and least against *Candida albicans*. And lastly, the combination EOs group was most efficacious against *S. aureus* and least with *E. faecalis*.

So, the overall results show that Cinnamon bark EO had the strongest efficacy against *S. aureus* (0.33 ± 0.14 mg/ml) and *E. faecalis* (0.41 ± 0.14 mg/ml), but not against *C. albicans* (2.85 ± 2.11 mg/ml). Cajeput EO shows the least efficacy against all the

groups; whereas the combination of Essential oils has proved to be the most efficacious and shows good antimicrobial activity against these most commonly encountered microorganisms in head and neck cancer post-radiotherapy.

Conclusion:

Cinnamon and Cajeput Essential Oils in combination have proved to be effective against the most common microorganisms encountered in head and neck cancer patients post-radiotherapy. Although the results of the present in-vitro study are promising, extensive research is still required to prove the capability of such herbal products against other causative microorganisms present in oral cancer-treated patients post-radiotherapy.

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INTRODUCTION

It is better to prepare and prevent than it is to repair and repent.

- Ezra Taft Benson

Head and neck cancers rank sixth among all the cancers occurring worldwide. Each year, it causes around 600,000 new cases and around 350,000 fatalities globally.¹ According to the worldwide agency for research on cancer, the number of cancer cases in India is expected to rise from 1 million in 2012 to more than 1.7 million by 2035.² Despite of many efficient treatment modalities for oral cancer, such as aciurgy, irradiation, chemo, or a combination of these, local and pervasive immunity decreases amongst the treated patients. An unbalanced oral environment may be the result of hyposalivation, swallowing issues, and differences in the quality, quantity, and complexity of oral bacteria.³ Moreover, precancerous diseases and malignancies have been linked to increased levels and altered microbial composition in the oral cavity.⁴

Human mouth is home to more than 700 distinct bacterial species and at least six billion other sorts of microbes. Intraorally, the bacterial population is dynamic and changes quickly when exposed to the outside world. An inflammatory reaction brought on by an altered oral microbiome is the cause of periodontitis, which also brings about tooth and bone loss. The oral mucosa benefits from immune cells infiltrating it when exposed to inflammatory circumstances. The hallmarks of malignant transformation have long been recognized to be inflammation and immune-related mediators.⁵

The majority of oral cavity organisms engage in commensalism, which means that both the

host and the bacteria benefit biologically from one another. But within this largely harmless flora, there are dangerous germs that can spread disease.⁶ Even normally benign organisms can become aggressive in the presence of immunocompromised state and cause a variety of issues, such as inflammation, degeneration, cancer, or temporary illnesses.⁶ *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* are the most prevalent and numerous phyla associated with oral neoplasm.^{6,7,8} *Streptococcus* species are the most prevalent oral bacteria found in healthy persons, while anaerobic *Prevotella*, *Veillonella*, *Neisseria* and *Haemophilus* are less common. Before radiotherapy, *Staphylococcus* species was shown to be significantly abundant in oral cancer patients, while *Candida albicans*, *Klebsiella* species, and *Pediococcus* species were the important pathogens isolated from cancer patients after radiotherapy.^{6,8}

According to studies linking microbial flora and mouth cancer, carcinoma sites had higher densities and distributions of microbial flora. According to variations in bacterial tropisms towards specific oral localities, certain intra-oral surfaces and bacterial species may have particular receptors and adhesion molecules that regulate the colonisation of particular oral surfaces.⁶

Oral hygiene is frequently overlooked in patients who have endured resection for oral tumors and after radiotherapy. Sometimes, patients may be non-compliant or too unwell to implement conventional oral hygiene practices on their own and it may only be possible with the assistance of nursing staff, family members and allied health professionals. Post-radiation therapy causes decreased saliva production which may

cause an alteration in oral microflora, resulting in the disruption of the balance between bacterial load and a compromised immune status.

This causes infection, which allows some bacteria and *Candida* species to proliferate and exterminate other local microorganisms. Numerous studies show that bacteria can affect the development of chronic systemic inflammation, dental disorders, and the risk of developing cancer.

Following radiotherapy, the patient may develop drug-resistant opportunistic infections from oral cavity microorganisms like *Candida albicans*, *Staphylococcus aureus*, and *Enterococcus faecalis*, which may lead to systemic problems and significant morbidity in immunocompromised people. *Staphylococcus aureus* causes epidermal and soft tissue infections such as furuncles, abscesses (boils) and cellulitis. They are also responsible for serious infections such as bloodstream infections, pneumonia or bone and joint infections. *Enterococcus faecalis* can cause septicemia, meningitis, urinary tract infections and a multitude of other infections. It has been found to colonize dental implants to cause peri-implantitis.

Candidiasis is a mycotic infection caused by a yeast (a type of fungus) called *Candida* - the most common of which is *Candida albicans*. If the infection spreads or if it enters deep into the body (for example, the bloodstream or internal organs like the kidney, heart, or brain) it causes systemic Candidemia, which may prove to be fatal.

A thorough literature review shows the antimicrobial activity of essential oils obtained from plant materials which have the potential to inhibit the growth of such opportunistic microorganisms in the oral cavity. More than 3,000 Essential Oils (EOs), of which roughly 300 are commercially significant and employed by the flavor

and fragrance industries, are thought to exist among these secondary metabolites. Terpenes, Nitrogen and Sulphur containing compounds, phenylpropanoids are the three primary categories of secondary metabolites.⁹ While certain hydrocarbons also have antibacterial properties, the oxygenated terpenoids (such as phenolic terpenes and alcohols) make up the majority of the antimicrobial activity in EOs.⁹

Cinnamon bark EO and Cajeput EO are known to show antimicrobial efficacy against varied microorganisms which include *S. aureus*, *E. coli*, *A. baumannii*, *E. faecalis*, *C. albicans* etc. and *P. aeruginosa* *M. luteus*, *S. aureus*, *S. epidermidis*, *S. faecalis*, *Klebsiella* sp. *S. aureus*, *S. capitis* etc. respectively.

Chlorhexidine (CHX) has a broad spectrum of dose-dependent antibacterial action. It is bacteriostatic at low concentrations (0.02%-0.06%), but bactericidal at higher quantities (>0.12%). The cationic compound CHX interacts in a unidirectional manner with the bacterial membrane phospholipids that are negatively charged. CHX alters the osmotic balance of the bacterial cell at low doses. Potassium, phosphorus, and other low-weight molecules are released as a result, and the protein structure of the cell is destroyed along with the essential intracellular components, such as nucleotides. Gram-positive bacteria are more susceptible to the bactericidal effects of CHX, while Gram-negative bacteria are less susceptible. Additionally, it has anti-fungal and anti-viral properties. It has equivalent efficacy against methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) strains of *Staphylococcus aureus*. *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans* were the endodontic pathogens that were the focus of a study conducted by Vianna et al. *Actinomyces israelii* and *Enterococcus faecalis* have been found to be resistant to CHX solution in ex vivo examinations of infected root canal systems.¹⁰

Because of its diverse activity, Chlorhexidine has been the gold standard antiseptic and disinfectant for ages. Despite of this, it carries numerous disadvantages such as alteration of taste, staining of teeth, dry mouth, mucositis induced by radiation therapy etc.

Thus, it is imperative to develop other means of oral hygiene practices in such situations. Therefore, the rationale of the present study was to assess and contrast the effectiveness of Cinnamon bark oil (*Cinnamomum verum*), Cajeput oil (*Melaleuca leucadendron*) and their synergism against the most common opportunistic microorganisms seen in post-radiation therapy in head and neck cancer patients.

AIM & OBJECTIVES OF THE STUDY

AIM OF THE STUDY:

- To assess the antimicrobial efficacy of Cinnamon bark oil (*Cinnamomum verum*), Cajeput oil (*Melaleuca leucadendron*), their combination and Chlorhexidine gluconate against the standard strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*.

OBJECTIVES OF THE STUDY:

- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Cinnamon oil, Cajeput oil and their combination against standard strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*.
- To evaluate and compare the antimicrobial efficacy of Cinnamon oil, Cajeput oil and their combination with chlorhexidine against standard strains of *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*.

REVIEW OF LITERATURE

Definition of essential oil

The term “essential oil” or “ethereal oil” is defined as the volatile oil obtained by the distillation of plants.¹¹

Classification of essential oils

The essential oils are classified into four main groups, which are representative of the majority of essential oils.

1. Terpenes
2. Straight chain components
3. Benzene derivatives
4. Miscellaneous

The word terpenes implies to compounds which are similar to the simple C_5H_8 molecule, and compounds with 10 carbon atoms derived from $C_{10}H_{16}$.

The second major group of oil components contain: Alcohol, Aldehydes, Ketones, Acids, Ethers and Esters

The third major group of essential oil belongs to compounds originated from benzene and n-propyl benzene.

The fourth group consists of the compound which does not come under the first three groups.¹¹

CINNAMON BARK ESSENTIAL OIL:

ACTIVE COSTITUENTS	ACTIONS
Cinnamaldehyde	Antibacterial, Antioxidant, Anti inflammatory
Alpha -pinene	Antimicrobial, Anti inflammatory
Beta-Ocimene	Antioxidant, Anti inflammatory
Eugenol	Anti-bacterial

CAJEPUT ESSENTIAL OIL:

ACTIVE COSTITUENTS	ACTIONS
1,8-cineole	Antimicrobial, Anti inflammatory
Linalool	Antimicrobial, Anti-inflammatory, Analgesic, Anti-hyperalgesic
Terpinen-4-ol	Antibacterial, Anti-viral, Anti-fungal

1. **Kamat et al, (2020)** found that *Staphylococcus*, *Bacillus*, *Enterococcus*, and rest other microbes like *Klebsiella*, *Staphylococcus kloosi*, and *Staphylococcus hominis* increased after six months of radiation therapy. In a study on the impact of radiotherapy on the carcinoma of the head and neck and oropharyngeal flora. He also reported that *S. aureus*, *Pseudomonas*, *Bacteroides*, and *Candida albicans* increased after radiation treatment, *Streptococcus pneumoniae* considerably decreased.⁴
2. An investigation by **Irani S et al, (2020)** revealed that when oral infections attack the gingival tissues, they can result in inflammation. The immune-inflammatory responses have a substantial impact on the patient's susceptibility to periodontal diseases. According to a significant wealth of evidence, chronic inflammation increases the risk of developing chronic systemic disorders including autoimmune diseases and various malignancies, and periodontitis increases the risk of developing malignancies in the injured oral epithelium. Periodontitis, an inflammatory response to the oral microbiome, is connected to bone and tooth loss. When exposed to inflammatory conditions, immune cells penetrate the oral mucosa and support it. Inflammation and immune-related variables have been identified as the hallmarks of malignant transformation.⁵
3. The study conducted by **Yumiko Kawashita, (2020)** aimed to show that an oral management strategy should include the removal of infected teeth before the start of radiotherapy to prevent osteoradionecrosis, oral care for the duration of radiotherapy to support patient completion of radiotherapy, and oral care to prevent dental caries followed by

osteoradionecrosis after radiotherapy. According to the study's findings, dental care providers must encourage their patients to practice strict plaque management. Additionally, drugs should be administered to increase saliva production and counselling should be made available to reduce cariogenic meals. These steps will improve the quality of life for head and neck cancer survivors while reducing the number of dental caries caused by radiotherapy.¹²

4. A review by **Katarzyna et al. (2019)** revealed the origins, chemical makeup, and antibacterial capabilities of several essential oils. Transcinnamaldehyde, orthomethoxycinnamaldehyde, cinnamylaldehyde, benzaldehyde, phenyl ethanol, borneol, cinnamic acid, eugenol and coumarin were the active constituents in cinnamon essential oil.¹³
5. **Rather et al (2019)** reported that essential oils have a broad range of antibacterial, antifungal, and even antiviral activities. The current review paper covers all the different facets of essential oils' antibacterial action in great depth. It is abundantly obvious from the literature on the antimicrobial activity of essential oils that they have special antibacterial, antifungal, and antiviral activities and can thus be used as antimicrobial agents.¹⁴
6. **Rai et al (2017)**: The objective of this review analyzed numerous studies on the essential oil's broad-spectrum antibacterial effectiveness when used alone or in conjunction with nanoparticles. According to the review, multi-drug-resistant diseases are spreading rapidly, as shown by

the high rates of morbidity and mortality. Finding new and effective medications to treat the issues has become necessary due to the ineffectiveness of existing medical treatments. Due to this feature, EOs have been employed in food, medications, and cosmetics. EOs contain significant volatile molecules with a variety of bioactivities, including antibacterial potential.¹⁵

7. According to a study by **Benbelad et al., (2014)**, *E. faecalis* is to blame for up to 90% of human enterococcal infections; its pathogenicity ranges from life-threatening illnesses in vulnerable people to less severe conditions, especially as a result of a variety of virulence factors. Multidrug-resistant (MDR) enterococci pose significant treatment problems since they are resistant to the majority of antimicrobial medicines used to treat human infections. *E. faecalis* is not regarded as a normal component of the oral microbiota in the oral cavity. The main cause of several oral illnesses caused by *E. faecalis* is recurrent endodontic infections, which can lead to life-threatening consequences. EOs are effective substitute antiseptics for Chlorhexidine or NaOCl, particularly for fighting MDR *E. faecalis* biofilm.¹⁶

8. A study conducted by **Varshitha A (2015)** provides information about the incidence, prevalence, geographical variations in India, the causes, symptoms, and prevention of oral cancer. Both males and females in India are at a 61% lifetime risk of dying from cancer. Statisticians report that 36,463 men and 15,361 women died in 2012 as a result of oral cancer. Preventive actions must be performed to lower the prevalence

and mortality of oral cancer and improve survival. The prevention of cancer should be given top priority in India due to the country's large population to make the best use of the country's few resources. Oral mucosa screening is necessary to detect oral cancer in those under the age of 40 who routinely consume alcohol, smoke cigarettes, or chew betel nut.²

