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Design, Development and Evaluation of Herbal Antiseptic Wound Plaster

by

Zaki ul Hasan

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

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I dedicated this thesis to Almighty, His Beloved Prophet (SAW), my Parents, my Teachers Dr. Sahar Fazal and my supervisor Dr. Erum Dilshad who are the source of inspiration and motivation for me.

CERTIFICATE OF APPROVAL

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Abstract

Acrinol is widely used globally as antimicrobial agent in plasters. There is a risk of developing resistance in microbes for acrinol. Herbal antimicrobial compound can be a better replacement for acrinol in plasters. Eugenol is a herbal compound found in clove oil having antimicrobial characters. This study was aimed to design, develop and evaluate the antiseptic activity of herbal wound pad. This product contains clove oil (*Syzygium aromaticum*), eucalyptus oil and tea tree oil (*Melaluca terpenin-4-ol)*. These plants have been reported in the literature as having good antimicrobial, anti-oxidant and anti-inflammatory activity. Formulated batch was fully tested for physical, chemical and microbiological parameters. It is a very good attempt to establish the herbal antiseptic wound pad containing natural compounds having antiseptic and anti-inflammatory effects. The pilot trial were conducted on a commercial scale machines in order to observe behavior and feasibility of machine with respect to new product formulation and also initiated analytical studies(qualitative quantitative and microbiological determination) in order to get the physical and chemical compatibility of formulation with the wound pad material. Current good manufacturing practice(cGMP) was followed during all the manufacturing and packaging process. Antiseptic wound plaster was successfully designed and developed after extensive manufacturing and evaluation process by specialized techniques for evaluation of antiseptic activity in vitro.

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Abbreviations

ATCC	American Type Culture Collection
EDTA	Ethylenediamine Tetraacetic Acid
GC	Gas Chromatography
MIC	Minimal Inhibitory Concentration
MLT	Microbial Limit Test
MRSA	*Methicillin Resistance Staphalococcus aureus*
TAMC	Total Aerobic Microbial Count
TTO	Tea Tree Oil
TYMC	Total Least and Mold Count
VcE	*Vancomycin Resistance Entercocci*

Chapter 1

Introduction

An open injury can be dressed by utilizing a norm of biomaterial, for example a wrap to keep it shielded from contaminations and furthermore to recuperate the influenced region. A cement gauze, moreover called a staying mortar(and furthermore known by generalized brand names) is a little dressing utilized for wounds not serious enough to require a full-site gauze. The glue swathe shields the cut from erosion, microorganisms, dirt and damage [1]. Adhesive gauze is otherwise called staying plaster, medical plaster or simply plaster. It is fundamentally a little and adaptable sheet of material which is tacky on one side, having a more modest, non-sticky, permeable cushion adhered io the tacky side. The plaster ia putted against the injury covering edges of the tacky material aid smoothed down so they adhere to the encompassing area. Plaster wraps are available in a bundled sealed, sterile bags, with a support for the sticky side; the packing should be removed as the gauze is applied. They are accessible in an assortment of shapes and sizes. Fundamental oils have the capability of novel antimicrobial action particularly against bacteria [2]. It is ordinary that the use of edible plants for remedial, normal or flavors, for instance, oregano, rosemary, thyme, sage, basil, turmeric, ginger, garlic, nutmeg, clove, mace, appealing aid fennel have been applied viable either alone or in blend in with other defending procedures as antimicrobial trained professional [3]. Antimicrobial properties of large oils from plants have been used for a long time; however, they have been reduced in size over the years. Recently, a huge number of assessment is being finished seeming antimicrobial activity of

various plant oil isolates and their specific fragments for possible use in fields going from food industry to dentistry [4, 5]. Clove oil depicts biological activities, for instance, antibacterial, antifungal, insecticidal, cell strengthening properties and is used as spaces and antimicrobial material in food [6–8]. Also, clove oil is furthermore used as a disinfectant in oral infections [5, 9].

The compounds of major oils of *Melaleuca alternifolia* (tea tree oil) include large cyclic monoterpenes, about half of which are oxygenated and about other half are hydrocarbons. Its widespread antimicrobial action is actually due tc terpinen-4-ol [10]. Tea tree oil can hold respiration and increase leakage of microbial cells and recommends its deadly functions mainly due to the membrane located metabolic events inhibition and the loss of chemiosmotic control. Different microorganisms like *E. coli*, *Staphlococus aureus* and some what *C. albicans* showed change susceptibility which can be explained by contrasts in the level of monoterpene induced cell film hurt. Furthermore, in the case of *C. albicans* the absence of K^+ efflux bound to tea tree oil and the presence of abnormal cells with insoluble plasma levels in PI indicate that the retained tissue damage may change from that observed by bacteria [11]. Microorganisms showed resistance for manor antibiotics known to man and it is a risk to general prosperity, which can be the reason to an increase in loss. Methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *Enterococci* (VcE) are the rule known safe life forms among Gram-positive microorganisms concerning nesocomial diseases. Resistance development in Gram-negative microbes (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomanas aeruginosa* and *Acinetobacter*) has also been accounted for. The past has seen that different antibiotics that have regularly been utilized have now gotten less active on these microorganisms [12].

Henceforth, there is a rush to locate the elective antimicrobial compounds for the treatment of pathogenic microorganisms. Albeit, logical reasons for therapeutic significance were not known for some basic oils counting those from eucalyptus but they have been utilized in society medication all through the world for quite a long time, and their medicinal properties have been examined [13]. It has been demonstrated that fundamental oils from eucalyptus showed antibacterial, antifungal, pain easing and anti-inflammatory properties and accordingly it has been

for the most part used in medication, food, likewise, beautifiers things [14–16]. *Eucalyptus globulus Labill* (*Myrtaceae*) is the major origin of eucalyptus oil on the planet and has been used as an antiseptic and for relieving of cool, sore throat and various illnesses [17, 18]. The oil, outstanding as 'eucalyptus oil', has been conveyed at business level from leaves [19]. Additionally, natural properties of the essential oil of regular items from *E. globulus* have not been very inspected, while the manufactured structure of the natural item oil has been settled [20]. Taking everything into account of *E. globulus*, fundamental oils from *E. radiataSieber* ex. DC (*Myrtaceae*), *E. citriodora* and *Hook (Myrtaceae)* are by and large used for fragrant healing. *Eucalyptus radiata* oil has moreover been shown to be significant for issues of the respiratory system [13]. *Eucalyptus citriodora* is another critical eucalyptus with antibacterial, antifungal, pain relieving and antagonistic to inflammatory properties [15, 19]. In any case, no report is available till now on its activity against multidrug resistant organisms.

1.1 Aims and Objectives

1.1.1 Aims

There is an emerging demand for first aid plaster traditionally. Acrinol is used in plasters as an antiseptic agent. Moreover, antimicrobial resistance to acrinol can be increased; therefore, it is necessary to have other antiseptic agents in the plaster. As natural compounds are better than synthetic ingredients, so eugenol may be a better substitute for acrinol. As microbial resistance is a continuous phenomenon and their is always a need for new antimicrobial compound to combat this resistance. The natural compounds are biodegradable having fever side effects as compared to synthetic compounds so they are helpful in bioremediation. Eugenol is antibacterial and antifungal at the same time while Acrinol and others compounds being used in plasters are only antibacterial. Thus the study aims to develop a wound healing antiseptic plaster with herbal extract as active ingredient by using essential clove oil (Eugenol) to treat minor wounds.

1.1.2 Objectives

The study includes following objective.

- To prepare a herbal antimicrobial batch
- To check chemical, physical and microbiological parameters
- To impregnate the herbal solution with wound pad
- Striping of plasters
- Analyzing the physical parameters of bandage

Chapter 2

Literature Review

2.1 Medicinal Plant

The history of wound healing is related to human history. One of the oldest medical manuscripts in existence is a clay table that dates from 2200 BC, Which describes for the first time the "three acts of healing" that are wash wounds, paint, and bind wounds. The first people to use bandages and honey for the first time were the Egyptians. Honey, grease and clay were the mainstays of Egypt's popular concrete system. While honey has been shown to be an effective antiseptic, it has many other cooling properties. Honey has been used for thousands of years and is still part of the wound healing process.These evidences provide us the proof that the medicinal plants are being used since centuries for the human cure and protection. The Egyptians, Romans, Greek, Indus all civilizations used these [20]. At the turn of the century modern medicine came to the fore. Currently, there are more than 5,000 wound care products available. Most modern wear contains substances with high absorption, such as alginates, foam, or carboxy methyl cellulose. These exist as existing garments and casual clothing. There are growths factors, improved honey-based wear and hypochlorous acid-based cleansers. Chemical tissue, chemotherapy and oxygen-based care have changed the way we tend to treat several chronic wounds these days [20].

2.2 Herbal Bioactive Compound

A bioactive compound found in minor amount in foods and medicinal plants(for example organic matter, vegetables, nuts, oils, whole grains and medicinal plants). The compounds produced contain body building substances that can promote amazing prosperity. They are mainly focused on preventing the threat, coronary heart disease and various diseases. These compounds include lycopene, resveratrol, lignan, tannins and indole [21].

Home grown beverages, devoured as a component of nutritious eating routine, can improve the cell reinforcement status and improve in general wellbeing. Home grown teas/drinks are rich wellsprings of characteristic bioactive items for example carotenoids, phenolic corrosive, flavonoids, coumarins, alkaloids, poly acetylenes, saponins and terpenoids among others. The plentiful logical proof accessible shows that regular mixes offer various impacts, for example, cancer prevention agent, antibacterial, antiviral, mitigating, hostile to unfavorably susceptible, antithrombotic and vasodilatateurs activity just as against mutagenicity, against cancer-causing nature and maturing impacts. Numerous natural teas are utilized worldwide and some are considerably more mainstream than others. Nonetheless, in the time of globalization and racial obstructions have progressively been taken out and such things. In spite of the fact that from different sources are presently accessible worldwide as public wellbeing items. Herbal beverages have been used as natural part of the food culture in countries where traditional medicines are widely used [22].

2.3 Eugenol

Eugenol is also called clove oil, a fragrant oil that is widely used as flavor of food, tea, as an herbal oil and is also taken orally to treat intestinal and respiratory complaints. Clove or *Eugenol* oil is often used by dentists because it is antibacterial and anti-inflammatory. They are often used in gums to kill germs and to relieve the pain of dental surgery such as tooth extraction, filling, and roots. In addition clove oil along with cinnamon, basil, and nutmeg all of which contain *eugenol* are

a common ingredient in oral hygiene, toothpaste, soap, insect repellent, perfumes, food and various animal medicines. The different studies reviewed in the work confirm that the traditional use of clove as food preservative and medicinal plant standing out the importance of this plant for different applications [23].

Fundamental investigations propose that *eugenol* may have extra results which stretch out a long ways past the therapeutic worth. For instance, a few examinations showed that *eugenol* battles microbes and represses the development of numerous parasites including *Candida albicans*, a microorganism that is liable for some human yeast contaminations. *Eugenol* or clove oil has just been utilized to battle contagious contaminations of the skin and ears in non-western nations, yet this treatment can cause disturbance in skin so should not be attempted without a specialist's solution [24].

Eugenol is the alkyl chain, an individual from the alkyl benzene class of chemical compounds. It has yellow, fragrant cola-based oil removed from certain fundamental oils particularly clove, nutmeg, cinnamon, basil, and inlet leaf. It is found in a centralization of 80-90% in clove bud oil and 82-88% in clove leaf oil. *Eugenol* has a sweet, unpleasant, clove-like fragrance. The name is derived from *Eugenia caryophyllata*, the previous name of the Linnean terminology of cloves(The name presently embraced is *Syzygium aromaticum*) [24].

2.3.1 *Syzygium aromaticum*

Cloves the blossom buds of the clove tree, are the evergreen and are otherwise called *Syzygium aromaticum*. Found in both entire and ground frames, this flexible zest can be utilized to prepare pot broils, add flavor to hot refreshments, and carry zesty warmth to treats and cakes. Cloves are most popular as a sweet and sweet-smelling product; however they have additionally been utilized in conventional medication. Indeed, researchers have discovered that the compounds in cloves may have a few medical advantages including supporting liver wellbeing and balancing out glucose levels. Traditionally Eugenol has been used from ancient ages as spices and medicines. It was well known to humanity from a long time. Colves are mostly sweet and sweet-smelling [25].

2.3.1.1 Surprising Health Benefits of *Syzygium aromaticum*

- Contain important nutrients [26]
- High antioxidants quality [27]
- May assist with giving protection against malignant growth [28]
- Has the ability to kill various types of bacteria [29]
- May improve liver capacity [30]
- May help control glucose level [31]
- May advance bone/joint wellbeing [32]

2.3.1.2 Taxonomy of *Syzygium aromaticum*

Order: *Myrtales*

Family: *Myrtaceae myrtles, myrtaces*

Genus: *Syzygium P. Br. ex Gaertn.*

Species: *Syzygium aromaticum (L.) Merr. & L.M. Perry clove* [33]

2.3.2 Clove Oil

Clove essential oil is a naturally occurring anticancer and antimicrobial agent that can be used in many fields at the modern level [34]. Minimum inhibitory concentration (MIC) was determined using a published strategy for viral development and mass learning in the working class [35]. The MIC is believed to be similar to a small specialist test equipped to suppress bacterial growth after 24 hours of anointing. The tests were performed on Gram-positive(*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Streptococcus mutans* ATCC 5175) and gram-negative bacteria (*Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922 and *Salmonella choleraesuie* ATCC 10708). Minimum Inhibitory Concentration (MIC) of free clove oil and nanoemulsion (NE 4) stacked with clove oil against gram-negative and gram-positive microorganisms was tested.

Among the different compounds in clove oil, a compound called eugenol is one of its fundamental constituents [35].

2.3.2.1 Traditional Uses of Clove Oil

Clove oil has been used for a variety of purposes including:

- As an antimicrobial activity, to kill bacteria and fungi [36]
- As a pain killer for conditions such as headache, toothache and muscle pain [37]
- For digestive/stomach problems [38]
- To treat respiratory tract conditions like cough and asthma [39]
- Clove essential oil may also help to prevent dental cavities and relieve oral pain [40]
- In a test tube, clove oil appears to be effective at killing cancer cells or stopping them from growing. However, further research is needed to determine clove oils anticancer properties [28]

2.4 *Melaleuca alternifolia*

Melaleuca alternifolia is known as the tea tree, is a kind of tree or bush tall in the myrtle family and produces advantageous oils with a new camphoraceous fragrance. The name tea tree is utilized for various plants mainly from Australia and New Zealand from the *Myrtaceae* family [41]. Tea tree oil(TTO) is protected as a skin treatment and can be applied directly to the skin. When applied to the skin with its most suitable formula (100% oil). The name tea tree is utilized for various plants mainly from Australia and New Zealand. The TTO is composed of tarpene hydrocarbons, basically monotarpenes, sesquiterpene. It is used as essential oil for many skin products as well.

2.4.1 Surprising Health Benefits of Tea Tree

Tea tree oil is a well-known solution for skin rashes due to its thinning. It is thought to reduce redness, stretch and enlargement. It can similarly help prevent and reduce cuts, leaving you with smooth and clear skin. Tea tree oil(TTO) is protected as a skin treatment and can be applied directly to the skin on a daily basis. When applied to the skin with its most suitable formula (100% oil). TTO does not cause irritation. However, few people develop contact dermatitis [42].

