**KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,**

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**FACULTY OF PHYSICAL SCIENCES**

**DEPARTMENT OF CHEMISTRY**

# ANTIMICROBIAL ACTIVITY PROFILE OF THE CONSTITUENTS OF FOUR GHANAIAN AROMATIC MEDICINAL PLANTS

**A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY, FACULTY OF PHYSICAL SCIENCES, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN ORGANIC CHEMISTRY.**

**BY**

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**FEBRUARY, 2010**

**CERTIFICATION**

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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## ABSTRACT

The study was conducted to investigate the antimicrobial activities of four Ghanaian aromatic medicinal plants and their respective major antimicrobial constituents. The petroleum ether (40 -60$^{o}$C), ethanol, aqueous extracts and as well as the essential oils from the leaves of four Ghanaian aromatic medicinal plants [*Cinnamomum zeylanicum Nees.(*Cinnamon*), Psidium guajava Linn.(*Guava*), Ocimum gratissimum Linn.(*Ocimum), *Xylopia aethiopica A. Rich (*Xylopia*)]* were tested using the Agar Diffusion method for their antimicrobial activity against two gram positive bacteria (*Bacillus subtilis* and *Staphylococus aureus*), two gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and one fungus (*Candida albicans).* The ethanolic extracts of the four (4) samples were fractionated according to Mistscher's scheme of fractionation and the resulting fractions were also tested against the five microorganisms by the Agar diffusion test. All the petroleum ether extracts were active against all the microorganisms except *Xylopia aethiopica* which showed activity against *P.aerugionsa* and *C. albicans* only. The petroleum ether extract of *Ocimum gratissimum* leaves showed the highest zones of inhibition against all five test organisms followed by *Cinnamomum zeylanicum*, *Psidium guajava* and *Xylopia aethiopica*. The ethanolic extracts of the four samples showed zones of inhibition poorly compared to those of the petroleum extract except for *Psidium guajava.* The ethanolic extract of *Psidium guajava* inhibited strongly the growth of all test organisms compared to its corresponding petroleum ether extract. The aqueous extract of *Psidium guajava* was the only aqueous extract which showed activity against the microorganisms. The ethanolic extract of *Cinnamomum zeylanicum* was active against *E. coli* and *C. albicans*. However, the ethanolic extracts of *Ocimum gratissimum* and *Xylopia*

*aethiopica* were not active at all against the test organisms. The essential oil from *Ocimum gratissimum* was the most active of the four (4) essential oils followed by *Psidium guajava*, *Cinnamomum zeylanicum* and lastly *Xylopia aethiopica* essential oils. The *Ocimum gratissimum* essential oil had minimum inhibition concentration (MIC) range of $1.0 \times 10^{-5}$ % to 0.158%, *Psidium guajava* had $3.98 \times 10^{-3}$ to 0.251%, *Cinnamomum zeylanicum* had $6.31 \times 10^{-2}$ to 0.251% whiles *Xylopia aethiopica* oil had $3.98 \times 10^{-2}$ to 1.26%. All the acidic or phenolic fractions (components or constituents) except *Xylopia aethiopica* were active against the test organisms. The terpenoidal, waxes and alkaloidal fractions or components showed various levels of activity against the test microorganisms. It was only the water soluble quaternary alkaloidal fractions which could not reveal any significant activity against the test microorganisms.

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## DEDICATION

This work is dedicated to my parents, Mr. Emmanuel & Mrs. Mercy Osei-Wusu

and

my siblings.

# CHAPTER ONE

## 1. INTRODUCTION

The World Health Organization (WHO) recognizes traditional medicine, particularly plant medicine, as an important alternative healthcare delivery system for most of the world's population. In Ghana, traditional medicine, especially plant medicine, provide many citizens with affordable healthcare services. (Nyarko et. al., 2005)

Since prehistoric times man has used plants for various purposes and he will continue to do so as long as life continues on this planet. (Abbiw, 1990).

Man's symbiotic relationship over time with plants has given the world many invaluable benefits. Apart from the raw materials that go to form our variety of foods, the most important plant products are medicines, cosmetic and flavour products, as well as other pharmaceuticals. (Sofowora, 1996)

Even in an age of substitute man-made materials, plants and plant products are still in great demand. The living world depends on plant life. Plants purify the air we breathe and serve as food for both man and beast; they are a source of fuel for cooking, lighting, heating and provide materials for building and construction. (Abbiw, 1990)

It was estimated in 1987 by Anon that, more than two thirds of the world's population relied on plant derived drugs. (Anon, 1987).

It is estimated that local communities have used about ten percent (10%) of all flowering plants on Earth to treat various infections, although only one percent (1%) have gained recognition by modern scientist. (Kafaru, 1994).

The Centre for Research into Plant Medicine has identified one thousand medicinal plants in Ghana and forty (40) of them are used in treatments of thirty-three diseases such as: malaria, jaundice, asthma, diabetes, epilepsy, typhoid fever, hypertension and anaemia. (Yidana *et al.*, 2002).

Many medicinal plants have other economic uses, supplying fruits, and vegetables, browse for livestock and timber for fuel and tool handles (Abbiw, 1990).

Medicinal plants therefore have a high potential of contributing to enhanced rural health care and in poverty reduction from sale of processed products from herbal plants. Unfortunately, supply of medicinal plants is entirely dependent on wild sources. (Yidana *et al*., 2002).

In the rural areas of Ghana, elderly people and herbalists apply their knowledge of plant medicine as a responsibility to household and community members. (Yidana *et al*., 2002).

The use of plants and their extracts for healing by fetish priests, native doctors, and other 'specialists' was the main method of treating various illness before the advent of Western medicine. The practice continues still, especially among rural communities who, in any case, may not have access to a hospital or health post. (Abbiw, 1990).

The skill of healing with herbs is acquired informally and improved upon with practice. The ingredients or constituents of a particular prescription, and its preparation, are usually the herbalists' copyright which is secretly and jealously guarded. (Abbiw, 1990). Illiterate herbalists die, regrettably, with this wealth of secret knowledge.

The efficacy or otherwise of herbal medicine depends on the active part or parts in it and their pharmacological effect. (Abbiw, 1990).

An ethno -botanical survey which was conducted between August and September 2001 in Ghana, selected six localities and eighty-six (86) registered herbalists. A total of 339 medicinal plant species were recorded during the period under review. Leaves of the plants dominated the plant parts used. Other parts frequently used were roots, stems and bark. The use of whole plants, flowers and buds were also noted. About 80% of the plant parts used were common in all of the communities.

The ethno botanical survey enumerated the social aspects that can affect the distribution and subsequent conservation status, of individual species. They include the following:

- Modernization and the changing patterns of housing in rural areas;
- Changing patterns of agriculture have resulted in the increasing clearance of wild lands for crops and also the abandonment of older fields used for declining crops;
- Changing values and beliefs may also have an impact on plant distribution.
- There are also external factors that encroach on the traditional patterns of harvesting. In Ghana today there is an increase in tourism and the export of handicrafts from the villages. (Agbovie et al., 2002).

The multi-purpose uses of medicinal plants have subjected them to over-exploitation which, coupled with increasing desertification has led to severe scarcity of the species. (Yidana et al., 2002).

In the Northern Region of Ghana, it is now extremely difficult to locate mature trees of many species except in forest reserves which are over fifty (50) kilometers from settlements. (Yidana et al., 2002).

Furthermore, rapid methods for propagating medicinal plants are unknown. There is therefore real danger that the increasing awareness and usage of herbal medicine in recent times, if not backed by concerted efforts to propagate and cultivate medicinal plant species, many of them will be lost in the near future. (Yidana et al., 2002).

The usage of herbs as medicines is determined mostly by the community and environment in which one grows up. Addo (2007) carried out a study to determine the socio-demographic characteristics and pattern of use of herbal medicines by women admitted to the Obstetrics and Gynaecology Department in the Komfo Anokye Teaching Hospital (KATH), a teaching hospital serving the Northern part of Ghana and made the following observations:

More than fifty percent (50%) of patients used herbal medicines which were mostly unknown to the attending health workers. The less educated as well as the unskilled/ semi-skilled used herbal medicines more frequently compared to their more skilled and educated counterparts. Herbal medicine use is thus more prevalent in the groups who usually have poor socio-economic facilities and carry most of the burden of social deprivation. It is possible that their disease conditions may be adversely affected.

Herbal drugs are often promoted as ‘natural’ and ‘safe’ and these claims may especially attract pregnant women who are often concerned about their unborn child’s well-being.

Media liberalization, especially of the airwaves has provided avenues for widespread advertising of herbal medicines. It is common to hear advertisements on the numerous FM (Frequency Modulation) Radio stations, whose broadcasts cover large areas of the country, about herbal preparations which can "melt" fibroids and treat various diseases including cancers and infertility.

Concluding on a positive note, Addo (2007) ended that, there are encouraging strategies to make the use of herbal medicines safe. The Ministry of Health in Ghana has produced a manual to harmonize procedures for assessing the safety, efficacy and quality of plant medicines (Addo, 2007).

The Ghana Herbal Pharmacopoeia (2007) has documented authentic herbal tradition in Ghana. Prior to this, scattered and scanty information existed about Ghanaian Medicinal plants. The Pharmacopoeia contains literature on the indications, pharmacological actions as well as the secondary metabolites present in fifty (50) medicinal plants in Ghana (Ghana Herbal Pharmacopeia, 2007).

However, the exact secondary metabolites responsible for the stated actions such as antimicrobial activity is absent in the pharmacopoeia for some of the plants.

This study seeks to:

Determine the antimicrobial activities of four (4) aromatic medicinal plants found in Ghana listed in the Ghana Herbal Pharmacopoeia (G.H.P.) 2007;

Determine the families of secondary metabolites responsible for the known or reported antimicrobial activities of these plants.

**OBJECTIVES OF STUDY:**

a. To obtain aqueous, ethanolic and petroleum ether extracts and the essential oils from the leaves of four (4) aromatic medicinal plants.
b. To screen the crude extracts and essential oils for their relative antimicrobial potencies.
c. To fractionate the crude extracts into components of related polarity.
d. Determine and compare the antimicrobial potencies of the fractions (components or constituents).

# CHAPTER TWO

# 2 LITERATURE REVIEW

## 2.1 THE DISTRIBUTION AND USES OF THE SELECTED PLANTS IN GHANA

### 2.1.1 *Xylopia aethiopica* (Dunal) A. Rich.

Common names: Ethiopian pepper, Spice tree, Poivrier or Piment Noir de Guinee.

Twi: Hwentia (i.e. “slender nose”, referring to the fruits).

Ga, Krobo: Soo; Ewe (Aw.): Tsuo; Hausa: Kimba

Habitat: On upper edge of mixed Deciduous Forest and Fringing Forest.

The fruits and seeds are hot to the taste and are commonly sold in local markets as a spice and as a substitute for pepper. They were formerly used as such in Europe.

In Ghana they are crushed together with other spices and the mixture called “kurobow” in Twi, is applied to the skin, to which is imparts a whitish color and a fragrant odor. The ground seeds are added to local snuff to give it a good scent. The dried and powdered fruits are used in coffee and sometimes in food. The crushed fruits, with Capsicum pepper, mixed with kola nuts prevent attacks of kola weevils and are sometimes added to water to purify it, or to palm-wine to flavour it.

The wood is white, the heartwood being pale yellowish brown. Fairly hard and heavy, it is used for house posts and scantlings; and being flexible, it is useful as masts, oars and paddles and for bows.

It is reputed to be termite-proof. It also makes good fuel. From the inner bark cordage can be made. The bark is scented and is used for partitions in huts.

This plant is used to assist action of other local remedies and as a counter-irritant. The root is used as a cough medicine. An extract of the bark is used by the Hausas as an ointment for sores. A decoction of the bark is used for bronchitis, dysentery, and biliousness. (Irvine, 1961)

A decoction of the leaves is used for rheumatism and as emetic. A leaf poultice is sometimes applied for headache and neuralgia. The spicy fruits and seeds have many local medicinal uses both external and internal, e.g. as a cough medicine, carminative, purgative and stimulating addition to other medicines (Agbo pot (Yoruba)) (Irvine, 1961)

A decoction of the fruit is used for bronchitis, dysenteric conditions, and biliousness, and as a women's remedy to encourage fertility. It is used as a lotion for boils and eruptions, and as a liniment for lumbago. Sometimes an oily extract of the seeds is used. The pounded fruit, with tobacco, is burned and smoke inhaled for respiratory complaints in Liberia. A poultice of then fruit and leaves is used for headache and neuralgia. The crushed seeds rubbed on the forehead cure headache and neuralgia. (Irvine, 1961)

A seed extract is used for round worm, and as an emetic for biliousness. The fruit and seeds are used as a restoration after delivery, and a decoction is used as vermifuge. Some of the uses are also applied to cattle and other domestic animals. (Irvine, 1961).

The fruit and seed contain avoceine, a "ruberosole" fat and a resin rich in essential oil. (Irvine, 1961).

### 2.1.2 *Cinnamomum zeylanicum* Nees

Common name: Ceylon cinnamon

Vernacular names: Twi – Anoatre dua; Fante – Anoater dua

**Plant description and geographical distribution**

A small tree with smooth bark; opposite dark green, coriaceous and shiny leaves, obovate, with 3-5 basal nerves, up to 15 cm long and 10 cm broad; flowers unisexual, cream, in auxillary and terminal panicles; fruit small drupe.

A small evergreen tree native to tropical southern India and Sri Lanka; now cultivated extensively in Sri Lanka, the coastal regions of India and many tropical countries in gardens and compounds. (Ghana Herbal Pharmacopeia, 2007)

Grown widely in tropics, and occasionally seen in Ghana. Commercial cinnamon is obtained by peeling the bark from actively growing young twigs. After slight fermentation the outer bark is scraped and the inner rolls in on itself, forming ‘quills’ which are dried in the sun and sold as the well-known flavouring. The ‘quills’ chips, leaves, and bark scrapings or ‘featherings’, cinnamon oil and essence are prepared. (Irvine, 1961)

The bark of the young shoots is carminative, astringent, and because of its volatile oil, antipyretic. It is largely used as an intestinal astringent in diarrhoea and for catarrh and colds (Irvine, 1961).

### 2.1.3 *Psidium guajava* Linn

Common name: Guava.

Twi: Oguawa; Fante: Eguaba; Ga: Gowa; Ewe: Goa; Nzema: Aduoba

Native to Tropical America, now inter-tropical, planted as a fruit tree in West Africa, but growing wild in Ghana, sometimes forming thickets behind sea beach.

The sticky and edible fruits are often used for making the famous Guava Jelly, and the Fantes use the roots in soups. (Irvine, 1961)

The young fruits are used medicinally. The bark contains 11 – 30% of tannin, and the leaves have been used for local tanning in India. The pounded roots, with water, are used for diarrhoea and dysentery. The roots and bark are astringent. The strongly scented leaves are chewed to relieve toothache, and a leaf infusion is widely used for stomach complaints, e.g. constipation. A leaf decoction, boiled with lemon grass, is taken for coughs. The leaves (and fruits) are astringent and the leaves are used in Gabon mainly as an infusion. The ripe fruits are laxative (Irvine, 1961).

### 2.1.4 *Ocimum gratissimum* Linn

Common Names: Fever plant; fever leaf; tea bush; mosquito plant

Vernacular Names:

Twi- Nunum; Onunum; Fante- Nunum; Onunum; Ga-Dangme -Suro; Sulu; Sro; Gbekono

Nzema -Amaloko; Ameloko; amaliko; Ewe –Bebusui; Hausa -Dai'doya ta gida

**Plant description and distribution**

Erect glabrous herb, up to 2 m high; old stems glabrescent (young stem pubescent); leaves petiolate, up 12 cm long, 4 cm broad, ovate or obovate, cuneate or asymmetric base, apex acute or acuminate, margin toothed; white flowered; pedicel puberulous, calyx two-lipped, upper lip ovate, lower lip oblong, two-teethed.

The plant is cultivated in home gardens but also spontaneous; widely distributed in the tropics.

(Ghana Herbal Pharmacopeia, 2007)

## 2.2 SECONDARY METABOLITES FROM HIGHER PLANTS WITH ANTIMICROBIAL ACTIVITY

Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, flavonoids, resins fatty acids gums which are capable of producing definite physiological action on body. (Joshi et al., 2009).

Plants with anti-bacterial effect are rich in polyphenolic substances such as tannins, catechins, alkaloids, steroids and polyphenolic acids. The anti-bacterial activity also could be due to various chemical components and the presence of essential oils in adequate concentrations, which damage microorganism. The insolubility of essential oils and non-polar extracts make it very difficult for them to be used in an aqueous medium during the study of anti-microbial activity (Samy et al., 2008).

A great number of factors can influence the results such as the extraction method, volume of media, culture composition and incubation temperature (Samy et al., 2008).

### 2.2.1 Solvent Extraction

Soxhlet extraction, a method of separation, relies on the solubility characteristics of the particular species involved. In this method, the grinding process assists the penetration of the solvent to the cellular structure of the plant tissues, thereby helping to dissolve the secondary metabolites and increase the yields of extraction. Generally, the smaller the particle sizes of the plant material the more efficient the extraction (Silva et al., 1998).

Also in this procedure, ether is often used as a solvent for extraction due to its low boiling point, relatively non-toxic nature (when compared to chloroform or methanol), and of course because it is quite non-polar (Bergeron & Benning, 2010 ). Petroleum ether is more non-polar, cheaper and less flammable than diethyl ether. Due to its greater non-polarity, petroleum ether will yield a more specific extract than diethyl ether (Bergeron & Benning, 2010). Diethyl ether is rarely used for plant extractions because of its volatility, flammability, toxicity, as well as its tendency to form explosive peroxides (Silva et al., 1998).

From the scheme of fractionation i.e. from Mistcher et al. (1987), (using the soxhlet extraction) all the dry powdered plant samples were first extracted with a non-polar solvent thus petroleum ether 40 – 60$^{o}$C. The residue was subsequently extracted with 70% ethanol and finally the residue was extracted with water.

According to Silva et al. (1998), alcoholic solvents efficiently penetrate cell membranes, permitting the extraction of high amounts of endocellular components but in contrast, less polar solvent such as chloroform, etc may wash out mostly extracellular material.

Petroleum ether being non-polar solvent is believed to have extracted the extracellular materials of the leaves plus any other non-polar components present.

### 2.2.2 Plant – derived Antimicrobial Agents

Traditional medicinal plants have an almost maximum ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most of these are secondary metabolites, of which 12,000 plant-derived agents have been isolated in the recent past (Samy et al., 2008).

### 2.2.3 Major groups of Antimicrobial Compounds from plants

Many of these substances serve as plant defense mechanism against invasion by micro-organisms, insects and herbivores. Some of the plants substances such as terpenoids are responsible for odor (quinines and tannins) plus pigment of the plant. Many compounds are responsible for plant flavor (e.g. the terpenoid capsaicin from chili pepper), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Samy et al., 2008).

The useful major groups of antimicrobial Phytochemicals can be divided into several categories that include: alkaloids, flavonoids (flavones, flavonols, Quinones), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids (Samy et al., 2008).

#### 2.2.3.1 Phenolics and Polyphenols

The term phenolic compounds embrace a wide range of plant substances which possess in common aromatic ring bearing one or more hydroxyl substituents. Phenolic substances

tend to be water-soluble, since they frequently occur combined with sugar as glycosides and they are usually located in the cell vacuole (Harborne, 1984).

#### 2.2.3.1.1 Simple phenols and phenolic acids

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state (Fig. 2.1) (Cowan, 1999).

The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses, bacteria, and fungi (Cowan, 1999).

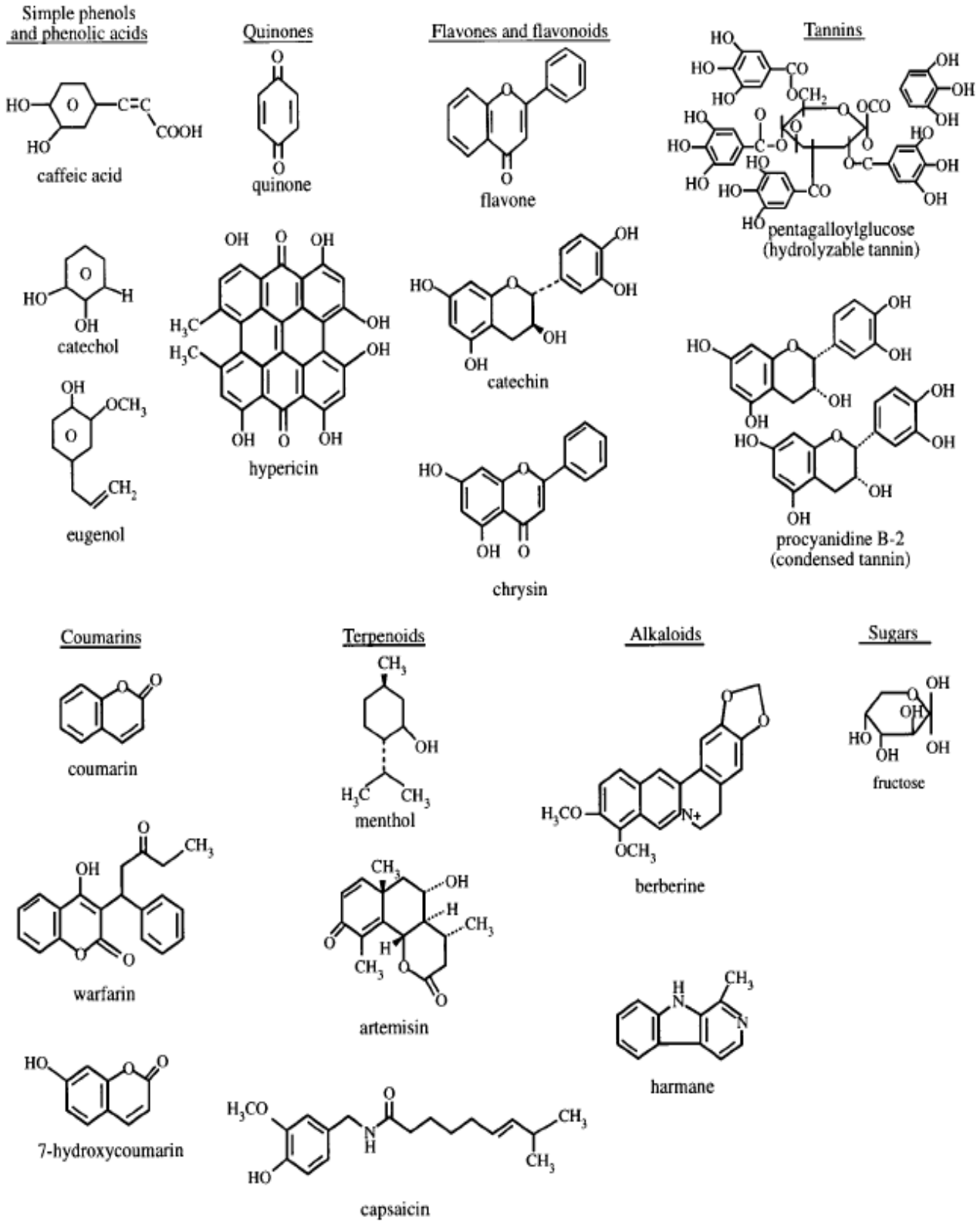


**Fig. 2.1: Structures of some secondary metabolites**

Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two (2) OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, some authors have found that more highly oxidized phenols are more inhibitory. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999).

Phenolic compounds possessing a C3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well-characterized representative found in clove oil (Fig. 2.1). Eugenol is considered bacteriostatic against both fungi and bacteria (Cowan, 1999).

#### 2.2.3.2 Quinones

Quinones are aromatic rings with two ketone substitutions (Fig. 2.1). They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin. Their presence in henna gives that material its dyeing properties (Cowan, 1999).

The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular

quinone- hydroquinone pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in mammalian electron transport systems. Vitamin K is a complex naphthoquinone. Its antihemorrhagic activity may be related to its ease of oxidation in body tissues. Hydroxylated amino acids may be made into quinones in the presence of suitable enzymes, such as a polyphenoloxidase (Cowan, 1999). The reaction for the conversion of tyrosine to quinone is shown in Fig. 2.

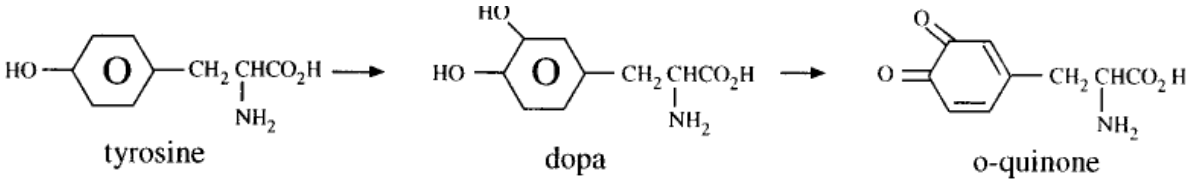


**Fig. 2.2: the conversion of tyrosine to quinone**

In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Cowan, 1999), often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined. Kazmi et al. (1994) described an anthraquinone from *Cassia italica*, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis, Corynebacterium pseudodiphthericum, and Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae.* Hypericin, an anthraquinone from St. John's wort (*Hypericum perforatum*), has received much attention

in the popular press lately as an antidepressant, and has been reported that it had general antimicrobial properties (Cowan, 1999).