9. A Study conducted by **R. Metgud, K. Gupta, J. Gupta, et al. in (2014)**, showed that the mouth cavity possesses a unique and diversified microbiome. It was thought that the majority of these organisms exhibit commensalism, changes in the dynamics of the bacterial population result in pathogenic changes in the mouth cavity and other places. Immunocompromised cancer patients are at risk from oral bacteria, which are typically found in biofilms, if they get into the bloodstream or spread to nearby tissues. To control oral infections, a variety of clinical guidelines and practices have been devised, although the evidence for these protocols is scant. All cancer patients must maintain good dental health, including those with oral cancer.⁶

10. A study by **Pushalkar S et al., (2012)** proved that several bacterial species or phylotypes discovered in this study may contribute to ongoing oral inflammation and may be related to different cancer stages. This could be brought on by a damaged oral mucosal surface, which would then permit bacterial infiltration and possibly function as a location of entry to the neighborhood lymph nodes. This demonstrates that, even if the bacteria in the oral cavity were commensals, they can

still become harmful when their equilibrium is disturbed. Microbial shift, also known as dysbiosis, has been several diseases because of an imbalance between beneficial symbionts and pathogens. Eighty bacterial species or phylotypes were discovered, 57 of which were in tumors of tumors and 59 of which were not.⁷

11. A study by **Bassolé et al, (2012)** proved that essential oils are effective antibacterial agents because they are organic plant products with a complex blend of components. The majority of the antimicrobial activity in EOs seems to come from oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids, and alcohols. Combinations can be utilized to increase the bioactivities of other components, such as hydrocarbons, which generally display modest activity. These components may interact to have negative, positive, or cooperative impacts. Essential oils (EOs) have long been known to have antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties.⁹

12. The study conducted by **Singh et al. (2012)** was based on findings from several clinical trials, and the ADA has approved the use of essential oils, mouthwash, and chlorhexidine mouthwash as an addition to regular mechanical oral hygiene measures. However, both of these products have side effects, so the current study was conducted to compare the antiplaque effectiveness of a new herbal formulation to those of EO and chlorhexidine rinse. Although the herbal formulation in this study significantly reduced the amount of plaque that returned when compared

to the benchmark and negative controls, it was not as effective as the gold standard (CHX) antiplaque mouthwash. The difference in reduction seen between the herbal mouthwash and chlorhexidine reached statistical significance. The herbal mouthwash had a promising plaque-inhibitory potential, but longer-term studies are required to determine the true effectiveness of this mouthwash and its place among the other rinses that are used in addition to mechanical oral hygiene procedures. These studies must assess the product's anti-gingivitis activity in addition to its antiplaque property, as well as safety and microbiological considerations.¹⁷

13. In a study by **Feng et al. (2011)**, it was found that the EO from the cinnamic aldehyde-rich bark of *C. zeylanicum* had a better effect than other EOs used and had very good action against *S. aureus*, *E. coli*, and other infections (including clove EO, lemongrass, tea tree, ginger, and basil). 1,8-cineole (15–60%), linalool, and terpinen-4-ol are some of the most distinctive elements of Cajeput EO. At a concentration of 0.2–0.4%, Cajeput oil inhibits the growth of Gram-positive bacteria like *S. aureus*, *E. faecalis* etcetera and at a higher concentration of 0.4–0.6 % it inhibits yeast such as *Candida albicans*.¹⁸

14. According to a study by **Bik EM, Long CD, et al. (2010)**, while each person has a distinct community of bacterial species in their mouth, these communities tend to be more similar when they are classified at the genus level. The study of complex bacterial communities, such as those found in the human body, will be substantially aided by ecological tools created for larger organisms, such as co-occurrence analysis. This

will deepen our comprehension of the role of the microbiome in health and disease.⁸

15. According to **Ferlay J et al (2010)**, there were 7.6 million cancer-related deaths and 12.7 million new cases of the disease in 2008. Of these, 63% of cancer-related fatalities and 56% of newly diagnosed cases will, respectively, take place in less developed regions of the globe. The most common malignancies diagnosed globally are colorectal (1.23 million, 9.7%), lung (1.61 million, 12.7% of the total), and breast (1.38 million, 10.9%). The three most common cancers are lung cancer (1.38 million fatalities, 18.2% of all cancer deaths), stomach cancer (738,000 fatalities, 9.7% of all deaths), and liver cancer (696,000 fatalities, 9.2% of all deaths). Neither rare nor restricted to countries with plenty of resources, cancer affects people everywhere. The patterns of cancer differ noticeably from place to region.¹

16. According to **Jukka H. Meurman (2010)** The oral microbiome of other patients with the malignant disease has been addressed in this study's clinical guidelines and recommendations, although there is insufficient scientific support for these recommendations. Organisms can enter the bloodstream or neighbor neighboring tissues, the oral microbiota, which contains hundreds of microbial species and is primarily found in biofilms, poses a concern to immunosuppressed counts. To control several, several clinical recommendations and regimens have been developed. Though not evidence-based, the procedures are derived from extensive clinical experience. Therefore, it is essential for all cancer patients, including those with oral cancer, to maintain good oral health,

which frequently calls for the assistance of a regular professional healthcare team.¹⁹

17. **Vianne Mi et al. (2004)** carried out a study to examine the efficiency of sodium hypochlorite (0.5%, 1%, 2.5%, 4%, and 5.25%) and chlorhexidine gluconate (CHX gel and CHX liquid) against endodontic infections (Sodium hypochlorite). The timing for irrigants to kill microbial cells was quantified and statistically assessed following a broth dilution test. *Staphylococcus aureus* and *Candida albicans* were eliminated by the 2.0% gel and liquid formulations in 15 seconds each, while *Enterococcus faecalis* was eliminated by the gel formulation in 1 minute. In less than 15 seconds, all of the investigated irrigants efficiently eradicated *Porphyromonas gingivalis* and *Prevotella intermedia*. Liquids containing 1.0%, 2.0%, and 5.25% NaOCl eliminated all germs in the same amount of time.¹⁰

MATERIAL AND METHODS

The current study aims to assess the antimicrobial activity of commercially available Cinnamon essential oil, Cajeput essential oil and their combination against standard strains of *Staphylococcus aureus*, *Enterococcus Faecalis* and *Candida albicans*.

Study Design: An in-vitro study

Study Setting:

1. Department of Public Health Dentistry, KAHER's KLE VKIDS, Belagavi.
2. KLES' Dr. Prabhakar Kore Basic Science Research Centre.

Steps associated for the current in-vitro study are as follows:

- I. Procurement of materials and instruments used for the study.
- II. Procurement of essential oils and active ingredients identification by Certificate of Analysis (COA) (**ANNEXURE V & VI**)
- III. Procurement of microorganisms for the study.
- IV. Brief Methodology (Flowchart)
- V. Preparational Procedures:
 - a. Inoculum preparation
 - b. Preparation of BHI medium
 - c. Preparation of stock solution
 - d. Preparation of Positive control
 - e. Preparation of Growth control

- VI. Determination of Minimum Inhibitory Concentration (MIC).
- VII. MIC Methodology - Brief Flowchart
- VIII. Preparation of BHI Agar Medium
- IX. Determination of Minimum Bactericidal Concentration (MBC)

I. Procurement of materials and instruments used for the study:

A. Instruments

- Agar culture plates
- Eppendorf microcentrifuge tubes
- Conical flasks
- Microtiter plates
- Micropipettes
- Sterile 96 multi-well Tissue culture plates

B. Machines and Apparatus

- Incubator
- Inoculation Loop Sterilizer
- Anaerobic jar
- Electronic Weighing machine
- Water Bath Sonicator
- Laminar Air Flow Chamber

C. Culture media and chemicals

- Sterile saline
- Brain Heart Infusion (**BHI**) broth {HiMedia®}
- BHI-Agar
- Sterile distilled water

- Dimethyl Sulphoxide (DMSO)
- Resazurin dye
- McFarland solution
- Ethanol (99.8%)

D. Test Materials used for the study

- Cinnamon bark Essential oil
- Cajeput Essential oil
- Chlorhexidine hydrochloride salt (CHX) {BP grade, ICPA Health Products Ltd., Mumbai, India }

II. Procurement of Essential Oils and their active ingredients:

- The essential oils – namely Cinnamon bark oil (*Cinnamomum verum*) and Cajeput oil (*Melaleuca leucadendron*), were purchased from *Nishant Aromas Private Limited*.
- The authentication of these essential oils was obtained from *Nishant Aromas Private Limited* as a Certificate of Analysis (COA).

III. Procurement of microorganisms for the study:

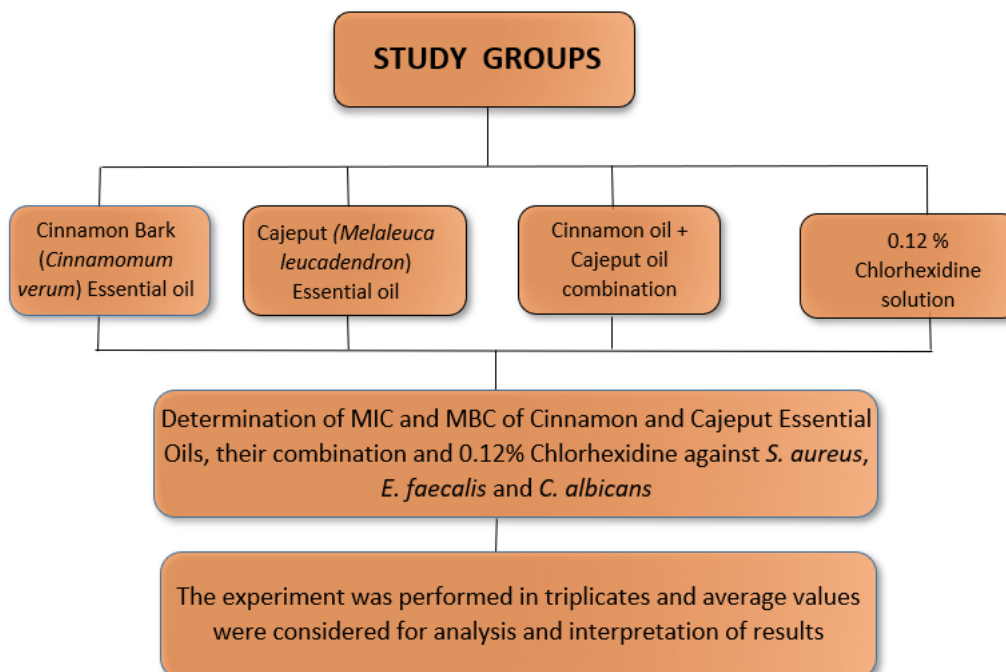
BACTERIAL STRAINS: The standard strains of the following microorganisms were procured from the microbiological depository at KLE's Dr. Prabhakar Kore Basic Science Research Centre (BSRC) Belagavi, Karnataka.

- 1) *Staphylococcus aureus* (MTCC 12598)
- 2) *Enterococcus faecalis* (MTCC 35550)

3) *Candida albicans* (MTCC 2091)

The laboratory procedures were carried out at KLE's Dr. Prabhakar Kore Basic Science Research Centre (BSRC), Belagavi, Karnataka.

IV. Brief Methodology



V. Preparational Procedures

a) Inoculum preparation:

- Standard microbial strains of *Staphylococcus aureus* (MTCC 12598) and *Candida albicans* (MTCC 2091) were obtained from the microbiological repository of BSRC. They were revived and sub-cultured using Brain Heart Infusion broth (BHI), supplemented with horse serum and maintained on selective blood agar plates at 37° C under aerobic conditions for 24-48 hours for the growth of bacteria.

- Anaerobic cultures (facultative anaerobes like *E. faecalis*) were incubated in an anaerobic jar where a mixture of chemicals with cold catalase produced 80% N₂, 10% CO₂ and 10% H₂. Carbon dioxide released from the reaction stimulated the growth of the anaerobes which were incubated for 48 to 72 hours.

b) Preparation of BHI Medium:

- The medium formulation was in liquid form. According to the manufacturer's instructions, 37 grams of BHI (HiMedia®) can be dissolved in one litre of distilled water. So, to prepare 100 ml. of BHI broth solution 3.7 grams of BHI powder was weighed and dissolved in 100 ml. of distilled water in the conical flask.
- Then the solution was autoclaved at 121°C at 15 pounds per square inch for 20 minutes. This preparation was freshly made every time to carry out the study procedure.

c) Preparation of stock solutions:

- The cinnamon bark essential oil and Cajeput essential oil were procured and checked for their solubility in DMSO. The oils were found to be miscible with each other as well as with DMSO. The final ratio at which the oils were found to be soluble was at 1:1 ratio (i.e. Essential Oil: DMSO).
- This ratio was used thereafter for carrying out the MIC. The preparation for two stock solutions of Cinnamon Essential Oil and Cajeput Essential Oil with DMSO was in the ratio of 1:1; and their Combination was prepared in the ratio of (0.5:0.5:1 i.e. Cinnamon EO: Cajeput EO: DMSO respectively).¹³

- The concentration in the 1st well was taken as 500 mg/ml. Serial dilution was repeated up to 10⁻¹² for the Essential oils to reach the concentration of 0.244 mg/ml.

d) Preparation of Positive control (0.12% CHX solution):

To prepare 50 ml of the standard solution of 0.12% CHX solution, 6 mg of 100% CHX salt was mixed in 50 ml of distilled water. The solution was kept in Ultrasonic Bath Sonicator for 2 min. to ensure proper dissolution of the CHX salt.

e) Preparation of Growth Control (Broth + Organisms):

A hundred microlitres of microbial strains were inoculated along with BHI broth without the essential oils.