2.4.2 Dermal Toxicity

TTO can cause increased responses and sensitivity. An estimated irritancy score of 0.25% was obtained based on test results of 311 volunteers. One study in which 217 patients from a dermatology center were tested with a 10% TTO fix did not receive a negative response. Since aggressive responses can always be prevented by the use of low-energy environments, this strengthens the case to reduce the use of clean oil and improve the use of high-performance materials. Allergic reactions have been reported a range of components have been suggested as responsible. Researches indicate that they are mainly caused by oxidations occurring in old persons or use of not properly stored oils. There is little sensible help with the view that 1,8-cineole is the most annoying TTO. No evidence of concern was detected during the investigation of the rehabilitated of red and brown rabbits, guinea pigs and humans including people who had a previous positive response to the TTO [43].

2.4.3 Taxonomy of *Melaleuca alternifolia*

The following taxonomy was followed of Plantae:

Order: *Myrtales*

Family: *Myrtaceae*

Genus: *Melaleuca*

Species: *M. alternifolia*

2.4.4 Medicinal Properties of Tea Tree Oil(TTO)

Alternative medicines for example tea tree oil (*Melaleuca*) became popular in recent decades. This essential oil has been used for almost 100 years in Australia but is now fully accessible as complete oil and as an active ingredient in a variety of products. Significant recruitment of tea tree oil has been achieved with certainty in antiseptic and calming activities. Major in vitro data currently maintain the belief that *Melaleuca alternifolia*(tea) essential oil has antimicrobial and anti-inflammatory properties [44, 45]. Various examinations have upheld the promising antibacterial impacts of *Melaleuca alternifolia* or tea tree fundamental oil. One examination reported in 2004 utilized a dressing model on petri dishes to check the antimicrobial impacts of the fumes of tea tree basic oil. This investigation utilized a similar dressing model with patients who had wounds infection with *Staphylococcus aureus*. Ten members participated in test study, and four of the 10 were utilized as coordinated members to think about injury mending times between regular treatment alone and ordinary treatment in addition to fumes of tea tree fundamental oil. The outcomes showed diminished recuperating time in everything except one of the members treated with tea tree oil. The contrasts between the coordinated members were huge. The after effects of this little investigational study show that extra examination is justified. The TTO is composed of terpenes hydrocarbons basically monoterpenes, sesquiterpenes and their related alcohol. Terpenesare volatile, contains fragrant hydrocarbons and can be considered as isoprene polymers with the C5H8 formula. Initial reports on a TTO piece consists of 48 components. The Brophy Basic Paper and Partners analyzed more than 800 TTO tests with gas chromatography and gas chromatography-mass spectrometry and contained details of approximately 100 parts and their concentration range [46].

2.4.5 Traditionally Uses of Tea Tree Oil

- Hand sanitizer containing tea tree is an ideal natural hand sanitizer [41]
- Insect repellent tea tree oil may help keep pesky insects away [47]

- Natural deodorant for removal of sweating smell [48]
- Antiseptic for minor cuts, wounds and Scrapes [41]
- Boost fast wound healing [49]
- Fight with acne and pimples [50]
- Get rid of nail fungal infection [51]
- Chemical-free/natural mouthwash [52]
- Tea tree oil makes a great all-purpose surface cleaner that also sanitizes surfaces [53]
- Soothing effects on skin Inflammation [54]
- Control hair dandruff [55]
- Treat athletes foot/fungal infection [56]
- Expel mold/fungi on fruits and vegetables [57]

2.5 Eucalyptus

Eucalyptus is a genus of more than 700 species of flowering trees, shrubs or *Malle* in the *myirtile* family, the *Myrtaceae.* Along with a distinct species from the *Eucalypteae* family, including *Corymbia* commonly known as eucalyptus. The various plants of eucalyptus have smooth, sinewy, hard or red bark, oily leaves, sepals and leaves combined to form a "cap" or operculum over the stamens. A living product is a wooden container that is often referred to as "gumnut" [58].

2.5.1 Surprising Health Benefits of Eucalyptus

- Insecticide/insect repellent
- Pain killer

- Immune system stimulating in arthritis patientpotentially due to its anti-inflammatory properties of Eucalyptus
- Diabetesmight help lower blood sugar [59]

Eucalyptus oil is financially classified into three broad types for their composition and final use: aromatic, medical and industrial [60]. The best known is "eucalyptus oil" at the level of cineole, a bitter (yellow and old) liquid with a deep odour, combined with a pleasant aroma [61].

2.5.2 Taxonomy of Eucalyptus

Kingdom: *Plantae*
Clade: *Tracheophytes*
Clade: *Angiosperms*
Clade: *Eudicots*
Clade: *Rosids*
Order: *Myrtales*
Family: *Myrtaceae*
Subfamily: *Myrtoideae*
Tribe: *Eucalyptea*
China produces about 75% of the world's exchange, however quite a bit of this depends on the fundamental oils of camphor trees as opposed to genuine eucalyptus oil. An assortment of oil-in-water emulsions have been set up from the fundamental oil of eucalyptus, to balance out and complete antimicrobial action against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This plan was planned utilizing a test configuration done in the reaction zone and examined with Design-Expert ®10 programming. Emulsions are set up in a colloid pit and the emulsion plan is performed utilizing a potential scattering of zeta (ζ), bead size conveyance and stage partition. Antibacterial impacts are determined by the energy of death. The bead size and force of 16 emulsions went from 1.071 to 1.865μm (in light of Feret reach) and 34.8 to 36m and 24 mV individually. Three factors(14, 15, and 16) indicated the most elevated adjustment boundaries(no stage division)

inside 28 days of testing. Fundamental emulsions of eucalyptus show antibacterial movement against *E. coli*, *S. aureus* and *P. aeruginosa* under 1 moment [62].

2.5.3 Plaster Applications and Uses

Antimicrobial plaster is often used in the treatment of cuts or skin injuries. It is used to prevent and treat cuts, scratches, rankings, fraudulent chomps and minor injuries. Adhesive tape of some sterile polyester texture as the original texture is prepared for use. For maximum benefit each swathe is enclosed independently in a waterless bag. The outer layer is a solid cardboard box to maintain the best storage condition [63]. It shows therapeutic properties for example analgesic, antibacterial, antifungal, and reducing properties [64, 65]. Clove oil has antibacterial properties (Dorman HJ, Deans SG, 2000), (Halcon L, Milkus K., 2004). Eucalyptus oil contains soothing, analgesic and antibacterial properties impregnated with plant extract [66].

2.6 Synergistic Effect of Essential Oils

Essential oils are widely used especially as a general oral therapeutic for the treatment of oral diseases including periodontal infections [67]. The interaction between the essential oil can create synergism, an additional element, a split (non-interaction) or opposite effects. The synergistic effect is undoubtedly seen when the impact of compounds is significantly greater than the number of individual impacts. An extra factor is the point at which the joined effect is equivalent to the quantity of individual effects. The opposite effect is seen when the effect of one or two items is less when used together than when used separately [68]. Clove oil has a pleasant aroma and is as important as the taste of basic foods, drugs and dentists. It has long been used by dentists as its basic remedy, eugenol, primarily responsible for acting as a bacterial or bacteriostatic specialist in combating dental disease [69]. It is commonly used in many societies to obtain teeth, painful gums and mouth sores without wearing teeth for minor injuries [70]. Cinnamon

(*Cinnamomum zeylanicum*) Blume oil also has been considered to have high anti-cancer activity and anti-bacterial properties [71]. It is widely used in toothpaste or chewing gum to prevent tooth decay [72]. The antibacterial activity of *E. caryophyllata* and *C. zeylanicum* oil has been extensively tested; few studies have been conducted on the antimicrobial effect of the combined use of *E. caryophyllata* and *Coil. zeylanicum*. However, only a few studies have shown the action of combining *E. caryophyllata* or *C. zeylanicum* with other essential oils. For example, a mixture of *E. caryophyllata* with rosemary oil [73]. *C. zeylanicum*, Thyme or clove oil [74], *C. zeylanicum* and *Lavandula angustifolia* oil [75]. These studies have shown antimicrobial effects of Gram-positive and Gram-negative strains. In addition they have also blocked data on rational knowledge of essential oils that include *Eugenia caryphyllata L.* in addition *Cinnamomum zeylanicum* Blume which is effective against oral infections. As a result the interaction of *Eugenia caryphyllata L.* also, *Cinnamomum zeylanicum Blume* oil is made as an alternative treatment option for possible oral diseases [76].

Fundamental oils are a flexible drink, portrayed by a solid smell, extricated from plants or flavours. Their business creation is fundamentally an approach to drink water with smoke. Not withstanding, measures for maturation, openness or extraction of dissolvable are likewise utilized [77]. The interest in fundamental oils utilized in different enterprises for example food, scents, fragrance based treatment and medication is because of their likely advantages [4, 78]. In expansion to their antibacterial and antifungal exercises, basic oils contain antiviral, insecticidal, pain relieving, hostile to diabetic, mitigating, against tumor and cancer prevention agent [79–86]. As indicated by the literature, the antibacterial impacts of basic oils are discovered basically in poly phenols and terpenes, typically monoterpenes, eugenol, cinnamaldehyde, carvacrol and thymol decides its antimicrobial movement [66]. Numerous basic oils, melaleuca oil and clove oil are known for their high restorative properties. The basic oil of *Melaleuca alternifolia* likewise called tea oil(TTO) is an unpredictable mix of terpene hydrocarbons with high liquor content. This oil has been utilized effectively in the therapy of oral candidiasis in AIDS patients(determined to have immunodeficiency condition) and other oral/contagious diseases in patients with cutting edge malignancy. This is on the

grounds that TTO speaks to quite possibly the most generally utilized mitigating drugs, since yeast regularly shows protection from them. Clove oil(*Syzygium aromaticum*) is a major oil unfriendly to bacterial and cell fortification activities, which contains active including eugenol, eugenol acetate and -caryophyllene for anti-oxidant, against allergic and antimicrobial activity of the oil. Clove oil can be utilized in numerous enterprises for an assortment of purposes for example food, wellbeing and individual consideration items. Melaleuca oil and cloves have been broadly examined for their promising advantages to human wellbeing. The composition and quality depends on the extraction method, plant analytical conditions and solvent used the testing of their antimicrobial properties against the Gram-positive microorganism of *Staphylococcus aureus*, the Gram-negative organisms of *Escherichia coli* and *Candida albicans* was done. Moreover, as these oils are volatile unstable compounds which can be effortlessly diminished (by oxidation, volatilization, heat, light, and so forth) when utilized in free structure, they are added to chitosan as a compelling method to reinforce their solidness and increment the term of their activity. As per the writing, the consolidation of clove oil or eugenol utilizing nano particles or nano emulsions improves substance solidness. The joining of clove oil and salvia oil utilizing nano liposomes improves oil soundness before treatment [87].

Chapter 3

Materials and Methods

3.1 Collection of Ingredients/Materials

The ingredients to be used in the batch were collected from local market. Clove oil, eucalyptus oil, croduret LD 40, EDTA(disodium) & polysorbate 20 were purchased from local chemical suppliers.

3.2 Preparation Of Antiseptic Solution

3.2.1 Formulation

Each 100 ml solution Contains:

Table 3.1: Table shows the ingredients used to formulate per 100ml of the solution.

S. No.	Ingredients	Qty/100 ml
01.	Tea Tree oil(*Melalucaterpenin-4-ol*)	250 μl
02.	Eucalyptus oil(*Eucalyptus globulus*)	300 μl
03.	Clove oil(*Syzygium aromaticum*)	300 μl
04.	Croduret LD40	750 mg
05.	Polysorbate 20	750 mg
06.	EDTA(Di-Sodium)	100 mg
07.	Purified Water	Make volume to 100 ml

3.2.2 Mixing of Ingredients

With the help of a calibrated cylinder 12.5 ml of tea tree oil was taken into a 5 liter volumetric flask. 15 ml of eucalyptus oil and 15 ml of clove oil were added to this flask. All ingredients were mixed for five minutes. Some amount of purified water was added while stirring. 5 gm of EDTA was added to this solution. Stirring was continued till solution became clear. Thereafter, 37.5 g of polysorbate 20 was transferred to the solution and stirred for 15 minutes later; volume was filled to 5000 ml with purified water. Solution was mixed well for 15 minutes.

3.3 Analysis

Chemical and microbiological analyses were performed on prepared antiseptic solution(Pilot Batch) while physical perimeters were checked on finished product.

3.3.1 Chemical Analysis

The batch was analysed on gas chromatography for identification and quantification of eugenol.

3.3.1.1 Standard Preparation

0.2 g of standard eugenol was taken to a 100 ml volumetric flask and volume was made to 100 ml with ethyl alcohol. Standard solution was sonicated for 20 min. This solution was filtered through 0.45 μm filter.

3.3.1.2 Sample Preparation

Prepared antiseptic solution was taken as such for chemical analysis on gas chromatography. This solution was filtered through 0.45μm filter. Bulk stage(prepared antiseptic solution) was tested for identification and quantitative analysis of eugenol.

Clarity software was utilized to work on gas chromatography, 100 m long column was used for analysis. The infusion port and detector temperature were set to 225 °C and 280 °C respectively. Infusion of 2 μl sample was infused. After infusion, the oven temperature was expanded from 150 °C and afterward programmed within 10 min to 280 °C at a increase of 13 °C/min. Nitrogen flow rate of 1.0 ml/min, synthetic air 100 ml/min and hydrogen (25 ml/min) were setted to the FID. All gases utilized were pharmacopoeial grade.

3.3.2 Microbiological Analysis

Microbiologically the prepared antiseptic batch was analyzed to check the antimicrobial property of eugenol. All media were tested for growth promotion and inhibition properties.

3.3.2.1 Growth Promoting, Inhibiting and Indicating Properties of Media

Test Strain Preparation

All ATCC strains were prepared according to seed lot technique that gave cultures not more than 5 passages removed from the first culture. Buffered sodium chloride peptone pH 7.0 and phosphate buffer solution pH 7.2 were used to suspend the cultures. To suspend A-niger, 0.05% of polysorbate 80 was added to the buffer. The suspension of cultures hadnot more than 100 cfu/0.1 ml.

Growth Promotion of Media

Petri sterile plates were labeled the name of media, date and culture name.

Inoculum was poured in small amounts(not more than 100 cfu) on the appropriately labeled plates. Melted agar at a temperature of 45 °C was poured into the appropriately labeled plates; which were gently rotated clockwise and counter clockwise to mix the inoculum with the media. The media was allowed to tighten at room temperature. Bacterial cultures were incubated at 30 - 35 °C for <3 days, and yeast/mold cultures at 20 - 25 °C for <5 days.

In solid media, the growth achieved did not differ by a factor greater than 2 from

the calculated value of the standard inoculum. It means that the effect was 2 times higher or lower than the inoculum. For example per 100 cfu has an acceptable count calculated 100/2 = 50 cfu up to 100 x 2 = 200 cfu.

The liquid media was inoculated with not more than 100 cfu populations and incubated properly at specific temperatures.

After incubation plates were observed for growth. The numbers of CFUs were counted for agar while visual growth/turbidity were observed in broths.

For inhibitory property of liquid or solid media, inoculate 100 cfus was distributed on the media with a sterile colonial spreader. Media were incubated at the specified temperature not below the maximum specified time in the test. Results were observed after incubation.

3.3.2.2 Microbiological Limit Test(MLT)

MLT is a test method for enumeration of total aerobic microbial count (TAMC) and total yeast and mold/fungal count (TYMC). Pour Plate count method and membrane filtration method were used for this test.