**2.2.3.3 Flavonoids**

Flavonoids are hydroxylated phenolic substances that occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms (Cowan, 1999).

Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999).

The leaves of guava are rich in flavonoids, in particular, quercetin. Much of guava's therapeutic activity is attributed to these flavonoids. The flavonoids have demonstrated antibacterial activity. Quercetin is thought to contribute to the anti-diarrhea effect of guava; it is able to relax intestinal smooth muscle and inhibit bowel contractions (Taylor, 2005).

Catechins, the most reduced form of the C3 unit in flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in Oolong green teas. It was noticed some time ago that teas exerted antimicrobial activity and that they contain a mixture of catechin compounds. These compounds inhibited in vitro *Vibrio cholerae* O1, *Streptococcus* mutans, *Shigella*, and other bacteria and microorganisms. The catechins inactivated cholera toxin in Vibrio and inhibited isolated bacterial glucosyltransferases in *S. mutans*, possibly due to complexing activities described for quinones above. This latter activity was borne out in in vivo tests of conventional rats. When the rats were fed a diet containing 0.1% tea catechins, fissure caries (caused by *S. mutans*) was reduced by 40% (Cowan, 1999).

Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside, glycyrrhizin (from licorice), and chrysin against HIV. More than one study has found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV). Kaul et al. (1985) provide a summary of the activities and modes of action of quercetin, naringin, hesperetin, and catechin in in vitro cell culture monolayers. While naringin was not inhibitory to herpes simplex virus type 1 (HSV-1), poliovirus type 1, parainfluenza virus type 3, or RSV, the other three flavonoids were effective in various ways. Hesperetin reduced intracellular replication of all four viruses; catechin inhibited infectivity but not intracellular replication of RSV and HSV-1; and quercetin was universally effective in reducing infectivity. The authors propose that small structural differences in the compounds are critical to their activity and pointed out another advantage of many plant derivatives: their low toxic potential (Cowan, 1999).

An isoflavone found in a West African legume, alpinumisoflavone, prevents schistosomal infection when applied topically. Phloretin, found in certain serovars of apples, may have activity against a variety of microorganisms. Galangin (3,5,7-trihydroxyflavone), derived from the perennial herb *Helichrysum aureonitens*, seems to be a particularly useful compound, since it has shown activity against a wide range of gram-positive bacteria as well as fungi and viruses, in particular HSV-1 and coxsackie B virus type 1 (Cowan, 1999).

Delineation of the possible mechanism of action of flavones and flavonoids is hampered by conflicting findings. Flavonoids lacking hydroxyl groups on their b-rings are more active

against microorganisms than are those with the 2OH groups; this finding supports the idea that their microbial target is the membrane. Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect; i.e., the more hydroxylation, the greater the antimicrobial activity. This latter finding reflects the similar result for simple phenolics. It is safe to say that there is no clear predictability for the degree of hydroxylation and toxicity to microorganisms (Cowan, 1999).

Anti-microbial flavonoids have been reported from *E. latissima*. Dimethoxyflavone and bonducellin were isolated from the aerial parts of *Caesalpinia pulcherrima*. Isobonducellin was found to be a homoisoflavanoid containing a cis (Z)-double bond possessing anti-microbial activity. Compounds of *C. pulcherrima* with anti-viral activities were derived from the flavonoid of quercetin. Moreover, the flavonoids, acacetin-7-o-b-D-galactopyranoside of *C. morifolium* was found to be active as towards HIV. A wide variety of flavonoids, sesquiterpenoid alcohols, triterpenoids and quinic acid caffeates product from plants may also be useful as anti-microbials (Samy et al., 2008).

#### 2.2.3.4 Terpenoids and Essential Oils

An enormous range of plant substances are covered by the word ‘terpenoid’, a term which is used to indicate that all such substances have a common biosynthetic origin. Thus, terpenoids are all based on the isoprene molecule and their carbon skeletons are built up from the union of two or more of these $C_5$ units. Chemically, terpenoids are generally lipid-soluble and are located in the cytoplasm of the plant cell. Essential oils sometimes occur in special glandular cells on the leaf surface, whilst carotenoids are especially associated with

chloroplast in the leaf and with chromoplasts in the petal. Terpenoids are normally extracted from plant tissues with light petroleum, ether or chloroform and can be separated by chromatography on silica gel or alumina using the same solvents (Harborne, 1984).

The mainly terpenoids essential oils comprise the volatile steam-distillable fraction responsible for the characteristic scent, odor or smell found in many plants. They are commercially important as the basis of natural perfumes and also spices and flavorings in the food industry. Plant families particularly rich in essential oils include the Compositae, *Matricaria*, Labiatae, e.g. the mints, *Mentha* spp., Myrtaceae, *Eucalyptus*, Pinaceae, *Pinus*, Rosaceae, 'altar' of roses, Rutaceae, *Cirus* oils and Umbelliferae, anise, caraway, cumin, dill, etc. Chemically, the terpene essential oils can be divided into two classes, the mono- and sesquiterpenes, $C_{10}$ and $C_{15}$ isoprenoids, which differ in their boiling point range (monoterpenes b.p. 140 – 180$^{o}$C; sesquiterpenes b.p. >200$^{o}$C). (Harborne, 1984)

Essential oils are steam-volatile or organic-solvent extracts of plants used traditionally by man for many centuries for the pleasant odour of the essence, its flavour or its antiseptic and/or preservative properties. (Wallace, 2004)

The anti-microbial properties of aromatic volatile oils from medicinal, as well as other edible, plants have been recognized since antiquity (Samy et al., 2008).

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure (Fig. 2.1). They are called terpenes, and they occur as diterpenes, triterpenes, and tetraterpenes (C20, C30, and C40), as well as hemiterpenes (C5) and

sesquiterpenes (C15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999).

Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Useful effects of essential oils have been demonstrated against pathogenic bacteria (Cowan, 1999).

Oils from *Cinnamomum osmophloeum* have been shown to possess antibacterial activity against *Escherichia coli, Enterococcus faecalis, and Staphylococcus aureas* (including the clinically problematic methicillin-resistant *S. aureus*), *Salmonella sp*. and *Vibrio parahemolyticus*; cinnamaldehyde is the main antibacterial component of the mixture (Chang et al., 2001).

Examples of common terpenoids are menthol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Artemisin and its derivative a-arteether, also known by the name qinghaosu, find current use as antimalarials (Cowan, 1999).

The seeds of *Nigella sativa* Linn. (Ranunculaceae) contain active constituents, e.g. volatile oil and thymoquinone showed protection against nephrotoxicity and hepatotoxicity induced by either disease or chemicals. The seed oil has anti-inflammatory, analgesic, anti-pyretic, anti-microbial and anti-neoplastic activity. Petroleum ether extract of *Melicope indica* afforded two unusual pentacyclic triterpenes and the ubiquitous steroids, stigmasterol and sitosterol (Samy et al., 2008). Pentacyclic tritepenes were isolated from *Combretum imberbe* that are novel glycosidic derivatives (hydroxyimberbic acid). *Terminalia stuhlmannii* Engl. Stem bark yielded two glycosides of hydroxyimberbic acid, several of

which had anti-bacterial activity. Imberbic acid showed potent activity against *Mycobacterium fortuitum* and *Staphylococcus aureus*. New cycloartane-type triterpenes isolated from the aerial parts of *Acalypha communis* exhibited moderate anti-microbial activity (MIC 8 and 32 mg/ml) against vancomycin-resistant enterococci (Samy et al., 2008). In 1977, it was reported that 60% of essential oil derivatives examined to that date were inhibitory to fungi while 30% inhibited bacteria (Cowan, 1999). The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Accordingly, Mendoza et al. (1997) found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity. Food scientists have found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes*. Oil of basil, a commercially available herbal, was found to be as effective as 125 ppm chlorine in disinfecting lettuce leaves. Chile peppers are a food item found nearly ubiquitously in many Mesoamerican cultures. Their use may reflect more than a desire to flavor foods. Many essential nutrients, such as vitamin C, provitamins A and E, and several B vitamins, are found in chiles (Cowan, 1999).

A terpenoid constituent, capsaicin, has a wide range of biological activities in humans, affecting the nervous, cardiovascular, and digestive systems as well as finding use as an analgesic. The evidence for its antimicrobial activity is mixed. Recently, Cichewicz and Thorpe (Cichewicz et al**.,** 1996) found that capsaicin might enhance the growth of *Candida albicans* but that it clearly inhibited various bacteria to differing extents. Although possibly detrimental to the human gastric mucosa, capsaicin is also bactericidal to *Helicobacter*

*pylori*. Another hot-tasting diterpene, aframodial, from a Cameroonian spice, is a broad-spectrum antifungal. The ethanol-soluble fraction of purple prairie clover yields a terpenoid called petalostemumol, which showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus* and lesser activity against gram-negative bacteria as well as *Candida albicans*. When it was observed that residents of Mali used the bark of a tree called *Ptelopsis suberosa* for the treatment of gastric ulcers, investigators tested terpenoid-containing fractions in 10 rats before and after the rats had ulcers chemically induced. They found that the terpenoids prevented the formation of ulcers and diminished the severity of existent ulcers (Cowan, 1999).

Whether this activity was due to antimicrobial action or to protection of the gastric mucosa is not clear (Cowan, 1999).

**2.2.3.5 Alkaloids**

There is no one definition of the term 'alkaloid' which is completely satisfactory, but alkaloids generally include "those basic substances which contain one or more nitrogen atoms, usually in combination as part of a cyclic system." Alkaloids are often toxic to man and many have dramatic physiological activities; hence their wide use in medicine. They are usually colorless, often optically active substances; most are crystalline but a few (e.g. nicotine) are liquids at room temperatures. A simple but no means infallible test for alkaloids in fresh leaf or fruit material is the bitter taste they often impart to the tongue (Harborne, 1984).

The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum*; the name morphine comes from the Greek Morpheus,

god of dreams. Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family, are commonly found to have antimicrobial properties. Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be useful against HIV infection as well as intestinal infections associated with AIDS (Cowan, 1999).

Berberine is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Cowan, 1999).

Bioassay-guided isolation studies done on the root extract of *Polyalthia longifolia* shows that it possesses significant anti-bacterial activity led to the isolation of three new alkaloids pendulamine A, pendulamine B and penduline along with stigmasterol 3-O-beta-D-glucoside, allantoin, the known diterpenoid kolavenic acid and the azafluorene alkaloid isoursuline. Compound pendulamine A and pendulamine B were found to be active. Micro-organism inhibition concentrations, abbreviated as MICs, are 0.02–20 mg against bacteria (Samy et al., 2008). The seed pods of *Erythrina latissima* yielded erysotrine, erysodine, syringaresinol, vanillic acid and a new erythrina alkaloid, (+)-10, 11-dioxoerysotrine that was lethal to brine shrimp. 2-(50-Hydroxy-30-methoxy phenyl)-6-hydroxy-5-methoxybenzofuran has strong anti-microbial activity against yeast spores (Samy et al., 2008). Ethanol extracts of the *Guatteria multivenia* root have furnished known alkaloids such as liriodenine, lysicamine, lanuginosine, guadiscine and O-methylpallidine. Lanuginosine possesses weak inhibitory effects against fungi and liriodenine was found to have anti-microbial activity against both bacteria and *Candida albicans* (Samy et al. 2008).

## 2.3 METHODS OF ASSESSING ANTIMICROBIAL ACTIVITY

### 2.3.1 Factors affecting antibacterial activity

The activity of antimicrobial agents against microorganisms depends on two major factors:

- the nature of the physical environment and
- the condition of the organism

### 2.3.2 Pretreatment Factors

The structure and functioning of bacterial cells and their sensitivity to antibacterial agents may be profoundly affected by the conditions under which they are grown (Gilbert, 1988). Thus, for mechanism of action studies, it is important that the conditions used for cultivation and those involved in the preparation of test suspensions be identical and rigorously controlled (Bloomfield, 1991).

### 2.3.3 Factors during treatment

Any number of environmental factors may affect the activity of an antibacterial agent. This may involve effects on the bacterial cell itself or alternatively (or possibly additionally) may result from direct effects on the agent.

1. Concentration of antibacterial agents

   The activity of antibacterial agents may be profoundly affected by relatively minor changes in concentration

2. Temperature

The activity of antibacterial agents usually increases geometrically with increasing temperature.

Number of organisms

In general the activity of an antibacterial agent decreases as the inoculum size increases. The extent of this effect varies with different antibacterial agents but it is important that studies of antibacterial activity are carried out with inocula which are similar in size to those used for biochemical and other studies (Bloomfield, 1991).

3. Environmental pH

Environmental pH can affect antimicrobial activity in a number of ways:

- It may affect the activity of the antimicrobial agents. Many antibacterial agents are weak acids or weak bases. Experience has shown that the majority of agents are more active in their unionized form. Activity of these agents will therefore depend on their pKa in relation to the pH of the suspending medium. The interaction of chemically reactive agents such as chlorine and glutaraldehyde with cell constituents may also be affected by pH.

It may produce changes in the distribution of the bacterial cell surface thereby affecting uptake of charged antibacterial agents (Bloomfield, 1991).

4. Other Constituents of the Suspending medium

All constituents of the suspending medium should be considered in terms of possible effects on antibacterial activity. Neutralizing effects of organic matter on the activity of disinfectant formulations have been widely studied. Surface

active agents which are common formulation components in pharmaceuticals, cosmetic and other formulations may also affect activity. In general, it would seem that at concentrations of surface active agent below the critical micelle concentration (cmc), activity of the antibacterial agent is potentiated due to effects of the surfactant on the cell surface which increase permeability. At concentration above the cmc, interaction and micellization produce a reduction in the aqueous phase concentration of 'free' antibacterial agent causing reduced activity (Bloomfield, 1991).

5. Other Factors

   Mechanism of action studies are usually carried out with aqueous suspensions of test organisms. Experimental observations with pharmaceutical and other formulations show that where a solid or non-miscible liquid phase is introduced, loss of activity may occur as a result of adsorption or partitioning. For mechanism of action studies which are invariably carried out in vitro, the possibility of interaction with glass or other container surfaces must be considered. Laboratory investigations suggest that loss of activity can result from adsorption of agents on container. Reduced sensitivity to antibacterials may also be associated with growth of organisms as biofilms on glass or PVC surfaces (Bloomfield, 1991).

### 2.3.4 Tests for Determining Bacteriostatic Activity

In determining the minimum growth inhibitory concentration (MIC) of a bacteriostatic agent, the organisms is introduced into the system which contains the antimicrobial agent but which also provides optimum nutrients and environmental conditions for growth.

Following an incubation period (usually 18 – 24 hours) the culture is examined either visually or by other means to assess whether there is an increase in number of viable cells (Bloomfield, 1991).

In relating the results of MIC determination to biochemical studies, two major problems are encountered:

1. Whereas biochemical or other studies are usually carried out with aqueous, saline or buffered suspensions, for MIC determinations nutrient materials which may range from simple salts to undefined bacteriological media constituents such as meat extract and peptone must be included. As mentioned previously both the growth media and growth environment (e. g. incubation temperature) may affect activity.
2. Experimental investigations show clearly that MIC values will depend on inoculums size, MIC increasing with increasing inoculum size. For mode of action studies, the sensitivity of many biochemical assays dictates the use of suspensions containing about 1- $5x10^9$ organisms/ml (c. 1mg dry weight organic material per mL), whereas MIC's are normally determined with inoculums sizes of $10^3$ - $10^5$ organism/mL, which in batch culture will grow to a maximum of about 5 x $10^8$ organisms / mL, as determined by availability of nutrients and oxygen, the production of toxic metabolites and other inhibitory factors.

In the laboratory, Bacteriostatic assays generally involve either agar diffusion techniques in which inhibition zones are used for assessment of activity or serial dilution methods in which MIC is determined (Bloomfield, 1991).

### 2.3.4.1 Agar Diffusion Technique

These tests are relatively simple and may be either ‘qualitative’ (i.e. simple screening for activity against a range of micro-organisms) or ‘quantitative’ (i.e. determination of the concentrations of antibacterial agent required to inhibit growth).

For qualitative assay, a nutrient agar plate is inoculated with micro-organism either by adding the organism to the agar before it is poured, or by streaking the organisms across the surface of the plate.

For seeded plates a solution of the antibacterial is introduced into cups which can be cut into the surface of the plate using a sterile cork borer or impregnated on sterile filter paper discs which are then placed on the agar surface (Bloomfield, 1991).

For streak plates the antibacterial solution is pipette into a trough cut into the agar at right angles to the streaks or again impregnated on a filter paper strip which is placed on the agar surface. One of the advantages of using filter paper discs or strips is that the paper can be dried before placing on the agar surface. This is useful for low-water-soluble antibacterial agents. These can be dissolved in organic solvents which can be evaporated off to avoid any toxic effects which may occur from the solvent itself. Evaporation of solvent from filter paper discs is usually done using an infrared lamp bulb. It must be borne in mind however, that agents with low water solubility may show limited agar diffusion (Bloomfield, 1991).

Following storage for a short period (2-3 hours) to allow diffusion of the antibacterial agent into the agar without microbial growth, plates are then incubated and the radius of the inhibition zone measured. Zone diameters may be used to give a measure of the relative activity against a range of micro-organisms. Since the size of the diffusion depends on the rate of diffusion, quantitative assessment of MICs by this method requires knowledge of the diffusion rate of the compound under investigation (Bloomfield, 1991).

#### 2.3.4.2 Serial Dilution Methods

For determination of MICs by serial dilution methods, graded concentrations of the agent are added to nutrient broth or nutrient agar. For achieving the required concentration range of water-soluble compounds, concentrated solution may be added to double- or triple-strength broth and made up to volume with water as appropriate. For compounds with low water solubility, intermediary solvents may be required to achieve the desired concentration range in which case the system must be checked to ensure that the quantity of solvent added is not inhibitory. Laboratory experience suggests that volumes of 50 – 100 µl per 10 ml broth of solvents such as chloroform, acetone, methyl and ethyl alcohol are satisfactory, although the sensitivity to these solvents may vary from one species to another and should be checked. Mode of action studies with the chlorinated phenolic compound Fentihlor (Hugo & Bloomfield, 1971) indicated that for compounds of low water solubility, antibacterial activity may continue to increase at concentrations above saturation.

For determining MICs, media are the inoculated with test organisms, incubated and the MIC determined as the lowest concentration which inhibits visible growth. One of the advantages of using agar as opposed to broth dilution is that a number of test organisms can be inoculated on to a single series of plates. Although serial dilution provides a direct quantitative assessment of active concentrations, it must be remembered that, in effect, the MIC is a concentration which lies between the observed maximum inhibitory concentration and the minimum inhibitory concentration. Thus the accuracy of the end-point will depend on the range of concentrations used. In practice, for initial screening it is usual to employ a series of 10-fold or doubling concentrations, e.g. 0.001, 0.01, 0.1, 1.0 …10% (w/v), or 0.01, 0.02, 0.04, 0.08… % (w/v). Once the approximate MIC is known, an arithmetical series of initially not less than eight dilutions is employed, e.g. 0.1, 0.2, 0.3, 0.4… % (w/v). In practice the choice of dilution should take into account the concentration exponent of the agent under consideration (Bloomfield, 1991).

# CHAPTER THREE

## 3 METHODOLOGY

### 3.1 MATERIALS: PLANT SAMPLES, EQUIPMENTS AND REAGENTS

#### 3.1.1 Plant samples:

The leaves of the following plants were used in the study:

1. *Cinnamomum zeylanicum Nees.(*Cinnamon*)*
2. *Psidium guajava Linn.(*Guava*)*
3. *Ocimum gratissimum Linn.(*Ocimum)
4. *Xylopia aethiopica A. Rich (*Xylopia*)*

All plant samples were collected from the residence of Mr. G. K. Tuani, a natural product organic chemist at 54 Okodee road on the KNUST campus. The plants had already been identified by Botanists at the College of Agriculture and Natural Resources, Department of Horticulture, aKNUST, Kumasi.

#### 3.1.2 Equipments

Soxhlet extractors, Buchi Rotavpor R-114, microbiological studies at the Kwame Nkrumah University of Science and Technology (KNUST) Faculty of Pharmacy and Pharmaceutical Sciences, Department of Pharmaceutics Microbiology lab.

#### 3.1.3 Chemicals and reagents:

Ethanol, methanol, petroleum ether 40 – 60$^{o}$C, citric acid, chloroform, hydrochloric acid, sodium hydroxide, and dimethyl sulphoxide (DMSO).

## 3.2 PREPARATION AND EXTRACTION OF PLANT SAMPLES

### 3.2.1 Preparation of Plant Samples

All the plant samples, i.e. leaves, were dried under shade thus air drying.

**Fig. 3.1: Picture of some sample during drying**

Dried samples were ground at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi.

### 3.2.2 EXTRACTION

Extraction was done using the Soxhlet apparatus. Serial or successive extraction whereby the same sample was first extracted with a non polar solvent, thus Petroleum ether (40 – 60$^{o}$C), followed by 70% ethanol and lastly extracted with distilled water. The essential oils were obtained by steam distillation.

About one hundred grams (100g) of the dried powered sample was first refluxed with Petroleum ether (500 ml) in a Soxhlet Apparatus (1000 ml round bottom flask) for about

six (6) hours, followed by the extraction of the residue with 500 ml of 70% ethanol and successively followed by the extraction with 500 ml of distilled water.

Each of the extracts after refluxing was evaporated, using Buchi Rotavpor R-114, at 40$^{o}$C and 60$^{o}$C for Petroleum ether and ethanol extracts(about 350 to 400ml of extract) respectively at relatively mild rotation per second (speed of the rotary evaporator,).

The resulting crude extracts (except the Petroleum ether extracts) were dried further using evaporating dishes on water baths. Petroleum ether extracts which were left in the fume hood for some hours for all the solvents to evaporate. The resulting crude extracts were kept in the glass bottles in a refrigerator at 4$^{o}$C until use.

**Fig. 3.2: A picture of Soxhlet apparatus during an extraction**

**Fig. 3.3: A picture of Buchi Rotavpor R-114**

### 3.2.2.1 Fractionation of Crude Extracts

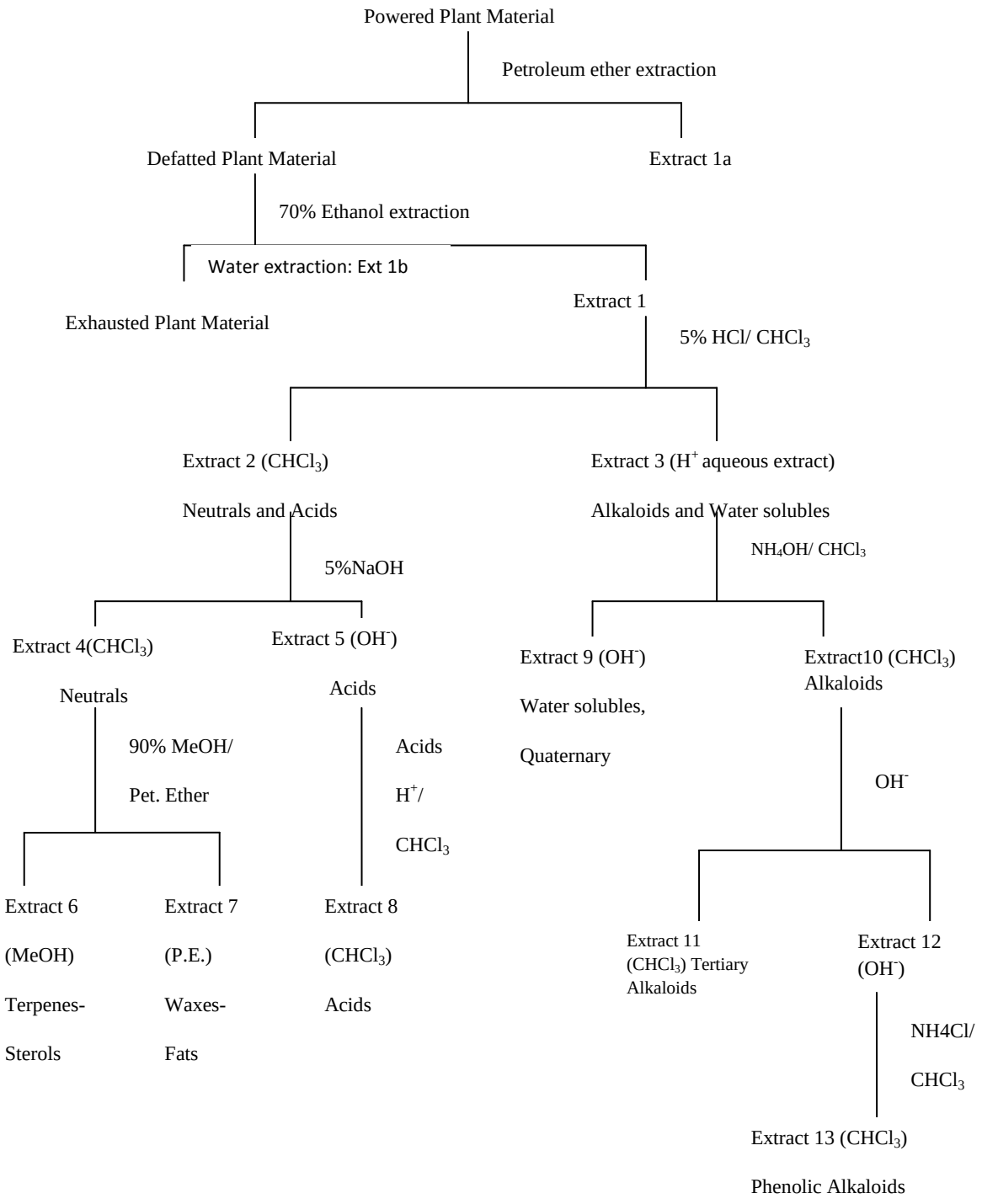


**Fig. 3.6: The generalized scheme for isolation of Antimicrobial Agents from Higher plants**

The generalized scheme for isolation of antimicrobial agents from higher plants, (fig. 3.6) proposed by Mistcher et al. (1987) was adopted in the fractionation.

