Chapter one

1. Introduction

Cinnamomum zeylanicum is one of the oldest herbal medicine known, having been mentioned in Chinese texts since 4 000 years ago

Cinnamomum zeylanicum is an evergreen tropical tree, belonging to the Lauraceae family. Cinnamon barks and leaves are widely used as spice and flavoring agent in foods and for various applications in medicine

Cinnamon is often used for medicinal purposes due to its unique properties. The essential oil from its bark is rich in trans-cinnamaldehyde with antimicrobial effects against animal and plant pathogens, food poisoning and spoilage bacteria and fungi . The bark and leaves of Cinnamomum sp. are commonly used as spices in home kitchens and their distilled essential oils are used as flavoring agent in the food and beverage industries .

The bark of the tree is dried and used for spice. In the United States, cinnamon is used to flavor cereals, grain-based dishes, and fruits. Cinnamon is among the world's most widely used spices and is relatively inexpensive. Cinnamon contains antioxidants and other active ingredients which are found in the water-soluble portions of cinnamon, and not the cinnamon oil. It is through these components that

cinnamon is believed to produce its associated health effects.

Until now more than 300 volatiles were found as constituents of essential oils of cinnamon. It has been established that the oils and extracts from cinnamon possess a distinct antioxidant activity, which is especially attributed to the presence of phenolic and polyphenolic substances.Furthermore, plant essential oils are widely available and some are relatively inexpensive compared to plant extracts

1.1 Main component of Cinnamomum zeylanicum

Cinnamon bark contains up to 4% of essential oil consisting primarily of cinnamaldehyde (60-75%), cinnamyl acetate (1-5%), eugenol (1-10%) (WHO Vol. 1, 1999), β-caryophyllene (1-4%), linalool and (1-3%) and 1.8-cineole (1-2%). (ESCOP, 2003). Other constituents are oligopolymeric procyanidins, cinnamic acid, phenolic acids, pentacyclic diterpenes cinnzeylanol and its acetyl derivative cinnzeylanine and the sugar mannitol, L-arabino-D-13

xylanose, L-arabinose, D-xylose, α-D-glucose as well as mucilage polysaccharides (Hänsel et al., 1992; ESCOP, 2003).
The essential oil of the bark is described in the European Pharmacopoeia, 2009. There exists a summary report on the essential oil of cinnamon bark by the Committee for Veterinary Medicinal Products. According to this information, the oil mainly contains cinnamaldehyde (55-76%), eugenol (5-18%) and saffrole (up to 2%). This document refers also to human use (CVMP 2000).

1.1.1. Cinnamaldehyde

3-Phenyl-2-propenal is known as cinnamaldehyde, is a pale yellow liquid with a warm, sweet,spicy odor and pungent taste reminiscent of cinnamon. It is found naturally in the essential oils of Chinese cinnamon (Cinnamomum cassia, Blume) (75–90%) and Ceylon cinnamon (Cinnamomum zeylanicum, Nees) (60–75%) as the primary component in the steam distilled oils. It also occurs in many other essential oils at lower levels.
Greater than 95% of the consumption of cinnamaldehyde occurs in flavor uses where a spicy, cinnamon character is needed. It is used in a large range of products including bakery goods, confection, and beverages and also in toothpastes, mouthwashes and chewing gum. Furthermore, it is also used effectively in air fresheners where odor neutralization 14

can be achieved by reacting with sulfur and nitrogen malodorants. Other uses include its capability as an animal repellent, its use in compositions to attract insects and demonstration of a positive antifungal activity. Cinnamaldehyde has been efficiently isolated in high purity by fractional distillation from cassia and cinnamon bark essential oils and also used in manufacturing protocols for the preparation of natural benzaldehyde through a retro-aldol process.

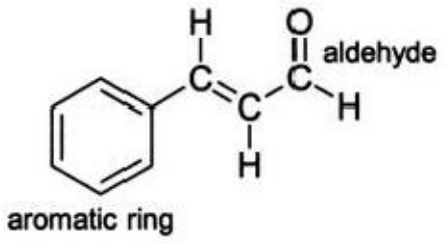


Figure 2.1 Cinnamaldehyde

1.1.2. Eugenol

According to Wikipedia, eugenol is a phenylpropene, an allyl chain-substituted guaiacol. Eugenol is a member of the phenylpropanoids class of chemical compounds. It is a clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil, cinnamon, basil and bay leaf. It is slightly soluble in water and soluble in organic solvents and has a spicy, clove-like aroma. The name is derived from the scientific name 15

for clove, *Eugenia aromaticum* or *Eugenia caryophyllata*. Eugenol is responsible for the aroma of cloves**.** Eugenol is used in perfumeries, flavorings, essential oils and in medicine as a local antiseptic and anesthetic. It was used in the production of isoeugenol for the manufacture of vanillin, though most vanillin is now produced from phenol or from lignin. Eugenol derivatives or methoxyphenol derivatives in wider classification are used in perfumery and flavoring. They are used in formulating insect attractants and UV absorbers, analgesics, biocides, and antiseptics. Eugenol possesses significant antioxidant, anti-inflammatory and cardiovascular properties, in addition to analgesic and local anesthetic activity. (Pramod, Ansari and Ali, 2010).