VI. Determination of Minimum Inhibitory Concentration (MIC):

- The MIC, which is a key indicator of an antimicrobial agent's potency, is defined as the concentration (ml⁻¹) at which visible growth of bacteria is prevented under defined growth conditions (Wiegand et al. 2008).
- MIC of Cinnamon bark oil (*Cinnamomum verum*) and Cajeput oil (*Melaleuca leucadendron*) against *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans* was determined by serial dilution method using **Resazurin dye**. The test was conducted in triplicates.

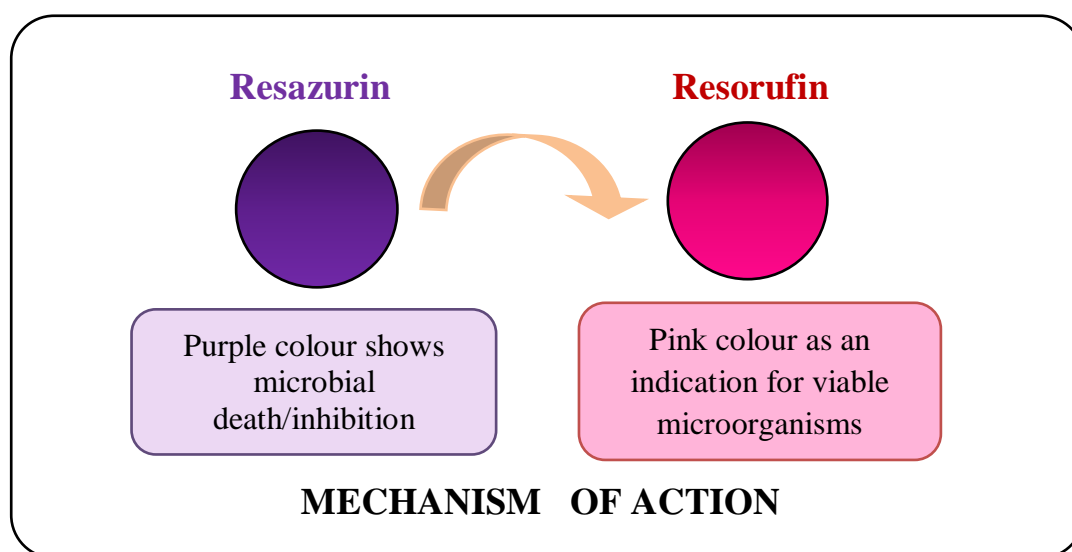
ANTIMICROBIAL PREPARATION:

- Following CLSI (Clinical and Laboratory Standards Institute) guidelines, broth dilution method was carried out in 96 multi-well microtiter plate containing 8 rows and 12 columns.
- Each 96-well plate was used for one single microorganism.
- The 1st, 2nd and 3rd row were assigned for Cinnamon EO, Cajeput EO and their combination respectively. 4th row was left unused.
- To the 5th row broth and diluent were added to ensure that diluent did not prove to be fatal for the microorganisms.
- The sixth row tested the efficacy of the positive control CHX against the microorganisms, whereas the 7th row comprised of Growth control, which had broth and microorganisms.

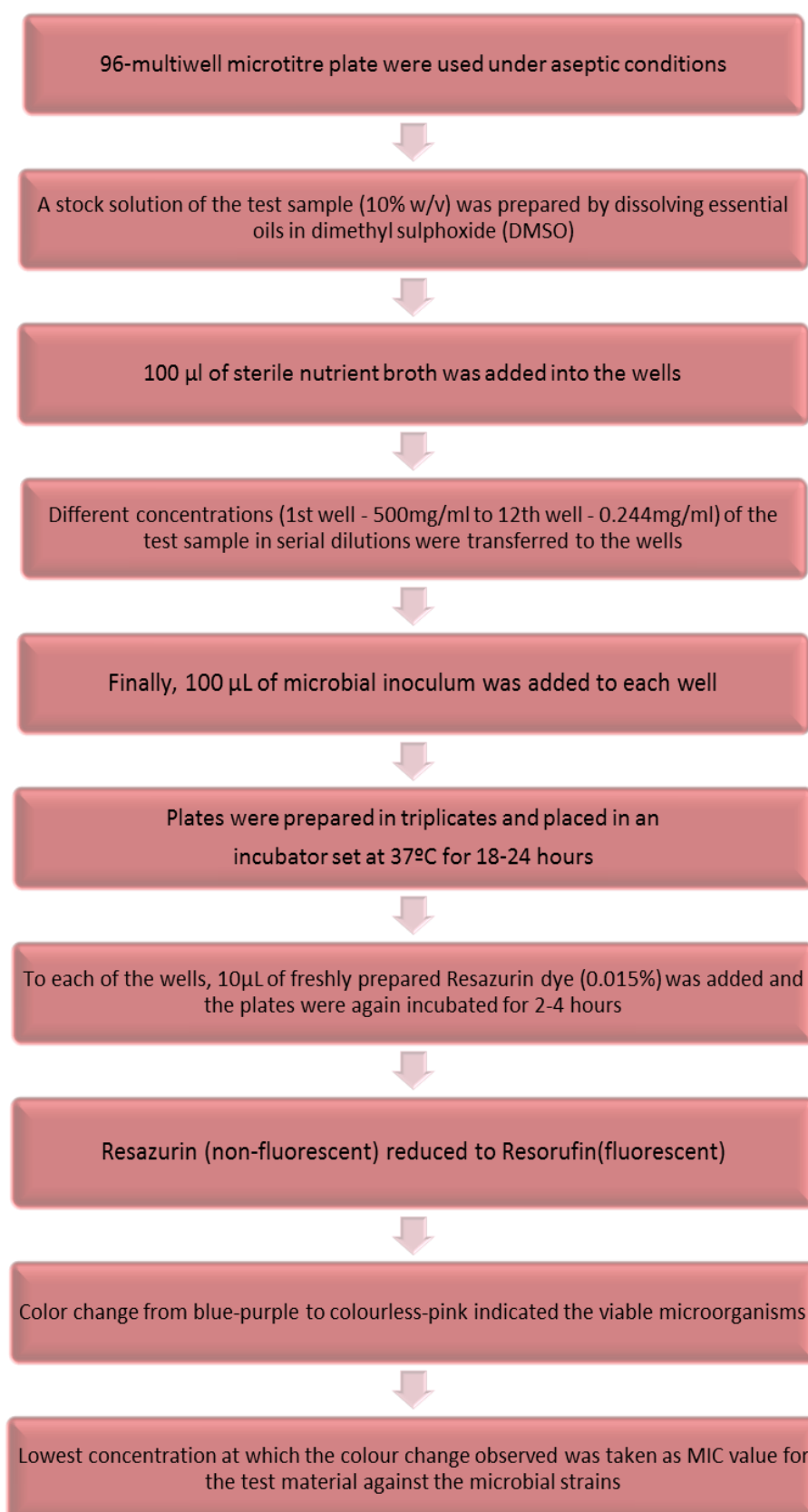
PROCEDURE:

- Hundred microlitres of sterile nutrient broth was added to all the above-mentioned wells. Different concentrations (1st well - 500mg/ml to 12th well - 0.244mg/ml) of the test sample was transferred to the wells to attain the two-fold serial dilutions up to 10^{-12} . Finally, 100 μ l of microbial inoculum was added to each well. Plates were prepared in triplicates for *S. aureus*, *E. faecalis* and *C. albicans*. The 96-well microtiter plates were sealed and were placed in the incubator set at 37° C for 18-24 hours. After ~24 hours, 10 μ l of freshly prepared Resazurin dye (0.015%) was aseptically added to each well under the UV light laminar air flow. The plates were incubated for 4 hours and

any change in colour from blue/purple to pink/red indicated viable microorganisms (Resazurin reduced to Resorufin) and no change of Resazurin dye indicated inhibition of microbial growth. Lowest concentration at which the colour change observed was taken as MIC value of the respective essential oil and their combination against *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*.



VII. Brief Methodology - MIC



VIII. Preparation of BHI Agar medium:

To obtain 100 ml agar mix, 5.2 grams of BHI Agar was dissolved in 100 ml. of distilled water and was mixed well until it was evenly dispersed. (As per manufacturer's instructions, 52 grams of BHI Agar to be dissolved in 1000 ml of sterile water). It was autoclaved at 121-37°C, at 15 pounds per square inch of pressure for 20 minutes and then cooled. The medium was transferred to the culture plates prior to its solidification which were autoclaved and dried to avoid contamination. These plates were used for the determination of MBC.

IX. Determination of Minimum Bactericidal Concentration (MBC):

- The Minimum Bactericidal Concentration (MBC) for all Essential oils was determined by *Spread Plating* method. Prior to streaking, the 96 well plates containing the essential oils were blended with pipette, followed by transferring a full loop culture onto the BHI Agar plates and streaked in a zig zag fashion.
- The MBC streaked plates were incubated for 16 to 18 hours and the temperature was set at 37°C. The plates were taken out of the incubator and observed for bacterial growth. These observations were compared with 96 well microtiter plate employed for MIC determination.
- The concentration at which the microorganisms were completely killed was taken as the MBC. All the tests were performed in triplicates for all microorganisms.

ETHICAL CLEARANCE:

- Ethical clearance for the study was obtained from the Institutional Ethical Committee with reference number: 1452, dated: 05-05-2021. (ANNEXURE I)

STATISTICAL ANALYSIS:

Data obtained were entered in Microsoft Excel (2019) 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 \pm Standard deviation for continuous variables. The following univariate analysis was performed: Kruskal Wallis Test followed by the Bonferroni Post-Hoc test to compare the differences in the antibacterial activity of individual essential oils, their combination and 0.12% CHX. *P-value* ≤ 0.05 was considered statistically significant.

PHOTOGRAPHS



Photograph no. 1 - Laminar Air Flow Chamber with UV Light



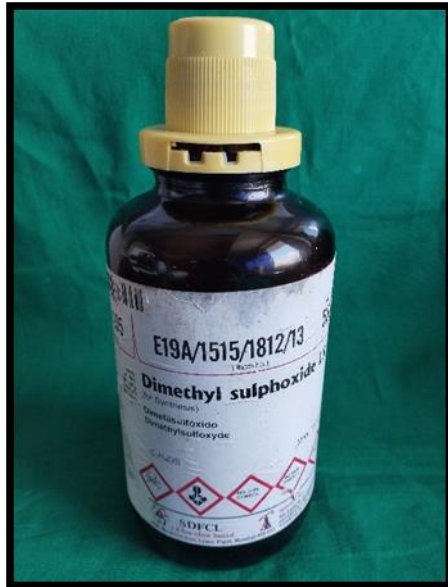
Photograph no. 2 – Digital Weighing Balance



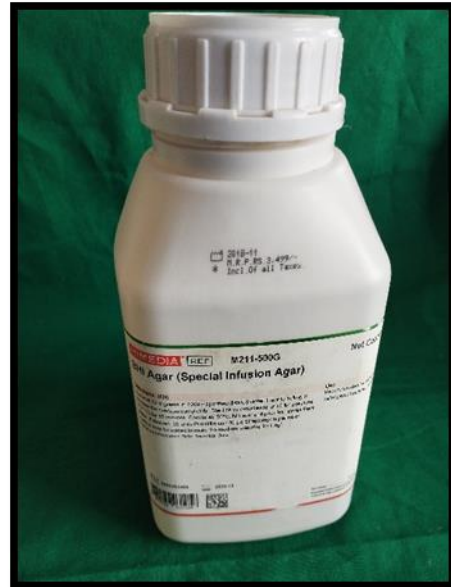
Photograph no. 3 – Ultrasonic Water Bath Sonicator with 0.12% Chlorhexidine solution



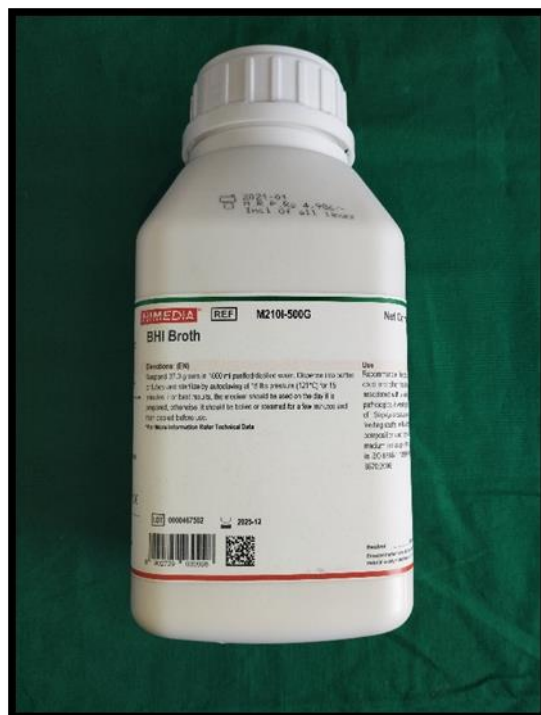
Photograph no. 4 – Microbiological Incubator, used for incubation of microorganisms.