1. The powdered plant material was first extracted with Petroleum ether 60-40$^{o}$C using the Soxhlet apparatus.
2. The plant material after defatting with Petroleum ether was also extracted with 70% ethanol to give Extract 1. The plant material was subsequently extracted with water. Some portions of Extract 1 was evaporated using the Buchi Rotavpor R-114 and was subsequently used for the determination of the antimicrobial activities.
3. The volume of the remaining portion of Extract 1 was reduced to about one tenth the initial volume.
4. The resulting extract from Extract 1was acidified with 5% HCl. The acidity of the extract was carried out using litmus, which turned blue litmus red.
5. The acidified Extract 1 was extracted with chloroform ($CHCl_3$) thrice in a separatory funnel.
6. The chloroform extract (i.e. Extract 2) was extracted with 5% Sodium hydroxide (NaOH) in a separatory funnel. The NaOH phase was labeled Extract 5.
7. The acidified extract from Extract 1 after the separation was labeled Extract 3.
8. The chloroform extract i.e. Extract 4 was partitioned between equal amounts 90% methanol and Petroleum ether.
9. The methanol extract (Extract 6) was separated and the solvent evaporated.
10. The Petroleum ether extract (Extract 7) was also separated and solvent evaporated.

11. The basic (OH-) extract (Extract 5) from Extract 2 was neutralized with 5% HCl and was extracted with chloroform. The chloroform extract was separated and the solvent evaporated and stored as extract 8.
12. Extract 3 was partitioned between ammonium hydroxide (ammonia solution) and chloroform.
13. The chloroform extract was separated and labeled as Extract 10. The solvent was evaporated.
14. The ammonium hydroxide extract was also separated and labeled as Extract 9 and the solvent evaporated. All extracts were kept in the refrigerator until use.

## 3.3 ANTIMICROBIAL ACTIVITY DETERMINATION OF CRUDE EXTRACTS

### 3.3.1 Agar Diffusion Technique for MIC Determination

#### Test organism

Gram positive bacteria used are *Bacillus subtilis* and *Staphylococus aureus* and gram negative bacteria used are *Escherichia coli* and *Pseudomonas aeruginosa* and one fungus *Candida albicans.* The organisms were clinical strains or isolates from the Komfo Anokye Teaching Hospital (KATH), School of Medical Sciences, Kumasi.

**Materials:**

Nutrient Agar (Oxoid powder CM 3), sterile Petri dishes with diameter of 90mm, analytical balance, New Brunswick Scientific (Edison N.J. USA) reciprocal water bath shaker model R76, Gallenkamp plus II incubator, Becton Dickinson 2ml sterilized syringes, autoclave.

**Determination of Antimicrobial activity**

1. Preparation of four (4) different concentrations of the extracts

    - Leaves extracts

These solvents were used as solubilizers: Methanol, water and occasionally DMSO for the petroleum ether extracts. One gram (1 g) of the crude extract was dissolved in ten milliliters (10 mL) of the solvents thus making 10%w/v. The petroleum ether extract was dissolved in methanol (occasionally DMSO) whiles ethanol and water extracts were dissolved in distilled water. The 10%w/v solution was serially diluted to produce 5%w/v, 2.5%w/v and 1.25%w/v solutions respectively.

    - Essential oils

Dimethyl surphoxide (DMSO) was used as solublizer. One millilitre (1mL) of the essential oil was dissolved in one milliliter (1 mL) of the DMSO thus making 50%v/v. Subsequent concentrations of 25%v/v, 12.5%v/v and 6.25%v/v were prepared by serially diluting the 50%v/v using DMSO.

2. Some quantities of the nutrient agar (20 g) were weighed at different sessions of the test. The weighed nutrient agar was suspended in some amount of distilled water and was heated to dissolve completely. 20 mL of the agar was poured into the needed test tubes and plugged with cotton wool. The agar was then sterilized by autoclaving at 121$^{o}$C for 15 minutes after which it was stabilized in the New Brunswick Scientific (Edison N.J. USA) reciprocal water bath shaker model R76 at 45$^{o}$C for 15 minutes.
3. A twenty-four (24) hour broth culture of E. coli was diluted to contain $10^5$ viable cells present. 0.1mL of this dilution was added to each of the stabilized agar in test

tubes, rolled in the palm to mix thoroughly and contents poured into sterilized Petri dishes and allowed to set. Each Petri dish was labeled with the test organism.

4. By means of a cork borer (number 6) four (4) cups were bored well separated and equidistant from each other in the agar.
5. The cups were labeled appropriately with the concentrations of the extracts and each cup was filled with its corresponding concentration to about three quarters full.
6. The Petri dishes and its contents were kept on the bench at room temperature for 60 minutes for the extracts to diffuse in to the agar. The procedure was repeated twice using E. coli.
7. The whole process was repeated using each of the other organisms.
8. They were then incubated at $37^{o}C$ for 24 hours. The mean zones of growth inhibition were determined after incubation.
9. Graphs of mean zones of growth inhibition (mm) as ordinates versus log concentration as abscissa were plotted. The antilog of the intercept on the log concentration axis is the MIC.


**Fig. 3.4: A picture of Gallenkamp plus II incubator**

**Fig. 3.5: A picture of New Brunswick Scientific (Edison N.J. USA) reciprocal water bath shaker model R76**

### 3.3.2 Antimicrobial Activity Determination of Fractions

1. Preparation of solutions from fractions: Solubilizers used:

Extracts 6 – Methanol Fractions: methanol

Extracts 7 – Petroleum ether Fractions: DMSO

Extracts 8 – Chloroform Fractions: DMSO

Extracts 9 – Aqueous Ammonia Fractions: Water

Extracts 10 – Chloroform Fractions: DMSO

**Table 3.1: A table showing the respective amounts of fractions**

| Ethanolic Crude extract | Amount of Fractions | | | | |
|---|---|---|---|---|---|
| | Extract 6 | Extract 7 | Extract 8 | Extract 9 | Extract 10 |
| Cinnamon | 0.1g | 0.1g | 0.1g | 0.1g | 0.1g |
| Guava | 1 ml | 0.2g | 0.2g | - | 0.2g |
| Ocimum | 0.2g | 0.2g | 0.1g | 1ml of Ext 9 | 0.2g |
| Xylopia | 1ml of Ext 6 | 0.2g | 0.1g | 1ml of Ext 9 | 0.1g |

**Table 3.2: A table showing the respective amounts of Solubilizers added to each fraction**

| Ethanolic Crude extract | Volume of Solubilizers Used | | | | |
|---|---|---|---|---|---|
| | **Extract 6** | **Extract 7** | **Extract 8** | **Extract 9** | **Extract 10** |
| Cinnamon | 1.5 ml methanol | 1 ml DMSO | 1 ml DMSO | 1.5 ml water | 1 ml DMSO |
| Guava | 1 ml methanol | 2 ml DMSO | 2 ml DMSO | - | 1.5ml DMSO |
| Ocimum | 2 ml methanol | 2 ml DMSO | 1.5ml DMSO | 1ml water | 2 ml DMSO |
| Xylopia | 1ml methanol | 2 ml DMSO | 1ml DMSO | 1ml water | 1ml DMSO |

2. Some quantities of the nutrient agar (20g) were weighed at different sessions of the test. The weighed nutrient agar was suspended in some amount of distilled water (200ml) and was heated to dissolve completely. 20 mL of the agar was poured into the needed test tubes and plugged with cotton wool. The agar was then sterilized by autoclaving at 121$^{o}$C for 15 minutes after which it was stabilized in the New Brunswick Scientific (Edison N.J. USA) reciprocal water bath shaker model R76 at 45$^{o}$C for 15 minutes.
3. A twenty-four (24) hour broth culture of *E. coli* was diluted to contain $10^5$ viable cells present. 0.1mL of this dilution was added, to each of the stabilized agar in test tubes, rolled in the palm to mix thoroughly and contents poured into sterilized Petri dishes and allowed to set. Each Petri dish was labeled with the test organism.
4. By means of a cork borer (number 6) four (4) cups were bored well separated and equidistant from each other in the agar.

5. The cups were labeled appropriately with the concentrations of the extracts and each cup was filled with its corresponding concentration to about three quarters full.
6. The Petri dishes and its contents were kept on the bench at room temperature for 60 minutes for the extracts to diffuse in to the agar. The procedure was repeated twice using *E. coli.*
7. The whole process was repeated using each of the other organisms.
8. They were then incubated at $37^{o}$C for 24 hours. The mean zones of growth inhibition were determined after incubation.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

**Table 4.1: Mean values of triplicate zones of inhibition (mm) of crude extracts and essential oil of *Cinnamomum zeylanicum* against five test organisms**

| PLANT EXTRACTS/ ESSENTIAL OIL | CONC./% w/v, v/v | ZONES OF INHIBITION/mm | | | | |
|---|---|---|---|---|---|---|
| | | *E. coli* | *P. aeruginosa* | *B. subtilis* | *S. auerus* | *C. albicans* |
| *Cinnamomum zeylanicum* (Pet. Ether extract) | 10 | 8 | 6 | 7 | 7 | 6 |
| | 5 | 6 | 5 | 6 | 0 | 5 |
| | 2.5 | 4 | 4 | 4 | 0 | 4 |
| | 1.25 | 4 | 0 | 3 | 0 | 3 |
| *Cinnamomum zeylanicum* (ethanol Extract) | 10 | 8 | 0 | 0 | 0 | 9 |
| | 5 | 7 | 0 | 0 | 0 | 7.5 |
| | 2.5 | 0 | 0 | 0 | 0 | 0 |
| | 1.25 | 0 | 0 | 0 | 0 | 0 |
| *Cinnamomum zeylanicum* (aqueous extract) | 10 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 0 | 0 | 0 | 0 | 0 |
| | 2.5 | 0 | 0 | 0 | 0 | 0 |
| | 1.25 | 0 | 0 | 0 | 0 | 0 |
| *Cinnamomum zeylanicum* (Essential oil) | 50 | 10.5 | 8.5 | 8.5 | 9 | 9.5 |
| | 25 | 9.5 | 7 | 8 | 7 | 8.5 |
| | 12.5 | 8.5 | 6 | 7 | 6 | 7.5 |
| | 6.25 | 7 | 5.5 | 5.5 | 5.5 | 6.5 |

**Table 4.2: mean values of triplicate zones of inhibition (mm) of crude extracts and essential oil of *Psidium guajava* against five test organisms**

| PLANT EXTRACTS/ ESSENTIAL OIL | CONC./ % w/v, v/v | ZONES OF INHIBITION/mm | | | | |
|---|---|---|---|---|---|---|
| | | *E. coli* | *P. aeruginosa* | *B. subtilis* | *S. auerus* | *C. albicans* |
| *Psidium guajava* (Pet Ether extract) | 10 | 7.5 | 5 | 6 | 6 | 6 |
| | 5 | 7 | 4 | 6 | 5 | 5.5 |
| | 2.5 | 6 | 0 | 4 | 4 | 5.5 |
| | 1.25 | 5 | 0 | 4 | 3.5 | 4.5 |
| *Psidium guajava* (Ethanol Extract) | 10 | 8.5 | 6 | 8 | 6.5 | 10.5 |
| | 5 | 7 | 5 | 5 | 5 | 8.5 |
| | 2.5 | 6.5 | 4 | 4 | 4 | 7 |
| | 1.25 | 5.5 | 4 | 3.5 | 4.5 | 6.5 |
| *Psidium guajava* (Aqueous Extract) | 10 | 8 | 5 | 5 | 6 | 9.5 |
| | 5 | 7 | 5 | 4 | 5 | 8.5 |
| | 2.5 | 6.5 | 4 | 0 | 5 | 7 |
| | 1.25 | 6 | 4 | 0 | 4.5 | 6.5 |
| *Psidium guajava* (essential oil) | 50 | 10.5 | 7.5 | 8 | 7.5 | 12 |
| | 25 | 10 | 7 | 7.5 | 7 | 11.5 |
| | 12.5 | 9 | 6 | 6.5 | 6 | 10.5 |
| | 6.25 | 6 | 5.5 | 5 | 6 | 9 |

**Table 4.3: mean values of triplicate zones of inhibition (mm) of crude extracts and essential oil of *Ocimum gratissimum* against five test organisms**

| PLANT EXTRACTS/ ESSENTIAL OIL | CONC./ % w/v, v/v | ZONES OF INHIBITION/mm | | | | |
|---|---|---|---|---|---|---|
| | | *E. coli* | *P. aeruginosa* | *B. subtilis* | *S. auerus* | *C. albicans* |
| *Ocimum gratissimum* (Pet. Ether extract) | 10 | 10.5 | 7.5 | 6.5 | 9 | 7 |
| | 5 | 10 | 7 | 5 | 5 | 6 |
| | 2.5 | 6 | 5 | 4.5 | 0 | 6 |
| | 1.25 | 5 | 4 | 3.5 | 0 | 5 |
| *Ocimum gratissimum* (ethanol Extract) | 10 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 0 | 0 | 0 | 0 | 0 |
| | 2.5 | 0 | 0 | 0 | 0 | 0 |
| | 1.25 | 0 | 0 | 0 | 0 | 0 |
| *Ocimum gratissimum* (aqueous Extract) | 10 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 0 | 0 | 0 | 0 | 0 |
| | 2.5 | 0 | 0 | 0 | 0 | 0 |
| | 1.25 | 0 | 0 | 0 | 0 | 0 |
| *Ocimum gratissimum* (essential oil) | 50 | 11 | 9.5 | 8 | 10.5 | 10 |
| | 25 | 9 | 9 | 7.5 | 9.5 | 8.5 |
| | 12.5 | 8 | 8.5 | 7 | 9 | 8 |
| | 6.25 | 7 | 8 | 7 | 8 | 7 |

**Table 4.4: mean values of triplicate zones of inhibition (mm) of crude extracts and essential oil of *Xylopia aethiopica* against five test organisms**

| PLANT EXTRACT/ ESSENTIAL OIL | CONC./ % w/v, v/v | ZONE OF INHIBITION/mm | | | | |
|---|---|---|---|---|---|---|
| | | *E. coli* | *P. aeruginosa* | *B. subtilis* | *S. auerus* | *C. albicans* |
| *Xylopia aethiopica* (Pet Ether extract) | 10 | 0 | 5 | 0 | 0 | 6 |
| | 5 | 0 | 4 | 0 | 0 | 5 |
| | 2.5 | 0 | 4 | 0 | 0 | 4 |
| | 1.25 | 0 | 4 | 0 | 0 | 0 |
| *Xylopia aethiopica* (ethanol Extract) | 10 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 0 | 0 | 0 | 0 | 0 |
| | 2.5 | 0 | 0 | 0 | 0 | 0 |
| | 1.25 | 0 | 0 | 0 | 0 | 0 |
| *Xylopia aethiopica* (aqueous Extract) | 10 | 0 | 0 | 0 | 0 | 8 |
| | 5 | 0 | 0 | 0 | 0 | 7 |
| | 2.5 | 0 | 0 | 0 | 0 | 7 |
| | 1.25 | 0 | 0 | 0 | 0 | 0 |
| *Xylopia aethiopica* (essential oil) | 50 | 6 | 0 | 0 | 0 | 12 |
| | 25 | 5.5 | 0 | 0 | 0 | 9 |
| | 12.5 | 3 | 0 | 0 | 0 | 8.5 |
| | 6.25 | 3 | 0 | 0 | 0 | 8.5 |

The P.E. extracts showed higher activity than other solvent extracts of the selected aromatic leaves (except *Psidium guajava*). This can be attributed to the fact that P.E. being non-polar can extract most of the terpenes of the leaves, hence having activities close to that of the essential oils. However the P.E. extracts cannot have the same activity as the essential oils because some of the volatile components could have been lost during evaporation of the P.E. extracts. Also leaves samples for obtaining the essential oils were not powdered prior to steam extraction but leaves samples for obtaining P.E extracts were first grinded before extraction started. According to Silva et al. (1998), when the secondary metabolites to be extracted are thermolabile or volatile, the milling stage may be omitted to avoid losses by heat generated during grinding. Owing to this assertion, it is possible that some of the secondary metabolites were lost especially the volatile components during grinding of the leaves and supports the reason why the P.E. extracts were not as active as the essential oils.

Increase in the concentration of all the active extracts (including the essential oils) yielded their corresponding increase in the zones of inhibition. This linear relationship between the concentrations of extracts (as well as essential oils) and zones of inhibition could be that the extracts were able to diffuse into the inoculated nutrient agar.

It can be deduced from table 4.1 to 4.4 that all the petroleum ether extracts were active against the test microorganisms except the P.E. extract of *Xylopia aethiopica* which showed activity against *P.aerugionsa* and *C. albicans* only.

The petroleum ether extract of *Cinnamomum zeylanicum* showed highest zone of inhibition against *E. coli* followed by *B. subtillis, C. albicans, P. aeruginosa* and lastly *S. auerus.* The

latter microorganism was quite resistant to the *Cinnamomum zeylanicum* petroleum ether extract.

The petroleum ether extract of *Psidium guajava* was active against all five test organism in the decreasing order of susceptibility as: *E. coli* > *B. subtillis* > *C. albicans* > *S. auerus* > *P. a-eruginosa*.

The petroleum ether of *Ocimum gratissimum* was active against all the test organisms. The trend of susceptibility of the microorganisms to the P.E extracts was: *E. coli* > *P. aeruginosa* > *C. albicans* > *B. subtillis* > *S. auerus.*

Of all the four plants, the P.E. extract of *Ocimum gratissimum* leaves showed the highest zones of inhibition against all five test organisms followed by *Cinnamomum zeylanicum, Psidium guajava* and *Xylopia aethiopica.*

The ethanolic extracts of the four samples showed zones of inhibition poorly compared to those of the P.E extract except *Psidium guajava.* The ethanolic extract of *Psidium guajava* inhibited strongly the growth of all test organisms compared to its corresponding P.E. extract. This trend could suggest that most of the active agents of *Psidium guajava* are soluble in polar solvents. The most susceptible test organism to the ethanol extract of *Psidium guajava* was *C. albicans* followed by *E. coli, B. subtillis, S. auerus and* lastly *P. aeruginosa.* The essential oil of *Psidium guajava* also showed similar susceptibility trend against test organisms but with higher zones of inhibition than its ethanol extract. Gonçalves et al. (2008) screened the antimicrobial effect of essential oil and methanol, hexane, ethyl acetate extracts from guava leaves. The extracts were tested against diarrhoea-causing bacteria: *Staphylococcus aureus, Salmonella* spp. and *Escherichia coli*. Of the bacteria tested, *Staphylococcus aureus* strains were most inhibited by the extracts. The methanol extract showed greatest bacterial inhibition. The essential oil extract showed

inhibitory activity against *S. aureus* and *Salmonella* spp. (Gonçalves et al., 2008). This supports the finding that the essential oil and solvent extracts of *Psidium guajava* are active against diarrhoea-causing bacteria: *Staphylococcus aureus.* This explains why *Psidium guajava* is used in the treatment of diarrhoea in folk medicine in Ghana and other tropical countries (G.H.P., 2007; Mahfuzul Hoque et al., 2007).

The aqueous extract of *Psidium guajava* was the only aqueous extract with activity against all microorganisms and buttresses the idea that most of the active components of *Psidium guajava* are soluble in polar solvents. The most susceptible test organism to the aqueous extract of *Psidium guajava* was *C. albicans* followed by *E. coli, S. aureus, P. aeruginosa, and* lastly *B. subtillis.*

The ethanolic extract of *Cinnamomum zeylanicum* was active against *E. coli* and *C. albicans*. It has been reported by Khan *et al*., (2008) that the ethanol extracts of *Cinnamomum zeylanicum were* active against multidrug resistant (MDR) strains of *Escherichia coli, Klebsiella pneumoniae* and *Candida albicans.* Khan et al. (2008) concluded that *C. zeylanicum (*together with *A. nilotica* and *S. aromaticum)* could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms from community as well as hospital settings.

Oboh (2006) in an attempt to explain the scientific basis for the medicinal and nutritional benefits of *Ocimum gratissimum* leaves as leafy vegetable assessed the phytochemical constituents, antioxidant and antimicrobial activity of *Ocimum gratissimum.* The outcome of the study revealed that the ethanol extract of *Ocimum gratissimum* contained tannin,

saponin, anthraquinone, alkaloid and glycosides. Additionally, Oboh (2006) stated that the ethanol extract of *Ocimum gratissimum* (at 1.0 mg/ml) inhibited the growth of *P. aeruginosa, S. aureus, C. albicans* and other microorganisms. The antimicrobial activity of the ethanol extract of *Ocimum gratissimum* of this study differs from the findings of Oboh (2006), thus, the ethanolic extract of *Ocimum gratissimum* was not active at all against the test organisms. The loss of activity of the ethanolic extract of *Ocimum gratissimum* could be due to the fact that the active agent was lost during extraction and/or evaporation processes.

The acidic and alkaloidal fractions (table 4.7) of *Ocimum gratissimum* (ethanol extract) were active against the test organisms. These fractions were obtained from a non-polar solvent (i.e. chloroform) during fractionation of the ethanolic extract of *Ocimum gratissimum*. Though the crude ethanol extract of *Ocimum gratissimum* had no activity, the acidic and alkaloidal fractions of the same extract had activity against the test organisms. Literature supports the presence of alkaloids in the ethanol extract of *Ocimum gratissimum* (Oboh, 2006).

The P.E. extract of *Xylopia aethiopica* was active against *P. aeruginosa* and *C. albicans.* However, the ethanol extract of *Xylopia aethiopica* had no activity the test organisms. The aqueous extract of *Xylopia aethiopica* had activity against *C. albicans* only. It has been reported by Ezeifeka et al., (2004) that the ethanol extract of *Xylopia aethiopica* leaves had activity against *S. aureus, E. coli, P. aeruginosa and C. albicans* but the aqueous extract of the same had no effect on all the bacteria except *S. aureus.* Ezeifeka et al., (2004)

concluded that the ethanol extracts of the plant parts showed more inhibitory effects than the water extracts which tends to show that the active ingredients of the plants were better extracted with ethanol than water. This observation was also seen in the extracts of *Cinnamomum zeylanicum,* i.e. the ethanol extract of *Cinnamomum zeylanicum* showed activity against *E. coli and C. albicans* while the aqueous of the same had no activity. This trend was not observed for the ethanol and aqueous extracts of *Ocimum gratissimum, Psidium guajava* and *Xylopia aethiopica.* Both the ethanol and aqueous extracts of *Ocimum gratissimum* had no activity against the test organism. For *Psidium guajava,* both the ethanol and aqueous extract had approximately the same effect on the test organisms. The ethanol extract of *Xylopia aethiopica* showed no effect on the test organisms while the aqueous extract of the same inhibited the growth *C. albicans.*

The ability of the essential oils and extracts of *Cinnamomum zeylanicum, Psidium guajava, Ocimum gratissimum* and *Xylopia aethiopica* to inhibit microorganisms explains why these are used in folk medicines in Ghana and other tropical countries (G.H.P., 2007).

Though the ethanol extract of *Xylopia aethiopica* was not active against the test organisms, the waxes fraction of *Xylopia aethiopica* (table 4.7) was active against *E. coli* only whiles the alkaloidal fraction was active against all test microorganisms except *C. albicans*. The waxes fraction was obtained from P.E phase whiles alkaloidal fraction came from chloroform phase.

The *Cinnamomum zeylanicum* (table 4.7) active fractions were terpenes, waxes, acids and alkaloidal fractions. This reveals that the point noted earlier that some of the active

component were soluble in non-polar solvents whiles others soluble in polar solvents is explainable from the results of their respective fractions.

