QUALITATIVE COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ETHANOL EXTRACT OF *thymus vulgaris* ON SOME BACTERIAL ISOLATES

BY

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TO

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CERTIFICATION

This document serves to certify that the project submitted in partial fulfillment of the requirements for the award of a Bachelor of Science degree in Microbiology was executed by **Osazuwa Ighodaro Franklin** (Matriculation Number: **LSC2104013**) under the direct supervision of Prof. E.A. Ophori.

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(HEAD OF DEPARTMENT)

DEDICATION

This work is dedicated to God Almighty, my all-sufficient Father, whose infinite mercy and grace have sustained me throughout this journey. I also dedicate it to my wonderful family for their unwavering love, moral support, and financial encouragement during my stay at the University of Benin, Benin City, Edo State, Nigeria.

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ABSTRACT

The antibacterial activities of thyme (Thymus vulgaris) against some Gram positive isolates such as *Staphylococcus aureus* and *Enterococcus faecalis* were assayed using ethanol extract. The agar-well and paper disc diffusion method were used to determine the inhibitory effect of the thyme leaves on the tested microorganism. The extract produced different zone of inhibition on the tested organism. The ethanol extract had the highest antibacterial effect against *Enterococcus faecalis* (21.6±3.51 mm) at 100% and *Staphylococcus aureus* has the lowest zone of inhibition (21.3±3.2l mm) at 100%, while at 75% *Enterococcus faecalis* had the highest zone of inhibition (l 7±2.64 mm) and *Staphylococcus aureus* has the lowest zone of inhibition (12.3±2.5l mm), while at 62% *Enterococcus faecalis* showed the highest zone of inhibition (12±3.46 mm) while *Staphylococcus aureus* has the lowest zone of inhibition (11.3±1.52 mm). The ethanol extract had the highest antibacterial effect against *Staphylococcus aureus (*20.6±4.04 mm) at 87% and *Enterococcus faecalis* has the lowest zone of inhibition (20.3±1.52 mm) at 87% while at 50% *Staphylococcus aureus* showed the highest zone of inhibition (6.33±1.15 mm) while *Enterococcus faecalis* has the lowest zone of inhibition (5±0 mm). Among these isolates, *Enterococcus faecalis* were highly sensitive to the thyme oil at different percentage (100%, 75%, 62%) while *Staphylococcus aureus* were little more sensitive to the thyme oil at 87% and 50% using the same solvent (ethanol). The increase in multidrug resistant strains of pathogenic microorganism has led to extensive phytochemical and pharmacological studies of *Thymus vulgaris* as an important source of medicinal substances which possess antibacterial properties and their effective medicinal application, as well as use in Pharmaceutical, food and cosmetic industries.

CHAPTER ONE

INTRODUCTION

Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities. The local people have a long history of traditional plant usage for medicinal purposes. The medicinal use of plants is very old. The writings indicate that therapeutic use of plants is as old as 4000 – 5000 BC and Chinese used first the natural herbal preparations as medicines. In India, however, earliest reference of use of plants as medicine appear in rig-Veda, which is said to be written between 1600 – 3500B.C. Later the properties and therapeutic uses of medicinal plants were studied in detail and recorded empirically by the ancient physicians (an indigenous system of medicine) which are a basic foundation of ancient medical science in India (Prakash *et al.,* 2025). Medicinal plant is an important element of indigenous medical systems in all over the world. The ethno botany provides a rich resource for natural drug research and development (Hosseinzadeh *et al.,* 2015). "Traditional" use of herbal medicines implies substantial historical use, and this is certainly true for many products that are available as "traditional herbal medicines". Although modern medicine may exist side-by-side with such traditional practice, herbal medicines have often maintained their popularity tfurt v for historical and cultural reasons (Vishwakarma *et al.,* 2013).

Natural products have played an important role throughout the world in treating and preventing human diseases. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates and its importance in modern medicine has been discussed in different reviews and reports (Hosseinzadeh *et al.,* 2015). Thymus vulgaris is a species of flowering plant in the mint family Lamiaceae, native to southern Europe from the western Mediterranean to southern Italy. It is bushy, woody-based evergreen sub shrub with

small, highly aromatic, grey-green leaves and clusters of purple or pink flowers in early summer. Thyme grows well during a temperate to heat, dry sunny climate, and wherever the plants don't seem to be shaded. Thyme species do best in coarse, rough soils that may be unsuitable for several alternative plants. Many pharmacological in vitro experiments carried out during the last decade revealed well defined pharmacological activities of both, the thyme essential oil and the plant extracts. The non-medical use of thyme is worthy of attention, because thyme is used in the food and aroma industries; it is widely used as culinary ingredient and it serves as a preservative for foods especially because of its antioxidant effect. The dried herbal substance contains up to 25% essential oil; the main components are thymol, carvacrol, p-cymene, -terpinene, linalool, -myroene, terpinen-4-ol Dauqan and Abdullah (2017). Oils are very complex natural mixtures which can contain about 30-60 components at quite different concentrations. Generally, these major components determine the biological properties of the essential oils Fatimah (2014).

Thyme essential oil constitutes raw material in perfumery and cosmetic due to a special and characteristic aroma. Thymus vulgaris oil is a combination of monoterpenes and those will acts as anti-oxidative, antimicrobial, medicinal drug, anti-tissue, antispasmodic, and antibacterial activities. Thyme is herbaceous plant of the platoon species, grows in mountainous areas, used as a beverage instead of or with tea, added to some food to give it an acceptable flavor, the plant is used in folk medicine frequently where it is prescribed to treat mouth infections, stomach, intestine and airways, coughing and gastroenteritis and expel intestinal worms as well as to strengthen the heart (Mohamed *et al.,* 2013). Extracts from Thyme have been used in traditional medicine for the treatment of several respiratory diseases like asthma and bronchitis and for the treatment of other pathologies thanks to several properties such as antiseptic, antispasmodic, antitussive antimicrobial, antifungal, antioxidative and antiviral Dauqan and Abdullah (2017)

1.1 Aims and Objectives

The aim of this study is to determine the antibacterial properties of common thyme found in Edo state on some clinical isolates.