Gloves and masks were used prior to microbiological testing and media handling. 10 ml of sample was transferred into a 250 ml glass bottle containing 90 ml of Fluid Soybean Casein Digest Medium (TSB) or 0.1% bacteriological peptone to make a 1:10 dilution(master dilution). Sample was mixed by vortex before further inoculation. Gloves, table and media bottles were disinfected with 70% isopropyl alcohol (IPA).

Pour Plate Method

1 ml of final dilution(master dilution) prepared was pipetted on all sterile petri plates with the help of a sterile pipette. Two petri plates were quickly poured with about 15-20 ml of sterile TSA and another two plates were poured with SDA at 45 °C. Petri plates were closed with lids and mixed sample with the media by tilting or rotating the plates and letting the contents freeze at room temperature. The plates of Tryptic Soy Agar(TSA) were converted & incubated for 72 hour at 32.5 ± 2.5 °C and Sabouraud Dextrose Agar Medium (SDA) for 5 to 7 days at 22.5 ± 2.5 °C. After incubation, plates were checked for growth, numbers of colonies were calculated e.g. average number of colonies were multiplied with dilution factor

and showed the results according to the colony forming units per ml(cfu/ml) of sample. If colonies were not found on the plates representing of first dilution 1:10, stated the results as less than ten colonies forming unit per ml($<$ 10cfu/ml) of sample. Results were recorded.

For Membrane Filtration Method

A membrane filter was applied with a designated pore size not exceeding 0.45 μm and 47-50 mm in diameter. Filter paper was placed on the filter assembly with the help of sterile forceps. 10 ml of liquid sample was poured into the filter assembly under LFH. After filtering the 10 ml sample, membrane filter was washed by pouring 100 ml of the sterile peptone water. Two samples were filtered by same process. One of the membrane filters transferred, mainly intended for the calculation of total aerobic microbial count (TAMC) to the plate of the Soybean Casein Digest Agar medium (TSA) and other for yeast and mold count (TYMC), to the surface of the plate with the descendant of Sabouraud Dextrose Agar (SDA). TSA plates were incubated for 30 to 35 °C for 72 hours and SDA plate for 20 to 25 °C for 5 to 7 days; results were recorded by counting the number of colonies per ml.

Test Method for Specific Pathogens

Staphylococcus aureus* and *Pseudomonas aeruginosa

From master dilution 1:10, 10 ml or 1 ml equal amount was transferred to 90 ml of Fluid Soybean Casein Digest Medium (TSB) and incubated at 30 °C to 35 °C for 18 hours to 24 hours. Then Fluid Soybean Casein Digest Medium (TSB) were checked for growth, then streak a loop to locate a specific part of the surface of the agar surface of pre-formed petri dishes:

1. Mannitol Salt agar medium for *Staphylococcus aureus*
2. Cetrimide Agar (CA) for *Pseudomonas aeruginosa*

Dishes were closed, turned over and incubated at 30 °C to 35 °C for 18 to 24 hours. Number of colonies and colonial characters of colonies were checked against the characteristics listed in Table No 3.2. Gram staining was performed on positive growth for verification of tests.

Coagulase Test for *Staphylococcus aureus*

Suspected growth from MSA plate was tested for coagulase test. With the help of a loop suspected colonies were transferred from MSA to a tube containing 0.5 ml of mammals plasma. The tubes were checked at least three hours later for up to 24 hours on water bath at 37 °C. On negative coagulase test, results were considered the absence of *Staphylococcus aureus*.

Oxidase test for *Pseudomonas aeruginosa*

Suspected growth from cetramide agar was tested for oxidase test. With the help of a sterile loop appropriate colonies were transferred to a previously implanted filter with N, N dimethyl-p-phenylenediamine dichloride, there was no improvement as the pink color turns purple the sample met the need for the absence of *Pseudomonas aeruginosa*.

Escherichia coli

From master dilution 1:10, 10 ml or 1 ml equal amount was transferred to 90 ml of Fluid Soybean Casein Digest Medium (TSB) and incubated at 30 °C to 35 °C for 18 hours to 24 hours. After incubation 1 ml of TSB was transferred to sterilized pre incubated 100 ml MacConkey broth and incubated it at 42 °C - 44 °C for 24-48 hours. Than using a loop from MacConkey broth streaking were carried out on MacConkey's Agar. MacConkey's agar plates were incubated at 30 °C to 35 °C for 18-24 hours. In the experiment, the colonies on MacConkey's Agar had no characteristics listed in Table No.3.2. Test was negative for *E. coli*.

***Salmonella* Species**

From master dilution 1:10, 10 ml or 1 ml equal amount was transferred to 90 ml of Fluid Soybean Casein Digest Medium(TSB) and incubated at 30 °C to 35 °C for 18 hours to 24 hours. After incubation 0.1 ml of broth was transferred to 10 ml of Rappaport Vassiliadis Salmonella enrichment broth and incubated at 30 to 35 °C for 18-24 hours.

Streaking was carried out on xylose lysine deoxycholate (XLD) agar plate and incubated at 30 to 35 °C for 18-24 hours. Colonies on XLD agar had no characteristics listed in Table No.3.2. Test was negative for *Salmonella* test.

Test for Bile Tolerant Gram Negative Bacteria

Sample was prepared at 1:10 in TSB. This was incubated at 20 - 25 °C for approx. 2-5 hours to resuscitate the bacteria. 1 ml sample from TSB was injected

into *Enterobacter* Enrichment Broth Mossel and incubated at 30-35 °C for 24 - 48 hours.

Following samples of incubation sub culturing of this media was carried out on Violet Red Bile Glucose Agar. These plates were incubated at 30 - 35 °C for 18 to 24 hours. Colonies on VRBG agar had no characteristics listed in Table No.3.2. Test was negative for bile tolerant gram negative bacteria.

Table 3.2 shows all the colonial character of microorganism on different media. This was the criteria to check the morphological and biochemical character of bacteria. All the tests performed for the isolation and identification of microbes were checked against the Table 3.2.

Positive Control

All culture media were inoculated 100 cfu population by providing the same conditions as the media used in test.

Negative Control

Diluent Negative Controls

1 ml of diluent(peptone water) was transferred aseptically to two sets of sterile petri plates. 15 - 20 ml of melted TSA was poured to one set of petri plates and SDA to another set of petri plates. All plates were rotated clock and anti-clock wise to mix diluent evenly with agar. After solidification plates were incubated appropriately with product plates.

Media negative control

15 - 20 ml of melted TSA were added to one set of petri plates and SDA to another set of petri plates and incubated appropriately with product plates.

Following the incubation these plates served as negative control of the total count in preparation for the test. There was no growth in negative controls.

Interpretation Criteria

When determining the status of microbiological quality, it was interpreted as follows:

- 10^1 CFU: Maximum acceptable range = 20 CFU
- 10^2 CFU: Maximum number = 200 CFU
- 10^3 CFU: Maximum Value = 2000 CFU and so on.

Table 3.2: Morphological Characteristics of Specified Pathogenic Bacteria on Selective Agar Media.

Pathogenic Bacteria	**Selective Medium**	**Characteristic Colonial Morphology**	**Micros copy**	**Coagulase/ Indole/ Oxidase test**
Staphylococcus aureus	Bairkd-Parker	Black, shiny, surrounded by clear zone 2 to 5 mm	Positive Cocci	Coagulase +ve
	Mannitol Salt Agar	Yellow colonies with yellow zone		
Pseudomonas aeruginosa	Cetrimide Agar (CA)	Yellow-green or yellow-brown in color.	Negative rods	Oxidase +ve
Escherichia coli	Mac Conkeys Broth	Color change to yellow with gas production in Durhams tube	Negative rods (cocco-bacilli)	Indole +ve
	Mac Conkeys Agar Medium	Brick red; may have surrounding zone of precipitated bile		
Salmonella typhi	Xylose-Lysine Deoxycholate Agar Medium	Red, with or without black centers	Negative rods	---
	Triple Sugar Iron Agar (TSIA) medium	Alkaline (red) slants and acid (yellow) butts (with or without concomitant blackening of the butt from hydrogen sulfide production)		
Bile Tolerant Gramve Bacteria: E. coli	Violet Red Bile Glucose agar	Purple pink, surrounded by pink precipitation zones.	Negative rods	Indole +ve
Pseudomonas aeruginosa		Straw to Brown with green pigmentation.	Negative rods	Oxidase +ve

The table 3.3 shows the acceptance criteria for the substances used in manufacturing of a drug s to meet United States Pharmacopoeia requirements. As we are working on a medical product so we followed pharmacopeial instructions.

TABLE 3.3: Acceptance Criteria for Microbiological Quality of Non-Sterile Dosage Forms

Route of Administration	**Total Aerobic Microbial Count (cfu/ml)**	**Total Combined Yeasts and Molds Count**	**Specified Microorganism(s)**
Cutaneous Use	10^2	10^1	Absence of *E. Coli* (1 ml) Absence of *S. aureus* (1 ml) Absence of *P. aeruginosa* (1 ml) Absence of *Gram Negative Bile Tolerant Rods* (1 ml)

3.3.2.3 Antimicrobial Activity

Requirements

- Tryptic soya agar(TSA)
- Tryptic soya broth(TSB)
- Mueller-Hinton agar(MHA)
- *Bacillus subtilis* ATCC 6633
- *S. aureus* ATCC 6538
- *C. albicance* ATCC 10231

- *E. coli* ATCC 8739

Method Antimicrobial Activity

- Prior to prepare of the media it was ensured that the work place and glass equipment to be used to prepare the media were thoroughly cleaned
- Manufacturers guidelines provided on the label of each cultural media were followed because several media types may have different preparation requirements (e.g. temperature, additives and pH adjustment)
- The following information was recorded on the medium i.e name of media, autoclave temperature, time and pressure or other special commands
- Quantity of medium to be prepared was determined and calculation of the quantity of dehydrated media to water was determined
- Required amount of purified water was added to the flask or clean dry bottle at least half of the final volume
- Right amount of dehydrated medium or individual ingredients were carefully weighed and added a small amount of water. Note: Calibrated balance was used
- Cleaned tools(such as a spatula) were used to prevent the ingress of foreign substances
- Agar free media dissolved with a slight vibration
- The flask and screw capped bottle were marked with the media name, the scheduled date, the expiration date and the initials of the person who prepared the medium
- A medium with agar was placed in a bath of boiling water for dissolution
- Caps of bottles were untightened. Care was taken to avoid overheating as all traditional media, to a greater or lesser degree is sensitive to heat

- The media that does not require autoclaving was kept for pre incubation after boiling or as instructed by the manufacturer
- Media were sterilized in a calibrated and validated autoclave at a temperature of 121 °C, 15 PSI pressure for 15 minutes
- In the centre of the sterilization load, as a reference an autoclave indicator tape was putted on a bottle/tube to be sterilized
- On the completion of the sterilization cycle, autoclave was not handled until the jacket pressure became zero, sterile media were removed carefully
- Media was cooled t to 50- 550C before moving to avoid condensation
- Pre incubated the media according to their time & temperature

Handling

- Heat-resistant gloves were used when handling any hot objective
- Capped of screw capped bottles were tightened immediately after sterilization
- *Bacillus subtilis* ATCC 6633, *S. aureus* ATCC 6538, *C. albicance* ATCC 10231 and *E. coli* ATCC 8739, were serially diluted from 1:10^1 to 1: 10^8
- 1ml of each culture from 6 X10^3 CFUs/0.1ml tube were poured into 200 ml melted Muller Hilton Agar at 40 °C - 45 °C. Culture and agar were mixed
- Plate were covered and allowed it to solidify
- 5 bores were made on each Petri plate with the help of a sterile borer
- 50 μl of samples were added to their marked wells
- 5 μg/ml Nystatin was used as standard against yeast cells
- 3 μg/ml Levofloxacin was used as standard against bacterial cells
- Buffer pH 7.2 was used as diluent

- After that, all the plates were incubated for 2-3 days at 30 to 35 °C for bacterial cultures and 5 days at 20 to 25 °C for fungal culture
- Zone of inhibitions were measured with the help of vernier caliper. Results were recorded

3.4 Machines used for Plasters Impregnation and Stripping

- Impregnation Machine
- Medicated plaster shape cutting & Packing Machine
- Bulk Product: Herbal oil solution
- Batch No: Pilot
- Finished product: medicated Plaster strips

3.4.1 Impregnation Machine Process and Behavior

Impregnation machine was used to impregnate the wound pad with antiseptic solution. Pad roll, smooth rolls and rims were easily attached to the impregnation machine rollers and were connected easily by adjusting and stretching them on one side of the rollers. After this the process of product(solution) holding was carried out.

3.4.2 Product(Solution) Holding Pocket/Duct

Impregnation of solution carried out in "product (Solution) holding pocket/duct" of impregnation machine. Solution was easily filled in the pocket to the level. After this we went for dryer chamber. After the holding duct the process of dry chamber was carried out, which dried out the impregnated wound pad.

3.4.3 Dryer Chamber

Dryer chamber was used to dry the impregnated wound pad. No any problem faced in the drying room, all the wheels were moving smoothly with minimal adjustment to one wheel. After this we went for paper packing and positioning

3.4.4 Medicated Plaster Shape Cutting and Packing Machine Process and Behavior

Dry pregnant roll, extruded paper, printed and unpublished rolls cuts the shape & cement of the installation of machine were carried out without any problem. After this we went for shaping of plasters.

3.4.5 Plaster Shaping

Slightly by cutting the roller pad. No problem faced.

3.4.6 Paper Packing and Positioning

It was done just accurately within a few minutes, without the hazel.

3.4.7 Sealing and Cutting

It was done well with a cut-punch type cutter. After sealing and cutting the optical test was performed.

3.5 Optical Test

The strips that were finished got tested under a white fluorescent lamp and found to be aligned with the area.

Figure 3.1 shows the work flow of the process by using impregnation & medicated plasters shape cutting & packing machine. Cotton wound pad were installed on pad rolls. Antiseptic solution was filed to the level in solution holding pocket. Pad moved to solution holding pocket where pad impregnated with antiseptic solution. Impregnated pad passed through dry section were impregnated pad dried. Dry impregnated pad was collected in pad rollers. This roll of pad was installed in plaster shape cutting & packing machine. This machine stripped the plaster.

