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INTERACTION OF TERPENES AND OXYGENATED TERPENES WITH SOME DRUGS

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Supervisors

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Declaration

I hereby declare that this dissertation, submitted to the University of Fort Hare (UFH) for the award of Masters of Science (M.Sc.) in Chemistry, in the Faculty of Science & Agriculture, School of Science and the work contained therein, is my original work with exemption to the available citations. I further declare that this present work has not been submitted at any university, in partial or in its entirety for the award of any degree.

Name: _____

Signature: _____

Date: _____

Acknowledgements

If I was dead, either at the inception of this academic program, half-way into it or even at the very end, I would not have been able to write all that is contained in this work. That special gratitude goes to the giver of life, the Almighty God and my Savior, Jesus Christ. I am indeed, grateful to the ability He gave to me to reach, thus far.

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Dedication

To my lovely and beautiful wife, MRS. OLUWAPONMLE DEBORAH AJAYI and my angelic daughter, MISS LERATO REBECCA AJAYI. I love you guys and nothing is to be compared with you.

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List of abbreviations

CFU	Coliform forming unit
EDA	Ethylene diamine
EI	Electron ionization
Et	Ethyl
EUCAST	European Committee for Antimicrobial Susceptibility Testing
GC MS	Gas chromatography – mass spectrometry
HOMO	Highest occupied molecular orbital
HD	Hydrodistillation
LUMO	Lowest unoccupied molecular orbital
MAE	Microwave-assisted extraction
Me	Methyl
MIC	Minimum inhibitory concentration
PTFE	Polytetrafluoroethylene
SFME	Solvent-free microwave extraction
TCNE	Tetracyanoethylene
TLC	Thin layer chromatography

List of Journals Abbreviation

AA	Anaerobe
AAM	Advances in Applied Microbiology
ABB	Archives of Biochemistry and Biophysics
ABC	Analytical and Bioanalytical Chemistry
ACA	Analytica Chimica Acta
ACR	Accounts of Chemical Research
ACS	Acta Chemica Scadinavica
ADD	Advanced Drug Delivery Reviews
ADOC	Advances in Organometallic Chemistry
AGE	Angewandte Chemie, International Edition
AJB	African Journal of Biotechnology
AJP	African Journal of Pure and Applied Chemistry
AN	American Naturalist
AOB	Archives of Oral Biology
AP	Acta Pharmaceutica
ARJC	Arab Journal of Chemistry
AS	Archives of Surgery
AX	Acta Crystallographica
BMB	Biochemistry and Molecular Biology International
BMS	Biological Mass-Spectrometry
CB	Chemistry and Biodiversity

CES	Chemical Engineering Science
CJC	Canadian Journal of Chemistry
CLC	Clinical Chemistry
CMI	Clinical Microbiology and Infection
CPB	Chemical and Pharmaceutical Bulletin
CPL	Chemical Physics Letters
CRV	Chemical Reviews
EC	Euro Cosmetics
FC	Food Chemistry
FCT	Food and Cosmetics Toxicology
FFJ	Flavor and Fragrance Journal
FJA	Fresenius Journal of Analytical Chemistry
FM	Food Microbiology
FT	Fitoterapia
HH	Herba Hungarica
IC	Inorganic Chemistry
ICA	Inorganica Chimica Acta
IJF	International Journal of Food Microbiology
IJG	International Journal of Geriatric Psychiatry
IJM	International Journal of Molecular Sciences
IJP	International Journal of Pharmaceutics
IJPS	Iranian Journal of Pharmaceutical Sciences

JA	Journal of the American Chemical Society
JAB	Journal of the Applied Bacteriology
JAC	Journal of Antimicrobial Chemotherapy
JAF	Journal of Agricultural and Food Science
JAM	Journal of Applied Microbiology
JAS	Journal of Applied Science Research
JB	Journal of Burns
JBC	Journal of Burn Care and Rehabilitation
JC	Journal of Chromatography
JCE	Journal of Chemical Education
JCEL	Journal of Chemical Ecology
JCR	Journal of Controlled Release
JCSP	Journal of the Chemical Society of Pakistan
JEO	Journal of Essential Oil Research
JFS	Journal of Food Science
JEO	Journal of Essential Oils Research
JEP	Journal of Ethnoparmacology
JH	Journal of Hygiene (Cambridge)
JIC	Journal of the Iranian Chemical Society
JME	Journal of Medical Entomology
JMM	Journal of Medical Microbiology
JPC	Journal of Physical Chemistry

JSC	Journal of the Society of Cosmetic Chemistry
JSF	Journal of Supercritical Fluids
JT	Journal of Trauma
LAM	Letters in Applied Microbiology
MB	Microbiology
MC	Monatshefte für Chemie
MI	Miscellaneous
MJ	Microchemical Journal
MOL	Molecules
NFJ	Nigerian Food Journal
NL	Nano Letters
NT	Nursing Times
OM	Organometallics
PAH	Pharmaceutica Acta Helvetica
PC	Phytochemistry
PCCCP	Physical Chemistry Chemical Physics
PF	Perfumer and Flavorist
PGR	Plant Growth Regulation
PH	Pharmazie
PLM	Plasmid
PM	Planta Medica
PMP	Plantes Medicinales Phytotherapia

PR	Phytotherapy Research
PRC	Pharmacology Research Communications
PRCL	Proceedings of the Royal Chemical Society, London
RA	Risk Analysis
RCR	Russian Chemical Reviews
RIC	Rassegna Internazionale di Clinica e Terapia
RIE	Rivista Italiana EPPOS
RJG	Russian Journal of General Chemistry
RNM	Reviews of National Medicine
SA	Spectrochimica Acta
SGO	Surgery Gynecology and Obstetrics
SP	Surgical Practice
SPC	Soaps, Perfumery, and Cosmetics
SPH	Scientia Pharmaceutica
TAC	Trends in Analytical Chemistry
TL	Tetrahedron Letters
USP	U. S. Pharmacist
WHO	World Health Organization
WJC	World Journal of Chemistry
ZN(C)	Zeitschrift für Naturforschung C A Journal of Biosciences

Abstract

SFME and HD for the extraction of essential oil in *Lavandula officinalis* in Alice have been reported. A total of 59 compounds were identified with the major compound being 1,8-cineole, an oxygenated monoterpene, with 46.89% and 44.84% yield obtained for HD and SFME respectively.

Charge transfer (CT) complexes formed between α-pinene, 1,8-cineole and camphor as electron donors with iodine as the electron acceptor have been studied spectrophotometrically in methylene chloride solution. The Benesi-Hildebrand equation has been applied to estimate the formation constant (K_f) and molecular extinction coefficient (ε_{CT}). The value of K_f is the highest in camphor-I_2 complex compared to the other two complexes.

Antibacterial assessment was carried out on the various reagents, determining the MIC of individual reagents and in combination. The results show an improvement, on combination of the various reagents than when tested alone.

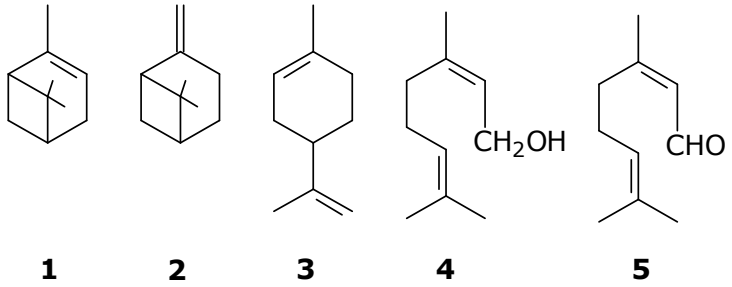
I. Introduction

Interaction means a kind of action that occurs when two or more objects have an effect upon one another. In nature, substances interact one with another to give a product, which sometimes and in most cases have properties and characteristics, different from the initial substances coming together. In this particular context, physico-chemical interaction refers to the charge-transfer formation between terpenes and oxygenated terpenes with some drugs. The research is sequel to the honors project work, where two isolation methods were employed and compared for the extraction of essential oil from *Lavandula offinalis*, in which a total of 59 compounds were identified with the major compound being 1,8-cineole, an oxygenated monoterpene, with 46.89% and 44.84% obtained for HD and SFME extraction techniques, respectively.

A. Terpenes

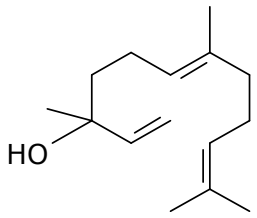
Terpenes are biogenic hydrocarbons present in large amounts in conifers and in smaller amounts in large variety of other plants (06MI1). Structurally, terpenes may be regarded as derivatives of isoprene (2-methylbutadiene). Monoterpenes ($C_{10}H_{16}$) predominate among the volatile terpenes. Sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$) and triterpenes ($C_{30}H_{48}$) have been identified in large numbers in plant material. The chemical properties of the terpenes depend much on the presence of one or more olefinic double bonds in structure. Terpenes are widespread in nature, main-

ly in plants as constituents of essential oils. Many of them are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones (terpenoids) are also found. When terpenes are modified chemically by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Terpenes and terpenoids are the primary constituents of the essential oils of many plants and flowers. Essential oils are widely used as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. Examples of monoterpenes are α-pinene **1**, β-pinene **2**, limonene **3**, nerol **4**, citral **5**, 1,8-cineole **6**, camphor **7**, menthol **8**, geraniol **9**, and others. Examples of sesquiterpenes are nerolidol **10** or farnesol **11**. Examples of diterpenes are phytol **12** or vitamin A1 **13**. Squalene **14** is an example of a triterpene, and carotene (provitamin A1) **15** is a tetraterpene.



1 **2** **3** **4** **5**

6 **7** **8** **9**



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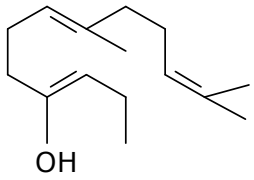

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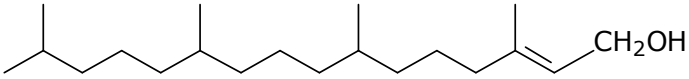

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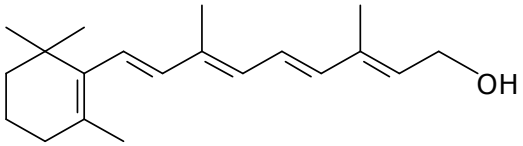

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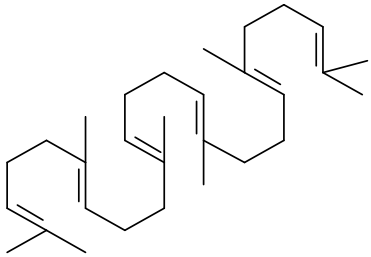

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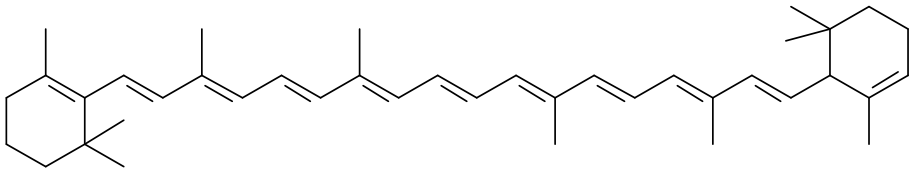

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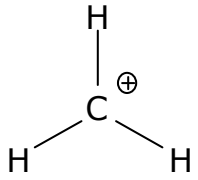
The terpenes used in this study are α-pinene **1**, 1,8-cineole **6**, and camphor **7**.

1. α-Pinene

α-Pinene is an organic compound of the terpene class. It is an alkene and it contains a reactive four-membered ring. It is found in the oils of many species of many coniferous trees, notably the pine. It is also found in the essential oils of rosemary (*Rosmarinus officinalis*) and lavender (*Lavandula officinalis*). It is a monoterpene that may be a significant factor affect-

ing bacterial activities in nature. It is a clear, colorless liquid with the molecular formula $C_{10}H_{16}$, having the molar mass of 136.23 g/mol. It has a very low solubility in water.

α-Pinene is a very reactive reagent as a result of its four-membered ring. The reactivity of α-pinene is described in Scheme 1. The four-membered ring in α-pinene makes it a reactive hydrocarbon, prone to skeletal rearrangements such as the Wagner-Meerwein rearrangement. Wagner-Meerwein rearrangement is a class of carbocation 1,2-rearrangement reactions in which a hydrogen, alkyl or aryl group migrates from one carbon to a neighboring carbon (00JCE858). A carbocation is an ion with a positively-charged carbon atom. Such a structure **16** plays the role of reactive intermediates in many organic reactions. Wagner-Meerwein rearrangement was first discovered in bicyclic terpenes, for example (Scheme 2), the conversion of *iso*-borneol to camphene (07MI1).



