STUDY ON NUTRITIONAL COMPOSITION, BIOACTIVE COMPOUNDS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF THE CLOVE (*SYZYGIUM AROMATICUM*)

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Roll No: 0118/05

Registration No: 540

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Food Chemistry and Quality Assurance

Department of Applied Chemistry & Chemical Technology

Faculty of Food Science Technology

Chattogram Veterinary and Animal Sciences University

Chattogram-4225, Bangladesh

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects and that all revisions required by the thesis examination committee have been made.

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Dedication

I dedicate this small piece of work

to my beloved parents and teachers

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The Author

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List of abbreviations

Abbreviation	Elaboration
AOAC	Association of Official Analytical Chemists
AOA	Antioxidant Activity
ABTS	Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BBS	Bangladesh Statistics Bureau
BDS	Bangladesh Standard
Kg	Kilogram
CHO	Carbohydrate
CAC	Codex Alimentarius Commission
CEO	Clove essential oil
COE	Clove Oil Extract
DPPH	diphenyl-2-picrylhydrazyl
DF	Dilution factor
DDM	Disc diffusion method
FC	Folin–Ciocalteu
FA	Fatty Acid
FDA	Food and Drug Administration
GC-MS	Gas chromatography-mass spectrometry
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
mg/L	Milligram/liter
meq	Mill equivalent
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
NFE	Nitrogen Free Extract
ORAC	Oxygen Radical Absorption Capacity
%	Percentage
ppm	Parts per million
ROS	Reactive Oxygen Species
UVS	UV-visible Spectrophotometer

Abstract

The nutritional composition, antioxidant, bioactive compound and antimicrobial characteristics of 3 brands clove and clove essential oil (CEO) were investigated. Few phytocomponents were identified in essential oils by GC-MS analysis like- eugenol, eugenol acetate, p-picoline, caryophyllic acid, caryophylene oxide which are widely used as flavoring agent, medicinal, nutritional and cosmetics products. The physicochemical characteristics of CEO were carried out and acid value found to be 5.213, 6.087 and 6.457; Saponification value: 37.257, 40.670, 38.056; peroxide value: 4.61, 6.667, 5.127; Iodine value: 51.457, 49.507, 50.157 of cloves essential oil of the Indonesia (CIs), India (CId) and Sri Lanka (CSl) respectively. Density & Viscosity for three brands clove essential oil were measured at six different temperatures between 25^{o}C and 50^{o}C maintaining 5^{o}C interval. Refractive Index for 3 brands clove essential oil were measured at T = 30^{o}C, 40^{o}C & 50^{o}C. In each sample ρ, η and n_D decreased with the increased of temperatures. In this study proximate composition of 3 brands clove were determined the: moisture contents were 12.287%, 13.653%, 11.553%; ash contents: 4.877%, 5.177%, 5.253%; protein contents: 4.213%, 4.127%, 4.557%; fat contents: 9.817%, 10.187%, 11.187%; fiber contents: 16.694%, 15.821%, 15.875%; carbohydrate contents: 52.21%, 51.53%, 51.587% of the CIs, CId and CSl clove essential oil respectively. The antioxidant activities of the cloves essential oil were analyzed by DPPH method and results were found to be 70.66, 70.14 and 69.74 ppm of the CIs, CId and CSl clove respectively. Bioactive compounds like total flavonoids content (TFC), total polyphenol content (TPC) and total anthocyanin content (TAC) were also analyzed by UV-Visible spectrophotometer and TFC content was found to be 244.36, 161.13 and 321.95 ppm of the CIs, CId and CSl clove essential oil respectively. TPC content was found to be 159.44, 142.82 and 135.11 ppm of the CIs, CId and CSl clove essential oil respectively. TAC content was found to be 16.071, 18.156 and 17.942 ppm of the CIs, CId and CSl clove essential oil respectively. The inhibitory effects of cloves oil were detected for progress of 3 bacteria, including *E. coli*, *Staph. aureus* and *P. aeruginosa*. This study has shown the dignity of clove and clove essential oil and indicated that these can be used as antimicrobial, antiseptic, antioxidant and preservative agent.

Keywords: *Syzygium aromaticum*, *Myrtaceae*, antimicrobial activity, antioxidant activity, anthocyanin and phytocomponents.

Chapter-1

Introduction

Cloves (*Syzygium aromaticum*) are the aromatic dried flower buds of a tree in the family of *Myrtaceae* (Chaieb et al., 2007a). Actually cloves are used as a spice all over the world. The term 'Clove' is derived from the 'Clou' (French word) and the 'Clout' (English word). Cloves are local to the Maluku Islands in Indonesia. Cloves are attainable throughout the year due to various harvest seasons in several countries. Cloves are a flavour made from the flower buds of an evergreen tree. Clove has been used as food preservative and for many medicinal purposes. Clove flower buds are harvested in their immature state and then cloves are dried. Whole cloves are shaped like a small, reddish-brown cornstalk, usually around 1 cm in length, with a bulbous top. Cloves have a very potent, pungent flavor and aroma (Parle and Khanna, 2011).

Plant essential oil is used in human health such as food additives, functional food, nutritional supplements, cosmetic manufacturing and medicine. Antioxidants are effective for inhibiting different human diseases because of their antimicrobial, antiradical and antioxidant properties (Zengin and Baysal, 2014). Cloves contain a compound called eugenol. It has been shown to act as a natural antioxidant. Therefore, investigation of bioactive compounds particularly polyphenol from natural plant sources including herbs and spices have an increasing trend. Plant essential oils have antimicrobial, antioxidant and antimutagenic activities and potential beneficial effects on certain health conditions. These generally recognized as safe nature substances inhibited lipid oxidation in foods, thereby serving as natural additives in foods and food products (Burt, 2004; FDA, 2013).

Approximately 72-90% of the necessary oil extracted from cloves has Eugenol. Other essential oil ingredients of clove oil are acetyl eugenol, vanillin, beta-caryophylene, tannins, methyl salicylate, gallotannic acid, triterpenoid, oleanolic acid, Kaempferol, and flavonoids eugenin. The dried buds of cloves contain about 15- 20% of essential oils. Eugenol is the important bioactive compound. One kg of dried buds provides about 150 ml of eugenol (Debjit et al., 2012). Whilst many different chemicals have been recognized in clove oil, a composite called eugenol is one of the elementary

components. Like many essential oils, researchers have been working to evaluate the potential health benefits of clove oil and its components (Debra and Seladi, 2019).

Plant essential oils are composed by highly volatile organic molecules which contribute special flavors and fragrances (Kurt Bauer et al., 2011). They have main physical feature namely-odor, color characteristic and high refractive index. The fragrance oils contribute a major role in the commercial production of cosmetics, bath soaps and perfumery items (Husnu and Gerhard, 2010). Aside from these they are extensively used in food beverages, flavoring and pharmaceuticals. Ordinary products are main plant metabolites, used as a source of medicine from primitive times worldwide. Since 1981, natural products isolated from plants were used in the discovery of new drugs to cure disease e.g. antimicrobial, anticancer, antibiotics, antihypertensive, antituberculosisetc (David & Gordon, 2012). The natural products based on plant origin produce flavors and fragrances. The knowledge of physicochemical properties like acid value, ester value, saponification value, iodine value, density, viscosity, refractive index and presence of natural products in essential oils decide their utilization in eating, pharmaceuticals and industrial making (Ajay Kumar, 2014). Among those, density as an equilibrium property and viscosity as a transport property seem to be quite significant for liquids.

Cloves have many therapeutic uses: control nausea and vomiting, relieve pain, and improve digestion. It protects against internal parasites and act as antimicrobial agents against fungi and bacteria cause uterine contractions and strong antiseptic (Burt and Reinders, 2003). In common medicine, clove is used as strength against gastro-intestinal spasm, stomach distension and flatulence (Elujoba et al., 2005). The clove oil is necessary for medicating rheumatoid arthritis and it has antispasmodic, antiseptic and analgesic properties. About four grams of clove are boiled in three liters of water until half the water has evaporated. This water, taken in drawers, will slow down acute symptoms of cholera (Kalemba and Kunieka, 2003). Chewing clove with a crystal of usual salt relieves the irritation in the throat and pause cough in the pharyngitis. Clove bud oil is a clear, discolored to yellow mobile liquid. It becomes browner with age or contamination with iron or copper (Weiss, 1997). Clove oil is applied in the manufacture of detergents, soaps, shampoo and perfumes. Clove bud oil, has been used for a lengthy time by Dentists; as a dressing in Dentistry, for

medicating minor oral wounds; as an analgesic in stinging and infective diseases of the oral cavity and pharynx as well as for common hygiene (Elujoba et al., 2005).

Nutrients base in some plants extracts do major than just prevent insufficiency diseases. Certain vitamins in produce, notably vitamin B, C and carotene as well as polyphenol are potent anti-oxidants. Carbohydrate contains Carbon (C), Hydrogen (H), and Oxygen (O) with H and O in the same proportion as in water. They form largely of hexosans. These are made up of 6-carbon atom molecules. Some examples are 1.Monosaccharides e.g. galactose, glucose, fructose. 2. Disaccharides e.g. sucrose, maltose. 3. Polysaccharides e.g. starch, glycogen, cellulose. Fat contains Carbon (C), Hydrogen (H), and Oxygen (O) with more C and H in proportion to the O than with carbohydrates. 1. Saturated fat e.g. stearic, palmatic. 2. Unsaturated fat e.g. oleic, linolenic. Dietary fats are important not only because of their high energy value but the fat-soluble vitamins and essential fatty acids contained in the fat of natural foods. Fats and oils aid to regulate blood pressure and play useful role in the synthesis and repair of vital cell parts (Dutta, 2003). Protein always contains Carbon (C), Hydrogen (H) and Oxygen (O), Nitrogen (N) and sometimes Iron (Fe), Phosphorus (P) and S. The only macronutrient which contains N. Feed proteins on the average contain 16% N. Formed by variant combinations of amino acids of which there are some 25+ to be found in proteins. Amino play as organic acids which carry the amino group (NH_2). Protein can help in cell division as well as growth (Okeke and Elekwa, 2006).

Antioxidants are compounds that can inhibit the oxidation of lipid or other molecules by inhibiting the initiation of oxidizing chain reactions (Velioglu et al., 1998). All aerobic organisms have antioxidant defenses inclusive antioxidant enzymes and foods to except or repair the damaged molecules (Cakır et al., 2006). Antioxidant composite can scavenge free radicals and increase shelf life by obstructing the system of lipid peroxidation. It is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage (Halliwell, 1997). Antioxidants can save the human body from free radicals and Reactive Oxygen Species (ROS) virtue. They repress the progress of many ancient diseases as well as lipid peroxidation (Gulcin, 2007). Hence, the requirement for identifying substitute natural and safe sources of food antioxidants has been generated and the search for natural antioxidants, especially of plant origin has noticeably increased in recent years

(Skerget et al., 2005). Antioxidants have been extensively used as food additives to provide defense against oxidative deterioration of foods (Gulcin, 2005). At actual, the most usually used antioxidants are BHT, BHA propyl gallate and tertbutylhydroquinone. Except this BHA and BHT have been supposed of being liable for liver damage and carcinogenesis (Sherwin, 1990). Therefore, there is an increasing interest on natural and certain antioxidants (Gulcin et al., 2006b).

Antioxidants conduct by: removing O_2 or reducing local O_2 concentrations, removing catalytic metal ions, removing key ROS, e.g. O^{2-} and H_2O_2, scavenging instituting radicals, e.g. OH^-, RO^- , RO^{2-} breaking the chain of an initiated sequence, scavenging singlet oxygen, enhancing endogenous antioxidant defenses by up-regulating the expression of the genes encoding the antioxidant enzymes. It repairs oxidative damage caused by radicals, increasing elimination of damaged molecules and not repairing excessively damaged molecules in order to minimize introduction of mutations (Mahfuz et al., 2010). Antioxidants helps prevent molecular damage caused by oxidation in that protection offered may help fend off diseases such as cancer, cardiovascular diseases and muscular degeneration (Islam et al., 2002). Nowadays, an extensive research is being focused on extraction and isolation of natural dietary antioxidant comprising, especially plant polyphenol for the improvement of anticancer agents, chemo- preventive drugs and other nutraceuticals to supplement and fortify the physiological fence mechanisms of human body (Fu et al., 2007).

A bioactive compound is just a constituent that has a biological action. Bioactive compounds are not nutrients they are contained in foods or their constituents. These constituents have an effect on human health (Biesalski et al., 2009). They are essential and non-essential composites that occur in nature. Bioactive compounds like total flavonoids content (TFC), total phenolic content (TPC) and total anthocyanin content (TAC). These composites are responsible for alternatives in health status (Studdert et al., 2011). Flavonoid and phenolic compounds may be useful as antioxidant from natural sources (Ghalem et al., 2014). Focusing on systems of relevance in biological processes or in food science, the materials to save are most commonly proteins, lipids, carbohydrates and to a minor extent. Another organic molecule that compose animal or vegetal tissues. Their oxidation occurs by a radical chain reaction mediated by peroxyl radicals (ROO•) that parallels the autoxidation of hydrocarbons (Valgimigli

& Pratt, 2012). These characteristics are due to the inherent capability of some of their consisting, particularly phenols, to delay the aerobic oxidation of organic matter, although the procedure by which the oil is obtained from the raw material (distillation) limits the amount of phenolics in the final matrix because many such compounds are nonvolatile. However, there is phenol-free EOs that expresses antioxidant behavior (Valgimigli et al., 2012; Foti, 2007).

Clove oil also applied in food and beverages as preservative versus bacteria or fungi and also flavoring agent (Pundir et al., 2010). It is noteworthy that Pande and Singh, (2011) reported the compound Eugenol, having molded and bacterial inhibiting activity in bakery food items. Ground beef i.e. cattle meat usually spoiled by psychotropic bacteria but can be prohibited by the use of clove oil (Oliveira et al., 2013). Recently, the antibacterial activity of *Syzygium aromaticum* reported against many microorganisms like *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella typhi* (Kumar et al., 2014) and antifungal activity against *Fusarium oxysporum* (Haminiet et al., 2014; Shrivastavaet et al., 2014). The synergistic antimicrobial actions of ethanol and acetone extract of clove plant positively assess with cultures of *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* (Reji and Rajshekharan, 2015).

Natural products have been used to treat microbial growth and numerous essential oils have demonstrated the ability to inhibit the growth of various pathogens. The effect of clove essential oil and eugenol on the growth of Gram-positive (*Bacillus cereus, B. subtilis, Staphylococcus aureus, Enterococcus faecalis*and *Listeria monocytogenes*) and Gram-negative (*Escherichia coli*; *Salmonella typhi*; *S. choleraesuis*; *Yersinia enterocolitica, Pseudomonas aeruginosa*) bacterial strains was investigated (Singh et al., 2007). At 1000 ppm, eugenol prohibited the enhancement of the bacteria and complete prohibition was obtained against *P. aeruginosa* at a high concentration of 2000 ppm, which was high in comparison to ampicillin 1mgml/1 used as a positive control. The antibacterial action of eugenol against several pathogens such as *E. coli, B. cereus, Helicobacter pylori, S. aureus, S. epidermidis, Streptococcus pneumoniae* and *S. pyogenes* was confirmed (Van Zyl et al., 2006; Leite et al., 2007).

Additionally, the combination of cinnamate and eugenol produced a bactericidal synergistic effect against *E. coli* O157:H7, *S. typhi*and *L. monocytogenes* and an

additive effect against *S. aureus* (Rico-Molina, 2012; Fu et al., 2007) reported that the clove essential oil showed inhibitory activity against *S. aureus* and *E. coli*. The biological action of essential oils is usually investigated without property on the mechanism of action. In an *in vitro* study, it is revealed that eugenol induced cell lysis through leakage of protein and lipid contents. Both cell wall and membrane of the treated Gram-negative and Gram-positive bacteria were significantly damaged (Oyedemi et al., 2009). This synergistic effect could be explained by the fact that eugenol is able to damage the membrane of Gram-positive and Gram-negative bacteria. It was found that a concentration of 1 mm damaged nearly 50% of the bacterial membrane allowing increased penetration antibiotics and therefore a greater antimicrobial effect (Hemaiswarya & Doble, 2009).

However, studies on the nutritional composition and bioactive compounds and antibacterial activity of clove oil are limited. The present study was carried out to investigate bioactive compounds (TPC, TFC and TAC), antioxidant activity were determined by DPPH methods by using UV Spectrophotometer and antimicrobial activity by agar disc diffusion method. Antibacterial activity was evaluated by inhibition of *in vitro* cell cultures of bacteria, namely, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonus aeruginosa* and to identify unknown compounds in clove essential oil.

1.1 Aims and Objectives

- Extraction and proximate analysis of three brands clove.
- To quantify the bioactive compounds (TPC, TFC and TAC) and antioxidant activity (AOA) of clove essential oil.
- To determine antimicrobial activity of essential oil of clove.
- To identify the unknown compounds in clove oil by GC-MS.

1.2 Anticipated Outcomes

- ✓ The nutritional composition of three brands clove were extracted and analyzed and several unknown compounds were identified.
- ✓ The bioactive compounds and antioxidant activity of clove essential oil (CEO) were quantified.
- ✓ The antimicrobial activities of clove essential oil were estimated.

Chapter-2

Review of Literature

2.1 Clove

Syzygium aromaticum belongs to family Myrtaceae is a small medium evergreen tree, 2-6 m tall. Usually, plant found medium sized, crown base lower, branches semi erect and manifold. Flowers in ultimate cymosely cluster, each pedicle bear 3- 4 stalked flowers at the end while sepals minute with triangular limb. Fruits found replicable olive shaped, one seeded popularly mentioned to as mother of clove with explicit aroma. The brown, dried, unrevealed flower buds called cloves, a name coming from French word “clou” mentioning nail. Plant arranged in south-west and central Indian subcontinent and under cultivation in large since primitive time. Mostly, the aromatic or spices plants contain composite that empower confirmed potential anti-oxidative properties. Antioxidants are very significant to human health, involving lowering the risk of cancer. Antioxidants obstruct the effect of free radicals. Antioxidant process prevents these reactive oxygen species from being made at optimum level. They play scavengers to neutralize effect of free radicals (Wankhede; 2015).

Parle and Khanna (2011) explained that, clove is an excellent spice. Clove may be looked upon as a better of all the antioxidants known till date. ORAC test is a scale promoted by U.S. Department of Agriculture for comparing antioxidant action. The ORAC score, of clove is more 10 million. A drop of clove oil is 400 times over powerful as an antioxidant than wolf berries or blueberries. In adjunct to its culinary uses, the clove buds have an affluence of medicinal and recreational uses. However, commercial use of the clove is for the produce of clove oil that contain active components, which possess antioxidant, anti-fungal, anti-viral, anesthetic, anti-diabetic, antithrombotic, anti-microbial, anti-inflammatory, pain reliving and insect repellent properties. Eugenol is the important components responsible for the medicinal features of the clove bud.

2.2 Essential oil of cloves

Clove oil is a mixture of different compounds. Clove oil contains three main active ingredients such as eugenol, eugenol acetate and caryophylene.

Griffiths and Hasseth (2007) explained By measuring a sample's absorption of infrared light at various wavelengths, FTIR analysis is able to pinpoint the molecular composition and structure of a sample, making it an incredibly useful quality control and troubleshooting tool for manufacturers that need help identifying unknown materials, additives, contaminants, and other material mysteries.

Nazrul et al. (2010) analyzed clove oil by GC-MS and thirty one components were identified in bud oil with the main components being eugenol (49.7%), caryophyllene (18.9%), benzene, 1-ethyl-3-nitro (11.1%) and benzoic acid, 3-(1-methylethyl) (8.9%). The clove oil from Bangladesh was found to be compared in terms of its eugenol extent. It is expressed that clove can be grown as an economically stable crop in Bangladesh.

Alma and Bristi (2013) analyzed chemical composition of clove bud oil by GC and GC-MS which obtained from steam-distillation method. The results showed that the essential oils mainly include about 3.56% β-Caryophyllene, 8.01% eugenyl acetate and 87.00% eugenol.

Hassan (2014) examined the antioxidant and antimicrobial activities of clove (*Syzygium aromaticum L.*) buds essential oil. Clove essential oil (CEO) developed high content of total phenolic composites with high radical scavenging activity toward DPPH, Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and linolenic acid radicals as well as iron chelating action. The composition of CEO exhibit high eugenol content (80.19%) over 16 identified components by GC-MS analysis. Furthermore, CEO exhibited antibacterial action *in vitro* at low concentrations versus tested food borne pathogens appealing disc diffusion and micro dilutions assays. MICs values for tested bacteria which were impressible to CEO were in the range of 1400–3600 μg/ml. The phenolic composite of CEO are most active and arrive to act principally as membrane permeabilizers which based using staining-DNA fluorescence dye assay. Therefore, the CEO can be probably used in commercial applications as antibacterial, antioxidant and flavoring agent in isolate or in combination with general preservatives for controlling the undesirable organoleptic and microbial degradation in foodstuffs.

2.3 Proximate composition of clove

Abdel M. (2007) determined the proximate chemical composition of clove as follows: moisture (10±0.006)%, fiber (20±0.1)%, ash (5.2±0.01)%, protein (1.2±0.02)%, fat (12.1±0.45)% and carbohydrates (51.5±0.02)%. The physico-chemical features of clove essential oil were carried out and the acid amount was found to be (3.843), saponification value (42.07), ester value (38.22), free fatty acids (1.92) and the refractive index was obtained to be (1.5310 at 30°C). The prohibitory effect of clove oil was discovered for growth of eight microorganisms. The results revealed that clove oil has a potential antimicrobial activity against all tested organisms.

2.4 Physicochemical properties of clove essential oil:

Ajay (2014) tested physicochemical properties and some natural products. The oil was isolated by hydrodistillation method with a yield of 0.52% (v/w). The physicochemical properties namely physical appearance, odor, density, refractive index, acid value, free fatty acids, saponification value, ester value, glycerol contents and iodine value were determined and found to be yellow liquid, aromatic, 0.8791 g/cm^3, 1.478, 1.12 mg KOH/g, 0.56%, 190.77 mg KOH/g, 189.65 mg KOH/g, 10.38%, 2.28 g/100g respectively. The results showed the oil is compatible for making bath soaps, cosmetics and perfumery products.

2.5 Antioxidant Activity of clove essential oil:

Essential oils are liquid mixtures of volatile composites obtained from aromatic plants. The use of fundamental oils as natural antioxidants is a field of growing interest due to some synthetic antioxidants such as BHA and BHT are now suspected to be potent harmful to human health.