The objectives are as follow;

1. To extract thyme oil from common thyme.
2. To extract the antimicrobial effect of the extract on known clinical isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

Thymus vulgaris is a flowering plant in the mint family Lamiaceae. It is growing up to 15-30 cm tall by 40cm (Prasanth *et al.,* 2014). Thyme is cultivated in most of the European countries together with France, Svizzera, Spain, Italy, Bulgaria, Portuguese republic and Elias. Yield and quality of oil varies in lie with the genetic make-up, crop_ maturity at harvest, setting and distribution follow.

2.1 Plant Description

Thyme is a tiny perennial shrub, with a semi evergreen ground cover that seldom grows quite to (40 cm) with horizontal and upright habits (Saleh *et al.,* 2015). The stems become woody with age. Thyme leaves are very little, usually 2.5 to 5 mm long and vary significantly in form and hair covering, depending on the variety, with every species having a rather completely different scent. Thymus vulgaris leaves are oval to rectangular in form and somewhat fleshy aerial components are used for volatile oil production, principally by steam distillation. The contemporary and dried herb market uses it for cooking (Prasanth *et al.,* 2014). Thyme grows well in a temperate to heat, dry, sunny climate, and wherever the plants don't seem to be shaded. The dried product should be processed to get rid of the leaves from the stems, and so sieved to get rid of dirt and to provide a consistent product (Prasanth *et al.,* 2014). Many strategies exist from sun to classy driers. The employment of sun-drying strategies leads to poor quality of the volatile oil. Artificial drying strategies permit higher management of product quality. Thyme should to be dried at temperatures not more than 40°C to cut back loss of flavor through volatilization of volatile oil, and to keep up a decent inexperienced color (Prasanth *et al.,* 2014).

Thyme grows well during a temperate to heat, dry, sunny climate, and wherever the plants don't seem to be shaded. It desires full sun to grow to its best potential. Thyme doesn't like

excessive wet as a result of its condition it will get rot diseases. Thyme prefers lightweight, well-drained soils with a pH of 5.0 to 8.0. Thyme species do best in coarse, rough soils that may be unsuitable for several alternative plants.

The dried product should be processed to get rid of the leaves from the stems, and so sieved to get rid of dirt and to provide a consistent product. Many strategies exist from sun to classy driers. The employment of sun-drying strategies leads to poor quality of the volatile oil. Artificial drying strategies permit higher management of product quality. A forced air-flow drier could be a appropriate system to dry better-quality leaves. Thyme should to be dried at temperatures not up to 40°C to cut back loss of flavor through volatilization of volatile oil, and to keep up a decent inexperienced color. Once drying, the leaves should be separated clear of the stems, sieved and hierarchic. Fresh turn out has got to be clean of foreign material and looking out recent and tender with a decent color and flavor. There is an International Standard (ISO 6754:1996), prescribing quality necessities for dried thyme. The quality prescribes bound necessities of the finished product. The volatile oil content of the dried herb is a vital issue contributory to the flavor intensity. Whole thyme leaves should contain a minimum of 0.5% essential oil, that equals 5 ml/ kg dried herb, and ground thyme should contain a minimum of 0.2% volatile oil to satisfy the wants. Thyme volatile oil should be hold on in a very cool, dry space till it's used. Keep it in dark, air-tight glass bottles and don't expose it to heat or serious metals. Once opened, refrigeration and tightly closing the cap can prolong its shelf-life. Deterioration begins if the liquid is far darker or a lot of viscous than traditional

TAXONOMIC CLASSIFICATION

Kingdom: plantae

Subkingdom: tracheobionta

Superdivision: Spermatophyta

Divisiion: magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Lamiales

Family: Lamiaceae

Genus: *Thymus* L.

Species: *Thymus volgaris* L.

2.2 Background and History of Traditional Herbal Medicine

The use of plants as medicine goes back to early man. Fossil records date human use of plants as medicines at least to the middle Paleolithic age. Evidences of this early association have been found in the grave of a Neanderthal man buried 60,000 years ago. Pollen analysis indicated that the numerous plants buried with the corpse were all of medicinal value. The earliest known medical document is a 4000-year –old. Sumerian clay tablet that recorded plant remedies for various illnesses. By the time of the ancient Egyptian civilization, a great wealth of information already existed on medicinal plants. Among the many remedies prescribed were man drake for pain relief, and garlic for the treatment of heart and circulatory disorders. This information, along with hundreds of other remedies, was preserved in the papyrus about 3500 years ago. Ancient China is also a source of information about the early medicinal uses of plants (Hosseinzadeh *et al.,* 2015). Although animal and mineral materials

have used, the primary source of remedies is botanical. Of the more than 12,000 items used by traditional healers, about 500 are uncommon use. Botanical products are used only after some kind of processing, which may include, for example, stir-frying or soaking in vinegar or wine. In clinical practice, traditional diagnosis may be followed by the prescription of a complex and often individualized remedy.

Traditional Chinese medicine is still in common use in China. More than half the population regularly uses traditional remedies, with the highest prevalence of use in rural areas. About 5000 traditional remedies are available in China; they account for approximately one fifth of the entire Chinese pharmaceutical market (Hosseinzadeh *et al.,* 2015). Many herbal remedies found their way from China into the Japanese systems of traditional healing. Herbs native to Japan were classified in the first pharmacopoeia of Japanese traditional medicine in the ninth century (Hosseinzadeh *et al.,* 2015). In India, herbal medicine dates back several thousand years to the Rig-Veda, the collection of Hindu sacred verses. One useful plants from this body of knowledge is snakeroot, used for centuries for its sedative effects. The number of higher plant species on this planet is estimated at 250,000, with a lower level at 215,000 (Hosseinzadeh *et al.,* 2015) and an upper level as high as 500,000. Of these, only about 6% have been screened for biologic activity and a reported 15% have been evaluated phytochemically. With high through put screening methods becoming more advanced and available, these numbers will change, but the primary discriminator in evaluating one plant species versus another is the matter of approach to finding leads. There are some broad starting points to selecting and obtaining plant material of potential therapeutic interest. However, the goals of such an endeavor are straight forward. Plants have an advantage in this area based on their long-term use by humans (often hundreds or thousands of years) (Hosseinzadeh *et al.,* 2015).