*Psidium guajava* (table 4.7) had the terpenes, waxes and acids fractions showing activity against the test microorganisms. This fact goes to buttress the point that some of the active components are soluble in polar solvents whiles others are soluble in non-polar solvents. *Psidium guajava* aqueous extract showed activity against all the test organisms while *Xylopia aethiopica* exhibited activity against *C. albicans.*

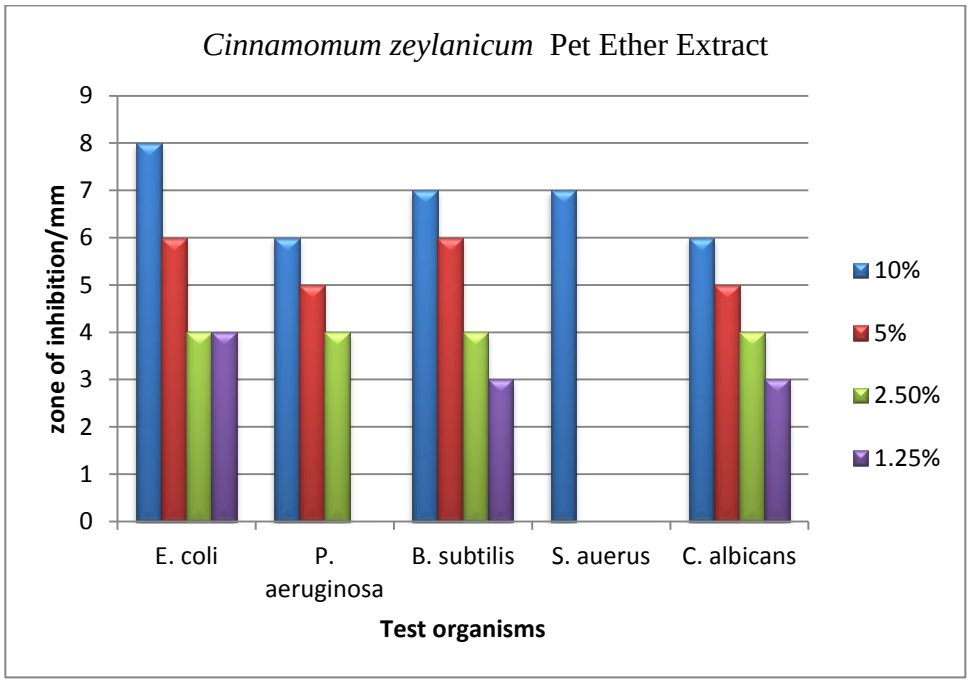


**Figure 4.1: A bar chart showing the zones of inhibition of the various concentrations of *Cinnamomum zeylanicum* Petroleum ether extract against test organisms**

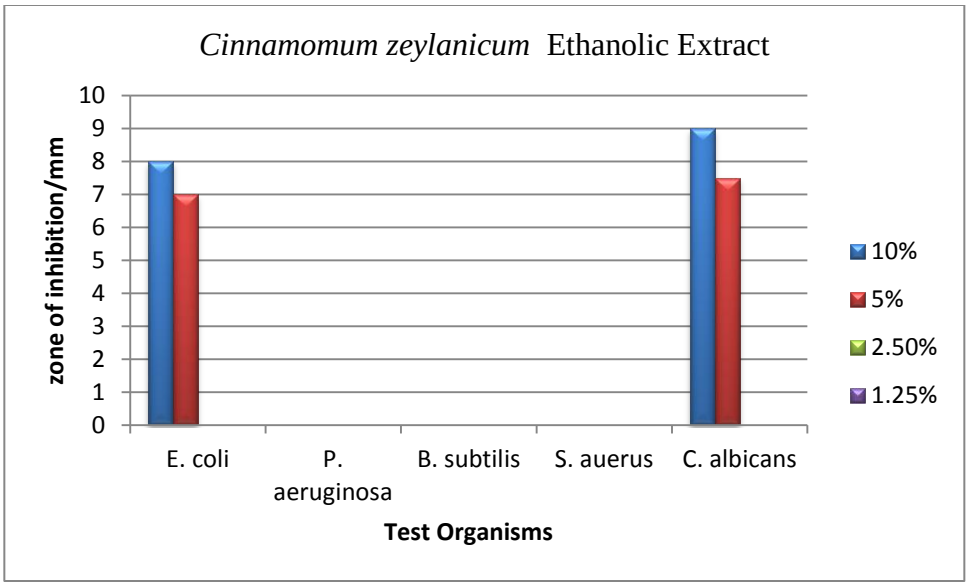


**Figure 4.2: A bar chart showing the zones of inhibition of the various concentrations of *Cinnamomum zeylanicum* ethanolic extract against test organisms**

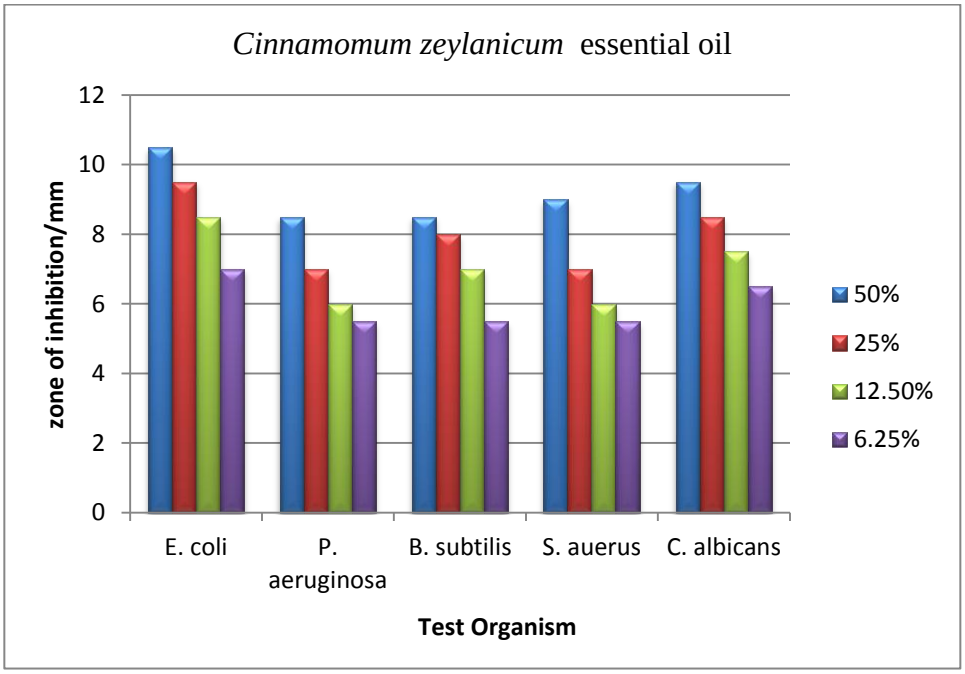


**Figure 4.3: A bar chart showing the zones of inhibition of the various concentrations of *Cinnamomum zeylanicum* essential oil against test organisms**

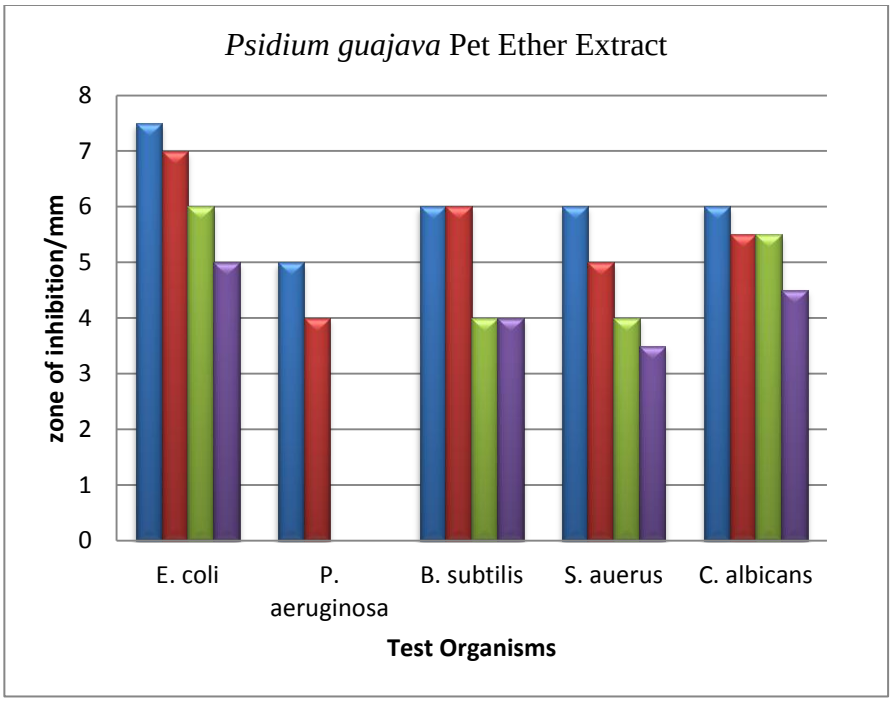


**Figure 4.4: A bar chart showing the zones of inhibition of the various concentrations of *Psidium guajava* Petroleum ether extract against test organisms**

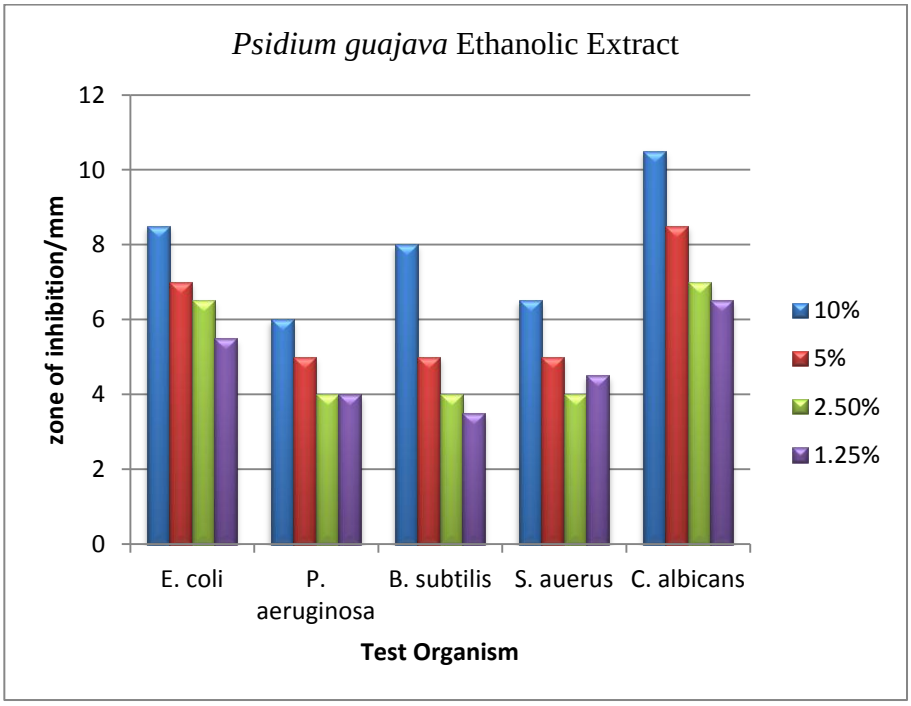


**Figure 4.5: A bar chart showing the zones of inhibition of the various concentrations of *Psidium guajava* Ethanolic extract against test organisms**

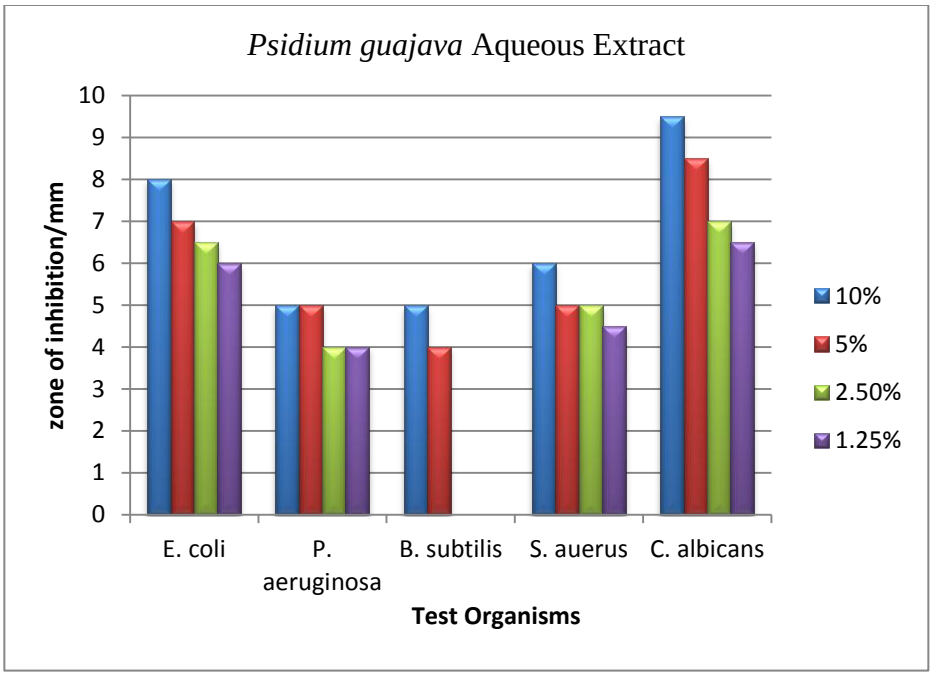


**Figure 4.6: A bar chart showing the zones of inhibition of the various concentrations of *Psidium guajava* Aqueous extract against test organisms**

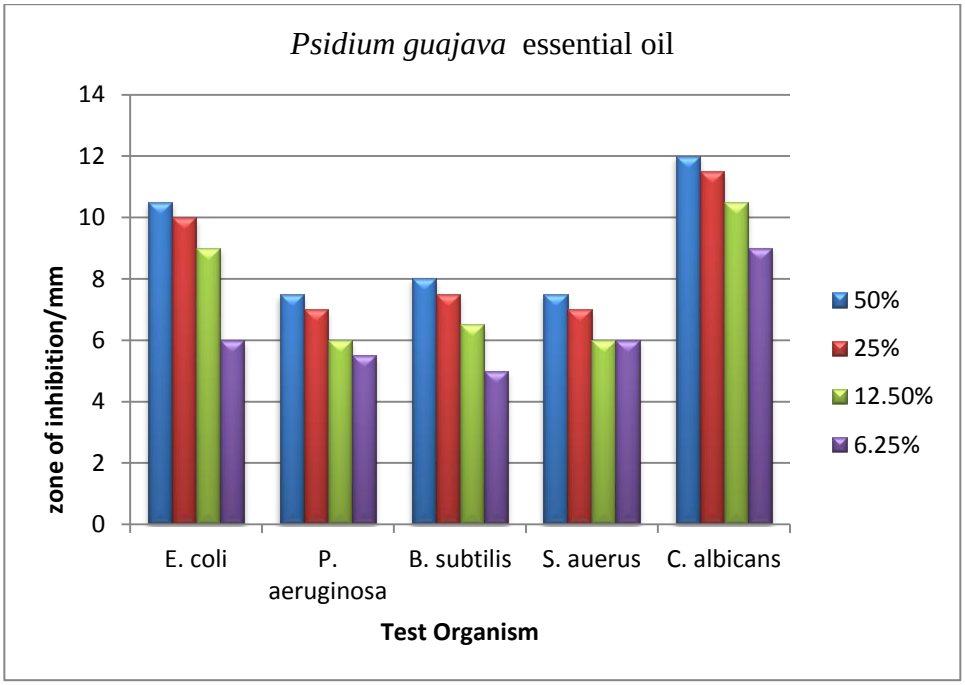


**Figure 4.7: A bar chart showing the zones of inhibition of the various concentrations of *Psidium guajava* essential oil against test organisms**

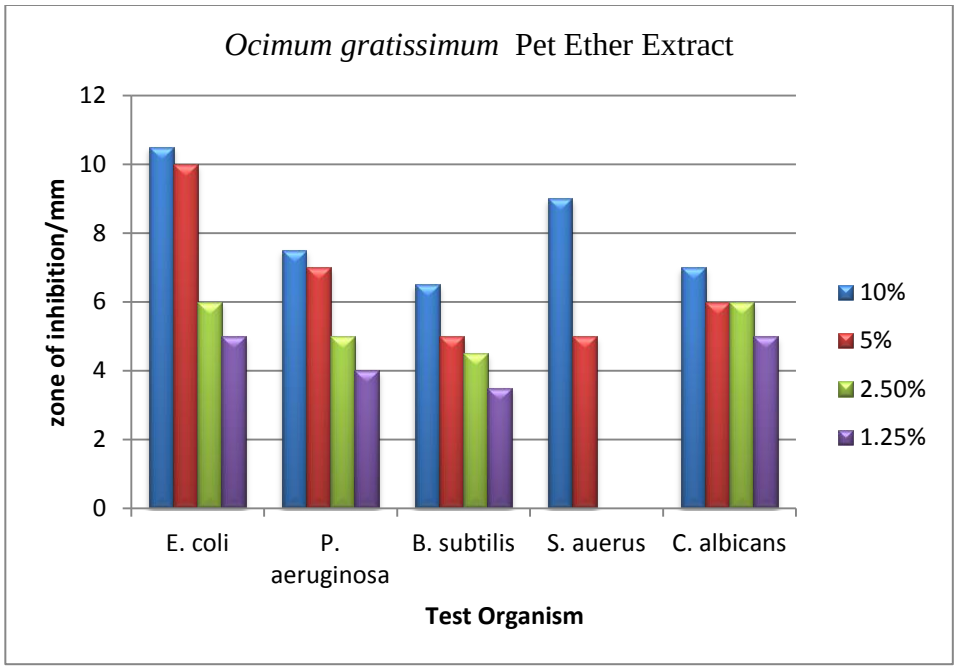


**Figure 4.8: A bar chart showing the zones of inhibition of the various concentrations of *Ocimum gratissimum* Petroleum ether extract against test organisms**

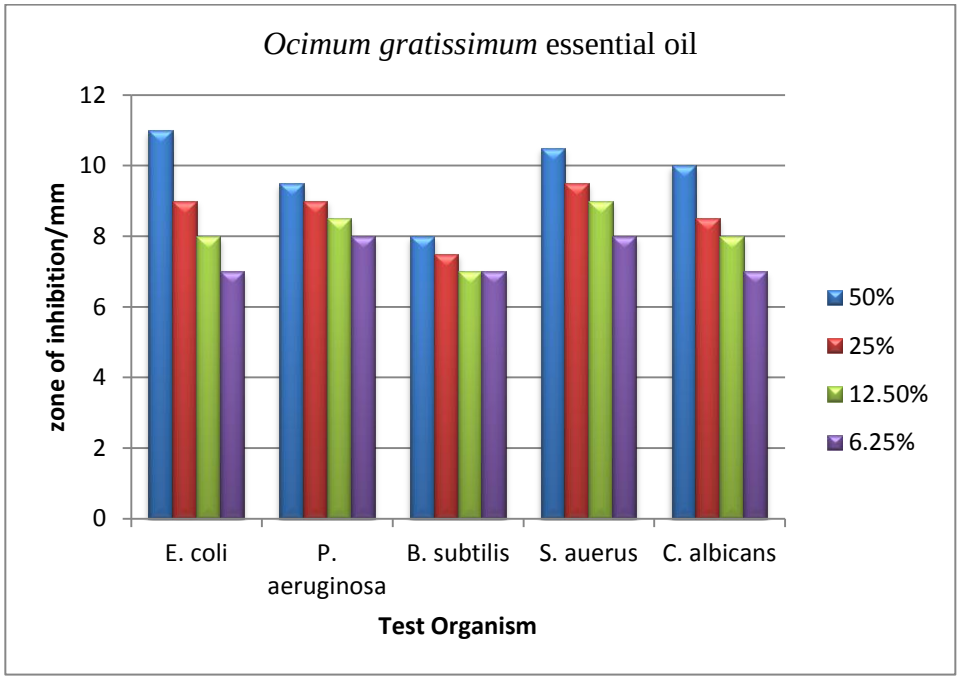


**Figure 4.9: A bar chart showing the zones of inhibition of the various concentrations of *Ocimum gratissimum* essential oil against test organisms**

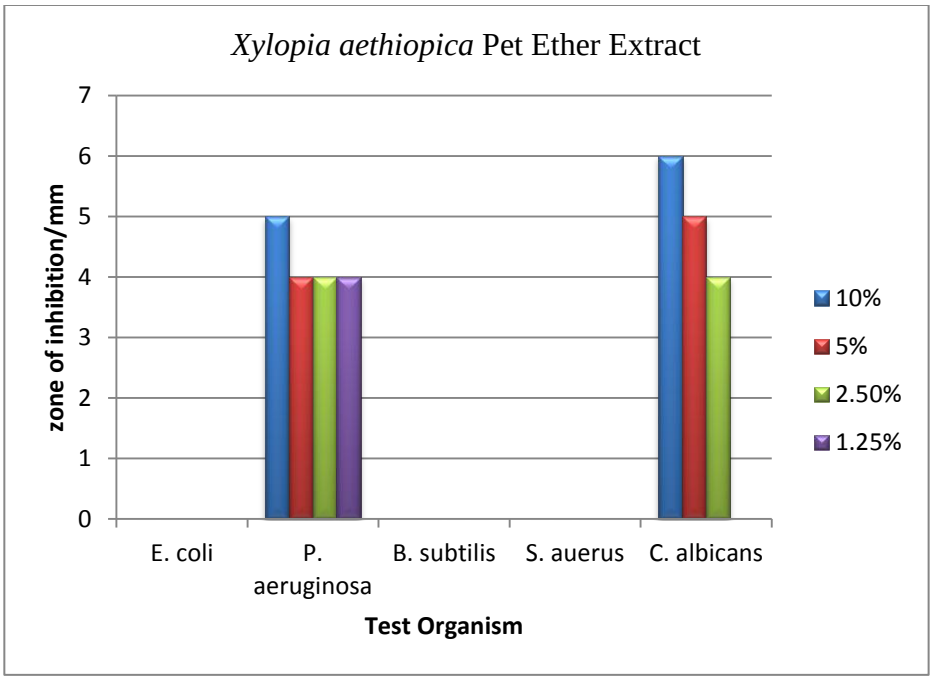


**Figure 4.10: A bar chart showing the zones of inhibition of the various concentrations of *Xylopia aethiopica* Petroleum ether extract against test organisms**

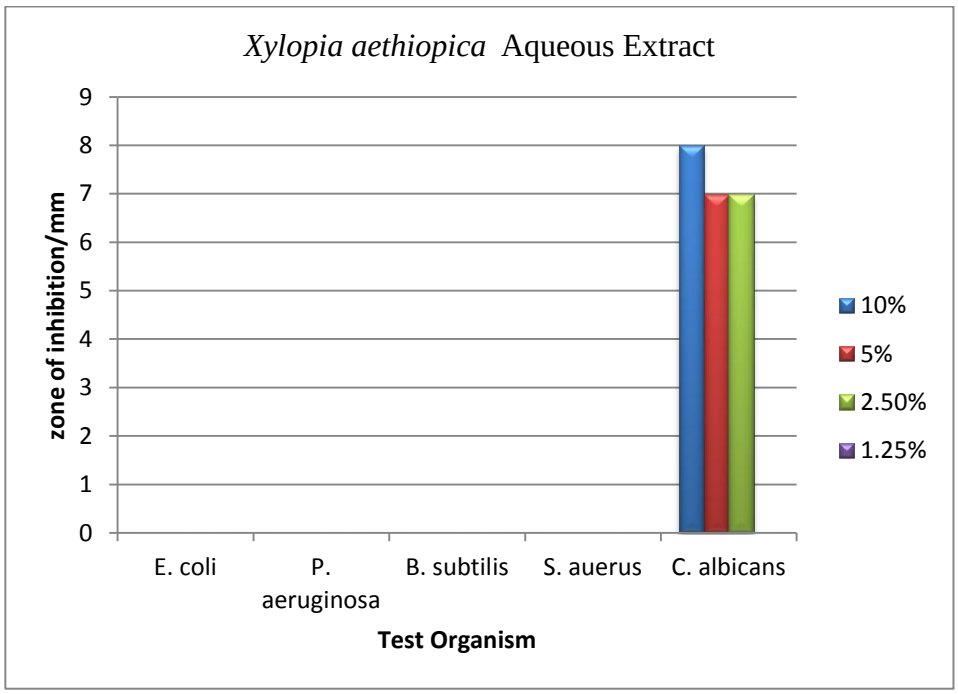


**Figure 4.11: A bar chart showing the zones of inhibition of the various concentrations of *Xylopia aethiopica* Aqueous extract against test organisms**

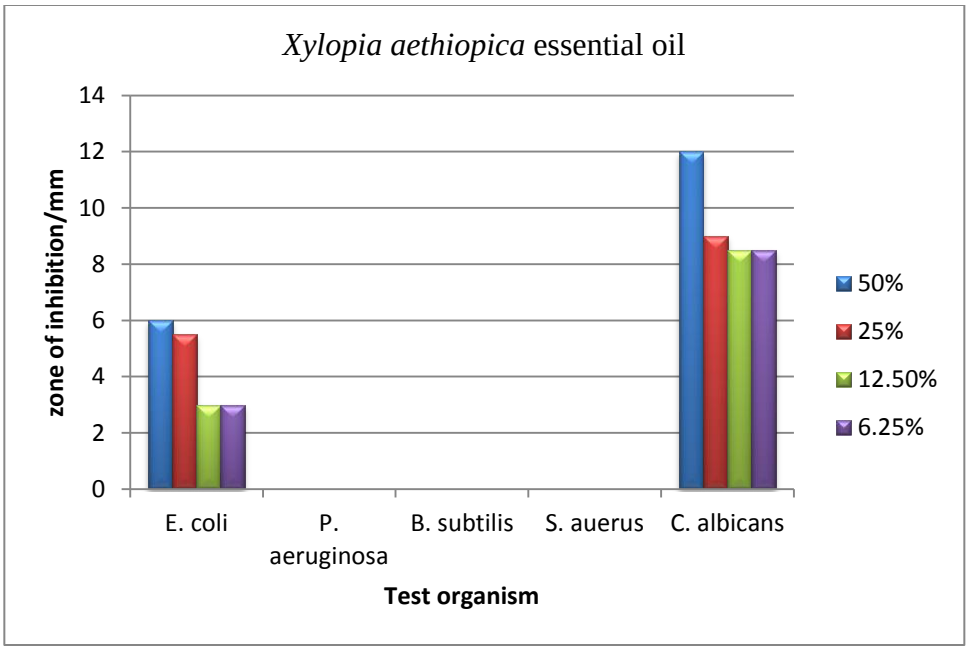


**Figure 4.12: A bar chart showing the zones of inhibition of the various concentrations of *Xylopia aethiopica* essential oil against test organisms**

Essential oils sometimes occur in special glandular cells on the leaf surface (Harborne, 1984). Extraction by steam distillation was used to obtain the essential oils from the leaves of the four (4) selected aromatic plants. The essential oils are basically volatile terpenes from the leaves.