Amorati et al. (2013) found that estimation of EOs to edible products either by direct mixing or in active packaging and edible coatings, may therefore illustrate a valid alternative to prevent autoxidation and develop shelf life. The evaluation of the antioxidant performance of necessary oils is however, a crucial issue, because many generally used “tests” are unsuitable and give contradictory results that may mislead future research. The chemistry explaining EO antioxidant action is discussed along with an analysis of the potent in food protection.

Miguel (2009) reported capitally attributed to the chemical composition of the necessary oils and the diversity of tests used. In fact, various *in vitro* assays for the determination of antioxidant capability have been used. They can be classified into two main groups: those that evaluate lipid peroxidation and that those that measurement free radical scavenging capability. In the present study, a brief description of the mechanism included in the majority of methods used for the evaluation of antioxidant action of essential oils is given. At the same time, the antioxidant activities of some necessary oils, measured through various methodologies are presented and linked.

Gulcin et al. (2012) estimated clove oil by employing several in vitro antioxidant assay such as a $DPPH^-$ scavenging, $ABTS^-$ radical scavenging action, total antioxidant activity determination by ferric thiocyanate, total reducing capability determination by $Fe^{3+} \rightarrow Fe^{2+}$ transformation system, superoxide anion radical scavenging by riboflavin/methionine method, hydrogen peroxide scavenging and Fe^{2+} chelating activities. Clove oil prohibited 97.3% lipid peroxidation of linoleic acid emulsion at 15 mg/mL concentration. However, under the same situation, the standard antioxidant composite such as BHA, BHT, a-tocopherol and Trolox demonstrated prohibition of 95.4, 99.7, 84.6 and 95.6% on peroxidation of linoleic acid emulsion at 45 mg/mL intentness respectively. In addition, clove oil had an effective $DPPH^-$ scavenging, $ABTS^-$ scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, Fe^{3+} decreasing power and Fe^{2+} chelating activities. Also, these several AOAs were linked to BHA, BHT, a-tocopherol and Trolox as reference antioxidant composite.

Johanna et al. (2015) describe a deflavored extract with standardized polyphenol profile (Clovinol) and incorporated in several foods. Antioxidant efficiency of Clovinol on healthy human volunteers who control and answer official emails were observed by analyzing their endogenous antioxidant enzymes and content of lipid peroxidation upon consumption of Clovinol either as capsules or as several food at 250 mg/serving/day for 30 days. It was observed that Clovinol can be conveniently incorporated in several food matrices without flavour issues and the consumption of such food/beverages may support an effective detoxification method with an average

elevation of 33±3% in catalase, 66±8% in SOD, 56±5% in GPx, 167±21% in GSH levels and 81±11% attenuation in membrane lipid peroxidation equality.

2.6 Bioactive Compounds of clove essential oil:

A type of chemical found in small quantity in plants and particular foods such as-fruits, vegetables, oils, nuts and whole grains. Bioactive compounds have actions in the body that may promote good health. They are being studied in the preclusion of cancer, heart disease and other diseases. Food bioactive composites in antimicrobial mobility and food control. Examples of bioactive compounds involve indoles, tannins lycopene, lignin etc. Bioactive composites in plants can be identified as secondary plant metabolites eliciting pharmacological or toxicitycal effects in man and animals.

Deepshikha G. (2013) analyzed TPC, TFC and AOA using DPPH assay and FRAP assay. The spices used were clove (*Syzygium aromaticum*), cinnamon (*Cinnamamum verum*), cumin (*Cuminum cyminum*), black pepper (*Piper nigrum*), ajwoin (*Trachyspermum ammi*), black cumin (*Nigella sativa*), coriander (*Coriander sativum*), star anise (*Ilicium verum*) and fennel (*Foeniculum vulgare*). The methanoic extracts of the prior spices were used for all the studies. The TPC noted the following sequence: maximum for cloves > cinnamon > ajwoin > star anise > cumin > black pepper > fennel > coriander > black cumin. The maximum TFC found was in clove > cinnamon > ajwoin > black cumin > star anise > fennel > coriander > black pepper. The spice exhibition maximum AOA was moreover clove followed by cinnamon. The results showed that cloves and cinnamon have the best AO potent and further analysis can be carried out to utilize their potent.

Chandrima et al. (2019) highlighted that the herbal readiness of tea and clove, when compound in very high conc. (1000mg), contained the highest volume of phenolic content mg/GAE (1007.25 ± 1.75), flavonoids content mg/CE (158.17 ± 2.14) free radical scavenging potency in terms of % prohibition (96.81 ± 0.16) along with promoted antimicrobial characteristic with respect to the others. Hence, we can accomplish that drinking tea mixed with clove can surely be a choice of medicinal beverage.

2.7 Antimicrobial Activity of clove essential oil:

Antimicrobial mobility refers to the process of destroying or inhibiting the disease causing microbes. Several antimicrobial agents are used for this purpose. Antimicrobial may be antiviral, antifungal, anti-bacterial. They all have various modes of action by which they act to suppress the infection. Antibiotics are used against bacteria. Antifungals are used against fungi.

Okmen et al. (2018) observed the biological activities of *S. aromaticum* several extracts. The various extracts were screened for antibacterial mobility. The bacteria were isolated from oral flora by traditional systems. The plant extracts were tested by Kirby-Bauer system. Other antibacterial mobility tests are MIC and MBC. In addition to, the antioxidant activities of plant extracts were screened by the calm DPPH free-radical. The antimutagenicity of the plant extracts was determinate by Ames test using *Salmonella typhimurium* strains. The highest antibacterial mobility was destined as 20 mm prohibition zone from methanol extracts. The highest DPPH scavenging activity was found as 82% from aqueous extract. *S. aromaticum* extracts have antimutagenic, antioxidant and antibacterial potent. Our results support the use of this plant in traditionary medicine and exhibition that some of the plant extracts possess composites with well biological activities.

Sana and Ifra (2012) tested the antibacterial mobility against *E.coli* (ATCC 25922) and *Bacillus subtilus* (DSM 3256) at various conc. of extracts of spices by using disc diffusion system. According to the results among the elect spices garlic had the best prohibitory activity showing maximum zone of 26mm against *Bacillus subtilis* DSM and a zone of 22mm against *E. coli* ATCC 25922. The aqueous extracts of garlic were more efficient than ethanolic extract. In the case of cinnamon and turmeric, the ethanolic extracts were major effective exhibiting zones of 16mm against *B. subtilis* DSM 3256 and 17mm against *E. coli*, which exposed that the cinnamon ethanolic extracts are equally efficient against both Gram negative and Gram positive bacteria. The largest zones formed by ethanolic extract of turmeric against *B. subtilis* were measurable as 14mm and it was 11mm for *E. coli* ATCC 25922. The results exhibited that *B. subtilus* is major susceptible to test spices as likened to *E. coli.*

Mandal (2011) obtained the zone diameter of prohibition (ZDP) due to CIN, CLV and CMN array between 22-27 mm, 19-23 mm and 9-15 mm, respectively; whilst the

MICs for the isolates were in the range of 64-256, 64-512 and 128-512 µg/ml respectively. When tested for their MIC levels; the CIN and CLV were found to be bactericidal after 6 hrs of incubation, whilst CMN showed bactericidal mobility after 24 hrs. However, when tested at different concentrations; CIN, CLV and CMN showed bactericidal activity against *S. aureus*, afterward 24 hours of incubation, at 200, 200 and 300 µg/ml, respectively. The *C. zeylanicum* and *S. aromaticum* displayed the strongest *in vitro* antibacterial mobility.

Ayoola et al. (2008) observed that the constituents were eugenol, eugenol acetate, caryo-phyllene and alpha-humelene, with eugenol being the important component. The antimicrobial sensitivity of the volatile oil against some Gram-negative bacteria (*E. coli*, *E. coli* ATCC 35218, *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Citrobacter* spp. and *Enterobacter cloacae*), a Gram-positive bacterium (*Staph. aureus* ATCC 25923), and a fungus (*Candida albicans*) showed a broad spectrum of mobility. MIC was determined for each organism as 2.4, 1.6, 0.27, 0.016, 0.23, 1.63, 0.73 and 0.067 mg/ml for *S. aureus* ATTC 25923, *E. cloacae*, *S. paratyphi*, *K. pneumoniae*, *E. coli* ATTC 35218, *E. coli*, *Citrobacter* spp. and *C. albicans*, respectively. AO screening of clove oil with DPPH was positive, initiative the presence of free radical scavenging molecules which can be assigned to the presence of eugenol, a phenolic compound.

Yu Jie (2007) analyzed the compositions of the oils by GC/MS. Minimum inhibitory concentrations (MIC) against three Gram-positive bacteria, three Gram-negative bacteria and two fungi were conditioned for the fundamental oils and their mixtures. Furthermore, time-kill progressive processes of clove and rosemary essential oils against *Staphylococcus epidermidis*, *Escherichia coli* and *Candida albicans* were tested. Both essential oils possessed important antimicrobial effects against all microorganisms tested. The MICs of clove oil ranged from 0.062% to 0.500% (v/v), whilst the MICs of rosemary oil ranged from 0.125% to 1.000% (v/v). The antimicrobial mobility of combinations of the two essential oils particular their synergistic, or antagonistic, additive effects against isolate microorganism tests. The time-kill curves of clove and rosemary essential oils toward three strains exposed clearly bactericidal and fungicidal processes of 1/2× MIC, MIC, MBC and 2× MIC.

Chapter-3

Materials & Methods

3.1 Site and period of Study

The study was conducted in the laboratory of the department of Applied Chemistry and Chemical Technology, Animal Science and Nutrition, Food Processing and Engineering, Microbiology and Veterinary Public Health laboratory of Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram-4225 and Training Institute for Chemical Industries (TICI), Polash, Narsingdi-1611, Bangladesh. The study was conducted for a period of six month from 1st July to 30th December, 2019.

3.2 Collection and Processing of cloves

Cloves were collected from the local market of Chattogram City (Khatunganj). These samples were cleared and removed dust materials and then dried. Finally the dried cloves were ground by Fritsch mortar grinder (Germany) for one hour.

3.3 Equipment

Digital analytical balance was used to measure oil sample. Beakers, watch glass, pipette, and conical flasks were used in the assessment and preparation of sample and standard solutions. Electric balance, Hot air oven, Desiccator, Metal tongs, Crucible were used for moisture determine and other.

3.4 Extraction of Essential Oil

There are a number of methods employed for the extraction of essential oil or volatile oil from the plant. In the present study steam distillation method was used. In the process, definite amount of sample (dirt free clove powder) were taken in a distillation flask (Clevenger's apparatus). Then distilled water was added two third of its volume to the flask. Then the flask was heated by electric heating mental for 4 hours. Water condenser condensed volatile substances of three brands clove and generated steam in the flask. The essential oils were lighter than water and so could be separated out. The steam distilled essential oil layer, which was collected over water, was extracted (AOAC, 1984).

3.5 Determination Physicochemical Properties of essential oil

Physical properties of extracted essential oil like refractive index, density, optical rotation and solubility in alcohol were determined according to the standard methods (Pharmacopoeia, 2004) with three replications. Again different chemical properties of extracted oil like acid value, free acid value, saponification value, peroxide value, iodine value after acetylation and phenol content were determined with three replications using cited standard methods (Bahl et al., 1997; Scholastica, 1998).

3.5.1 Determination of Acid Value

Acid Value is the Number of mg of KOH needed to neutralize the free fatty acids present in1g of oil. Acid value indicates the proportion of free fatty acids in the oil. The free fatty acid is generated by the hydrolytic decomposition of the oil. Acid Value of oil samples were determined by standard method described in AOAC (2016) for oils and fats.

Reagents:

Ethanol (95%), 0.1 N aqueous NaOH solution, Phenolphthalein indicator: 1% solution in 95% (υ/υ) ethanol.

Procedure:

At first 25 ml ethyl alcohol was taken in 250 ml conical flask and added 0.5 ml of phenolphthalein indicator solution. The mixture was boiled for about five minutes. Then 1.5 ml of sample was taken in a flask and boiled mixture again. 0.5 ml of phenolphthalein indicator was added. Finally titration was carried out with 0.1 N alkali solutions. While titrating with 0.1 N of KOH until a faint pink color which persisted for at least 30 seconds was observed.

The Acid value was estimated using the following equation:

$$\text{Acid Value} = \frac{\text{N of alkali (0.1)} \times \text{ml of alkali} \times 56.1}{\text{Wt of sample (g)}}$$

3.5.2 Determination of Saponification Value

The saponification value of oil or fat explained the weight of KOH expressed in mg, required to saponify of 1 g of the oil or fat. Saponification values of oil samples were estimated according to AOAC (2016) Official Method.

Reagents: Alcoholic 0.5 N KOH solutions, aqueous hydrochloric acid solution, Phenolphthalein indicator.

Procedure:

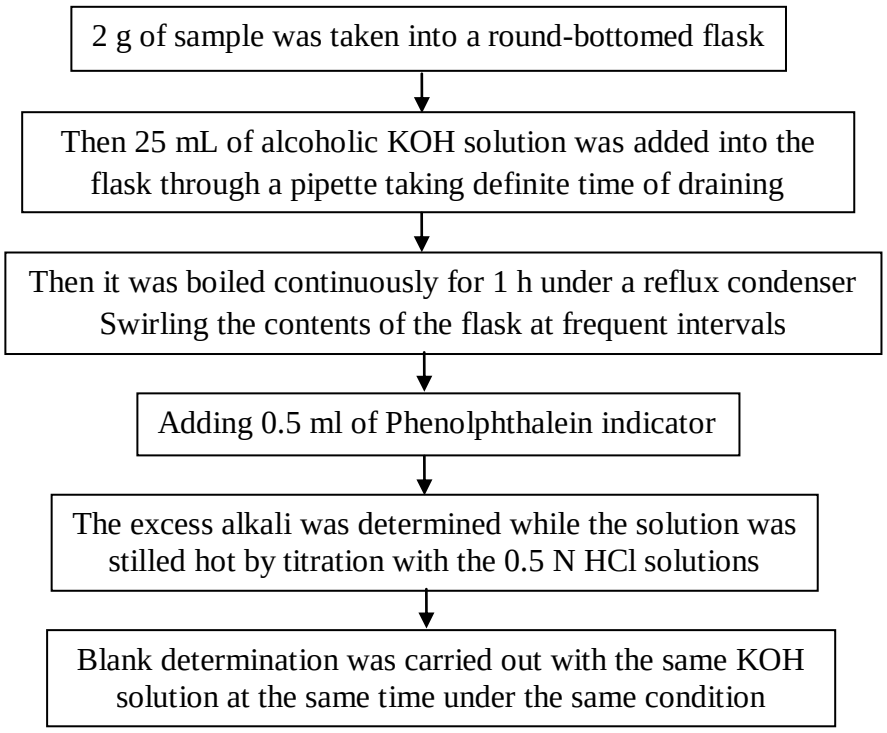


Figure 3.1: Saponification value determination procedure flow diagram

The Saponification value was estimated using the following equation:

$$\text{Saponification value} = \frac{N\times (b-a) \times 56.1}{\text{Wt of sample (g)}}$$

Where, b is blank titer value,

a is sample titer value and

N is 0.5 normality of HCl.

3.5.3 Determination of Peroxide Value

The peroxide value explained the content of peroxide oxygen per 1 kilogram of fat or oil. The peroxide value (PV) was determined according to AOAC Official Method. Chemical reactions-

$$H\text{-}O\text{-}O\text{-}H + H_2O + I^- (KI) = H\text{-}O\text{-}H + 2OH^- + I_2$$

$$I_2 + S_2O_3^{2-} = S_4O_6^{2} + 2I^-$$

Reagents:

Acetic acid (glacial), Chloroform, 15% potassium iodide solution, 0.I N sodium thiosulphate solution, Starch solution - 1%, Potassium dichromate (0.01 N)

Procedure: Peroxide value (PV) is a very sensitive indicator of the early stages of oxidative deterioration of fat and oils.

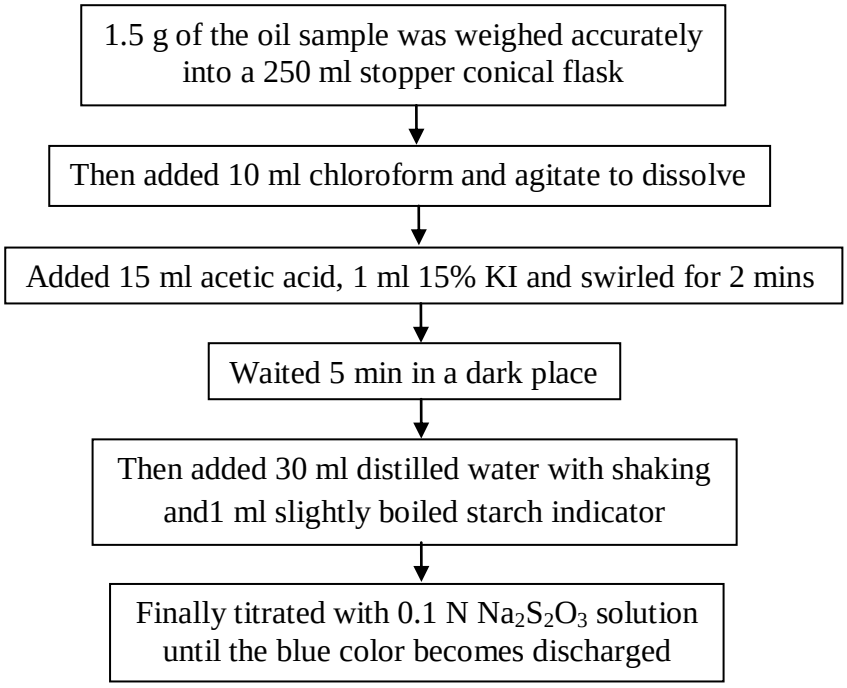


Figure 3.2: Peroxide value determination procedure flow diagram

The peroxide value was estimated using the following equation:

$$PV\ (meq/kg) = \frac{N\times (V_s - V_b) \times 1000}{Wt\ of\ sample\ (g)}$$

Where, N = normality of $Na_2S_2O_3$,

V_s = $Na_2S_2O_3$ consumed by sample (ml) and

V_b = $Na_2S_2O_3$ consumed by blank (ml).

3.5.4 Determination of Iodine value

Iodine value is expressed in g of iodine absorbed by 100 g of oil. The iodine value is a measure of the unsaturation of the oil and is expressed in terms of the number of centigrams of iodine absorbed per gram of sample. Iodine value was determined according to AOAC (2016) Official Method 920.159.

Reagents:

Pure resublimed iodine, glacial acetic acid, pure bromine, Chloroform, 15% KI solution, 0.1 N of $Na_2S_2O_3$ solutions, 1% Starch solution.

Procedure:

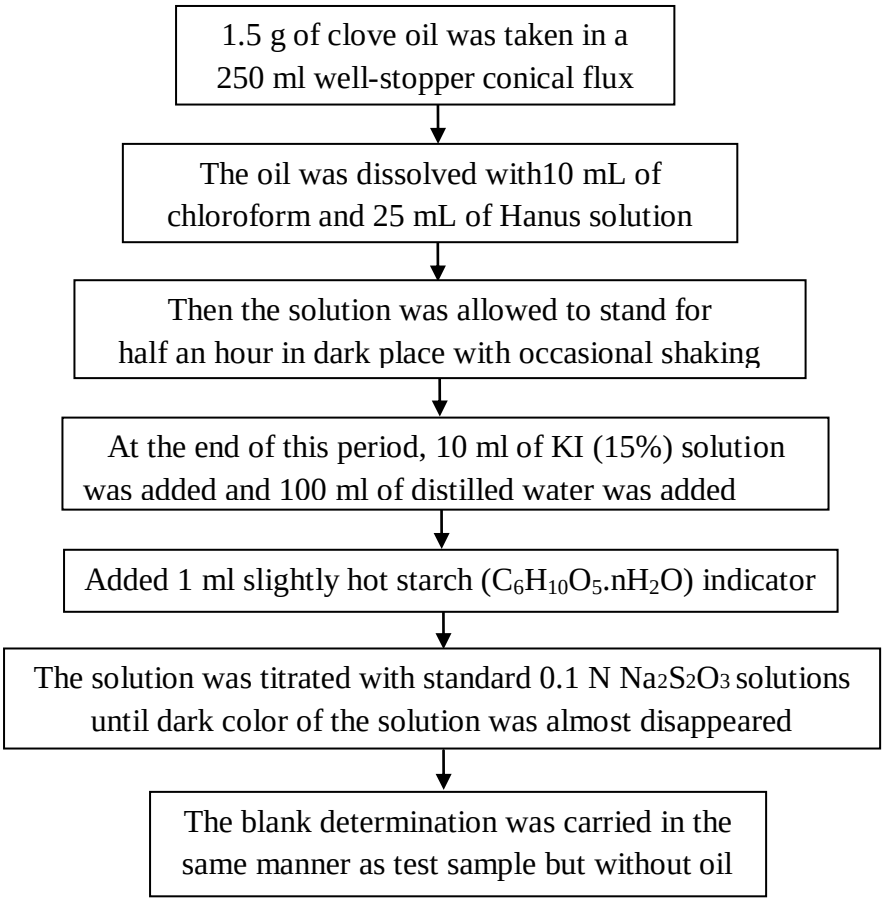


Figure 3.3: Iodine value determination procedure flow diagram

The iodine value was estimated using the following formula:

$$\text{Iodine value} = \frac{(b-a)\times N\times 1.267\times 100}{\text{Wt of sample (g)}}$$

3.6 Proximate composition analysis

Before proximate analysis, sample was dried in sun and used for determination of the percentages of moisture, ash, protein, fat, crude fiber and carbohydrate according to the standard methods (Hossain et al., 2015) with three replications. Total energy was

calculated by (Saleh and Roy, 2007). Moisture, ash, protein, fat and contents of clove samples were measured in triplicate according to AOAC methods.

3.6.1 Determination of moisture:

Moisture is one of the most important factor influencing clove quality and storability. The moisture in clove is removed as vapor. It is measured by the following methods-

A definite quantity of food sample was weighed in a flat bottom crucible with a cover. Empty weight of the crucible with cover was also taken earlier. Then the crucible was placed in an air oven (thermostatically controlled) and dried at a temperature of 105^{o}C for 24 hrs. After drying the crucible was removed from the oven and cooled in desiccators. It was then weighed with the cover glass. The crucible was again placed inside the oven, dried for 30 minutes, took out of the dryer, cooled in desiccators and weighed. Drying, cooling and weighing were repeated until the two consecutive weights were the same. From these weights the per cent moisture content in sample was calculated.