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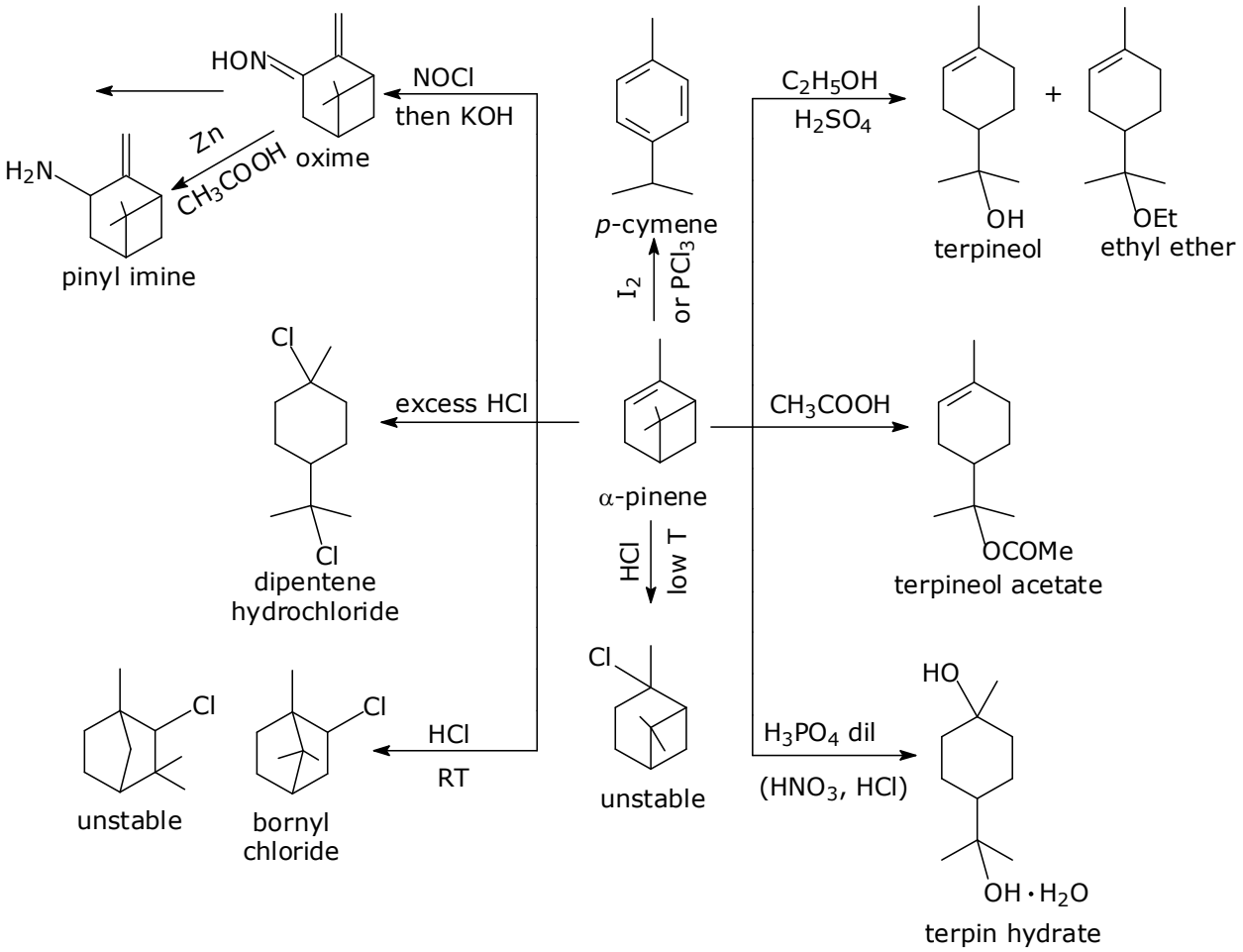
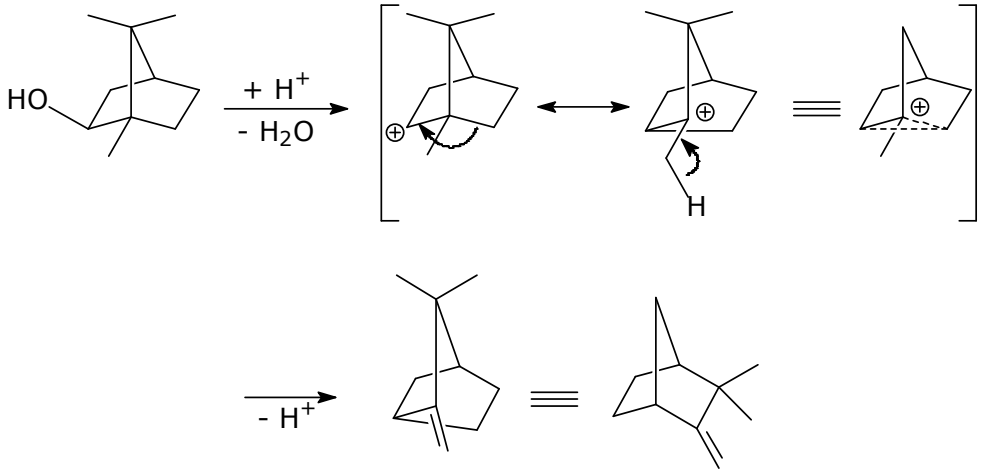


Scheme 1



Scheme 2

2. 1,8-Cineole

1,8-Cineole, **6**, the monoterpene cyclic ether known as eucalyptol, is one of the components in essential oils from *Lavandula officinalis*. It is a terpenoid oxide present in many plant essential oils and it was found to display an inhibitory effect on some types of inflammation in rats (00PR240). This component has a characteristic fresh and camphoraceous fragrance and pungent taste and is used for pharmaceutical preparations as an external applicant, a nasal spray, a disinfectant, an analgesic, or a food flavoring. It is also used for cosmetics. Furthermore, it has been reported that 1,8-cineole is used to treat cough, muscular pain, neurosis, rheumatism, asthma, and urinary stone (55RIC577, 99MI1). Although it can be used internally as a flavoring and medicine ingredient at very low doses, eucalyptol is toxic if ingested at higher than normal doses (MSDS). Eucalyptol forms crystalline adducts with halogen acids, *o*-cresol, resorcinol, and phosphoric acid. Formation of these adducts are useful for purification. Cineole-based *eucalyptus* oil is used as a flavoring at low levels (0.002%) in various products, including baked goods, confectionery, meat products, and beverages (01MI1). Eucalyptol is used as an insecticide and insect repellent (87JEC2131).

3. Camphor

Camphor **7** is a waxy, white, or transparent solid with a strong, aromatic odor (94MI1). It is a terpenoid with the chemical formula $C_{10}H_{16}O$. It was found in the wood of the camphor laurel (*Cinnamomum camphora*), a large

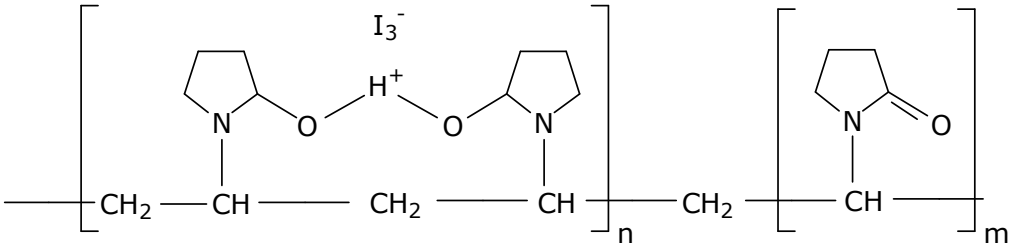
evergreen tree found in Asia. Camphor can be produced from α-pinene, which is abundant in the oils of coniferous trees and can be distilled from turpentine. It is used an insect repellent, in the manufacture of films, plastics, lacquers, explosives, and in medicine, mainly in external preparations to relieve mild pain and itching.

4. Drugs

Drugs are substances of natural or synthetic origin, which can alter the emotional state, perception, body functioning or behavior of an organism. They are substances used to treat or prevent diseases, relieve pains, control mental or physical ailments and to help diagnose illnesses. Broadly speaking, it is any substance which, when absorbed into the body of a living organism, alter normal bodily function (69WHO1). In pharmacology, a drug is "a chemical substance used in the treatment, cure, prevention, or diagnosis of disease or used to otherwise enhances physical or mental well-being" (07MI2). Without drugs, many people would not live as long as they do or enjoy life from diseases and pains. The drugs that are referred to in this study are betadine (iodine-based), resorcinol (a polyphenol distributed in South Africa as eskamel) and colloid silver modeled as silver perchlorate in this work. All these have been proven to be active as antiseptics or bactericides.

In one way or another, many of us have probably used an iodine-based disinfectant to clean a minor skin wound. Betadine cream is a brand of con-

sumer-available povidone-iodine topical antiseptics. It is used in treating minor wounds and infections, as well as killing bacteria. It is an antiseptic combination, working by killing sensitive bacteria. The ointment involves a stable complex of polyvinylpyrrolidone (povidone) and elemental iodine **17** (11SP79). It contains from 9.0% to 12.0% available iodine, calculated on a dry basis.



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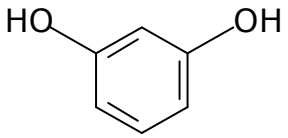
Free iodine, slowly liberated from the povidone-iodine complex in solution, kills eukaryotic or prokaryotic cells through iodination of lipids and oxidation of cytoplasmic and membrane compounds. This agent exhibits a broad range of microbicidal activity against bacteria, fungi, protozoa, and viruses. Slow release of iodine from the complex in solution minimizes iodine toxicity towards mammalian cells. The Betadine used was purchased in one of the local pharmaceutical shop in the University of Fort Hare environment.

Resorcinol (or resorcin) **18** is a dihydroxybenzene. It is the 1,3-isomer of benzenediol with the formula $C_6H_4(OH)_2$. It is a white crystalline compound with a weak odor and a bittersweet taste (00MI1). Resorcinol can be re-garded as a phenol derivative in which a hydrogen atom is substituted by a hydroxyl group in the *meta*-position with respect to the OH.



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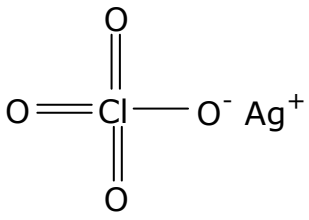
Resorcinol exists in at least two crystalline modifications (phases). At normal pressure, the α-phase is stable below about 71°C, whereas the β-phase is stable above that temperature up to the melting point (00MI1). Crystalline resorcinol is hygroscopic. The water solubility data indicate that resorcinol is almost completely miscible in water. Resorcinol is an important chemical intermediate in the manufacture of specialty chemicals, such as hexylresorcinol, *p*-aminosalicylic acid, and light screening agents for the protection of plastics from exposure to UV light. Other uses include the manufacture of dyestuff, pharmaceuticals, flame retardants, agricultural chemicals, fungicidal creams and lotions, explosive primers, antioxidants, a chain extender to urethane elastomers, and a treatment to improve the mechanical and chemical resistance of paper machine fabrics (00MI1). Resorcinol is used in pharmaceutical preparations for the topical treatment of skin conditions such as acne, seborrhoeic dermatitis, eczema, psoriasis, corns, and warts. An emerging use of resorcinol is as a template molecule in supramolecular chemistry. The –OH groups on resorcinol form hydrogen bonds to target molecules holding them in the proper orientation for a reaction. Many such reactions are able to be carried out in the solid state thereby reducing or eliminating the use of solvents that may be harmful to the environment.

Silver perchlorate is a chemical compound with the formula $AgClO_4$, **19**. It is a white solid, forms a monohydrate, and is mildly deliquescent. It is a source of the Ag^+ ion, although the presence of perchlorate presents risks.



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Silver ions and silver compounds show a toxic effect on some bacteria, viruses, algae and fungi, typical for heavy metals like lead or mercury, but without the high toxicity to humans that are normally associated with these other metals. Its germicidal effects kill many microbial organisms in vitro, but testing and standardization of silver products is difficult (07JAC987). Silver is widely used in topical gels and impregnated into bandages because of its wide-spectrum antimicrobial activity. The anti-microbial properties of silver stem from the chemical properties of its ionized form, Ag^+. This ion forms strong molecular bonds with other substances used by bacteria to respire, such as molecules containing sulfur, nitrogen, and oxygen (92LM72). When the Ag^+ ion forms a complex with these molecules, they are rendered unusable by the bacteria, depriving them of necessary compounds and eventually leading to the bacteria's death. Silver perchlorate is used to model the effects of colloidal silver, a well-known bactericide, antiseptic, and antimicrobial agent.

This research work centers on investigating the charge-transfer complexation between iodine and various donor compounds spectrophotometrically in dichloromethane, interactions of the donors with phenol by hydrogen bonding as well as formation of the molecular complexes with silver perchlorate. Extensive work has been done on the charge transfer complexation with iodine and various donor compounds. Very little work has been done on charge transfer complex formation involving iodine and pure compounds from essential oil, acting as the electron donors.

5. Objectives of the research

To determine and analyze the various chemical components of lavender, using two different extraction techniques, hydrodistillation and solvent-free microwave extraction and, identifying the oil components, using GC-MS.

To study the charge transfer complexation, spectrophotometrically between iodine and major extracted components α-pinene, camphor, and 1,8-cineole, including stability constants of the complex and kinetics of complex formation.

To study the hydrogen bonding formation between phenol and aforementioned components of the medicinal plant essential oils including evaluation of the basic thermodynamic functions $\Delta H°$, $\Delta S°$ and $\Delta G°$, determination of the stability constants.

To synthesize and characterize solid complexes of α-pinene, camphor, and 1,8-cineole with silver perchlorate.

To assess the antimicrobial activities of α-pinene, camphor, and 1,8-cineole on interaction both with model substances (molecular iodine, phenol, resorcinol, and silver perchlorate) and with betadine, eskamel, and colloidal silver in order to assess whether the effect of their interactions is antagonistic, synergistic, or indifferent on tested bacteria.

II. Review of Literature

A. *LAVANDULA OFFICINALIS*

Africa, and especially Southern Africa, has a rich diversity of plants. Recent statistics show that about 25% of the total number of higher plants in the world is found in Africa south of the Sahara. According to the African plant checklist and database project (06MI2), a total of 50,136 angiosperm taxa occur in tropical Africa and southern Africa. This figure is based on a recent merging of two major data bases EPFAT (Enumeration des plantes a fleurs d 'Afrique) and FSA (Flora of Southern Africa). The combined checklist shows a total of 32,424 taxa in tropical Africa and 22,755 taxa in southern Africa. A useful reference book on the medicinal plants of the whole African continent (00MI2) lists more than 5,400 medicinal plant taxa and over 16,300 medicinal uses. About 3,000 medicinal plants are regularly used in southern Africa (00MI3, 05JEP127). An annotated checklist of traditional medicinal plants of southern Africa (02MI1) gives a total of 3,481 plant taxa, of which 2,942 are administered to people only.

One of such plants that found its use in medicinal and household use is *Lavandula officinalis* (Fig. 1). Known as the true lavender, other synonym for this species is *Lavandula angustifolia*. *Lavandula officinalis* is a flowering plant in the family *Lamiaceae*, primarily in the Pyrenees and other mountains in northern Spain. The thin, lanceolate leaves have a characteristic grey-green color. When touched, the leaves emit an aromatic, pleasant fra-

grance. However, the plant is best known for the fresh fragrance of its flowers, which bloom with 6 to 10 blossoms on terminal spikes. *Lavandula* is probably reference to its use as an alternative to bathing; *angustifolia* means narrow leaf. Cosmetic and therapeutic applications of lavender date back at least to the time of the ancient Greek (00MI4, 00MI5). It is one of the most useful medicinal plants. Commercially, lavender, as it is normally called, provides several important essential oils to the fragrance industry, including soaps, colognes, perfumes, skin lotions and other cosmetics (04JEO347). In food manufacturing, lavender essential oil is employed in flavoring beverages, ice cream, and candies, baked goods and chewing gum (02JC(A)31). Recently, aromatherapy has become increasingly popular, and lavender is used in aromatherapy as a relaxant (99PM700, 99PR540). Many members of the botanical family *Lamiaceae* produce considerable amount of essential oils. Within this family, the genus *Lavandula* comprises 30 known species among which three are economically important: *Lavandula angustifolia*, *Lavandula latifolia*, and the hybrid *lavandin L. angustifolia* x *L. latifolia*. The essential oil of the highest quality is distilled from the flowering tips of *L. angustifolia*, the true lavender, and its characteristics scent has been prized since ancient times. Lavender is regarded as a pharmaceutical plant with predominantly sedative effects employed in aromatherapy (07ABB417).

Figure 1: *Lavandula officinalis*

1. Chemical constituents and various uses of Lavandula officinalis

The chemical composition of lavender essential oil depends largely on the species from which it was obtained (69SPC883, 75PAH373). Within the same species, the biochemical contents of the essential oil found in the flowers, stem, or leaves differs significantly depending on where and under what conditions the plant was grown (87PC995, 96PGR277). The presence and concentration of certain chemical constituents also fluctuates according to the season (95FFJ93) and the maturation of the plant when harvested (97AP133). Even the extraction process used to collect the oil introduces variation in the concentrations of biochemical compounds present in the distillate. Steam-distilled extracts have a characteristically higher ratio of α-terpineol and linalool to linalyl acetate compared to supercritical fluid extracts (95JAF1654, 96FFJ157, 06ACA157). Burning lavender oil does not affect its composition, implying that inhaling smoke from lavender aromatherapy candles may have the same impact as inhaling the vapor of the un-

heated essential oil (93JPS660). The outline below shows the potentially active chemical components in lavender (10JC(A)1530):

- Monoterpenes: α-pinene, β-pinene, β-ocimene, camphene, limonene, *p*-cymene, sabinene, terpinene.
- Monoterpene alcohols: α-terpineol, borneol, lavandulol, linalool, *p*-cymen-8-ol, *trans*-pivocarveol.
- Monoterpene aldehydes: cumin aldehydes
- Monoterpene ethers: 1,8-cineole (eucalyptol)
- Monoterpene esters: linalyl acetate, terpenyl acetate
- Monoterpene ketones: carvone, coumarin, cryptone, fenchone, methylheptenone, *n*-octanone, nopinone, *p*-methylacetophenone
- Benzenoids: eugenol, coumarin, cavacrol, hydroxycinnamic acid, rosmarinic acid, thymol.
- Sesquiterpenes: caryophyllene, caryophyllene oxide, α-photosantanol, α-norsantalenone, α-santalal.