According to Hili et al. (1997), the mechanism of action of essential oils or their components is unclear, but the findings of their study revealed that the antimicrobial action of Cinnamon oil was 50-fold higher in terms of activity when DMSO was not used as the dispersing solvent (using the broth micro dilution assay). The effect of solubilizers on the activity of essential oils in broth micro dilution assay may be due to the fact that there could be partitioning of the essential oil between the aqueous phase and DMSO, thus distancing the essential oil from the cells of the microorganisms (Hili et al., 1997). Randhawa (2006) has stated that higher concentrations of DMSO (thus 10% - 1.25%)

inhibited the growth of fungi. Additionally, Randhawa stated that the final concentrations of DMSO after serial dilutions are made in the culture changes from 100% of the stock to 1% or below in most studies and that the effect of less than 1% DMSO is negligible on the growth of yeast and some dermatophytes (the method used was the agar diffusion method). The effect of DMSO in agar diffusion could thus be control when concentration of DMSO is kept below 1%.

Comparatively, the essential oils inhibited the growth of the test organisms more than all the solvent extracts of the leaves. During the assessment and comparative analysis of the antimicrobial activities and effectiveness of cinnamon (*Cinnamomum zeylanicum*) extract (50% ethanol) and its oil against ten bacteria (seven Gram-positive and three Gram-negative) and seven fungi by agar well diffusion assays, Gupta *et al.* (2008*)* concluded that cinnamon oil is a more potent antimicrobial agent than cinnamon extract and that it has the potential to be used as food biopreservative.

Matasyoh *et al.* (2007) evaluated the antimicrobial activities of the essential oils of *Ocimum gratissimum* against both Gram positive (*Staphylococcus aureus, Bacillus* spp.) and Gram negative (*Escherichia coli, Pseudomonas aeruginosae, Salmonella typhi, Klebisiella pneumoniae, Proteus mirabilis*) bacteria and a pathogenic fungus *Candida albicans*. The oil had pronounced antibacterial and antifungal activities on all the microorganisms (Matasyoh *et al.*, 2007).

The essential oil from *Ocimum gratissimum* (figure 4.17) was the most active of the four (4) essential oils (table 4.5) because it exhibited the widest zones of inhibition followed by

*Psidium guajava*, *Cinnamomum zeylanicum* and lastly *Xylopia aethiopica* essential oils. MIC was defined as the lowest concentration of extracts at which the microorganisms tested did not demonstrate visible growth, the lower the value of MIC the greater the activity of the antibacterial agent. *Ocimum gratissimum* essential oil (Figure 4.15) had MIC range of 1.0 $x10^{-5}$ % to 0.158%, *Psidium guajava* essential oil (Figure 4.14) had 3.98 $x10^{-3}$ to 0.251%, *Cinnamomum zeylanicum* essential oil (Figure 4.13) had $6.31x10^{-2}$ to 0.251% whiles *Xylopia aethiopica* essential oil (4.16) had $3.98x10^{-2}$ to 1.26%.

**MIC VALUES**

**Table 4.5: MIC values of plant extracts and their respective essential oil against five test organisms**

| PLANT EXTRACTS | MIC/% | | | | |
|---|---|---|---|---|---|
| | *E. coli* | *P. aeruginosa* | *B. subtilis* | *S. auerus* | *C. albicans* |
| Cinnamon Pet Ether extract | 0.100 | 0.794 | 0.316 | 1.995 | 0.158 |
| Ethanolic Cinnamon extract | 1.585 | - | - | - | 1.585 |
| Aqueous Cinnamon extract | - | - | - | - | - |
| Cinnamon Essential oil | 0.100 | 0.158 | 0.126 | 0.251 | 0.0631 |
| Guava Pet ether extract | 0.0199 | 1.585 | 0.0501 | 0.0794 | 0.001 |
| Ethanolic Guava extract | 0.141 | 0.0316 | 0.316 | 0.0251 | 0.050 |
| Aqueous Guava extract | 0.00199 | 0.00158 | 1.585 | 0.00126 | 0.199 |
| Guava Essential oil | 0.251 | 0.0316 | 0.158 | 0.00398 | 0.010 |
| Ocimum Pet ether extract | 0.158 | 0.158 | 0.100 | 1.585 | 0.00398 |
| Ethanolic Ocimum extract | - | - | - | - | - |
| Aqueous Ocimum extract | - | - | - | - | - |
| Ocimum Essential oil | 0.158 | 0.0001 | 0.00001 | 0.00631 | 0.0398 |
| Xylopia Pet ether extract | - | 0.00199 | - | - | 0.891 |
| Ethanolic Xylopia extract | - | - | - | - | - |
| Aqueous Xylopia extract | - | - | - | - | 0.708 |
| Xylopia Essential oil | 1.259 | - | - | - | 0.0398 |

The aqueous extract of *Psidium guajava* was very active against *E. coli* with MIC of 0.00199% and the least active solvent extract against *E. coli* was ethanol extract of *Cinnamomum zeylanicum* (1.585%). The ability of the aqueous extract of *Psidium guajava* to inhibit the growth of *E. coli*, a diarrhoea causing bacteria, explains why it is used in folk medicine to treat diarrhoea in Ghana and other tropical countries. The essential oils of the

four plants were active against *E. coli*. The most active essential oil against *E. coli* was that of *Cinnamomum zeylanicum* (0.100%) followed by the oil of *Ocimum gratissimum* (0.158%), *Psidium guajava* (0.251%) and lastly followed by the oil of *Xylopia aethiopica* (1.259%). The Pet ether extracts of *Cinnamomum zeylanicum* (0.100%), *Psidium guajava* (0.0199%) and *Ocimum gratissimum* (0.158%) were active against E. coli whereas the Pet ether extract of *Xylopia aethiopica* was not active. The Pet ether extract of *Psidium guajava* with MIC of 0.0199% was the most active of the three active Pet ether extracts. The aqueous extract of *Psidium guajava* was the only aqueous extract with activity against *E. coli*. The ethanol extracts of *Cinnamomum zeylanicum* and *Psidium guajava* were the only ethanol extracts of the four with activity *E. coli*.

The essential oil of *Ocimum gratissimum* was the most active extract against *P. aeruginosa* with 0.0001% followed by the aqueous extract *Psidium guajava* (0.00158%) and petroleum ether extract of *Xylopia aethiopica* (0.00199%). The four (4) Pet ether extracts were all active against *P. aeruginosa*. The Pet ether extract of *Xylopia aethiopica* was the most active extract of the four Pet ether extracts with MIC of 0.00199%. The Pet ether extract of *Xylopia aethiopica* was the only extract out of the three (3) extracts of *Xylopia aethiopica* with activity against *P. aeruginosa* i.e. the ethanol, aqueous as well as the essential oil were not active against *P. aeruginosa*. The three (3) extracts and oil of *Psidium guajava* were able inhibit the growth of *P. aeruginosa*. Among the extracts of *Psidium guajava*, the aqueous extract inhibited strongly the growth of *P. aeruginosa* with MIC of 0.00158% followed by the ethanol and essential oil with the same MIC of 0.0316% and lastly followed by the Pet ether extract with MIC of 1.585%. The Pet ether extracts and essential oils of *Cinnamomum zeylanicum* and *Ocimum gratissimum* were active against *P.*

*aeruginosa* while the ethanol and aqueous extracts of the same had no activity against *P. aeruginosa*.

The essential oil of *Ocimum gratissimum* was the most active against *B. subtillis* with MIC of 0.00001%. Among the essential oils *Ocimum gratissimum* inhibited strongly the growth of *B. subtillis* followed by the oil of *Cinnamomum zeylanicum* (0.216%) followed by the oil of *Psidium guajava* (0.1585%) whereas the oil of *Xylopia aethiopica* had no activity against *B. subtillis.* The Pet ether extracts of three (3) out of the four (4) plants were active against *B. subtillis*. The three (3) active Pet ether extracts according to the order of activity were *Psidium guajava* (0.0501%), *Ocimum gratissimum* (0.100%) and *Cinnamomum zeylanicum* (0.316%). The three (3) extracts and essential oil of *Psidium guajava* were all active against *B. subtillis*, with the Pet ether extract (0.0501%) being the most active followed by the essential oil (0.158%), the ethanol extract (0.316%) and the aqueous extract (1.585%). The Pet ether extracts and essential oils of *Cinnamomum zeylanicum* and *Ocimum gratissimum* were active against *B. subtillis* while the ethanol and aqueous extracts of the same had no activity against *B. subtillis.* None of the extracts of *Xylopia aethiopica* were active against *B. subtillis.*

The aqueous extract of *Psidium guajava* was very active against *S. auerus* with MIC of 0.00126% followed by the essential oil of *Psidium guajava* with 0.00398%. Next in the order of most active extract was the oil of *Ocimum gratissimum* with MIC of 0.00631%. Among the essential oils that of *Psidium guajava* was the most active against *S. auerus* with MIC of 0.00398% followed by the oil of *Ocimum gratissimum* (0.00631%) and *Cinnamomum zeylanicum* (0.251%) whereas the oil of *Xylopia aethiopica* had no activity

against *S. auerus.* All the extracts and essential oil of *Psidium guajava* were all active against *S. auerus.* The inhibition of the growth of *S. auerus* by *Psidium guajava* further explains why it is used in the treatment of diarrhoea in folk medicines. The Pet ether extracts and essential oils of *Cinnamomum zeylanicum* and *Ocimum gratissimum* were active against *S. auerus* while the ethanol and aqueous extracts of the same had no activity against *S. auerus.* None of the extracts of *Xylopia aethiopica* were active against *S. auerus.*

Petroleum ether of extract *Psidium guajava* with MIC of 0.001% was the most active extract against *C. albicans* followed by the essential oil of *Psidium guajava* (0.010%). The Pet ether extracts and the oils of all the four (4) plants were all active against *C. albicans.* The Pet ether extract of *Psidium guajava* was the most of all the Pet ether extracts followed by that of *Ocimum gratissimum* (0.0398%), *Cinnamomum zeylanicum* (0.158%) and *Xylopia aethiopica* (0.891%). Among the essential oils that of *Psidium guajava* was the most active with MIC of 0.010% followed by the oils of *Ocimum gratissimum* and *Xylopia aethiopica* with MIC of 0.0398% and lastly *Cinnamomum zeylanicum* (0.0631%). The ethanol extracts of *Cinnamomum zeylanicum* (1.585%) and *Psidium guajava* (0.050%) were the only active ethanol extracts against *C. albicans* while the ethanol extracts of *Ocimum gratissimum* and *Xylopia aethiopica* had no activity against *C. albicans.* The aqueous extracts of *Psidium guajava* (0.199%) and *Xylopia aethiopica* (0.708%) were active against *C. albicans*. AllP the extracts and essential oil of *Psidium guajava* were all active against *C. albicans*.

The most active solvent extract against all microorganisms was petroleum ether extract of *Psidium guajava* and least active extract was observed for the ethanolic extract of *Cinnamomum zeylanicum.*

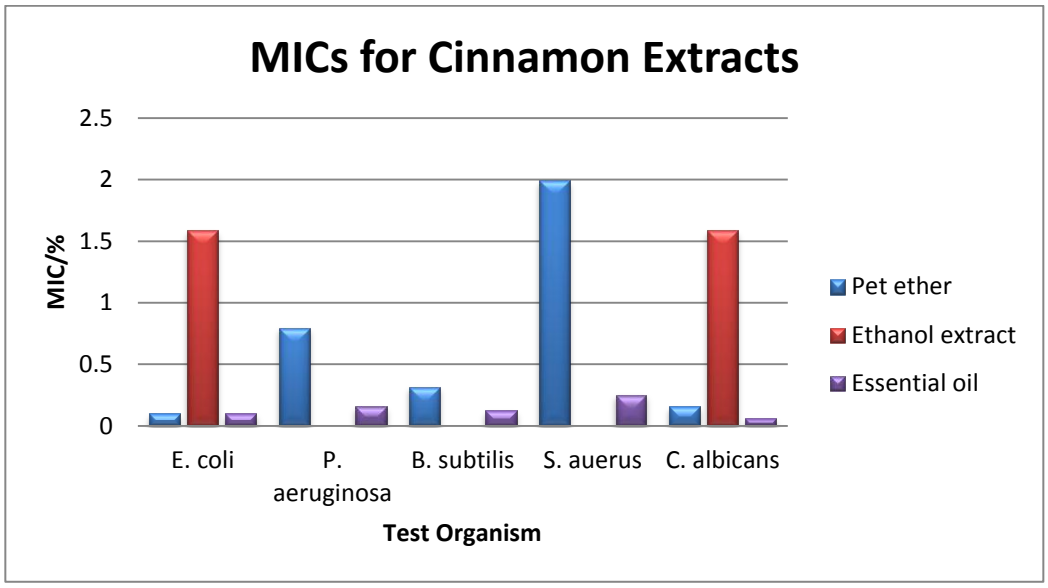


**Figure 4.13: A bar chart showing the MIC (%) of Cinnamon extracts against test organism**

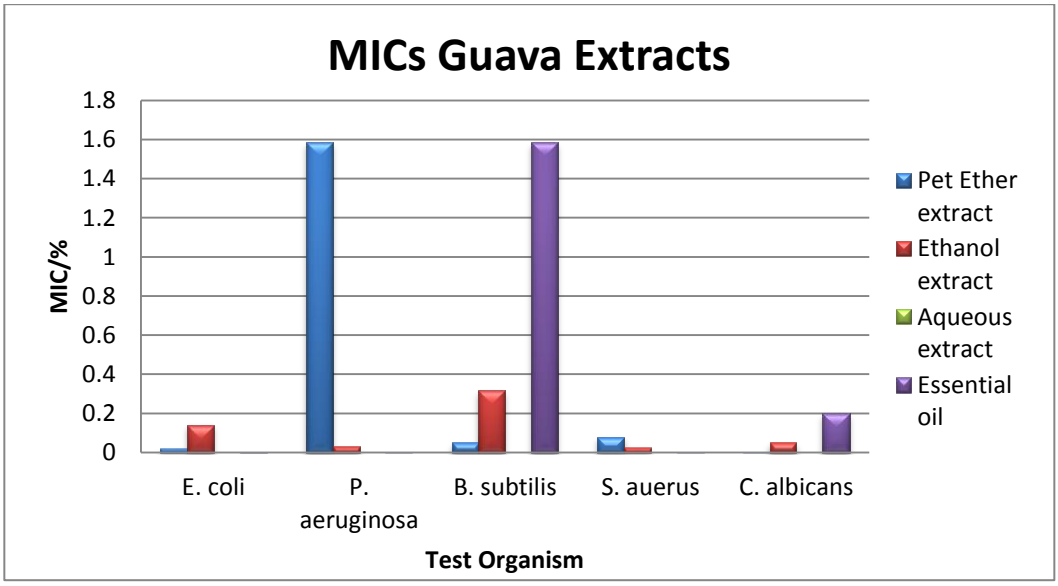


**Figure 4.14: A bar chart showing the MIC (%) of Guava extracts against test organisms**

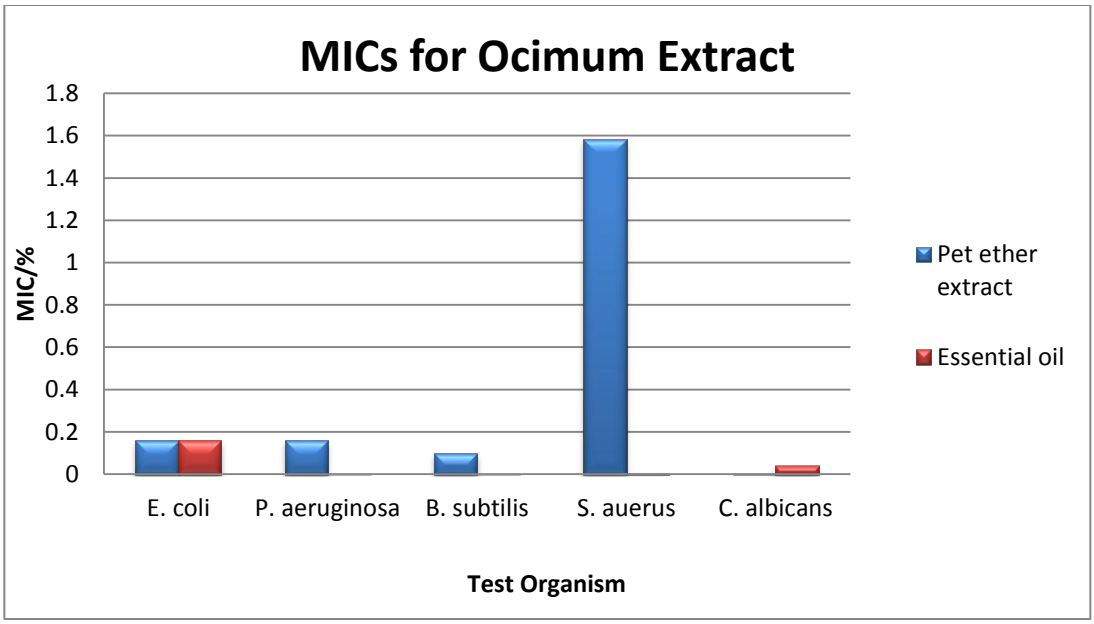


**Figure 4.15: A bar chart showing the MIC (%) of Ocimum extracts against test organisms**

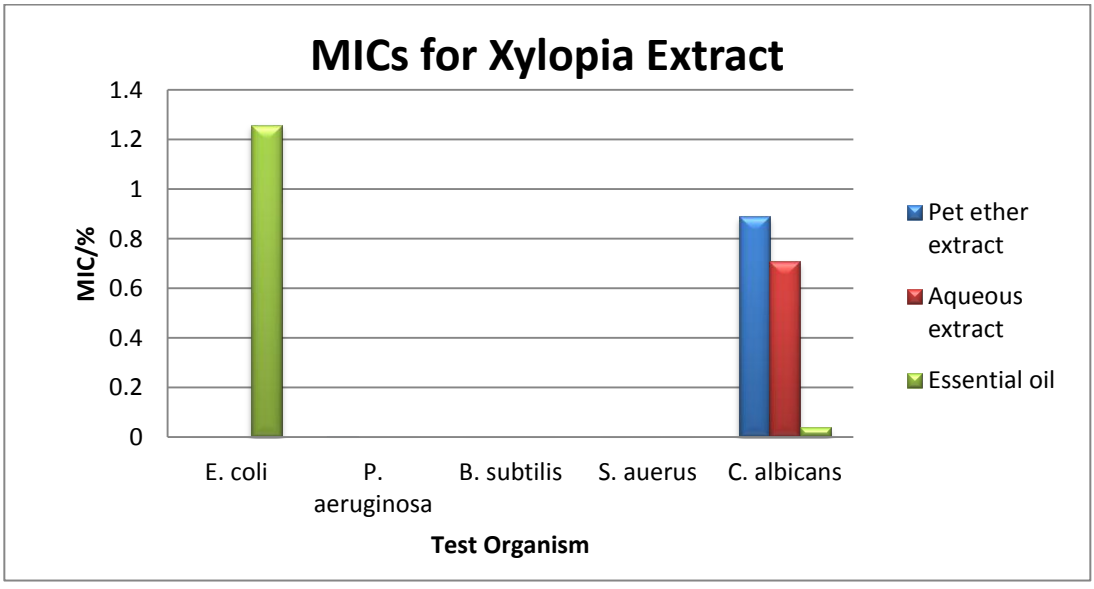


**Figure 4.16: A bar chart showing the MIC (%) of Xylopia extracts against test organisms**

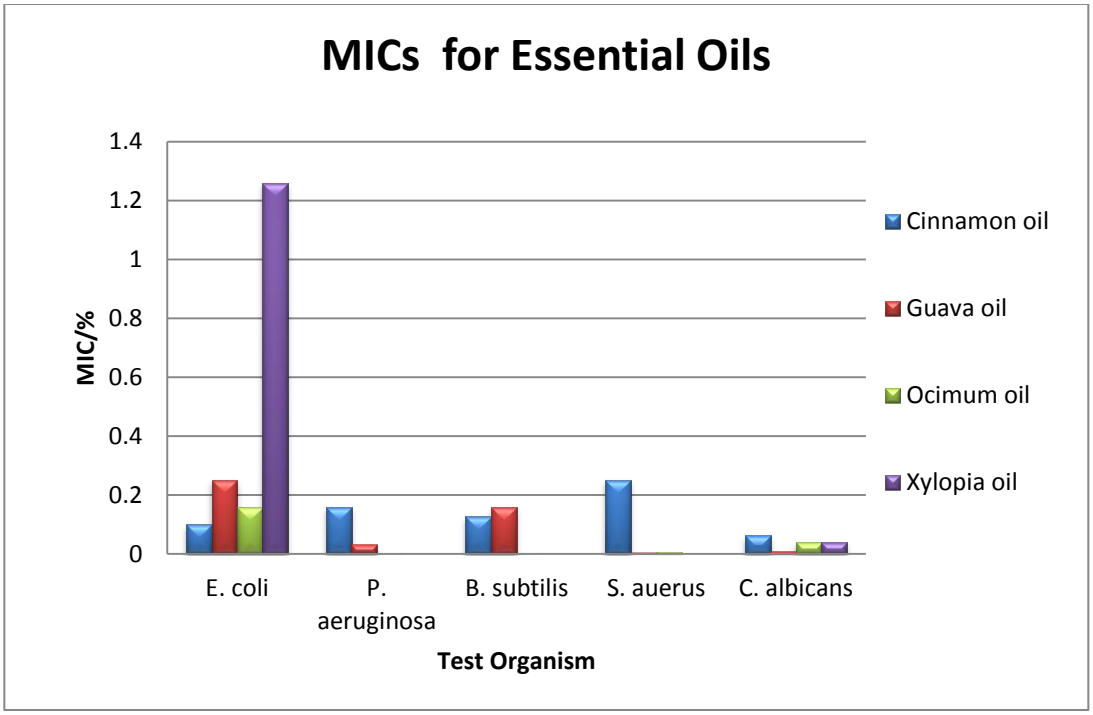


**Figure 4.17: A bar chart showing the MIC (%) of the four (4) essential oils against test organism**

**FRACTIONATION RESULTS**

| Crude extracts (70% ethanol) | Colors of Fractions | | | | |
|---|---|---|---|---|---|
| | **Fraction 6 Terpenes-sterols** | **Fraction 7 Waxes-fats** | **Fraction 8 Acids** | **Fraction 9 Water soluble, quaternary alkaloids** | **Fraction 10 Alkaloids** |
| *Cinnamomum zeylanicum* - brown | Pale brown | Colorless | Colorless | Brown | Colorless |
| *Ocimum gratissimum* – dark green | Dark green | Yellowish green | Colorless | Dark brown | Pale yellow |
| *Psidium guajava* – brown | Brown | Pale yellow | Colorless | Dark brown | Very pale brown |
| *Xylopia aethiopica* – dark brown | Pale brown | Pale dark green | Colorless | Brown | Pale dark brown |

**Table 4.6: Colors of fractions from the various ethanolic crude extract**

The terpene fractions (table 4.6) of the four plant samples had colors similar to that of the ethanolic extract. The wax fraction of *Cinnamomum zeylanicum* was colorless whereas wax fractions of the other three plant samples had colors. These colors were faded compared to the colors of the terpene fractions. The acidic fractions of the four (4) plant samples were all colorless. The water soluble, quaternary alkaloid fractions were generally brown for all the samples.

None of the water soluble, quaternary alkaloid fraction (table 6) was active against the test organisms. On the other hand, all acidic fractions except *Xylopia aethiopica* were active

against the test organisms. This is consistent with the fact that phenolic acids are active. Although the leaves samples were defatted with non-polar solvent, some waxes fractions were obtained. The waxes fraction of *Cinnamomum zeylanicum* was active against all test microorganisms. *Psidium guajava* and Xylopia *aethiopica* waxes fractions were only active against *E. coli*.

Alkaloidal fractions of *Cinnamomum zeylanicum*, *Ocimum gratissimum*, *Xylopia aethiopica* exhibited activity against the test organisms. The alkaloids as a group are distinguished from most other plant components by their basic (cationic) nature. Therefore, they normally exist in plants as the salts of various organic acids. These salts, and frequently the free alkaloids, are colourless crystalline compounds. A few alkaloids are liquids, and colour ones are even more rare (berberine and serpentine are yellow) (Robinson, 1963). The colours of the alkaloidal fractions confirm literature (i.e. Robinson, 1963).