The moisture content was estimated using the following formula:

$$\% \text{ DM} = \frac{\text{Loss of wt } (W_1) \times 100}{\text{Wt of sample } (W)}$$

$$\% \text{ Moisture} = 100 - \% \text{ DM}$$

3.6.2 Determination of ash:

Ash is measured by the following methods-

For the determination of ash, clean empty crucible was placed in a hot air oven at 105^{o}C. Cooled in desiccators and then weight of empty crucible was noted (W_1). 3 gm of each of sample (W_2) was taken in crucible. The sample was burned with crucible up to no smoke. Then the crucible was placed in muffle furnace at 600^{o}C for 6-8 hr. until white ash. The appearances of gray white ash indicate complete oxidation of all organic matter in the sample. Allowed the furnace to cool at 150^{o}C and transferred it to the Desiccator. The crucible was cooled and weighed (W).

Calculate the percent ash by following formula:

$$\% \text{ Ash content} = \frac{W - W_1}{W_2} \times 100$$

3.6.3 Determination of Crude Protein

Analysis of compound for its protein content by Kjeldahl method is based upon the determination of the amount of reduced nitrogen present in its $-NH_2$ and = NH nitrogen.

Chemicals Required:

1. Concentrated H_2SO_4
2. Digestion mixture:
3. Potassium sulphate (K_2SO_4) = 100 gm
4. Copper sulphate ($CuSO_4$) = 10gm
5. Selenium dioxide (SeO_2) = 2.5gm
6. Boric acid solution: 2% solution in water.
7. Alkali solution: 400 gm sodium hydroxide dissolved in water and diluted to 1 litre.
8. Mixed indicator solution: Bromocresol green 0.1 gm and methyl red 0.02 gm dissolved in 100ml ethyl alcohol.
9. Standard HCl: 0.1N.

Procedure:

Total process was conducted by the following steps-

- **Digestion:** At first, 2g sample, 3g digestion mixture and 25 ml H_2SO_4 was taken in a Kjeldahl digestion flask. It was heated for 4 hours in a Kjeldahl digestion and distillation instrument. The digestion was finished when the color of the substance was pale yellow.

  Chemical reaction occur-

  $$\text{Protein} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + CO_2 + H_2O$$

- **Distillation:** After digestion 100 ml water, 100ml 40% NaOH and glass blitz were added to Kjeldahl flask which containing about 10ml 2% boric acid and 2-3 drops mixed indicator. About 100ml distillate was culled just before the

distillation was stopped. The take in flask was moved so that the tip of the distilling tube was out the distillate. Some distillate was excerpted in this way to make sure the condenser tube was free from vestiges of ammonia.

Chemical reaction occur-

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

$$3NH_3 + H_3BO_3 \longrightarrow (NH_4)_3BO_3$$

- **Titration:** The ammonia culled was titrated with 0.1N HCl solution and titer value was recorded.

Chemical reaction occur-

$$(NH_4)_3BO_3 + 3HCl \longrightarrow NH_4Cl + H_3BO_3$$

The calculation of the percent of crude protein in the sample using protein factor 6.25.

$$\% \text{ Nitrogen} = \frac{(T_S - T_b) \times \text{Normality of acid} \times \text{meq.}N_2}{\text{Wt of sample (g)}} \times 100$$

Where,

Ts= Titer value of sample (ml)

T_b = Titer value of Blank (ml)

meq.N_2= 0.014

% Protein = % Nitrogen × 6.25

3.6.4 Determination of Crude Fat

The dried sample standing after moisture determination was dispelled to a thimble and plugged the top of the thimble with a wad of fat free cotton. The thimble was dropped into the fat extraction tube attached to a Soxhlet flask. Approximately 75ml or more of anhydrous ether was poured into a flask. The top of the fat extraction tube was linked to the condenser. The sample was extracted for 16 hrs or longer on a water bath at 70-80^oC. At the end point of the extraction period, the thimble was moved from the apparatus and distilled off most of the ether by allowing it or collected in Soxhlet tube. The ether was poured off during the tube was nearly full. When the ether reached a small quantity, it was poured into a

small, dry beaker through a small funnel involving a plug of cotton. The flask was rinsed and filtered thoroughly with using ether. The ether was evaporated on a steam bath at low heat; then it was dried at 100^{o}C for 1hr, cooled and weighed. The distinction in the weights gave the ether soluble components present in the sample.

The presence of crude fat was expressed as follows:

$$\% \text{ fat} = \frac{\text{Loss of ether soluble materials}}{\text{Wt of sample (g)}} \times 100$$

3.6.5 Determination of crude fiber

A moisture free and ether extracted sample of crude fiber formed of cellulose was first digested with dilute H_2SO_4 solution and then with dilute NaOH solution. The undigested residue culled after digestion was ignited and loss in weight after ignition was registered like crude fiber.

Reagents: Solution of H_2SO_4, NaOH, Acetone (foam suppresser)

Procedure:

1. Weighed 2g sample (W) weighed and shifted to porous crucible. Then placed the crucible into Dosi-fiber unit and kept the valve in "OFF" position.
2. After that added 150 ml of preheated H_2SO_4 solution and some drops of foam-suppresser to each column.
3. Then opened the cooling circuit and turned on the heating elements (power at 90%). When it started boiling, reduced the power at 30% and left it for 30 min.
4. Valves were opened for drainage of acid and rinsed with distilled water thrice to fully ensure the remove of acid from sample.
5. The same process was used for alkali digestion by using NaOH instead of H_2SO_4. Dried the sample in an oven at 105^{o}C for 24 hrs.
6. Then allowed the sample to cool in a Desiccator and weighed (W_1).
7. Kept the sample crucibles in muffle furnace at 600^{o}C for 3-4 hrs.

Calculations were done by using the formula:

$$\% \text{ Crude fiber} = \frac{W\text{-}W_1}{W_2} \times 100$$

3.6.6 Determination of Nitrogen Free Extract (NFE)

Nitrogen Free Extract (NFE) was calculated by difference after analysis of all the other items process in the proximate analysis.

NFE = 100 - (% moisture + % ash + % crude protein + % crude fat + % crude fiber)

3.6.7 Energy calculation

The percent calories in select samples were calculated by multiplying the percentage of carbohydrate and crude protein with 4 and crude fat with 9. The values were then converted to calories per 100gm of the sample.

3.7 Sample preparation for GC-MS analysis

Essential oil was mixed to 7% by chloroform. An inert gas (i.e. nitrogen) was initiated from a large gas cylinder through the injection part, the column and the detector. The flow rate of the carrier gas was synthesized to ensure reproducible retention time and to reduce detector dirt. Then the sample was injected by a micro syringe through a heated injection part when it was vaporized and steered into the column. The long tube of the column was tightly packed with solid atoms. The solid confirmation was uniformly covered with a thin film of a high boiling liquid (the stationary phase). The mobile and stationary phases were then partitioned by the samples and it was separated into the individual constituents. The carrier gas and sample constituent was then emerging from the column and passed through a detector. The amount of each constituent as concentration by the device and creates a signal which was registered electrically. The signal passed to a detector.

3.7.1 Determination of unknown compounds in clove oil by GC-MS

The analysis was carried out by GC-MS electron impact ionization (El) method on GC-17A gas chromatograph (Shimadzu, Japan) coupled to a GC/MS-QP 5050A Mass spectrometer (Shimadzu): fused silica capillary column 30m x 0.25mm i.d. coated with DB-1 (J & W), 0.25 µm film thickness; column temperature 40°C (2min) to 250°C at the rate of 5° C/min; injection port temperature 250°C, carrier gas (Helium) at constant pressure of 100 KPa. Flow rate 20 ml/min; acquisition parameters full scan; range 40-450 amu. Sample dissolved in n.hexane (Aziz et al., 2012).

3.8 Extract preparation for measuring antioxidant activity and bioactive compounds

For the preparation of methanoic extract, oil was mixed with absolute methanol and left for 72 hours with shaking. Again, for the ethanoic extract, oil was mixed with absolute ethanol and left for 72 hours with shaking. The filtrates were collected and stored at room temperature while the residues were re-extracted twice, each time with fresh solvent. Finally, centrifuged at 5000 rpm for 5 min. The supernatant was collected and the precipitate was extracted again. Supernatants were stored at 4°C in the refrigerator for further analysis (Leite-Legatti et al., 2012).

3.8.1 Determination of antioxidant activity by DPPH scavenging method:

Antioxidant mobility of the extracts was determined using DPPH assay as the process described by Azlim Almey et al. (2010) with slight modifications. About 6 mg of DPPH was dissolved in 100 mL absolute methanol and prepared methanoic DPPH solution. Then 1 ml methanoic extract was diluted with of 2 ml DPPH solution. Then the mixture was mildly shaken and left for 30 min in dark at room temperature. The absorbance was read at wavelength 517 nm using UV-VIS spectrophotometer (UV-2600, Shimadzu Corporation, USA). Control prepared by mixing 1 mL of methanol with 2 mL of DPPH solution whilst methanol was used like a blank. The scavenging mobility was measured as the decrease in absorbance of the samples in comparison with the DPPH standard solution.

Antioxidant capability based on the DPPH free radical scavenging mobility of extracts calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \text{ \% } 100$$

Trolox used as standard and TEAC composite (Trolox equivalent antioxidant mobility) was used for the calibration standard curve. The results were revealed in mg/ 100 g of Trolox equivalents per gram of powder on a dry weight (DW) base.

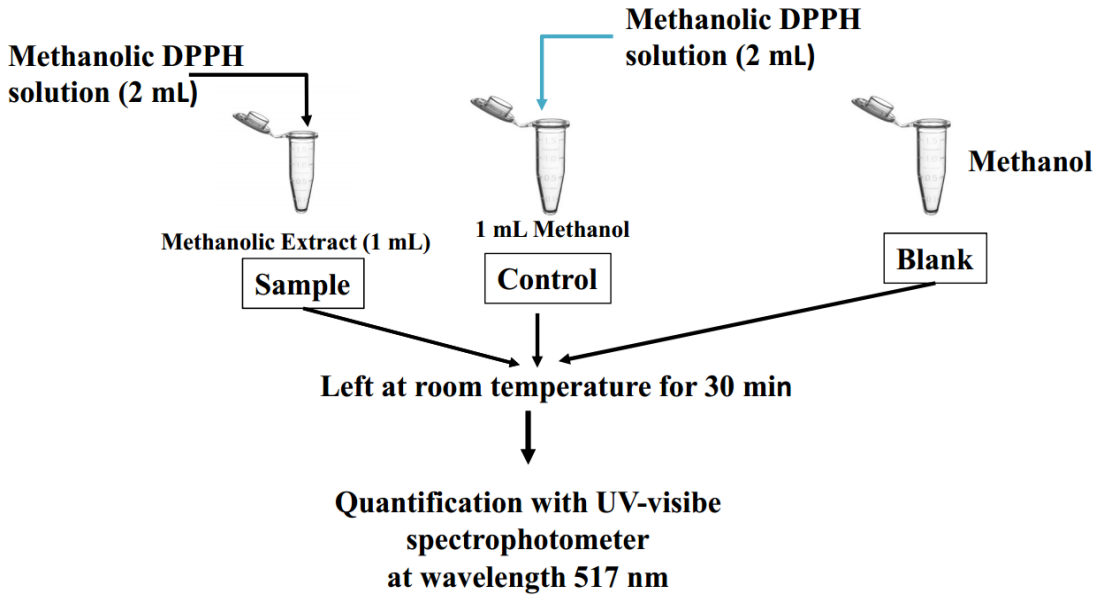


Figure 3.4: Antioxidant activity (AOA) determination procedure flow diagram

3.9 Bioactive Compounds

Bioactive compounds like total flavonoids content (TFC), total phenolic content (TPC) and total anthocyanin content (TAC) were also analyzed by UV-Visible spectrophotometer.

3.9.1 Determination of Total Flavonoid Contents (TFC)

Total flavonoids content (TFC) of the clove oil samples were determined by using the aluminum chloride colorimetric process expressed with slight modifications (Chang et al., 2002). Stock solution (1 mg/mL) of extracts was prepared and aliquots of 0.5 mL of diluted extract diluted with 1.5 mL of 95% C_2H_5OH in a cuvette. Then 0.1 mL of 10% $AlCl_3$, 0.1 ml of 1 mol/L potassium acetate and 2.8 mL of distilled water ($D.H_2O$) were added to the immixture in the cuvette. The immixture left at room temperature for 30 min. The absorbance was read at wavelength 415 nm in UV-visible spectrophotometer (UV-2600, Shimadzu Corporation, USA) and 10% aluminum chloride substituted with $D.H_2O$ of the same quantity were used as the blank. Total flavonoids amount in the sample was calculated by comparing absorbance of the sample extracts with a quercetin standard curve. TFC estimated and revealed as mg quercetin equivalents (QE) per gram of extract (mg QE/g).

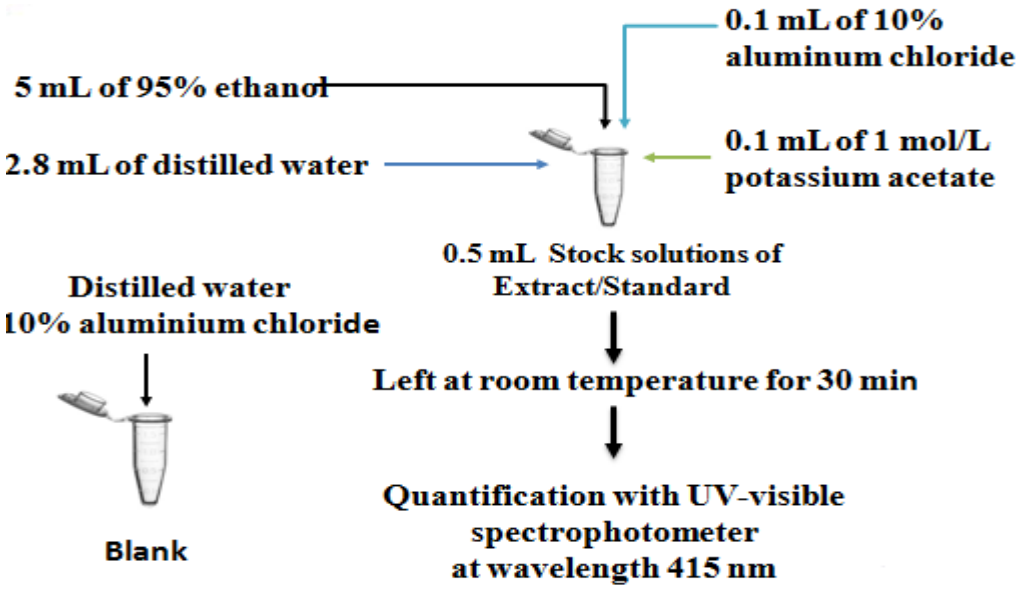


Figure 3.5: Total flavonoids content determination procedure flow diagram

3.9.2 Determination of total polyphenol contents (TPC)

Total polyphenol content (TPC) of the clove essential oil determined according to the Folin-Ciocalteu method expressed with slight modifications (Al-Owaisi et al., 2014, Parthasarathy et al., 2009). 1 ml ethanoic extract was taken in a falconer tube and added 1.5 ml of FC reagent and left for 3 mins at room temperature. Then 1.5 ml Na_2CO_3 (7.5%) was added into the mixture and left for 60 minutes. The absorbance was read at wavelength 765 nm using a UV-VIS Spectrophotometer (UV-2600, Shimadzu Corporation, USA) and C_2H_5OH was used as the blank. TPC was calculated and revealed as mg of gallic acid equivalents (GAE) per gram of extracts (mg GAE/g).

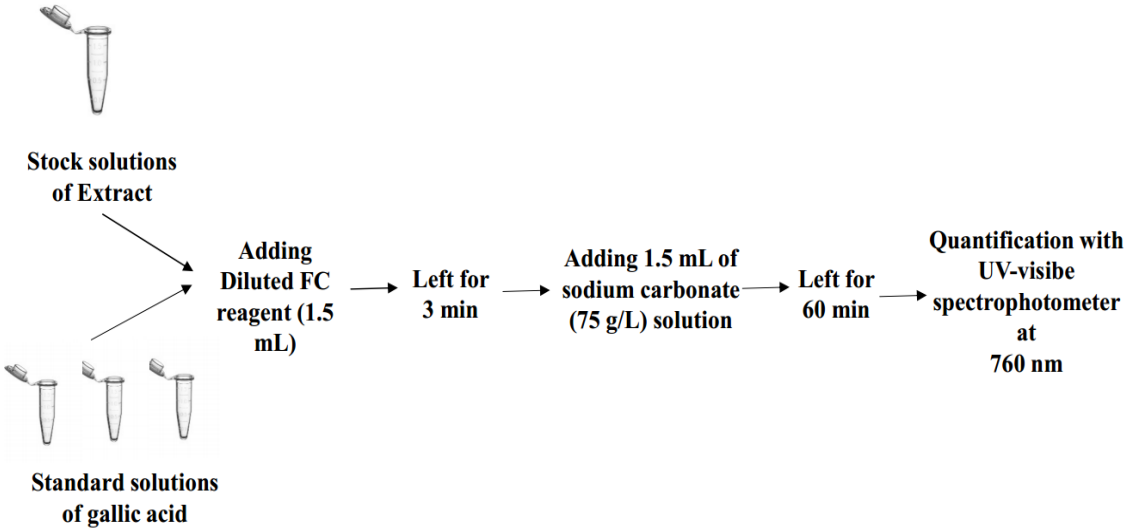


Figure 3.6: Total polyphenol content determination procedure flow diagram

3.9.3 Determination of Total Anthocyanin Contents (TAC)

Total anthocyanin content (TAC) of clove oil extracts will be determinate colorimetrically following the process expressed with slight modifications (Selim et al., 2008). About 3 mL of ethanoic extract was pippetted into a cuvette and predominance of color was measured at wavelength 520 nm using UV-visible spectrophotometer (UV-2600, Shimadzu Corporation, USA). Ethanol was used as a blank. TAC was calculated and expressed as mg per 100 g (mg/100 g) using the following equation:

TAC= Absorbance of sample×DF×100/m×E

Where,

DF = dilution factor

m = means the weight of sample

E = refers to extinction coefficient (55.9).

Procedure:

10 ml ethanoic stock solution was prepared. 3 ml extract solution was taken into cuvette. Quantification with UV-visible Spectrophotometer at wavelength 520.

3.10 Antimicrobial activity of three brands clove essential oil

The media used for antimicrobial assays were Mueller-Hinton agar for bacteria. All were incubated appropriately as specified for each organism (Aibinu et al., 2007). Labeled media plates were uniformly seeded with the different test microorganisms, by means of a sterile swab rolled in the suspension and streaked onto the agar's surfaces. Wells of 10mm in diameter were punched using a sterile cork borer. Into each well, 100 µl of the oil extract was dropped. Ciprofloxacin, diluted to a concentration of 0.005% (w/v) was dropped into each well to a volume of 100 µl.

3.10.1 Boiling Method (DNA Extraction):

It is essential to completely denature the template DNA at the beginning of PCR to ensure efficient utilization of the template during the first amplification cycle. A DNA denaturation time of 30 seconds per cycle at 95°C is normally sufficient. For GC-rich DNA templates, this step can be prolonged to 3-4 min (Ali Dashti et al., 2009; www. thermofisher.com).

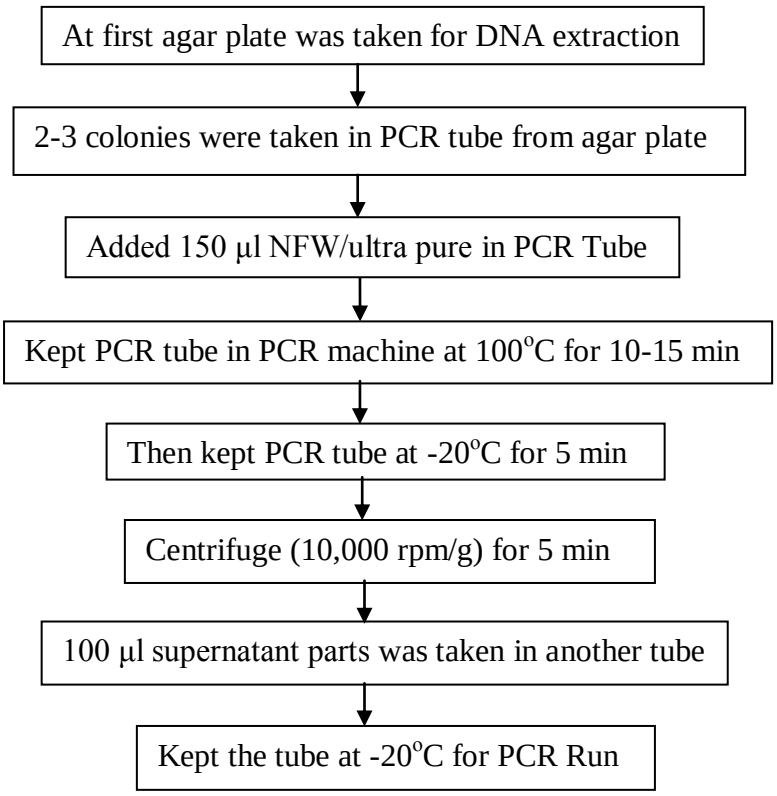


Figure 3.7: Procedure of Boiling Method (DNA Extraction)

3.10.2 Identification of *E. coli* /Molecular confirmation of *E. coli*

All culture positive *E. coli* isolates were further topic PCR using *E. coli* specific primers are uspA Up, uspA Down, uidA Up, uidA Down described by Godambe et al., 2017. Extracted bacterial DNA were preliminarily heated at 94°C for 5 min and sequentially amplified for 30 cycles where each cycle followed 94°C for 2 min, 70°C for 1 min and 72°C for 1 min. At last, the PCR products were then run in 1% agarose gel with ethidium bromide, incorporating 100 bp size markers and visualized under UV light. All positive *E. coli* (cultural test) isolates outcome expected bands at 585 bp regions assuring the isolates to be *E. coli.* The results found from PCR assay for *E. coli* is observed in gel electrophoresis.