Trace components of many other compounds, such as flavonoids, have been identified (86FT199, 97AP133, 04PF53).

Although the chemical composition of the oils is complex, the biological activities of some of the major chemical species present have been evaluated. Linalyl acetate and linalool have sedative (91ZN(C)1067, 98ACA93) and local anesthetic effects (99PM700). Linalool also has antibacterial (88PRC77, 97MB39, 99LAM130), antifungal (97MB39, 98JAF1739), and in-

secticidal (88JME1, 98RNM178) effects. These two compounds are the most prominent chemical constituents in the essential oil of *L*. *angustifolia*, accounting for up to 90% of the oil by volume (84PF53, 94MI2). Linalool also comprises over 70% of the essential oil of *L*. *hybrid*, a species commonly used by the perfume and pharmaceutical industries (89HH37, 93NT32). Relative concentrations of *linalyl acetate* and *linalool* are lower in *L. latifolia* (99USP24), and their concentrations range as 5–70% in *L. dentate* (76PH650, 84PF53, 90PH69). Following topical application of the essential oil of *L. angustifolia*, linalyl acetate and linalool can be detected in the blood within 5 minutes, peak at 19 minutes, and are cleared within 90 minutes (75FCT653, 92JSC49). They are also detected in the blood following inhalation of lavender oil (90FJA922, 91ZN(C)1067) and in exhaled air following massage (91JCEL499, 04EC30, 05SPH27).

1,8-Cineole has antispasmodic (90PH69) and antifungal (94PC139, 97MB39) properties. It comprises over 50% of the essential oil of *L. dentate* (84PF53, 91BMS801). 1,8-Cineole has also been detected in *L. angustifolia*, *L. latifolia,* and *L. hybrida*, although in much lower concentrations (69SPC739, 94FFJ11, 94MI2). Eugenol also has spasmolytic activity (84AOB611), as well as local anesthetic effects (84AOB611, 91AN137, 99PM700). The antibacterial properties of *L. angustifolia* have partially been attributed to the action of eugenol, which is also found in *L. latifolia* (69SPC739, 97JAC305) and in rosmarinic acid (99JAF2937), and it has also

been detected in *L. hybrida* (94MBI809). Other constituents of lavender with antibacterial effects include α-terpineol and terpene-4-ol, found in *L. dentate*. *L. latifolia* and *L. hybrida* (69SPC739, 90P69, 97AP133), and camphor present in each but found in high concentrations in *L. latifolia* compared to the other varieties (69SPC739). α-Pinene, 1,8-cineole, β-pinene, and *p*-cymene have some antifungal activity (98FFJ98, 98JAF1739). Terpineol, α-pinene, and camphene have anti-lice activity (98RNM178). Coumarin found in *L.angustifolia*, *L. latifolia* and *L. dentate* (79PH564, 84PF53, 94MI2) and caryophyllene oxide found in *L. latifolia* and *L. angustifolia* (84PF53, 94MI2) have anti-inflammatory effects (90CPB2283).

A total of 59 compounds were identified in the leaves of *Lavandula officinalis*, growing in Alice, Eastern Cape, South Africa, with the major being 1,8-cinole, an oxygenated monoterpene, with 46.89% and 44.84% obtained for HD and SFME respectively. The chemical constituents of the essential oil of flowers of *Lavandula officinalis* growing in Isfahan, Iran, found using TLC and gas chromatography-mass spectrometry (GC-MS) methods, include 12 components constituting 94.8% of the examined oil: linalool (34.1%), 1,8-cineole (18.5%), borneol (14.5%), camphor (10.2%), terpineol (4.5%), linalyl acetate (3.7%), α-bisabolol (3%), α-terpineol (2.2%) and (Z)-β-farnesen (2.2%).

The data on chemical composition of lavender in various countries (Table 1) show a diversity of different components. In most cases, linalool is the

principal component, including lavender collected in Pakistan (11PJB1315). However, fenchone, 1,8-cineole, and pinene types occur in some cases.

Table 1: Chemical Composition of Lavender Samples Collected in Various Countries

Component	Percentage	Method of ex-traction	Country	Reference
Borneol 1,8-Cineole	11.3 25.7	HD	Iran	11AJB196
1,8-Cineole Linalool Camphor Borneol	18.5 34.1 10.2 14.5	HD	Iran	06IJP169
1,8-Cineole Linalool Camphor Borneol	7.23 47.8 11.8 4.15	MASD	France	06ACA157
1,8-Cineole Linalool Camphor Borneol	7.29 46.9 10.2 4.07	SD	France	06ACA157
1,8-Cineole Fenchone Camphor Virdiflorol	8.71 37.5 17.1 7.38	HD	Algeria	11CB937
α-Pinene β-Pinene 1,8-Cineole Linalool Pincarveol	8.38 30.1 5.47 4.46 8.59	HD	Morocco	10WJC103
Sabinene 1,8-Cineole	13.89 41.28	HD	Morocco	09IJA113
Linalool Linalyl acetate	26.9 22.8	HD	Greece	10ICP
1,8-Cineole Camphor α-Pinene Linalool	34.6 13.4 9.00 7.90	HD	Portugal	11JMM

B. CHARGE-TRANSFER COMPLEXES

A charge-transfer complex is any aggregate (containing two or more molecules, molecular entities, or functional groups) in which an electric charge is transferred from a donor to an acceptor. The concept of charge-transfer was introduced by Mulliken (52JPC801) as a description of complex formation in which new electronic absorption bands, attributable to neither the donor, nor the acceptor is observable. The formation of electron donor-acceptor (EDA) charge-transfer complexes between two molecules involves the transfer of an electron from the highest occupied molecular orbital (HOMO) of the donor to the lowest unoccupied molecular orbital (LUMO) of the acceptor (98MJ175). Charge–transfer complexes are molecular complexes formed by the weak interaction between electron donors and electron acceptors. An electron donor may be defined as a molecule possessing a relatively high localized electron density. Conversely, an electron acceptor is relatively deficient in electrons. Charge-transfer complexes are also termed electron donor-acceptor complexes. In electron donor-acceptor complexes, there is always a donor and an acceptor molecule. The donor may donate an unshared pair (an *n*-donor) or a pair of electrons in a π orbital of a double bond or aromatic system (a π donor). One of the tests for the presence of an EDA complex is the electronic spectrum. These complexes generally exhibit a spectrum (called a charge transfer spectrum), which is not the same as the sum of the spectra of the two individual molecules. Because the first excited

state of the complex is relatively close in energy to the ground state, there is usually a peak in the visible or near-UV region, and electron donor-acceptor complexes are often colored. These charge transfer complexes have unique absorption bands in the ultraviolet-visible region. The compositions of donor-acceptor complexes could not be isolated at ordinary temperatures in pure state since they are mostly unstable. However, they exist only in solutions in equilibrium with their components. The rates of formation of complexes in solution are generally so rapid that kinetic studies of the reactions cannot be made, at least by ordinary procedures. The values of heat of interaction are generally smaller than the forces of coordination and are much feebler than those established in the formation of covalent bonds (64MI1). That is, the degree to which electron transfer from the donor component to the acceptor component takes place is much less than that ordinarily occurs when new compounds are formed (52JPC801). Aromatic hydrocarbons are rich in π-electron and favor the formation of charge-transfer complexes with electron deficient molecules. This may be the due to the intermolecular attractive forces or through dipole-dipole interaction with electron deficient molecules.

Charge-transfer complexes exist in two states: a ground state and an excited state. In the ground state, the two molecules composing the complex undergo the normal physical forces expected between two molecules which are in close proximity to each other. These include London dispersion forces and any electrostatic interactions, such as between dipole moments. In ad-

dition, a small amount of charge is transferred from the donor to the acceptor. This contributes some additional binding energy to the complex (71MI1). The excited state of the complex occurs when the ground state complex absorbs a photon of light having appropriate frequency. In the excited state, the electron which had only been slightly shifted towards the acceptor in the ground state is almost totally transferred. Depending on the structural features of both the donor and acceptor, the wavelength of light absorbed may be in the visible range of the electromagnetic spectrum. In many cases, therefore, charge-transfer complexes are colored substances. Examples of such charge-transfer complexes include the complex formed between a metal ion and a π orbital of a double bond or an aromatic system (73CRV163, 76ADOC33), the complex formed between polynitro aromatics, such as picric acid (62RCR8) as well as *n*-orbital-containing molecules, and complexes of I_2 and Br_2 with amines, ketones, aromatics, *etc*. Phenols and quinones also form charge-transfer complexes (74MI1).

The ground state of the complex is described by the wave function, Ψ_N, which is the hybrid of two wave functions, $\psi(A, D)$ and $\psi(A^-D^+)$ (69MI2). The wave function $\psi(A,D)$ is termed as the no-bond function and represents the wave function of the two molecules in close proximity to each other but with no charge-transfer between them. $\Psi(A, D)$ may include, however, the normal electrical interactions between molecules. Consequently, the ground state wave function for a weak complex can be described as:

$$\Psi_N = a\Psi(A,D) + b\Psi(A^-D^+)$$

Here a>>b. The wave function $\Psi(A^-D^+)$ is called the dative function and represents two molecules held together by total transfer of an electron from the donor, D, to the acceptor, A. The excited state of the complex can then be described by:

$$\Psi_E = b^*\Psi(A^-D^+) - a^*\Psi(A,D)$$

Here b*>>a*.

Charge transfer complexes may conveniently be classified according to the types of orbitals in the donor and acceptor molecules which are undergoing the interaction. Donor and acceptor molecules may each be divided into three classes (03MI1), as shown in the Table 2 below. The *v*-acceptors refer to metal atoms possessing a low-lying vacant valence-orbital. Hypothetically, there are nine possible types of complexes. However in practice, the *n*-donors do not form complexes with metal ions but form covalent bonds instead (07MI3).

Table 2: Charge-Transfer Complexes: Donor and Acceptor Types

Donor type	Examples
σ	R-X, cyclopropane
n	R_2O, R_3N, pyridine, dioxane
π	Aromatic and unsaturated hydrocarbons, especially if containing electron releasing substituent (hexamethylbenzene or phenols)

Acceptor type	Examples
v	Ag^+, certain organometalics
σ	I_2, Br_2, ICl
π	Aromatic and unsaturated hydrocarbons, especially if containing electron withdrawing substituents (TCNE, halogenated quinones).

π-Donors include alkenes, alkynes, aromatic hydrocarbons and their substitution products. The adducts which they form are called π-complexes. Here the electron transfer takes place when a π-donor coordinates with an acceptor. The term 'outer complex' has also been used (52JPC801) in describing adducts of this kind to emphasize that the acceptor does not make deep penetration into the π-orbital.

n-Donors include a large group of substances in which there are non-bonded electrons (lone pairs) available for coordination. Alcohols, organic iodides and nitrogen bases are examples.

Complexes of a wide variety of inorganic acceptors have been reported (61MI1). Some of this type of acceptors include chlorine, bromine, iodine, iodine monochloride, oxygen, salt of copper (I), silver (I), and mercury (II), aluminum bromide and hydrogen halides.

π-Acids and other organic acceptors embrace a variety of organic compounds which function as dienophiles in the Diels-Alder reaction, such as ethylene with highly electronegative substituents, chloranil. Tetracyanoethy-

lene, polynitro aliphatic and aromatic substances, tetranitromethane in very dilute solution, nitrobenzene, 2,4,7-trinitrofluorenone, and 1,3,5-tricyanobenzene are some of the acceptors. Iodine, bromine, or even chlorine accepts electrons from both *n*-donors and π-donors. Such complexes are formed with amines, aromatic hydrocarbons, ketones, *etc*. This is the reason why iodine is not its normal purple color in solvents such as acetone, ethanol, and benzene. IBr and ICl also form complexes, in which the iodine end of the molecule is the acceptor (65ACS807).

Charge-transfer complexes can exist as intermolecular complexes, where the interaction takes place between two molecules, or as intramolecular complexes, where both the donor and acceptor moieties are contained within one molecule. For the charge-transfer interaction to occur, the donor and acceptor components must be close enough, for their differences in electron density to be felt. For unrestrained complexes, distance between the components of 3.0-3.4 Å, or slightly less than the van der Waals' distance, are common (71MI1).

Most charge-transfer complexes exist in a 1:1 stoichiometric ratio of donor to acceptor. For some π-π types of complexes, there is evidence for the existence of higher order complexes in equilibrium with the 1:1 complexes (80JPC2135). For the 2:1 complex involving two donors, D_2A, support has been found for the D-A-A form rather than the D-D-A form. The crystal

structures of phenol-benzoquinone complexes have been found to have both 1:1 and 2:1 stoichiometries (69MI1).

The occurrence of a charge-transfer usually requires some amount of overlap between the molecular orbitals of the donor and acceptor. Normally, the interaction is between the highest occupied molecular orbital (HOMO) of the donor with the lowest unoccupied molecular orbital (LUMO) of the acceptor. This overlap principle is certainly true for intermolecular complexes. However, for intramolecular complexes, examples are also known of indirect, through-bond interactions, besides the direct, through-space interactions (77AGE799, 82JA5127).

The amount of orbital overlap between the donor and acceptor plays a critical role in the magnitude of the charge-transfer interaction observed. Constraints resulting from steric hindrances are major factors in this regard. For instance, the binding energies for the charge-transfer complexes between phenol and hydroquinone with *p*-benzoquinone were found to be larger than those for anisol and hydroquinone dimethyl ether with *p*-benzoquinone (55JA2644). This was attributed to the steric influence of the methyl groups. For highly substituted molecules, steric hindrance may well prevent the close approach necessary in order for charge-transfer interactions to take place. For most complexes, however, the charge transfer contribution to the complex stability appears to be minor or negligible, and

these complexes may be considered to be non-covalently bound, despite their possession of intense electronic absorption spectra.