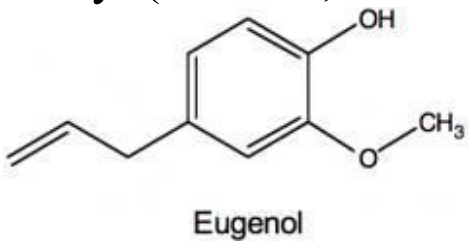


Figure 2.2: Eugenol

1.2. Steam distillation

Steam distillation is a special type of distillation (a separation process) for *temperature sensitive* materials like natural aromatic compounds. It once was a popular laboratory method for purification of organic compounds, but has become obsolete by vacuum distillation. Steam distillation remains important in certain industrial sectors.

Many organic compounds tend to decompose at high sustained temperatures. Separation by distillation at the normal (1 atmosphere) boiling points is not an option, so water or steam is introduced into the distillation apparatus. The water vapor carries small amounts of the vaporized compounds to the condensation flask, where the condensed liquid phase separates, allowing for easy collection. This process effectively allows for distillation at lower

temperatures, reducing the deterioration of the desired products. If the substances to be distilled are very sensitive to heat, steam distillation may be applied under reduced pressure, thereby reducing the operating temperature further.

After distillation the vapors are condensed. Usually the immediate product is a two-phase system of water and the organic distillate, allowing for separation of the components by decantation, partitioning or other suitable methods.

The advantage of steam distillation is that it does not require any solvent and is safer than other methods. And the properties of the oils produced by this method are not altered. As steam reduces the boiling point of a particular component of the oil, it never decomposes in this method. Apart from being economical, it is also relatively faster compared to other methods

1.2.1. Principle

When a mixture of two practically immiscible liquids is heated while being agitated to expose the surface of each liquid to the vapor phase, each constituent independently exerts its own vapor pressure as a function of temperature as if the other constituent were not present. Consequently, the vapor pressure of the whole system increases. Boiling begins when the sum of the vapour pressures of the two immiscible liquids just exceeds the atmospheric pressure (approximately

101 kPa at sea level). In this way, many organic compounds insoluble in water can be purified at a temperature well below the point at which decomposition occurs. For example, the boiling point of bromobenzene is 156 °C and the boiling point of water is 100 °C, but a mixture of the two boils at 95 °C. Thus, bromobenzene can be easily distilled at a temperature 61 °C below its normal boiling point

1.2.2. Applications

Steam distillation is employed in the isolation of essential oils, for use in perfumes, for example. In this method, steam is passed through the plant material containing the desired oils. Eucalyptus oil and orange oil are obtained by this method on the industrial scale. Steam distillation is also sometimes used to separate intermediate or final products during the synthesis of complex organic compounds.

Steam distillation is also widely used in petroleum refineries and petrochemical plants where it is commonly referred to as "steam stripping".

Steam distillation also is an important means of separating fatty acids from mixtures and for treating crude products such as tall oils to extract and separate fatty acids, soaps and other commercially valuable organic compounds.

1.2.3. Equipment

On a lab-scale steam distillations are carried out using steam generated outside the system and piped through macerated biomass or steam generation in-situ using a Clevenger-type apparatus

1.3. acid value

In chemistry, **acid value** (or "neutralization number" or "acid number" or "acidity") is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of chemical substance. The acid number is a measure of the amount of carboxylic acid groups in a chemical compound, such as a fatty acid, or in a mixture of compounds. In a typical procedure, a known amount of sample dissolved in organic solvent (often isopropanol), is titrated with a solution of potassium hydroxide (KOH) with known concentration and with phenolphthalein as a color indicator.

The acid number is used to quantify the amount of acid present, for example in a sample of biodiesel. It is the quantity of base, expressed in milligrams of potassium hydroxide, that is required to neutralize the acidic constituents in 1 g of sample.

V_{eq} is the volume of titrant (ml) consumed by the crude oil sample and 1 ml of spiking solution at the equivalent point, b_{eq} is the volume of titrant (ml) consumed by 1 ml of spiking solution at the equivalent point, and 56.1 is the molecular weight of KOH. W_{Oil} is the mass of the sample in grams.

The molar concentration of titrant (N) is calculated as such:

In which W_{KHP} is the mass (g) of KHP in 50 ml of KHP standard solution, V_{eq} is the volume of titrant (ml) consumed by 50 ml KHP standard solution at the

equivalent point, and 204.23 is the molecular weight of KHP.
There are standard methods for determining the acid number, such as ASTM D 974 and DIN 51558 (for mineral oils, biodiesel), or specifically for biodiesel using the European Standard EN 14104 and ASTM D664 are both widely used worldwide. Acid number (mg KOH/g oil) for biodiesel should to be lower than 0.50 mg KOH/g in both EN 14214 and ASTM D6751 standard fuels. This is since the FFA produced may corrode automotive parts and these limits protect vehicle engines and fuel tanks.
As oil-fats rancidify, triglycerides are converted into fatty acids and glycerol, causing an increase in acid number. A similar observation is observed with biodiesel aging through analogous oxidation processes and when subjected to prolonged high temperatures (ester thermolysis) or through exposure to acids or bases (acid/base ester hydrolysis).