2.3 Herbal Medicines in Developed Countries

Plants and their metabolites constitutes have a long history of use in modern "western" medicine and in certain systems of traditional medicine, and are the sources of important drugs such as atropine, codeine, dioxin, morphine, quinine. Use of herbal medicines in developed countries has expanded sharply in the latter half of the twentieth century. In recent years, the use of traditional medicine information on plant research has again received considerable interest. While the western use of such information has also become acknowledged by most academic and industrial researchers. Meanwhile, the need for basic scientific investigations on medicinal plants using indigenous medical systems becomes imminent (Hosseinzadeh *et al.,* 2015). The desire to capture the wisdom of traditional healing systems has led to a resurgence of interest in herbal medicines (Hosseinzadeh *et al.,* 2015) particularly in Europe and North America, where herbal products have been incorporated into so-called alternative, "complementary", "holistic" or "integrative" medical systems. Monographs on selected herbs are available from a number of sources, including the European Scientific Cooperative on Phototherapy and the World Health Organization (Hosseinzadeh *et al.,* 2015). The WHO monographs, for example, describe the herb itself by a number of criteria (including synonyms and vernacular names) and the herb part commonly used, its geographical distribution, tests used to identify and characterize the herb (including macroscopic and microscopic examination and purity testing), the active principles (when known), dosage forms and dosing, medicinal uses, pharmacology, contra-indications and adverse reactions. During the latter part of the twentieth century, increasing interest in self-care resulted in an enormous growth in popularity of traditional healing modalities, including the use of herbal remedies; this has been particularly true in the USA (Hosseinzadeh *et al.,* 2015) in the European market there are a lot of products derived from natural plants, which are

recognized to possess different biological properties, such as antioxidant, antiseptic, diuretic, stimulating the central nervous system, sedative, expectorant, digestive, etc. Some of these plants have been used in traditional medicine since ancient times and are available on market as infusions, tablets and/or extract s. Consumers have reported positive attitudes towards these products, in large part because they believe them to be of "natural" rather than " synthetic" origin, they believe that such products are more likely to be safe than are drugs, they are considered part of a healthy lifestyle, and they can help to avoid unnecessary contact with conventional "western" medicine (Hosseinzadeh *et al.,* 2015).

2.4 Modern and Traditional Prescription of Herbal (*Thymus vulgaris*)

The pharmacological treatment of disease began long ago with the use of herbs. Although herbalist waned in the eighteenth and nineteenth centuries, many of the remedies employed by the herbalists provided effective treatment. Some of these became useful prescriptions as physicians began experimenting with therapeutic agents. William withering was the first in the medical field to scientifically investigate a folk remedy. His studies (1775-1785) of foxglove as a treatment for dropsy (congestive heart failure) set the standard for pharmaceutical chemistry. In the nineteenth century, scientists began purification of the active extracts from medicinal plants.

One breakthrough in pharmaceutical chemistry came when friedrich isolated morphine from the opium poppy (*Papaver somniferum*) in 1806. Continuing this progress, Justus von Liebig, a German scientist became a leader in pioneering the field of pharmacology. With increased knowledge of active chemical ingredients, the first purely synthetic drugs based on natural products were formulated in the middle of the nineteenth century. In plants, these compounds are mostly secondary metabolites called bioactive compounds such as alkaloids, steroids, tannins, and phenol compounds, flavonoids which are capable

of producing definite physiological action on body. The medicinal plants are rich in secondary metabolites and essential oils of therapeutic importance.

The important advantages claimed for therapeutic uses of medicinal plants in different ailments are their safety besides being economical, effective and their easy availability. Because of these advantages the medicinal plants have been widely used by the traditional medical practitioners in their day-to-day practice. Among all plant secondary metabolites which act as antioxidants phenol compounds form a large and varied group. Phenol compounds contribute significantly to the antioxidant potential of several plant species. Among different thymus species. *Thymus vulgaris* is cultivated in many countries by most people especially in rural areas which depend on herbal medicines to treat many diseases including inflammation related ailments such as rheumatism, muscle, swelling, insect bites and pains. *Thymus vulgaris* is used more in; pharmaceutical dosage forms because it contains more essential oil than other species with high amount of thyme which exhibits considerable anti-tissue and expectorant effects (Hosseinzadeh *et al.,* 2015). Considering the fact that *Thymus vulgaris* is more expensive, some herbal industries tend to use other species of *Thymus* with different components. So it is crucial to develop a suitable and reliable identification method to confirm the quality of extracts and herbal drugs. Separation and detection of different constituents in plants have been always complicated. *Thymus vulgaris* has approved expectorant, anti-tissue and antispasmodic activities. Its antiseptic property is estimated to be 25 times more effective than phenol, with less toxicity (Hosseinzadeh et al.2015), Different species of *Thymus* are different in content and type of components. Generally they contain thyme, carvacrol, flavonoids and phenol compounds such as rosmarinic acid which may have antiedemic and macrophage-inhibiting effects. *Thymus vulgaris* shows a polymorphic variation in monoterpene production, the presence of intra-specific chemotype variation being common in the genus

Thymus. Each of the six chemotypes, geraniol (G), α - terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C) and thymol (T), is named after its dominant monoterpene (Hosseinzadeh *et al.,* 2015). Many pharmacological in vitro experiments carried out during the last decade revealed well defined pharmacological activities of both, the thyme essential oil and the plant extracts. The non-medicinal use of thyme is worthy of attention, because thyme is used in the food and aroma industries; it is widely used as culinary ingredient and it serves as a preservative for foods especially because of its antioxidant effect. Thyme essential oil constitutes raw material in perfumery and cosmetics due to a special and characteristic aroma. *Thymus vulgaris* oil is a combination of monoterpenes and those will acts as anti-oxidative, antimicrobial, medicinal drug, anti-tissue, antispasmodic and antibacterial activities (Hosseinzadeh *et al.,* 2015).

2.5 Origin and Distribution

Thyme is the general name for the many herb varieties of the Thymus species, all of which are native to Europe and Asia. Common or garden Thyme is considered the principal type and is utilized commercially for flowering and ornamental purposes (Nadiya *et al.,* 2016). Thyme is native to the Western Mediterranean region, extending to south-eastern Italy.

The name thyme, in its Greek form, was first given to the plant by the Greeks as a derivative of a word which meant “to fumigate,’ either because they used it as incense, for its balsamic odour, or because it was taken as a type of all sweet-smelling herbs. Others derive the name from the Greek words thyo, meaning perfume or *Thymus,* signifying courage, the plant being held in ancient and medieval days to be a great source of invigoration, its pleasant qualities inspiring courage. Another source quotes its use by the Sumerians as long ago as 3,500BC and to the ancient Egyptians who called it than. Dauqan and Abdullah (2017).