C. HYDROGEN BONDING

This is the second class of specific interactions to be considered in this study. It occurs during the complexation of phenol with the various pure compounds (terpenes) under study. Hydrogen bonds are a type of electrostatic interaction which arises from dipole-dipole interactions between a weakly acidic donor group and an acceptor group that bears a lone pair of electron (04MI1). The absolute importance of hydrogen bonding in the description of a large number of chemical and biological systems cannot be denied. Although the hydrogen bond is not a particularly strong bond, it has a great significance in determining the properties of substances. As such, several reports and reviews of the fundamental nature of this important bonding mechanism have been performed (59MI1, 60MI1, 60MI2, 71MI2, 91MI1). These intermolecular interactions govern many important processes, such as the base pairing of DNA and secondary structure of proteins, and have great influence over the bulk properties of many substances, such as the high boiling point of water. Scientists have been looking at hydrogen bonding interactions dating back to the 1920s when the hydrogen bonding phenomenon in water using varying temperatures was postulated. Pauling later postulated that hydrogen bonding is fairly ionic in nature and forms between only the most electronegative atoms. He then mentioned the fact that hydro-

gen bonding is of great importance in the determination of properties of substances and today, we can bear witness to this statement. Pimentel and McClellan defined a hydrogen bond between a functional group A-H and an atom, or a group, B in the same or different molecule when there is evidence of bond formation, be it association or chelation, there is evidence that this new bond linking A-H and B specifically involves the hydrogen atom already boded to A. This definition fails to account for the chemical nature of the participants, including the polarities, and net charges are equally unspecified. The definition has no restrictions on the interaction geometry; it simply makes the assumption that a hydrogen atom must be involved.

Steiner (02AGE48) later gave a definition of hydrogen bonding, stating the following: An A-H···B interaction is called a hydrogen bond, if it constitutes a local bond, and A-H acts as a proton donor to B. This definition has been widely accepted as the general definition for hydrogen bonding as it addresses the acid/base properties of A-H (donor) and B (acceptor), which in principle can be understood by the incipient proton-transfer reaction from A-H to B. In a particular hydrogen bond, A-H···B, the group A-H is called the proton donor and B is called the proton acceptor. Some authors refer to it as electron-acceptors and electron-donors. The nature of single hydrogen bonds has been examined for many simple systems which contain only one hydrogen bond donor and acceptor. There has also been exploration of the hydrogen bond donor and acceptor capabilities of various chemical functional

groups and their dependence on upon neighboring functional groups within a molecule (02PCCP490).

Hydrogen bonds are relatively common in chemistry, and occur when a hydrogen atom is bonded to an electronegative or electron deficient atom and is in close proximity to an acceptor atom which is electron rich. Hydrogen bonds are defined as the sharing of the hydrogen nucleus, or proton, between the donor and acceptor. Hydrogen bonds typically have strengths in the range of 5-50 kJ mol^{-1} (02ACR565), which is significantly less than a covalent bond. Thus energy of specific interaction of α-pinene with phosphorylated nucleocapsid protein is 8.8 kJ mol^{-1} (11MOL1044). Hydrogen bonding between 2-phenylethanol and α-pinene or camphor was studied photophysically (99JPC(A)1991).

The hydrogen bond has been widely investigated by spectroscopic tools such as IR and NMR spectroscopy and neutron/X-ray diffraction. Figure 2 shows the main types of hydrogen bonds which have been characterized (00MI6). The strength of a hydrogen bond can vary from the weakest (~4 kJ/mol) to the strongest (~120 kJ/mol) in intermolecular non-covalent interactions, depending on the interaction direction.

For the hydrogen bonding between phenols and cyanides and the shifts of the bonded OH group frequency were determined for a range of cyanides (66PRCL460). Equilibrium constants for the formation of the complexes were equally determined and correlated with the frequency shifts. In find-

ings, the C≡N group frequency is displaced to higher frequency when bonded to phenol. This effect was thought to be unusual, suggesting that the bonding occurs through the lone pair electrons on the nitrogen atom.

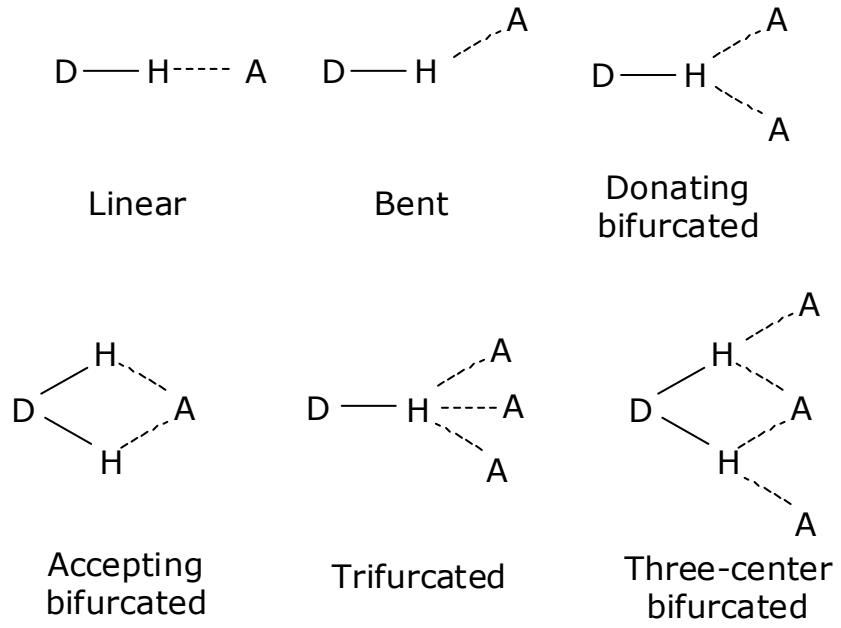


Figure 2: Main Types of Hydrogen Bond Geometry

The possibility and extent of the hydrogen bond formation between various substituted phenols and *p*-benzoquinone and chloranil (09AJP72) were investigated by FT-IR spectroscopy. A conclusion was reached that there is weak hydrogen bonding between the different types of phenol with p-benzoquinone and chloranil respectively, since the extent of lowering of frequency in the formation of hydrogen bonds indicates the strength of the bonds. It was also established that there is a complexation between phenols and quinines *via* weak hydrogen bonding.

D. Silver perchlorate complexes

The usefulness of silver as an antimicrobial agent has been known for a long time. It is an effective agent with low toxicity, which is especially important in the topical antibacterial treatment of burn wounds, where transient bacteremia is commonly cited (97JT1066). Silver sulfonamides, particularly silver sulfadiazine (94JBC213), have been used as the standard treatment for burns, either alone or in combination with other antibiotics (88JBC359), cerium (77SGO668), and zinc compounds (90JBC112). It was suggested that the basic function of the almost insoluble silver sulfadiazine may be the slow release of silver into the superficial wound environment (70AS281). The application of silver-binding membranes has recently been suggested to further reduce the likelihood of silver toxicity to retard the movement of silver ions and minimize silver absorption at a healing wound (97CLC43, 00JB117).

Silver compounds are used as disinfectants and microbiocides. Silver has germicidal effects and kills many lower organisms effectively without harm to higher animals. Silver has been known to be capable of rendering stored drinking water potable for a long period of time (several months). Water tanks on ships and airplanes are often silvered. Disposal of even small quantities of silver nitrate, $AgNO_3$, connected to a septic tank is guaranteed to destroy the septic bacteria and require pumping out, flushing and seeding with fresh bacteria. Silver nitrate antiseptic properties are known and a very

dilute solution is sometimes dropped into newborn babies' eyes at birth to prevent contraction of gonorrhea or Chlamydia from the mother. Silver chloride, AgCl is used to help prevent bacteria from growing on glass (when melted into the glass). Silver carbonate, Ag_2CO_3 is used as an antibacterial agent for concrete. It is also used as abiocide against bacteria, yeasts and molds in some paints and resins. In today's marketplace, Samsung has introduced washing machines that inject silver ions into the wash and rinse cycles to kill 99.9% of odor causing bacteria. Silver atoms, electrolytically stripped of an electron, are injected during the wash and rinse cycles, allowing 100 quadrillion silver to penetrate deep into the fabric, sanitizing clothing without the need for hot water or bleach. On the other hand, Kohler has introduced a line of toilet seats that include an antimicrobial agent formed into the plastic which inhibits the growth of odor causing bacteria, mold and mildew. American Standard, StayClean™ Whirlpool systems have plastic pipes manufactured with antimicrobial Alphasan® which are blue in color. This revolutionary material uses silver's natural antimicrobial characteristics to help the whirlpool free of 99.9% of microbes, mold and mildew. Electrical ionization units impregnate water with silver and copper ions. These sanitize pools, spas and fountains without the harsh effects of chlorine. AgION® Silver-based antimicrobial protects Logitech wireless keyboard and mouse against a broad spectrum of bacteria. In medicine, silver or silver compounds are used externally in wound and burns treatment: Curad®'s natural

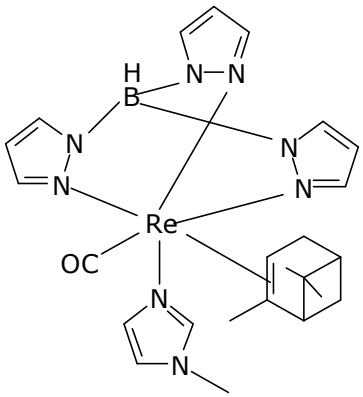
silver antimicrobial bandages, through laboratory testing showed that silver reduced bacterial growth like *Staphyloccocus aureaus*, *E. coli*, *E. hirae* and *Pseudomonas aeruginosa* (a powerful germ that does not respond to many antibacterials) in the dressing for 24 hours. Elastoplasts®'s Silver Healing™ bandages contain silver ions which kill over 150 different bacteria, fungi, yeast and other harmful germs. Silver sulfadiazine is an antibacterial and antifungal agent used as a topical cream to prevent and treat skin infections on areas of burned skin. Silver chloride has the ability to eliminate or flush out mercury from the body. It is also used as an antimicrobial agent in some infection resistant surgical fabric materials. Silver carbonate is used as a biocide against bacteria, yeast and molds in some cosmetics. It is also used in some tooth surface treatments to prevent cavities. However, silver plays no known natural biological role in humans, and possible health effect of silver has been a subject of dispute. Silver itself is not toxic but most silver salts are, and some may be carcinogenic. Silver and compounds containing silver (like colloidal silver) can be absorbed into the circulatory system and become deposited in various body tissues, leading to a condition called argyria which results in a blue-grayish pigmentation of the skin, eyes, and mucous membranes. Although this condition does not harm a person's health, it is disfiguring and usually permanent (11AAM115).

In the present work, silver perchlorate serves as a source of silver which forms complexes with various organic donors, to form solid complexes.

These complexes are then tested on wound healing by carrying out the antibacterial assessments on bacteria that has been implicated in wounds. Silver perchlorate is an effective reagent for replacing halides ligands with perchlorate, which is a weak or non-coordinating anion. However, the use of silver perchlorate in chemical synthesis has declined due to safety concerns about explosiveness of perchlorate salts.

Silver perchlorate is a chemical compound with the molecular formula $AgClO_4$. As earlier mentioned, it is a source of silver, responsible for antibacterial properties in wound healing. Dendrimer silver complexes and nanocomposites serve as antimicrobial agents, tested *in vitro* against *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli* bacteria (01NL218). They include silver complexes of poly(amidoamine) dendrimers. Their findings showed that both silver complexes and nanocomposites displayed considerable antimicrobial activity.

α-Pinene is a unique ligand and in the coordination compounds is coordinated basically in an η^2-manner, *e. g.*, **19** (02JA15099, 05OM1786) or dinuclear **20** (06RJG1288), as well as in silver perchlorate complexes, although no structural information is given (71CJC2760). Another way of coordination of α-pinene is σ-alkyl-π-allyl mode **21** followed by restructuring of the terpene to the so-called *seco*-pinene system including opening of the four-membered internal ring (91TL6101, 02OM2504). η^3-Allyl coordination is noted in the palladium complexes of β-pinene (99ICA246).



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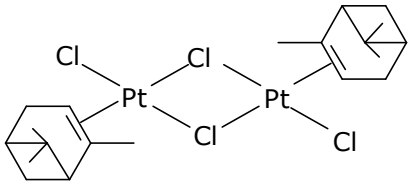

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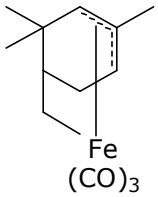

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E. MICROBIOLOGICAL ASPECT

One of the properties of essential oils is in their possession of antimicrobial properties which include antibacterial, antiviral and antifungal properties. The antibacterial properties of essential oils have been well recognized for many years (99JAM985). They have been found applicable as naturally occurring antimicrobial agents in pharmacology, pharmaceutical botany, phytopathology, medical and clinical microbiology, and food preservation. Essential oils of aniseed, lavender, lemon, orange, lime, rosemary, and basil have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties. Some of them have been reportedly used in cancer and HIV treatments, while others have been used in food preservation, aromatherapy and fragrance industries (94FM334). Some researchers have concluded that cinnamon, clove, and rosemary oils had antibacterial and antifungal activity (05MI1). Anti-inflammatory activity has been found in basil, anti-diabetic property in cinnamon oil, and antioxidant property in

lemon and rosemary oils. Orange was reported to have anticancer properties while lavender oil antibacterial and antifungal activity (02PR301).

Plant essential oils exhibit antibacterial activity by interfering and destabilizing the phospholipids bilayer of the cell membrane, enzyme systems, and genetic materials of bacteria (95JAF2839). The antibacterial properties of these compounds are in part associated with their lipophilic character, leading to accumulation in membranes and energy depletion in a membrane-associated event. In addition, a number of constituents of essential oils (98JAB211) exhibit significant antibacterial properties when tested separately by interfering at different levels of mechanism. It is evident that essential oils are more strongly antibacterial than is accounted for by the additive effect of their major antimicrobial components as a result of their minor components that appear, to play a significant role synergistically (94RIE13). Some essential oil components such as carvacrol, thymol and eugenol (phenolic compound) have been proven to be antibacterial. Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart and cancer is also raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects (91MI2). The antioxidant activity of phenolics is mainly due to their

redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential (95FRR375).

Microbiological tests will be carried out on the terpenes and the drugs to see their effects on interaction. It will be shown and reported if their interaction is synergistic, indifferent or antagonistic. Not only is this aspect of the interaction of essential oil components with drugs of interest. Terpenes may also enhance penetration of certain drugs through the skin directly into the blood stream, thus bypassing gastrointestinal and hepatic elimination pathways (04ADD603). Terpenes may be hydrophobic permeants, while oxygenated terpenes may act by a hydrophilic pathway using ability of their oxygen-containing functional groups (epoxy, hydroxyl, keto, carboxyl, or ester) to form hydrogen-bonded or charge-transfer complexes (04JCR113). Their formation could weaken intermolecular forces in the drugs, and they act at a submolecular, perhaps nanosized, level. The strength of the complexes could influence on the penetration ability of a drug. Thus, for 5-fluorouracil permeability in the presence of 1,8-cineole is about 90 times higher than the permeability in the presence of α-pinene in accord with their hydrogen bonding ability (08IJP40).

III. Experimental

A. Essential oil extraction methods

Extraction is needed in order for the oil, lodged in the hidden parts of the plant, to be released. Every part of the plant contains oil, and the only way to release and utilize it, is through the process of extraction. The essential oils are obtained from various parts of the plants and from the seeds. Cinnamon oil, for example, is obtained from the bark and the leaves. The yield and the quality of the oil largely depend on the handling and storage conditions of aromatic plant materials. As soon as the plant material is harvested, certain changes start taking place and these include: loss of water content of the plant material, loss of volatile oil, changes in the physico-chemical values of the oil, and deterioration of the oil present in the plant material. Therefore, it is advisable to extract the essential oil from the plant material as soon as it is harvested. The essential oils are enclosed in oil glands or oil sacs, present in the cellular structures of the plant materials. The extraction of the essential oil depends entirely on the rate of diffusion of the oil through the plant tissues to an exposed surface from where the oil can be removed by a number of processes.