1.4. Saponification value

Saponification value (or "saponification number"/"Koettstorfer number", also referred to as "**sap**" for short) represents the number of milligrams of potassium hydroxiderequired to saponify 1g of fat under the conditions specified. It is a measure of the average molecular weight (or chain length) of all the fatty acids present. As most of the mass of a fat/tri-ester is in the 3 fatty acids, it allows for comparison of the average fatty acid chain length. The long chain fatty acids found in fats have a low saponification value because they have a relatively fewer number of carboxylic functional groups per unit mass of the fat as compared to short chain fatty acids. If more moles of base are required to saponify N

grams of fat then there are more moles of the fat and the chain lengths are relatively small, given the following relation:
Number of moles = mass of oil/relative atomic mass
The calculated molar mass is not applicable to fats and oils containing high amounts of unsaponifiable material, free fatty acids (>0.1%), or mono- and diacylglycerols (>0.1%).
Handmade soap makers who aim for bar soap use NaOH (sodium hydroxide, lye). Because saponification values are listed in KOH (potassium hydroxide) the value must be converted from potassium to sodium to make bar soap; potassium soaps make a paste, gel or liquid soap. To convert KOH values to NaOH values, divide the KOH values by the ratio of the molecular weights of KOH and NaOH (1.403).
Standard methods for analysis are for example: ASTM D5558 for vegetable and animal fats, ASTM D 94 (for petroleum) and DIN 51559.

1.5. disc diffusion antimicrobial sensitivity testing

The disc diffusion antibiotic sensitivity testing or agar diffusion test (Kirby–Bauer antibiotic testing, KB testing) is a test of the antibiotic sensitivity of bacteria. It uses antibiotic and antibiotic-like substances-impregnated wafers to test the extent to which bacteria are affected by those antibiotics. In this test, wafers containing antibiotics are placed on an agar plate where bacteria have been placed, and the plate is left to incubate. If an antibiotic stops the bacteria from growing or kills the bacteria, there will be an area around the wafer where the

bacteria have not grown enough to be visible. This is called a zone of inhibition.
The size of this zone depends on how effective the antibiotic is at stopping the growth of the bacterium. A stronger antibiotic will create a larger zone, because a lower concentration of the antibiotic is enough to stop growth.
The bacteria in question are swabbed uniformly across a culture plate. A filter-paper disk, impregnated with the compound to be tested, is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar. The concentration of the compound will be highest next to the disk, and will decrease as distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition. This along with the rate of antibiotic diffusion is used to estimate the bacteria's sensitivity to that particular antibiotic. In general, larger zones correlate with smaller minimum inhibitory concentration (MIC) of antibiotic for that bacteria. Inhibition produced by the test is compared with that produced by known concentration of a reference compound. This information can be used to choose appropriate antibiotics to combat a particular infection.

1.6. Infrared spectroscopy

Infrared spectroscopy (IR spectroscopy or Vibrational Spectroscopy) is the spectroscopy that deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light. It covers a range of

techniques, mostly based on absorption spectroscopy. As with all spectroscopic techniques, it can be used to identify and study chemicals. For a given sample which may be solid, liquid, or gaseous, the method or technique of infrared spectroscopy uses an instrument called an infrared spectrometer (or spectrophotometer) to produce an infrared spectrum. A basic IR spectrum is essentially a graph of infrared light absorbance (or transmittance) on the vertical axis vs. frequency or wavelength on the horizontal axis. Typical units of frequency used in IR spectra are reciprocal centimeters (sometimes called wave numbers), with the symbol cm^{-1}. Units of IR wavelength are commonly given in micrometers (formerly called "microns"), symbol μm, which are related to wave numbers in a reciprocal way.

1.6.1. practical IR spectrophotometry

The infrared spectrum of a sample is recorded by passing a beam of infrared light through the sample. When the frequency of the IR is the same as the vibrational frequency of a bond or collection of bonds, absorption occurs. Examination of the transmitted light reveals how much energy was absorbed at each frequency (or wavelength). This measurement can be achieved by scanning the wavelength range using a monochromator. Alternatively, the entire wavelength range is measured using a Fourier transform instrument and then a transmittance or absorbance spectrum is generated using a dedicated procedure.
This technique is commonly used for analyzing samples with covalent bonds. Simple spectra are obtained from samples with few IR active bonds and high levels of
purity. More complex molecular structures lead to more
absorption bands and more complex spectra

1.6.2. Sample preparation

Liquid samples can be sandwiched between two plates of a salt (commonly sodium chloride, or common salt, although a number of other salts such as potassium bromide or calcium fluoride are also used). The plates are transparent to the infrared light and do not introduce any lines onto the spectra.

In photoacoustic spectroscopy the need for sample treatment is minimal. The sample, liquid or solid, is placed into the sample cup which is inserted into the photoacoustic cell which is then sealed for the measurement. The sample may be one solid piece, powder or basically in any form for the measurement. For example, a piece of rock can be inserted into the sample cup and the spectrum measured from it.