*Ocimum gratissimum* alkaloidal fraction was active against all microorganisms, *Cinnamomum zeylanicum* alkaloidal fraction showed activity against all microorganisms except *E. coli* whiles *Xylopia aethiopica* alkaloidal fraction was active against all microorganisms except *C. albicans*

**Table 4.7: Antimicrobial activity of fractions**

| Extracts | Fractions | Antimicrobial Activity of Fractions of 70% Ethanolic Extracts against micro-organism | | | | |
|---|---|---|---|---|---|---|
| | | ***E. coli*** | ***P. aeruginosa*** | ***B. subtilis*** | ***S. auerus*** | ***C. albicans*** |
| *Cinnamomum zeylanicum* | Terpenes-sterols | - | + | - | - | + |
| | Waxes-fats | + | + | + | + | + |
| | Acids | + | + | + | + | + |
| | Water solubles, quaternary alkaloids | - | - | - | - | - |
| | Alkaloids | - | + | + | + | + |
| *Psidium guajava* | Terpenes-sterols | - | - | + | + | - |
| | Waxes-fats | + | - | - | - | - |
| | Acids | + | + | + | - | + |
| | Water solubles, quaternary alkaloids | - | - | - | - | - |
| | Alkaloids | - | - | - | - | - |
| *Ocimum gratissimum* | Terpenes-sterols | - | - | - | - | - |
| | Waxes-fats | - | - | - | - | - |
| | Acids | + | + | + | + | + |
| | Water solubles, quaternary alkaloids | - | - | - | - | - |
| | Alkaloids | + | + | + | + | + |
| *Xylopia aethiopica* | Terpenes-sterols | - | - | - | - | - |
| | Waxes-fats | + | - | - | - | - |
| | Acids | - | - | - | - | - |
| | Water solubles, quaternary alkaloids | - | - | - | - | - |
| | Alkaloids | + | + | + | + | - |

"+": present "-": absent

# CHAPTER FIVE

# CONCLUSION

The antimicrobial activities of the leave extracts of four (4) Ghanaian aromatic medicinal plants have been examined against five (5) selected microorganisms. The essentials oils of all plants were the most active. Among the solvent extracts, the petroleum ether extracts were the most active followed ethanolic extracts, aqueous extracts except for *Psidium guajava*. The families of secondary metabolites responsible for the activities of the crude solvent extracts have also been determined.

All the acidic or phenolic fractions (components or constituents) except *Xylopia aethiopica* were active against the test organisms. The terpenoidal, waxes and alkaloidal fractions or components showed various levels of activity the test microorganisms. It is only the water soluble quaternary alkaloidal fractions which could not reveal any significant activity against the test microorganisms.

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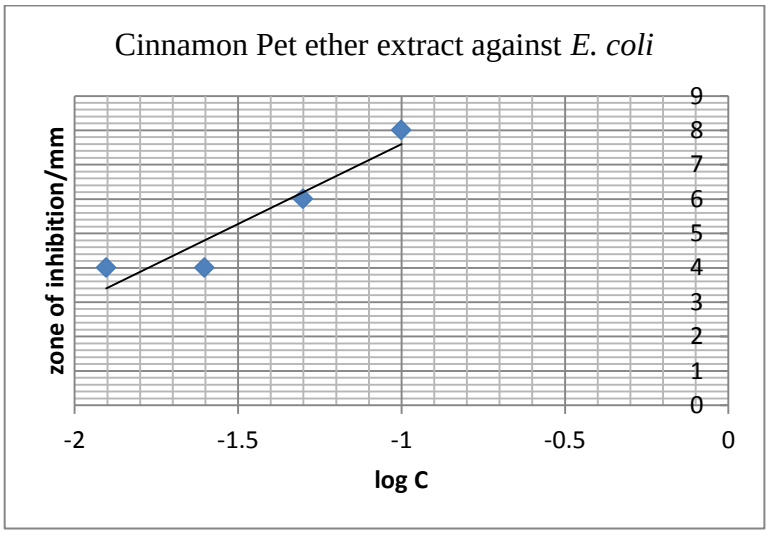
## APPENDICES

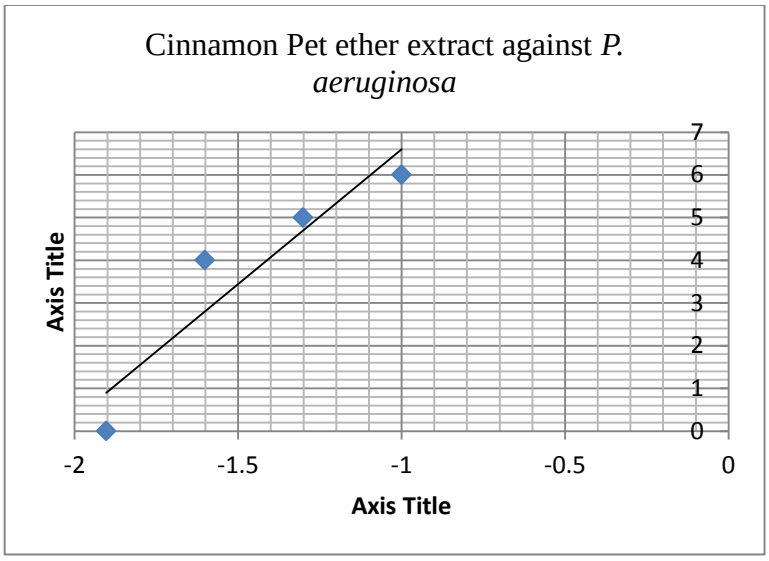
### APPENDIX ONE:

Graphs Showing the Plots of Zones of Inhibition (mm) against log of Concentrations of Extracts

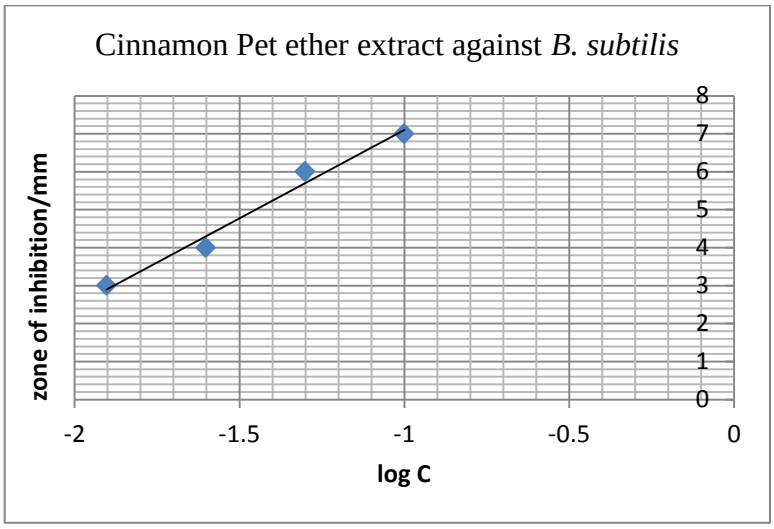



intercept on x-axis: -3

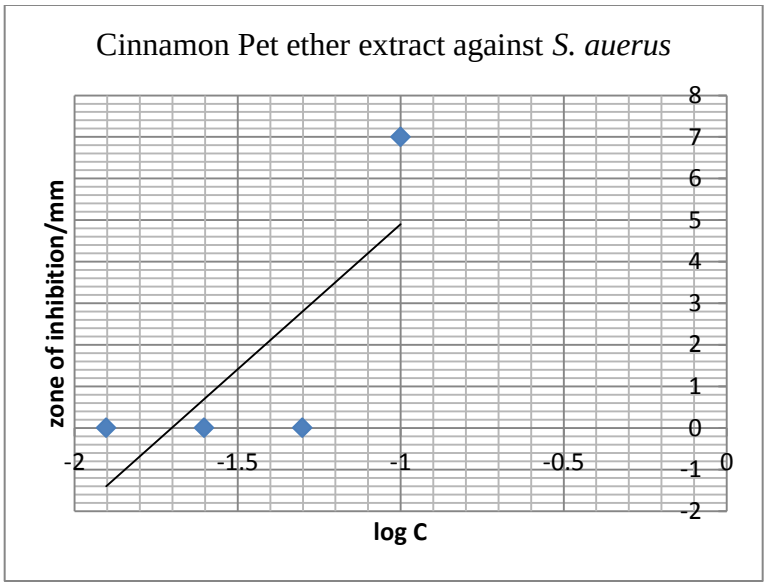
MIC is antilog(-3): 0.1%



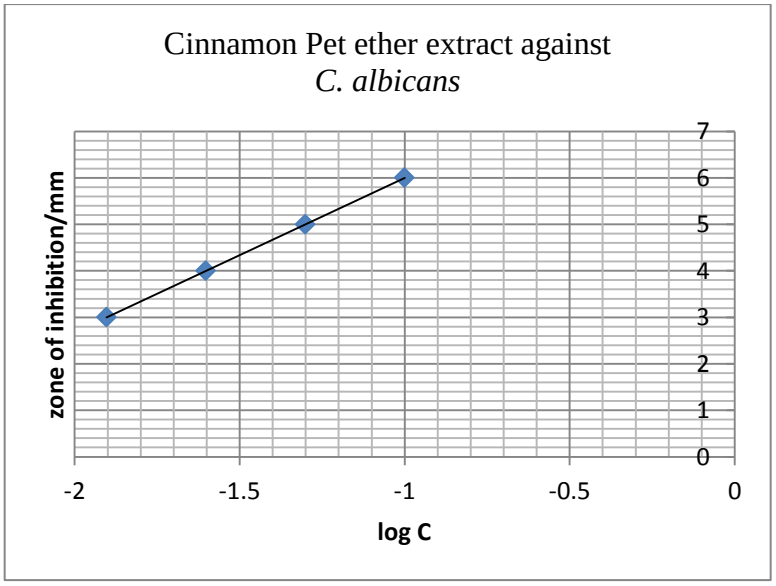

intercept on x-axis: -2.1
MIC is antilog(-2.1): 0.794%



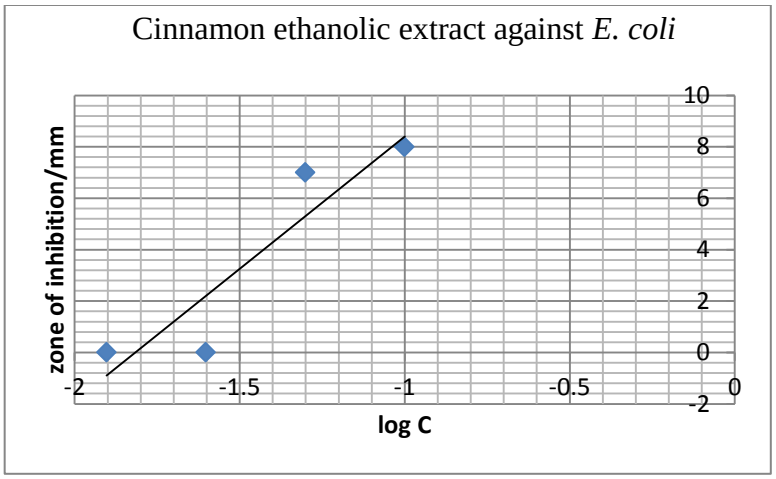

intercept on x-axis: -2.5
MIC is antilog(-2.5): 0.316%



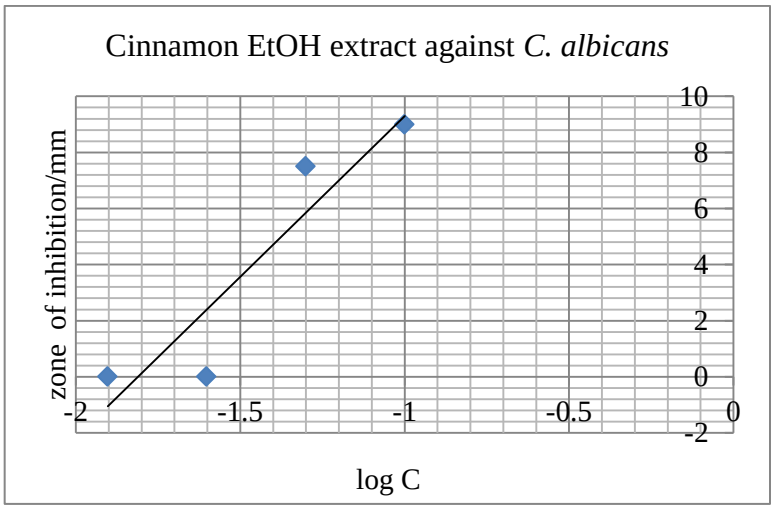

intercept on x-axis: -1.70
MIC is antilog(-1.70): 1.995%



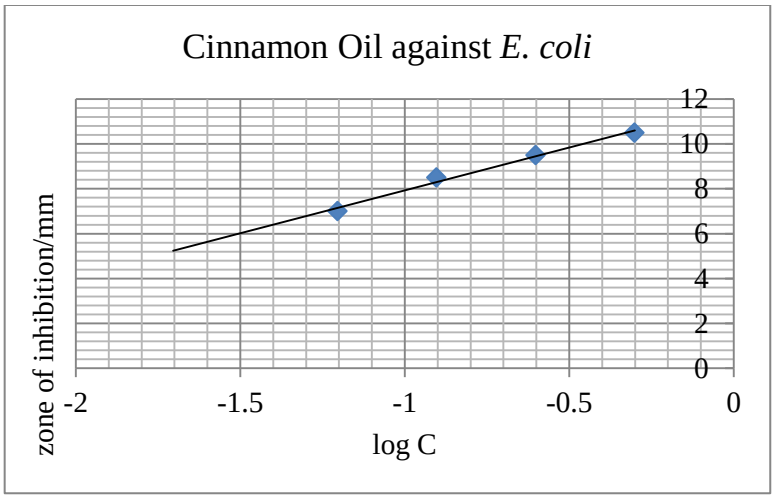

intercept on x-axis: -2.8
MIC is antilog(-1.70): 0.158%



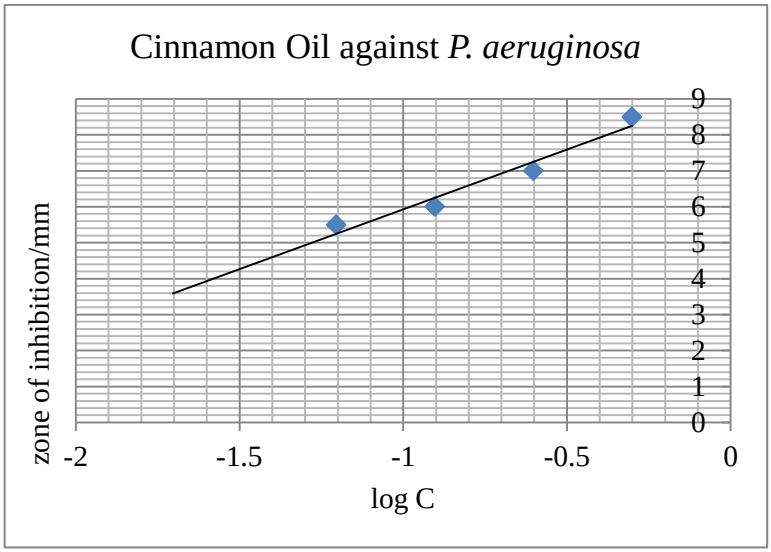

Intercept on x-axis: -1.8
MIC is antilog(-1.8):1.585%



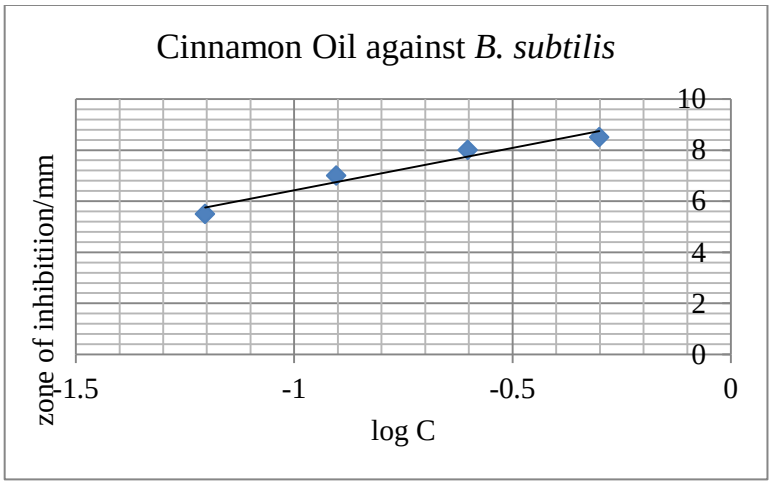

Intercept on x-axis: -1.8
MIC is antilog(-1.8):1.585%



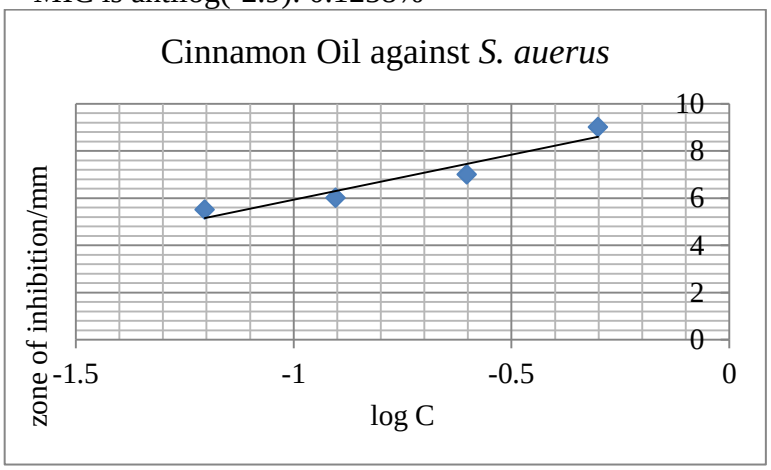

intercept on x-axis: -3
MIC is antilog(-3): 0.1%



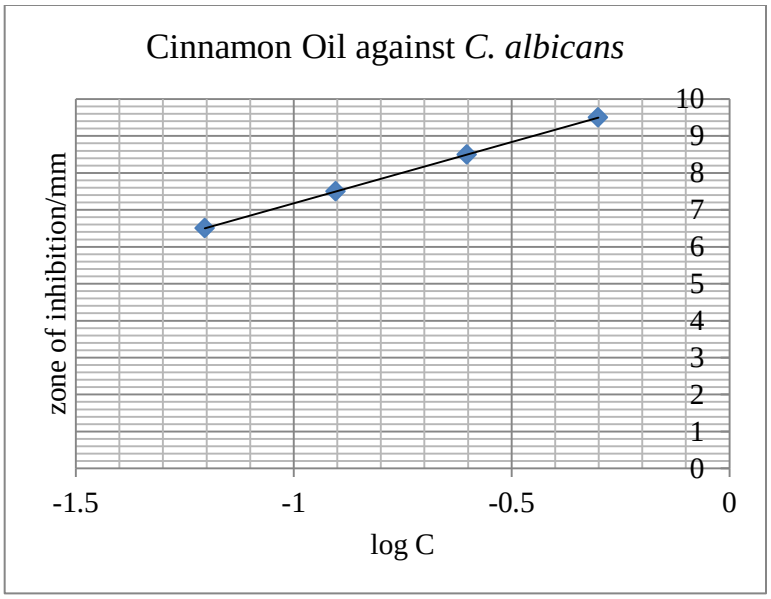

intercept on x-axis: -2.8
MIC is antilog(-2.8): 0.158%




Intercept on x-axis: -2.9
MIC is antilog(-2.9): 0.1258%

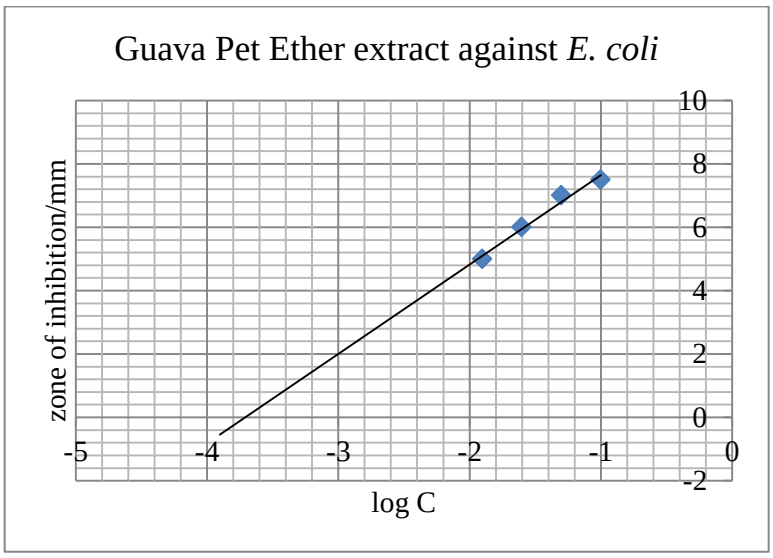



intercept on x-axis: -2.6
MIC is antilog(-2.6): 0.251%

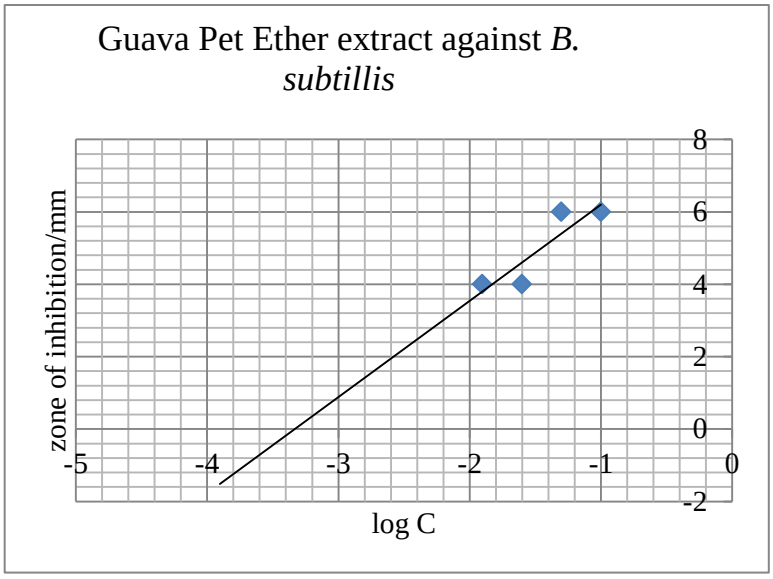



intercept on x-axis: -3.2
MIC is antilog(-3.2): 0.0631%

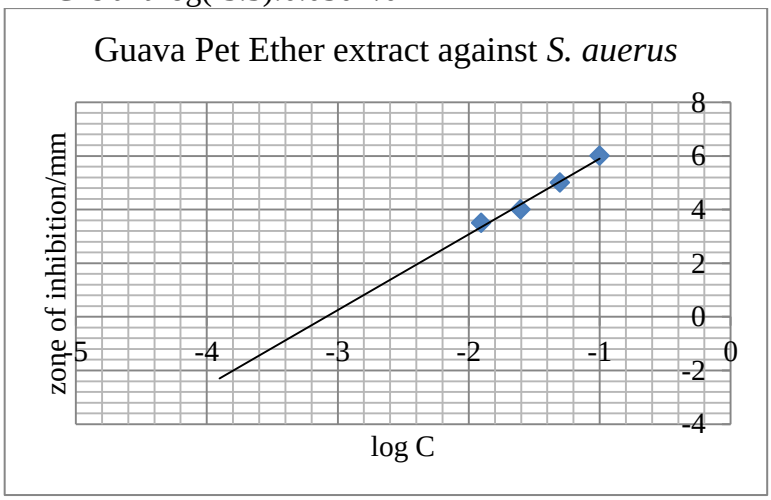
*Psidium guajava Linn* EXTRACTS



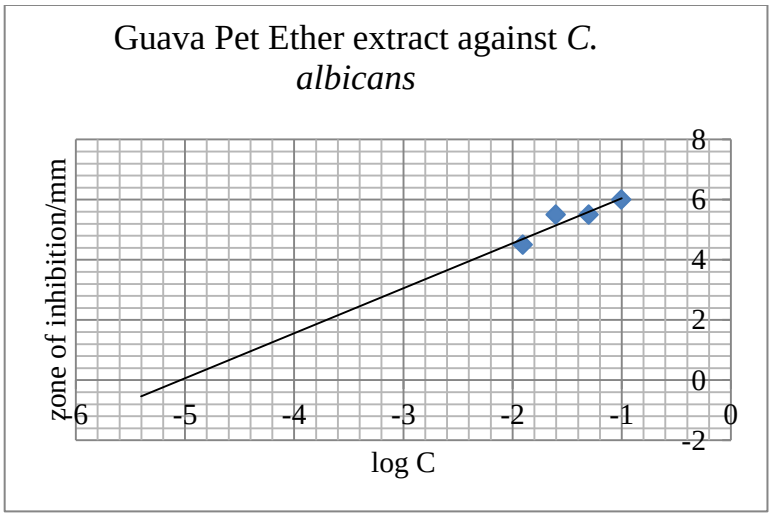
intercept on x-axis: -3.7
MIC is antilog(-3.7):0.0199%



