Sudan University of Science and Technology

College of Graduate Studies

Antimicrobial and Antioxidant Activity of *Rosmarinus officinalis* L. Flowers Pods and *Thymus vulgaris* L. Leaves Ethanolic Extract on *Escherichia coli* Urinary Isolates

فعالية النشاط المضاد للميكروبات والمضاد للاكسدة للمستخلص الايثانولي لبراعم زهور اكليل الجبل واوراق الزعتر على عزلات الايشريكية القولونية من البول

Dissertation Submitted for the Partial Fulfillment of the Requirement of M.sc Degree in Microbiology

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الآيـة

بسم الله الرحمن الرحيم

قال تعالى:

((قُلْ هَلْ يَسْتَوِي الَّذِينَ يَعْلَمُونَ وَالَّذِينَ لا يَعْلَمُونَ إِنَّمَا يَتَذَكَّرُ أُوْلُوا الأَلْبَابِ))

صدق الله العظيم

(سورة الزمرالآية 9)

DEDICATION

To my great parents.

To my brothers and sister

To all my family and my friends

Our home land Sudan

The Sudan University of Science & Technology

I dedicate this research.

ACKNOWLEDGEMENTS

In the name of Allah, the most Merciful, all praise is to Allah, the lord of the World. First and foremost, I must acknowledge my limitless thanks to Allah, the Ever-magnificent, the Ever - thankful, for His help and bless. I have to thank my Parents for giving me strength to reach for the stars and chase my dreams. I'm grateful to all people, who worked hard with me from the beginning till the completion of the present research particular my supervisor **Dr.Hind Haidar** for the guidance and support thought this study.

Last but not least, deepest thanks go to all people who took part in making this thesis real especially **Dr.Ayat Ahmed** who truly helped me.

ABSTRACT

The indiscriminate use of antibacterial agents has led to one of the largest recent global health problems which is the emergence of bacterial resistance. The present study was aimed to investigate the antimicrobial and antioxidant activity of ethanolic extracts of the two medicinal plants; *Rosmarinus officinalis* L. and *Thymus vulgaris* L.on *Escherichia coli (E.coli*).

Antibiotic susceptibility test was applied against *E.coli* urinary isolates using Chloramphenicol, Gentamycin, Amoxicillin , Ceftriaxone and Ciprofloxacin. According to the result, 88.7% of isolates were sensitive to Chloramphenicol, 39.6% were sensitive to Ceftriaxone, 87% were sensitive to Gentamycin , 0% were sensitive to Amoxycillin and 71.7% were sensitive to Ciprofloxacin and there was 7(13%) *E.coli* isolates which were multi drug resistant (MDR).

Plant extraction and *E.coli* suspension were prepared. The ethanolic extracts of *R. officinalis* and *T. vulgaris* were examined with concentrations of 100, 50, 25and 12.5 mg/ml against 53 isolates of *Escherichia coli*. The antioxidant activity through the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) method and chemical analysis of both extracts by Gas Chromatography Mass Spectrometry (GC/MS) was identified.

The inhibitory zone of *R. officinalis* was found between 8-23mm and for *T. vulgaris* was between (8-20mm), For *R. officinalis* extract 100mg/ml the inhibition zone was between 9-23mm for 91% of *E.coli* with mean (16±7 mm), 50mg/ml inhibition zone was between (8-19mm) for 64% of *E.coli* with mean (13.5±6 mm), 25mg/ml inhibition zone was between (8-12mm) for 34% of *E.coli* with mean (10± 2 mm) and 12.5mg/ml inhibition zone was between (0-8mm) for 2% with mean (4±4 mm).

T. vulgaris extract 100mg/ml showed inhibition zone between (8-20mm) for 94% of *E.coli* with mean (14±6 mm), 50mg/ml inhibition zone was between (8-15mm) for 77% of *E.coli* with mean (11.5±3.5 mm), 25mg/ml inhibition zone was between (8-12mm) for 54% of *E.coli* with mean (10± 2 mm) and

12.5mg/ml inhibition zone was between (8-12mm) for 28% of *E.coli* with mean (10±2 mm). The MIC of of *R. officinalis* and *T. vulgaris* against (MDR) isolates between 12.5 to 50 mg/ml and the MBC between 25to 100 mg/ml.

Also both extracts showed high antioxidant activity, the DPPH radical scavenging potential of *T. vulgaris* was 83.09% followed by *R. officinalis* (81.26 %).

The GC/MS analysis of both plants revealed presence of different chemical constituents which have different biological activities. The chemical analysis of *R. officinalis* showed the most abundant compounds were, Eeucalyptol, Bicycloheptan and Octahydrodibenz anthracene, for *T. vulgaris* the most abundant compounds were Thymol**,** phytol and Hexadecanoic acid.

In conclusion, *Rosmarinus officinalis* and *Thymus vulgaris* ethanolic extracts have antimicrobial properties and antioxidant activity *(T. vulgaris* showed higher antioxidant activity than *R. officinalis*) which might justify the use of those herbs in traditional medicine.