Chapter two

2. Experimental:

-sample origin and preparation
-collection : the sample is collected from Alsoog Elarbi
-weighting
-distillation

2.1. Equipment Apparatus:

Sensitive balance Burette pipette conical flask beaker measuring cylinder dropper

2.2. Chemicals

Oil sample ph.hp indicator Diethyle ester potassium hydroxide (0.1M)

2.3. methods of calculating of some chemical properties of cinnamon oil

2.3.1. Acid value:

The acid value is the number that expresses, in milligrams the quantity of potassium hydroxide required to neutralize the free acids present in 1 g of the substance.

10.00 g (m) of each sample was dissolved in 50 ml of a mixture of equal volumes of 96 % ethanol and light petroleum, *previously* neutralized with 0.1 M potassium hydroxide and titrate with 0.1 M potassium hydroxide until the pink colour persists for at least 15 second (n ml of titrant Acic value was calculated as followed

$$I_A = \frac{5.610n}{m}$$

2.3.2. Saponification Number

2 g of each sample was weighted into a 200-ml flask, 25.0 ml of the ethanolic solution of potassium hydroxide

was added and boiled under a reflux condenser for 1 hour and rotated the contents frequently. While the solution is still hot, the excess of alkali was titrated with 0.5M hydrochloric acid VS using 1 ml of phenolphthalein solution as indicator. Operation was repeated without the substance being examined.

Saponification value was calculated from the expression

$$Sample\ value = \frac{28.05 \times V_{ml}}{W_{sample}}$$

where v is the difference, in ml, between the titrations and w is the weight, in g, of substance taken.

2.3.3. Ester value:

Ester value was calculated by as followed:

$$Ester\ value = Saponification\ value - Acid\ value$$

2.4. Methods of obtaining biological activity of cinnamon oil against some bacteria and fungi

2.4.1 studied micro-organisms

Bacterial microorganisms:

Bacillus subtilis (BS) (Gram + ve bacteria)

Staphylococcus aureus (SA) (Gram +ve Bacteria)

Escherichia coli (EC) (Gram -ve bacteria)

Pseudomonas aeruginosa (PS) (Gram -ve bacteria)

Fungal microorganisms:

Candida albicans (CA)

2.4.2 Preparation of the test organisms:

2.4.2.1. Preparation of bacterial suspensions:

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used.

The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique (**Miles and Misra, 1938**). Serial dilutions of the stock suspension were made

in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.4.2.2. Preparation of fungal suspension:

The fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspension in 100ml of sterile normal saline, and the suspension were stored in the refrigerator until used.

2.4.3. Testing of antibacterial susceptibility

Disc diffusion method

The paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 1999). Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extracts.The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured.