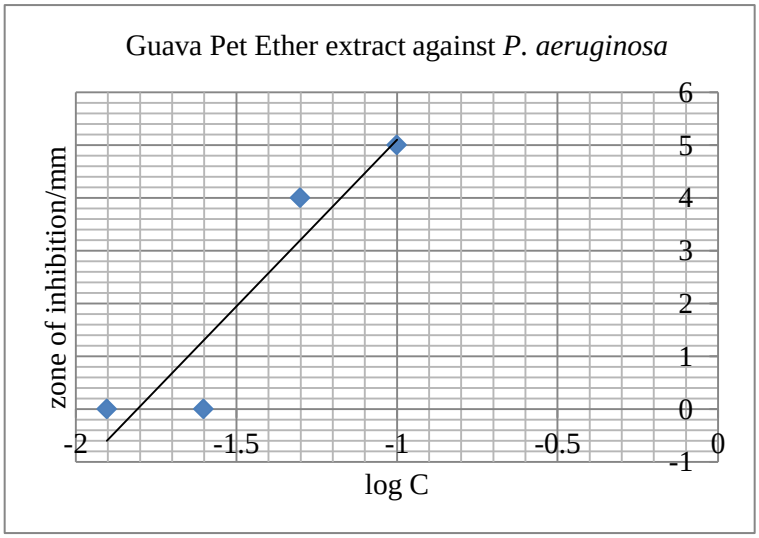

intercept on x-axis: -3.3
MIC is antilog(-3.3):0.0501%

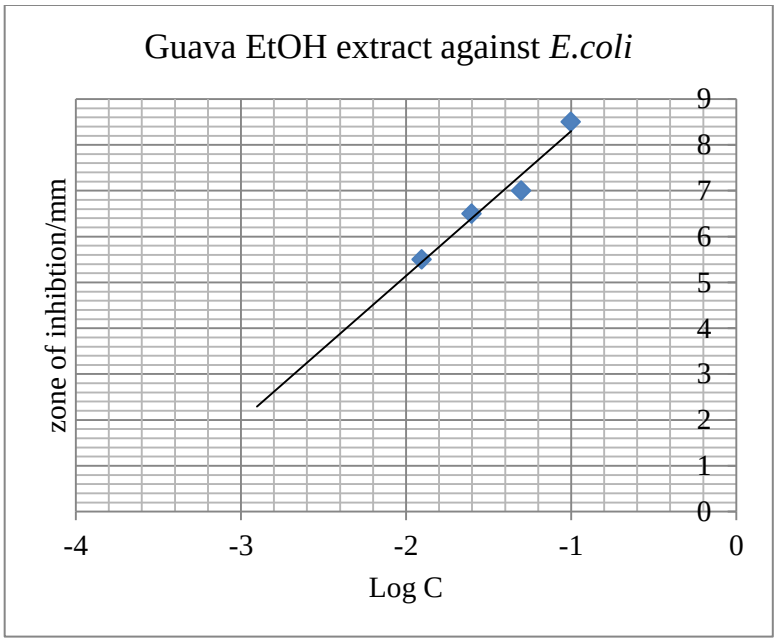



intercept on x-axis: -3.1
MIC is antilog(-3.1):0.0794%

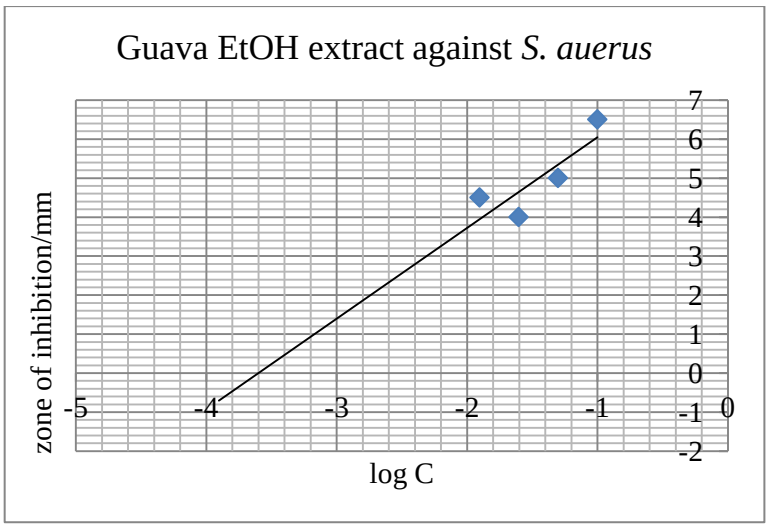



intercept on x-axis: -5
MIC is antilog(-5):0.001%

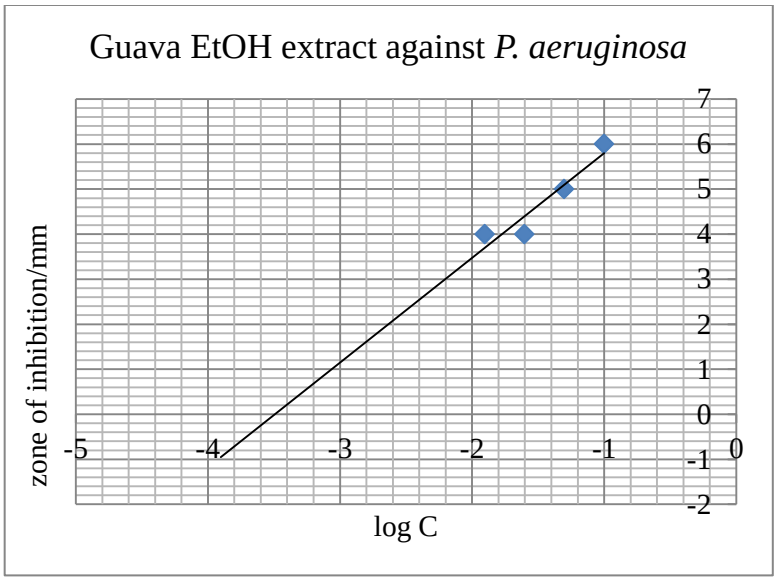




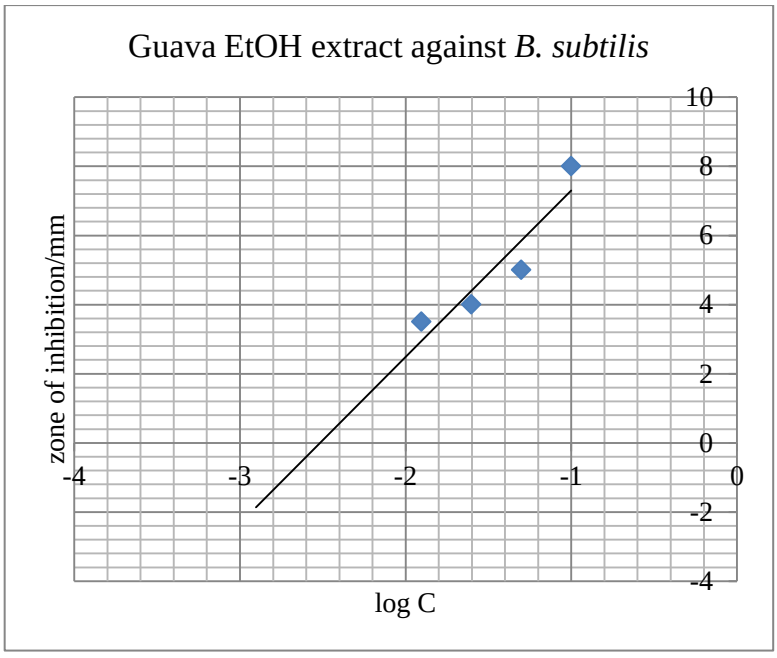

intercept on x-axis: -2.85
MIC is antilog(-2.8) : 0.141%



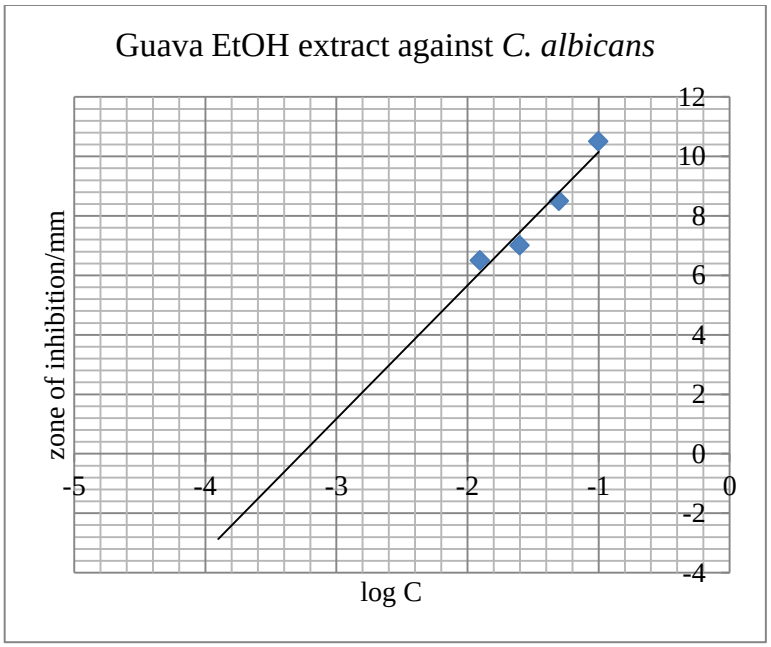

intercept on x-axis: -3.6
MIC is antilog(-3.6): 0.0251%



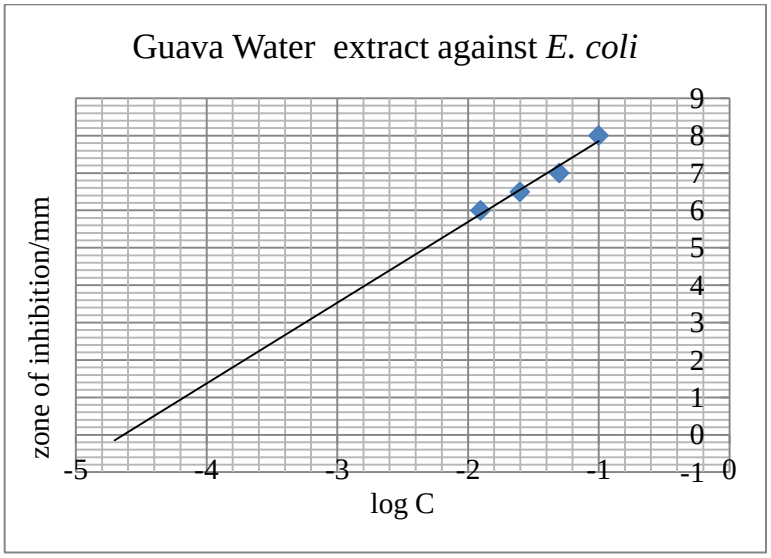

intercept on x-axis:-3.5
MIC is antilog(-3.5): 0.0316%



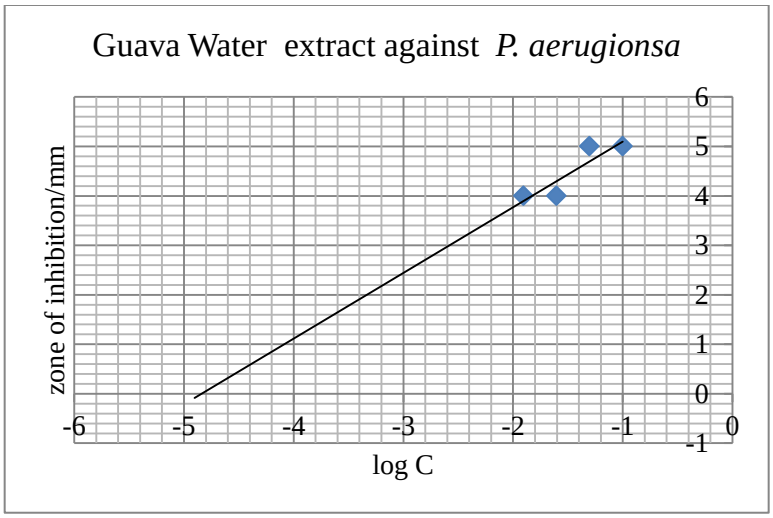

intercept on x-axis: -2.5
MIC is antilog(-2.5): 0.316%



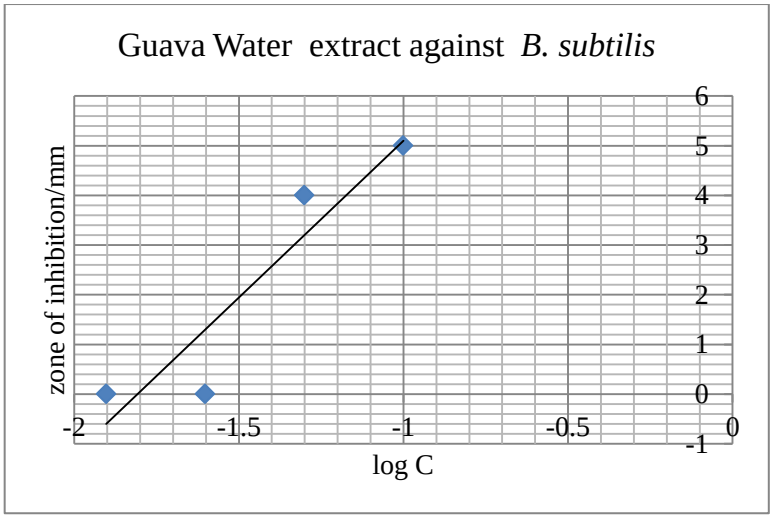

intercept on x-axis: -3.3
MIC is antilog(-3.3):0.05%



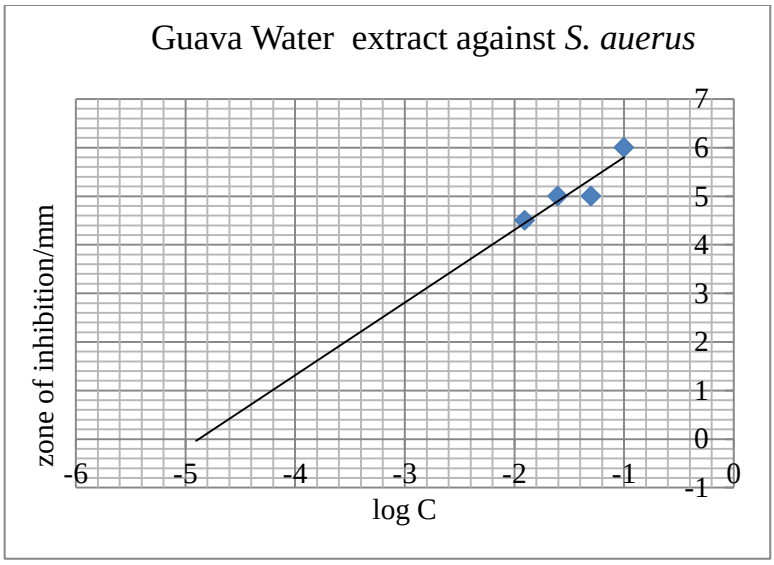

intercept on x-axis:-4.70
MIC is antilog (-4.70):0.00199%



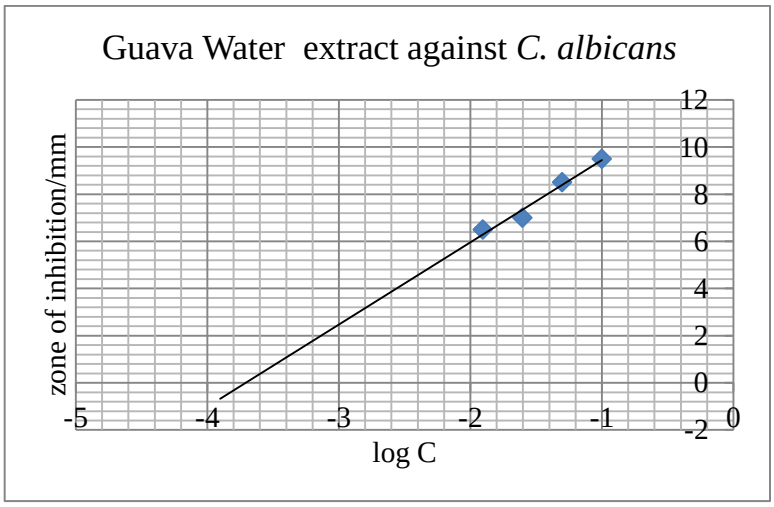

intercept on x-axis:-4.80
MIC is antilog (-4.80):0.00158%



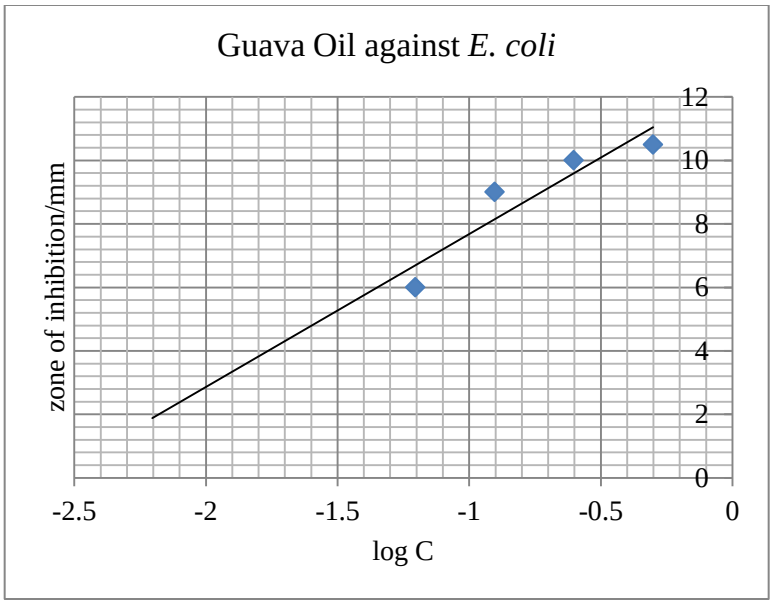

intercept on x-axis: -1.8
MIC is antilog(-1.8): 1.585%



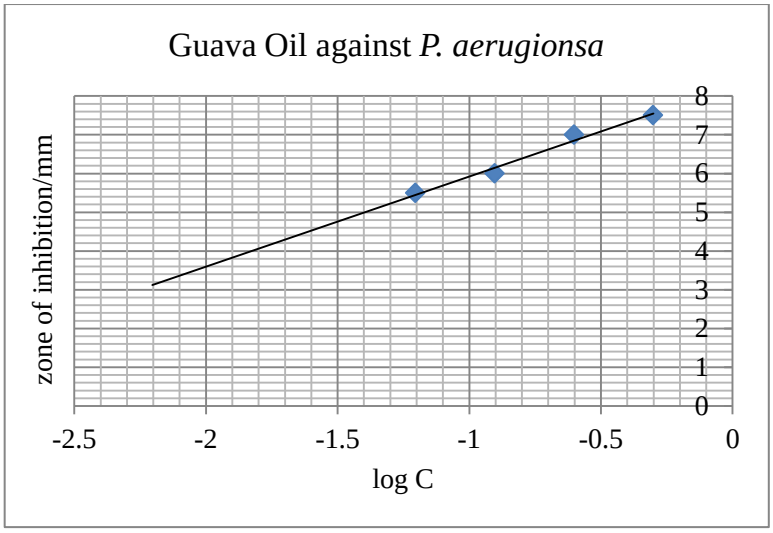

intercept on x-axis: -4.9
MIC is antilog(-4.9): 0.00126%



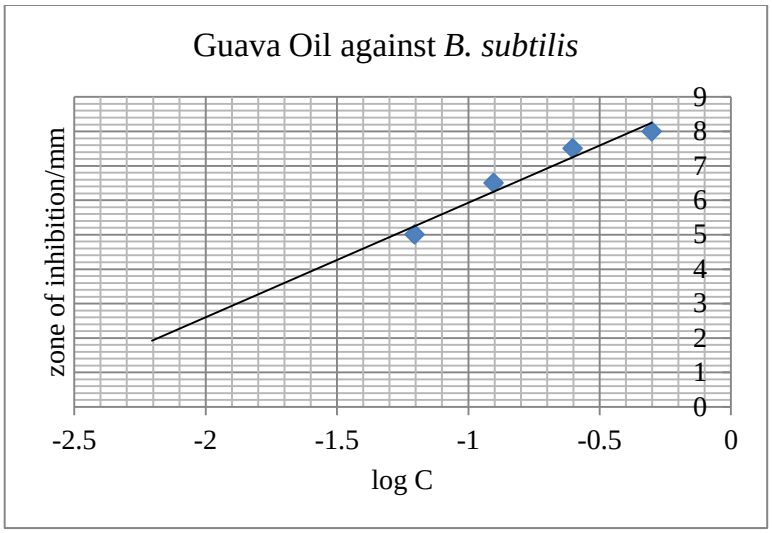

intercept on x-axis:-3.70
MIC is antilog (-3.70):0.199%



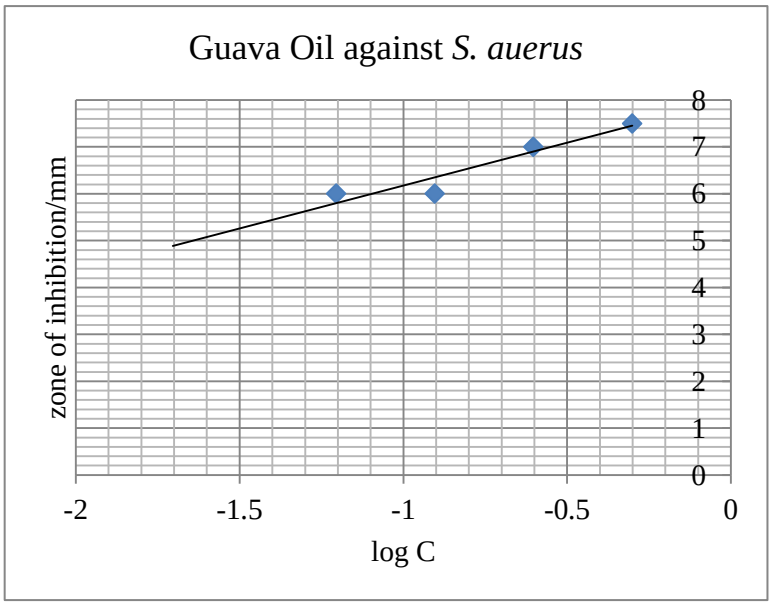

intercept on x-axis: -2.6
MIC is antilog(-2.6): 0.251%



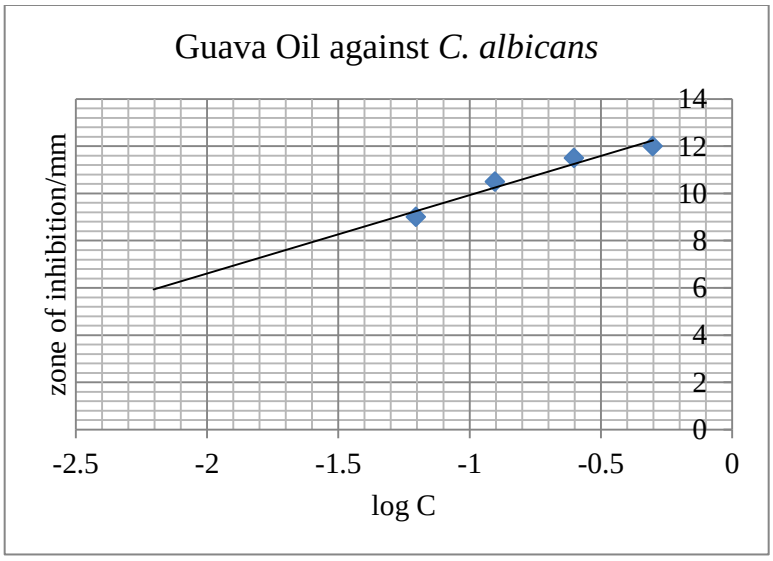

intercept on x-axis: -3.5
MIC is antilog(-3.5): 0.0316%




intercept on x-axis: -2.8
MIC is antilog(-2.8): 0.158%

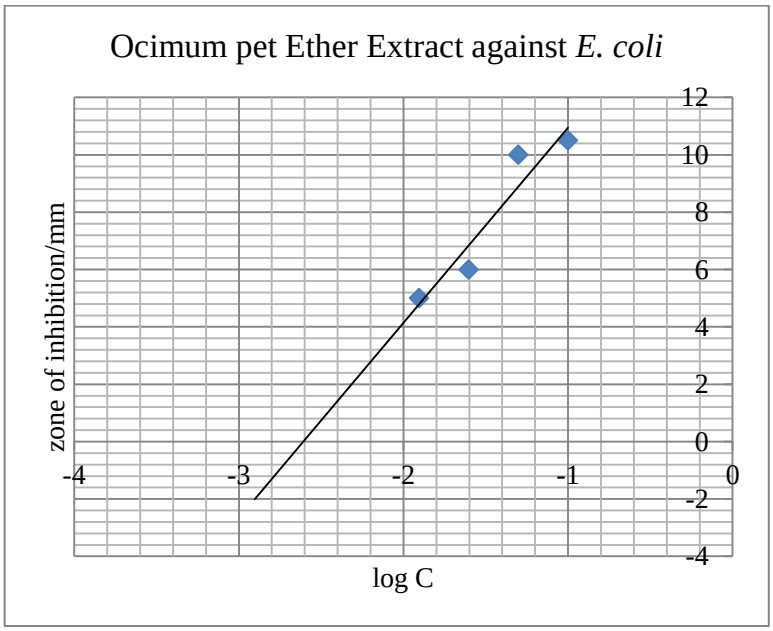



intercept on x-axis: -4.4
MIC is antilog(-4.4): 0.00398%

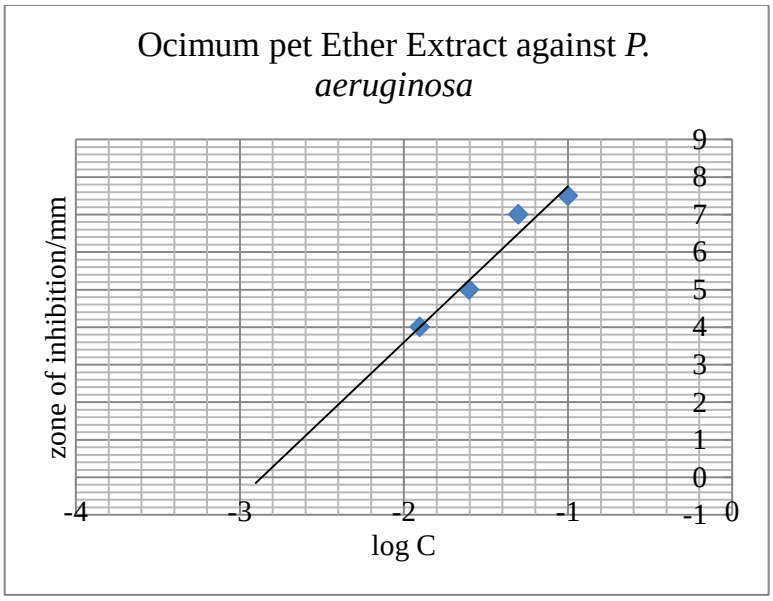



intercept on x-axis: -4
MIC is antilog(-4): 0.01%

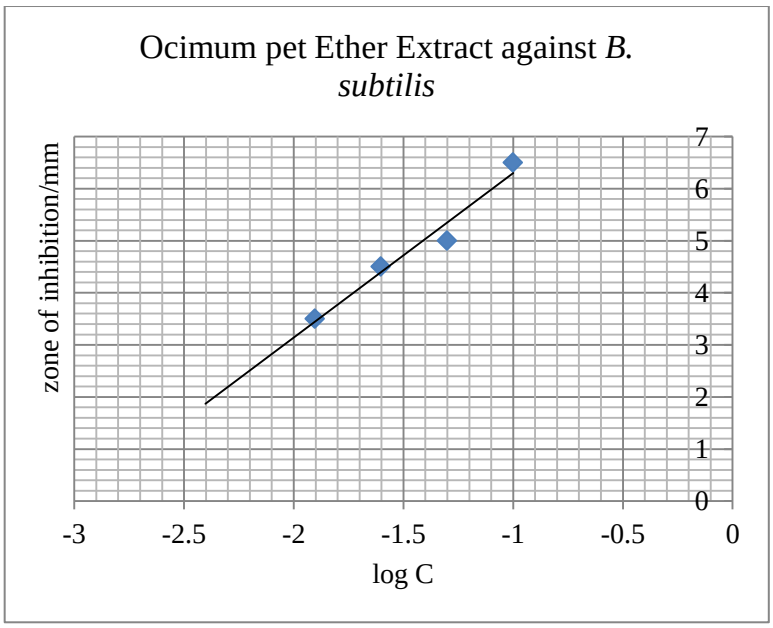
*Ocimum gratissimum Linn* (OCIMUM)



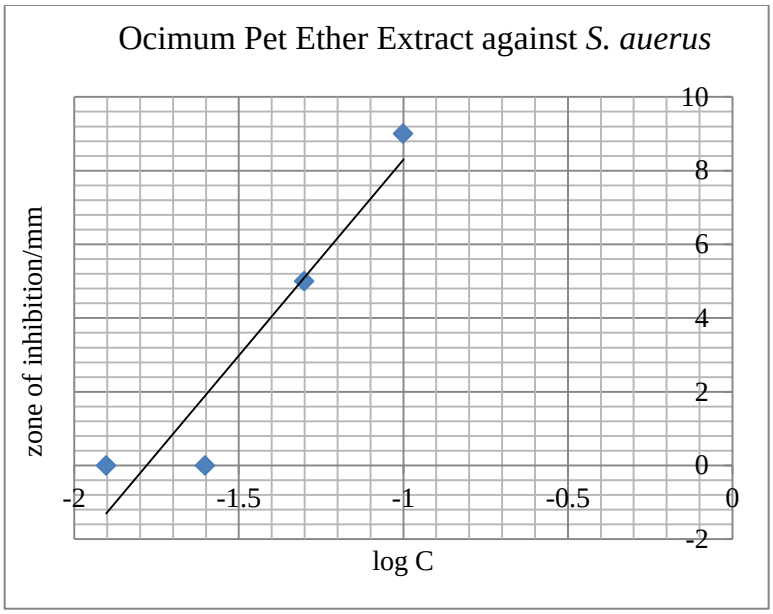

intercept on x-axis -2.8
MIC is antilog(-2.8): 0.158%



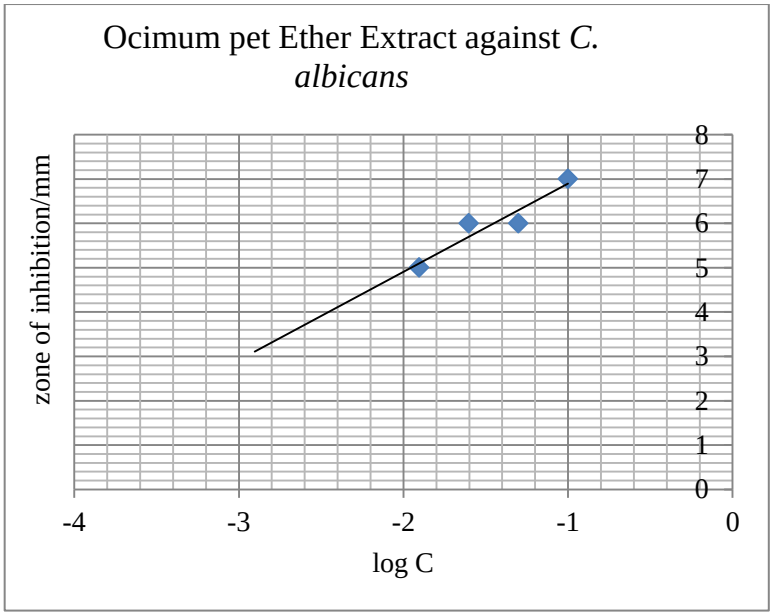

intercept on x-axis -2.8
MIC is antilog(-2.8): 0.158%



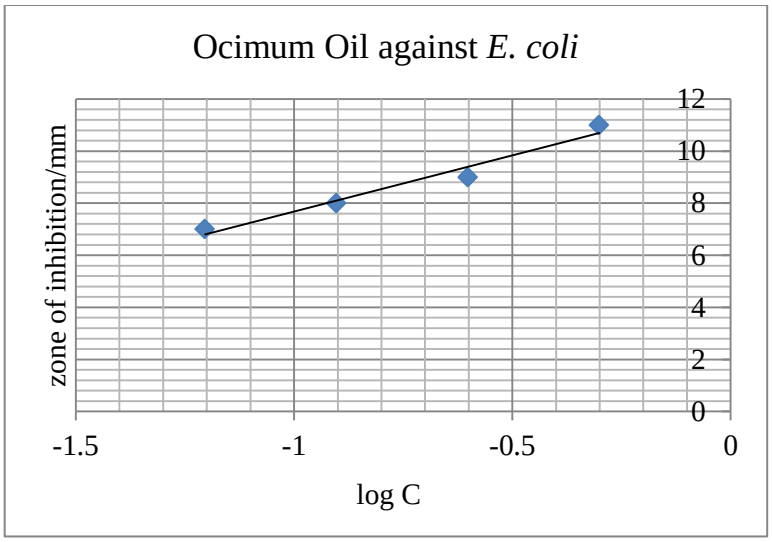

intercept on x-axis -3
MIC is antilog(-3): 0.100%



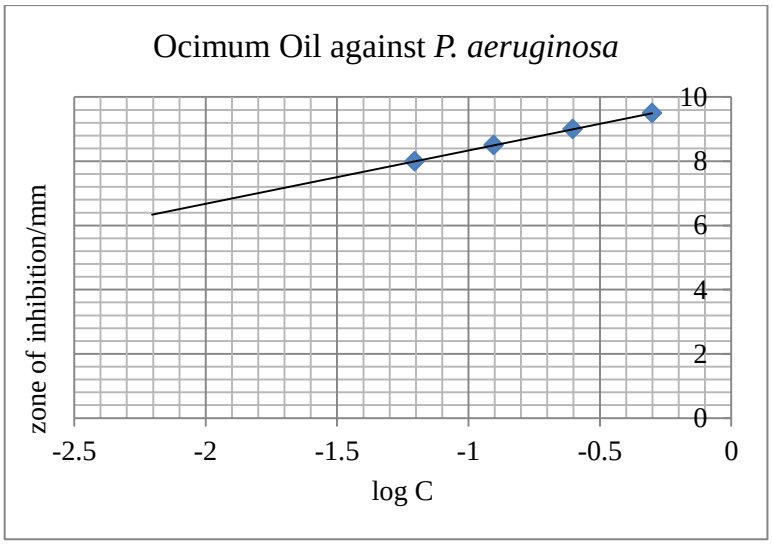

intercept on x-axis -1.8
MIC is antilog(-1.8): 1.585%



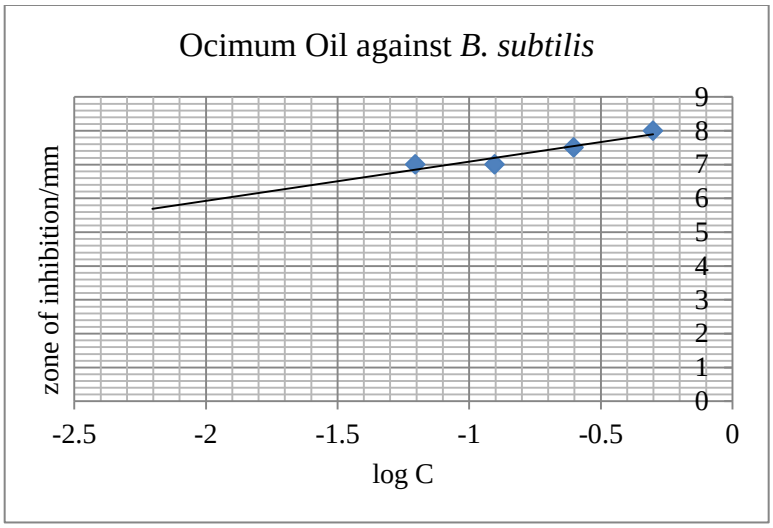

intercept on x-axis -4.4
MIC is antilog(-4.4): 0.00398%