The chemical composition of oil has been a subject of considerable studies (86PM96, 94PF33, 98JEO119). Different studies showed that the chemical composition depends on many parameters as the technique of extraction (04JC(A)323), geographical origin of the plant (02PR769) and the part of the

plant analyzed (96IJG1063, 02PR769). Although distillation is a very convenient method for extracting essential oils, some disadvantages are caused by the artifacts produced during the process, especially when thermolabile components are involved (94MI2). Supercritical fluid extraction is an interesting technique to resolve the problem encountered by the hydrodistillation, and different studies have been reported in the literature (96IJG1063, 97CES3421, 99JSF235).

Another important aspect in the essential oil industry is the enhancement of the quality of the oil. As essential oils used in the food and perfume industries are commonly isolated by cold pressing, they contain more than 95% of monoterpene hydrocarbons, mainly limonene. The industrial practice of deterpeination of 'folding' is to remove some of the limonene along with other unsaturated terpenes and to concentrate the oxygenated fraction. This process is carried out in order to improve the oil stability, increase the solubility of the oil in low proof alcohol, in food solvent and in water and to reduce storage and transport costs (99TAC708). The commercial method currently used for folding essential oils is fractional vacuum distillation (83JFS1197), selective solvent extraction (86JFS1180), and chromatographic separation (98MI1). All these methods have important drawbacks, such as low yields, formation of by-products (owing to the time of exposure to high temperatures) and the presence of toxic organic residues in the extracts (99TAC708). Microwave-assisted extraction (MAE) is a popular and

developing method which has been proved to have many advantages such as rapidity, simple instrumentation, *etc*. (05JC(A)275, 06ABC810). A novel method, solvent-free microwave extraction (SFME) has been developed in recent years (04JC(A)323). For fresh plant materials, SFME can be used to extract essential oils directly. However, for dried plant materials, it is necessary to moisten the samples before extraction which makes the process, complex and time-consuming. Two types of extraction methods employed in this study are discussed below.

1. Solvent- free microwave extraction

With increasing energy prices and drive to reduce carbon dioxide emission, concerned institutions are being challenged to find new technologies in order to reduce energy consumption, to meet legal requirements on emissions, and for cost reduction and increased quality. For instance, existing extraction techniques have considerable technological and scientific bottlenecks to overcome: often requiring more than 70% of total process energy used (08JC(A)229).

Solvent-free microwave extraction was carried out with a Milestone Dry DIST (2008) microwave apparatus (Fig. 3). The multimode microwave reactor has a twin magnetron (2,800 W, 2,450 MHz) with a maximum delivered power of 1000 W in 10 W increments. A rotating microwave diffuser ensures homogenous microwave distribution throughout the plasma-coated PTFE cavity (35cm x 35cm x 35cm). The temperature is monitored by a shielded

thermocouple (ATC-300) inserted directly into the corresponding container. Temperature is been controlled by feedback to the microwave power regulator. In a typical SFME procedure, a 100 g of the leaves of *Lavandula officinalis* were placed in the reactor. The essential oil is collected after the extraction process.

Figure 3: Solvent-Free Microwave Extraction Setup

2. Hydrodistillation

Hydrodistillation is a traditional method of extraction. It is one of the oldest and easiest methods being used for the extraction of essential oil. In this method, the plant is fully dipped in the water. The plant material is placed in water and heated directly in a Clevenger type distillation apparatus (Figure 4). The essential oils whose boiling points normally range up to 300^0C are distilled with steam and both are condensed and separated. The distillation is usually done at atmospheric pressure, although vacuum processes are used if the oil is subject to hydrolysis (12MI1). Since most

essential oils are prepared by this method, and have a specific gravity less than water some essential oils processed in this form may have a small amount of water at the base of the flask. This is a form of disadvantage. Hence it is necessary to dry the oil over anhydrous sodium sulfate. However, the advantage of this method over isolation method such as solvent extraction is that the isolates obtained do not include non-volatile matter, which may interfere with chromatographic analysis. It should also be noted that the yield of essential oil, using hydrodistillation process, is very small (96NFJ78).

Figure 4: Hydrodistillation Setup

B. GC-MS ANALYSIS AND IDENTIFICATION OF COMPONENTS

GC MS is standard equipment used to analyze essential oils (Fig. 5). Gas chromatography-mass spectroscopy is a method that combines the features of gas-liquid chromatography and mass-spectrometry to identify different substances within a sample. The GC-MS analyses was carried out using

Hewlett-Packard HP 5973 mass-spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. The following conditions were used: initial temperature 70°C, maximum temperature 325°C, equilibration time 3 min, ramp 4°C/min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30 mL/min, purge time 0.20 min, gas type helium; column: capillary, 30 m × 0.25 mm, film thickness 0.25 μm, initial flow 0.7 mL/min, average velocity 32 cm/s; MS: EI method at 70 eV.

Figure 5: GC-MS Setup

Components of the oils were identified by matching their mass spectra and retention indices with those of the Wiley 275 library (Wiley, New York) in the computer library and literature (87MI1). Yield of the oil was calculated per gram of the plant material, while the percentage composition was calculated from summation of the peak areas of the total oil composition.

4. UV/VIS SPECTROSCOPY

Pure compounds used in the experiments are α-pinene, camphor and 1,8-cineole [Sigma-Aldrich (For R&D use only), 1,8-cineole: 99%, α-pinene: 98% camphor: 99%. They were purchased as pure substances and used without further purification. The pure compounds in this case are acting as the donors while iodine is an acceptor. The UV-VIS analysis was carried out using the UV-3000 PC spectrometer.

Methylene chloride (CH_2Cl_2), a solvent for spectrophotometric studies, was purified according to the following procedure (09MI1). Methylene chloride was washed with some portion of concentrated sulfuric acid, H_2SO_4 until the acid layer remains colorless. Afterwards, the solvent is washed with distilled water. The solution is then washed with 20% sodium carbonate solution (Na_2CO_3) and with distilled water and later dried over calcium chloride ($CaCl_2$). The solution is filtered off and distilled over CaH_2 before use. On distilling, the fraction boiling point 40-41°C was collected. Solution was then stored in a brown bottle, away from light over Type 3A molecular sieves.

The iodine supplied is purified by procedure of re-sublimation. Iodine crystals in a beaker are heated, causing it to sublime onto an evaporating dish cooled by ice.

Two kinds of UV-VIS experiments were performed, one with the purpose to determine stability constants of the charge-transfer complexes, and another to characterize kinetics of their formation.

Charge transfer complexes formation between pure compounds and iodine were studied in the following manner. 1.0×10^{-3} M stock solution of iodine was prepared. Series of solution of α-pinene, camphor and 1,8-cineole were prepared in CH_2Cl_2, 8.0×10^{-3} M, 7.0×10^{-3} M, 6.0×10^{-3} M, 5.0×10^{-3} M, 4.0×10^{-3} M, 3.0×10^{-3} M, 2.0×10^{-3} M, and 1.0×10^{-3} M. Solutions were freshly prepared just before recording spectra. Equal volumes of the iodine solution and each of the series of the pure compounds in methylene chloride were mixed in the cuvette. Pure solvent methylene chloride was used at the reference cuvette. The work cuvette is rinsed with methylene chloride before proceeding from solution to solution. Spectra are recorded in the range 300-600 nm. For measurements of kinetics of complex formation, one of the solutions of 1,8-cineole, for example, 6.0×10^{-3}M, is taken (not discarded after the first recording of spectrum). Spectra are recorded after every 3 – 8 minutes until no changes in the spectrum is observed. Normally, 10-12 measurements sufficed. Same procedures apply to other donors (α-pinene and camphor).

From the first series of measurements, the stability constant is determined for the equilibrium below was determined:

$$D + A \underset{}{\overset{K_f}{\rightleftharpoons}} [D\text{-}A]_{CT}$$

Benesi-Hildebrand analysis of K_f involves the measurement of the $[D\text{-}A]_{CT}$ absorbance (Abs) as a function of varied [A] when [A]>>[D]. A plot of y =

$[D]_0$/Abs *vs*. x = $1/[A]_0$ gives an intercept = 1/ε and slope = ($1/K_f\varepsilon$) as defined by the Benesi-Hildebrand equation:

$$\frac{[D]_0}{abs} = \frac{1}{[A]_0}\frac{1}{K_f\varepsilon} + \frac{1}{\varepsilon}$$

Here $[D]_0$ = total donor concentration (fixed); abs = CT absorption of the DA complex at a wavelength λ; $[A]_0$ = total concentrations of an acceptor (varied); K_f = equilibrium constant for DA complex formation; ε = molar absorptivity of DA complex at λ. The linear dependences were treated using the least squares' method realized as the Microsoft Excel spreadsheet (04MI1).

D. IR SPECTROSCOPY

Purification of the solvent CCl_4: One liter (1 L) of CCl_4 was treated with potassium hydroxide, KOH, and the mixture, shaken vigorously for 30min at 50-60^oC. After washing with water, the process is repeated with half the quantity of KOH. Ethanol is then removed by shaking, several times with 500 mL of water, followed by shaking with small portion of concentrated H_2SO_4 until no further coloration was observed. The CCl_4 is then washed with water, dried over anhydrous $CaCl_2$ and distilled. Further purification was effected by passing the distilled solvent through a column of alumina and then allowing to stand in the presence of a type 5A molecular sieve and finally distilled before use. Pure solvent of CCl_4 was collected at the boiling point of 76.5^oC.

One of the approximate methods for the determination of the electron donor ability of the ligands is the IR-spectroscopic method. Using one of the standard donors (phenol, methanol, chloroform, phenylacetylene, and others), it is possible to determine the frequency shift under hydrogen bonding, for example, for phenol,

$$\Delta\nu(OH) = \nu_{free}(OH) - \nu_{bound}(OH)$$

Comparison of the obtained value for a compound under investigation with the literature data allows to place a ligand in a series of the other electron donor molecules. Using various empirical dependences, it is possible to calculate all the thermodynamic parameters of the hydrogen bond formed (ΔH°, ΔS°, and ΔG°), or, in other words, all the parameters for the equilibrium

$$XH + L \leftrightarrows XH \cdots L$$

Approximately 20 mL of the stock 0.01 M phenol solution in CCl_4 was prepared. 3 mL of this solution were diluted with 3 mL of CCl_4 up to the concentration of 0.005 M. IR spectrum of this solution was recorded in the range 2600-3700 cm^{-1} at a thickness of the absorbing layer of 4-5 mm, and $\nu(OH)$ of the non-associated phenol molecules were recorded. 3 mL of 0.04 M solutions of the ligands under study in CCl_4 (α-pinene, 1,8-cineole, camphor) were prepared and mixed with 3 mL of the stock phenol solution, so that the solutions containing phenol and a ligand in the ratio 1:4 (concentration 0.005 and 0.02 M) are obtained. Spectra of these solutions are recorded in

the same range of frequencies. The values of $\Delta\nu$(OH) of the associated phenol molecules are calculated.

According to Badger-Bauer empirical equation (10MI1), the enthalpy of the hydrogen bond formation is

$$\Delta H^\circ = -67\Delta\nu(OH)-2638 \text{ (J/mol)}$$

Empirical equation for the entropy change is valid for a wide range of molecular complexes (07RCR1935):

$$\Delta S^\circ = 0.0297\Delta H^\circ + 33.5 \text{ (J/mol K)}$$

The free energy of complex-formation is

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \text{ (J/mol)}$$

The equilibrium constant is

$$K = e^{-\Delta G^\circ/RT}$$

Here R = 8.314 J/mol K.

Intensity of ν(OH) of the non-associated phenol molecules allows to calculate the equilibrium concentration of the free phenol under the following equilibrium (L is an organic base):

$$C_6H_5OH + L \leftrightarrows C_6H_5OH\cdots L$$

The association constant is

$$K_{ass} = \frac{[C_6H_5OH \cdot L]}{[C_6H_5OH][L]}$$

If c°_p and c°_L are the initial concentrations of phenol and ligand, and the equilibrium concentration of phenol is c_p,

$$K_{ass} = \frac{c^{\circ}_p - c_p}{c_p(c^{\circ}_L - c^{\circ}_p + c_p)}$$

To find phenol concentration accurately, the calibration graph D = f(c) should be plotted using the data on phenol solutions of different concentration.

30 mL of a stock phenol solution in CCl_4 was prepared with concentration at 0.01 M. By dilution, five working solutions of concentrations 0.001, 0.002, 0.004, and 0.06 M were prepared, 20 mL each. IR spectra of these solutions were recorded in the range of ν(OH). For ν(OH) of the free hydroxyl group (3610 cm^{-1}) the graph D = f(c) was plotted, which is a straight line passing through the coordinate origin.

By mixing 2 mL of 0.01 M solution of phenol in CCl_4 and 3 mL 0.01 M or 0.02 M of organic base, a working solution of the complex was obtained, in which c°_p and c°_L are twice less. IR-spectra of solutions were recorded in the range 2600-3700 cm^{-1}. From the optical density of ν(OH) of the free phenol molecules at 3610 cm^{-1}, the equilibrium concentration C_p was determined using the calibration graph. Stability constants were calculated using the formula above.

E. MICROBIOLOGICAL TESTS

1. Bacterial strains used in this study

Four reference strains of bacteria used in this study were chosen based on their pathological effects on human and deterioration of food products. Gram positive bacteria *Bacillus cereus*, *Staphlococcus feacalis,* and Gram

negative bacteria *Proteus vulgaris and Pseudomonas aeruginosa* were obtained from the Department of Microbiology, University of Fort Hare.

2. Culture media and growth condition

The bacterial stock cultures were maintained on nutrient agar (SAAR-CHEM, Gauteng, SA) plates. A loopful of bacterial cells from the nutrient agar plates was inoculated into 50 mL of a nutrient broth (DIFCO, California, USA) in a 250-mL sidearm Erlenmeyer flask and incubated at 37°C for 16 h with vigorous shaking (Orbital incubator, S150, UK). After incubation, the culture was diluted with fresh media to give an D_{600nm} of 0.1. One hundred microliters of the culture cells was added onto the plate and spread into a bacterial lawn using a sterile glass spreader.

3. Antimicrobial susceptibility test

The agar well diffusion-based method (87IJF165) modified later (09AJB1280) was used to determine the susceptibility of bacteria. A 100-µL volume of 18 h bacterial cultures were used to spread a bacterial lawn on nutrient agar. The cultures were adjusted to approximately 10^5 cfu/mL using McFarland standard. Twenty five microliters (25 µL) of various concentrations of plant extracts was added to each well (diameter of 4 mm) of nutrient agar plate under aseptic condition. The plates were left for 30 min at room temperature for the diffusion of the extracts and incubated at 37°C for 18 h. The zones of inhibition were measured after 18 h using ruler. Each concentration of the extract was repeated three times.

4. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the essential component and drugs was determined by the agar dilution method as described by the National Committee for Clinical Laboratory Standards (1994). Dilution of the plant extracts ranging from 0.195-100 mg/mL in a Mueller Hinton agar solution were prepared by incorporating the extract stock solution into molten agar at 50°C. After pouring into plates and allowing the agar to set, the plates were inoculated with standardized Inocula of the test bacteria of 10^5 CFU/mL. The plates were further incubated at 37°C for 24 h under aseptic conditions. The MIC's were the lowest concentrations of the extract resulting in complete inhibition of visible growth of the test organisms.