2.6 Chemical Composition of Thyme Essential Oil

Oils are very complex natural mixtures which can contain about 30-60 components at quite different concentrations. Generally, these major components determine the biological properties of the essential oils Fatimah (2014). Essential oil: there are at least 6 chemotypes of *Thymus vulgaris* with different compositions of the essential oil; only the 'thymol' type with thymol as predominant compound complies with the definition in the European Pharmacopoeia. The dried herbal substance contains up to 2.5% essential oil; the main components are thymol, carvacrol, p-cymene, γ-terpinene, linalool, β-myrcene, terpinene-4-ol. Some compounds occur partly as glycosides Dauqan and Abdullah (2017). Prasanth *et al.,* (2014) reported that the essential oil from *Thymus vulgaris* showed a high content of oxygenated monoterpenes (56.53%) and low contents of monoterpene hydrocarbons (28.69%), sesquiterpene hydrocarbons (5.04%) and oxygenated sequiterpenes (1.84%). The predominant compound among the essential oil components was thymol (51.34%) while the amount of all other components of the oil was less than 19% (Prasanth *et al.,* 2014)

Table 1: The chemical compositions of the essential oil of *Thymus vulgaris*

Component	Formula	Relative concentration (%)
Anisole	$C_7H_{18}O$	0.23
Geraniol	$C_{10}H_{18}O$	0.10
Citral	$C_{10}H_{16}O$	0.24
Thymol	$C_{10}H_{14}O$	54.26
Carvacrol	$C_{10}H_{14}O$	4.42
Octadiemoic acid	$C_{18}H_{12}O$	0.10
Geranic acid	$C_{10}H_{10}O_2$	0.30

(Dauqan and Abdullah, 2017)

Table 2: The List of selected chemical constituents in Thymus vulgaris

Chemical constituent	Biological activities
Thymol	Antiseptic, antibacterial, antifungal and antioxidant properties
Carvacrol	Antimicrobial, antithrombotic, anti-inflammatory, acctyl cholinesterase inhibitory properties
Linalool	Antiviral effect, anti-inflammatory, antioxidant, anti-nociceptive as well as analgesic activity
Apigenin	Anti-carcinogenic, anti-inflammatory, anti-progression, anti-inflammatory, anti-viral and anti-oxidant properties
Eugenol	Neuro-protective, anticancer, anti-bacterial and anti-anaphylactic activities
Rosanarinic acid	Astringent, anti-allergic, anti-mutagen, anti-oxidative and anti-inflammatory

(Hina *et al.,* 2013)

2.7 Thyme Nutritional Value

The amazing thyme benefits can be attributed to its rich nutritional value. The nutrients in Thyme have disease-preventing and health-promoting properties Dauqan and Abdullah (2017). Thus aromatic herb is loaded with phytonutrients, minerals and vitamins which are vital for good health Dauqan and Abdullah (2017).

Vitamins

Thyme is also a good source of vitamins. It is particularly rich in Vitamin A and Vitamin C. Vitamin A is an antioxidant, vital for maintaining healthy mucus membranes and skin as well as good vision Dauqan and Abdullah (2017). Vitamin C provides resistance against infectious diseases and fights harmful pro-inflammatory free radicals. Among the B-complex vitamins, it is a good source of vitamin B6 or pyridoxine with a 100against infectious diseases and fights harmful pro-inflammatory free radicals. Among the B-complex vitamins, it is a good source of vitamin B6 or pyridoxine with a 100 gram serving providing about 0.35mg or 27% of the daily recommended intake of this vitamin Daugan and Abdullah (2017). This vitamin assists in maintaining GABA levels in the brain and acts as a stress buster. Other vitamins found in this herb include Vitamin K, vitamin E and folic acid Dauqan and Abdullah (2017).

Minerals

Thyme is packed with minerals that are vital for optimum health. Its leaves are excellent sources of potassium, calcium, iron, manganese, magnesium and selenium. Potassium, being an important component of cells and body fluids, controls heart rate and blood pressure. Manganese is a co-factor for the antioxidant enzyme superoxide dismutase. Iron is involved in red blood cell formation Sharangi and Guha (2013).

Volatile Oils

Thymol is one of the most important essential oils found in thyme and known for its antiseptic and antifungal properties. It also contains other volatile oils such as carvacolo, geraneol and borneol Moghtader (2012).

Antioxidants

Thyme is a rich source of flavonoid phenolic antioxidants such as zea-xanthin, pigenin, lutein, luteolin and thymonin (Dauqan and Abdullah, 2017).

Antioxidant properties

An antioxidant is a molecule that inhibits the oxidation of different molecules. Oxidation is a chemical process that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions will produce free radicals Dipak (2013). In turn, these radicals will begin chain reactions. Once the chain reaction happens is a cell, it will cause damage or death to the cell. Antioxidants stop these chain reactions by removing free radical intermediates, and inhibit different oxidation reactions (Eqbal *et al.,* 2013). The leafy parts of Thyme and its oil are utilized in foods for the flavor, aroma and preservation and additionally in folk medicines (Saleh *et al.,* 2015). El-Nekeety et al. (2011) conducted an experiment to work out the elements of thymus vulgaris L. oil and to evaluate the protecting effects of this oil against aflatoxin induce oxidative stress in rats. The results indicated that the oil contains carvarcrol (45mg/g), Thymol (24.7mg/g), β -phellandrene (9.7mg/g), essential oil (4.1mg/g), Humuline (3.1mg/g), α -Phellandrene (2.3mg/g) and Myrcene (2.1mg/g). however, α and β -pinene, Myrcene, α -thyjone, Tricyclene, 1,8-cinole, and – β -sabinene were found in very lower concentrations. Treatment with aflatoxins alone disturbs lipid profile in blood serum, decreases total antioxidant

capability, increase creatinine, uric acid and nitric oxide in blood serum and lipid peroxidation in liver and excretory organ attended with a sever histological changes within the liver tissues (El-Nekeety *et al.,* 2011). The oil alone at the two tested doses didn't induce any important changes within the biochemical parameters or the histological image. The combined treatment showed important enhancements of tested parameters and histological footage within the liver tissues. Moreover, this improvement was more pronounced within the cluster that received the high dose of the oil (Dauqan and Abdullah, 2017).