Photograph no. 5 - Dimethyl Sulphoxide: diluent used with Essential oils, also acts as a vehicle



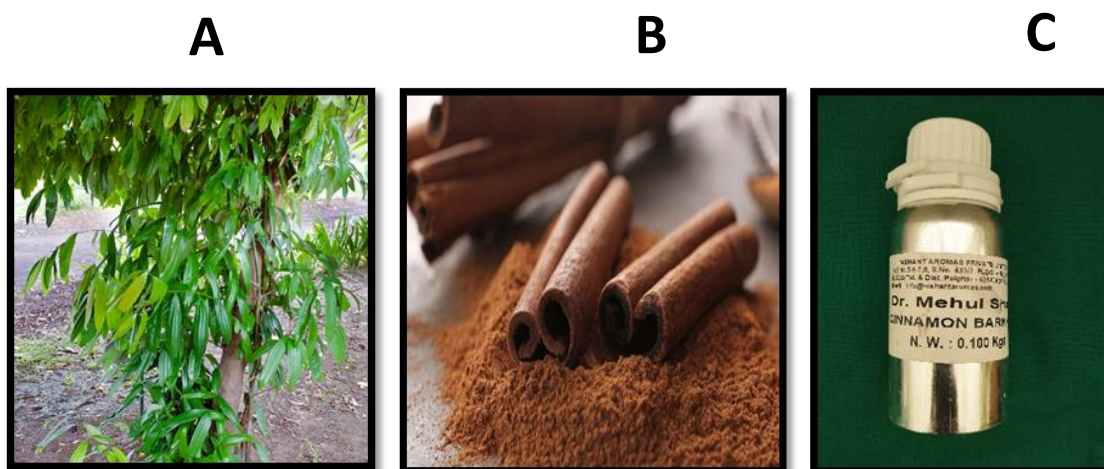
Photograph no. 6 - Brain Heart Infusion (BHI) Agar, used as a medium for the growth of microorganisms in the determination of MBC



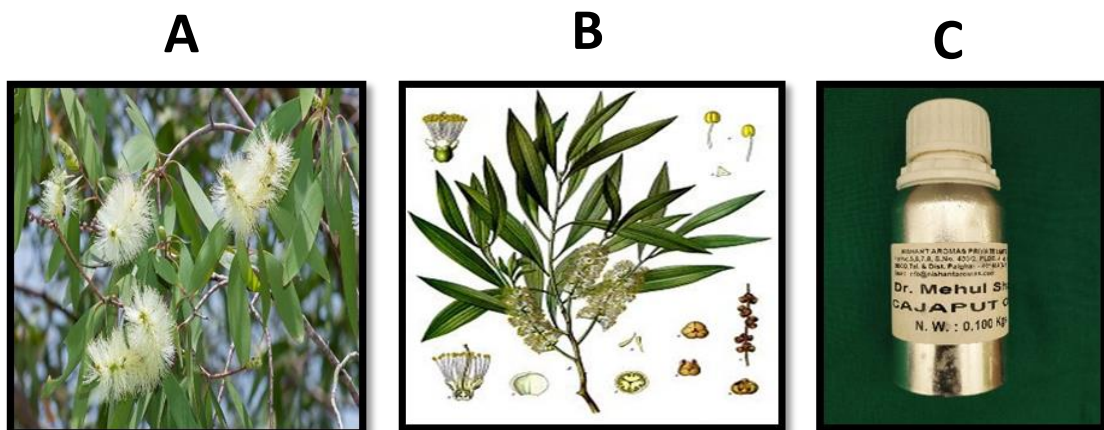
Photograph no. 7 - Brain Heart Infusion (BHI) Broth, used as a vehicle and medium for growth of microorganisms in determination of MIC



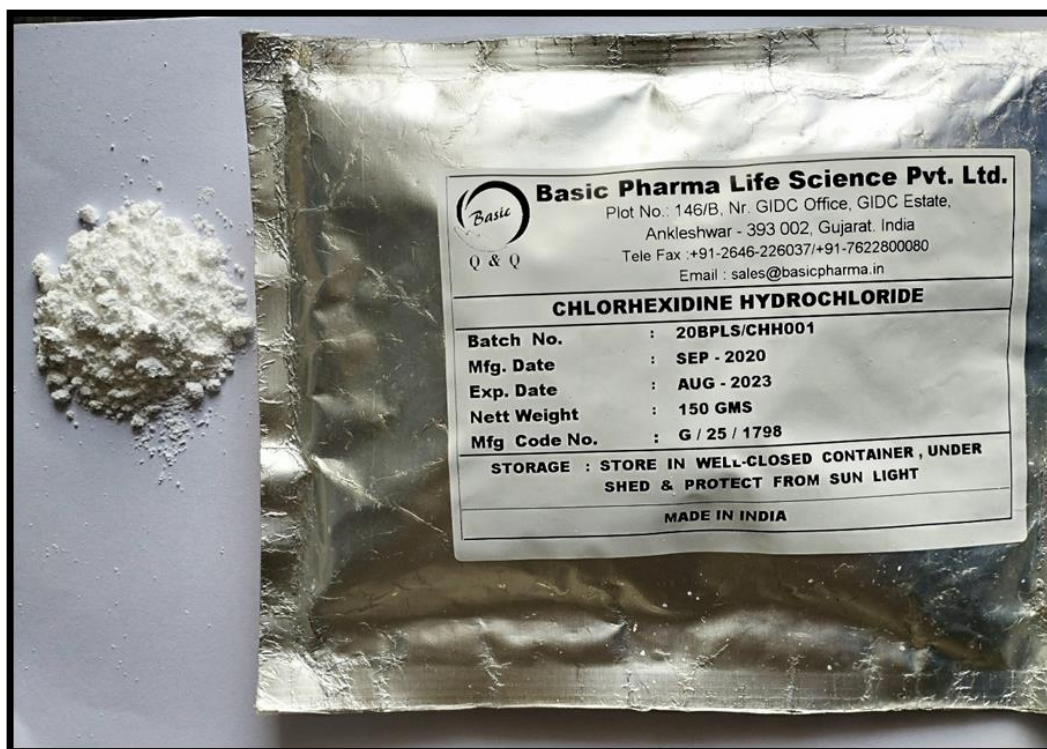
Photograph no. 8 - (A) Resazurin Dye Powder
(B) – 0.015% of Resazurin dye prepared and used as indicator for Determination of MIC



Photograph no. 9 – A. Cinnamon Tree B. Cinnamon bark C. Cinnamon Bark Essential Oil



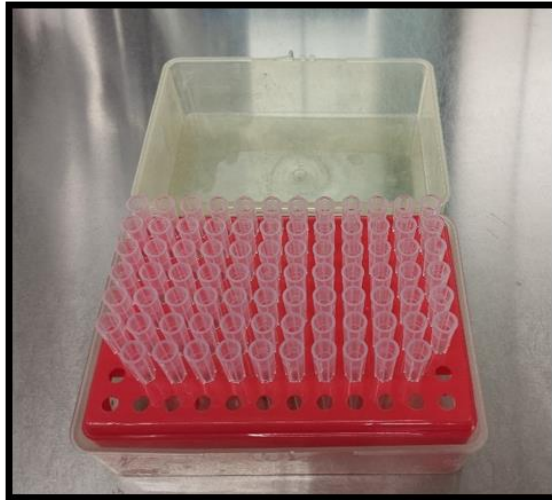
Photograph no. 10 – A. Cajaput tree B. Cajaput leaves C. Cajaput Essential Oil



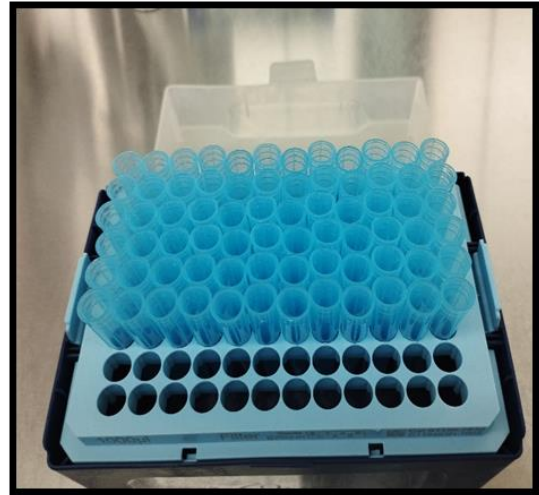
Photograph no. 11 - Pure 100% concentrated Chlorhexidine (CHX) Hydrochloride salt, used in the preparation of 0.12% CHX solution used as a positive control arm.



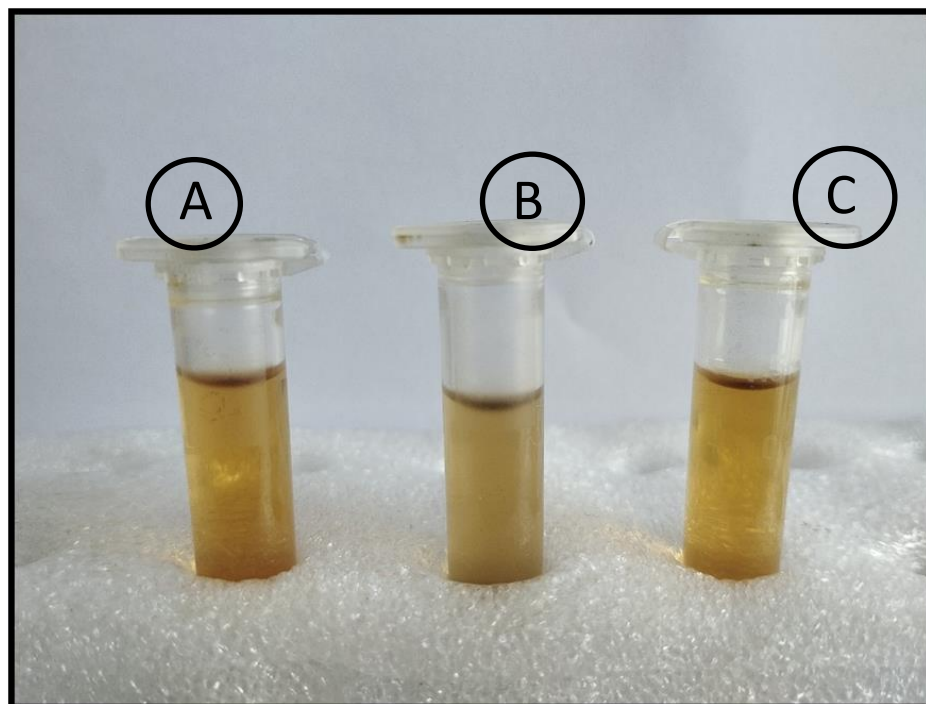
Photograph no. 12 – Sterile 96-well microtitre tissue culture plate, used for Broth Dilution Method to determine the MIC.



Photograph no. 13 – Sterile 100 microliter pipette tips, used to transport the reagents to 96-well microtitre plates

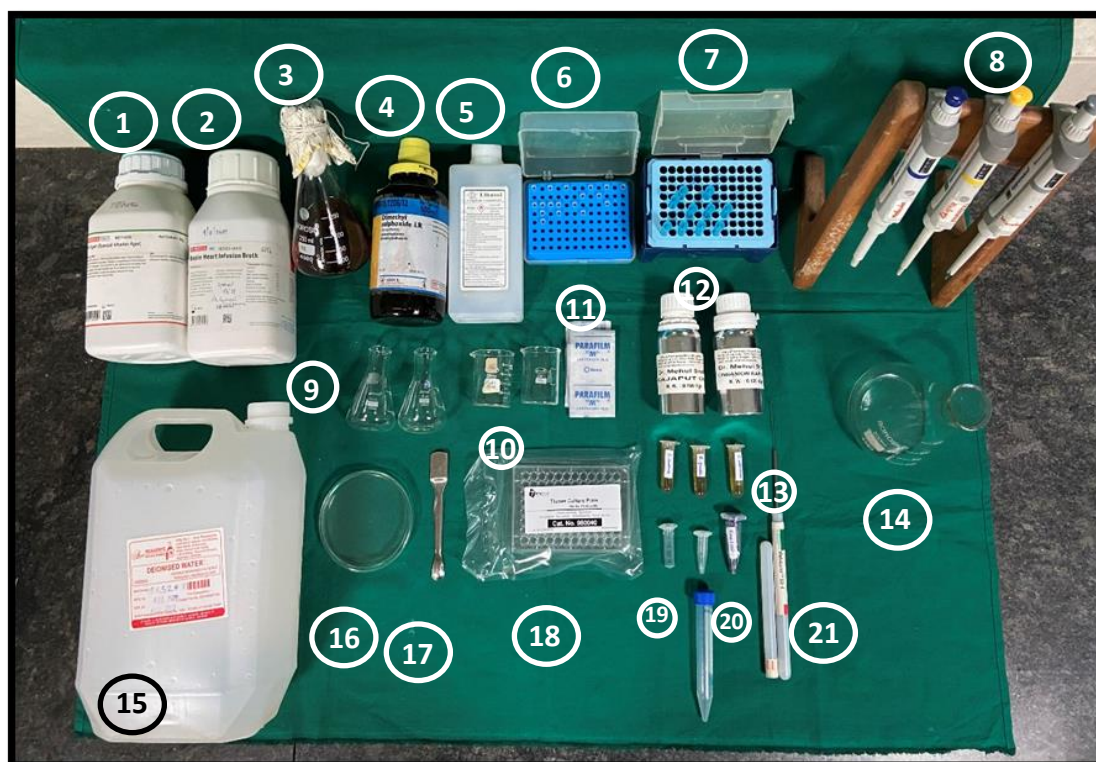


Photograph no. 14 - Sterile 1000 microliter pipette tips, used to transport the reagents to 96-well microtitre plates



Photograph no. 15 - Microbial Inoculates in 2 ml. Eppendorf tubes (A) *Staphylococcus aureus*

(B) *Enterococcus Faecalis* (C) *Candida albicans*



Photograph no. 16 - ARMAMENTARIUM : 1 - BHI Agar 2 - BHI Broth 3 – Sterile BHI broth prepared 4 - DMSO 5 – 99.8% Ethyl alcohol 6,7 - 100 µl and 1000 µl pipette tips 8 – Micropipettes(10 µl, 100 µl, 100 µl) 9 - Conical flasks 200ml. 10 – Glass Beaker 200ml. 11 – Paraffin wax sheet 12 - Cinnamon and Cajeput Essential Oils 13 – Microbial Inoculum 14 – Conical Flask 500 ml. 15 – Distilled water 16 – Agar Plate 17 – Spatula 18 – 96-well Tissue culture plate 19 – Eppendorf tubes 20 – Resazurin dye 21 – Inoculation loop



Photograph no. 17 - Investigator performing MIC. The image shows addition of microbial suspension into the wells of microtitre tissue culture plates.