Figure 1

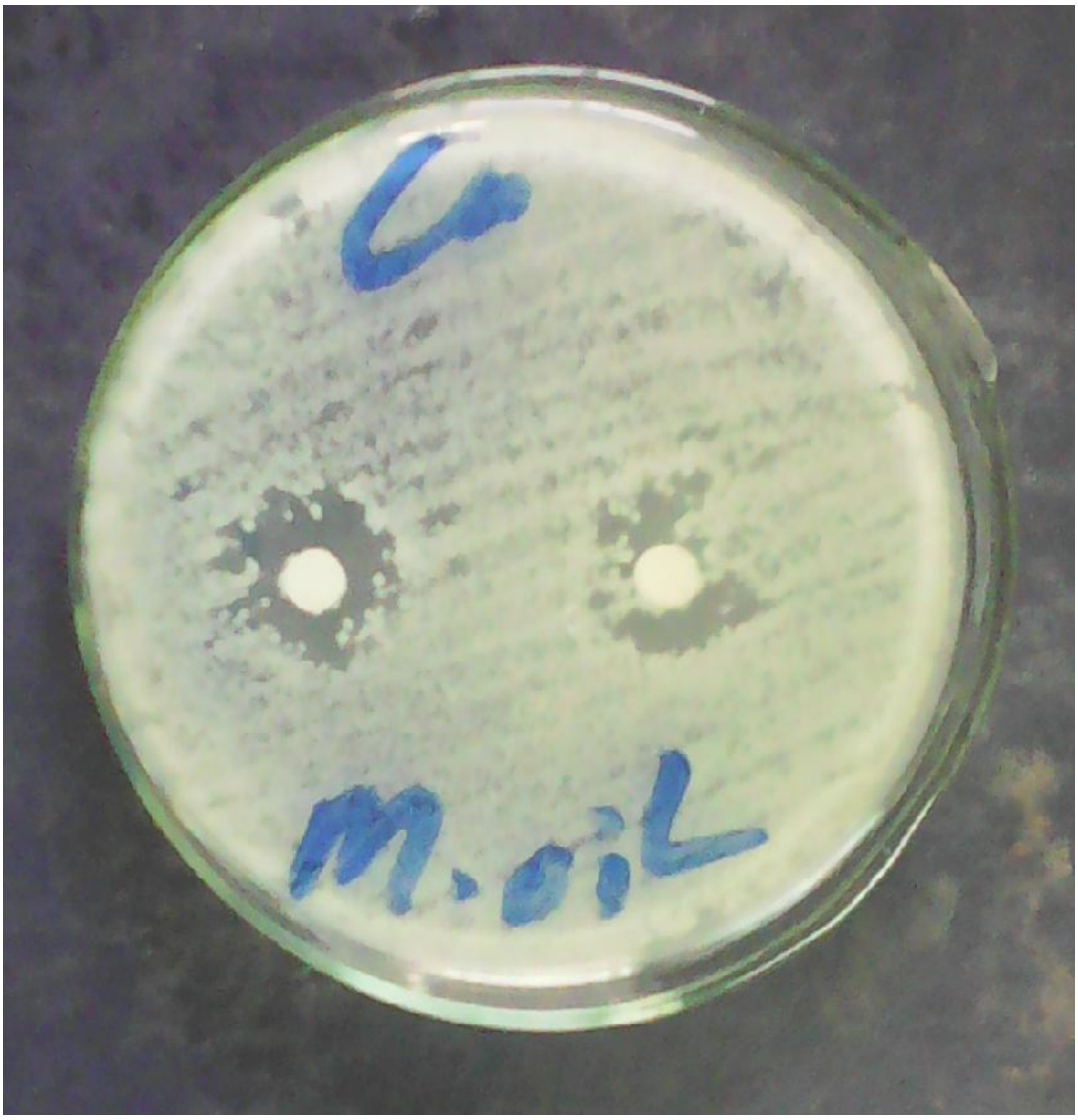


Fig. 2

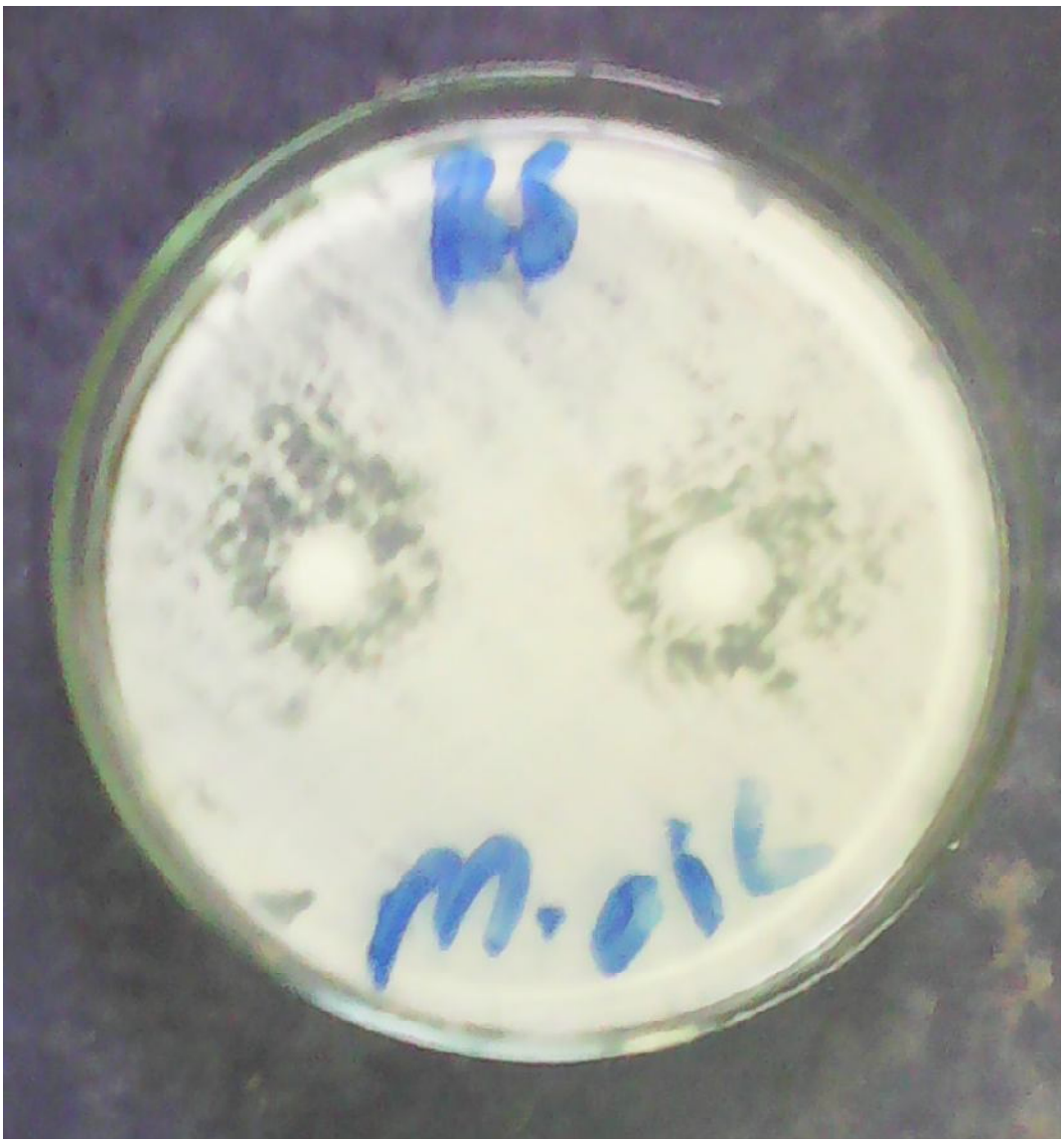


Fig. 3

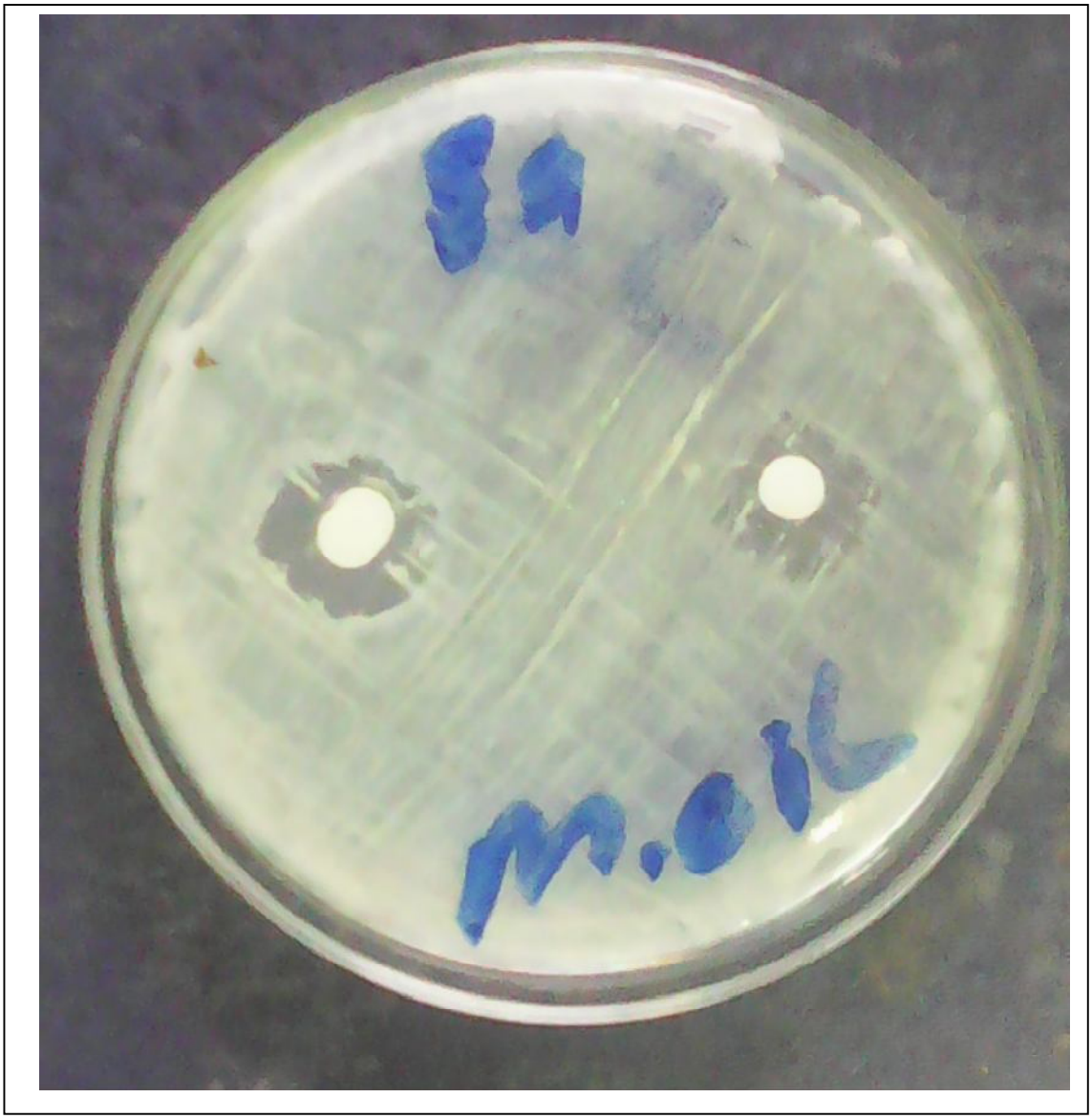


Fig.4

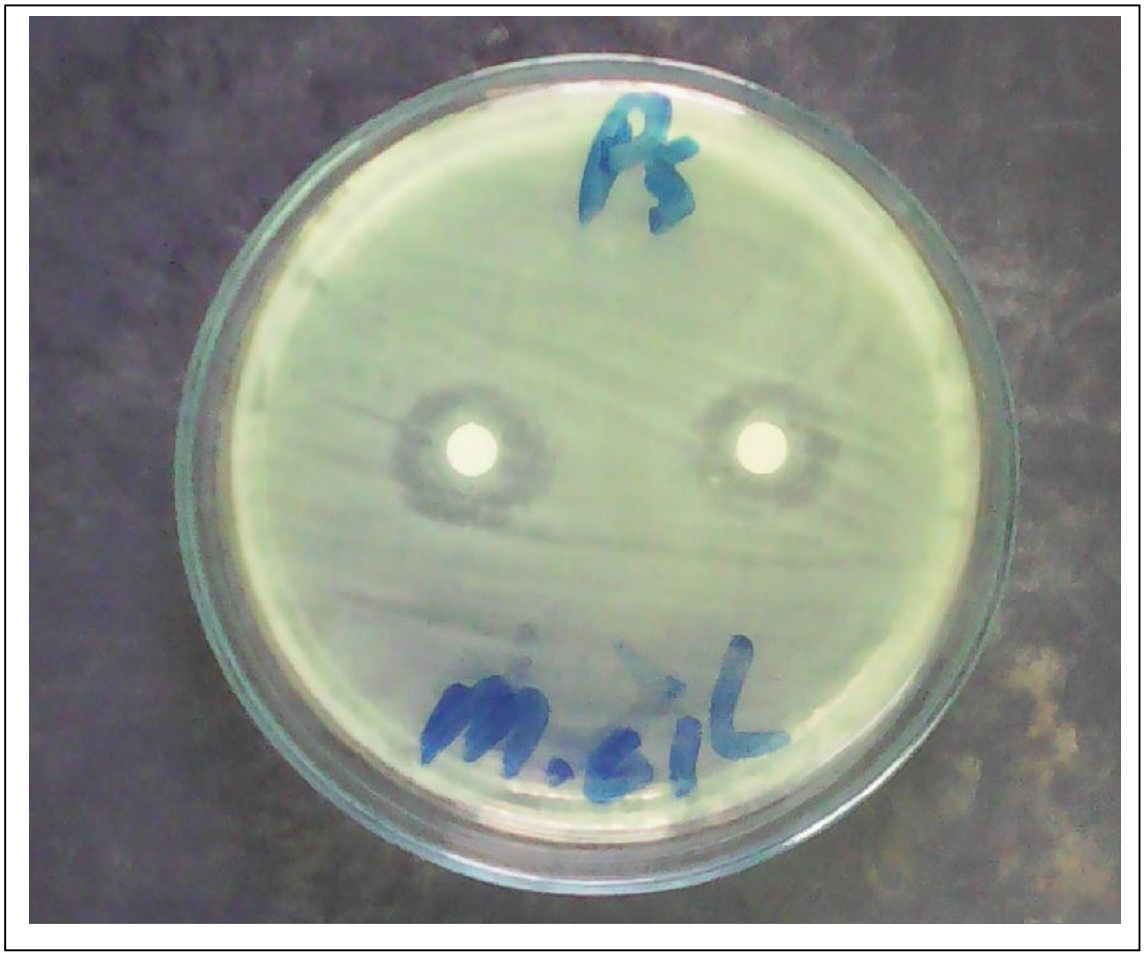


Fig. 5

2.5. Infrared spectra (IR)

Instrumentation

IR spectra were reoded in K BR or per Kim . Elmer spcrtroum BX series FT –IR spectrometer

Name of instrument FT-IR spectrometer

Make: thermo nicolet (IR-300) spectrometer

Date issue: 2006 USA

Scans : 8

Resolution 4.000