2.8 Pharmaceutical Importance of Thymus vulgaris

Thyme has long history of being used in traditional medicine for treatment of various diseases for instance to treat respiratory diseases (whooping cough, bronchitis and asthma), in the form of tea, ointment, tincture, syrup or by steam inhalation (Hina *et al.,* 2013). It is also used to prevent hardening of the arteries, treatment of toothache, urinary tract infection and dyspepsia (Hina *et al.,* 2013). It also expels fungus from stomach and intestine and it has the ability to increase appetite because of its important component thymol, which has the ability to kill bacteria and parasites. Different studies were carried out in the last decades to reveal reported pharmacological activities of thymus vulgaris bot of plant extracts and essential oil. In mice analgesic and antipyretic properties were reported for thyme extracts. Thyme has changed from a traditional herb to a serious drug rational phototherapy. It is an incredible source of iron, calcium, manganese, vitamin K and likewise upgrades blood flow and pushes an invigorating impact for the entire system (Moshin *et al.,* 1986).

This herb invigorated activity on anxious framework made it as a cute for physical and mental weakness and additionally for diminishing insomnia. The remedial potential of Thymus vulgaris is due to the presence of flavonoids, thymol, carvacrol, eugenol and phenols. Its controls numerous valuable effects, such as, antispasmodic, bactericides, antiseptics, antioxidants, anthelmintic properties and has lately been recommended as substitute for cancer prevention agent (Monira and Naima, 2012). Among different thymus species, Thymus vulgaris is cultivated in many countries by most people especially in rural areas who depend on herbal medicines to treat many diseases including inflammation-related ailments such as rheumatism, muscle swelling, insect bites and pains (Namsa *et al.,* 2009).

Thymus vulgaris is used more in pharmaceutical dosage forms because it contains more essential oil than other species with high amount of thymol which exhibits considerable antitussive and expectorant effects Verpoote, (2000). Considering the fact that Thymus vulgaris is more expensive, some herbal industries tend to use other species of Thymus with different components (Saleh *et al.,* 2015).

2.9 MEDICINAL APPLICATION

The main component of the essential oil of thyme, thymol, is active against salmonella and staphylococcus bacteria. The antiseptic and tonic properties of thyme make it a useful tonic for the immune system in chronic, especially fungal infections as well as an effective remedy for chest infections such as bronchitis, whooping cough, and pleurisy (Marina *et al.,* 2010). Thyme and thyme oil have been used as fumigants, antiseptics, disinfectants, and mouth washes. The pleasant-tasting infusion can be taken for minor throat and chest infections, and the fresh leaves may be chewed to relieve sore throats. Thyme is prescribed with other herbs for asthma, hay fever, and is often used to treat

worms in children (Dauqan and Abdullah 2017).

Thyme has been though to be antiseptic, antimicrobial, astringent, anthelmintic, carminative, disinfectant, medicinal drug and tonic (Prasanth *et al.,* 2014). Thyme is incredibly useful in cases of assorted intestinal infections and infestations, like hookworms, acarida, gram positive and Gram negative bacteria, fungi and Yeasts such as Candida albicans. Its active constituent, thymol, is active against enterobacteria and coccid bacteria. Thyme may also improve liver function and act as an appetite stimulant. It will be used in treatment of cartilaginous tube, bronchial and urinary infections (Hompson *et al.,* 2003). Thyme is helpful in treatment of laryngitis and inflammation. The main component of the volatile oil of thyme, thymol, is active against enterobacteria (Saleh *et al.,* 2015). It is used for skin issues like oily skin, sciatica, acne, dermatitis and bug bites. A corrected products, 'white thyme oil' is also used and its milder on the skin. Applied to the skin, thyme relieves bites and stings and relieves neuralgia and rheumatic aches and pains (Prasanth *et al.,* 2014).

2.9.1 Anti-inflammatory property

Thymus vulgaris oil is a combination of monoterpenes, the most compounds of this oil are the natural terpenoid thymol and its phenol chemical compound carvacrol (Prasanth *et al.,* 2014) that have antioxidative, antimicrobial, medicinal drug, antitussive, antispasmodic, and antibacterial effects (Hoferl *et al.,* 2009). Terpenoids, flavonoid agalycones, flavonoids glycosides, and synthetic resin acids were additionally found in Thymus species.

2.9.2 Antibacterial activity of thymus vulgaris

Prasanth *et al.,* (2014) and Salehn *et al.,* (2015) reported that the essential oils obtained from thymus vulgaris harvested at 4 biological process stages were evaluated for their

biological activity and chemical components. The thyme volatile oils were analyzed for their inhibition effects against 9 strains of gram-negative bacteria and 6 strains of gram-positive bacteria.

The bioimpedance methodology was chosen for finding out the antibacterial activity of the essential oils and also the parameter chosen for outlining and quantifying the antibacterial activity of the thyme oils was the detection time (Prasanth *et al.,* 2014). The plate counting technique was used to study the inhibitory effect by direct exposure. All the thyme essential oils examined had a significant bacteriostatic activity against the microorganisms tested. This activity was more pronounced against the gram-positive bacteria (Marino and Bersina, 1999).

The oil from thyme fully flower was the foremost effective at stopping the growth of the microorganism species examined (Marino and Bersina 1999). The oils tested were conjointly shown to possess smart antibacterial activity by direct contact, that gave the impression to be a lot of marked against the gram-negative microorganism. Some species were capable of recovering a minimum of 50% of their metabolic function once contact with the inhibitor whereas most of the strains were shown to have been inactivated almost completely Prasanth *et al.,* (2014) and Saleh *et al.,* (2015)

2.9.3 INSECTICIDAL ACTIVITY

The insecticidal activity of thyme volatile oil, thymol and carvacrol was evaluated in laboratory against completely different larval stages of lesser mealworm. The sooner and later larval stages were reared on diets containing one or two acetone solutions of tested compounds. Insecticidal activity of thyme volatile oil and pure monoterpenes against A. diaperinus larvae relied on the dose and age of larvae. The growth of younger larvae was considerably affected, whereas those of older larval stage were less influenced and only by pure oil components. In young larvae the application 1% thyme oil, thymol and

carvacrol, caused mortality of 50.0%, 86.67% and 85% respectively (Szczepanik *et al.,* 2012)

2.9.4 ANTIMICROBIAL ACTIVITY OF Thymus vulgaris

Boruga *et al.,* (2014) reported that the antimicrobial activity of essential oils depends on their chemical constituents. Apparently, the antimicrobial activity of the essential oil analyzed is related to the presence of phenolic compounds (thymol) and terpene hydrocarbons (y-terpinene), respectively (Boruga *et al.,* 2014). p-Cymene, the third major element according to percentage, does not show antibacterial efficacy when used alone, synergistic effects being however attributed to it in relation to thymol and y-terpinene, respectively, which might represent another cause of the antimicrobial activity recorded. On the other hand, a number of studies have shown that essential oils exhibit stronger antimicrobial activity than that of their major constituents or their mixtures, respectively, which suggests synergistic effects of the minor components, but also the importance of all components in relation to the biological activity of Essential oil Gill *et al.,* (2002); Rota *et al.,* (2008) and Dorman and Deans (2000).