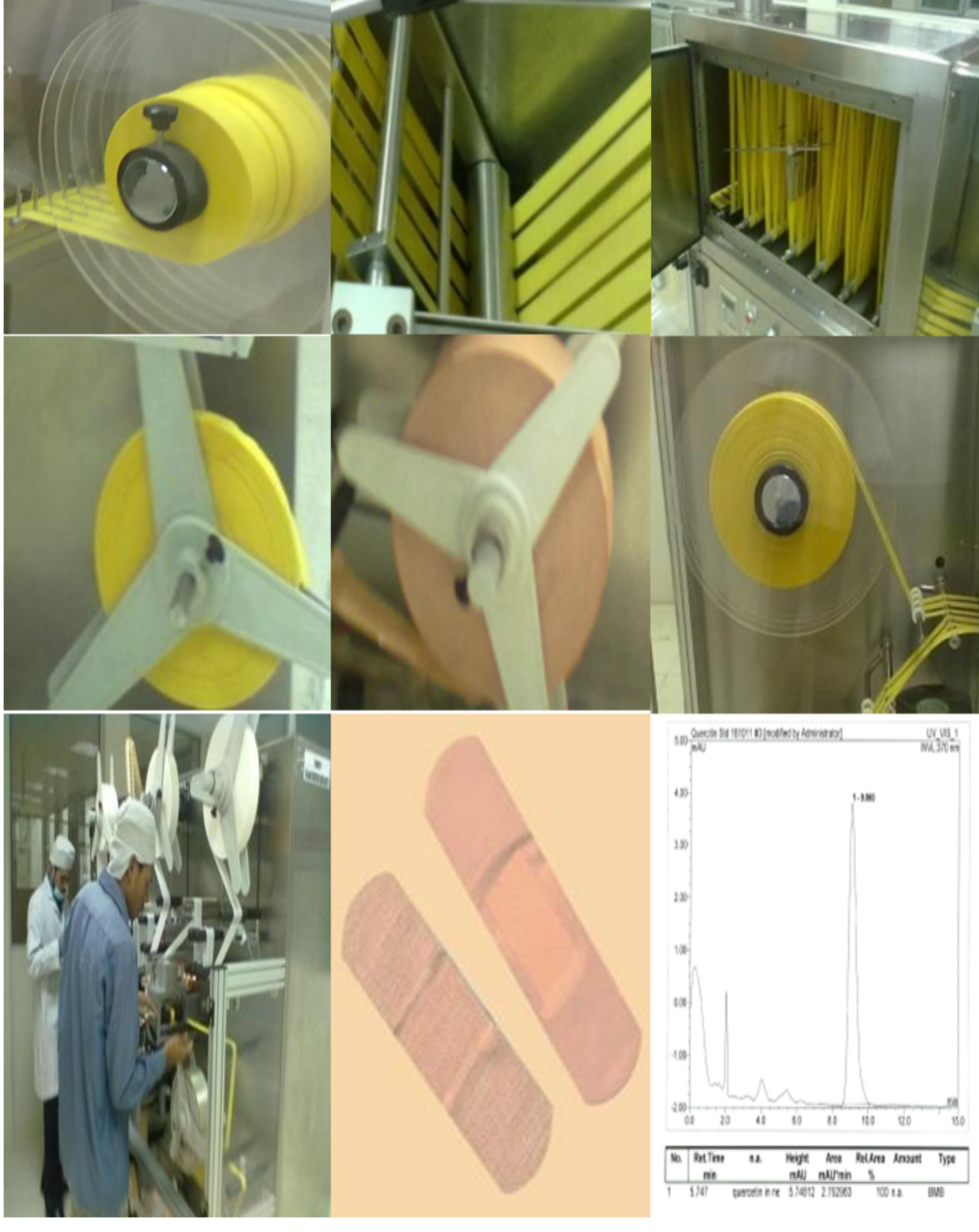

FIGURE 3.1: Flow chart of the process by using impregnation & medicated plasters shape cutting & packing machine.

3.6 Physical Parameters of Prepared Antiseptic Plaster

The table 3.4 explain the physical parameters of antiseptic plaster in finished form which should be remain same throughout the process. These are standard limits for plaster and taken from other research papers [88] and local brands of antiseptic plasters.

TABLE 3.4: Physical Parameters of Prepared Antiseptic Plaster.

S. No.	Parameters	Results
1	Description	Light brown film ventilated bandage, pad impregnated with Eugenol oil which is covered by paper.
2	Identification	Eugenol (against Eugenol Standard by GC)
3	pH	5.0-6.0
4	Adhesive skin test	Can be easily removed from skin without any adhesive deposition
5	Average weight of strip with releasing and packing paper (g)	Medium0.55 g 5%
6	Average Weight (with releasing paper only)	0.236 gm+ 5% (0.220gm 0.250gm)
7	Wound pad length (only) (mm)	Length 72 mm 1 mm
8	Wound pad width (only) (mm)	Width 19 mm 1 mm
9	Bandage Length	72mm + 1mm
10	Bandage Width	19mm + 1mm

Chapter 4

Results and Discussion

Eugenol based plaster developed and analyzed for physical, chemical and microbiological parameters.

On bulk stage physical appearance of bulk (prepared antiseptic solution), pH, microbial enumeration, antimicrobial activity, identification and quantification were carried out. While on the finished stage of plaster physical parameters were checked.

Testing of raw materials used for manufacturing of antiseptic plaster was relied on the manufacturer's certificate of analysis. All certificates are attached.

All media used for microbial testing were tested for their growth promoting, indicating and inhibiting properties to eliminate false microbial results.

4.1 Certificate of Analysis for Raw Materials Used in Batch Formulation

4.1.1 COA of Tea Tree Oil

The results of tea tree oil used in batch were considered according to manufacturers certificate of analysis (Figure 4.1).

Certificate of Analysis

Tea Tree Oil *(MELALEUCA ALTERNIFOLIA)*

Product Name	Tea Tree
Scientific Name	Melaleuca Alternifolia
Extraction Method	Steam Distillation
Origin	Australia
Product Code	EOTTE
Batch Number	51264-01

TESTING RESULTS

Analytic Test	Specification Range	Result
Appearance	Colourless to Pale Yellow Liquid	Conforms
Odour	Characteristic	Conforms
Relative Density @ 20°C	0.882 - 0.903	0.892
Refractive Index @ 25°C	1.475 - 1.482	1.479
Optical Rotation @ 20°C (Degrees)	5° to 15°	11.2°

STABILITY

Manufacturing Date:	04/2019
Testing Date:	05/2019
Commercial Expiry Date:	04/2022

FIGURE 4.1: Certificate of Analysis for Tea Tree Oil.

4.1.2 COA of Eucalyptus Oil

The results of eucalyptus oil used in batch were considered according to manufacturers certificate of analysis (Figure 4.2).

SHANGHAI YEN PERFUME CO., LTD CHINA.

CERTIFICATE OF ANALYSIS

COMMODITY	EUCALYPTAS OIL
APPEARANCE AT 25 C	COLOURLESS TO PALE YELLO LIQUID
STANDARDS	COMPLIES WITH BP/USP
ASSAY	PURITY: 75-80% MIN AS 1.8 CINEOLE (EUCALIPTOL)
ANGULAR ROTATION	(-5 TO +10) °C
REFRACTIVE INDEX @ 25°C	1.458 - 1.470
SPECIFIC GRAVITY @ 25 °C	0.905 - 0.925
STORAGE	TO BE KEPT IN COOL & DRY PLACE
PACKING	50 KG DRUM
PRODUCTION DATE	JAN 2020
EXPIRY DATE	JAN 2023
BATCH NO	100193-9

ITEM	STANDARDS
HAZARD CODES	XI
RISK STATEMENTS	Oct-38
SAFETY STATEMENTS	16-26-36
RIDADR	UN 1993 3/PG 3
RTECS	LE2530000
FLASH POINT(F)	135 °F
FLASH POINT©	57 °C
CONCULUSION	ANALYTICAL RESULT CONFORMING TO STANDARD

FIGURE 4.2: Certificate of Analysis for Eucalyptus Oil.

4.1.3 COA Clove Oil

The results of clove oil used as main ingredient in batch were considered according to manufacturers certificate of analysis (Figure 4.3).

CERTIFICATE of ANALYSIS (COA)

COMMON NAME	Clove Bud - Organic
LATIN NAME	*Eugenia caryophyllata*
COUNTRY OF ORIGIN	Sri Lanka
CULTIVATION METHOD	Cultivated, Certified Organic
TYPE	Essential Oil
EXTRACTION METHOD	Steam Distilled
PLANT PART	Fruit
USE	Aromatherapy, Natural Perfumery

SKU	238
LOT #	12
MANUFACTURING DATE	July 2016
BEST BY DATE	July 2021

	SPECIFICATIONS (Range)	
SPECIFIC GRAVITY @20˚C	1.0323 – 1.0750	1.0410
REFRACTIVE INDEX @20˚C	1.5257 – 1.5380	1.5287
OPTICAL ROTATION @20˚C	-0˚70 to -1˚50	-1 ˚10

PHYSICAL APPEARANCE	Liquid	*Conforms*
COLOR	Pale yellow to yellow	*Conforms*
ODOR	Spicy, warm, sweet	*Conforms*
SOLUBILITY	Soluble in alcohol and fixed oils	
SPECIAL USE INSTRUCTIONS	Dilute before use.	

PRIMARY CONSTITUENTS	Beta-Caryophyllene, Eugenol, Eugenyl Acetate

COMPONENTS	Range %	%		COMPONENTS	Range %	%
BETA-CARYOPHYLLENE	10 - 18	14.11		EUGENYL ACETATE	2 - 9	7.03
EUGENOL*	65 - 78	72.66				
** EU Allergen*						

COMMENTS	Odor quality is excellent.

This Essential Oil is a 100% pure and natural product. It does not contain any artificial ingredients or adulteration of any kind to the best of our knowledge. The analysis and statements herein constitute the most complete information available to Eden Botanicals. This product is guaranteed by Eden Botanicals to be of excellent quality.

Eden Botanicals
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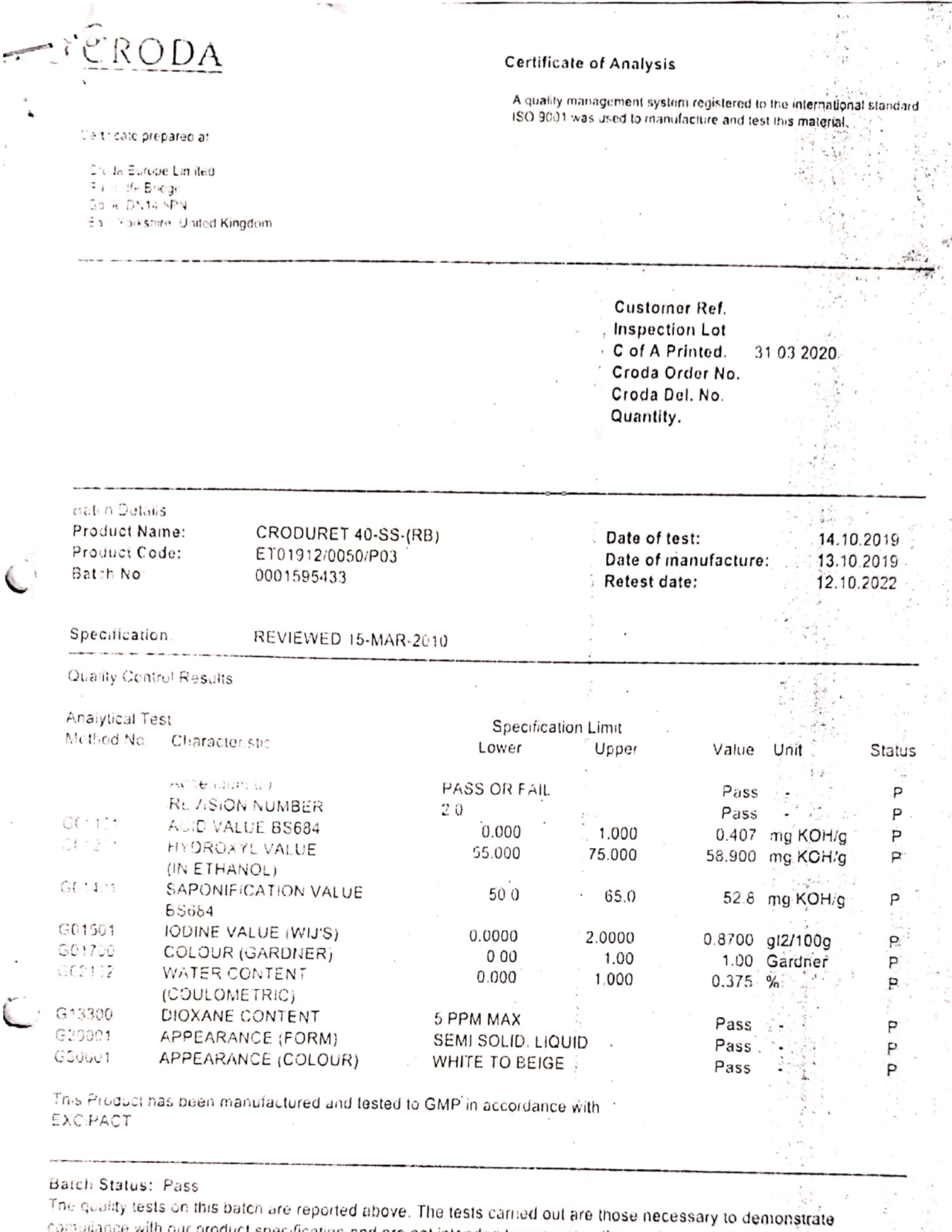
Document created: 07.26.16

Figure 4.3: Certificate of Analysis for Clove Oil.

4.1.4 COA of Crodurate 40

The results of crodurate 40 used in batch were considered according to manufacturer's certificate of analysis (Figure 4.4).



CRODA

Certificate of Analysis

A quality management system registered to the international standard ISO 9001 was used to manufacture and test this material.

Certificate prepared at

[illegible] Europe Limited
[illegible] Bridge
[illegible] DN14 [illegible]
[illegible] Yorkshire United Kingdom

Customer Ref.
Inspection Lot
C of A Printed. 31 03 2020
Croda Order No.
Croda Del. No.
Quantity.

Batch Details

Product Name:	CRODURET 40-SS-(RB)	Date of test:	14.10.2019
Product Code:	ET01912/0050/P03	Date of manufacture:	13.10.2019
Batch No	0001595433	Retest date:	12.10.2022

Specification REVIEWED 15-MAR-2010

Quality Control Results

Analytical Test Method No	Characteristic	Specification Limit Lower	Upper	Value	Unit	Status
	[illegible]	PASS OR FAIL		Pass	-	P
	REVISION NUMBER	2 0		Pass	-	P
[illegible]	ACID VALUE BS684	0.000	1.000	0.407	mg KOH/g	P
[illegible]	HYDROXYL VALUE (IN ETHANOL)	55.000	75.000	58.900	mg KOH/g	P
[illegible]	SAPONIFICATION VALUE BS684	50 0	65.0	52 8	mg KOH/g	P
G01501	IODINE VALUE (WIJ'S)	0.0000	2.0000	0.8700	gI2/100g	P
G01700	COLOUR (GARDNER)	0 00	1.00	1.00	Gardner	P
[illegible]	WATER CONTENT (COULOMETRIC)	0.000	1.000	0.375	%	P
G13300	DIOXANE CONTENT	5 PPM MAX		Pass	-	P
G30001	APPEARANCE (FORM)	SEMI SOLID. LIQUID		Pass	-	P
G30001	APPEARANCE (COLOUR)	WHITE TO BEIGE		Pass	-	P

This Product has been manufactured and tested to GMP in accordance with EXCIPACT

Batch Status: Pass

The quality tests on this batch are reported above. The tests carried out are those necessary to demonstrate compliance with our product specification and are not intended to guarantee the product as suitable for any application beyond those contained in the specification. We recommend you perform your own quality and or identification checks

Page 1 of 1

FIGURE 4.4: Certificate of Analysis for Crodurate 40.

4.1.5 COA of Polysorbate 20

The results of polysorbate 20 used in batch were considered according to manufacturers certificate of analysis (Figure 4.5).

杭州中包进出口有限公司

HANGZHOU ZHONGBAO IMP.& EXP. CORP., LTD.