5. Time killed assay of combined drugs and essential oil compounds

The effect of combinations of essential oil components with selected drugs was evaluated using the time-kill assay method in accordance with the descriptions (05AJB946). Inocula were prepared following the described guidelines of EUCAST (03CMI1). The essential oil components (α-pinene, camphor, and 1,8-cineole) were incorporated into 50 mL of nutrient broth at 0.0625 × MIC. Both the test and control were inoculated with each organism to a final inoculum density of 10^5 CFU/mL. One hundred microliter, 100 µL of cultured bacteria were taken from the flask and serially diuted in steriled nutrient broth and plated on the nutrient agar in order to determine the zero hour counts and later incubated overnight at 37°C. The flasks containing the

organisms were further incubated for 8 hrs with shaking at 120 rpm at 37°C. After 8 hrs of incubation, the samples were taken from the control and each test flask and transfered to the recovery medium (nutrient broth) containing 3% Tween 80 to neutralize the effects of the carry-overs drug and essential component from the test suspensions. The samples were serially diluted in sterile nutrient broth and one hundred microliter (100 µL) plated on nutrient agar plate in duplicate and later incubated at 37°C for 24 hrs under aerobic conditions. The number of colonies were counted after incubation and the mean of CFU/mL for each test and controls were determined and expressed as $\log_{10}$. The interactions of various essential oil components and drugs used in this study were considered synergistic, if there was a decrease of ≥ $2\log_{10}$ CFU/mL in colony counts, after 8 hrs. Additivity or indifferences was described as $<2\log_{10}$ CFU/mL in the average viable counts after 8 h for the combination, in comparison to the most active drugs. Antagonism was defined as ≥ $2\log_{10}$ CFU/mL increase in colony counts.

IV. Results and Discussion

A. Essential oils from *Lavandula officinalis*

After completion of the extraction processes for both hydrodistillation (HD) and solvent-free microwave extraction (SFME), using the formula for yield, the following values were obtained for two different methods employed: 0.9% and 0.7% for HD and SFME, respectively. These amounts of percent yields, when compared with literature, show an improvement in terms of quantity with respect to the initial amount of plant materials, distilled and microwaved, respectively. For instance, for a 500g of fresh plant materials distilled and microwaved the yields obtained were 0.35% and 0.33% respectively for *Rosmarinus officinalis* (09FC355). The higher yield in conventional hydrodistillation method over SFME calls for a critical examination of what might have happened. This could be attributed to two different reasons: firstly, to the loss of some volatile compounds during processing time, and secondly, to the manner in which the oils were collected in SFME. Collection of the oils in HD was made once but in the SFME oils were collected at intervals of hours, lasting into the following day. As the SFME set-up cools down, new oils were seen floating on top of the hydrosol and collected. Inaccuracy and loss of oil arise during the process of collecting and transferring into the bottle. However, after a visual inspection of the two oils, the oil for the SFME was found to be pure to the sight than those of the HD. This purity is attributed to the shorter time of extraction as compared to the HD.

The longer time and heat experienced in HD allows higher molecular compounds to be extracted, thereby impairing the purity of the oil.

One of the merits of the solvent-free extraction method (SFME) over the conventional hydrodistillation (HD) is rapidity. The yields obtained by the two methods are of the same order of magnitude, the only difference is the time taken for the extraction processes. The chemical components of essential oils obtained from *Lavandula officinalis* by SFME and HD are shown in Table 3. A total of 59 compounds were identified in *L. officinalis* essential oils using the two techniques. The methods allowed the detection of most volatile active compounds such as α-pinene, camphor, verbenone, and camphene. Slightly higher amounts of oxygenated compounds are present in the aromatic plant isolated by SFME, compared to HD. Monoterpene hydrocarbons are found to be present in equivalent amounts in the SFME and HD essential oils. Some essential oils are also found to be absent in either of the methods as seen in Table 3. α-Phellandrene, δ-3-carene and α-terpinene were absent in SFME but present, in a minute amount in HD. In the same way, *trans*-β-ocimene was present in the essential oil extracted by SFME but, absent in HD. The oil obtained by SFME is slightly concentrated in oxygenated compounds than as obtained in HD. β-Pinene was found to be the major and the dominant monoterpene in the essential oil extracted from *Lavandula officinalis* with the value 21.93% and 20.58% for HD and SFME respectively, followed by α-pinene, 8.50% and 6.74%.

1,8-Cineole was found to be the main oxygenated components in the essential oil isolated from leaves with a difference of 2.13% between the two extraction methods. 1,8-Cineole for SFME has 44.84% but 46.89% for HD. The next in value is α-terpineol with 5.23% for SFME and 3.90% in HD. *trans*-Sabinene hydrate, D-fenchyl alcohol, α-thujone, fenchone, α-campholene aldehyde, linalyl propionate, cumin aldehyde, and carvone were the oxygenated monoterpenes absent, using SFME method. They were found to be present in fairly low amount using HD. Some oxygenated monoterpenes are equally absent in HD but present in SFME. These include filifolone, *trans*-2-caren-4-ol, chrysanthenone, *trans*-pinocarveol, nopol, and myternyl acetate. Oxygenated monoterpene compounds are more valuable than the monoterpene hydrocarbons due to their contribution to the fragrance of the essential oil. Hence, the oxygenated compounds are highly odoriferous and most valuable. The greater proportion of the compounds detected and the proportion of the oxygenated compounds in SFME essential oils could be attributed to the less thermal and hydrolytic effects, as compared to HD which uses a large quantity of water. Essential oils contain organic compounds that strongly absorb microwave energy. Compounds with high and low dipole moments could be extracted in various proportions by microwave extraction. Organic compounds, like oxygenated compounds, having high dipole moment interact more vigorously with microwaves and can be extracted more easily in contrast with aromatic compounds having

low dipole moments (like monoterpene hydrocarbons). Percentages of the identified sesquiterpenes and sesquiterpenoid components were relatively low, being 1.73% and 2.31% for HD and SFME respectively. The main components of this fraction in the examined leaves of *L. officinalis* are α-guaiene and α-bisabolene. α-Guaiene is present only in the essential oil of SFME with a very low percentage of 0.26%. α-Bisabolene is also present in the amount of 0.26% in SFME and 0.25% in the HD essential oil. Occurrence of a very low content, as low as <2.5% for sesquiterpenes and sesquiterpenoids using microwave-assisted process and HD in *R. officinalis* has been reported (09FC355).

Table 3: Chemical Composition of *Lavandula officinalis* Essential Oils Obtained by SFME and HD

No.	Compounds	HD(%)	SFME(%)	RI[a]	RI[b]
	Monoterpenes	**33.8**	**30.7**		
1.	α-Pinene	8.50	6.74	1037	1040
2.	Camphene	0.52	1.26	1062	1245
3.	β-Pinene	21.9	20.6	1111	1091
4.	Myrcene	1.58	1.30	1108	1106
5.	α-Phellandrene	0.07	—	1129	—
6.	*δ*-3-Carene	0.07	—	1129	—
7.	α-Terpinene	0.29	—	1151	—
8.	γ-Terpinene	0.47	0.13	1197	1194
9.	α-Terpinolene	0.24	0.65	1249	1248
10.	*trans*-β-Ocimene	—	0.16	—	1443
11.	Naphthalene	0.08	0.09	1796	1682
	***Oxygenated* monoterpenes**	**66.4**	**68.5**		
12.	1, 8-Cineole	46.9	44.8	1178	1165
13.	*cis*-Sabinene hydrate	0.12	0.20	1207	1205

14.	*trans*-Sabinene hydrate	0.12	—	1207	—
15.	Linalool	0.24	0.65	1249	1248
16.	D-Fenchyl alcohol	0.04	—	1269	—
17.	Filifolone	—	0.26	—	1254
18.	α-Thujone	0.72	—	1240	—
19.	Fenchone	0.72	—	1240	—
20	α-Campholene aldehyde	0.20	—	1285	—
21.	Camphor	1.01	4.99	1311	1311
22.	Terpinen-4-ol	1.09	0.62	1357	1354
23.	α-Terpineol	3.90	5.23	1379	1374
24	Linalyl propionate	3.90	2.35	1331	1327
25.	Myrtenol	2.15	1.44	1385	1381
26.	Verbenone	0.26	2.01	1400	1400
27.	*trans*-Carveol	0.10	0.10	1411	1411
28.	Cuminaldehyde	0.03	—	1440	—
29.	*trans*-2-Caren-4-ol	—	0.10	—	1411
30.	Chrysanthenone	—	0.60	—	1283
31.	t*rans*-Pinocarveol	—	1.08	—	1303
32.	Carvone	0.06	—	1445	—
33.	Bornyl acetate	0.14	3.70	1428	1429
34.	Nopol	—	0.30	—	1348
35.	Myrtenyl acetate	—	0.07	—	1469
	Sesquiterpenes	**1.58**	**2.01**		
36.	*trans*-α-Bergamotene	0.07	0.22	1581	1579
37.	β-Sesqui-phellandrene	0.03	0.05	1598	1642
38.	*epi*-Bicyclosesqui-phellandrene	0.19	0.05	1598	1621
39.	*(Z,E)*-α-Farnesene	—	0.22	—	1579
40.	β-Cubebene	0.25	0.15	1612	1632
41.	β-Selinene	0.25	—	1646	—
42.	β-Bisabolene	0.07	—	1667	—
43.	Germacrene-D	0.05	0.15	1674	1632
44.	α-Guaiene	—	0.26	—	1646
45.	*cis*-Calaminene	0.08	0.09	1683	1682
46.	α-Bisabolene	0.25	0.26	1701	1701
47.	α-Humulene	0.25	—	1701	—
48.	β-Caryophyllene	—	0.17	—	1574
49.	*trans*-β-Farnesene	0.04	0.09	1643	1620

	Oxygenated sesquiterpenes	**0.15**	**0.30**		
50.	Caryophyllene oxide	0.05	0.08	1744	1744
51.	α-Bisabolol	0.07	—	1840	—
52.	β-Eudesmol	0.03	0.12	1813	1810
53.	Nerolidol	—	0.10	—	1655
	Other oxygenated compounds	**5.31**	**4.43**		
54.	Heptan-2-ol	0.04	—		
55.	Heptan-3-one	0.05	0.38		
56.	3-Cyclohexene-1-methanol	3.11	2.35		
57.	3-Cyclohexene-1-ol	—	0.62		
58.	Heptan-3-ol	2.11	1.08		
Extraction time (min)		180	45		
Yield (%)		0.9	0.7		
Total oxygenated compounds (%)		**66.2**	**69.3**		
Total non-oxygenated compounds (%)		**33.8**	**30.7**		

RI^a and RI^b – Retention index for HD & SFME, respectively.

In conclusion, SFME and HD of essential oils in *Lavandula officinalis* have been reviewed and studied with a lot of additional inputs to knowledge. Microwave extraction makes use of the physical and chemical phenomena that are differently compared with those applied in conventional isolation techniques. Microwave extraction method produces essential oil in a concentrated form, free from any residual solvents or contaminants. This piece of work has further added to knowledge and to the proof that solvent-free microwave extraction has an outstanding edge over conventional methods in providing shorter extraction time (45 min against 3 h), low cost (energy cost is greatly reduced in SFME), and cleaner features. The main component

found in the *Lavandula* essential oil was 1,8-Cineole, an oxygenated mono-terpene that has found its usefulness as possessing antispasmodic and anti-fungal properties. This component, 1,8-cineole, along with camphor and α-pinene are the three major pure compounds the project work centers on.

B. Molecular Iodine Charge Transfer Complexes

According to the procedure, the electronic absorption spectra of iodine (1.0×10^{-3} M) in the presence of increasing amounts of the various donors (α-pinene, 1,8-cineole, and camphor) in methylene chloride, CH_2Cl_2 at 25°C are shown in Figures 6-8. The spectra obtained for the donor-I_2 systems show new maximum absorption bands at wavelengths which are not due to the absorption of any of the reactants, and it was considered to be as a result of charge-transfer complex formation.