Aug. 19. 15:57:00. 2016 (GMT-07:00) oil sample

The sample is placed into the sample cup which is inserted into the photoacoustic cell which is then sealed for the measurement.

Chapter three

3. Results:

Among the 22 compounds identified by means of GC and CG/ MS analyses (Floris *et al*., 1996), cinnamaldehyde (79.3% w/w) and eugenol (11.9% w/w) are the main components, and some terpenic compounds, such us α– pinene and caryophyllene in a lesser proportion.

3.1. Table 3-1 (physical properties of cinnamon oil)

Odor	Sweet smell
Taste	sweet
Appearance	Pale yellow to green
Density	1.061 g/ml
Refractive index	1.6073
Specific gravity	
Usage	Flavor , perfume , antibiotics , cosmetics

3.2. Table 3-2 (chemical properties of cinnamon oil)

Acid value	1.122
Saponification value	179.52
Esterfication value	178.398
Free fatty acids	0.561

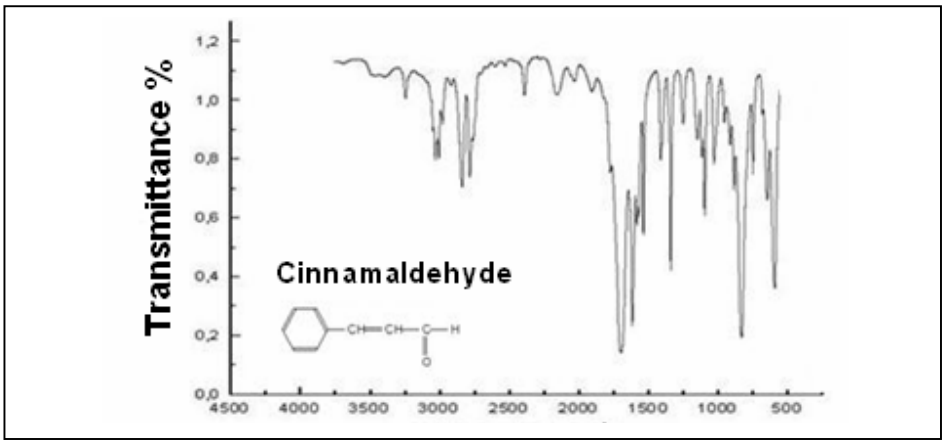
3.3. Table 3-3 (antimicrobial activity of cinnamon oil)