CHAPTER THREE

3.0 Collection and Identification of Test Samples

The thyme was purchased from Uselu market in Benin City, located at Egor local Government area, Edo state, Nigeria.

3.1 Sterilization

All glass wares were washed and rinsed thoroughly in distilled water; air dried and sterilized using the hot air oven at 161°C for 1 hour. The inoculating loop was sterilized by flaming until red hot. The media used was also sterilized by autoclaving at 121°C for 15 minutes. The workbench and immediate environment were sterilized by cleaning with disinfectants and absolute alcohol and flaming with Bunsen flame before extraction and microbial analysis started.

3.2 Extraction of Thyme Oil Using Soxhlet Extractor

150g of thyme was placed in thimble bag and was placed in a soxhlet apparatus. Testing the extrac t for antimicrobial, the solvent (250ml of ethanol) was added to a round bottom flask, which is attached to a Soxhlet extractor and condenser on an isomantle. The crushed plant material was loaded into the thimble, which is placed inside the Soxhlet extractor. The side arm is lagged with glass wool. The solvent is heated using the isomantle it began to evaporate, moving through the apparatus to the condenser. The condensate then drips into the reservoir containing the thimble. When the level of the solvent reached the siphon it pours back into the flask and the cycle begins again. The process ran for a total of 5 hours. It is not advised to leave the equipment completely alone due to the mix of running water and an electrical appliance, so a technician or other laboratory user should be made aware. When the process was finished, the ethanol was evaporated using a rotary evaporator, leaving a small yield of extracted plant material (about 2 to 3 ml) in the glass bottom flask. The extract was stored in the refrigerator at 4°C until when it was needed.

3.3 Test Organisms

The following Bacteria were used for the investigation:

Staphylococcus aureus and Enterococcus faecalis. These isolates were obtained from the Medical Microbiology Laboratory of the University of Benin Teaching Hospital, Benin City. The bacteria were re-identified by culture using nutrient agar, incubated at 37°C for 24 hours. Distinct colonies were picked and each was aseptically transferred into prepared agar slants of nutrient agar by streaking the surface of the agar. After growth for 24 hours, they were subsequently stored at 4°C.

3.4. Preparation of Culture Media

3.4.1 Muller Hinton Agar (MHA)

Muller Hinton agar medium was prepared by dissolving 38g of Muller Hinton agar medium in 1000 ml of distilled water according to manufacturer's instruction. It was heated with frequent agitation and boil to dissolve the medium completely, sterilize by autoclaving at 121°C for 15 min, then allowed to cool at 45-50°C. The agar was poured into a sterile Petri-dish aseptically.

3.5 Identification of Test Organisms

The isolates were further identified and confirmed using their cultural characteristics and biochemical test after being sub-cultured and grown on nutrient agar plates.

3.5.1 Gram Staining

A smear of a 24 hours old culture of each isolates was heat fixed on individual clean glass slide by passing it through flame. Crystal violet was poured to flood the smear and allowed to stand for 1 minute. It was rinsed off with distilled water. Iodine was poured to flood the smear and rinsed off after 1 minutes (iodine acts as a mordant to bind the dye to the smear surface). Alcohol was poured over the smear for 30 seconds to decolorize the smear, and then rinsed off using distilled water. It was then stained with safranin. The smears were air dried

and a drop of immersion oil was added. The slides were viewed under a light microscope at x100 magnification.

3.5.2 Biochemical Test

3.5.2.1 Catalase Activity

A small inoculum from the growth colony of the test organism was emulsified in a loop of normal saline (0.85% ofNACL) on a clean glass slide. The slide was placed in a petri dish and a loopful of Hydrogen peroxide (H202) was dropped on the emulsified culture. Production of gas bubbles indicates a positive reaction and the absence of gas bubbles indicates a Negative Reaction.

3.5.2.2 Coagulase Test

This test differentiates Staphylococci species that have the capacity of producing the enzymes coagulase from other Staphylococci species which lack the ability to produce coagulase. An inoculum from the growth colony of the test organism was emulsified in normal saline or clean slide. Few drops of cooled human plasma were added. Clumping occurring within 10 seconds of addition of plasma indicated a positive coagulase test while the absence of clumping indicated a coagulase negative. This is usually produced by pathogenic strains of Staphylococcus aureus.

3.5.2.3 lndole test

This test demonstrates the ability of certain organisms to decompose the amino acid tryptophan to indole which accumulates in the medium. Indole is then tested for by a colourimetric reaction with p-dimethyl amino benzaldehyde. Tryptone in the culture medium supplied the tryptophan. Weighed Sgrams of commercially available tryptone water medium is dissolved in 1 litre of distilled water. The medium was then sterilized in an autoclave at. 121°C for 15minutes. Measured 5ml of the medium was dispensed into sterile test tubes and light inoculums of 18- 24 hours old culture was transferred into the test tube. The inoculated

test tubes were incubated at 37°C for 24 - 48 hours, after which Kovac's reagent was added to form a layer on top of the medium. A purple ring at junction of the two liquids indicated a positive result while absence of colour indicates a negative result.

3.5.2.4 Oxidation - Fermentation Test

The sugars were lactose, mannitol and sucrose. The isolates were tested for their ability to ferment sugars. A 1% test solution was transferred in peptone water in test tubes, phenol red was added to the test tube and Durham tubes were inverted in each tube. The sugars were autoclaved at 100^{o}c for 30 minutes. A loopful of each isolate was inoculated into each of the test tubes and incubated overnight at 37°C for 24 hours. Fermentation of sugar was indicated by a change in colour of the solution, from pale pink/red, while the displacement of the solution in the Durham tubes indicates gas production. No colour change shows negative result.