3.10.3 Molecular confirmation of *Staphylococcus aureus*

Previously identified *Staphylococcus* isolates were further sub-cultured on blood agar and bacterial genomic DNA was extracted by using boiling lysis process expressed by Millar et al., 2000. At last, *Staphylococcus* isolates was assured by the PCR amplification of *nuc* (a thermonuclease gene characteristic of *S. aureus*) gene using following primers: au-F3 (Forward) 5' TCGCTTGCTATGATTGTGG 3' and au-nucR

(Reverse) 5' GCCAATGTTCTACCATAGC 3'. The PCR amplification situation were primary denaturation for 2 minutes at 95°C, followed by 30 cycles for 30s at 95°C, 35s at 56°C, and 60 s 72°C and final extent at 72°C for 2 minutes expressed by Sasaki et al. Previous known isolate was used as *nuc*-positive control and nuclease free water was used as negative control.

3.10.4 Molecular confirmation of *Pseudomonus aeruginosa*

Previously identified *Pseudomonus aeruginosa* isolates were further sub-cultured on blood agar and bacterial genomic DNA was extracted by using boiling lysis method.

3.10.5 Preparation of different media

3.10.5.1 Blood Base Agar (BBA) Media

Four grams of base was added to 100 ml of distilled water in a flask and heated until boiling to dissolve the medium fully. Then the medium was sterilized by autoclaving. The autoclaved materials were allowed to cool to a temperature of 45^{o}C in a water bath. Defibrinated 5% cattle blood was added to the medium sterilized and distributed to sterile petridishes and then allowed to cool at room temperature for solidification. After solidification of the medium, the plates were allowed to incubate at 37^{o}C for overnight to check their sterility and stored at 4^{o}C in the refrigerator until used.

3.10.5.2 Mueller-Hinton Agar (MHA) Media

In 200 ml of distilled water 7.6 grams powder of Mueller-Hinton agar base was added. The mixture was heated to boil for 5 minutes to dissolve all the powder fully with water. Then the medium was autoclaved for 30 minutes to make it sterile. After autoclaving the mixture was put into water bath setting temperature 50°C to cool down its temperature and allow the media not to solidify. From water bath 10-20 ml of medium was poured into medium sized sterile petridishes under safety hood or biological safety cabinet to make sterile nutrient agar plates. After solidifying the medium in the plates, the plates were then allowed to incubate at 37°C for overnight to cheek their sterility.

3.10.6 Determination of antimicrobial activity of clove essential oil

A primary antimycobacterial screening was performed using the disc diffusion technique according to National Committee for Clinical Laboratory Standards. Briefly, Petri dishes containing mueller-hinton agar culture medium were inoculated with previously prepared mycobacterial inoculums. The discs (filter paper, 6 mm of diameter) placed in the center of each plate were impregnated with 100 µl of each essential oil. Petri dishes were placed at 4^{o}C for 2 h to allow a better diffusion of molecules and then incubated at 37^{o}C for 24 h. The antimycobacterial mobility was evaluated by measuring the diameter of prohibition zone in mm. All experiments were condiment in triplicates. After confirmation of isolates as *E. coli,* antimicrobial susceptibility of the isolates were determined by using the micro disc diffusion technique, and the technique was used according to guidelines founded by Clinical and Laboratory Standards Institute (CLSI), 2010.

3.10.7 Culture Sensitive test at Muller Hinton agar

After confirmation of isolates as *E. coli,* antimicrobial susceptibility of the isolates were determined by using the micro disc diffusion technique, and the technique was used according to guidelines founded by Clinical and Laboratory Standards Institute (CLSI), 2010.

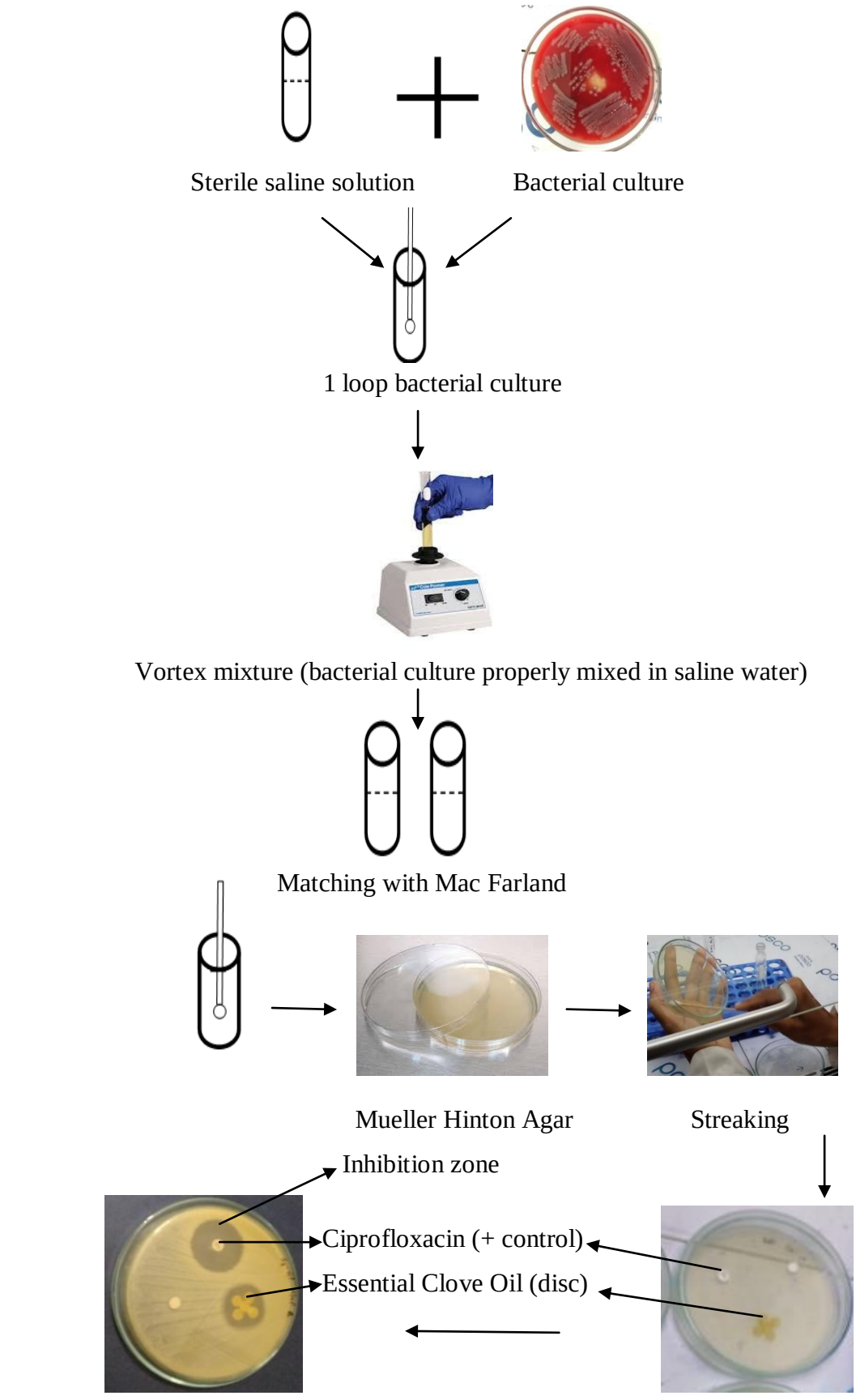


Figure 3.8: CS test at Muller Hinton agar procedure flow diagram

3.11 Statistical Analysis

The obtained data were stored in Microsoft Excel 2013 spread sheet to evaluate statistical analysis and then significant differences were determined by one-way analysis of variance (ANOVA). All samples were in three replicates. Descriptive analysis was performed by using mean and standard deviation for different variables. Data were sorted, coded and recorded in IBM SPSS Statistics 25. After that statistical analysis were conducted. Finally, one-way ANOVA and post-hoc test were used to compare the obtained data of three brands clove and clove essential oil. The statistical analysis was conducted for at 5% level of significant ($p \leq 0.05$).

Chapter-4

Results

In this chapter result of the study are given under the following headings. The sample of cloves of Indonesia, India, and Sri Lanka marked out as CIs, CId and CSl respectively. Dry clove powder and clove essential oil marked out as DCP and CEO respectively.

4.1 Proximate composition analysis:

The proximate chemical composition of the dried clove buds is shown in Table 4.1.The contents of moisture, ash, protein, fat, crude fiber, carbohydrates (CHO) and energy of clove bud (CIs) were: 12.23±0.01, 4.88±0.01, 4.21±0.01, 9.82±0.1, 16.694±0.01 and 52.12±0.314% and 313.71Kcal/g respectively.

Table 4.1: Proximate composition of three brands clove

Sample ID	% Moisture	% Ash	% Protein	% Fat	% Fiber	% CHO	Energy (Kcal/g)
CIs	12.287 ± 0.01^{b}	4.877 ± 0.01^{c}	4.213 ±0.01^{b}	9.817 ±0.01^{c}	16.694 ±0.01^{a}	52.210 ±0.01^{a}	313.71 ±0.01^{b}
CId	13.653 ± 0.01^{a}	5.177 ±0.01^{b}	4.127 ±0.01^{c}	10.187 ±0.01^{b}	15.821 ±0.01^{c}	51.53 ±0.01^{c}	312.35 ±0.01^{c}
CSl	11.553 ± 0.01^{c}	5.253 ±0.01^{a}	4.557 ±0.01^{a}	11.187 ±0.01^{a}	15.875 ±0.01^{b}	51.587 ±0.01^{b}	325.23 ±0.01^{a}

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a column are significantly different (P<0.05).

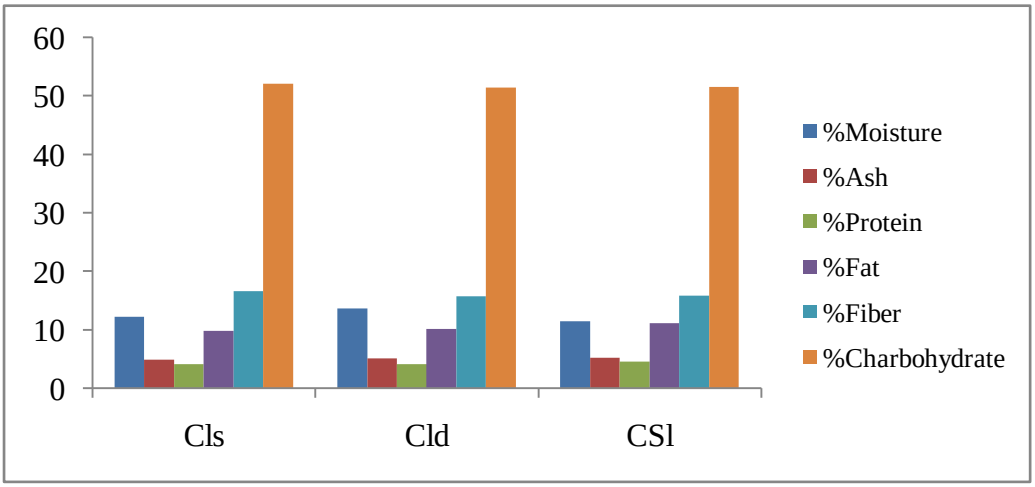


Figure 4.1: Proximate composition of three brands clove

4.2 Physicochemical properties of clove essential oil

The physicochemical properties of clove bud oil is presented in Table 4.2 the Acid value, Saponification value, Peroxide value, Iodine value of clove essential oil (CIs) were found to be 5.21, 37.26, 4.61, 51.46 respectively. The Density, Viscosity and refractive index of clove essential oil (CIs) were 1.021, 10.33 at 25℃ and 1.549 at 30℃ respectively (Table- 4.3, 4.4, 4.5).

Table 4.2: Physicochemical properties of three brands clove essential oil

Sample ID (n = 3)	Acid value (mg KOH /g oil)	Saponification value (mg /g oil)	Peroxide value (meq O_2/kg oil)	Iodine value (g I_2/100 g of oil)
CIs	5.213 ± 0.01^{c}	37.257 ± 0.01^{c}	4.610 ± 0.01^{c}	51.457 ± 0.01^{a}
CId	6.087 ± 0.01^{b}	40.670 ± 0.01^{a}	6.667 ± 0.01^{a}	49.507 ± 0.01^{c}
CSl	6.457 ± 0.01^{a}	38.056 ± 0.01^{b}	5.127 ± 0.01^{b}	50.157 ± 0.01^{b}

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a column are significantly different (P<0.05).

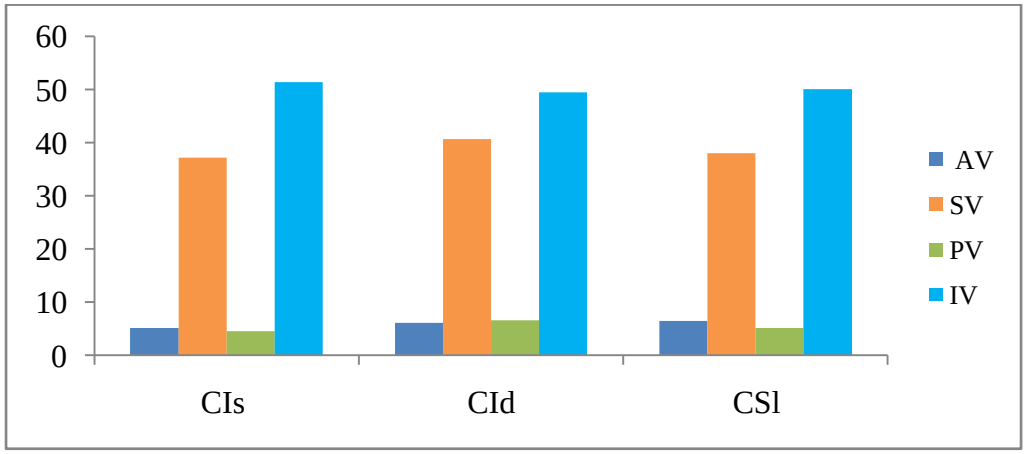


Figure 4.2: Physicochemical properties of three brands clove essential oil

Table 4.3: Density of three brands clove essential oil

Sample Id / Temp. in °C	CIs	CId	CSl
25	1.0213 ± 0.0005^{c}	1.0347 ± 0.0005^{a}	1.0337 ± 0.0005^{ab}
30	1.0173 ± 0.0005^{c}	1.0301 ± 0.0005^{a}	1.0295 ± 0.000^{b}
35	1.0127 ± 0.0005^{c}	1.0257 ± 0.0005^{a}	1.0253 ± 0.0005^{ab}
40	1.0083 ± 0.0005^{c}	1.0213 ± 0.0005^{a}	1.0208 ± 0.0005^{ab}
45	1.0037 ± 0.0005^{c}	1.0167 ± 0.0005^{a}	1.0167 ± 0.0005^{ab}
50	0.9990 ± 0.00^{c}	1.0127 ± 0.005^{a}	1.0123 ± 0.057^{ab}

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a row are significantly different (P<0.05).

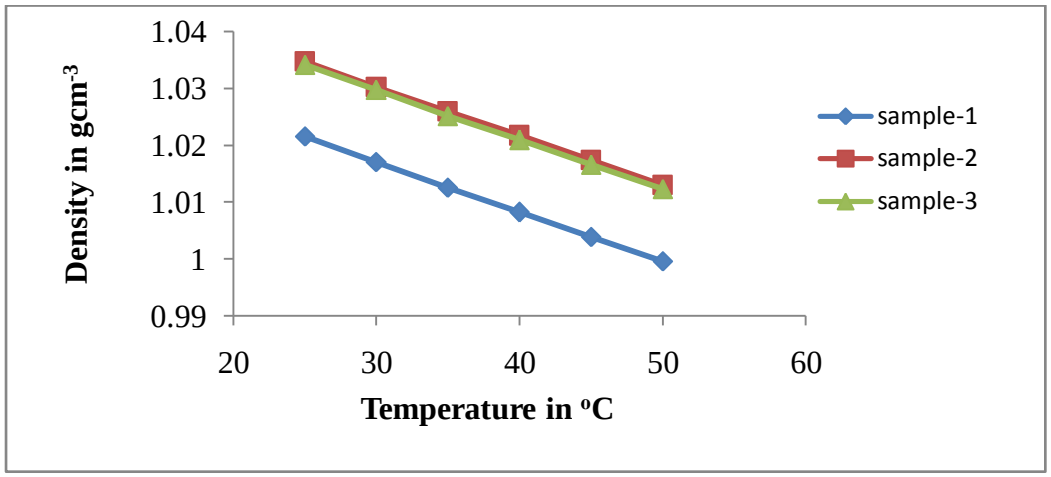


Figure 4.3: Comparative study of density of three samples at different temperatures

Table 4.4: Viscosity of three brands clove essential oil

Sample Id / Temp. in °C	CIs	CId	CSl
25	10.328±0.0005^{c}	14.437±0.0005^{b}	16.353±0.0005^{a}
30	8.3636±0.0005^{c}	11.349±0.0005^{b}	12.948±0.0005^{a}
35	6.6663±0.0005^{c}	9.1253±0.0011^{b}	10.532±0.0005^{a}
40	5.6347±0.0005^{c}	7.5613±0.0005^{b}	8.6753±0.0005^{a}
45	4.8233±0.0005^{c}	6.3607±0.0005^{b}	7.3167±0.0005^{a}
50	4.1783±0.0005^{c}	5.4427±0.0005^{b}	6.2663±0.0005^{a}

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a row are significantly different (P<0.05).

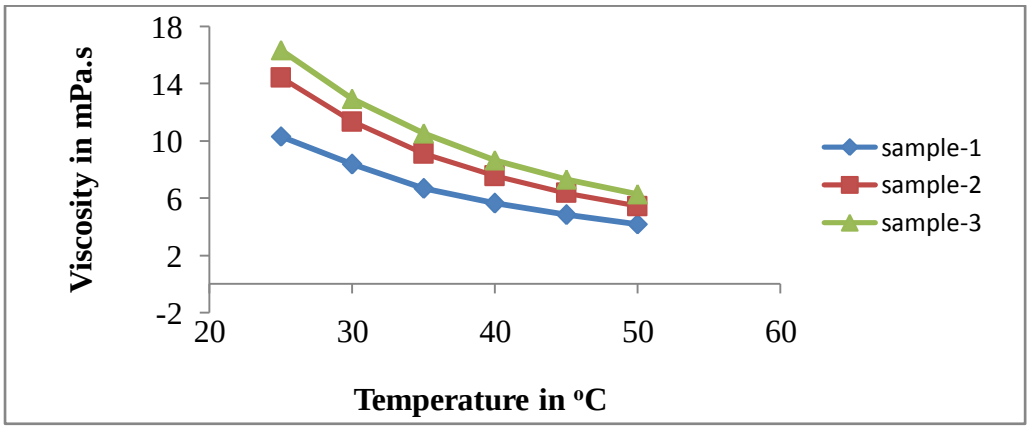


Figure 4.4: Comparative study of coefficient of viscosity (η) of three brands CEO at different temperatures

Table 4.5: Refractive Index of three brands clove essential oil

Sample Id / Temp in ^{o}C	CIs	CId	CSl
30	1.54886±0.0005^{c}	1.55049±0.0005^{a}	1.55046±0.0005^{ab}
40	1.54486±0.0005^{c}	1.5457±0.0005^{b}	1.5465±0.0005^{a}
50	1.5413±0.0005^{bc}	1.5417±0.0005^{b}	1.5427±0.0005^{a}

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a row are significantly different (P<0.05).

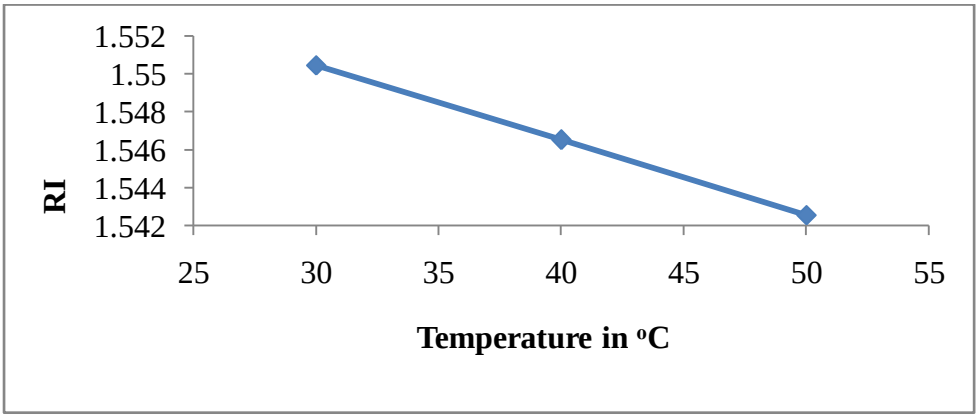


Figure 4.5: Refractive Index of CSl Sample at different temperatures

4.3 Phytocomponents/volatile compounds of the Clove essential oil

Phytocomponents identification were done by comparing the NIST library data of the peaks with those reported in literature, mass spectra of the peaks with literature data. Eugenol is the main components in cloves oil. Eugenol, Eugenol acetate, Caryophyllene, Alpha-picoline, Chavicol, Aniline, Copaene, Ylangene etc. are shown in table 4.6. The high concentration of eugenol in clove buds oil makes it potentially useful in the medicines because these exhibit antibacterial, antifungal, anti-inflammatory activity, insecticidal and antioxidant properties. These are used traditionally as flavouring agent and antimicrobial material in food (Velluti et al., 2003). Among 3 samples the potential activity of Indian clove essential oil sample was higher than other 2 samples.