	antimicrobial activity by diffusion method				
	Bacteria				Fungi
	EC	PS	SA	BS	CA
Test (1)	-	16	16	11	11
Test (2)	-	15	14	11	10

3.4. **The infrared spectroscopy spectrum**

It displayed characteristic bands corresponding to aromatic CH bonds, between 3000 cm-1 and 3100 cm-1; to CH alquenes, between 3020 cm-1 and 3080 cm-1; to C=C, between 1640 cm-1 and 1680 cm-1; and to the aldehydes C=O group between 1690 cm-1 and 1760 cm-1. The spectra similarity of the cinnamon essential oil and a cinnamaldehyde (SDBSWeb at http://www.aist.go.jp/RIODB/SDBS) are shown in fig 6

Fig 6



3.5. Discussion

The percentage of extracted oil from CZ was found to be 0.642% w/v. This percentage, however, is comparatively lower than the corresponding extractions found in many researches done worldwide (which range from 0.98-1.1%). The reason why the extracted percentage in this work is lower may be due to the fact that the sample from which the oil was extracted was not stored properly and for long period of time.

When compared with sesame oil, the saponification value of other oils such as sunflower, olive and corn oils which were found to be 192.5, 190 and 190, respectively. That means the cinnamon oil result agree with the standard values.

The acid value and the free fatty acid are virtually similar to standard results. However, its rancidity which is 1.1 is greater than 0.6, and hence limits its household use.

Comparing infrared spectrum of cinnamon essential oil and the individual spectra of the main compounds, i.e., cinnamaldehyde and eugenol (database of SDBS) virtually superimposed, both spectra are considerably similar despite of IR-device power and resolution. This could result from the high content of cinnamaldehyde as well as from the presence of eugenol in the essential oil analyzed.

Antimicrobial activity among the three bacteria tested, *PS* was found to be the most sensitive towards C*innamomum*

essential antimicrobial activity, followed by SA. Unlike for other bacteria tested, here *C. zeylanicum* developed bigger zone of inhibition at the highest concentration 10% -which is used in the experiment. However, *EC* showed the least sensitivity, almost none. The results of this work were found to be consistent with the work done by (Hoque et al. (2007)) who showed different effective concentration of essential oil of cinnamon against *Staphylococcus aureus,* and (Bowels et al. (1995)) who showed that the essential oil of cinnamon inhibit the growth of *Staphylococcus aureus* but against *(*Friedman et al. (2002)) who found that cinnamon oil was effective against *E. coli*. These findings are also quite similar with the results of (Chao et al. (2000))reporting that cinnamon oil fully inhibited the growth of some gram positive and gram negative bacteria, fungi and yeasts

The results of this study are showed the *Cinnamomum zeylanicum,* essential oils had higher inhibitory effect on gram positive , negative bacteria and fungi.
according to literature reviewed , the antibacterial activity has been attributed to the presence of some active constituents in the essential oils, the antibacterial activity of cinnamon was probably due to their major component, cinnamaldehyde and their constituents is also known to

inhibits bacterial *acetyl CoA carboxylase* enzyme and responsible for major antibacterial activity

The anti-microbial action is considered to arise mainly from the potential of hydrophobic essential oils to obstruct the bacterial cell membrane and its structures which leads to ion leakage and thus cell microbial death.

This could be explained by their hydrophobicity, an important characteristic that exists in EO and their fractions, and may allow them to partition the lipids of the bacterial cell membrane, turning them more permeable and leading to leakage of ions and other cell constituents

3.5. Conclusion

The percentage of CZ oil content was found to be 0.642%w/v. the acid value of oil was twice as sesame oil . by comparison , the saponification value was similar to sesame oil ,sunflower oil and corn oil.

IR spectra showed similar ranges as standard cinnamaldehyde reflecting the high content of cinnamaldehyde as a main constituent of CZ oil.

Antimicrobial study indicated the importance of both essential oil in diseases treatment to reduce drug resistance in microorganisms (given the fact that inhibition zone greater than 7 mm is considered positive results) .These herbs behave as antioxidant fight free radical in the body. In fact these herbs are very useful in pharmaceutical industries.

3.6. Recommendation

Further studies is needed using more sophisticated devices to know more about CZ EO minor components to investigates more important values and uses .

The CZ antimicrobial activity is now proved to be of paramount importance. however, further studies is needed to determine the doses and toxic concentrations to aid its use as future alternatives to the current antibiotics for which most bacteria and fungi have started to show variable resistances.

3.7. References:

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Appendix :