RESULTS

The present *in vitro* study showed the antimicrobial activity of commercially available Cinnamon Bark Essential oil and Cajeput Essential oil and their combination on *Staphylococcus aureus*, *Enterococcus Faecalis* and *Candida albicans*, which are the microorganisms associated with post-radiation therapy in head and neck cancer patients. The study was performed in triplicates and mean values were taken as MIC and MBC of the test agents against the microorganisms. The data obtained were entered in Microsoft Excel (2019) and analyzed using the SPSS® (IBM Corp. Released 2012 IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Gas Chromatography (GC):

Certificate of Analysis (COA) and Gas Chromatographic analysis (GC) of the EOs were obtained from *Nishant Aromas Pvt. Ltd. Mumbai*. The analysis of the EOs is generally required to evaluate (i) their composition through the characterization of their chemical pattern to be compared with Pharmacopoeia monographs or ISO norm; (ii) to evaluate the presence of an adulteration; (iii) to quantitate compounds that can be considered toxic or harmful for human use and/or (iv) suspected allergens limited by regulatory authorities.¹

Table 1: shows Gas Chromatographic analysis of Cinnamon Bark EO showed 20 active compounds. Cinnamaldehyde (82.85%) was the major component present.

S. No	CONSTITUENTS	PERCENTAGE
1	Benzaldehyde	2.22
2	β - Pinene	1.08
3	Camphene	0.59
4	Sabinene	0.72
5	Linalool	0.94
6	(E)- Cinnamyl alcohol	0.37
7	Camphor	0.86
8	Borneol	0.17
9	Benzenepropanal	0.62
10	α - Terpineol	0.40
11	(Z)- Cinnamaldehyde	0.43
12	Decanal	0.13
13	(E)-Cinnamaldehyde	82.85
14	Bornyl acetate	1.06
15	Eugenol	0.13
16	Geranyl acetate	0.29
17	(E)-Cinnamyl acetate	1.48
18	Caryophyllene	0.32
19	Ethylcinnamate	0.46
20	α -Calacorene	0.77

*Z - substituents ranked on the same side of the pi bond, *Zusammen* (German) which means 'together'.

***E** - substituents ranked on the opposite side of the pi bond, *Entgegen*, (German) which means 'opposite'.

Table 2: shows Gas Chromatographic analysis of Cajeput EO showed 13 active compounds. 1,8 Cineole (50.91%), Alpha Terpineol (7.11%) and Limonene (6.84%) were the major components present.

S. No	CONSTITUENTS	PERCENTAGE
1	Limonene	6.84
2	1,8 Cineole	50.91
3	Alpha Pinene	3.63
4	Beta Pinene	2.36
5	Myrcene	1.11
6	Alpha Phellandrene	0.26
7	Alpha Terpinene	0.52
8	Para Cymene	1.15
9	Gamma Terpinene	2.98
10	Terpinolene	1.41
11	Linalool	0.34
12	Alpha Terpineol	7.11
13	Beta Caryophyllene	2.50

Determination of MIC (Figures 1-9)

Broth Dilution method was performed in triplicates using Resazurin Dye for determining MIC of Cinnamon EO, Cajeput EO, their combination, and CHX against *S. aureus* (Fig1-3), *E. fecalis* (Fig.4-6) and *C. albicans* (Fig7-9). The first wells in all the columns were incorporated with highest concentration (500 mg/ml) of the test agents. Double-fold Serial dilution was achieved till the 12th well (0.244 mg/ml).

Determination of MBC (Figures 10-18)

Plate streaking method was performed in triplicates for determining MBC of Cinnamon EO, Cajeput EO, their combination, and CHX against *S. aureus* (Fig 10-12), *E. fecalis* (Fig.13-15) and *C. albicans* (Fig16-18). The even numbers; i.e. the well numbers (WN) marked on each BHI Agar plate were divided into sextants corresponding to the respective MIC wells of different test groups against that particular microorganism mentioned above in the 96-well microtitre plates. The sextant at which no growth was observed was taken as the MBC of that test agent against that particular microorganism.

MIC against *Staphylococcus aureus*

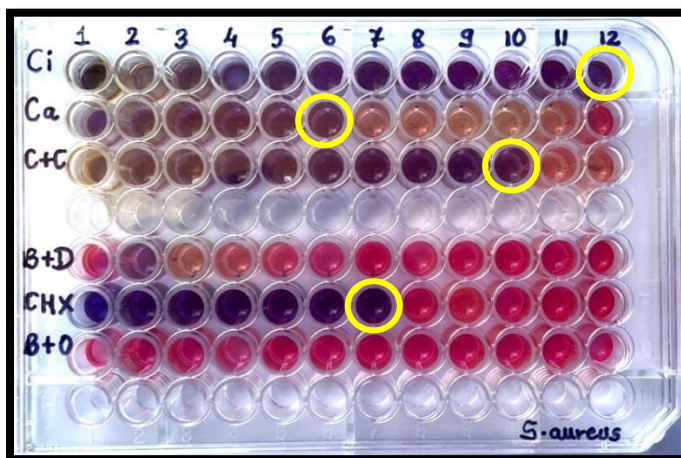


Fig. 1 shows **MIC 1** against *S. aureus*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.244mg/ml (12th well), Cajeput oil as 15.562 mg/ml (6th well), the combination of EO's as 0.977 mg/ml (10th well) and CHX as 7.812 mg/ml (7th well) respectively.

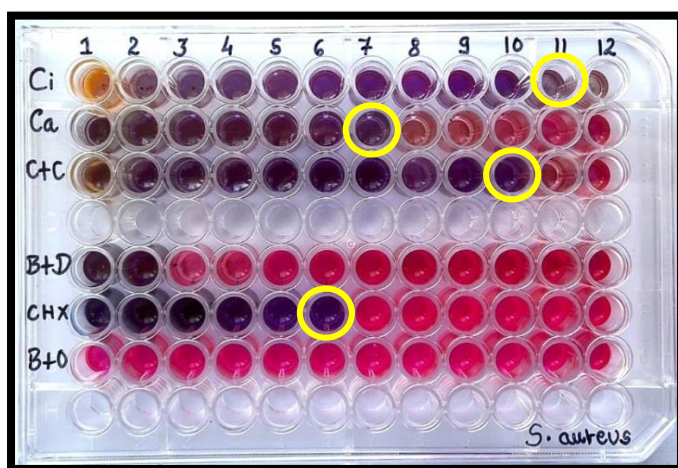


Fig. 2 shows **MIC 2** against *S. aureus*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.488 mg/ml (11th well), Cajeput oil as 7.812 mg/ml (7th well), the combination of EO's as 0.977 mg/ml (10th well) and CHX as 3.906 mg/ml (6th well) respectively.

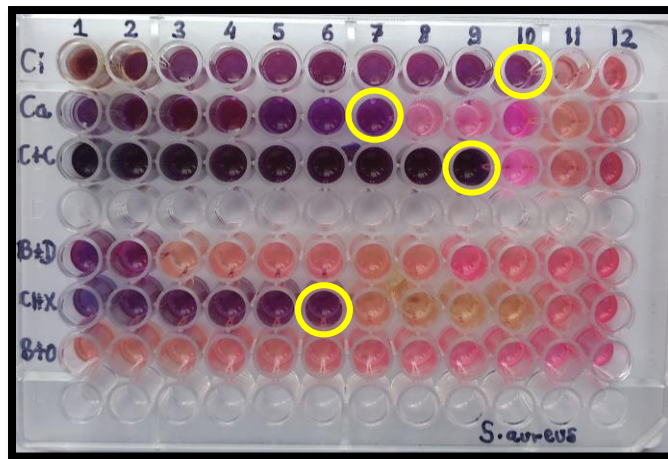


Fig. 3 shows **MIC 3** against *S. aureus*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.977 mg/ml (10th well), Cajeput oil as 7.812 mg/ml (7th well), the combination of EO's as 1.954 (9th well) and CHX as 15.624 mg/ml (6th well) respectively.

Fig. 1-3: Ci – Test for Cinnamon EO, Ca – Test for Cajeput EO, C+C – Combination of EO's, B+D – Broth + Diluent, CHX – Chlorhexidine, B+O – Broth + Organisms. The wells encircled in white color show the MIC for that particular test agent against *S. aureus*

MIC against *Enterococcus faecalis*

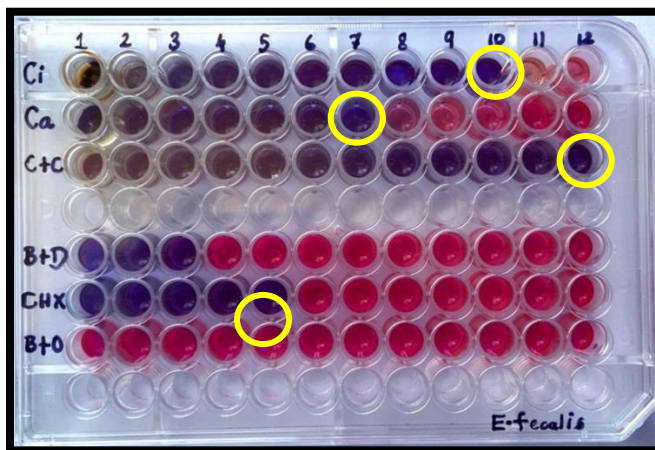


Fig. 4 shows **MIC 1** against *E. faecalis*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.977 mg/ml (10th well), Cajeput oil as 7.812 mg/ml (7th well), the combination of EO's as 0.244mg/ml (12th well) and CHX as 31.248 mg/ml (5th well) respectively.

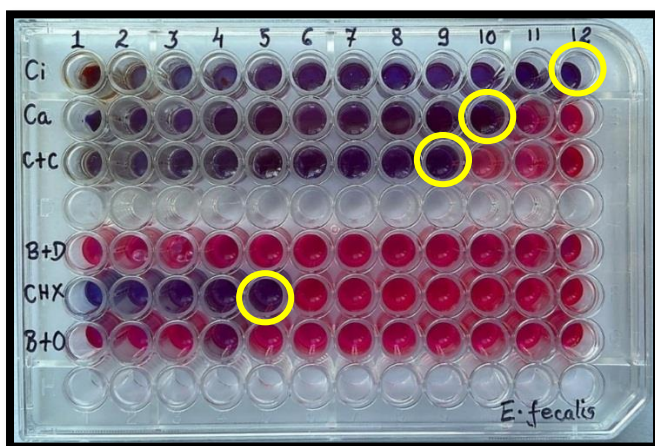
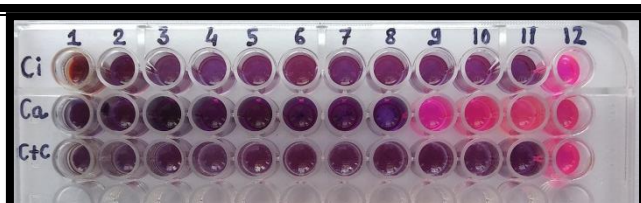


Fig. 5 shows **MIC 2** against *E. faecalis*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.244 mg/ml (12th well), Cajeput oil as 0.977 mg/ml (10th well), the combination of EO's as 1.954 mg/ml (9th well) and CHX as 31.248 mg/ml (5th well) respectively.



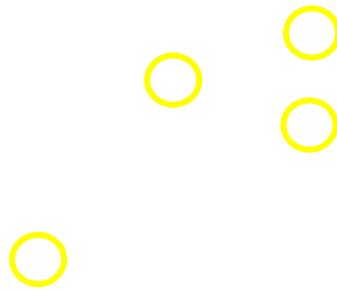


Fig. 6 shows **MIC 3** against *E. faecalis*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.488 mg/ml (11th well), Cajeput oil as 3.906 mg/ml (8th well), the combination of EO's as 1.954 mg/ml (9th well) and CHX as 31.248 mg/ml (5th well) respectively.

Fig. 4-6: Ci – Test for Cinnamon EO, Ca – Test for Cajeput EO, C+C – Combination of EO's, B+D – Broth + Diluent, CHX – Chlorhexidine, B+O – Broth + Organisms. The wells encircled in white color show the MIC for that particular test agent against *E. faecalis*

MIC against *Candida albicans*

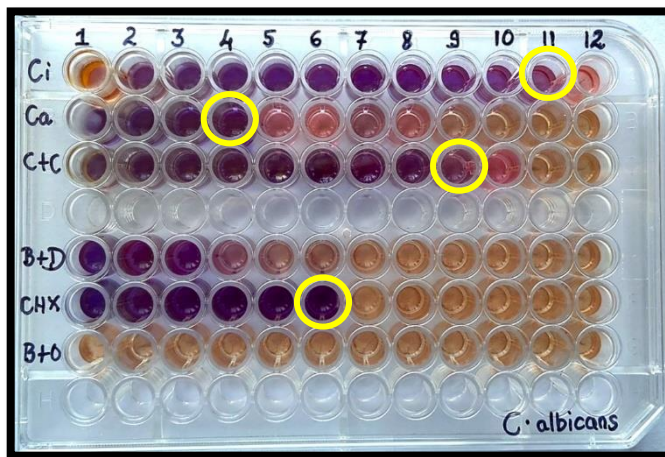


Fig. 7 shows **MIC 1** against *C. albicans*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.488 mg/ml (11th well), Cajeput oil as 62.496 mg/ml (4th well), the combination of EO's as 0.244mg/ml (12th well) and CHX as 3.906 mg/ml (6th well) respectively.