Table 4.6: Phytocomponents identified in clove essential oils

Sample ID	Phytocomponents name	Chemical formula	MW	Ret. Index
CIs	1. 2,5-Diethylideneoctahydropentalene	$C_{12}H_{18}$	162	1229
	2. 3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	$C_{10}H_{16}N_2$	164	0
	3. P-Picoline (Methylpyridine)	C_6H_7N	93	787
	4. Alpha-picoline (Methylpyridine)	C_6H_7N	93	787
	5. Aniline (Phenylamine)	C_6H_7N	93	992
	6. Chavicol (Hydroxyallylbenzene)	$C_9H_{10}O$	134	1203
CId	7. Trans-Z-alpha. Bisabolene epoxide	$C_{15}H_{24}O$	220	1531
	8. Spiro[androst- 5-ene-17,1cyclobutan] -2'-one,3-hydroxy-,(3β, 17β)-	$C_{22}H_{32}O_2$	328	2413
	9. Caryophyllene oxide	$C_{15}H_{24}O$	220	1507
	10. 1, 7, 7-Trimethylbicyclo [2.2.l] heptane-2, 5-diol	$C_{10}H_{18}O_2$	170	1326
	11. Eugenol (2-methoxy-4-(2-propenyl)	$C_{10}H_{12}O_2$	164	1392
	12. Eugenol (2-methoxy-4-(2-propenyl)	$C_{10}H_{12}O_2$	164	1392
	13. Phenol, 2-rnethoxy-3-(2-propenyl)-	$C_{10}H_{12}O_2$	164	1392
	14. Phenol, 2-Allyl-4-propenyl	$C_{10}H_{12}O_2$	164	1410
	15. Copaene	$C_{15}H_{24}$	204	1221
	16. Ylangene	$C_{15}H_{24}$	204	1221
CSl	17. Ethanone,1-(2,3,4-Trimethoxyphenyl)	$C_{11}H_{14}O_4$	210	1596
	18. 4-(2,6,6-Trimethyl-1-cyclohexen-l-yl) butanoic acid	$C_{13}H_{22}O_2$	210	1670
	19. 2-Arnino-5-ethyl-3-nitro-benzoic acid	$C_9H_{10}N_2O_4$	210	2070
	20. Eugenol acetate	$C_{12}H_{14}O_3$	206	1552
	21. Caryophyllic acid	$C_{10}H_{12}O_2$	164	1392
	22. 3-Allyl-6-methoxyphenyl acetate	$C_{12}H_{14}O_3$	206	1552

4.4 Antioxidant Activity:

The antioxidant activity of three brands clove essential oil were found 70.66 ppm, 70.14 ppm, 69.74 ppm of the CIs, CId and CSl clove essential oil respectively which shown in Table 4.7. The concentration of these 3 clove essential oil (CEO) samples was close to one another.

Table 4.7: Concentration and absorbance of Standard solution for AOA

Sample ID	Type	Ex	Conc. (ppm)	WL 517. 0	Wgt. Factor
1	Std1	Standard	0.500	0.272	1.000
2	Std2	Standard	1.000	0.221	1.000
3	Std3	Standard	1.500	0.185	1.000
4	Std4	Standard	2.000	0.133	1.000
5	Std5	Standard	2.500	0.092	1.000

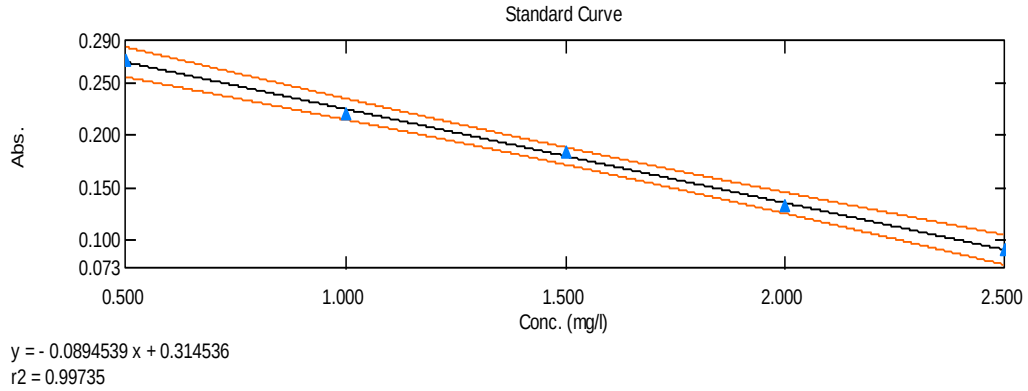


Figure 4.6: Standard curve (Trolox) for antioxidant capacity determination test

Table 4.8: Concentration and absorbance of sample solution for AOA

Sample ID	Type	Conc. (ppm)	WL 517. 0	Wgt. Factor
CIs	EAIs	70.66 ± 0.01^{a}	0.001	1.000
CId	EAI_d	70.14 ± 0.01^{b}	0.002	1.000
CSl	EAS	69.74 ± 0.01^{c}	0.003	1.000

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a column are significantly different (P<0.05).

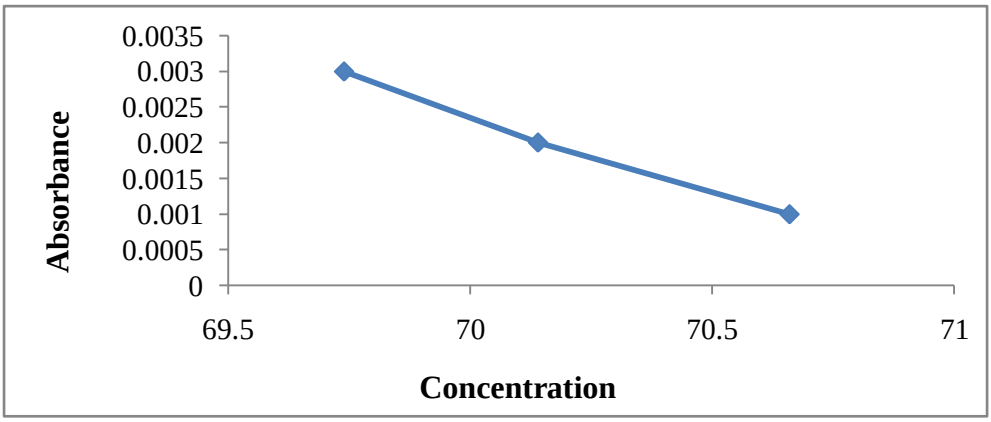


Figure 4.7: Absorbance vs. concentration curve of Sample solution for AOA

4.5 Bioactive Compound:

Results of total flavonoids, total phenolics and total anthocyanin contents of three brands clove essential oil were listed Table-4.9. Among these 3 samples the Flavonoid content of Sri Lankan clove essential oil (CEO) sample was highest. Phenolic content of Indonesian CEO sample was higher than other 2 samples. Among these 3 samples the Anthocyanin content of Indian CEO sample was highest.

Table 4.9: Concentration & absorbance of sample Solution for bioactive compounds

Sample ID	Type	TFC		TPC		TAC		Wt Factor
		Conc. (ppm)	WL 415nm	Conc. (ppm)	WL 760nm	Conc. (ppm)	WL 520nm	
CIs	EAIs	244.36±0.01^{b}	1.603	159.44±0.01^{a}	1.725	16.071±0.01^{c}	0.233	1.000
CId	EAId	161.13±0.01^{c}	1.057	142.82±0.01^{b}	1.854	18.156±0.01^{a}	0.338	1.000
CSl	EAS	321.95±0.01^{a}	2.112	135.11±0.01^{c}	1.912	17.942±0.01^{b}	0.326	1.000

n=3, All values are means ±SD. a-c means not sharing a common superscript letter with in a column are significantly different (P<0.05).

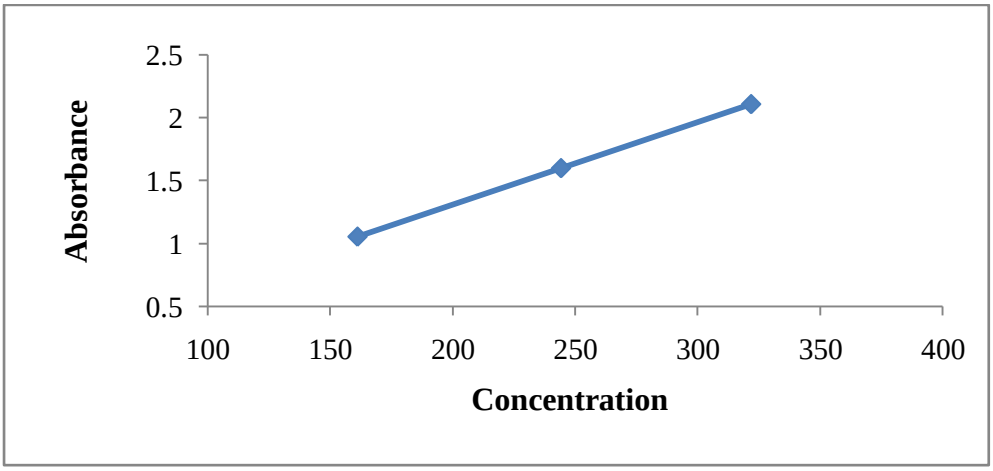


Figure 4.8: Absorbance vs. concentration curve of Sample solution for TFC

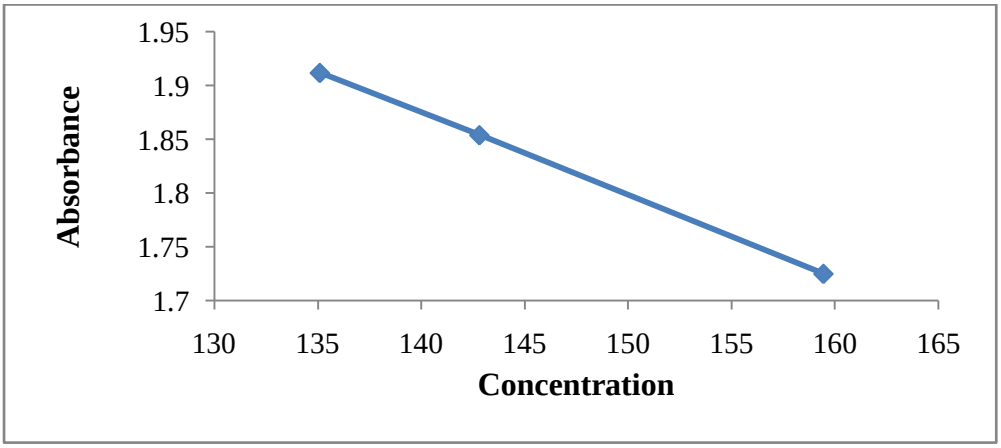


Figure 4.9: Absorbance vs. concentration curve of Sample solution for TPC

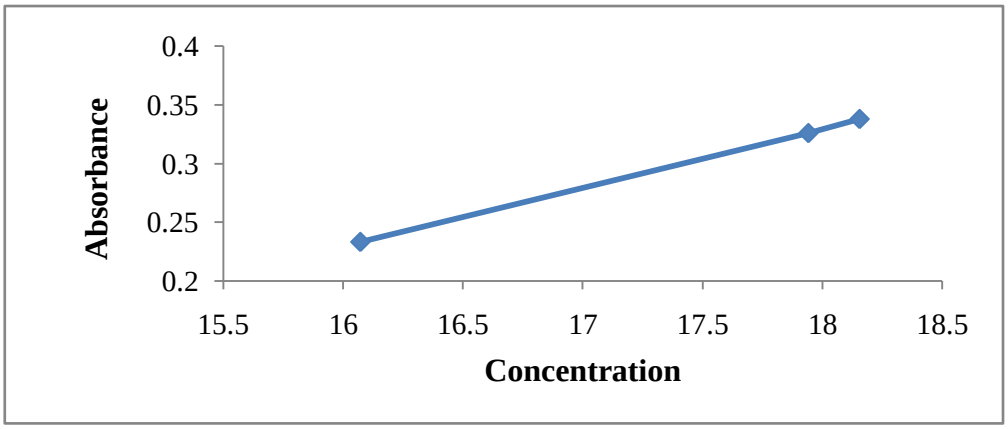


Figure 4.10: Absorbance vs. concentration curve of Sample solution for TAC

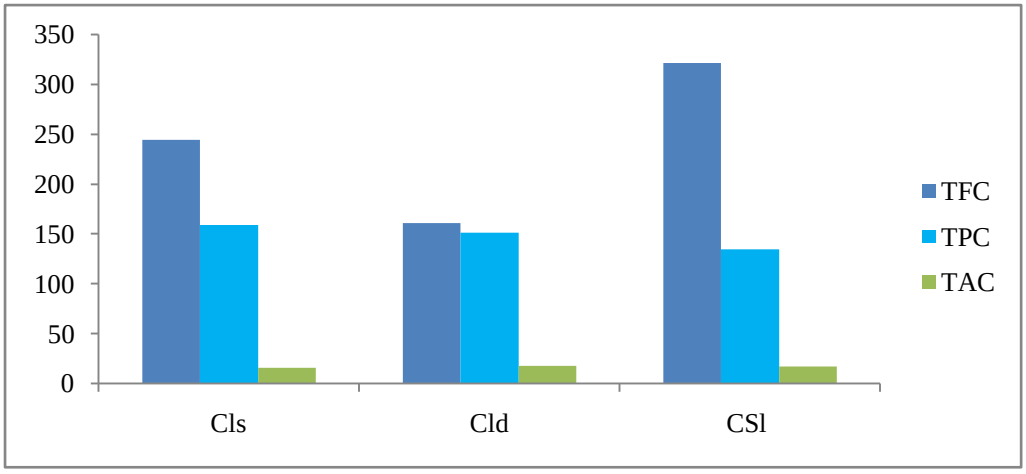


Figure 4.11: Bioactive Compounds of CIs, CId & CSl sample at different concentrations

4.6. Antimicrobial Activity:

4.6.1 Identification of *E. coli*

The PCR products were run in 1% agarose gel with ethidium bromide, incorporating 100 bp size markers and visualized under UV light. All positive *E. coli* (cultural test) isolates outcome expected bands at 585 bp regions assuring the isolates to be *E. coli.* The results obtained from PCR assay for *E. coli* is observed in gel electrophoresis.

Table 4.10: Zone of inhibition (mm) showing the antimicrobial activity of CEO

Test Organism: Gram +ve Bacteria: *Staphylococcus aureus*

Sample ID	COE (µg/ml)	Essential oil	(+) control: Ciprofloxacin	(-) control: SSS + FP
S_1	100	26 mm	30 mm	Nz
S_2	100	25 mm	28 mm	Nz
S_3	100	24 mm	29 mm	Nz

Test Organism: Gram -ve Bacteria: *Escherichia coli*

Sample ID	COE (µg/ml)	Essential oil	(+) control: Ciprofloxacin	(-) control: SSS + FP
S_1	100	25 mm	Nz	Nz
S_2	100	24 mm	Nz	Nz
S_3	100	27 mm	Nz	Nz

Test Organism: Gram -ve Bacteria: *Pseudomonus aeruginosa*

Sample ID	COE (µg/ml)	Essential oil	(+) control: Ciprofloxacin	(-) control: SSS + FP
S_1	100	Nz	36	Nz
S_2	100	Nz	27	Nz
S_3	100	Nz	33	Nz

Key: Clove Oil Extract (COE), sterile saline solution (SSS), Fiter paper (FP), No Zone of inhibition (Nz).

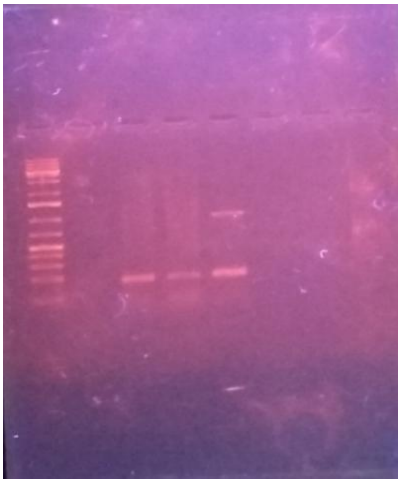


Figure 4.12: Identification of *E. coli*

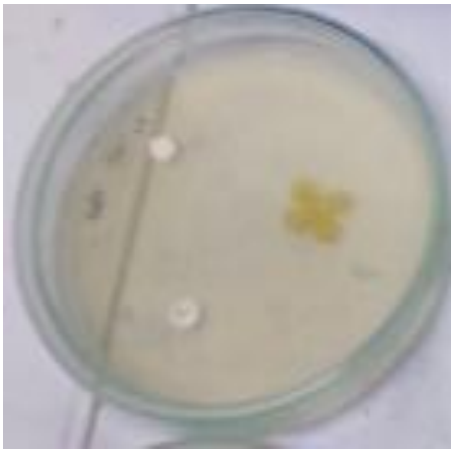


Figure 4.13: CST of clove essential oil on M-H Agar

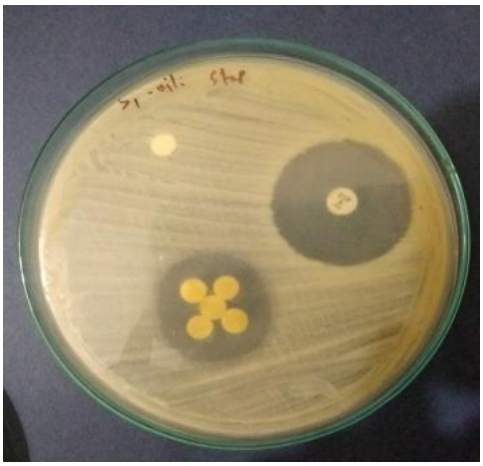


Figure 4.14: CST of *Staph. aureus* isolate by DDM for CEO (CIs)

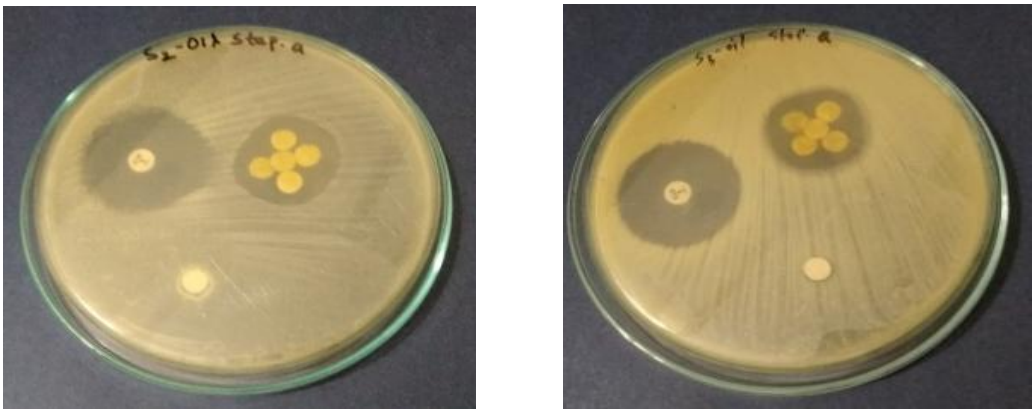


Figure 4.15: CST of *Staph. aureus* isolate by disc diffusion method (DDM) for CEO (CId & CSl)

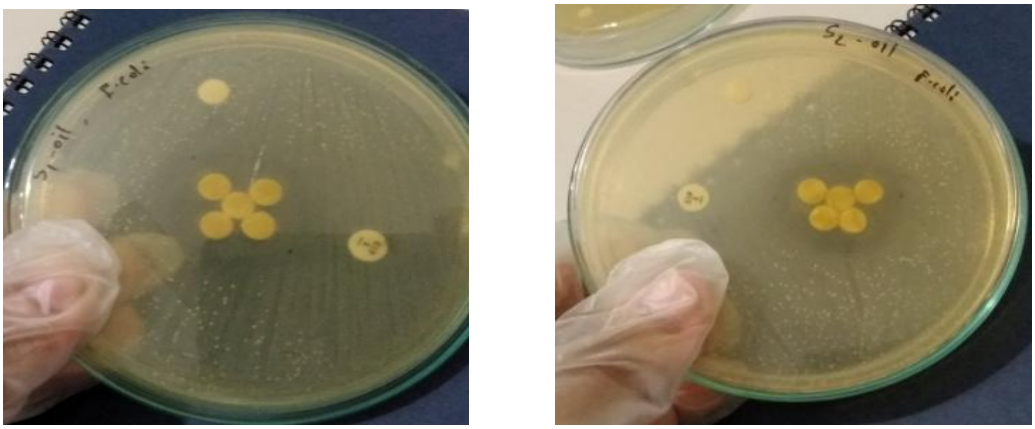


Figure 4.16: CST of *E. coli* isolate by disc diffusion method for CEO (CIs & CId)

Fig. 4.17: CST of *E. coli* isolate by DDM for CEO (CSl)

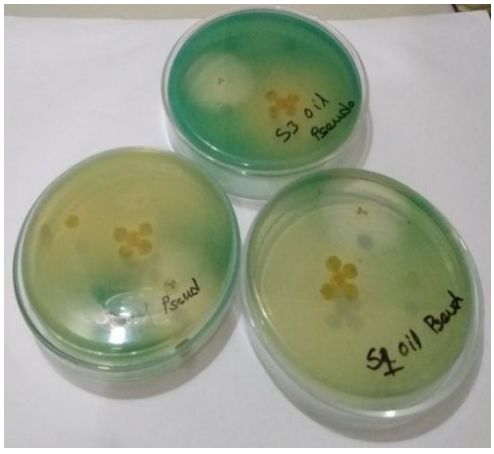


Fig. 4.18: CST of *P. aeruginosa* isolate by DDM for CEO

Chapter-5

Discussion

5.1 Proximate analysis of three brands clove

In this study the moisture contents were 12.287%, 13.653%, 11.553% of Indonesian (CIs), Indian (CId) and Sri Lankan (CSl) clove respectively which are more than 10% (Adom, Sorrells, and Liu; 2005). Moisture quantity of foods is influenced by type, diversity and storage condition. The low moisture content of clove powder would enhance its storage stability by avoiding bacterial growth and extend the shelf life of the final product.

The ash content of the clove powder exhibited 4.89%, 5.18%, 5.25% of the CIs, CId, and CSl respectively (Table 4.1). The ash content of clove powder was similar in accordance with this reported for clove buds by many investigators (Abdel Moneim et al; 2007). Ash content is an indication of nutritional content of a food.

The crude protein content of the cloves powder sample were found 4.21%, 4.13%, 4.56% of the CIs, CId and CSl respectively (Table 4.1). In this study the crude protein content of cloves powder were higher than the crude protein content of 1.20% clove (Abdel Moneim et al; 2007).

The crude fat content of cloves powder was found 9.82%, 10.18%, 11.19% of the CIs, CId and CSl respectively. In this study the fat content of the cloves powder were found lower than 12.1% cloves powder reported in (Abdel Moneim et al; 2007). Diets with high fat amount contribute effectively to the energy need for humans. High fat acts as good flavor enhancers and useful in improving palatability of foods.

The crude fiber content of brown clove powder 16.69%, 15.82%, 15.88% of the CIs, CId and CSl respectively. The crude fiber content of brown cloves was lower than 20% crude fiber of cloves powder reported in (Abdel Moneim et al; 2007). Crude fiber helps in the prevention of diabetes, colon cancer, toothache, heart diseases and muscle pain etc. Clove would be a better source of fiber content. Therefore, it will be useful if clove is added to meal diet and used in food formulation to help relieve constipation.