13F/E -8 BUILDING, WESTPORT NEW TERRITORIES, NO.206 ZHENHUA RD, HANGZHOU, 310030 CHINA

TEL: 86-571-85811826 FAX: 86-571-87357766

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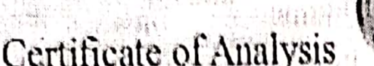

Certificate of Analysis

Consistent Quality

Product Name	POLYSORBATE 20		Analysis basis		
Batch Number	20190909	Date of Mfg.	2019-09-09	Date of Expiry	2021-09-08
Quantity	1400 kgs	Packages Size	25 kg		
Storage condition	Keep the container tightly closed in a dry and well-ventilated indoor area, away from light or heat or moisture				

ITEM	SPECIFICATION	RESULTS
Appearance	Yellow oily liquid	Conforms
Acid value	≤2.2 KOH mg/g	1.63KOH mg/g
Saponification Value	40~50 KOH mg/g	46.66 KOH mg/g
Hydroxyl Value	96~108 KOH mg/g	98.04 KOH mg/g
Water	≤3.0%	1.30%
Residue on ignition	≤0.25%	Conforms
Heavy metals	≤0.001%	Conforms

Conclusion: Pass.

Analysts:	Gezheng	Checker	Ren Yuehua	Supervisor	Chen Jinguo
Signature	[signature]	Signature	[signature]	Signature	[signature]

BANK CONTRACT NO:1014CT36505/2019 DATED:05/09/2019

FIGURE 4.5: Certificate of Analysis for Polysorbate 20.

4.1.6 COA of Eugenol.

Eugenol was used as standard for the identification and quantification of eugenol in bulk stage. The result of eugenol was relied on manufacturers certificate of analysis(Figure 4.6).

REFERENCE MATERIAL CERTIFICATE ISO 17034

Reference Material

This certificate is designed in accordance with ISO 17034 and ISO Guide 31. This reference material (RM) was designed, produced and verified in accordance with ISO/IEC 17025, ISO 17034 and a registered quality management system ISO 9001.

Product Name
Eugenol

Product Code
DRE-C13395000

Lot Number
G1071096

CAS No.
97-53-0

Format
Neat

Mol. Weight
164.20

Expiry Date
06 Apr 2026

Mol. Formula
$C_{10}H_{12}O_2$

Storage Temp
4°C ± 4°C

OH
O

CERTIFIED	CERTIFIED
Purity 99.20% (g/g)	Expanded Uncertainty (U) 0.30% (g/g)

Uncertainty
The certified value(s) and uncertainty(ies) are determined in accordance with ISO 17034 with an 95% confidence level (k=2). Uncertainty is based on the Total Combined Uncertainty, including uncertainties of characterisation, homogeneity and stability testing. Stability values are based on real evidence opposed to simulation.

The producer certifies that this reference material meets the specification stated in this certificate until the expiry date, provided it is stored unopened at the recommended temperature herein. Product warranties for this reference material are set out in the terms and conditions of purchase.

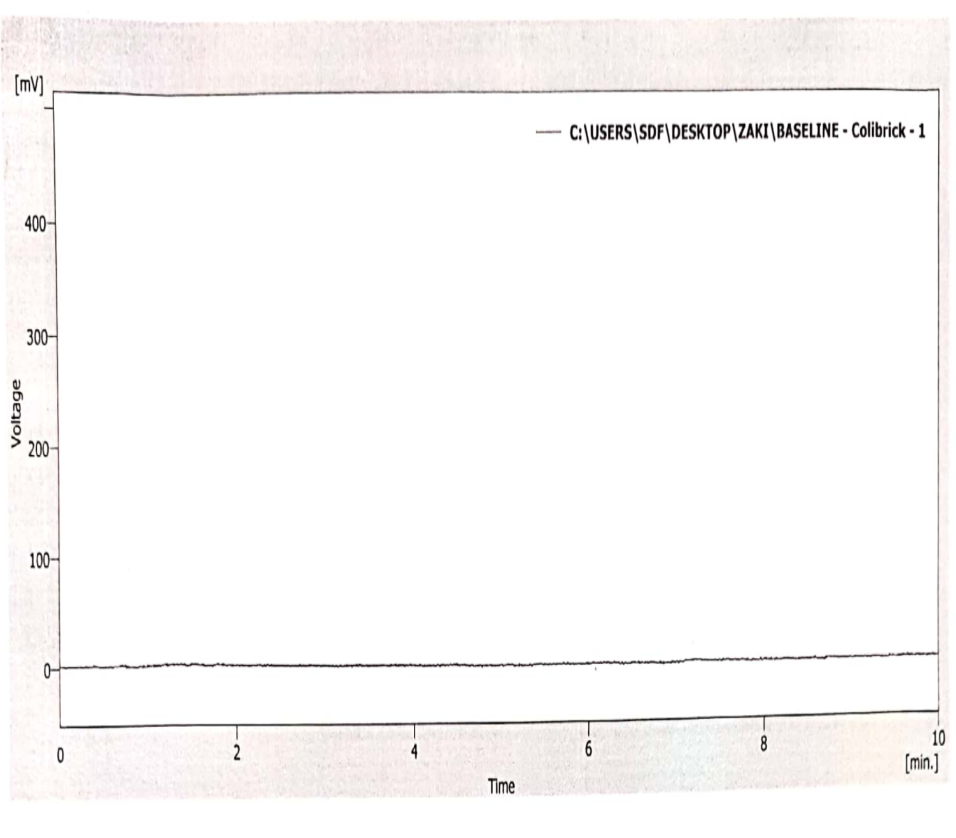
CERTIFIED BY	CERTIFIED ON		
D. Schmid	06 Apr 2020	D. Schmid	RM Release

Figure 4.6: Certificate of Analysis for Eugenol.

4.2 Chemical Analysis

Bulk stage was analyzed for qualitative and quantitative investigation of eugenol. Clarity software was utilized to work on Gas chromatography, 100 meters long column was utilized for analysis. The infusion port and detector temperature were set to 225 °C and 280 °C respectively. Infusion of 2 μl sample was infused. After infusion, the oven temperature was expanded from 150 °C and afterward programmed within 10 minutes to 280 °C at an increase of 13 °C/min. Nitrogen flow rate of 1.0 ml/min, synthetic air 100 ml/min and hydrogen (25 ml/min) were setted to the FID. All gases utilized were Pharmacopoeial grade.

Base line was settled to check the performance of gas chromatography (Figure 4.7). Five injection of standard and two injections of test were infused. RSD vale should not surpass 2%.

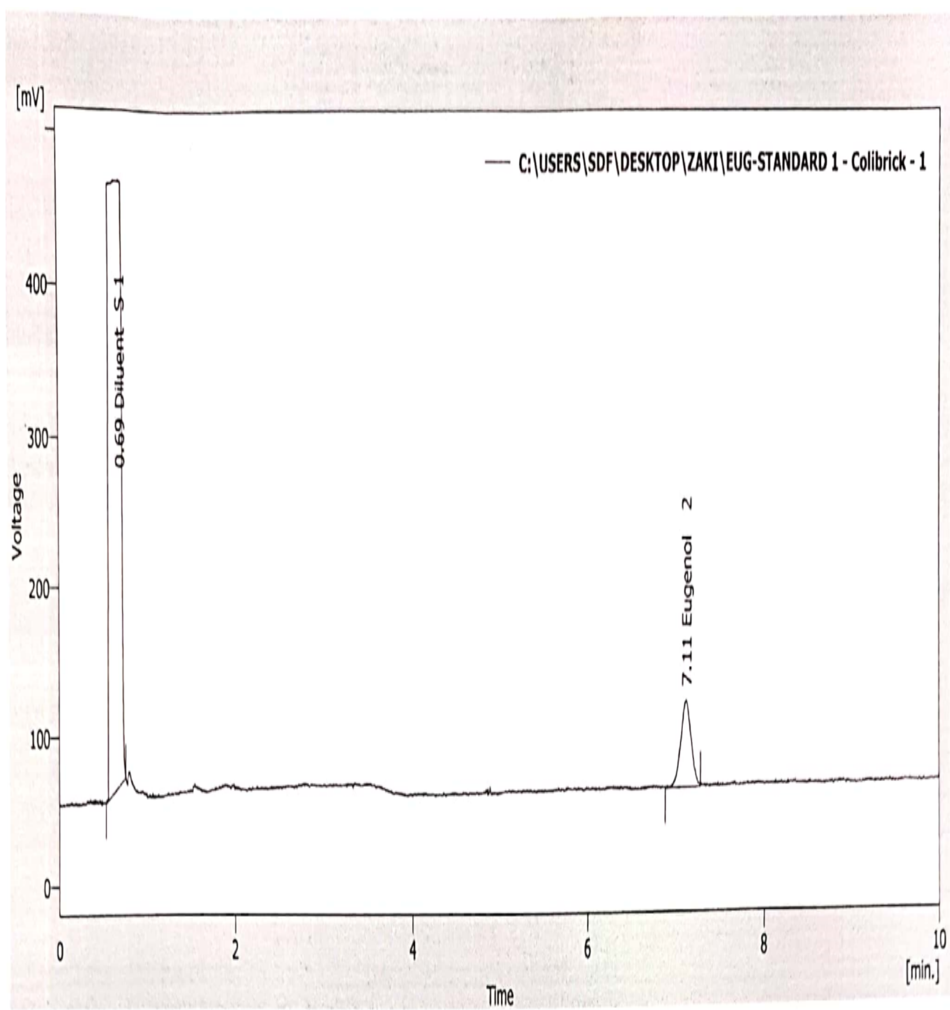


Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\BASELINE - Colibrick - 1)

Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
No peak to report						

FIGURE 4.7: Baseline of Chromatogram.

Standard chromatograms (injection one to injection five) are attached as Figure 4.8 to Figure 4.12 respectively. In Figure 4.8, Standard replicate 1, injection showed the retention time of eugenol 7.11 minutes. This injection showed the area for eugenol 470.111.

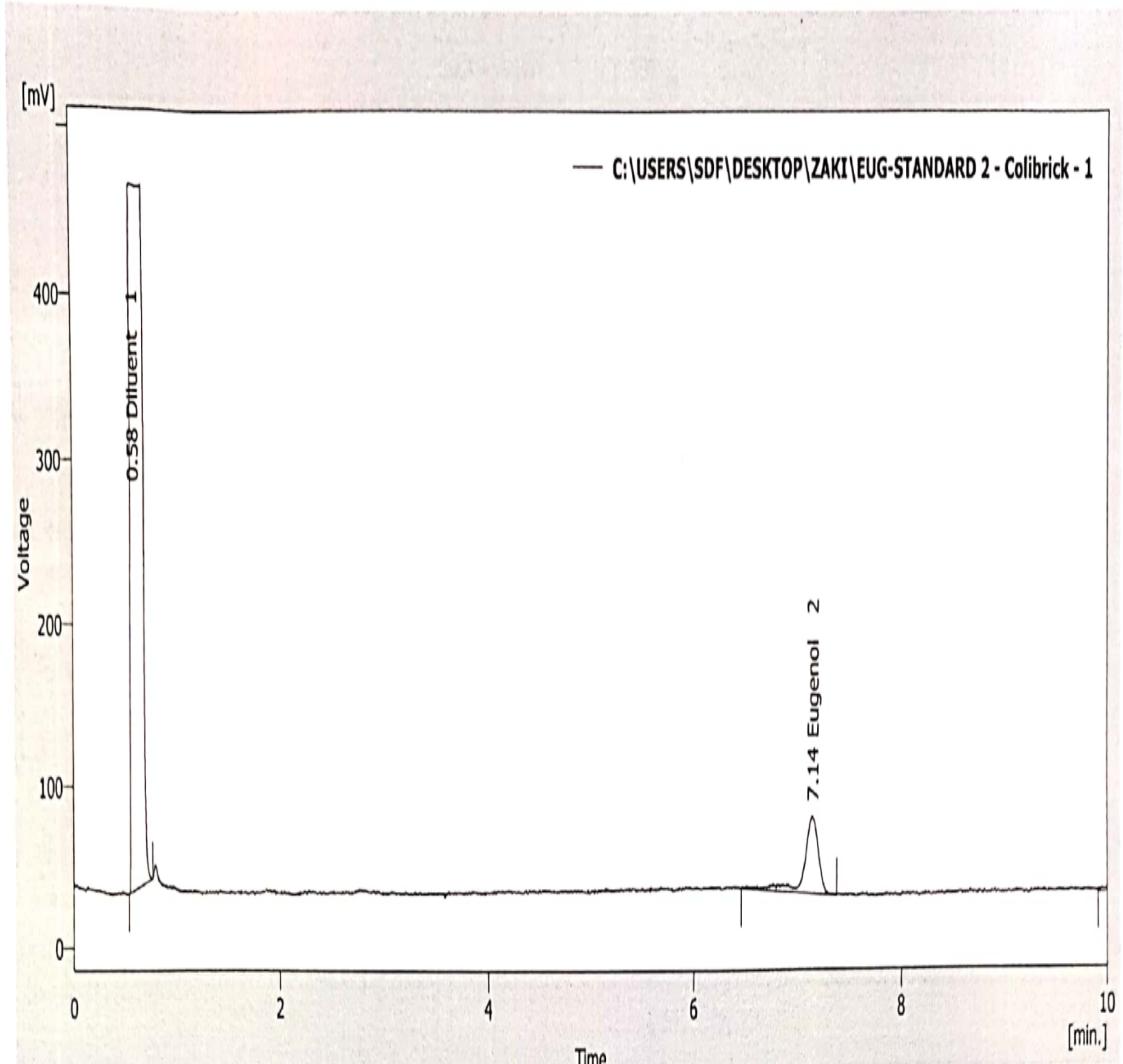


Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-STANDARD 1 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
2	7.108	470.111	57.390	100.0	100.0	0.13	Eugenol
	Total	470.111	57.390	100.0	100.0		

Figure 4.8: Standard Replicate 1

In Figure 4.9, Standard replicate 2, injection showed the retention time of eugenol 7.14 minutes. This injection showed the area for eugenol 467.040.