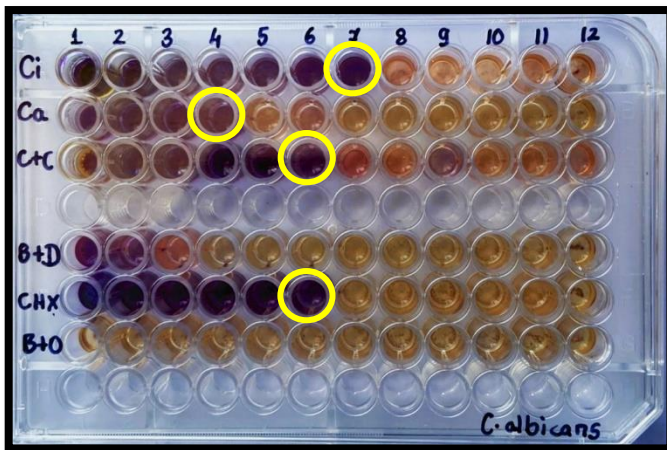


Fig. 8 shows MIC 2 against *C. albicans*. The first, second, third and sixth rows show MIC of Cinnamon EO as 7.812 mg/ml (7th well), Cajeput oil as 62.496 mg/ml (4th well), the combination of EO's as 3.906 mg/ml (6th well) and CHX as 3.906 mg/ml (6th well) respectively.

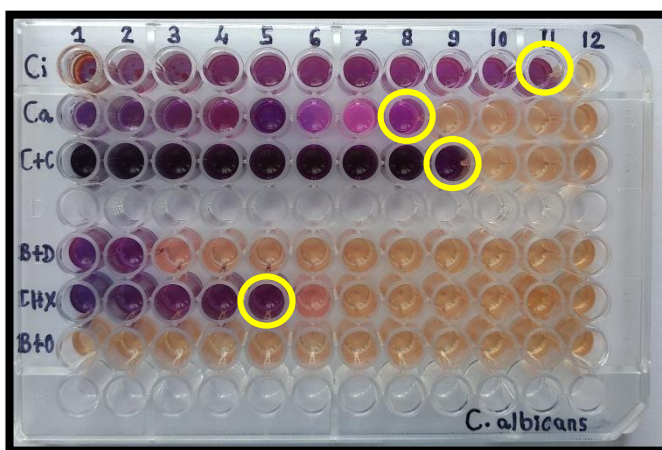


Fig. 9 shows MIC 3 against *C. albicans*. The first, second, third and sixth rows show MIC of Cinnamon EO as 0.488 mg/ml (11th well), Cajeput oil as 3.906 mg/ml (8th well), the combination of EO's as 1.954 mg/ml (9th well) and CHX as 31.248 mg/ml (5th well) respectively.

Fig. 7-9: Ci – Test for Cinnamon EO, Ca – Test for Cajeput EO, C+C – Combination of EO's, B+D – Broth + Diluent, CHX – Chlorhexidine, B+O – Broth + Organisms. The wells encircled in white color show the MIC for that particular test agent against *C. albicans*

MBC against *Staphylococcus aureus*



Fig 10. shows MBC of **Cinnamon bark EO** against *S. aureus*. Visible growth was seen in the WN 12. No growth was seen in the WN 10 (at the concentration of 0.488 mg/ml). This was taken as the MBC value of Cinnamon EO against *S. aureus*

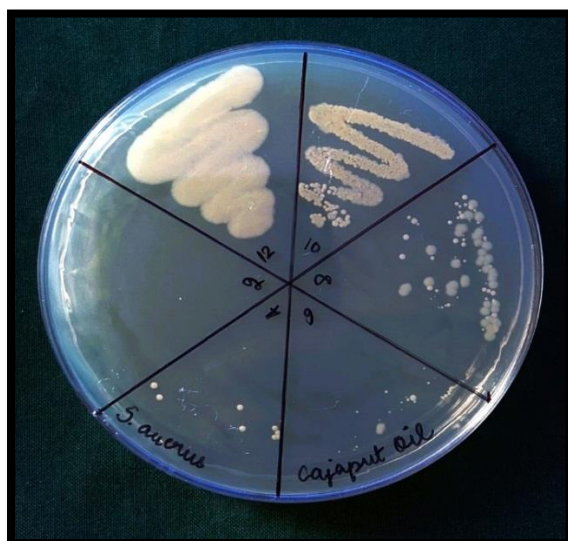


Fig 11. shows MBC of **Cajeput EO** against *S. aureus*. Visible growth was seen in the WN 12, 10 and 8. No growth was seen in the WN 6, (at the concentration of 15.624 mg/ml). This was taken as the MBC value of Cajeput EO against *S. aureus*



Fig 12. shows MBC of **Combination of EO's** against *S. aureus*. Visible growth was seen in WN 12 and sparse growth was seen in WN 10. No growth was seen in the WN 8, (at the concentration of 3.906 mg/ml). This was taken as the MBC value of the Combination of EOs against *S. aureus*

MBC against *Enterococcus faecalis*



Fig 13. shows MBC of **Cinnamon bark EO** against *E. faecalis*. Visible growth was seen in the WN 12. No growth was seen in the WN 10 (at the concentration of 0.488 mg/ml). This was taken as the MBC value of Cinnamon EO against *E. faecalis*.

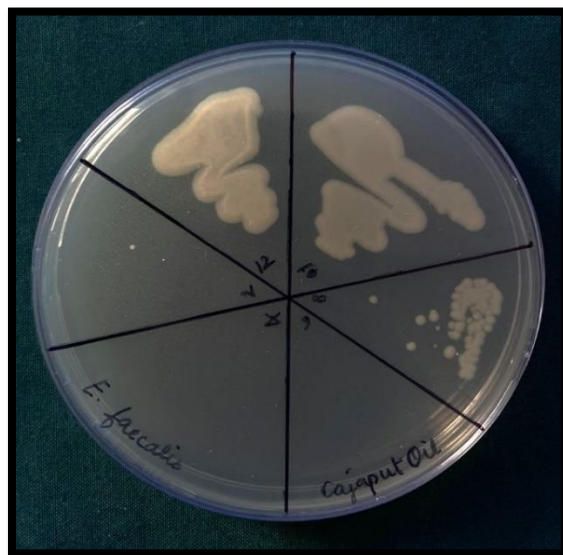


Fig 14. shows MBC of **Cajeput EO** against *E. faecalis*. Visible growth was seen in the WN 12, 10 and 8. No growth was seen in the WN 6, (at the concentration of 15.624 mg/ml). This was taken as the MBC value of Cajeput EO against *E. faecalis*

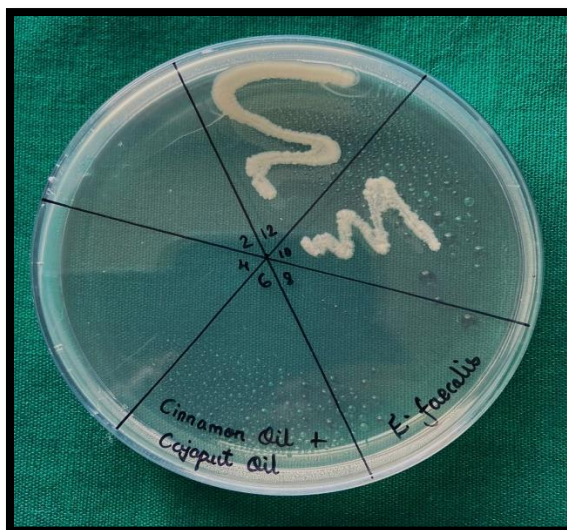


Fig 15. shows MBC of **Combination of EOs** against *E. faecalis*. Visible growth was seen in WN 12 and WN 10. No growth was seen in the WN 8, (at the concentration of 3.906 mg/ml). This was taken as the MBC value of the Combination of EOs against *E. faecalis*

MBC against *Candida albicans*



Fig 16. shows MBC of **Cinnamon bark EO** against *C. albicans*. Visible growth was seen in the WN 12 and WN 10. No growth was seen in the WN 8 (at the concentration of 3.906 mg/ml). This was taken as the MBC value of Cinnamon EO against *C. albicans*

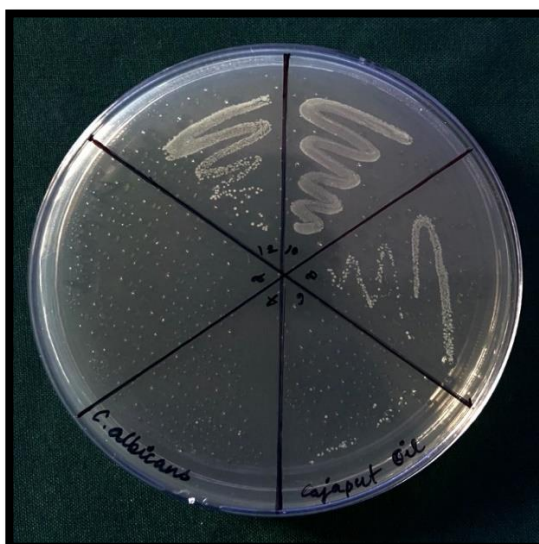


Fig 17. shows MBC of **Cajeput EO** against *C. albicans*. Visible growth was seen in the WN 12, 10 and 8. No growth was seen in the WN 6, (at the concentration of 15.624 mg/ml). This was taken as the MBC value of Cajeput EO against *C. albicans*.



Fig 18. shows MBC of **Combination of EOs** against *C. albicans*. Visible growth was seen in WN 12 and WN 10. No growth was seen in the WN 8, (at the concentration of 3.906 mg/ml). This was taken as the MBC value of the combination of EOs against *C. albicans*.

Table 3: Determination of MIC of Cinnamon Essential Oil, Cajeput Essential Oil and their combination, and 0.12% Chlorhexidine hydrochloride tested against *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*

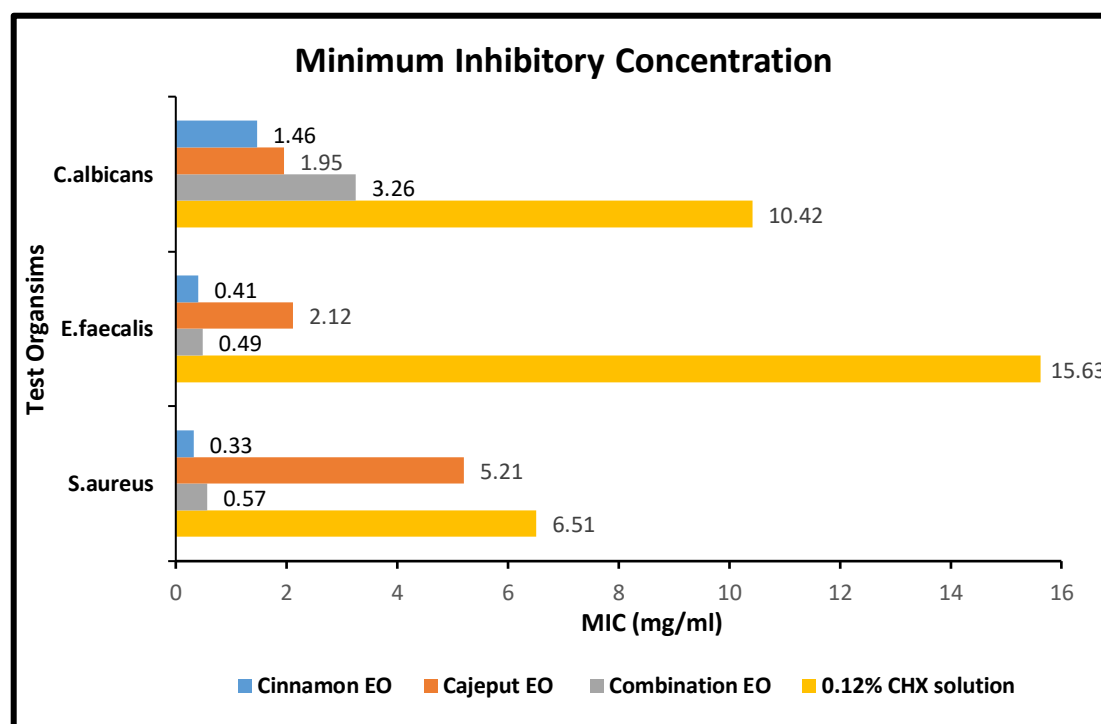
Sl No.	Test Microorganisms	MIC (mg/ml)				Statistics	
		0.12% CHX	Cinnamon Oil	Cajeput Oil	Combination	Z value	P value
1	<i>S. aureus</i>	6.51 ± 2.26 _a	0.33 ± 0.14 _b	5.21 ± 2.26 _a	0.57 ± 0.37 _c	9.039	0.029*
2	<i>E. faecalis</i>	15.63 ± 0.02 _a	0.41 ± 0.14 _b	2.12 ± 1.71 _a	0.49 ± 0.42 _c	8.484	0.037*
3	<i>C. albicans</i>	10.42 ± 4.51 _a	1.46 ± 2.11 _a	1.95 ± 0.01 _a	3.26 ± 3.95 _b	6.297	0.098

MIC: Minimum Inhibitory Concentration. CHX: Chlorhexidine hydrochloride solution

The results are shown as average values of triplicates in mg/ml (Mean ± SD)

Statistical test applied: Kruskal-Wallis test

Post-Hoc test applied: Bonferroni Post-hoc test ^{a,b,c}; Superscript with same letters (a) as that of the gold standard 0.12% CHX are not statistically significant. Whereas, those with different letters (b,c) in the same row show significant difference when compared to 0.12% CHX; **Level of significance:** * p ≤ 0.05 was considered statistically significant.