The utilizable carbohydrate content of the cloves powder were obtained 52.21%, 51.53%, 52.587%, of the CIs, CId and CSl respectively which was higher than that of 51.5% for clove; reported by Abdel Moneim et al (2007). Carbohydrates are good sources of energy and that a high concentration of this is desirable in meals.

The high proportions of carbohydrate, protein were noticeable characteristics of the clove. The high protein content (4.56%) of cloves bud may possess positive effect in human diet. But some asymmetry was also obtained due to some environmental factors such temperature, rate of photosynthesis, heat etc. influencing the carbohydrate and protein level of cloves (Prejeena, 2003; Sumesh, 2004; Shujari et al., 2005).

5.2 Physicochemical properties of three brands clove essential oil

The acid value (AV) is an important determinant of oil quality (Gharby et al., 2015). It is used to measure the extent to which glycerides in the oil has been decomposed by the lipase and other physical factor such as light and heat (Hilditch, 1949). The acid value was found 5.213 mg KOH/g oil, 6.09 mg KOH/g oil and 6.457 mg KOH/g oil of the CIs, CId, and CSl clove essential oil respectively. In this study acid value of all cloves essential oil samples were higher than the maximum limit. Low acid value is an indication of freshness and suitability for oil for edible purpose while high acid value indicate the rancidity which is caused by hydrolysis of ester link and oxidation of double bonds of triglycerides.

The saponification value (SV) describes the average molecular weight of the fatty acids in the lipids (Zahir et al., 2014). The saponification value was found 37.257 mg/g oil, 40.670 mg/g oil and 38.056 mg/g oil of the CIs, CId and CSl clove essential oil respectively. The SV of cloves was higher than 1.12mg KOH/g SV of clove essential oil (CEO) reported in (Ajoy, 2014). In this study saponification value of CId clove essential oil samples was higher than the other two samples.

Peroxide value (PV) is used as a dimension of the extent to which rancidity reactions have ensued during storage. Peroxide value is used as an indication of the stability and quality of oils and fats (Ekwu and Nwagu, 2004). Peroxide value reflected the formation of hydro peroxides which due to the primary oxidation. It may break down into nonvolatile secondary product, which decreases the quality of oil. The Peroxide

value (PV) was found, 4.61 meq O_2/kg oil 6.667 meq O_2/kg oil and 5.127 meq O_2/kg oil of the CIs, CId and CSl clove essential oil respectively. In this study PV of CIs clove essential oil samples was lower than other two samples.

The determination of iodine is effective as a measure of the degree of unsaturation of the oils. The Iodine value was found, 51.457 g I_2/100 g of oil, 49.507 g I_2/100 g of oil, 50.157 g I_2/100 g of oil of the CIs, CId and CSl clove essential oil respectively. The iodine value (IV) of clove essential oil was higher than 2.28 g/100g IV of CEO reported by Ajay (2004). In this study iodine value (IV) of CIs clove essential oil samples was lower than other two samples. The highest iodine value of CIs clove essential oil samples was found and the lowest iodine value of CId clove essential oil samples was found.

Saponification Value (40.670 ± 0.01^a) and Peroxide Value (6.667 ± 0.01^a) are the highest values of CId sample. Iodine Value (51.457 ± 0.01^a) is the highest value of CIs sample. Acid Value (6.457 ± 0.01^a) is the highest value of CSl sample.

Density of a liquid is the mass of unit volume of the liquid. An increase in temperature of a liquid slightly increases the volume of the liquid, thus decreasing its density somewhat. The temperature increase brings about an increase in molecular velocity. These energetically molecules then fly aside causing extra holes in the bulk of the liquid. This causes the expansion of the liquid, thereby decreasing the number of molecules per unit volume or the density.

Density (ρ) for three brands cloves essential oil are measured at six different temperatures between 25°C and 50°C maintaining 5°C interval. All the results of density (ρ) were listed in Table 4.3. Also, the comparative study of density (ρ) as a function of temperature is graphically represented by the Fig. 4.3 for CIs, CId and CSl sample at different temperatures.

The following are the observations regarding density (ρ):

a) At a particular temperature, ρ of the cloves essential oil decreases in the order: CId > CSl > CIs
b) The ρ vs. T curves for three brands cloves essential oil follow a similar trend - ρ decreasing almost linearly with the temperature.

c) Effect of temperature on ρ for the cloves essential oil is quite large, and as usual the more the temperature the less is the density.

All liquids possess certain resistance to flow, known as their viscosities. In general, viscosity is the property that opposes the relative motion of the adjacent layers of the liquid and can consequently be regarded as a type of internal function. The viscosity of a liquid generally decreases with the increase of temperature, i.e., a liquid become freer moving at higher temperature.

Viscosity (η) for three brands cloves essential oil are measured at six different temperatures between 25^{o}C and 50^{o}C maintaining 5^{o}C interval. All the results of viscosity (η) were listed in Table 4.4. Also, the comparative study of viscosity (η) as a function of temperature is graphically represented by the Fig. 4.4 for CIs, CId and CSl sample at different temperatures.

The following are the observations regarding viscosity (η):

a) At a particular temperature, η of the cloves essential oil decreases in the order: CSl > CId > CIs
b) The η vs. T curves for three brands cloves essential oil follow a similar trend - η decreasing slowly with the temperature.
c) Effect of temperature on η for the cloves essential oil is quite large, and as usual the more the temperature the less is the viscosity.

The velocity of light in a vacuum is always the same, but when light moves through any other medium, it travels more slowly since it is constantly being absorbed and re-emitted by the atoms in the material. Refractive Index (n_D) is the characteristic property of a liquid and varies with temperature and wavelength of the light used. It is defined as the ratio of the velocity of light in vacuum or air to that in the substance.

Refractive indices (n_D) for three brands cloves essential oil are measured at T = 30^{o}C, 40^{o}C & 50^{o}C. All the results of refractive indices (n_D) were listed in Table 4.5. Also, the values of refractive indices (n_D) as a function of temperature is graphically represented by the Fig. 4.5 for CSl sample at different temperatures.

The following are the observations regarding refractive indices (n_D):

a) At a particular temperature, n_D of the three brands cloves essential oil decreased in the order: CSl > CId > CIs.
b) The n_D vs. T curves for three brands cloves essential oil followed a similar trend - n_D decreased almost linearly with the temperature.
c) Effect of temperature on n_D is large, but with the rise of temperature, n_D decreased.

5.3 Phytocomponents of three brands clove essential oil (CEO)

Essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants, buds etc. Recently, those plants widely used in traditional medicines to cure diseases have been scientifically screening as potential sources of modern anticancer, antioxidant and antifungal agents (Al Nomaani et al., 2013). The objective of this topic was FTIR analysis of the essential oil from 3 brands of clove samples collected from Indonesia, India and Sri Lanka. In our present study, volatile compounds such as eugenol, eugenol acetate, caryophyllic acid, caryophyllene oxide etc. were identified in all essential oils (Table 4.6). These compounds are used as a flavoring agent and in medicine as a local anesthetic and antiseptic (Jadhav et al., 2004).

5.4 Antioxidant activity analysis of CEO

The antioxidant activity of the cloves powder sample were found 70.66ppm, 70.14ppm, 69.74ppm of the CIs, CId and CSl clove essential oil respectively which shows in Table 4.8. Many aromatic plants and spices especially clove buds and their fundamental oils have been known to support different biological activities such as antimicrobial and antioxidant features (Fu et al., 2007). The radical scavenging effects were determinate for clove buds extract and their components. The clove buds extracts or their components when diluted with DPPH decolorized it due to hydrogen donating ability. All the tested samples (methanol or ethanol extracts) revealed scavenging effects on DPPH (Gulcin et al., 2012). Antioxidants are suspected to neutralize the free radicals in lipid chains by availing a hydrogen atom usually from a phenolic hydroxyl group, which in turn assign phenolic groups into static free radicals that do not initiate or propagate further oxidation of lipids (Miguel, 2009).

5.5 Bioactive compounds analysis of CEO

The total flavonoids content (TFC) of the cloves essential oil were found 244.36 ppm, 161.13 ppm, 321.95 ppm of the CIs, CId and CSl clove essential oil respectively. In the present study, I have found that clove essential oil had higher total flavonoids content.

Total phenolic content (TPC) is another category of bioactive compounds as TFC (Salar, Purewal, & Bhatti, 2016). The total phenol content (TPC) of the cloves essential oil were found, 159.44 ppm, 142.82 ppm, 135.11 ppm of the CIs, CId and CSl clove essential oil respectively. Phenolic content was found 158.543 ppm showed that the oil had much antioxidant property than Salvia (51.19 ppm) (Ozcan and Ozkan, 2015) and Lamiaceae (54 ppm) (Stankovicet al., 2011). So essential oil of clove is good free radical scavenger for human health.

The mean value of TAC in anthocyanin- was found 16.07 mg /100 g, 18.156 mg /100 g, 17.94 mg /100 g of the Indonesia, India, and Sri Lanka clove essential oil respectively (Table 4.9).

5.6 Antimicrobial activity of three brands clove essential oil

Bioactive compounds are playing a main role for the treatment of several diseases. Eugenol is a phenolic compound. Phenols are known to have antiseptic properties (Pelczar et al., 1998), which is consistent with the antimicrobial data obtained for these compounds. Caryophyllene has also been shown to possess antimicrobial properties. As a result of findings, the *Syzygium aromaticum* contain bioactive compounds that explain the importance of *S. aromaticum* as medicinal plant. Results show that the *S. aromaticum* extracts inhibit bacterial growths. The highest inhibition zone was found against E. coli and the inhibition zone was 27 mm (Table 4.10) (Soni and Dahiya, 2003) reported that antimicrobial activities of *Syzygium caryophyllatum* essential oil were found between 7 to 22 mm inhibition zones. High levels of eugenol present in *S. caryophyllatum* fundamental oil is responsible for potent antimicrobial action. This phenolic compound can denature proteins and reacts with cell membrane phospholipids changing their permeability. Clove bud oil showed the no zone of inhibition (Nz) i.e.; no antimicrobial activity among the tested bacteria against *Pseudomonas aeruginosa*.

The cloves oil extract were found to have able antimicrobial activity against all the Grampositive and Gram-negative organisms tested. Clove oil exhibited a broad spectrum antimicrobial activity with a minimum zone diameter of 24 mm for *E. coli* and *Staphylococcus aureus* isolate and a maximum zone diameter of 36 mm for positive control Ciproflo-xacin (0.005% [w/v]) for bacteria was more active against *Pseudomonus aeruginosa* at 100 µg/ml concentration of the extract. However, the clove oil extract was more active against *Staphylococcus aureus* isolate at concentrations of 100µg/ml. Ciprofloxacin was inactive against *Escherichia coli*, but the clove oil extract appeared to have very potent activity against *E. coli* (Table 4.10). The positive control produced significantly sized inhibition zones against the test bacteria (ciprofloxacin). However, the negative control made no observable prohibitory effect.

Using agar disc assay, the tested clove extracts induced inhibition zones against 3 tested bacteria. Usually, there were no important differences between the Gram-positive (*Staph. aureus*) and Gram-negative bacteria (*E. coli*). Petroleum ether extract of clove exhibited inhibition zone diameter 24-27 mm with *Staph. aureus, E. coli*. The clove essential oil conc. was 100 µg/ml against Gram-positive and Gram-negative bacteria. The extracts that included high content of TPC showed relatively high antibacterial activity. Petroleum ether extract showed good inhibition zone diameter with Gram-positive and Gram-negative bacteria (Figure 4.14-4.18). It has been reported that the antibacterial potential of extracts is attributable to the concentration of phenolics in the plant extract.

Chapter-6

Conclusion

GC-MS analysis of the oil extract showed eugenol, caryophylene, eugenol acetate as the major constituent. Eugenol and caryo-phyllene are known to occupy antibacterial properties. Hence the antibacterial exhibited by the clove oil extract that can be attributed to the compounds identified.

Decrease in acid value (free fatty acid) reduces its decomposition to volatile compounds which causes rancidity in oil. Stability and shelf life of the oil was increased with decreased in peroxide value. However, low iodine value of the clove oil samples alluded mixing with other accessible oil products. At a particular temperature, viscosity (η) and Refractive Index (n_D) of the cloves essential oil decreases in the order: CSl > CId > CIs but density (ρ) of the cloves essential oil decreases in the order: CId > CSl > CIs. Density (ρ) and Refractive Index (n_D) are decreasing almost linearly with increasing temperature but viscosity (η) decreasing slowly with increasing temperature.

The result obtained showed that clove has high carbohydrate content. It was low in fat content as likened to carbohydrate content. It also provided dietary fiber. The moisture content is higher than the values pointed out by other scientists. The ash content, nitrogen free extract (NFE) was almost intimate to the values. The exchange in results may be due to the environmental situation.

The results expressed that clove essential oil extracted from clove buds may provide an significant means of prohibiting the progress and growth of all tested pathogens and indicator organisms especially *E. coli*. However, the highest inhibitory effect of clove oil was found against *Escherichia coli.* The study implied that clove and clove oils can be used as antibacterial and antiseptic agent. So that cloves can be used as a food preservative due to improve shelf life of foods. Some of the tested organisms are suspected as important food-borne pathogens and raising the possibility of using clove bud oil to prevent food-borne diseases.

Chapter-7

Recommendations and Future perspectives

This chapter provides the recommendations and future perspectives of the present study on the basis of the prosperity of nutritional composition, bioactive compounds and antimicrobial activity of clove and clove oil.

- ✓ Some intensive sampling and analysis, including sampling of three brands cloves of Indonesian (CIs), Indian (Cid) and Sri Lankan (CSl) may be carried out which would better describe nutritional composition, bioactive compounds and antimicrobial activity of clove and clove oil.
- ✓ Samples may be collected during dry and wet season to determine the seasonal variation of values.
- ✓ Chemical constituents, antifungal and antimutagenic activities may be considered for further analysis.
- ✓ Nevertheless, it is quiet impossible to do something without limitation. There were some limitations in this study such as storage time may affect the rate of loss of quality of clove oil. Minerals and vitamins that are not analyzed in this study and cultural sensitive test analysis can have significance.

References

Ajay K. 2014. Physico-chemical and natural products investigations of essential oil from the rhizomes of *Kaempferia galanga L*. Der Chemical Sinica. 5(2): 91-94.

Alam MN, Bristi NJ. 2013. Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal. 21(2): 143-152.

Aziz S, Naher S, Abukawsar M, Roy Sk. 2012. Comparative studies on physicochemical properties and GC-MS analysis of essential oil of the two varieties of the Black Pepper (Piper nigrum Linn.). International Journal of Pharmaceutical and Phytopharmacological Research. 2(2): 67-70.

Ali A Dashti, Mehrez M Jadaon, Abdulsamad M Abdulsamad, Hussein M Dashti 2009. Heat Treatment of Bacteria: A Simple Method of DNA Extraction for Molecular Techniques. Kuwait Medical Journal, 41 (2): 117-122.

Ayoola GA, Lawore FM, Adelowotan T, Aibinu IE, Adenipekun E, Coker HA. 2008. Chemical analysis and antimicrobial activity of the essential oil of *Syzygium aromaticum* (clove). Afr J Microbial Res. 2: 162-166.

Abdel ME. Sulieman MO, Boshra EI, Amin A, Khalifa EI. 2007. Nutritive value of clove (*Syzygium aromaticum*) and detection of antimicrobial effect of its bud oil. Research Journal of Microbiology. 2: 266-271.

Ali SM, Khan AA, Ahmed I, Musaddiq M, Ahmed KS, Polasa H. 2005. Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. Ann Clin Microbiol Antimicrob. 4: 20-24.

Adom KK, Sorrells ME, Liu RH. 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. Journal of Agriculture and Food Chemistry. 53: 2297-2306.

Agrawal SK, Rastogi RP. 1974. Triterpenoid saponins and their genius. Photochemistry. 13: 2623-2645.

Alain R. 1994. Le Robert micro-poche (2nd Ed) (in french). 14 and 126.

Anonymous. 1994. Larousse de poche (in french). Les Editions Francaises Inc: 8-16.

Antifungal action of Amphotericin B. by essential oil from *Thymus vulgaris*. Phytother. Res. 18(12): 990-995.

Aksel B. 2010. A brief review on bioactive compounds in plants. In: Bioactive compounds in plants - benefits and risks for man and animals. Oslo: The Norwegian Academy of Science and Letters. pp. 11-17.

Ayoola GA, Lawore FM, Adelowotan T, Aibinu IE, Adenipekun E, Coker HA. 2008. Chemical analysis and antimicrobial activity of the essential oil of *Syzygium aromaticum* (clove). African Journal of Microbiology Research. (2): 162-166.

Bernard G, Dromard A. 2011. Book of etymology and medical terminology: Lexicon etymology (in French). Livretd'etymologieet de terminologiemedicale: Lexiqued'etymologie. pp. 1-4.

Biesalski HK. 2009. Bioactive compounds: Definition and assessment of activity. Nutrition. 25(11): 1202-1205.

Burt SA, Reinders RD. 2003. Antibacterial activity of selected plant essential oils against *E. coli* 0157: 117. Lett. Applied Microbial. 363: 162-167.

Bouttwell RK. 1998. An Overview of the role of nutrition in carcinogenesis. In: Nutrition growth and cancer. Alan R. Liss, London. 6: 387-418.

Chandrima D, Shreya K, Abhineel M, Ahana D, Pranabesh G, Sirshendu C. 2019. Clove based herbal tea: development, phytochemical analysis and evaluation of antimicrobial property. J. Pharm. Sci. & Res. 11(9): 3122-3129.

Chaieb K, Hajlaoui H, Zmantar T, Nakbi K, Rouabhia M, Mahdouani K, Bakhrouf A. 2007. The chemical composition and biological activity of essential oil, Eugenia caryophyllatum (*Syzygium aromaticum L.* Myrtaceae): S short review. Phytother. Res. 21: 501-516.

Cakır A, Mavi A, Kazaz C, Yıldırım A. 2006. Antioxidant activities of the extracts and components of Teucriumorientale L. var. orientale. Turk. J. Chem. 30: 1-12.

Cammack R. 2006. Oxford Dictionary of Biochemistry and Molecular Biology (2nd Ed). Oxford University Press. pp. 74-75.

Debjit B, Sampath K, Akhilesh Y, Shweta S, Shravan P, Amit SD. 2012. Recent trends in Indian traditional herbs Syzygium aromaticum and its health benefits. 1(1): 13.

Debra S, Jill S. 2019. What you need to know about clove essential oil.

Deepshikha G. 2013. Comparative analysis of spices for their phenolic content, flavonoids content and antioxidant capacity. AIJRFANS.6: 13-323.

Dictionary of food science and technology (2nd Ed). 2009. International Food Information Service (IFIS Editor). pp. 47-48.

Dutta AC. 2003. Botany for Degree Students. 6th Edn. Oxford University Press, New Delhi, India. pp. 301-588.

Davidson SJF, Brock AS. 1975. Human nutrition and dietetics. Churchill living stone. Edinburgh, UK. 107: 221-224.

Donald E, Green H, Morgan G. 1998. Animal nutrition 5th Edu, Longman, London. Pages: 602.

Elujuba AA, Odelleye CM. 2005. Review: Traditional medicine developmennnt for medical and dental primary health care delivery system in Africa. Afr. J. Trad. CAM. 2: 46-61.

Ekwu FC, Nwagu A. 2004. Effect of processing on the quality of cashew nut oils. Journal of Science and Agriculture, Food Technology and Environment. 4: 105-110.

Fu YJ, Zu YG, Chen LY, Shi XG, Wang Z, Sun S. 2007. Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytother Res. 21(10): 989–994.

Foti M. 2007. Antioxidant properties of phenols. J. Pharm. Pharmacology. 59: 1673−1685.

Gulten O, Mahabbat M, Mustafa V. 2018. The antibacterial activities of Syzygium aromaticum merr. & perry against oral bacteria and its antioxidant and antimutagenic activities. IJPSR. 9(11): 4634-464

Gharby S, Harhar H, Bouzoubaa Z, Asdadi A, Yadini A, Charrouf Z. 2015. Chemical characterization and oxidative stability of seeds and oil of sesame grown in Morocco. Saudi Society of Agricultural Science.

Ghalem M, Merghache S, Belarbi M. 2014. Study on the antioxidant activities of root extracts of *Zizyphus lotus* from the western region of Algeria. Pharmacognosy J. 6(4): 32-42.

Gulcin I. 2007. Comparison of in vitro antioxidant and antiradical activities of l-tyrosine and l-Dopa. Amino Acids. 32: 431–438.

Griffiths P, Hasseth JA. 2007. Fourier transform infrared spectrometry (2nd ed.). Wiley-Blackwell.

Gulcin I. 2006. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology. 217: 213–220.

Gulcin I. 2005. The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. Int. J. Food Sci. Nutr. 56: 491-499.

Gulcin I, Sat IG, Beydemir S, Elmastas M, Kufrevioglu OI. 2004. Comparison of antioxidant activity of clove (*Eugenia caryophylata Thunb*) buds and lavender (*Lavandula stoechas L.*). Food Chem. 87: 393-400.

Gutteridge JM. 1994. Biological origin of free radicals and mechanisms of antioxidant protection. Chem. Biol. Interact. 91: 133-140.

Hatice Z, Ayse HB. 2014. Antioxidant and antimicrobial activities of thyme and clove essential oils and application in minced beef. Izmir. 5: 37-50.

Hassan B. 2014. Composition, antioxidant, antibacterial activities and mode of action of clove (*Syzygium aromaticum L.*) buds essential oil. British Journal of Applied Science & Technology. 4(13): 1934-1951.

Hemaiswarya S, Doble M. 2009. Synergistic interaction of eugenol with antibiotics against gram-negative bacteria. Phytomedicine. 16: 997–1005.

Halliwell B. 1997. Antioxidants in human health and disease. Annu. Rev. Nutr. 16: 33–50.

Hilditch TP. 1949. The industrial chemistry of fat and waxes. London. Ibrahim NA. 2013. Characteristics of Malaysia palm kernel and its products. Journal of oil palm research. 25(2): 24-352.

Ilhami G, Mahfuz E, Hassan Y. Aboul E. 2012. Antioxidant activity of clove oil–a powerful antioxidant source. Arabian Journal of Chemistry. 5: 489–499.

Ilhami G. 2011. Antioxidant Activity of Eugenol: a structure–activity relationship study. J Med Food. 14 (9): 975–985.