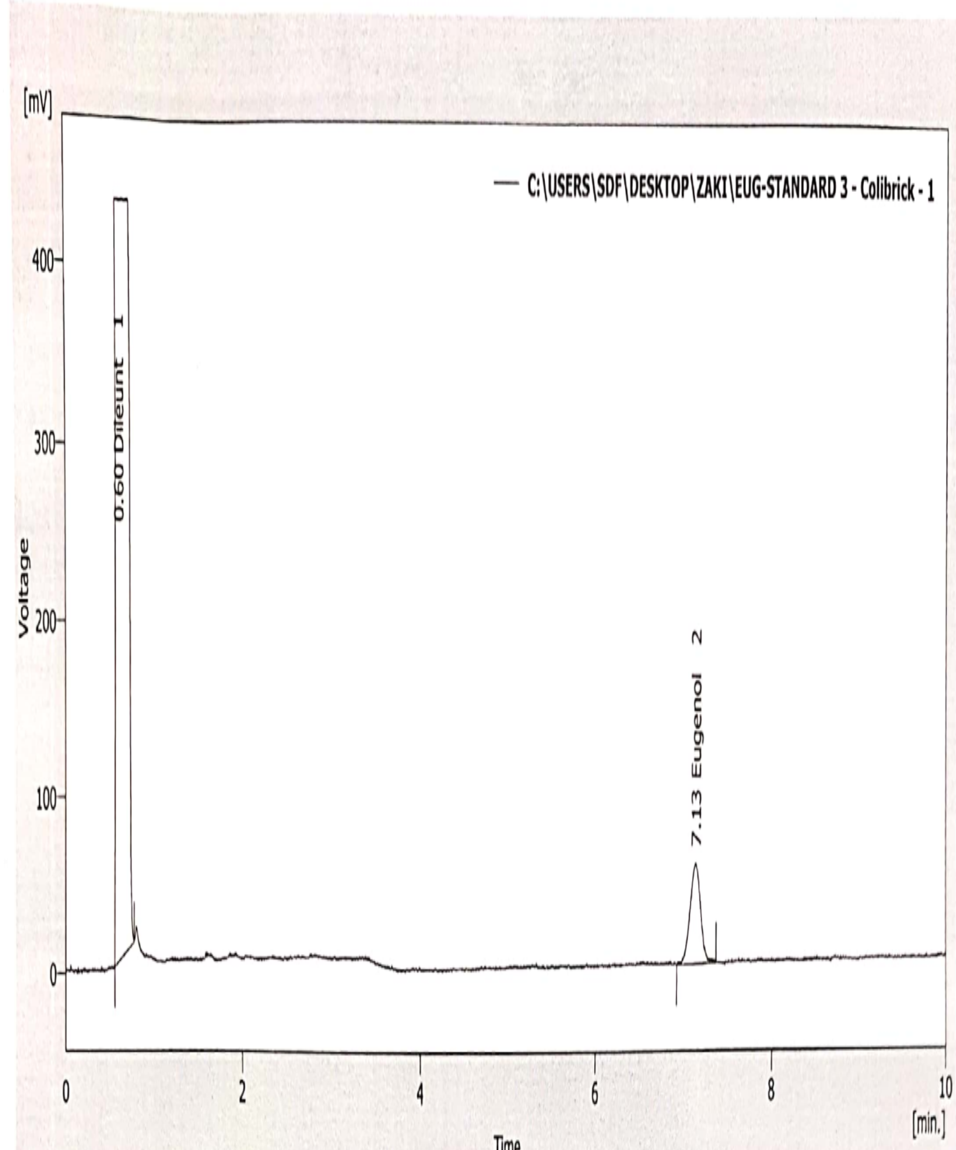


Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-STANDARD 2 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.576	3527.727	432.867	87.7	89.6	0.14	Diluent
2	7.136	467.040	47.408	11.6	9.8	0.13	Eugenol
3	10.100	26.443	2.570	0.7	0.5	0.02	
	Total	4021.210	482.846	100.0	100.0		

Figure 4.9: Standard Replicate 2

In Figure 4.10, Standard replicate 3, injection showed the retention time of euginol 7.13 minutes. This injection showed the area for euginol 473.661.

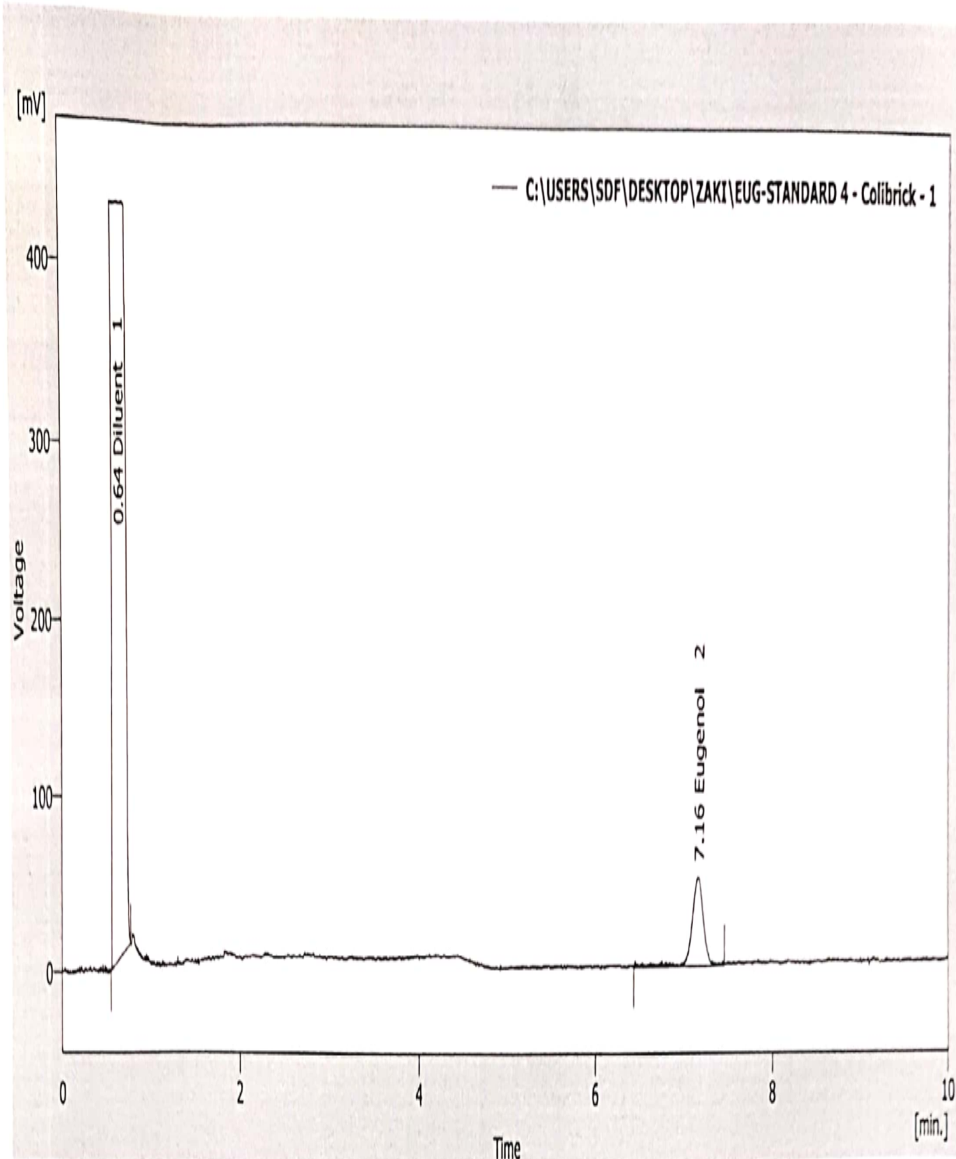


Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-STANDARD 3 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.596	4360.904	430.395	90.2	88.4	0.17	Dileunt
2	7.128	473.661	56.438	9.8	11.6	0.13	Eugenol
	Total	4834.566	486.833	100.0	100.0		

Figure 4.10: Standard Replicate 3

In Figure 4.11, Standard replicate 4, injection showed the retention time of euginol 7.16 minutes. This injection showed the area for euginol 472.232.

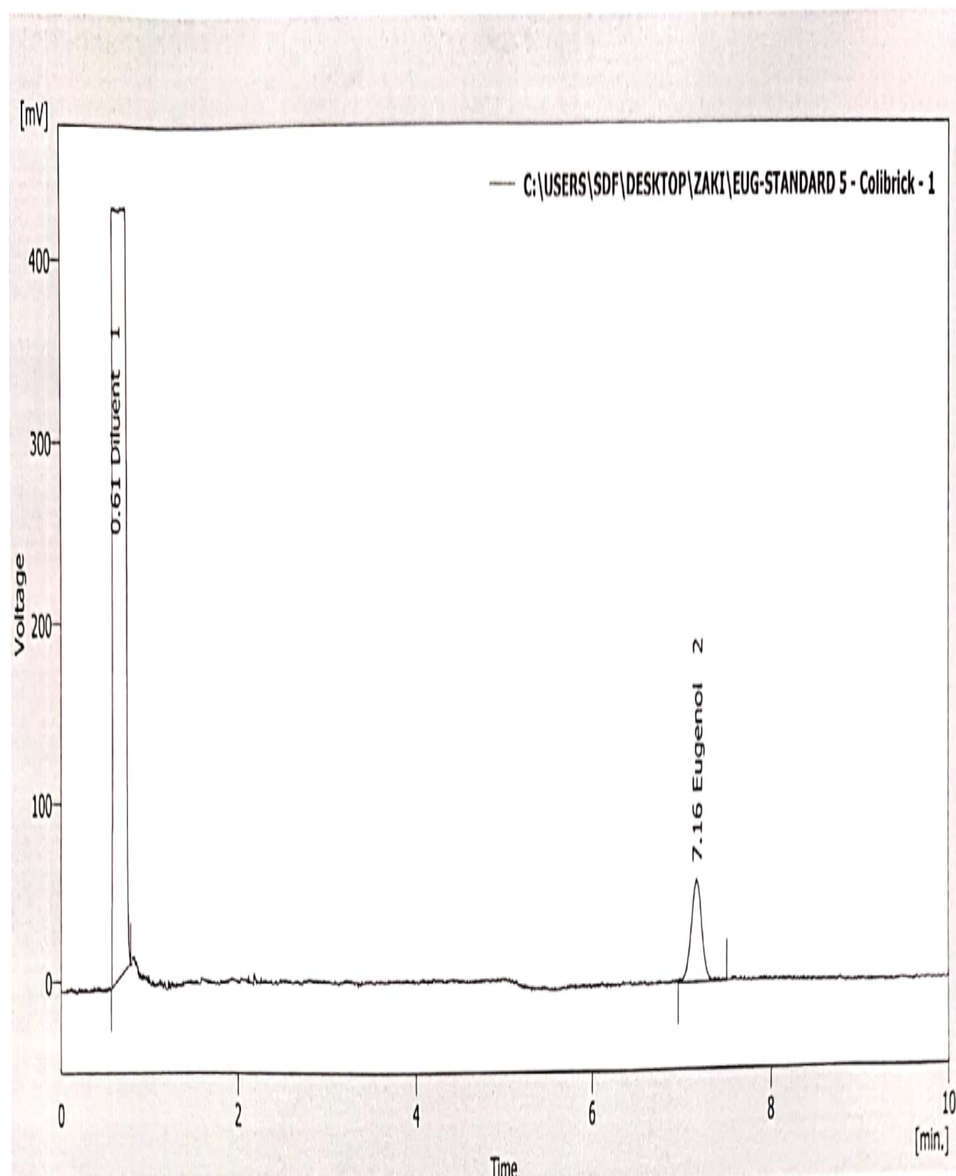


Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-STANDARD 4 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.640	4349.333	427.077	90.2	89.5	0.17	Diluent
2	7.160	472.232	50.038	9.8	10.5	0.13	Eugenol
	Total	4821.565	477.115	100.0	100.0		

Figure 4.11: Standard Replicate 4

In Figure 4.12, Standard replicate 5, injection showed the retention time of euginol 7.16 minutes. This injection showed the area for euginol 473.602.



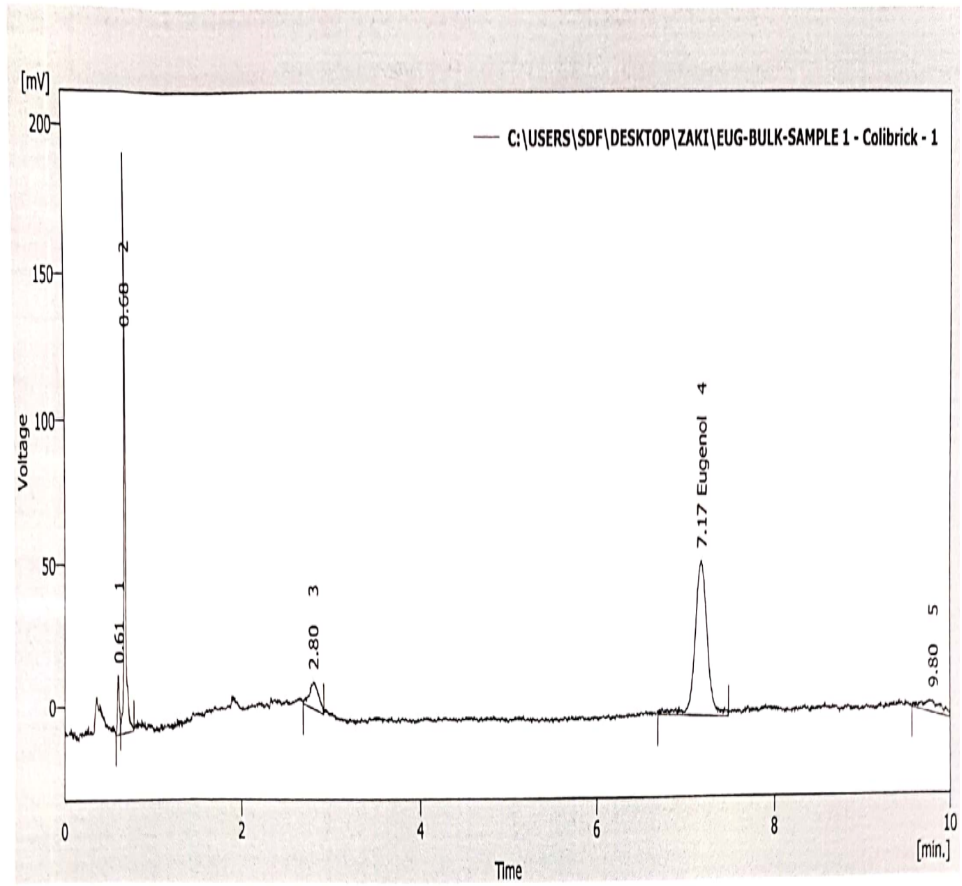
Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-STANDARD 5 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.612	4274.876	430.568	90.0	88.3	0.17	Diluent
2	7.160	473.602	56.931	10.0	11.7	0.13	Eugenol
	Total	4748.478	487.499	100.0	100.0		

FIGURE 4.12: Standard Replicate 5

Sample chromatograms (injection one to injection two) are attached as Figure 4.12 to Figure 4.13 respectively. In Figure 4.13, sample replicate 1, injection showed the retention time of eugenol 7.17 minutes. In this injection peak retention time

is matching with peak retention time of eugenol standards in Figure 4.8 to 4.12 standards. This injection showed the area for eugenol 520.981.