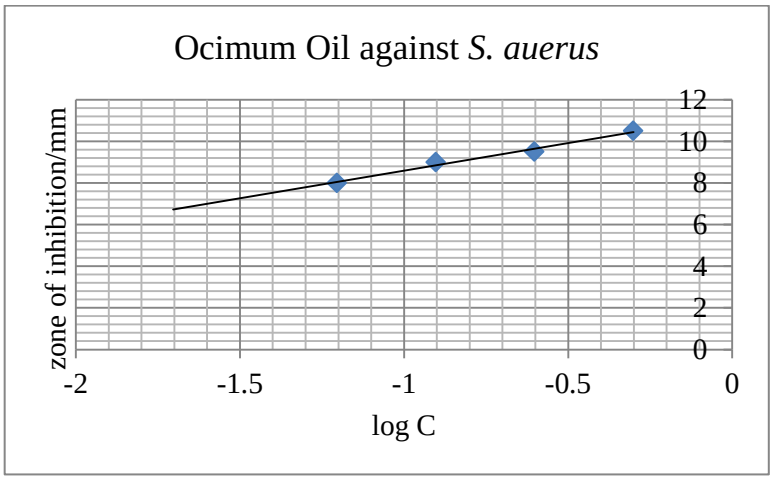

intercept on x-axis: -2.8
MIC is antilog(-2.8):0.158%



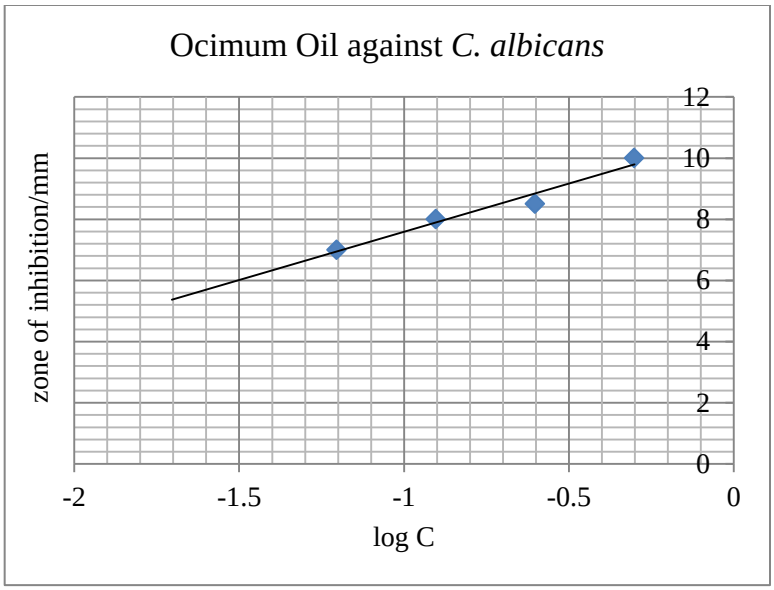

intercept on x-axis: -6
MIC is antilog(-6):0.0001%




intercept on x-axis: -7
MIC is antilog(-7):0.00001%

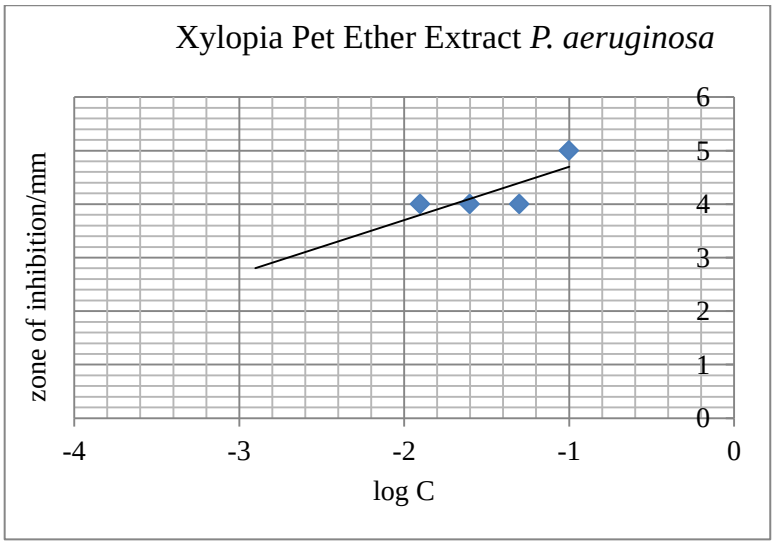



intercept on x-axis:- 4.2
MIC is antilog : 0.0063%

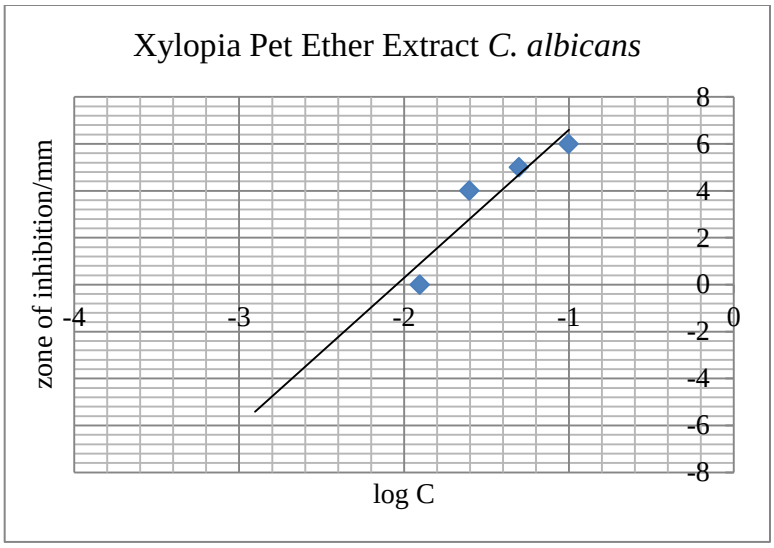



intercept on x-axis: -3.4
MIC is antilog(-3.4):0.0398%

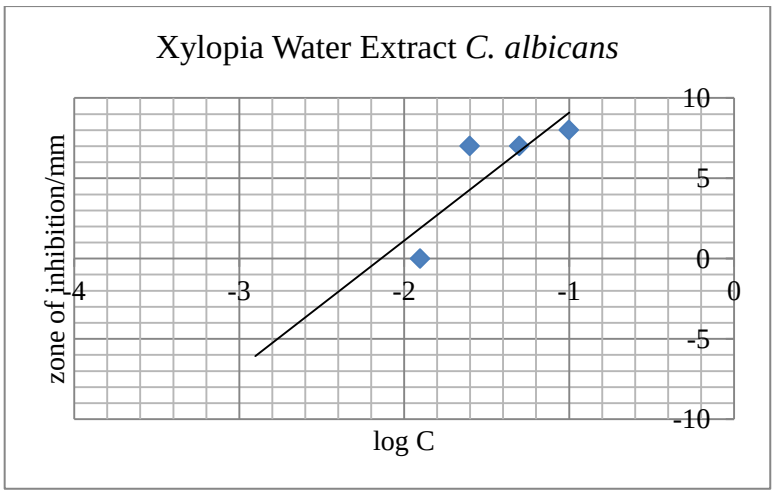
*Xylopia aethiopica A. Rich (*XYLOPIA) EXTRACT



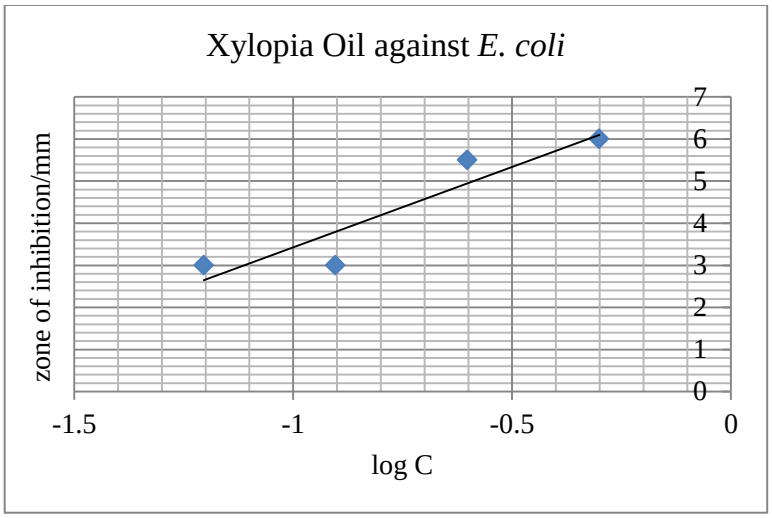

intercept on x-axis: -5.7
MIC is antilog(-5.7): 0.00199%



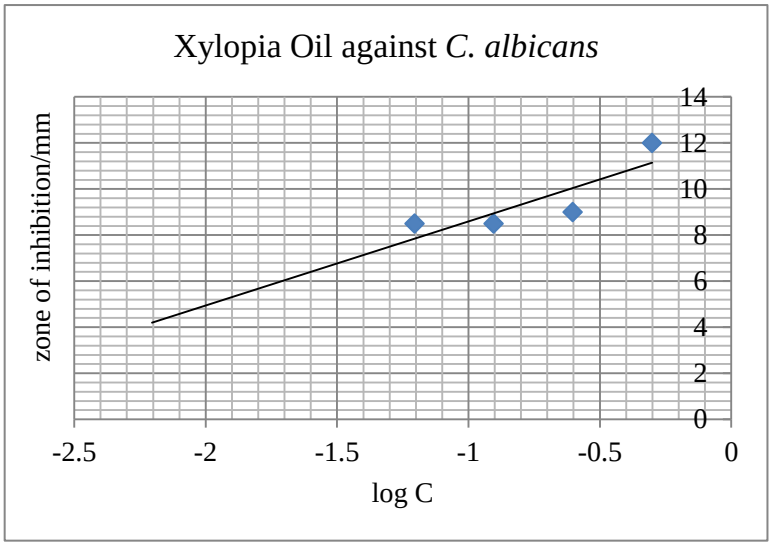

intercept on x-axis: -2.05
MIC is antilog(-2.05): 0.891%




intercept on x-axis:-2.15
MIC is antilog(-2.15): 0.708%




intercept on x-axis: -1.9
MIC is antilog(-1.9): 1.259%



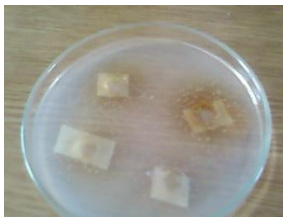

intercept on x-axis: -3.4
MIC is antilog(-3.4): 0.0398%

**APPENDIX TWO:**

**PICTURES OF ZONES OF INHIBITION OF THE CRUDE EXTRACTS AND THEIR ESSENTIAL OILS AGAINST 5 MICROBES**

CINNAMON LEAVES EXTRACTS

Cinnamon EtOH Extract against Pseudomonas



Cinnamon EtOH Extract against B. subt

Cinnamon EtOH Extract against Candida

Cinnamon Water Extract against E. coli

Cinnamon Water Extract against Pseudomonas

Cinnamon Water Extract against Staph

Cinnamon Water Extract against B. subt

Cinnamon Water Extract against Candida

GUAVA LEAVES EXTRACTS

Guava EtOH Extract against E.coli

Guava EtOH Extract against Staph

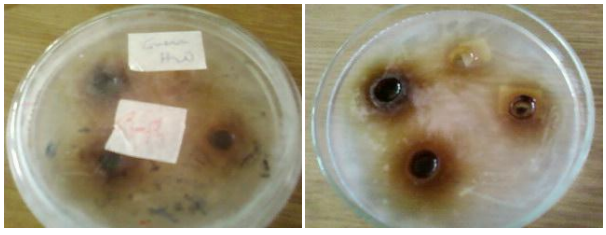
Guava EtOH Extract against B. subt

Guava EtOH Extract against Candida

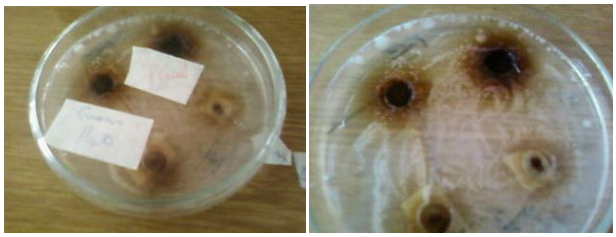
Guava Water Extracts

Guava Water Extracts against E. coli

Guava Water Extracts against Staph

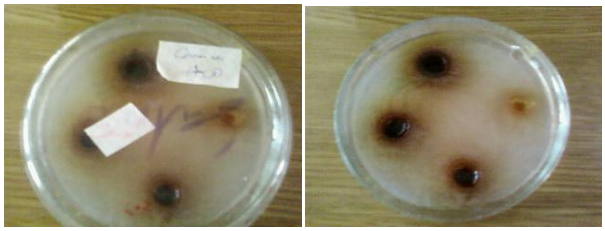


Guava Water Extracts against Pseudo



Guava Water Extracts against B. subt

Guava Water Extracts against Candida



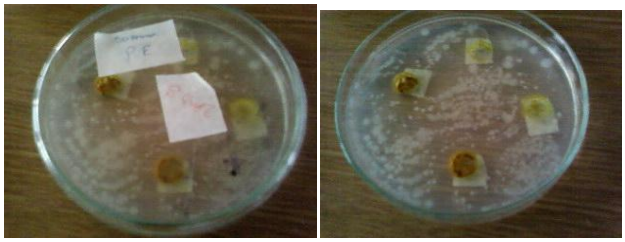
OCIMUM LEAVES EXTRACTS

Ocimum Pet Ether Extracts against E. coli

Ocimum Pet Ether Extract against Staph

Ocimum Pet Ether Extract against Pseudo

Ocimum Pet Ether Extract against B. subt



Ocimum Pet Ether Extract against Candida

Ocimum EtOH Extracts

Ocimum EtOH Extract against E. coli

Ocimum EtOH Extract against Staph

Ocimum EtOH Extract against Pseudo

Ocimum EtOH Extract against B. subt

Ocimum EtOH Extract against Candida