Graph 1. shows average MIC values of the triplicates of Cinnamon EO, Cajeput EO, their combination and 0.12% Chlorhexidine hydrochloride solution against *S. aureus*, *E. faecalis* and *C. albicans*

Table 4. Determination of MBC of Cinnamon Essential Oil, Cajeput Essential Oil and their combination, and 0.2% Chlorhexidine gluconate against *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*

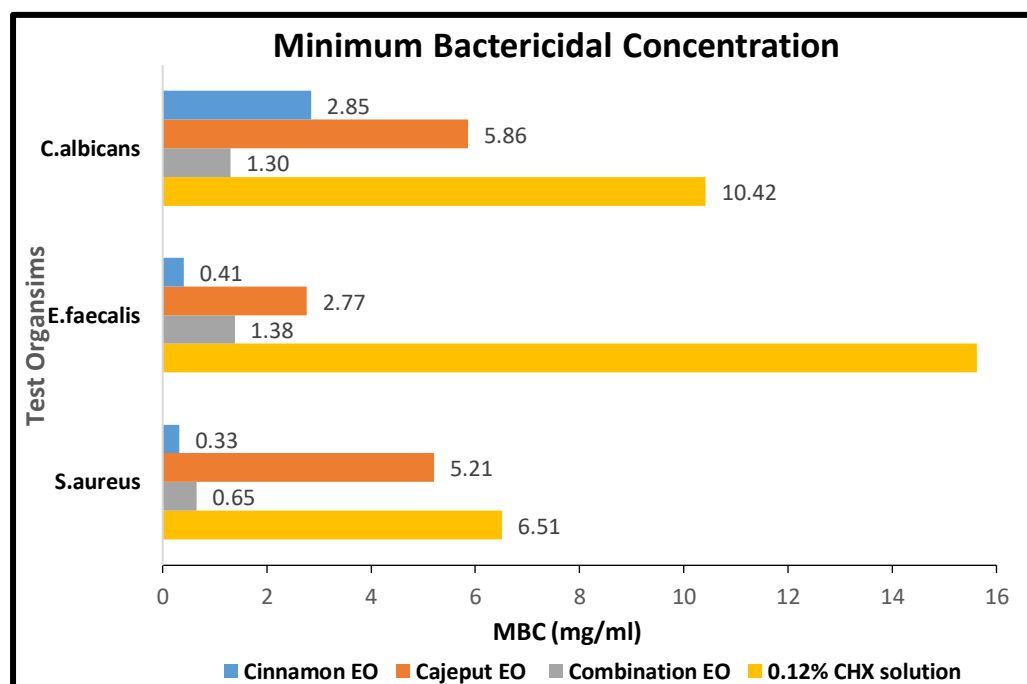
Sl No.	Test Microorganisms	MBC (mg/ml)				Statistics	
		0.12% CHX	Cinnamon Oil	Cajeput Oil	Combination	Z value	P value
1	<i>S. aureus</i>	6.51 ± 2.26 ^a	0.33 ± 0.14 ^b	5.21 ± 2.26 ^a	0.65 ± 0.28 ^c	9.482	0.024*
2	<i>E. faecalis</i>	15.63 ± 0.02 ^a	0.41 ± 0.14 ^b	2.77 ± 1.97 ^a	1.38 ± 0.99 ^c	8.253	0.041*
3	<i>C. albicans</i>	10.42 ± 4.51 ^a	2.85 ± 2.11 ^a	5.86 ± 3.38 ^a	1.30 ± 0.56 ^b	6.431	0.092

MBC: Minimum Bactericidal Concentration. CHX: Chlorhexidine hydrochloride solution

The results are shown as average values of triplicates in mg/ml (Mean ± SD)

Statistical test applied: Kruskal-Wallis test

Post-Hoc test applied: Bonferroni Post-hoc test^{a,b,c}; Superscript with same letters (a) as that of the gold standard 0.12% CHX are not statistically significant. Whereas, those with different letters (b,c) in the same row show significant difference when compared to 0.12% CHX; **Level of significance:** * p ≤ 0.05 was considered statistically significant.



Graph 2. shows average MBC values of the triplicates of Cinnamon EO, Cajeput EO, their combination and 0.12% Chlorhexidine hydrochloride solution against *S. aureus*, *E. faecalis* and *C. albicans*

The experiments were performed in triplicates. The mean MIC values of Cinnamon bark EO, Cajeput EO, their combination and 0.12% CHX solution against *S. aureus* were 0.33 ± 0.14 mg/ml, 5.21 ± 2.26 mg/ml, 0.57 ± 0.37 mg/ml and 6.51 ± 2.26 mg/ml respectively; which suggests that Cinnamon bark EO is the most effective bactericidal against *S. aureus* when compared to Cajeput EO and combination of EOs. (Fig1-3). The results obtained from MBC plates were in accordance with the MIC results (Fig.10-12). Cinnamon bark EO and combination EOs possess comparable antimicrobial activity when compared to 0.12% CHX solution, but amongst the test groups, Cajeput EO was the least effective against *S. aureus*.

The mean MIC values of Cinnamon bark EO, Cajeput EO, their combination and 0.12% CHX solution against *E. faecalis* were 0.41 ± 0.14 mg/ml, 2.12 ± 1.71 mg/ml, 0.49 ± 0.42 mg/ml and 15.63 ± 0.02 mg/ml respectively; which suggests that Cinnamon bark EO is the most effective bactericidal against *E. faecalis* when compared to Cajeput EO and combination of EOs. (Fig4-6) The results obtained from MBC plates were in accordance with the MIC results (Fig.13-15). Cinnamon bark EO and combination EOs possess comparable antimicrobial activity when compared to 0.12% CHX solution, but amongst the test groups Cajeput EO was the least effective against *E. faecalis*.

The mean MIC values of Cinnamon bark EO, Cajeput EO, their combination and 0.12% CHX solution against *C. albicans* were 1.46 ± 2.11 mg/ml, 1.95 ± 0.01 mg/ml, 3.26 ± 3.95 mg/ml and 10.42 ± 4.51 mg/ml respectively; which suggests that Cinnamon bark EO was the most effective fungicidal against *C. albicans* when compared to Cajeput EO and combination of EOs. (Fig7-9). The results obtained from MBC plates were in accordance with the MIC results (Fig.16-18), with the only

difference being the MBC of the Combination group (1.30 ± 0.56 mg/ml) was more efficacious when compared to the MIC (3.26 ± 3.95 mg/ml) results. Therefore, only the combination EOs possess comparable antimicrobial activity when compared to 0.12% CHX solution, but amongst the test groups, Cinnamon bark EO and Cajeput EO were the least effective against *C. albicans*

The highest concentration of the positive control (0.12% CHX solution) was required against *E. faecalis* (15.63 ± 0.02 mg/ml), whereas the lowest concentration was required against *S. aureus* (6.51 ± 2.26 mg/ml). The cinnamon bark EO was most effective against *S. aureus* and least against *C. albicans*. Similarly, Cajeput EO was most efficacious against *Enterococcus faecalis* and least against *Candida albicans*. And lastly, the combination EOs group was most efficacious against *S. aureus* and least with *E. faecalis*

So, the overall results show that Cinnamon bark EO had the strongest efficacy against *S. aureus* (0.33 ± 0.14 mg/ml) and *E. faecalis* (0.41 ± 0.14 mg/ml), but not against *C. albicans* (2.85 ± 2.11 mg/ml). Cajeput EO shows the least efficacy against all the groups; whereas the combination of Essential oils has proved to be the most efficacious and shows good antimicrobial activity against these most commonly encountered microorganisms in head and neck cancer post-radiotherapy.

DISCUSSION

Oral microbiota in patients with and without cancer:

It is a well-known clinical reality that oral cancer patients frequently have bad oral hygiene when they first come. According to studies, mouth microbiota may play a role in the emergence of cancer, and there may even be a causative relationship between oral microbes and cancer.²³ A common component of many chronic diseases, including cancer, is inflammation.^{24, 25} Inflammations brought on by infections are thought to contribute to the pathophysiology of about 15% to 20% of human malignancies.²⁶

Malignant and healthy oral mucosa appear to have different microbial populations. For instance, a number of upper gastrointestinal tract carcinomas have been linked to *Streptococcus anginosus* and *Treponema denticola*.²⁷ *S. anginosus* infection may be related to the development of head and neck squamous cell carcinoma, according to Shiga et al.²⁹ Additionally, it should be remembered that the sampling site itself may affect the outcomes, making it challenging to conduct the best possible sampling in people with oral cancer.³⁰

Impact of cancer treatment on oral microbiome

Chemotherapy alters the quantity, complexity, and quality of the oral microbiota in cancer patients, which causes a significant ecological imbalance, according to microbiological investigations.³¹ Radiotherapy also causes hyposalivation which may affect the oral microbiota. According to Almstahl et al, *Candida albicans* was discovered in one or more sites in 54% of the participants who

had had radiation to the head and neck, compared to 15% of the controls. In 38% of these individuals, compared to none of the controls, enterococci were also present.³² Although some bacterial or fungal species not often found in normal participants were also found among radiation patients, a thorough investigation by Leung WK et al. revealed that the predominant elements of the sub-gingival microbiota resembled gingivitis sites in the general population. *Peptostreptococcus*, *Staphylococcus*, *Streptococcus*, *Actinomyces*, *Eubacterium*, *Lactobacillus*, *Neisseria*, *Veillonella*, *Bacteroides*, *Campylobacter*, *Capnocytophaga*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, etc. were among these species of microorganisms.³³ Periodontal disease status and attachment loss were positively correlated with an increase in the proportional recovery of *Staphylococcus* sp. from supra-gingival sites and total yeasts from supra- and sub-gingival sites, according to a study by Reynolds MA et al on non-lymphocytic leukemic patients in Baltimore.³⁴

A study conducted by Anjali K et al showed increase in *Staphylococcus*, *Bacillus*, *Enterococcus*, *Pedococcus*, and other germs like *Klebsiella*, *Staphylococcus kloosi*, and *Staphylococcus hominis* following six months of radiation therapy. When Kamath et al. examined how radiotherapy affected the oropharyngeal flora in head and neck cancer, they came to the conclusion that *S. aureus*, *Pseudomonas*, *Bacteroides*, and *Candida albicans* increased while *Streptococcus pneumoniae* considerably decreased after the end of irradiation.⁴

Use of herbal essential oils for the prevention of oral microbiota-related cancer

Since the dawn of civilization, several naturally occurring herbs and spices, including garlic, cumin, cloves, cinnamon, thyme, mustard, and rosemary, have been used both in cuisine and as medicines.³⁵ More than 300 EOs are commercially

significant and employed by the flavor and fragrance industries, are thought to exist among these secondary metabolites. Terpenes, Nitrogen and Sulphur containing compounds, phenylpropanoids are the three primary categories of secondary metabolites.³¹ While certain hydrocarbons also have antibacterial properties, the oxygenated terpenoids (such as phenolic terpenes and alcohols) make up the majority of the antimicrobial activity in EOs.³¹ More recently, another propriety, not related with the biocidal activity of the essential oils, has been observed. These compounds demonstrated anticancer activity; more specifically, the constituents thymol and carvacrol present in some essential oils, such as oregano EO.³⁶ The study conducted by Kang et al reported that the patient's health status, the presence of other oral pathology, the timing of chemotherapy and radiation treatment, surgery, the stage of the disease, the type of malignancy, the patient's age, previous or ongoing antibiotic treatments, etc., may all have an impact on the prevalence of oral microbes in cancer patients. Local and systemic infections are brought on by changes in the oral microbial flora in oral cancer patients, which may enhance morbidity.⁶

A review conducted by Katarzyna Wińska et al, described the sources, chemical composition and antimicrobial properties of various essential oils. The main component of cinnamon EO is **trans-cinnamaldehyde** - which was also the primary constituent present in Cinnamon Bark EO which was tested in this study.¹³ O-methoxy-cinnamaldehyde, cinnamyl aldehyde, benzaldehyde, phenyl ethanol, borneol, eugenol, coumarin, and cinnamic acid are some of the other ingredients. Studies using EO from the cinnamic aldehyde-rich bark of *C. zeylanicum* were conducted by Intorasoot et al. and Feng et al. They demonstrated that cinnamon bark essential oil had a superior impact in comparison to other essential oils (such as clove, lemongrass, tea tree, ginger, and basil), with excellent action against *S. aureus*, *E.*

coli, and other pathogens. The present study also showed excellent bactericidal activity of Cinnamon bark EO against *Staphylococcus aureus* at a pretty much lower MIC of 0.244 mg/ml.³⁷

One of the most characteristic components of Cajeput EO is **1,8-cineole (15-60%)** – with antimicrobial and anti-inflammatory activity, **linalool** – with antimicrobial, anti-inflammatory, analgesic, as well as anti-hyperalgesic activity **and terpinen-4-ol** - with antibacterial, antiviral and antifungal activity are also the active compounds present in commercially available EO used in this study. According to the current study, Cajeput oil suppressed the growth of Gram-positive bacteria like *S. aureus* and *E. faecalis* at a concentration of 0.2-0.4%. It inhibits yeast like *Candida albicans* at a dosage of 0.4-0.6%. In the present study the MIC of Cajeput oil was found to be 5.21 mg/ml and 2.77 mg /ml against *S. aureus* and *E. faecalis* respectively; whereas MIC was higher at a concentration of 5.67 mg/ml against *Candida albicans* which was in accordance with the present study.⁶

According to Metgud et al. greater numbers of bacterial and candidal colonies were found on the cancer site. Although *Candida* sp., in particular *C. albicans*, has been linked to oesophageal and oral cancer, the pathogenic pathways and carcinogenic potential were unclear.¹³ Hence, in the present study *Candida albicans* was chosen as one of the most commonly found microorganism in oral cancer patients.