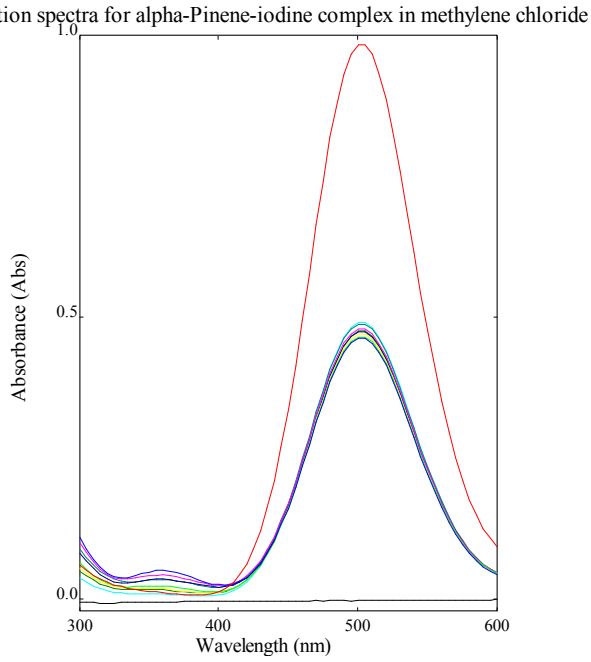


Figure 6: Absorption Spectra of α-Pinene-I_2 CT Complex at 25°C in CH_2Cl_2

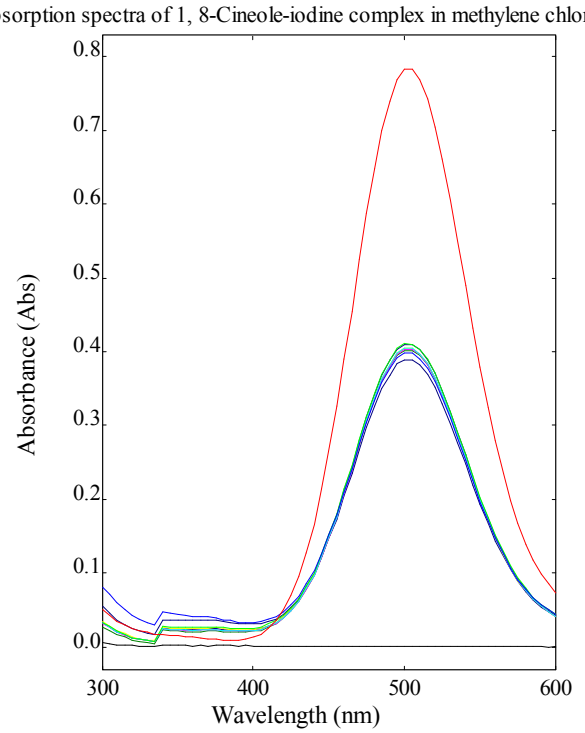


Figure 7: Absorption Spectra of 1,8-Cineole-I_2 CT Complex at 25°C in CH_2Cl_2

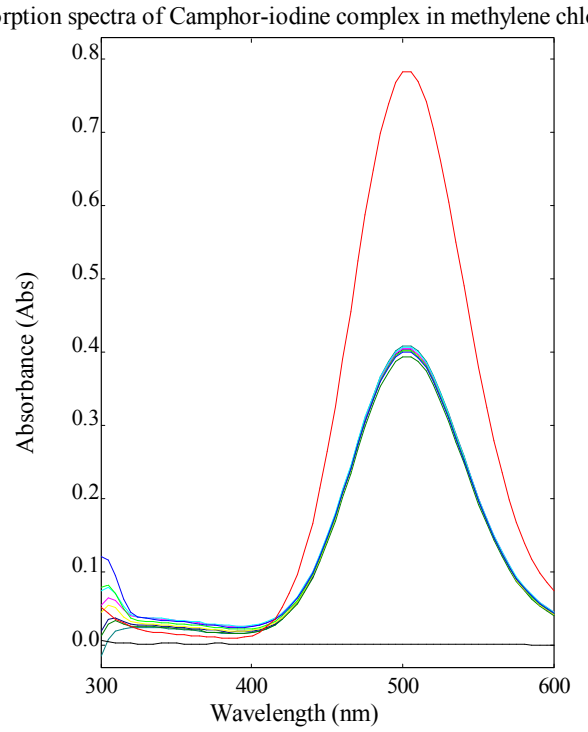


Figure 8: Absorption Spectra of Camphor-I_2 CT Complex at 25°C in CH_2Cl_2

For the α-pinene-I_2 reaction (Fig. 6), while none of the reactants show any considerable absorption in the range 330-390 nm, the spectra obtained immediately after mixing iodine with the alpha-pinene resulted in the absorption around 330-390 nm, presumably due to the complex formation of iodine with α-pinene. Spectra of this system (α-Pinene-I_2) are characterized by the appearance of a new band at 360 nm beside the presence of the blue shifted iodine band at 500 nm (Fig. 6). The intensity of the new band increases, as the concentration of the donor (α-Pinene) is increased. It is evident that the broad band located around 500 nm that is due to the electronic transition of free iodine (69MI2) exhibit a hypsochromic shift due to complexation with the added α-pinene. Thus, the new band located at 360 nm is assigned as a charge-transfer band. The new, low energy absorption observed in solution containing both a donor and an acceptor has been described by Mulliken (69MI2) as charge transfer transition involving the excitation of an electron on the donor to an empty orbital on the acceptor. The absorbance of the CT complex was then measured at its maximum absorption wavelength. Also, it should be noted that the band around 364 nm is well known to be characteristic for the formation of triiodide ion, I^-_3, in the process of complex formation between iodine and different electron pair donating ligands (81JPC1789, 91SA743). This was further supported by conductivity measurements of an 8 X 10^{-3} M solution of α-pinene in methylene chloride before and after addition of iodine. Just after the addition of the

iodine to the α-pinene solution (with negligible conductivity), the conductivity of the solution is 0.1 μS; after 300 seconds, the conductivity increases to 0.39 μS, indicating the gradual release of I^-_3 ion from its molecular complex with α-pinene into the solution. A similar observation is noted for the 1,8-cineole-I_2 and camphor-I_2 systems.

In a similar manner, the reaction of 1,8-Cineole with iodine resulted in a spectra, showing a completely new absorption band, different from the initial absorptions of the reactants. Two different bands of 300 nm and 340 nm were observed, extending to around 370 nm (Fig. 7). The absorption spectra of camphor-I_2 complex also gave rise to two different absorption bands at 300 nm and 340 nm, extending to around 370nm. These bands belong to neither of the reactants (Fig. 9). Similarly, within the same time frame, the conductivity of the 1,8-Cineole increases from 0.01 μS to 0.36 μS while that of camphor rose from 0.10 μS to 0.52 μS respectively.

The original Benesi-Hildebrand equation was modified into the following form:

$$\frac{[I_2]_0}{abs} = \frac{1}{[L]_0}\frac{1}{K_f \varepsilon_{CT}} + \frac{1}{\varepsilon_{CT}}$$

Here $[I_2]_0$ and $[L]_0$ are the total concentration of the acceptor and donor, respectively; abs is the absorbance of the complex at λ_{max}; ε_{CT} is the molar absorptivity of the complex; K_f is the association constant of the complex (L mol^{-1}). Modified Benesi-Hildebrand equation above is used to calculate the association constants K_f and molar absorptivity co-efficient ε_{CT}, as has been

done by other workers (05SA(A)2017, 08JCSP209, 08JIC610, 09AX(E)m1191, 11ARJC105). Values of K_f and ε_{CT} are calculated from the equation by varying the concentration of donor $[L]_0$ and keeping the concentration of acceptor $[I]_0$ constant such that $[L]_0 >> [I]_0$. Equation is of the form: Y = MX + C and the plot of $[I_2]_0$ Vs $1[L]_0$ is fairly linear and the sample linear plots are as shown in Fig. 9-11. From the intercepts and slopes, the values of ε_{CT} and K_f can be evaluated. Such a fair linear relationship in the resulting Benesi-Hildebrand plots is a good evidence for the 1:1 stoichiometry of the charge transfer complexes studied. The ΔG° values of the complex were also calculated from the Gibbs free energy of formation according to the equation below.

$$\Delta G° = -2.303\ RT \log K_{CT}$$

Here ΔG° is the free energy change of the complexes (KJ mol^{-1}), R is the gas constant (8.314 J mol^{-1} K), T is the temperature in Kelvin (273 + °C) and, K_{CT} is the association constant of the complexes, which is K_f in this case *i. e.* $K_{CT} = K_f$. The calculated ε_{CT}, K_f and ΔG° values are also included in the Table 4.

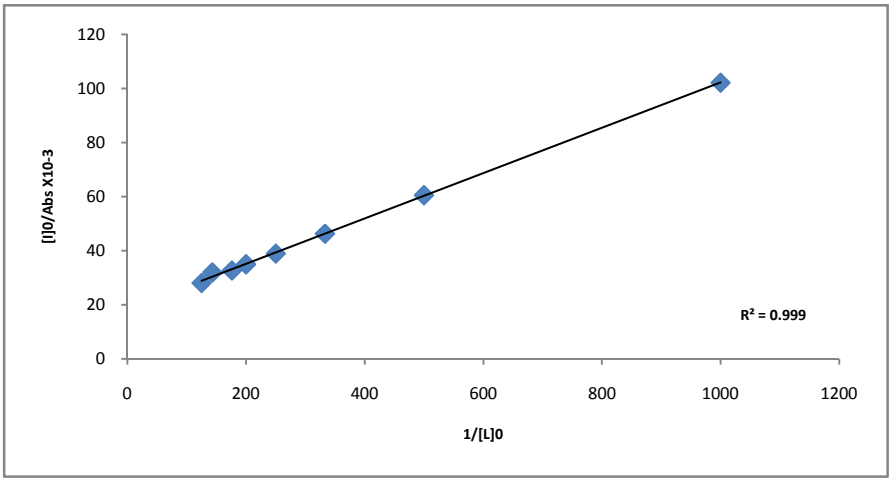


Figure 9: Benesi-Hildebrand Plot for α-Pinene-I_2 at λ = 360 nm

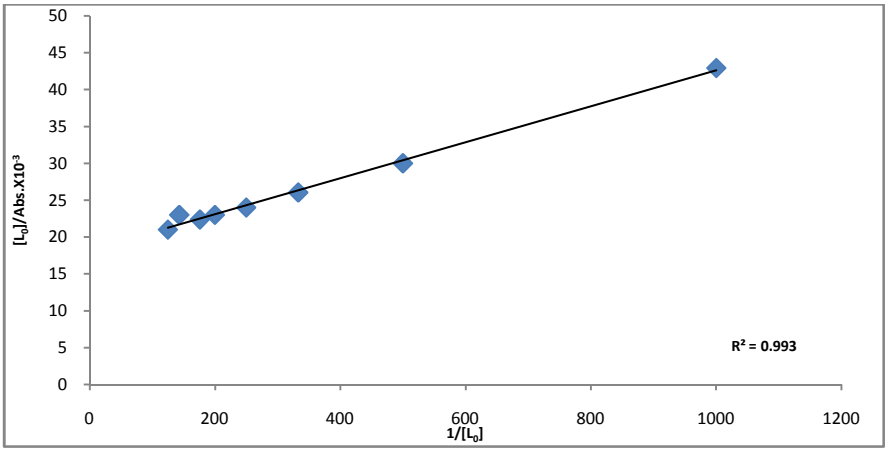


Figure 10: Benesi-Hildebrand Plot for 1,8-Cineole-I_2 at λ = 340 nm

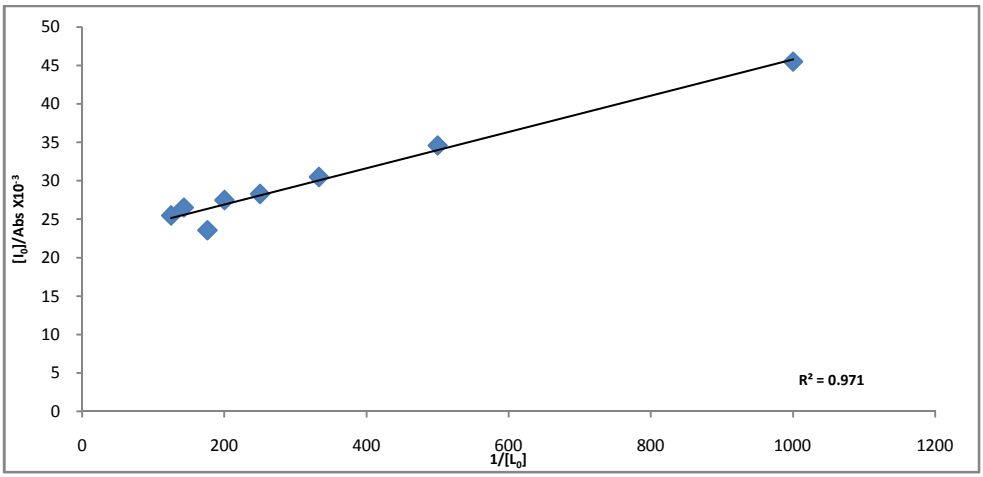


Figure 11: Benesi-Hildebrand Plot for Camphor-I_2 at λ = 320 nm

Table 4: Data for Benesi-Hildebrand Study of Various Complexes in CH_2Cl_2 at 25°C

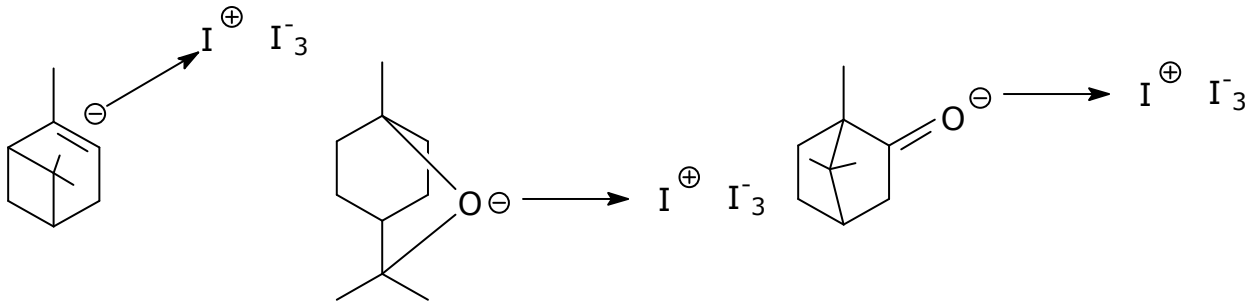
Complex	λ_{max} (nm)	ε_{CT} ($M^{-1}cm^{-1}$)	K_f	r^2	$-\Delta G°$ (KJ mol^{-1})
1 α-Pinene-I_2	360	55	220	0.999	13.37
2 1,8-Cineole-I_2	340	55	759	0.993	16.43
3 Camphor-I_2	320	45	966	0.971	17.03

A critical look at the Table 4 reveals the values of both K_f and ε_{CT} associated with the complexes in solution under study. The values of K_f and ε_{CT} obtained is smaller, compared to what has been previously recorded (05SA(A)205, 08JIC610), although, relatively lower values of K_f have also been reported (98MC121). Generally, relatively low K_f values suggest that the formed complexes are of the weak type, *i. e.*, the ground state wave function has predominantly a nonbonding structure. Lower values of K_f and ε_{CT} obtained in this case indicate low stabilities of the formed charge-transfer complexes as a result of the expected low donation of a donor nucleus. Complexes are expected to form instantaneously, depending on the environmental condition; they break apart, back into solution. In fact, the reaction is better explained as being in equilibrium with the complex formed in solution. The results listed in Table 4 clearly reveal that stabilities of the complexes of donors with the same σ-acceptor (I_2) are of the order 3 > 2 > 1. This behavior could be ascribed to the higher ability of the *n*-donors

compared to that for the π-donor α-pinene. High values of ε_{CT} however agree with the existence of triiodide species (72CPL151, 80IC463).

The values of the Gibbs free energy of formation, ΔG° obtained indicate that complexation is thermodynamically favored. The free energy change of the complexation also reveals that the charge transfer complex formation between used donors and acceptor is exothermic in nature. The values of ΔG° are generally more negative as the association constants of the molecular complex increase. As the bond between the components becomes stronger and thus the components are subjected to more physical strain or loss of freedom, the values of ΔG° become more negative.

In a similar manner, according to the procedure, kinetics of complex-formation in a solution was studied. The concentration of 6×10^{-3} M of various complexes was used, as a way of monitoring the change in absorption with time. The following graphs were obtained for the kinetics measurement (Figures 12-14). They are preceded by possible structures of the charge-transfer complexes formed.