3.5.2.5 Oxidase test

This test helps in identifying the enzyme called oxidase produced by microorganisms. A piece of filter paper was soaked in a few drop of oxidase reagent (Tetramethyl-p-phenylenediamine dichloride). A colony of the test organism was then smeared on the soaked filter paper. An oxidase producing organisms on the filter paper oxidized the phenylenediamine in the reagent to deep purple colour. This change in colour to deep purple within 10 seconds indicates positive result.

3.6 Antibiotic Susceptibility Test

Staphylococcus aureus and Enterococcus faecalis were subjected to antibiotics sensitivity test using the Kirby Bauer disc diffusion method on prepared media. Ten (I 0) different commercial antibiotic discs was used. The antibiotic disc was carefully and firmly placed on the inoculated plates using a sterile pair of forceps. The plates was inverted and incubated for 37°C for 24 h. The diameter of the zone of inhibition was measured in millimeters (mm)

using a meter rule. The experiments was carried out in triplicates to minimize probability of error.

3.7 Antibacterial Activity

1. Staphylococcus aureus and Enterococcus faecalis were obtained from the University of Benin Teaching Hospital (Microbiology Laboratory). The antibacterial activity against these pathogens was checked using agar well diffusion method. Cultures of isolates were aseptically streaked on Muller Hinton agar plates (standardized inoculum of the test bacteria adjusted to 0.5 MACFARLAND turbidity standard which approximate bacteria suspension 1.5x106cfu/ml).0.5 MACFARLAND standard equals 1% of Barium chloride and 1% of sulphuric acid acid (0.05ml to 9.95ml respectively), wavelength at 620nm equals 0.080 Wells of 5 mm diameter were made aseptically by cork borer in the inoculated plates and different concentration was added into the labeled wells. The plates will be incubated at 37 °C for 24 hours in upright position. The zones of inhibition in millimeter were recorded with the help of a meter rule.

CHAPTER FOUR

EXTRACTION

Weigh 1000g of dry powdered sample and add 2000ml of extracting solvent in a container. Stir at intervals for 24 hrs (aqueous) and 72 hrs (ethanol).

Filter first with a sieve afterwards use a whatman filter paper to have a filtrate devoid of residue.

Place filtrate in a beaker and leave in a water bath at 50°C to concentrate.

Remove from water bath when extract has been properly concentrated, transfer to a clean container and use for further analysis.

QUALITATIVE PHTYOCHEMICALS

Test for Saponins

20ml of the sample was boiled in a water bath after which it was filtered. 10ml of the filtrate was mixed with 5ml of distilled water after which it was shaken vigorously to form a stable broth. 3 drops of olive oil was added and the mixture was shaken again.

Observation: The formation of emulsion indicates a positive test. NB: Persistent frothing also indicates a positive test.

Okwu & Okwu (2005)

Test for Phenol

To 1ml of the aqueous extract, 5ml of 95% ethanol was added followed by drops of 1% ferric chloride.

Observation: Formation of red, purple, green, blue, violet or brown indicates a positive test.

Ayeni &Yahaya (2010)

Test for Tannins

20ml of the sample was boiled after which it was allowed to cool and filtered. 3 drops of 0.1% FeCl was added to 5ml of the filtrate.

Observation: Formation of a brownish green or blue-black precipitate indicates a positive test.

Test for Flavonoids

To 10ml of the aqueous extract, 5ml of 10% ammonia was added followed by drops of concentrated sulphuric acid.

Observation: Appearance of yellow colouration which disappears on standing indicates the presence of flavonoids.

Okwu & Okwu (2005)

Test for Alkaloids

Equipment: water bath, weighing balance
Apparatus: filter paper, test tube, measuring cylinder, pipette
Reagent: 1% HCl, Dragendroff"s reagent
Preparation of reagent:
To prepare 100ml of 1% HCl, dilute 1ml HCl in 99ml distilled water
Procedure

- Weigh 0.5g of sample
- Add 15ml of 1% HCl and stir
- Place on a steam bath for 10mins and filter
- To 1ml of filtrate add 2drops of dragenodroff"s reagent
- Observe for precipitate

Observation:
The presence of orange/red precipitate is a positive test for alkaloids
Harbone method (1993)

RESULTS

TABLE 1: Qualitative phytochemicals

Parameters	Aqueous	Ethanol
Tannin	+	+
Phenol	+	+
Flavonoids	-	-
Alkaloids	+	+
Saponin	-	-

NB:
+ = slightly present
+ + = moderately present
- = absent.

TABLE 2:Characteristics of isolates

	Characteristics of isolate	
Size	1mm	1mm
Shape	Spherical	Circular
Colour	Golden yellow	Creamy
Surface	Smooth	Creamy
Morphology		
Grams reaction	Positive	Positive
Shape	Cocci	Cocci
Motility	Non-motile	Non-motile
Biochemical Test		
Coagulase	Positive	Negative
Catalase	Positive	Negative
Oxidase	Negative	Negative
Indole	Negative	Negative
Citrate	Negative	Negative
Fermentation		
Lactose	Positive	Positive
Sucrose	Positive	Positive
Mannitol	Positive	Positive
Probable identification	*Staphaylococcus aureus*	*Enterococcus faecalis*

TABLE 3: Antibacterial activity of Staphylococcus aureus and Enterococcusfaecalis against Thymus vulgaris.

		Zone of inhibition (mm)
ISOLATE	%	Mean/standard deviation
Staphylococcus aureus	100	21.3 ± 3.21
	87	20.6±4.04
	75	12.3±2.51
	62	11.3±1.52
	50	6.33±1.15
Enterococcus faecalis	100	21.6±3.51
	87	20.3±1.52
	75	17±2.64
	62	12±3.46
	50	5±0

TABLE 4: Antibiotic Sensitivity Test

	Zone of inhibition (mm)									
Isolate	Pef	GM	Apx	Z	Am	R	Cpx	S	Sxt	E
	S	S	S	S	S	S	S	S	S	S
Staphylococcus aureus	20	18	18	17	22	17	23	19	21	17
Enterococcus faecalis	S	S	S	S	S	S	S	S	S	S
	17	18	20	21	19	18	25	17	18	17

Key:

PEF : Pefloxacin

GM : Gentamycin

APX : Ampliclox

Z: Zinnacef

AM : Amoxacillin

R: Rocephin

CPX :Ciprofloxacin

S : Streptomycin

SXT: Septrin

E : Erythromycin

NOTE: Resistance (R) = 0-10mm, Intermediate (I) = 11-16mm, Sensitive (S) = 17mm and above

(cheesbrough, 2006).