Several *C. zeylanicum* bark extracts were evaluated in vitro using the disk-diffusion method against *Klebsiella pneumonia* 13883, *Pseudomonas aeruginosa* ATCC 27859, *Staphylococcus aureus* 6538 P, *Escherichia coli* ATCC 8739, and *Streptococcus faecalis* DC 74 to determine their antibacterial effects. According to the findings, the antibacterial activity ranges from 7 to 18 mm for the application of 30 L,

which is consistent with the current investigation, indicating a significant antibacterial activity.¹³ According to Mandal et al., *methicillin-resistant S. aureus* clinical isolates were resistant to the antibacterial effects of an ethanolic extract of the stem bark of *C. zeylanicum* (MRSA). The researchers came to the conclusion that *C. zeylanicum* could be regarded as helpful since it could aid in the creation of possible antibiotic drugs to combat MRSA bacteria.³⁷ Cinnamon bark essential oil obtained through hydro-distillation was tested for antibacterial activity (expressed as MIC) against several pathogenic bacterial strains (*Salmonella typhi*, *Salmonella paratyphi A*, *E. coli*, *S. aureus*, *Pseudomonas fluorescens* and *Bacillus licheniformis*).³⁸ TLC and GC-MS analysis of the chemical composition showed that the most prevalent component was t-cinnamaldehyde.³⁹ One of the primary causes of pulp and periapical illnesses of the oral cavity, *Enterococcus faecalis*, was examined for its antibacterial activity against cinnamon fresh leaf extract more recently. The essential oil, whose principal component is cinnamaldehyde (97% by weight), shown antibacterial, antioxidant, and anti-inflammatory activity. The MIC value of 0.41 mg/ml, or 410 g/mL, was in accordance with the current investigation and ranged from 250 to 1000 g/mL.⁴⁰

Cytotoxicity of Cinnamon and Cajeput EOs:

According to a study by Geneviève Lebel et al. it concluded that cinnamon oil may be a promising substance to incorporate into oral hygiene products for controlling bad breath by inhibiting growth and killing biofilm. Moreover, at the effective concentrations, cinnamon oil was found to have no toxic effects on oral keratinocytes.⁴¹ Although there is lacunae in the literature regarding cytotoxicity of Cajeput EO, a study done by Hsiu-Fang Ye et al revealed that as a food grade EO, it can be used without any toxic effect on oral epithelial cells.⁴²

CONCLUSION

Cinnamon and Cajeput Essential Oils in combination possess comparable antimicrobial activity against *S. aureus*, *E. faecalis* and *C. albicans* when compared to 0.12% CHX solution. The synergistic effect of the combination of Cinnamon and Cajeput essential oils significantly showed effectiveness against *C. albicans*, when compared to *S. aureus*, and *E. faecalis*. Although the results of the present in-vitro study are promising, extensive research is still required to prove the capability of such herbal products against other causative microorganisms present in oral cancer-treated patients post-radiotherapy.

LIMITATIONS OF THE STUDY:

Oral cancer is a multifactorial disease due to the combination and interactions of agent, host, and environmental factors. It is also pivotal to remember that laboratory experiments do not exactly reflect the clinical conditions that exist in the cancer-treated patients post-radiotherapy. Although the present study emphasizes the most commonly isolated microorganisms

(*S. aureus*, *E. faecalis*, and *C. albicans*) in head and neck cancer-treated patients post-radiotherapy, it does not consider other numerous microorganisms which could be life-threatening and reduce the life expectancy in such individuals.

Although the present study emphasizes on the most commonly isolated microorganisms (*S. aureus*, *E. faecalis*, and *C. albicans*) in head and neck cancer-treated patients post-radiotherapy; other numerous microorganisms that could reduce the life expectancy and lead to serious infections in such patients are not considered.

RECOMMENDATIONS AND FUTURE IMPLICATIONS:

1. Due to low toxicity, abundant availability, less adverse effects and potential antimicrobial activity, herbal formulations and medications have been extensively used globally. Therefore, extensive research should be conducted in the herbal therapeutics.
2. Literature shows an abundant collection of essential oils that can be utilized in different forms and formulations to help inhibit the growth of harmful microorganisms that compromise the prognosis in treated oral cancer patients.
3. Studies on EOs and their specific mechanism of action when used in combination to understand the synergistic effects of their antimicrobial activity should be conducted.
4. Extensive research and in-vivo studies should be carried out to substantiate the results of the present in-vitro study.
5. It is imperative to develop biocompatible, potent mouthwashes, gum paints, therapeutic pastes in different combinations of herbal products that will be efficient, cost-effective with less adverse effects.

PUBLIC HEALTH SIGNIFICANCE

The increasing demand for herbal formulations in the present Indian scenario can be supported by the results of the present study. This novel research can be applied to conduct in-vivo studies and if the results come good, it can be used as an alternative and/or adjunct to the available treatment modalities which can cause various adverse effects when used on a long-term basis, as mentioned earlier. Due to the alarming increase in microbial resistance with the available chemical agents, herbal therapies will provide a wide variety of antimicrobial properties which will be relatively safe, efficient, cost-effective and acceptable by the community in the prevention and management of head and neck cancer patients.

SUMMARY

The present study was an *in-vitro* study that was conducted for evaluating antimicrobial activity using Cinnamon bark essential oil, Cajeput essential oil, their combination and 0.12% Chlorhexidine solution against *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*. Ethical clearance was obtained from the Institutional Review Board. The prominent active constituents of the essential oils which were responsible for antimicrobial activity were present in the commercially procured Essential oils used in this study.

Cinnamon Bark EO, Cajeput EO, and their combination were assessed for determination of Minimum Inhibitory Concentration (MIC) by Broth dilution method, using Resazurin dye and Minimum Bacterial Concentration (MBC) by Plate streaking method against three standard strains of microorganisms most commonly encountered in Head and neck cancer patients (*Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans*) in comparison of 0.12% CHX solution. Change in colour from blue/purple to pink/red indicated viable microorganisms (Resazurin reduced to Resorufin) and no change of Resazurin dye indicated inhibition of microbial growth. Lowest concentration at which the colour change observed was taken as MIC value of the respective essential oil and their combination against *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*.

The experiment was conducted in triplicates and the mean values were considered for determination of results. Kruskal Wallis Test followed by the Bonferroni Post-Hoc test were employed to compare the differences in the antimicrobial activity of individual EOs and their combination and 0.12% CHX against the test organisms. $p \leq .05$ was considered statistically significant.

When compared to both the oils, Cinnamon EO was most efficacious against all microorganisms with MIC and MBC at a very low conc. of 0.244 mg/ml ($p \leq .05$) whereas Cajeput EO had the least efficacy. Overall, the combination proved to be the best in killing all the microorganisms. CHX was also efficient against all the microorganisms but at relatively higher MIC and MBC. Thus, the combination of Cinnamon and Cajeput EOs can be used as an adjunct to the standard treatment modalities considering its long-term use and lesser side effects in the patients treated with head and neck cancer post-radiotherapy.

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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

FAX: 0831-2470640

Web: <http://www.kledental-bgm.edu.in>E-mail: principal@kledental-bgm.edu.inSI. No. : **1452****CERTIFICATE**

This is to Certify that the synopsis titled

*Antimicrobial efficacy of essential oils and their
 combination on microorganisms associated with
 post radiation therapy in head & neck cancer patients
 An- in vitro study. Submitted by*

Dr. REG. NO. IL0220001 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 :

[Signature]
Member Secretary
 Research and Ethical Committee
 KLEVK Institute of Dental Sciences
 Belagavi
MEMBER SECRETARY
 Research & Ethical Committee
 KLEVK Institute of Dental Sciences
 BELAGAVI.

[Signature]
Chairman
 Research and Ethical Committee
 KLEVK Institute of Dental Sciences
 Belagavi
Chairman
 Research and Ethical Committee
 KLE VK Institute of Dental Sciences
 Belagavi

ANNEXURE II – PLAGIARISM REPORT REQUISITION

To:

Dr. Punya V. Angadi

Member Secretary

Scientific Correspondence and Review Committee

KLE V.K Institute of Dental Sciences, Belagavi

24th December, 2022

Respected Madam,

Kindly find enclosed the similarity index report, from Turn-it-in, of **REG. NO. IL0220001** -Post Graduate Student from the Department of Public Health Dentistry with the title **"ANTIMICROBIAL EFFICACY OF ESSENTIAL OILS AND THEIR COMBINATION ON MICROORGANISMS ASSOCIATED WITH POST-RADIATION THERAPY IN HEAD AND NECK CANCER PATIENTS: AN IN-VITRO STUDY"** under the guidance of **Dr. ROOPALI SANKESHWARI M.D.S, Ph.D,** Associate Professor,, Department of Public Health Dentistry, KLE Academy of Higher Education and Research, KLE Vishwanath Katti Institute of Dental Sciences, Belagavi, Karnataka.

Plagiarism score is found to be 7% which is within acceptable norms checked on 24/12/22

I request you to kindly issue the clearance certificate

Thanking you



Associate Professor

Dept. of Public Health Dentistry

KLE V.K. Institute of Dental Sciences, Belagavi

590010, Karnataka

ANNEXURE III – PLAGIARISM, SIMILARITY INDEX

Antimicrobial efficacy of essential oils and their combination on microorganisms associated with post-radiation therapy in head and neck cancer patients: An in-vitro study

ORIGINALITY REPORT

7 %	%	7 %	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

- | | | |
|----------|---|------------|
| 1 | Laurent Raynaud, Christelle Nguyen Dac, Madeleine Beaussac, Thibault Martinez, Mathieu Boutonnet. "Evacuations sanitaires aériennes collectives du service de santé des Armées français - MoRPHEE et MEROPE - au profit de patients en syndrome de détresse respiratoire aigu lié à la COVID-19", Médecine de Catastrophe - Urgences Collectives, 2021
Publication | 2 % |
| 2 | Vianna, M.E.. "In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite", Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology, 200401
Publication | 1 % |
| 3 | Alberto Pispero, Niccolò Lombardi, Maddalena Manfredi, Elena Maria Varoni, Andrea Sardella, Giovanni Lodi. "Oral infections in oral cancer survivors: A mini-review", Frontiers in Oral Health, 2022 | 1 % |

ANNEXURE IV – COA OF CINNAMON BARK EO



NISHANT AROMAS PRIVATE LIMITED

Factory : Plot No. 5,6,7,8, Survey No. 433/2, PLDC, Near BIDCO, Palghar (W)-401 404, MH (INDIA).
 Email Id: lab@nishantaromas.com/dispatch@nishantaromas.com. Cont: 02525-254163/325643/730354163

CERTIFICATE OF ANALYSIS

DATE : 29/04/2021

REF. NO. : DS0769/21
 PRODUCT NAME : CINNAMON BARK OIL RECT
 CHEMICAL NAME : Cinnamomum zeylanicum Blume Bark Oils
 CAS NO : 8015-91-6
 BATCH NO. : CBO/04/21 QTY : 0.100 Kg
 MANUFACTURING DATE : APRIL - 2021 PO NO. : EMAIL
 EXPIRY DATE : MARCH - 2022 PO DATE : 28/04/2021
 BUYER NAME : REG. NO. IL0220001

PARAMETERS	SPECIFICATION	RESULT
APPEARANCE & COLOUR	PALE YELLOW TO YELLOW CLEAR LIQUID	PALE YELLOW
ODOUR	SWEET CINNAMIC SPICY WARM WOODY AROMATIC ODOUR	SWEET CINNAMIC SPICY WARM WOODY AROMATIC ODOUR
REFRACTIVE INDEX	1.545 TO 1.609 AT 20°C	1.602
SPECIFIC GRAVITY	1.035 TO 1.050 AT 25°C	1.044
PURITY (GC %)	CINNAMIC ALDEHYDE 75% TO 85%	81.51%

ANALYSED BY

Dehew
 DATE: 29/04/21

APPROVED BY

[Signature]
 DATE: 29/04/21

Admin Office : Unit No. 604,605, 606, Lotus Business Park, Ram Baugh Lane, Off. S.V.Road, Malad (W),
 Mumbai-400064. MH (INDIA) EmailD : info@nishantaromas.com / export@nishantaromas.com
 Contact. : 022-28806611 CIN: U74999MH2019PTC326261

ANNEXURE V – COA OF CAJEPUT EO



NISHANT AROMAS PRIVATE LIMITED

Factory : Plot No. 5,6,7,8, Survey No. 433/2, PLDC, Near BIDCO, Palghar (W)-401 404, MH (INDIA).
Email Id: ravi@nishantaromas.com / dispatch@nishantaromas.com. Cont: 02525-254163/325643/730354163

CERTIFICATE OF ANALYSIS

DATE : 29/04/2021

REF. NO. : DS0768/21
 PRODUCT NAME : CAJUPUT OIL
 BOTANICAL NAME : Melaleuca leucadendron
 CAS NO. : 8008-98-8
 BATCH NO. : CO/04/21
 MANUFACTURING DATE : APRIL - 2021
 EXPIRY DATE : MARCH- 2022
 BUYER NAME : REG. NO. IL0220001

QTY : 0.100 Kg
 PO NO. : EMAIL
 PO DATE : 28/04/2021

PARAMETERS	SPECIFICATION	RESULT
APPEARANCE & ODOUR	CLEAR LIQUID WITH SWEET, FRESH, HERBAL, ROSEMARY, EUCALYPTUS, GREEN ODOUR	COMPLIES
COLOUR	COLOURLESS TO PALE YELLOW	COLOURLESS
REFRACTIVE INDEX	1.455 TO 1.475 AT 20 °C	1.465
SPECIFIC GRAVITY	0.870 TO 0.925 AT 25 °C	0.917
OPTICAL ROTATION	-5° TO +3°	-4.52°
PURITY (GC %)	1,8-CINEOL : MIN 60.0 %	67.18%

ANALYSED BY

[Signature]
DATE: 29/04/21

APPROVED BY

[Signature]
DATE: 29/04/21

Admin Office : Unit No. 604,605, 606, Lotus Business Park, Ram Baugh Lane, Off. S.V.Road, Malad (W),
Mumbai-400064. MH (INDIA) Email ID : info@nishantaromas.com / export@nishantaromas.com
Contact. : 022-28806611 CIN: U74999MH2019PTC326261

ANNEXURE VI – COA OF 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	: <i>[Signature]</i>	Checked by	: <i>[Signature]</i>	Approved by	: <i>[Signature]</i>
Date	: 18/09/2020	Date	: 18/09/2020	Date	: 18/09/2020

AN ISO 9001:2015 COMPANY

Quantity is Must but Quality is First

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Skype ID : bplspl, Web : http://www.basicpharma.in
CIN : U24230GJ2005PTC046278