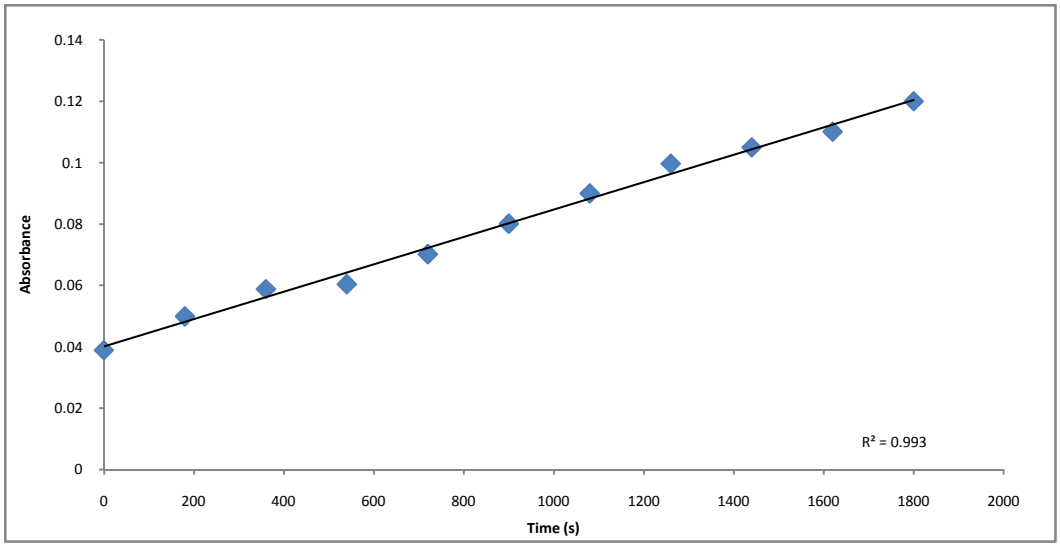


Figure 12: Absorbance-Time Plot at 25°C for a 6 x 10^{-3} M α-Pinene-I_2 Complex in CH_2Cl_2

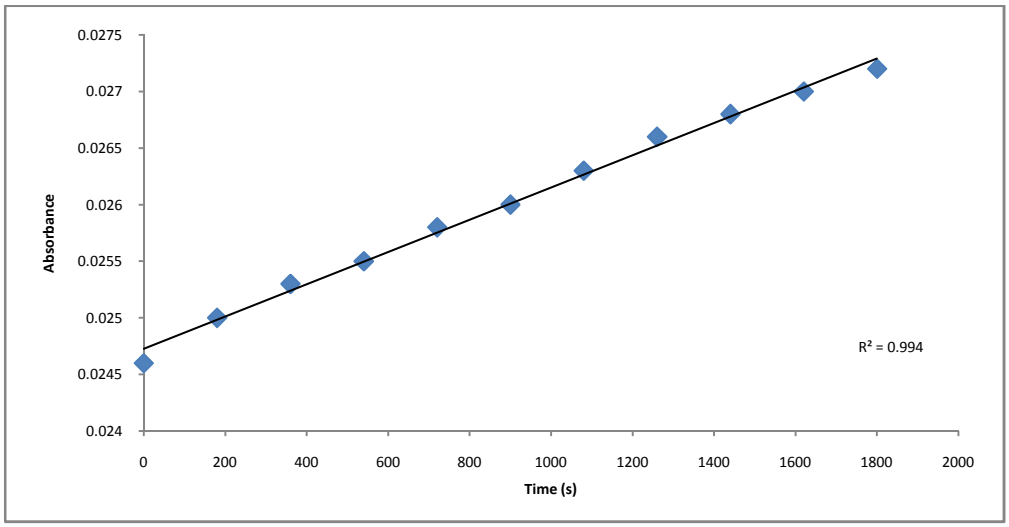


Figure 13: Absorbance-Time Plot at 25°C for a 6 x 10^{-3} M 1,8-Cineole-I_2 Complex in CH_2Cl_2

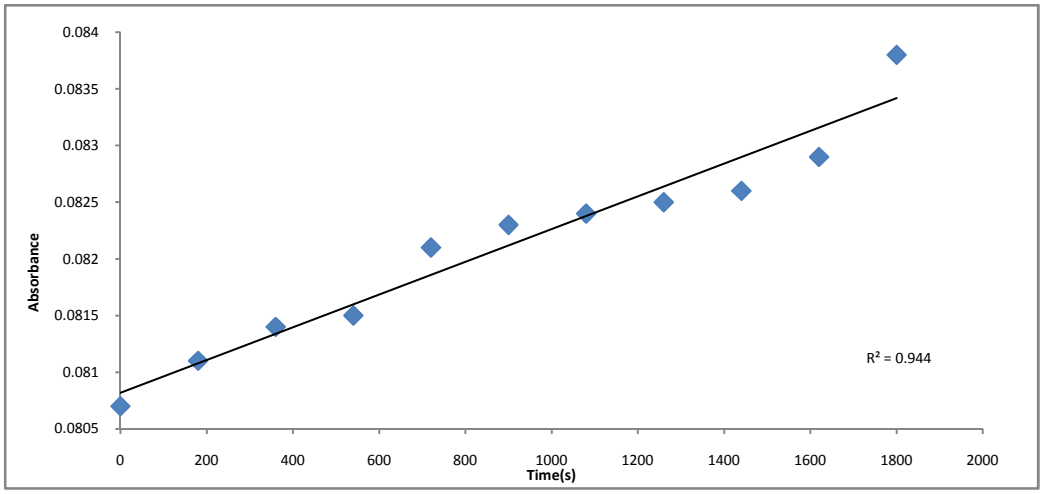


Figure 14: Absorbance-Time Plot at 25°C for a 6 x 10^{-3} M Camphor-I_2 Complex in CH_2Cl_2

A gradual increase in absorbance with time occurs in all cases in accord with similar results (08JIC610).

C. ANTIMICROBIAL TESTING OF ESSENTIAL OIL COMPONENTS AND THEIR MIXTURES WITH DRUGS

The result of the antimicrobial activity of the pure compounds from the essential oil components and the various drugs was presented in Table 5. At a glance, it can be observed that the pure compounds, camphor and α-pinene showed a very low activity and as such, all bacteria tested were resistant to their individual treatment with MIC ≥ 100 mg/mL. This observation was contrary to the report (92FFJ121), which demonstrated the potent antimicrobial activity of α-pinene and camphor against some bacteria. In a similar manner, 1,8-cineole was equally observed to have the same MIC values as the other two pure compounds on two different strains of bacteria –

P. *aeruginosa* and *B. cereus*. These results, showing the resistance of *B. cereus* and *P. aeruginosa* against the treatment of 1,8-cineole concurred with the report (93PMP331) on certain strains of bacteria, while *P. vulgaris* and *S. feacalis* were susceptible at the MIC values of 25 and 50 mg/mL, respectively. Among all the remaining drugs under study, resorcinol is the only one showing the least activity on all the bacteria strains with the MICs, equal in value to camphor and α-pinene. Following suit is the drug Eskamel with the MIC values of 100 mg/mL on two strains of bacteria tested, *S. feacalis* and *P. aeruginosa*. Eskamel, however showed a better MIC value of 0.3906 mg/mL on *P. vulgaris*, followed by MIC value of 25 mg/mL on *B. cereus*. Among all the drugs examined, silver perchlorate showed the greatest activity on all the bacteria strain. The highest potency was observed on the bacteria *P. vulgaris* with the MIC value of 0.781 mg/mL. MIC values of 1.563 mg/mL was obtained for two different strains – *S. feacalis* and *P. aeruginosa* while the least activity recorded for the drug was 3.125 mg/mL with respect to *B. cereus*. It would be interesting to know that the Gram negative bacteria tested in this study were susceptible to the treatment of silver perchlorate, despite their cell wall compositions. The data showed that silver perchlorate has a potential antibacterial activity, though the mechanism of action was not investigated in this study. Silver perchlorate is a very good source of silver ions in solution, which have been proven to have antibacterial properties (70AS281, 01NL218). The results obtained for the drug, Beta-

dine were alarming, and contrary to what has been reported (64JH509, 94JMM408, 10JAS70). The MIC values of >10 mg/mL was obtained for all the bacterial strains. On a repeat course, the same values were recorded.

On combination, the treatment of the various pure compounds with the drugs gave results, some of which are better than when tested on the bacterial strains, alone using the time-kill study (Table 6). The data obtained are presented in terms of the log CFU/mL change and are judged relative to the conventional definition of bacterial activity which is 3-log CFU/mL or greater reduction of the initial inoculums within 8 hrs (03AA1). The antibacterial activity of 1,8-cineole has been reported to have no antibacterial activity against selected bacteria, while camphor and α-pinene have been shown to possess potent inhibitory effect against the growth of some bacteria (93PMP331, 08RA1343). The results obtained from this present study showed a potent synergic biocidal effect of camphor plus silver perchlorate combination (0.0625 $\times$MIC each) against all tested bacteria. This combination resulted to log reduction of the bacteria between 4.25 and 3.64 as shown in Table 6. The individual combination of camphor with Eskamel and resorcinol showed additive effect against *P. aeruginosa* but synergic biostatic effect against *P. vulgaris* and synergic biocidal against *S. feacalis* and *B. cereus*. The log reduction of all the tested bacteria showed indifference or additive effect against the combination of α-pinene and resorcinol except *P. vulgaris* with synergic bacteriostatic effect. The log reduction of all the tested

bacteria showed indifference or additive effect against the combination of α-pinene and resorcinol except *P. vulgaris* with synergic bacteriostatic effect. Time kill of α-pinene combined with silver perchlorate against the bacteria tested was synergic against *S. feacalis* with an additive effect. Similarly, both *P. vulgaris* and *P. aeruginosa* show additivity to the combination of α-pinene and Eskamel but, showed biostatic synergy effect against other bacteria. Generally, most combinations of essential oil compounds and the drugs, used in this study were synergistic or additive against both Gram negative and Gram positive pathogenic bacteria, depending on the tested species and the interaction of combined agents as shown in Table 6. The antibacterial activity of 0.0625 × MIC of these compounds resulted to many fold reduction of bacterial growth compared with MIC values of the agents, alone. Our present study reported the antibacterial properties of Eskamel, resorcinol and silver perchlorate for the very first time. Among the drugs tested, silver perchlorate showed the strongest antibacterial activity against all tested bacteria with synergistic effect. The interaction of 1,8-cineole with the three tested drugs enhanced its antibacterial activity, synergistically against the bacteria used in this study. The synergy detected in this study was not specific to any group of bacteria but, could be related to the synergism of functional groups. The strong synergy observed between these compounds and drugs is a significant finding, demonstrating the therapeutic potentials of the essential oil components with lower antimicrobial properties

(11IJM4477). However, the synergy pattern of these compounds observed in this present study is suspected to depend on the interactive modes of action rather than concentrations of the two agents in combinations. The data obtained from this study indicate the major role played by the concentrations, relative to MIC. Meanwhile, further studies on the mechanisms of these compounds needed to justify the outcome of this experiment.

Table 5: Minimum Inhibitory Concentrations, MICs (mg/mL) of Reagents Used

	P. vulgaris	*S. faecalis*	*P. aeruginosa*	*B.cereus*
1. Resorcinol	100	100	100	100
2. Eskamel	0.3906	100	100	25
3. $AgClO_4$	0.7813	1.5625	1.5625	3.125
4. Betadine	>10	>10	>10	>10
5. Camphor	100	100	100	100
6. 1,8-Cineole	25	50	100	100
7. α-Pinene	100	100	>100	100

Table 6: Time-Kill Study for Combination of Essential Oil Compounds and Drugs

Reagent Combinations	*P. vulgaris*	*S. faecalis*	*P. aeruginosa*	*B. cereus*
Camphor + $AgClO_4$	3.65	4.01	3.90	4.52
Camphor + Eskamel	2.47	3.23	1.94	3.12
Camphor + $C_6H_4(OH)_2$	2.95	11.09	1.89	2.46
Camphor + Betadine	ND	ND	ND	ND
α-Pinene + $AgClO_4$	2.70	1.07	10.91	3.42
α-Pinene + Eskamel	1.67	2.63	1.32	2.00
α-Pinene + $C_6H_4(OH)_2$	2.75	1.26	1.38	1.71
α-Pinene + Betadine	ND	ND	ND	ND
1,8-Cineole + $AgClO_4$	3.65	2.83	2.91	10.72
1,8-Cineole + Eskamel	2.05	4.01	2.03	2.12
1,8-Cineole + $C_6H_4(OH)_2$	1.31	2.48	1.53	2.06
1,8-Cineole + Betadine	ND	ND	ND	ND

Log reduction index were interpreted as follows: <2.0 – additive; ≥2.0 **-** synergic.
$AgClO_4$ represents silver perchlorate; $C_6H_4(OH)_2$ represents resorcinol.
ND – Not Determined.

Figure 15: Some of the Colonies of Growth Observed, on Examination after 24 h of Incubation

Some of the colonies, on observation were photographed to depict the growth patterns of the microorganisms in the reagents with respect to time at a specified concentration (Figure 15). Take, for instance the slide labeled (7SF, 0H, 10^{-9}), (7SF, 4H, 10^{-9}) and (7SF, 8H, 10^{-9}). This set of slides represents a combination of reagents, represented with 7, on a particular organism, *Streptococcus feacalis* (SF), studying the rate of killing with time at time interval of 0 h, 4 h and 8 h, respectively at the maximum dilution index of 10^{-9}. As it can be observed on the three slides, there is an increase in the growth pattern with increasing time from 0 h to 8 h. After incubation,

the slides are counted to determine either the rate of growth or rate of killing with time.

D. ANTIMICROBIAL ACTIVITY OF SILVER-TERPENES COMPLEXES

The minimum inhibitory concentration of various silver-terpenes complexes was determined by agar dilution method as described by the National Committee for Clinical Laboratory Standards (1994). Dilution of the plant extracts ranging from 0.195- 100 mg/mL in Mueller Hinton agar solution were prepared by incorporating the extract stock solution into molten agar at 50^{o}C. After pouring into plates and allowing the agar to set, the plates were inoculated with standardized inocula of the test complexes of 10^5 CFU/mL. The plates were further incubated at 37^{o}C for 24 h under aseptic conditions. The MICs were the lowest concentrations of the extract resulting in complete inhibition of visible growth of the test organisms.

Table 7: Minimum Inhibitory Concentrations (MICs) of Silver-Terpene Complexes

Complexes	*P. vulgaris*	*S. feacalis*	*P. aeroginosa*	*B. cereus*
Ag-camphor	3.125	3.125	3.125	3.125
Ag-1,8-cineole	0.78125	0.78125	0.78125	0.78125
Ag-α-pinene	NA	NA	NA	NA

The results of the antimicrobial activity of the complexes of silver with various terpenes are shown in Table 7. All the test organisms are observed to be sensitive to the both the complexes of camphor and cineole but at dif-

ferent MICs values. Silver-camphor complex shows activity at the same MIC values on all the test organisms at 3.125 while the silver-cineole complex also showed constant values of MICs of 0.78125 on all the test organisms. The MIC for silver-pinene could not be determined due to the unavailability of the complex. The amount obtained was so small to be measured to get same amount as obtained for camphor and cineole respectively. In conclusion, it can be agreed that the silver-cineole showed better MIC results than those of silver-camphor.

IV. Conclusion

The SFME and HD of essential oils of *Lavandula officinalis* have been reviewed and studied. Microwave extraction makes use of the physical and chemical phenomenon that is different compared with those applied in conventional isolation techniques. To further add to knowledge, microwave extraction method was found to produce essential oil in concentrate form, free from any residual solvents or contaminants. The main component found in the *Lavandula* essential oil was 1,8-cineole, an oxygenated monoterpene that has found its usefulness as possessing antiplasmodic and antifungal properties.

Charge transfer complexation between iodine and α-pinene, 1,8-cineole and camphor has been studied, spectrophotometrically and the stability constants determined using the Benesi-Hildebrand equation and also identifying various new charge band different from the reactants in question. It was concluded that the relative low K_f values obtained suggest that the formed complexes are of the weak type, more so, in solution. Kinetics of complex formation was also studied to monitor the change in absorption with time. The study showed a gradual increase in the rate of absorption with time.

The antimicrobial activity of the various pure compounds of essential oil in this study, together with the various drugs was well treated. MIC of individual reagents were established which was later used to determine their combination, one with the other. Interesting results were obtained which will

go a long way to further add to existing knowledge. A particular of interest is the MIC for Betadine, a iodine-base compound, adjudged to be highly potent against many strains of bacteria. Further research will be put in place to ascertain the true identity of the Betadine, purchased from the local pharmacy in Alice and several analyses will be done on it, especially in combination with various pure compounds with the aim of publishing the findings in journals.

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