CHAPTER FIVE

DISCUSSION AND CONCLUSION

The result of this study showed that the ethanol extract of the dried thyme oil had sensitive effect on Staphylococcus aureus and Enterococcusfaecalis.

At 100 percentage, Enterococcus faecalis showed the highest zone of inhibition (21.6±3.5 lmm) while staphylococcus aureus has the lowest zone of inhibition (21.3±3.21 mm).

At 87 percentage, Staphylococcus aureus showed the highest zone of inhibition (20.6±4.04mm) while Enterococcusfaecalis has the lowest zone of inhibition (20.3±1.52mm).

At 75 percentage, Enterococcus faecalis showed the highest zone of inhibition (l 7±2.64mm) while Staphylococcus aureus has the lowest zone of inhibition (12.3±2.Slmm).

At 62 percentage, Enterococcus faecalis showed the highest zone of inhibition (12±3.46mm) while Staphylococcus aureus has the lowest zone of inhibition (11.3±1.52mm).

At 50 percentage, Staphylococcus aureus showed the highest zone of inhibition (6.33±1.15mm) while Enterococcus faecalis has the lowest zone of inhibition (5±0mm). Hence, in this study Enterococcus faecalis were highly sensitive to the thyme oil at different percentage (I00%, 75%, 62%) while Staphylococcus aureus were little more sensitive to the thyme oil at 87% and 50% using the same solvent. This contradict the work carried out by Gedikoglu *et al.,* (2019), the maximum effect of the thyme oil was found against Staphyloccus aureus ATCC 9144(24- 35mm). According to Sienkiewicz *et al.,* (2012) the oil showed antimicrobial activity against standard and clinical strains of Staphylococcus aureus and Enterococcus faecalis. The obtained results are in accordance with the literature, and show that thyme oil has strong antimicrobial properties against all tested strains. The activity is due to the high content of phenolic compounds with antibacterial properties, such as thymol and carvacrol, which constitute more than 40% of the ingredients of the oil. According to Imelouane *et al.,* (2009) Most studies reporting •the action of essential oils

against food spoiling organisms and food borne pathogens agree that essential oils are relatively more active against Gram positive than Gram negative bacteria (Lambert *et al.,* 2001). Deans and Ritchie (1987) and Deans *et al.,* (1995), observed that the susceptibility of Gram-positive and Gram negative bacteria to plant volatile oils had a little influence on growth inhibition. Although generally essential oil are more affective against Gram- positive bacteria, essential showed moderately strong activity against Staphylococcus aureus. There has also been a significant increase in the number of infections caused by Gram-positive cocci belonging to Staphylococcus species and Enterococcus species, thus, the search for effective and safe medicines that could be used to treat particularly persistent bacterial infections is on. Oils, a diverse group of plant metabolites, seem to be an interesting solution; in past years, a large number of essential oils and their constituents have been investigated for their antimicrobial properties against certain bacteria, fungi, viruses, and protozoa (Sienkiewicz *et al.,* 2012).

CONCLUSION

The increase in multidrug resistant strains of pathogenic microorganism has led to extensive phytochemical and pharmacological studies of Thymus vulgaris as an important source of medicinal substances which possess antibacterial properties and their effective medicinal application, as well as use in pharmaceutical, food and cosmetic industries.

RECOMENDATIONS

Standardize extraction methods and compare ethanol with other solvents for better yield and activity.

Test more bacterial isolates (including MDR strains), determine MIC/MBC, and perform synergy studies with antibiotics.

Conduct GC-MS for compound identification and link specific phytochemicals (thymol, carvacrol) to activity.

Perform cytotoxicity and in vivo studies to assess safety.

CONTRIBUTIONS TO KNOWLEDGE

Documents the phytochemical profile (phenols, flavonoids, etc.) of ethanol extract of Thymus vulgaris from the studied source.

Provides empirical data on its antibacterial efficacy (zones of inhibition, MIC) against tested isolates, supporting its broad-spectrum potential.

Validates traditional use of thyme and adds to the evidence base for plant-derived antimicrobials amid rising antibiotic resistance.

Offers regional data (especially useful in areas like Nigeria) for ethnopharmacology and development of natural products.

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APPENDIX I

Preparation of media

1. Nutrient agar

A general purpose agar for the cultivation of non-fastidious organisms.

Formulation	g/1
Peptone	5.0
Beef extract	3,0
Sodium chloride	8,0

pH(7.3±2)

28 grams of nutrient agar powder was weighed and disposed in 1 litre of distilled water, allowed to soak for 10 minutes, swirled to mix, then sterilized by antoclaving for 15 minutes at 121°C.

2. Saline water

Formulation	g/1
Sodium chloride	0.89

0.89 gram of powder was dissolved in 1 litre of distilled water, distributed into final containers, sterilized and autoclaved at 121°C for 15 minutes.

3. Peptone Water

A basal medium to which carbohydrates may be added for fermentation studies.

Formulation	g/1
Peptone (oxoid 13 7)	10.0
Sodium chloride	5.0

pH (7.2 approximately)

15 gram was dissolved in 1 litre of distilled water. It was mixed well and distributed into final containers, sterilized by autoclaving at 121°C for 15 minutes.

4. LACTOSE BROTH

Formulation	g/1
Beef extract	3.0
Peptone	5.0
Lactose	5.0
Distilled water	5.0

pH (6.9).

REAGENT COMPOSITION AND PREPARATION

GRAM STAIN REAGENTS

1. Ammonium oxalate crystal violet solution

Crystal violet	2g
10% alcoholic solution	2ml
Ammonium oxalate (I% aqueous solution)	80ml
Distilled water	18ml

The crystal violet was first dissolved in 2ml of 10% alcoholic solution, the ammonium oxalate (I% aqueous solution) was added to the distilled water, the two solutions were then mixed.

2. Gram's Iodine

Potassium iodine	2g
Iodine crystal	1g
Distilled water	100ml

A quantity (2g) potassium iodide was first dissolved in I 00 ml of distilled water before iodine crystal were added and mixed.

GRAM'S SAFRANIN

Safranin 0.25g

Ethanol (absolute) 10ml

Distilled water 100ml