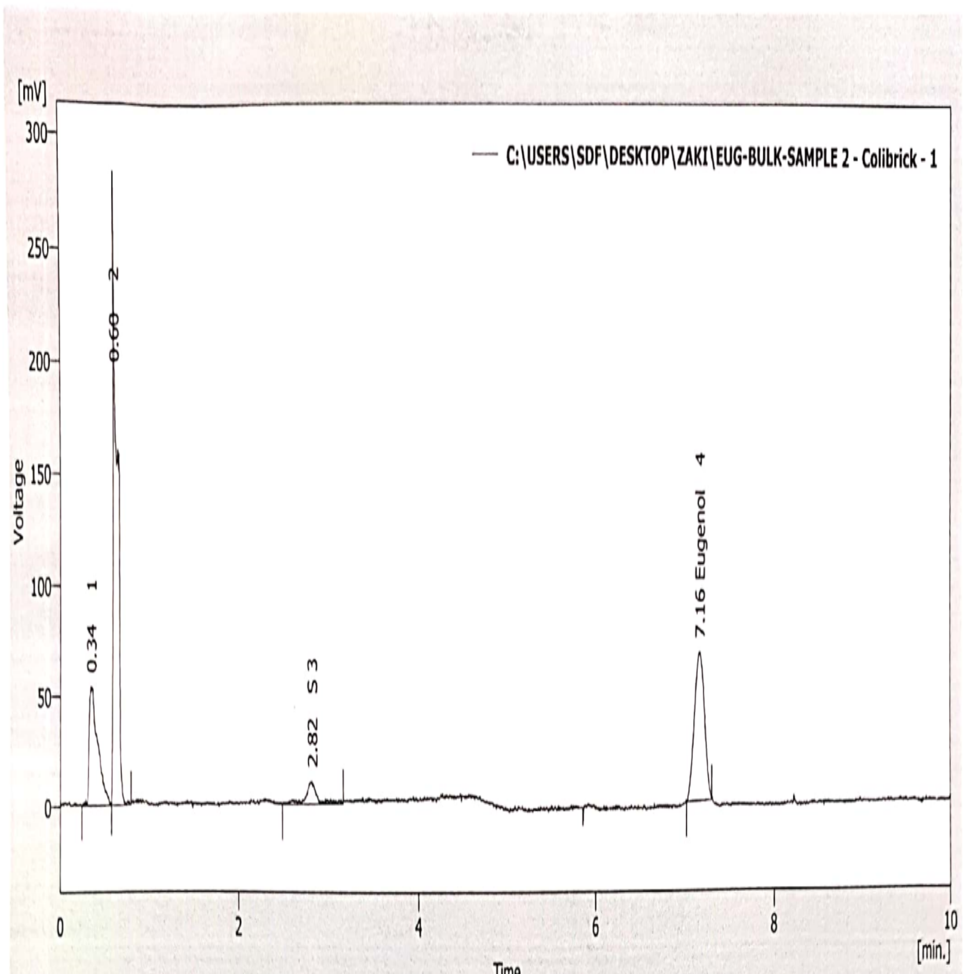


Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-BULK-SAMPLE 1 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.608	31.515	20.909	3.4	7.2	0.03	
2	0.676	245.863	200.542	26.3	69.4	0.02	
3	2.800	67.386	9.313	7.2	3.2	0.10	
4	7.168	520.981	53.722	55.8	18.6	0.14	Eugenol
5	9.804	68.702	4.349	7.4	1.5	0.13	
	Total	934.447	288.836	100.0	100.0		

FIGURE 4.13: Standard Replicate 1

In Figure 4.14, sample replicate 2, injection showed the retention time of eugenol 7.16 minutes. In this injection peak retention time is matching with peak retention time of eugenol standards in Figure 4.8 to 4.12 standards. This injection showed the area for eugenol 521.263.



Result Table (Uncal - C:\USERS\SDF\DESKTOP\ZAKI\EUG-BULK-SAMPLE 2 - Colibrick - 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.340	367.727	54.235	20.8	13.4	0.12	
2	0.596	880.920	283.739	49.8	70.2	0.08	
4	7.156	521.263	66.388	29.5	16.4	0.13	Eugenol
	Total	1769.910	404.362	100.0	100.0		

Figure 4.14: Standard Replicate 2

4.2.1 System Suitability Report

Table **??** shows the results of euginol present in batch of plaster.Total 0.219g/100ml euginol found in batch. The RSD value is less than 2 %. Five standard injections showed an average of 471.3292 area while two sample injections showed an average

of 521.122 area. 2 grams of standard euginol having potency of 99.20% w/w was used to determine the potency of Eugenol in sample.

Table 4.1: Results Calculation Sheet

Product Name	**Antiseptic Plaster**	**Batch**	**Pilot**
Instrument Used	**GC**	**Dated**	**30.11.2020**
Standard Injections	**Area**		
Replicate 1	470.111		
Replicate 2	467.040		
Replicate 3	473.661		
Replicate 4	472.232		
Replicate 5	473.602		
Mean	471.3292		
Standard Deviation	2.797284701		
Relative Standard Deviation	0.593488522		
RSD Limit NMT 2.0%			
Sample Injections	**Area**		
Replicate 1	520.981		
Replicate 2	521.263		
Mean	521.122		

4.2.1.1 Formula for Calculation

Quantity of eugenol in sample was calculated against the standard of eugenol having known potency. Following Formula was used to calculate the quantity.

$$\frac{SampleArea}{StandardArea} X \frac{StandardwtX100}{100XSampleVolume} X \frac{Purityofstandard}{100} X sampleconc$$

Where:

Sample Area: 521.122

Standard Area: 471.3292

Standard weight taken: 0.200g

Sample volume used: 0.3ml

Density of Eugenol: 1.04g/ml

Purity of Standard: 99.20 % w/w

$$= \frac{521.122}{471.3292} X \frac{0.2x100}{100X0.3} X \frac{99.20}{100} X0.3$$

$$Result = 0.219g/100ml$$

Identification and assay performed. Results yielded 0.219g/100ml. The method of gas chromatography was validated in other research papers [89]. Therefore this method is suitable for identification and assay of eugenol on gas chromatography [89, 90]. As we added 0.3 ml per 100 ml of clove oil having 72.66% eugenol. It means we have added 0.218 g of eugenol in 100 ml of newly prepared batch of antiseptic plaster. The quantity of eugenol calculated back after analysis on gas chromatography was 0.219 g per 100 ml. RSD value is 0.593488522% which must be less than 0.2%.

4.3 Microbiological Analysis

Microbiological analysis consists of three segments. First of all test for growth promotive and inhibitive properties were checked to check the health of media. Microbial limit test and test for antimicrobial properties were checked later.

4.3.1 Growth Promotion and Inhibition Test of Culture Media

Growth promotion and inhibition test of all freshly prepared media was performed for the true results of analysis. Test report of each media is given in table attached. Report for Mueller Hinton Agar Table 4.2, negative control for Mueller Hinton Agar Table 4.3, Tryptone Soya Agar Table 4.4, negative control for Tryptone Soya AgarTable 4.5, Bacteriological Peptone Table 4.6, negative control for Bacteriological Peptone Table 4.7, MacConkey Agar Table 4.8, negative control for MacConkey Agar Table 4.9, MacConkey Broth Table 4.10, negative control

for MacConkey Broth Table 4.11, Sabouraud Dextrose Agar Table 4.12, negative control for Sabouraud Dextrose Agar Table 4.13, Tryptone Soya Broth Table 4.14, negative control for Tryptone Soya Broth Table 4.15, Pseudomonas Agar Base Table 4.16, negative control for Pseudomonas Agar Base Table 4.17, XLD Agar Table 4.18, negative control for XLD Agar Table 4.19, Manitol Salt Agar Table 4.20, negative control for Manitol Salt Agar Table 4.21.

4.3.1.1 Muller Hinton Agar

Media: **Muller Hinton Agar** Manufacturer: Oxoid Limited
Lot No: 2114573 Expiry Date: 17/03/2022

Physical Parameters

Appearance: Straw Powder Specifications: pH Value: 7.3±0.2
pH: Before Sterlization: 7.2 pH: After Sterlization: 7.4
Microbial Challenge and Recovery

Table 4.2: Mueller Hinton Agar

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	Growth
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days	Growth
3	*Salmonella typhimurium* ATCC 14028	100cfu	32.5 °C	03 days	Growth
4	*Pseudomonas aeruginosa* ATCC 9027	100cfu	22.5 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Remarks: Satisfactory

Table 4.3: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	03 Days	No Growth
2	30 - 35 °C	03 Days	No Growth

4.3.1.2 Tryptone Soya Agar

Media: **Tryptone Soya Agar** Manufacturer: Oxoid Limited

Lot No: 2137633 Expiry Date: 05/2022

Physical Parameters

Appearance: Straw powder Specifications: pH Value: 7.3±0.2
pH: Before Sterlization: 7.2 pH: After Sterlization: 7.3
Microbial Challenge and Recovery

Table 4.4: Tryptone Soya Agar

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	Growth
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days	Growth
3	*Salmonella typhimurium* ATCC 14028	100cfu	32.5 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Table 4.5: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	03 Days	No Growth
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.3 Bacteriological Peptone

Media: **Bacteriological Peptone** Manufacturer: Oxoid Limited

Lot No: 1773177 Expiry Date: 20/09/2022

Physical Parameters

Appearance: Straw powder Specifications: pH Value: 6.2±0.2
pH: Before Sterlization: 6.3 pH: After Sterlization: 6.2
Microbial Challenge and Recovery

Table 4.6: Bacteriological Peptone

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	30 - 35 °C	03 days	Growth
2	*Staph aureus* ATCC 6538	100cfu	30 - 35 °C	03 days	Growth
3	*Salmonella typhimurium* ATCC 14028	100cfu	30 - 35 °C	03 days	Growth
2	*Candida albicans* ATCC 6538	100cfu	30 - 35 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Table 4.7: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	03 Days	No Growth
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.4 MacConkey Agar

Media: **MacConkey Agar** Manufacturer: Oxoid Limited

Lot No: 2457378 Expiry Date: 01/2024

Physical Parameters

Appearance: Straw powder
Specifications: pH Value: 7.1±0.2
pH: Before Sterlization: 7.3
pH: After Sterlization: 7.2
Microbial Challenge and Recovery

TABLE 4.8: MacConkey Agar

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	Growth
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days	No Growth
3	*Salmonella typhimurium* ATCC 14028	100cfu	32.5 °C	03 days	Growth
2	*Candida albicans* ATCC 6538	100cfu	32.5 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

TABLE 4.9: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	N/A	N/A
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.5 MacConkey Broth

Media: **MacConkey Broth**
Manufacturer: Oxoid Limited
Lot No: 1758297
Expiry Date: 30/10/2020

Physical Parameters

Appearance: Straw powder
Specifications: pH Value: 7.4±0.2
pH: Before Sterlization: 7.1
pH: After Sterlization: 7.3
Microbial Challenge and Recovery

Table 4.10: MacConkey Broth

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	Yellow Growth
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days	No Growth
3	*Pseudomonas aeruginosa* ATCC 9027	100cfu	32.5 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Table 4.11: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	N/A	N/A
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.6 Sabouraud Dextrose Agar

Media: **Sabouraud Dextrose Agar** Manufacturer: Oxoid Limited
Test Date: 29/06/2020 Observation Date: 06/07/2020

Physical Parameters

Appearance: Straw powder Specifications: pH Value: 5.6±0.2
pH: Before Sterlization: 5.5 pH: After Sterlization: 5.6
Microbial Challenge and Recovery

Sterility of Media

Growth (Positive) / No Growth (Negative)

Remarks: Satisfactory

Table 4.12: Sabouraud Dextrose Agar

Sr.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*C. albicans* ATCC 10231	100cfu	32.5 °C	07 days	Growth
2	*Brasillensis* ATCC 16404	100cfu	32.5 °C	07 days	Growth

Table 4.13: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	07 Days	No Growth
2	30 - 35 °C	N/A	N/A

Media: **Tryptone Soya Broth** Manufacturer: Oxoid Limited
Lot No: 2307241 Expiry Date: 04/2023

4.3.1.7 Tryptone Soya Broth

Physical Parameters

Appearance: Straw powder Specifications: pH Value: 7.3±0.2
pH: Before Sterlization: 7.4 pH: After Sterlization: 7.3
Microbial Challenge and Recovery

Table 4.14: Tryptone Soya Broth

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	Growth
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days	Growth
3	*Salmonella typhimurium* ATCC 14028	100cfu	32.5 °C	03 days	Growth
2	*Candida albicans* ATCC 6538	100cfu	22.5 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Table 4.15: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	N/A	N/A
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.8 Psedomonas Agar Base

Media: **Psedomonas Agar Base** Manufacturer: Oxoid Limited
Lot No: 1889737 Expiry Date: 07/2021

Physical Parameters

Appearance: Straw powder Specifications: pH Value: 7.1±0.2
pH: Before Sterlization: 7.1 pH: After Sterlization: 7.2
Microbial Challenge and Recovery

Table 4.16: Psedomonas Agar Base

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	White Growth
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days	No Growth
3	*Pseudomonas aeruginosa* ATCC 9027	100cfu	32.5 °C	03 days	Greenish Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Table 4.17: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	N/A	N/A
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.9 XLD Agar

Media: **XLD Agar** Manufacturer: Oxoid Limited
Lot No: 2393519 Expiry Date: 09/2021

Physical Parameters

Appearance: Straw/pink powder Specifications: pH Value: 7.2-7.6
pH: Before Sterlization: 7.3 pH: After Sterlization: 7.5
Microbial Challenge and Recovery

Table 4.18: XLD Agar

Sr. No.	**Culture with ATCC no.**	**Inoculum**	**Incubation Temp.**	**Incubation Time**	**Remarks**
1	*E. Coli* ATCC 8739	100cfu	32.5 °C	03 days	Conforms
2	*Staph aureus* ATCC 6538	100cfu	32.5 °C	03 days (No Growth)	Conforms
3	*Salmonella typhimurium* ATCC 14028	100cfu	32.5 °C	03 days	Conforms
3	*Pseudomonas aeruginosa* ATCC 9027	100cfu	32.5 °C	03 days	Conforms

Sterility of Media

Growth (Positive) / No Growth (Negative)

Table 4.19: Media Negative Control

Sr. No.	**Temperature**	**Duration**	**Observation**
1	20 - 25 °C	N/A	N/A
2	30 - 35 °C	03 Days	No Growth

Remarks: Satisfactory

4.3.1.10 Manitol Salt Agar

Media: **Manitol Salt Agar** Manufacturer: Oxoid Limited
Lot No: 2272392 Expiry Date: 30-10-2024

Physical Parameters

Appearance: Straw powder

Specifications: pH Value: 7.5±0.2

pH: Before Sterlization: 7.6

pH: After Sterlization: 7.5

Microbial Challenge and Recovery

TABLE 4.20: Manitol Salt Agar

Sr. No.	Culture with ATCC no.	Inoculum	Incubation Temp.	Incubation Time	Remarks
1	*E. Coli* ATCC 8739	100cfu	30 - 35 °C	03 days	No Growth
2	*Staph aureus* ATCC 6538	100cfu	30 - 35 °C	03 days	Growth

Sterility of Media

Growth (Positive) / No Growth (Negative)

Remarks: Satisfactory

TABLE 4.21: Media Negative Control

Sr. No.	Temperature	Duration	Observation
1	20 - 25 °C	N/A	N/A
2	30 - 35 °C	03 Days	No Growth

It was necessary to check the quality of prepared media to avoid false result of microbiological analyses. Growth promotion and inhibition test of all media used in research were checked for their health. Mueller Hinton Agar, Tryptone Soya Agar, Tryptone Soya Broth, Bacteriological Peptone and Sabouraud Dextrose Agar were checked for their growth promotion while MacConkey Agar, MacConkey Broth, Pseudomonas Agar Base, XLD Agar and Manitol Salt Agar were checked for growth promotion and growth inhibition properties. All media were challenged with 100 CFUs of relevant microorganism and recovered back. All the media used were in good condition. The quality check was the most important for any media thus, it was checked regularly for the quality assessment.

4.3.2 Microbial Limit Test

Bio burden of bulk sample was checked according to united state pharmacopeia 2019 specifications. Satisfactory results found. Microbial Limit test were performed to fulfill the requirements of United States pharmacopeia. Total number of aerobic bacterial count and total combined yeast and mold stayed below the limits (Table 4.23). USP pathogens were also absent (Table 4.24).

Product Name:	Antiseptic plaster (Bulk)	Sampled by:	Zaki Ul Hasan
Batch/Lot No:	Pilot	Batch Size:	5 Litre
MFG Date:	11-2020	Expiry Date:	NA

4.3.2.1 Sample Preparation:

10 ml of sample in 90 ml of 0.1% peptone water.