Johanna NM, Renny RM, Gopakumar G, Balu M, Sureshkumar D, Krishnakumar IM. 2015. Beyond the flavour: de-flavoured

polyphenol rich extract of clove buds (*Syzygium aromaticum L.*) as a novel dietary antioxidant ingredient.

Jadhav BK, Khannelwal KR, Ketkar AR, Pisal SS. 2004. Formulation and evaluation of mucoadhesive tablets containing eugenol for the treatment of periodontal diseases. Drug Dev Ind Pharm. 30(2): 195-203.

Kurt B, Dorothea G, Horst S, 2001. Common fragrance & flavor materials: preparation, properties & uses. 4th Edn, Wiley-VCH Verlag, Weinheim.

Husnu CB, Gerhard B. 2010. Essential oils: science, technology & applications. CRC Press, Taylor and Francis Group, Boka Raton. pp. 844-855.

Hakk A, Murat E, Hubert K. 2010. Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum L.*) Bio Resources. 2(2): 265-269.

Kalemba D, Kunicka A. 2003. Antibacterial and antifungal properties of essential oils. Curr. Med. Chemistry. 1010: 813-829.

Kris-Etherton PM. 2004. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. Annual Review of Nutrition. 24: 511-538.

Kris-Etherton PM. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. American Journal of Medicine. 113: 71S-88S.

Lobo V, Patil A, Phatak A, Chandra N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Rev. 4(8): 118-26.

Leite AM, Lima E, Souza EL, Diniz M, Trajano VN, Medeiros IA. 2007. Inhibitory effect of β-pinene. α-pinene and eugenol on the growth of potential infectious endocarditis causing gram-positive bacteria. Braz J Pharm Science. 43:121-126.

Lopez P, Snchez C, Batle R, Nern C. 2005. Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected food borne bacterial and fungal strains. J Agric Food Chem. 53(17): 6939–6946.

Lai LS, Chou ST, Chao WW. 2001. Studies on the antioxidative activities of Hsian-tsao (Mesona procumbens Hemsl) leaf gum. J. Agric. Food Chem. 49: 963–968.

Miguel D. 2009. Antioxidant activity of medicinal and aromatic plants, Published online in Wiley Online Library.

Moure A, Cruz JM, FrancoD, Dominguez JM, Sineiro J, Dominguez H, Nunez MJ, Parajo JC. 2001. Natural antioxidants from residual sources. Food Chem. 72: 145-171.

Mahfuz E, Ilhami G, Hassan Y. 2012. Antioxidant activity of clove oil – a powerful antioxidant source. Arabian Journal of Chemistry. 5: 489-499.

Mosby's Dictionary of Medicine. 2013. Nursing and Health Professions (9th Ed). Mosby Creator. P.83.

Miller K, Marie TO. 2005. Miller-Keane Encyclopedia and Dictionary of Medicine, Nursing and Allied Health (7th Ed). Saunders. 62.

Nomaani RS, Hossain MA, Weli AM, Riyami Q, Sabahi JN, Rahman SM. 2013. Chemical composition of essential oils and in vitro antioxidant activity of fresh and dry leaves crude extracts of medicinal plant of *Lactuca sativa* L. native to Sultanate of Oman. Asian Pac J Trop Biomed. 3(5): 353-357.

Nazrul IB, Jaripa B, Nemai CN and Farhana A. 2010. Constituents of the essential oil from leaves and buds of clove (*Syzygium caryophyllatum* (L.) Alston). African Journal of Plant Science. 4(11): 451-454.

Oliveira TLC, Cordoso MDG, Soares RA, Ramos EM Piccoli RH, Tebaldi VMR. 2013. Inhibitory activity of *Syzygium aromaticum* and *Cymbopogon citrates* (Dc.) Stapf. essential oil against *Listeria monocytogenes* inoculated in bovine ground meat. International Journal of Pure and Applied Bioscience. 44 (2): 33-40.

Oyedemi SO, Okoh AI, Mabinya LV, Pirochenva G, Afolayan AJ. 2009. The proposed mechanism of bactericidal action of eugenol, α-terpineol and γ-terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli*. Afr J Biotechnol. 8(7): 1280-1286.

Okeke CU, Izundu, Uzoechinda. 2008. Phytochemical and proximate study of female pawpaw (*Carica papaya L.*) Caricaecae. J. Sci. Eng. Technol. 15: 8207-8216.

Oktay M, Gulcin I, Kufrevioglu OI. 2003. Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Lebensm. Wiss.Technol. 36: 263- 271.

Parle M, Khanna D. 2011. Clove: a champion spice. International Journal of Research in Ayurveda & Pharmacy. 2(1): 47-54.

Pundir RK, Jain P, Sharma C. 2010. Antimicrobial activity of ethanolic extracts of *Syzygium aromaticum* and *Allium sativum* against food associated bacteria and fungi. Ethanobotanical Leaflet. 14: 34-60.

Pei RS, Zhou F, Ji BP, Xu J. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol and carvacrol against E. coli with an improved method. J Food Sci. 74:379–383.

Park MJ, Gwak KS, Yang I, Choi WS, Jo HJ, Chang WJ, Jeung EB, Choi IG. 2007. Antifungal activities of the essential oils in *Syzygium*

aromaticum (*L.*) Merr. Et Perry and Leptospermum betersonni Bailey and their constituents against various dermatiphytes. J. Microbial. 45: 460-465.

Pelczar MJ, Chan ECS, Krieg NR. 1998. Control of microorganisms, the control of microorganisms by physical agents. In: Microbiology. McGraw-Hill International, New York. 4: 469-509.

Reji R, Rajasekakran M. 2015. Evaluation of synergistic antimicrobial activity of *Cinnamonum zeylanicum*, *Trachyspermum amni* and *Syzygium aromaticum*. International Journal of Pharma Sciences and Research. 6(2): 304-310.

Riccardo A, Mario C, Luca V. 2013. Antioxidant activity of essential oils. J. Agric. Food Chem. 61: 10835−10847.

Rico MD, Aparicio OG, Dorantes AL, Hernández SH. 2012. Antimicrobial activity of cinnamate-eugenol: Synergistic potential, evidence of efflux pumps and amino acid effects. Am J Food Technol. 7: 289-300.

Raven PM, Johnson M. 1999. Biology. 2nd Edn., McGraw-Hill, London. p.1567.

Sumit S. 2019. Antioxidants and antimicrobial activity of some medicinally important plants. IJCIRAS. 2(4): 2581-5334.

Sana M, Ifra G. 2012. Antibacterial activity of aqueous and ethanolic extracts of garlic, cinnamon and turmeric against *E. coli* 25922 and *Bacillus subtilis* dsm 3256. Research Article.

Shyamapada M, Manisha DM, Krishnendu S, Nishith KP. 2011. In Vitro Antibacterial activity of three Indian spices against methicillin-resistant *Staphylococcus aureus*. Oman Medical Journal. 26(5): 319-323.

Singh G, Maurya S, De Lampasona MP, Catalan CAN. A. 2007. Comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. Food Chem.Toxicol. 45(9): 1650–1661.

Srivastava KC, Malhotra N. 1991. Acetyl eugenol a component of oil of cloves (*Syzygium aromaticum L.*) inhibits aggregation and alters arachidonic acid metabolism in human blood platelets prostaglandins. Leukot Essen. Fatty acids. 42: 73-81.

Skerget M, Kotnik P, Hadolin M, Hras AR, Simonic M, Knez Z. 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 89: 191–198.

Sherwin ER, Branen AL, Davidson PM, Salminen S. 1990. In: Food Additives. Marvel Dekker Inc., New York. pp. 139–193.

Studdert VP. 2011. Saunders Comprehensive Veterinary Dictionary (4th Ed). Elsevier Health Sciences, UK. P. 79.

The American Heritage Medical Dictionary. 2007. Houghton Mifflin Company. P. 47.

Valgimigli L. 2012. Essential oils: an overview on origins, chemistry, properties and uses. In essential oils as natural food additives. Ed. Nova Science Publishers: New York. p.24

Valgimigli L, Pratt DA. 2012. Antioxidants in chemistry and biology. In encyclopedia of radicals in chemistry, biology and materials. Wiley: Chirchester, UK. 3: 1623−1677.

Van Z, Seatlholo ST, Vuuren SF, Viljoen AM. 2006. The biological activities of 20 nature identical essential oil constituents. J Essent Oil Res. 18: 129–133.

Velluti A, Sanchis V, Ramos AJ, Mari'n S. 2003. Inhibitory effect of cinnamon, clove, lemongrass, oregano and palmarose essential oils

sin B1production by Fusarium proliferatum in maize grain. Int. J. Food Microbial.,, 89: 145-154.

Velioglu YS, Mazza G, Gao L, Oomah BD. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. 46: 4113–4117.

Wankhede TB. 2015. Evaluation of antioxidant and antimicrobial activity of the Indian clove Syzygium aromaticum L. Merr. & Perr. Int. Res. J. of Science & Engineering. 3(4): 166-172.

Weiss EA. 1997. Essential Oil Crops. Victoria, Australia. pp. 235-259.

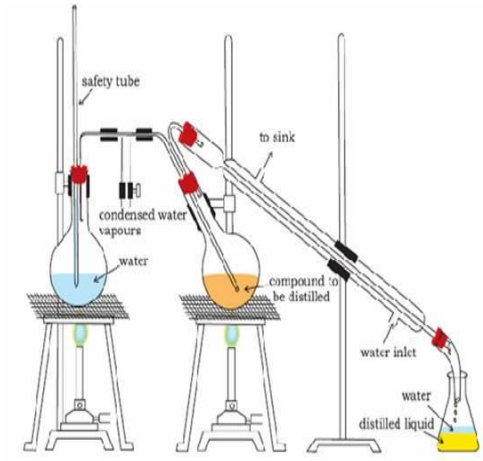
Wood LG, Gibson PG, Garg ML. 2006. A review of the methodology for assessing in vivo antioxidant capacity. J. Sci. Food Agric. 86: 2057–2066.

YuJie F, YuanGang Z, LiYan C, XiaoGuang S, Zhe Wang, Su S, Thomas E. 2007. Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytother. Res. 21: 989–994.

Appendix A: Analysis related pictures

Clove & Clove oil extraction:

Clove & Clove powder



Oil extraction at clevenger apparatus

Recovery during oil extraction

Collecting clove oil

Proximate analysis:

Moisture remove at 105^{o}C (oven)

Keeping sample in muffle furnace

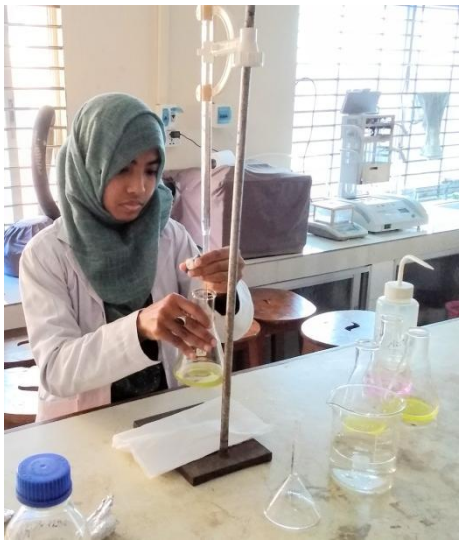
Boiling at 70^{o}C

Taking residue after 3 times washing

Collecting distillate from distillation flask

Titration with 0.1N HCl

Physicochemical properties analysis:



Titration with 0.1N KOH (AV)

Boiling for 1hr (SV)

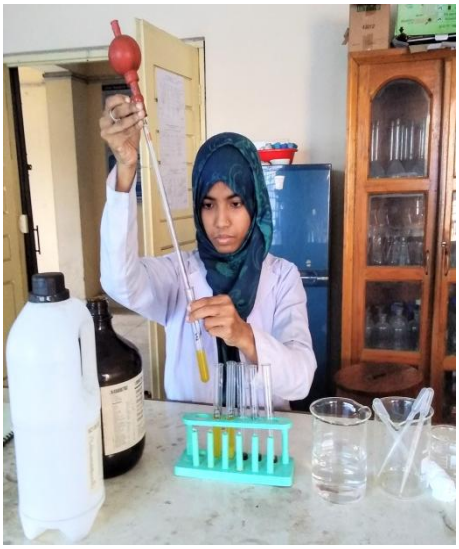
Adding acetic acid solution (PV)

Titration with unknown $Na_2S_2O_3$ solution

Adding Hanus solution (IV)

Titration with 0.1N $Na_2S_2O_3$ solution

Antioxidant activity & Bioactive compound analysis:



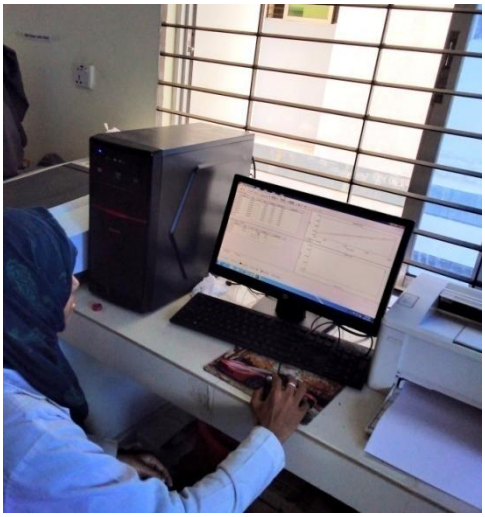
Adding C_2H_5OH

Adding reagents (FC, DPPH)

Sample preparation for AOA

Sample preparation for TPC

Sample attaching with
UV-visible Spectrophotometer



Quantification of bioactive
compounds & AOA

Antimicrobial activity analysis:

PCR tube in PCR machine

Gel electrophoresis

Agar in water bath

Taking sterile saline solution

Oil absorb with filter paper

Bacterial colony mixing with SSS

Streaking

Adding Ciprofloxacin

Appendix B: MS spectra of the Volatile compounds/ Phytocomponents

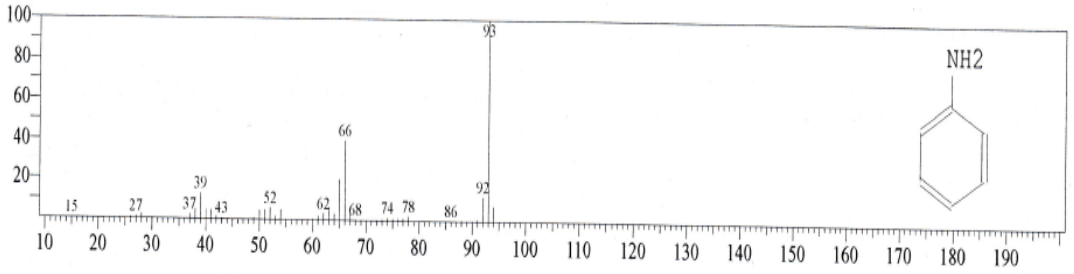


Figure: MS spectra of the Aniline (CIs)

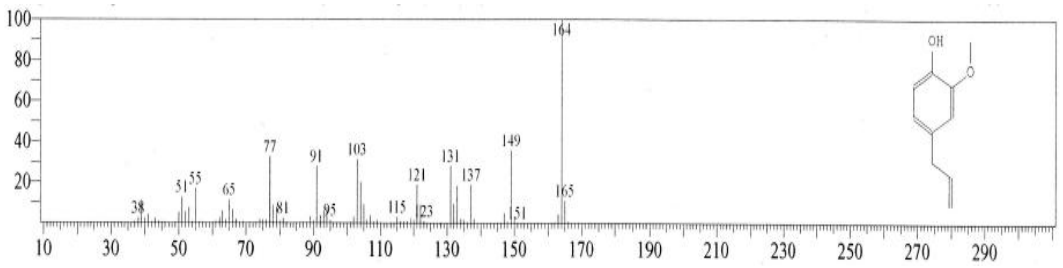


Figure: MS spectra of the Eugenol (CId)

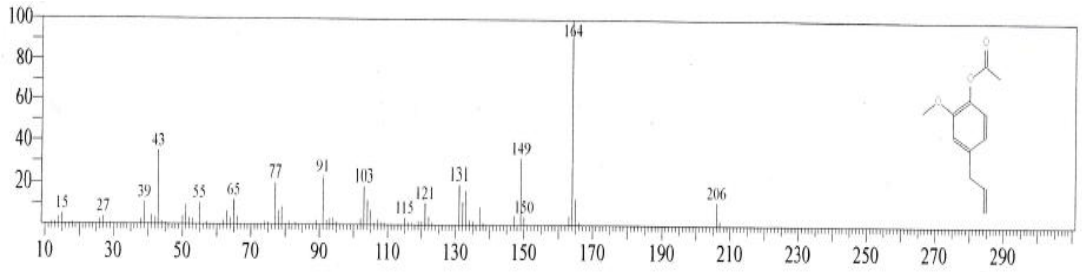


Figure: MS spectra of the Eugenol acetate (CSl)

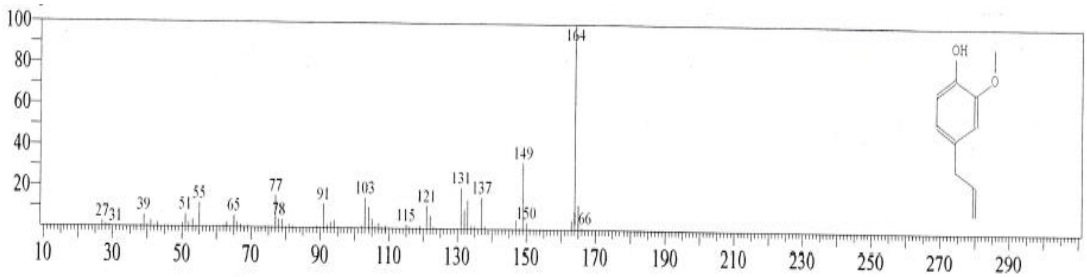
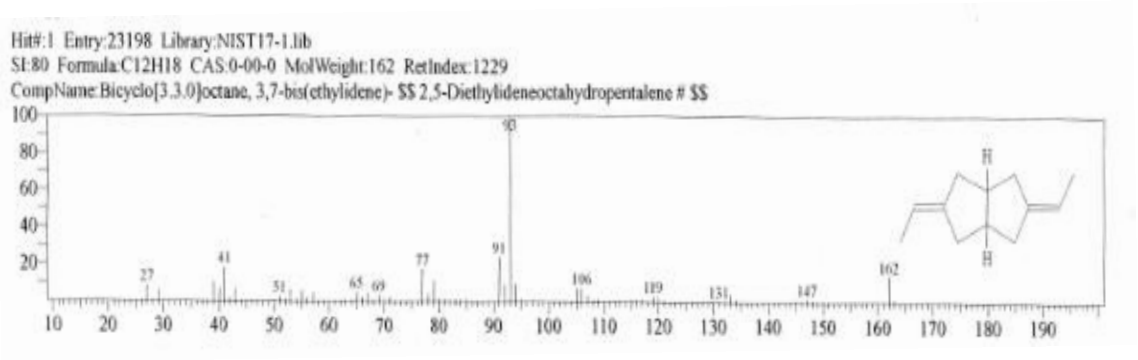
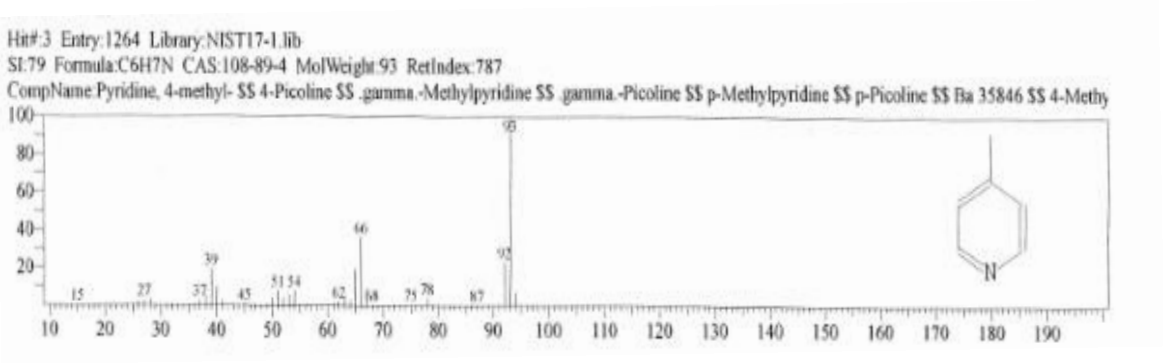
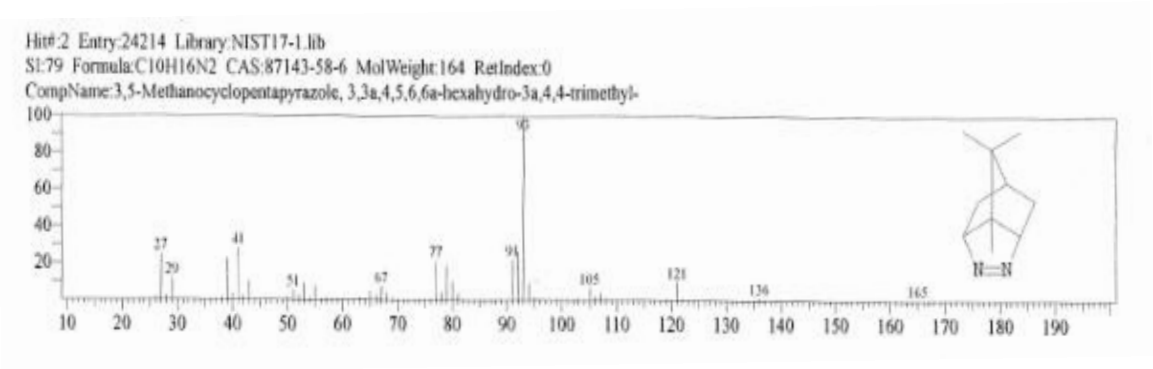