Table 4.22: Results of Total Microbial Count

Name of Test	**Media**	**Dilution Factor**	**Volume Tested**	**Accepta- -nce Limit**	**Results (cfu/ml)**
Total Aerobic Microbial Count	Tryptone Soya Agar	1:10	1ml	Not More than 10^2 CFU	Less than 10 CFU
Total Yeast and Mold Count	Sabouraud Dextrose Agar	1:10	1ml	Not More than 10^1 CFU	Less than 10 CFU

Table 4.23: Results of Specified Microorganisms

Name of Microorganism Test	**Media**	**Results (per ml)**	**Positive Control**	**Negative Control**
Escherichia coli	MacConkey Agar	Absent	Positive	Negative
Staphylococcus aureus	Mannitol Salt Agar	Absent	Positive	Negative
Pseudomonas aeruginosa	Pseudomonas agar base	Absent	Positive	Negative
Salmonella spp.	X.L.D agar	Absent	Positive	Negative

Bio burden of the product was very important as it might be applicable to minor wounds. Open wound come in direct contact with antiseptic plaster. Product with higher microbial load or having pathogenic microorganism can cause infection to the persons using the product. Total aerobic bacterial count, total yeast and mold count, test for *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella spp.* were performed [91]. According to United State pharmacopoeia a medically important substance that can be applied on cutaneous/skin must contain total aerobic microbial load less than 10^2 CFUs per gram, total yeast and mold count less than 10^1 per gram and absent of *Escherichia coli*, per gram. We have result under these limits. Controls of all tests were satisfactory.

4.3.3 Antimicrobial Activity

Following ATCC Cultures were used to check the antimicrobial activity of Eugenol.

- *Bacillus subtilis* ATCC 6633
- *S. aureus* ATCC 6538
- *C. albicance* ATCC 10231
- *E. coli* ATCC 8739

Six thousand cfu of each culture per plate were challenged against the product and checked for its activity. 5 μ/ml Nastatin and 3 μ/ml Levofloxicin were used as standard for fungal and bacterial cultures respectively. Bacterial cultures were incubated for 2 to 3 days at 30 to 30 °C while yeast culture was incubated at 20 to 25 °C for 5 days. Sample showed significant zones of inhibition against each ATCC culture. Figure 4.15 *Bacillus subtilis* ATCC 6633, Figure 4.16. *S. aureus* ATCC 6538, Figure 4.17 *C. albicance* ATCC 10231, Figure 4.18 *E. coli* ATCC 8739. Each culture showed a significant zone of inhibition for eugenol.

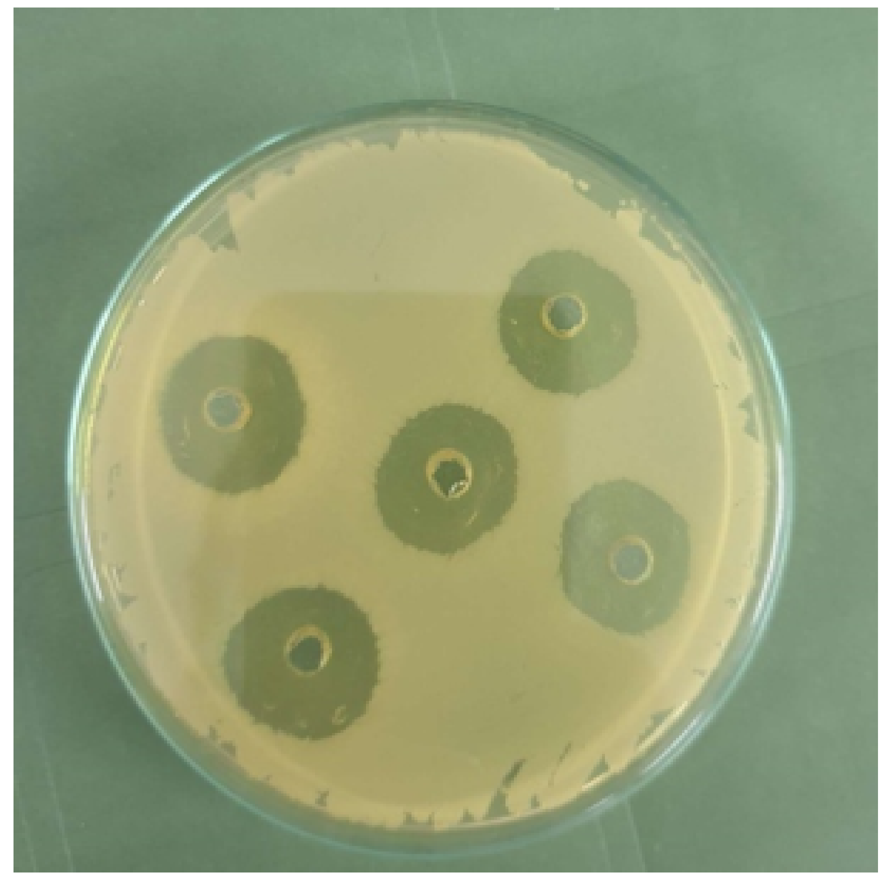


FIGURE 4.15: Zone of Inhibition Against *Bacillus subtilis* ATCC 6633

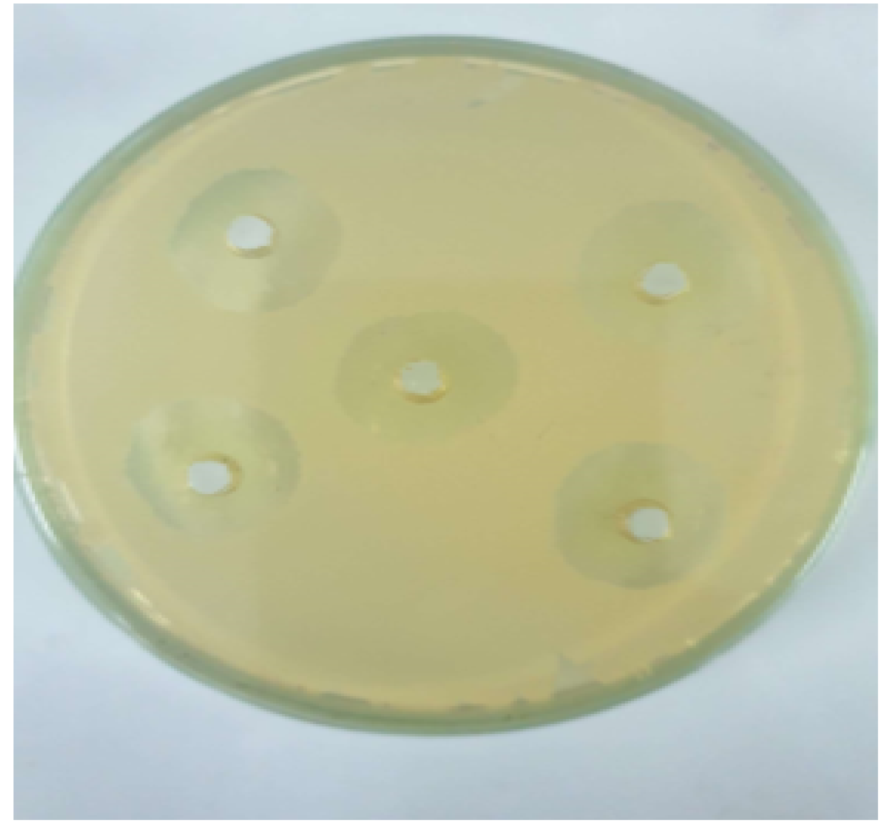


FIGURE 4.16: Zone of Inhibition Against *Aureus* ATCC 6538

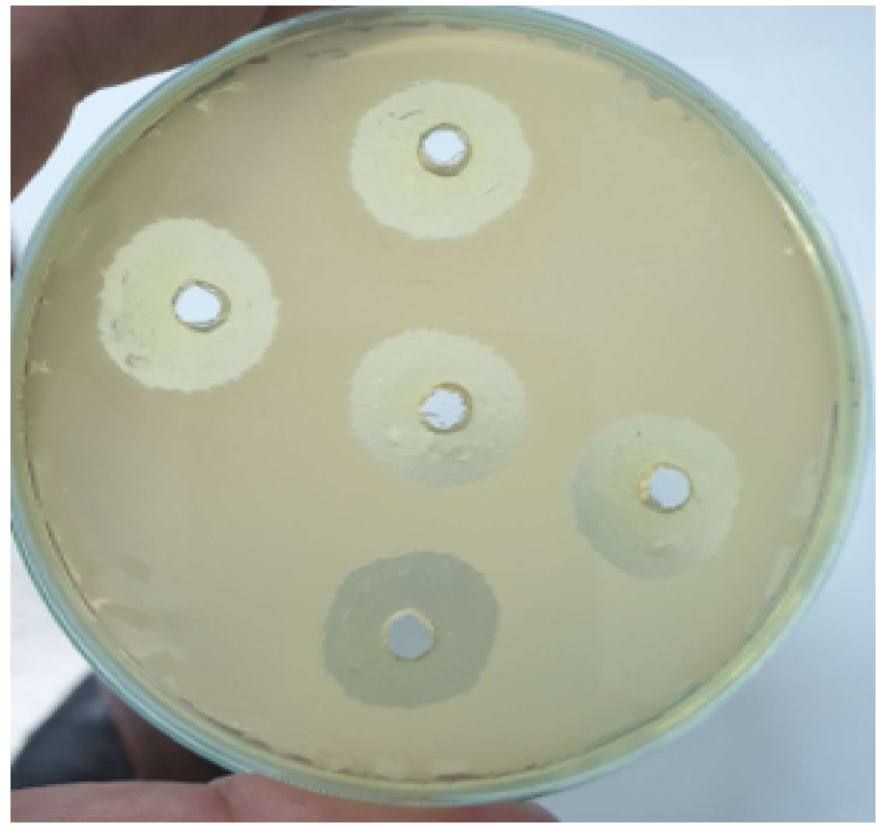


FIGURE 4.17: Zone of Inhibition against *C. albicance* ATCC 10231

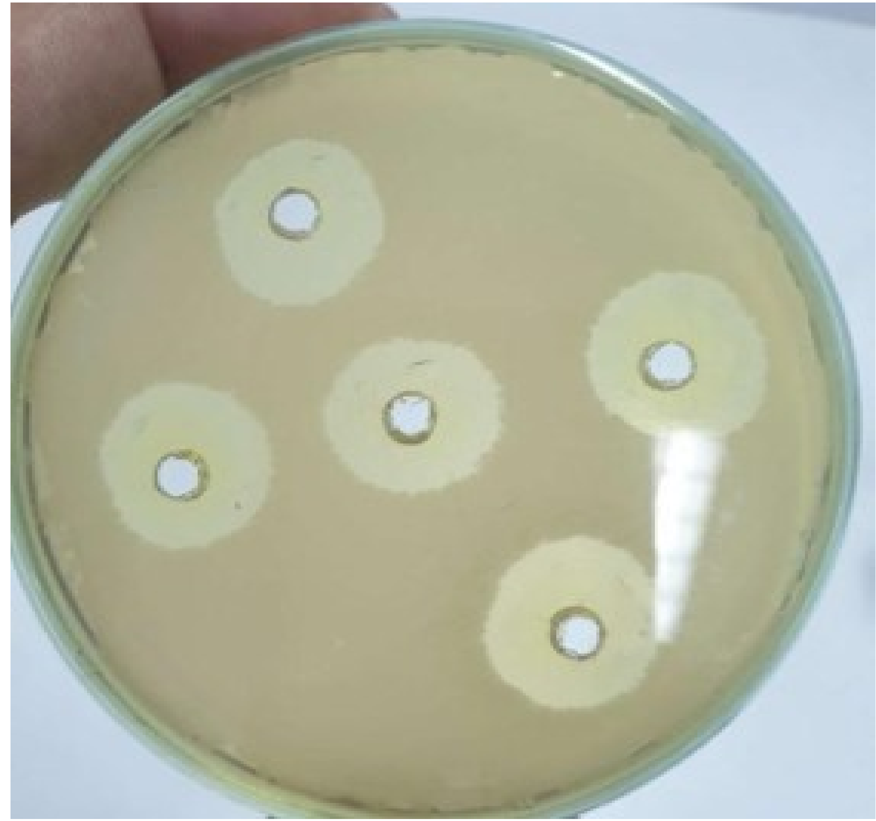


FIGURE 4.18: Zone of Inhibition Against *E. coli* ATCC 8739

Table 4.24: Zones of Inhibitions Against ATCC Cultures

Serial #	ATCC Cultures	Mean Zone of Inhibition of Eugenol (mm)	Mean Zone of Inhibition of Standard (mm)	Results in percentage
1	Bacillus subtilis ATCC 6633	20.4	20.7	98.55
2	S. aureus ATCC 6538	20.1	20.5	98.04
3	C. albicance ATCC 10231	19.8	20.0	99.00
4	E. coli ATCC 8739	20.3	20.6	98.54

Antimicrobial activity of eugenol was performed which yielded significant zones of inhibition. Eugenol is a strong antimicrobial agent [92]. Researches showed the antimicrobial property of Eugenol. Six thousand population of each culture, *Bacillus subtilis* ATCC 6633, *S. aureus* ATCC 6538, *C. albicance* ATCC 10231 and *E. coli* ATCC 8739 were uniformly distributed in different petri plates. 50 μl of prepared antiseptic solution was poured in five wells in each plate. Zone of inhibitions against these cultures was very significant. Therefore it can be used as antimicrobial agent. As the sample was herbal mixture so there may be other herbal compounds than eugenol having antimicrobial properties.

4.4 Finished Product Parameters

Physical dimensions [88] for antiseptic plaster were taken from research paper and two local brands of antiseptic plasters Neeemplast and Saniplast. 10 strips were used to check each perimeter and average reading was interpreted. All parameters were found satisfactory and within limits. Plaster was removed easily from skin without any adhesive deposition in a smooth pattern. Plaster is designed in a way to facilitate aeration to wound.

TABLE 4.25: General Specification of Plaster

S#	Parameters	Specification	Results
1	Description	Light brown film ventilated bandage, pad impregnated with eugenol which cover by paper	Complies
2	Identification	Eugenol (Same Retention time against Eugenol Standard by GC)	Complies
3	pH	5.0-6.0	5.7
4	Adhesive skin test	Easily removes from skin without any adhesive deposition	Complies
5	Average weight of strip with releasing and packing paper (g) (10 strips)	Medium 0.55 g 5%	0.56 g
6	Average Weight (with releasing paper only) (10 Safety)	0.236 gm + 5% (0.220gm 0.250 gm)	0.240 gm
7	Wound pad length (only) (mm)	Length 72 mm 1 mm	72 mm
8	Wound pad width (only) (mm)	Width 19 mm 1 mm	20 mm
9	Bandage Length	72mm + 1mm	72 mm
10	Bandage Width	19mm + 1mm	20 mm

Chapter 5

Conclusion

The clove contains active chemicals that can be used for medicinal purposes. Eugenol is a major component of clove essential oil and is a major component responsible for the antimicrobial activity of such extracts. This study was done under current good manufacturing practices. It is a good effort to use herbal antiseptic compounds in bandages which showed satisfactory antimicrobial effects against a variety of ATCC cultures. Identification and quantification of compound was done by gas chromatography. Batch was manufactured in a large scale to check the behavior of product components with each other and found satisfactory. We hope that these findings are encouraging against treatment failure and antibiotic resistance.

Further studies are needed, prior to the incorporation of eugenol into drug formulations. Additional in vivo studies and clinical trials may be required in order to justify and further analysis of these components used.

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