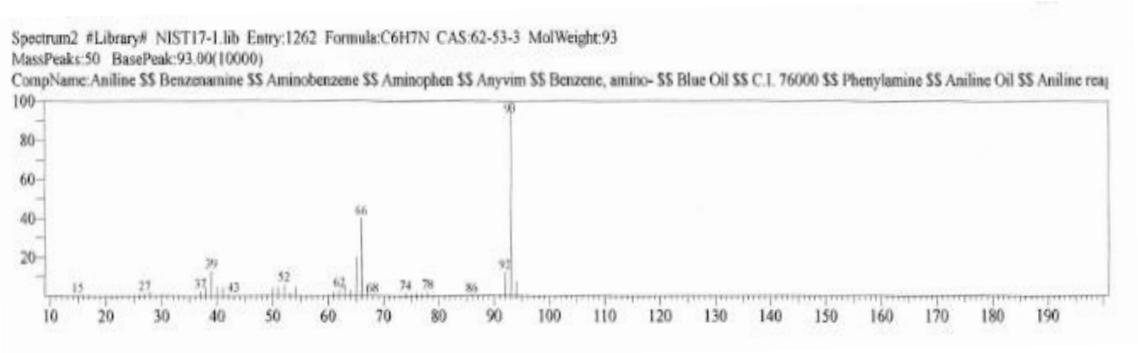
Figure: MS spectra of the Caryophyllic acid (CSl)

MS spectra of the Volatile compounds (CIs)

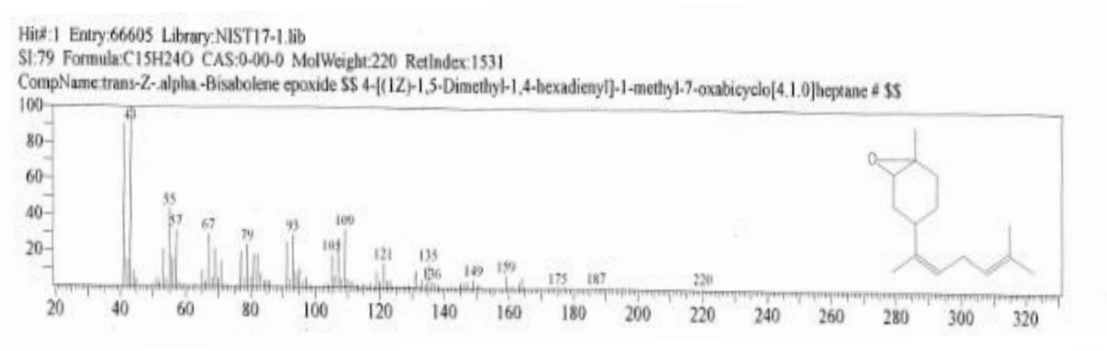


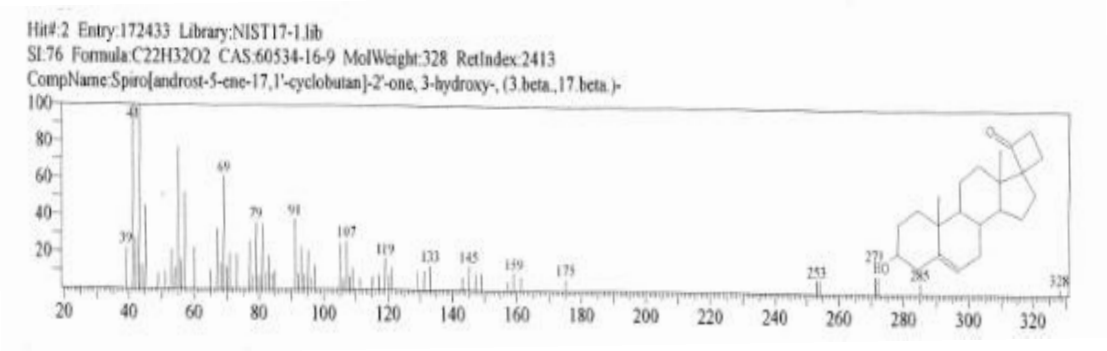


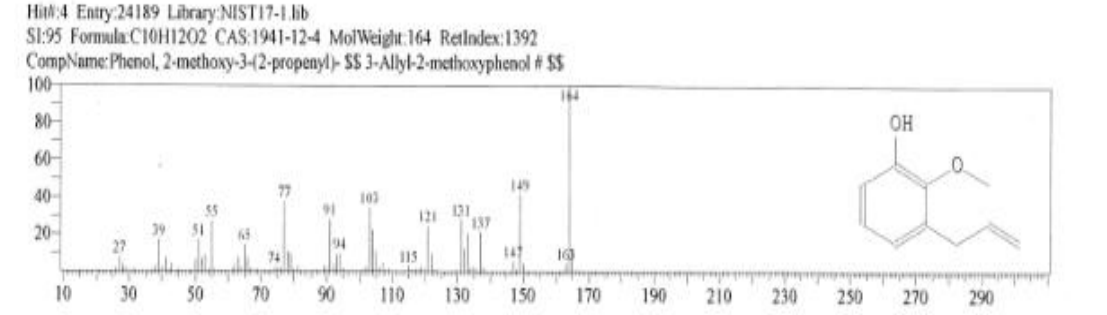


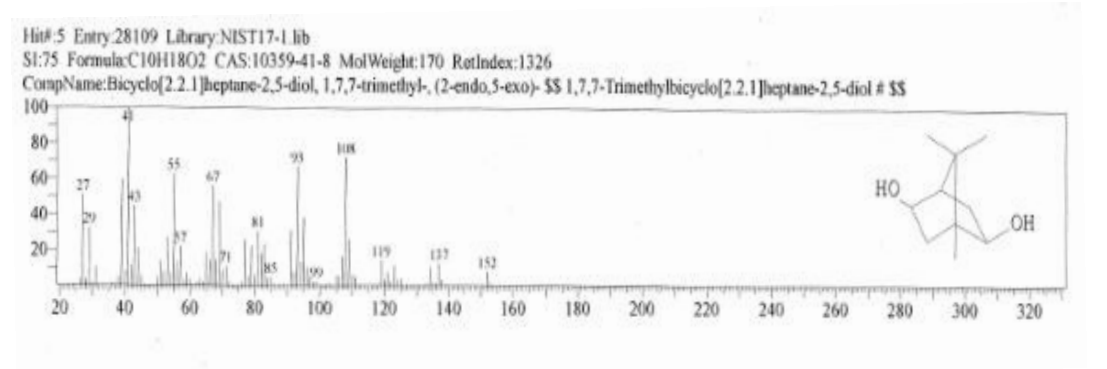


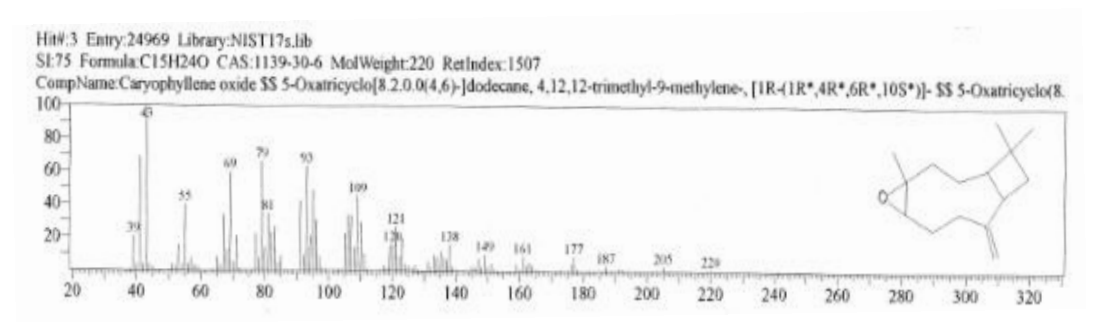
MS spectra of the Volatile compounds (CId)



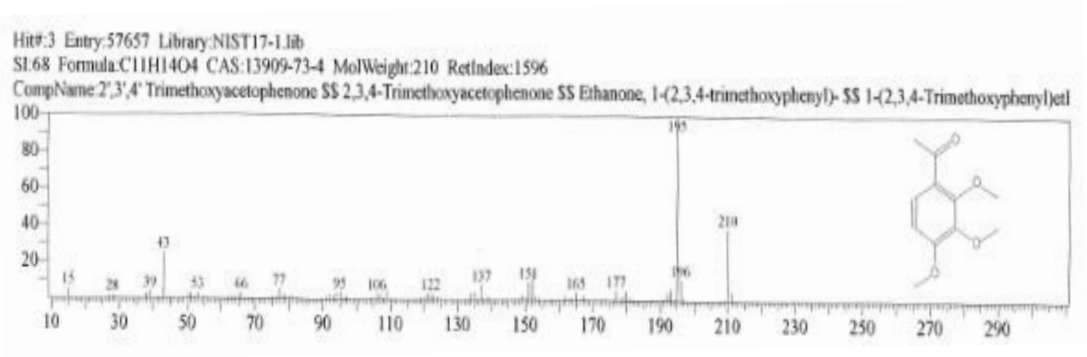


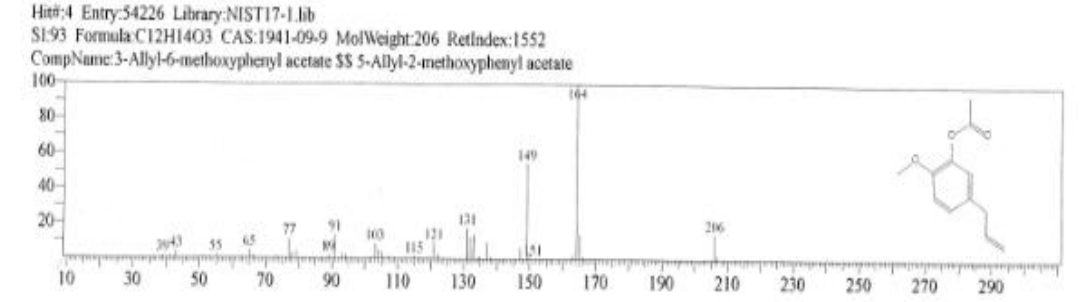


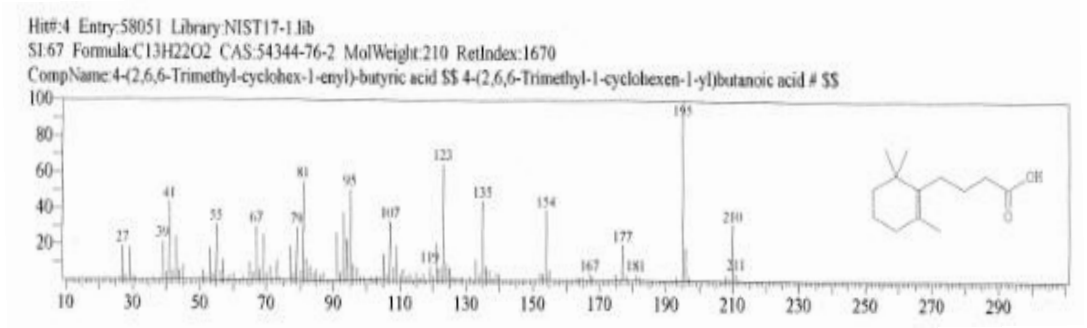


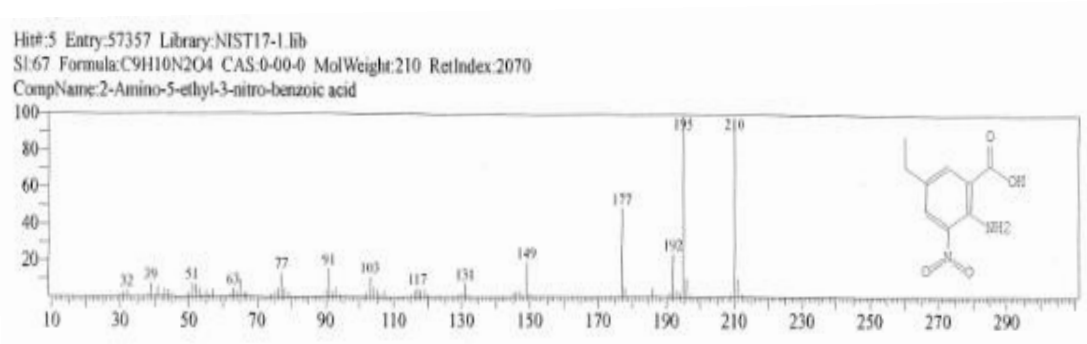


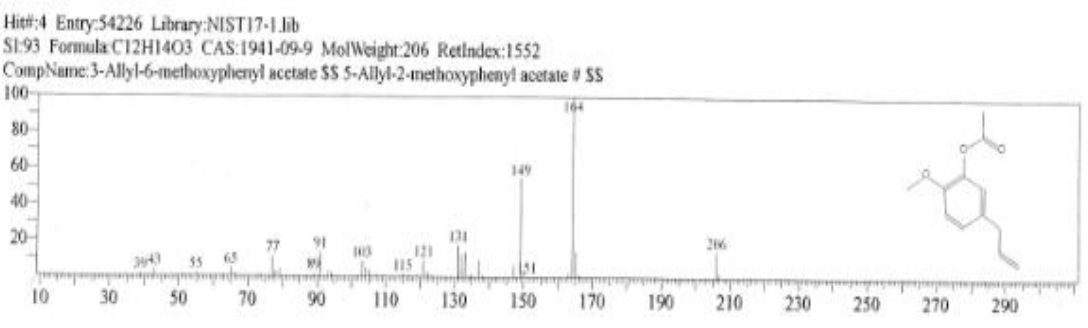
MS spectra of the Volatile compounds (CSl)



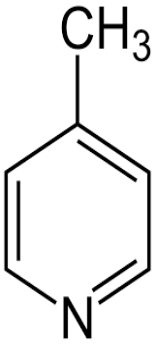




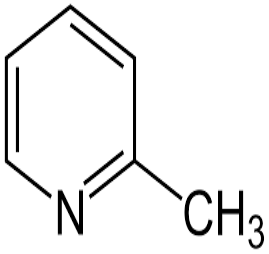




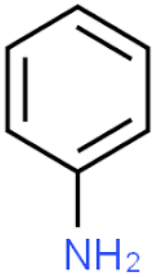
Chemical structure of volatile compounds



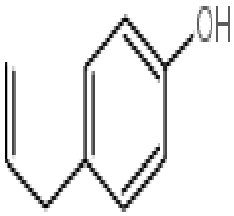
P-Picoline



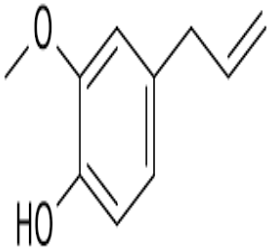
alpha-picoline



Aniline

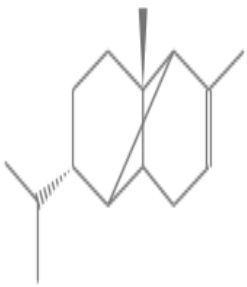


Chavicol

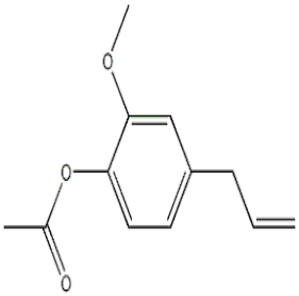


Eugenol

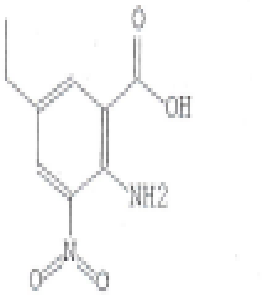
Copaene



Ylangene



Eugenol acetate



2-Amino-5-ethyl-
3 -nitro-benzoic acid

Appendix C: Sample and Standard Table and Curve

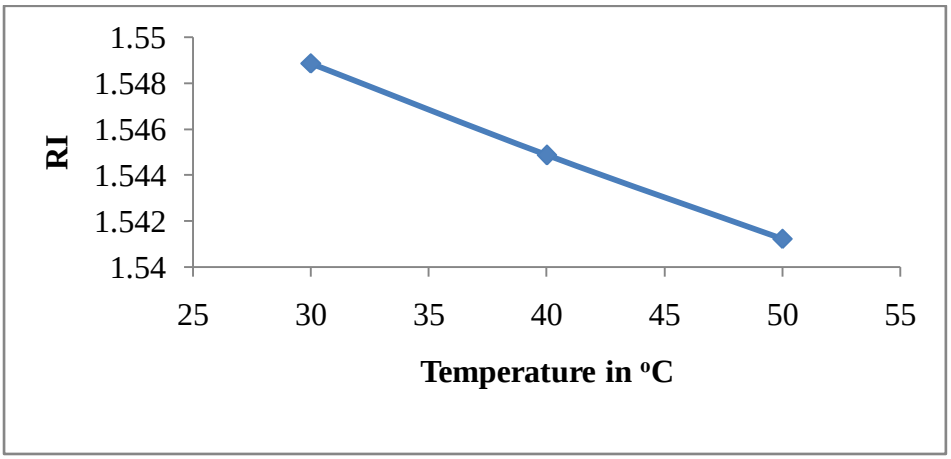


Figure: Refractive Index of CIs Sample at different temperatures

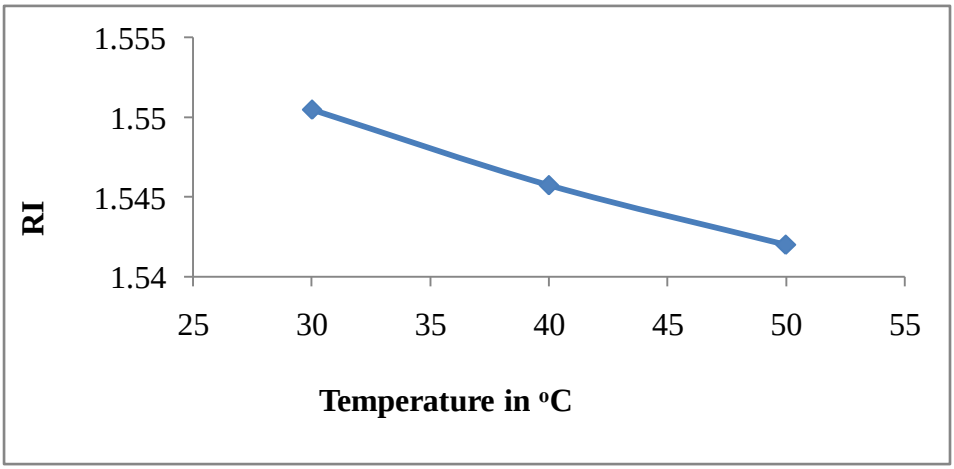


Figure: Refractive Index of CId Sample at different temperatures

Table: Concentration and absorbance for Standard solution for TFC

Sample ID	Type	Ex	Conc.	WL 517. 0	Wgt.Factor
1	Std1	Standard	1.000	0.041	1.000
2	Std2	Standard	3.000	0.088	1.000
3	Std3	Standard	5.000	0.171	1.000
4	Std4	Standard	7.000	0.234	1.000

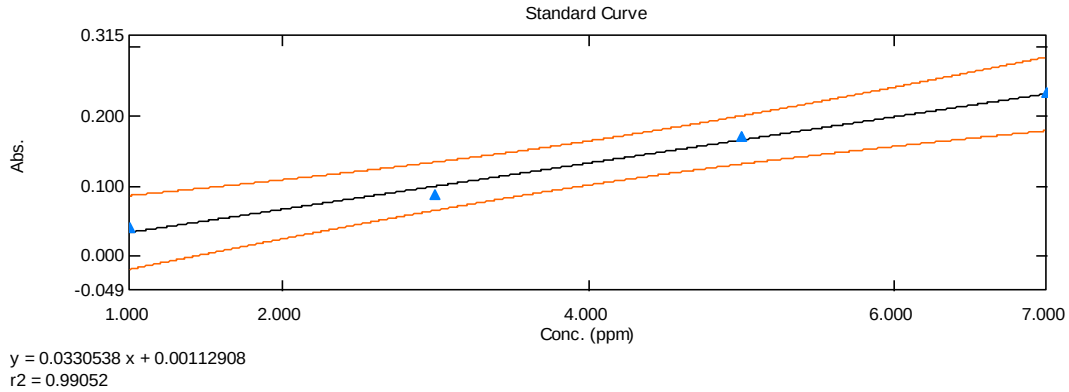


Figure: Standard curve for TFC capacity determination test

Table: Concentration and absorbance for Standard solution for TPC

Sample ID	Type	Ex	Conc.	WL 517. 0	Wgt. Factor
1	Std1	Standard	1.000	0.763	1.000
2	Std2	Standard	2.000	0.780	1.000
3	Std3	Standard	3.000	0.920	1.000
4	Std4	Standard	4.000	1.007	1.000
5	Std5	Standard	5.000	1.074	1.000
6	Std6	Standard	6.000	1.115	1.000
7	Std7	Standard	7.000	1.230	1.000
8	Std8	Standard	8.000	1.314	1.000

Standard Curve

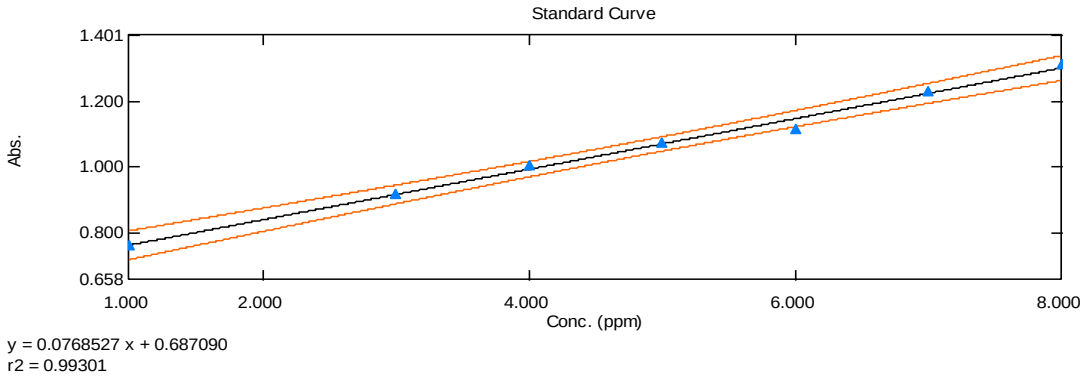


Figure: Standard curve for TPC capacity determination test

Brief Biography

Sharmin Hossen Jenia passed the Secondary School Certificate Examination in 2009 from Bakalia Adarsha Girl's High School, Chattogram and Higher Secondary Certificate Examination in 2011 from Bangladesh Mohila Samiti Girls High School and College, Chattogram. I also received Bachelor of Food Science and Technology (BFST) degree from Faculty of Food Science and Technology, Chattogram Veterinary and Animal Sciences University, Khulshi, Chattogram and receiving M.Sc. degree in Food Chemistry and Quality Assurance under the Department of Applied Chemistry and Chemical Technology of the same faculty. My research interests are in the areas of Analytical Food Chemistry, Food processing, Food Safety and Food toxicology.