الخلاصـــه

أدى الاستخدام العشوائي للمضادات البكتيريه الى واحده من اكبر المشاكل الصحيه العالميه الاخيره وهي ظهور مقاومه جرثوميه .

هدفت هذه الدراسه للكشف عن النشاط المضاد للبكتيريا والنشاط المضاد للاكسده لمستخلص الايثانول لكل من نباتي براعم زهرة اكليل الجبل واوراق الزعتر ضد بكتيريا الايشريكيه القولونيه. وتم اجراء اختبار الحساسيه للمضادات الحيويه الكلورامفينيكول ,الجينتاميسين, الاموكسيسيلين ,السيفترياكسون والسيبروفلوكساسين على53 عزله ايشريكيه قولونيه. تبعا لهذه الدراسه اظهرت 88%. 7 حساسيه للكلورامفينيكول. 6.39% اظهرو حساسيه للسيفترياكسون في حين ان18.9% اظهرو حساسيه متوسطه. واظهر87% حساسيه للجينتاميسين. واظهرت كل العزلات مقاومه للاموكسيسيلين. اما السيبروفلوكساسين فقد اظهرت 71.7% حساسيه له. وقد هنالك 7 عزلات اظهرو مقاومه لاكثر من 3 مضادات حيويه وكان اقل تركيز كافي لتثبيطهم بالمستخلص الايثانولي لكل من اوراق الزعتر وازهار اكليل الجبل هو بين 12.5 ال 50 مجم/ مل اما اقل تركيز لقتل البكتيريا بين 50 الى 100 مجم/ مل.

في هذه الدراسه تم تحضير مستخلص النبات و محلول الايشريكيه القولونيه وتم اختبار المستخلص الايثانولي لكل من الزعتر واكليل الجبل على 53 عزله ايشريكيه قولونيه بواسطة تقنية نشر القرص حيث تم استعمال التراكيز 100مجم/ مل و50 مجم/ مل و 25مجم/ مل و5. 12مجم/ مل لكل من النباتين لتحديد الخاصيه المضاده للايشريكه القولونيه وتم تحيد اقل تركيز كافي لتثبيط وقتل الايشريكيه القولونيه بواسطة التخيفيف .وتم حديد فعاليتهم المضاده للاكسده بواسطة طريقة DPPH. وتم عمل تحليل كيميائي لكل من اوراق الزعتر وزهرة اكليل الجبل بواسطة طريقة الاستشراب الغازي مطياف الكتله GC/MS.

اظهرت النتائج ان المستخلص الايثانولي لكل من الزعتر واكليل الجبل لهم نشاط مضاد ضد الاشريكيه القولونيه فكان اقل تركيز كافي لتثبيط الايشريكيه القولونيه لمستخلص اكليل الجبل هو 12.5مجم/ مل لعزله واحده اما البقيه فكان اقل تركيز كافي لتثبيط الايشريكيه القولونيه هو 25مجم/ مل اما بالنسبه لنبات الزعتر فكان اقل تركيز كافي لتثبيط عدد من العزلات هو 12.5مجم/ مل ووجد ان منطقة التثبيط التي اظهرها مستخلص اكليل الجبل تتراوح بين 8 الى 23ملم اما مستخلص الزعتر اظهر منطقة تثبيط من (8الى 20ملم).مستخلص اكليل الجبل بتركيز 100 مجم/مل اظهر منطقة تثبيط بين9 ال 23ملم في (91%)ومتوسط منطقة التثبيط كان (16 ± 7 ملم) وتركيز 50 اظهر منطقة تثبيط بين (8الى 19 ملم) في (64%)ومتوسط منطقة التثبيط

(13.5± 5.5 ملم) وتركيز25اظهر منطقة تثبيط بين (8 الى12 ملم) في (34%)ومتوسط منطقة التثبيط (10±2)وتركيز5. 12 مجم/ مل اظهر منطقة تثبيط بين(0 الى 8ملم) في (2%) ومتوسط منطقة التثبيط (4± 4 ملم). وكان اقل تركيز كافي لتثبيط العزلات المقاومه لاكثر من 3 مضادات حيويه بالمستخلص الايثانولي لكل من اوراق الزعتر وازهار اكليل الجبل هو بين 12.5 ال 50 مجم/ مل اما اقل تركيز لقتل البكتيريا بين 50 الى 100 مجم/ مل.

ايضا اظهر كلا المستخلصين خاصيه عاليه مضاده للاكسده حيث كانت القوه المضاده للاكسده لمستخلص اكليل الجبل (26%, 81) واوراق الزعتر (09%, 83).اما التحليل الكيميائي بطريقة الاستشراب الغازي مطياف الكتله GC/MS فقد اظهر ان مستخلص اكليل الجبل تحتوي على; ايوكالبتول,ثنائي سايكلوهيبتان و أوكتاهايدرو ثنائي بينزا انثراسين. ومستخلص اوراق الزعتر يحتوي على; ثايمول, فايتول وحمض الهكساديكانويك. خلصت هذه الدراسه ان المستخلص الايثانولي لكل من اوراق الزعتر و براعم زهرة اكليل الجبل لها خواص مضاده للبكتيريا الاشريكيه القولونيه ومضادات للاكسدة (اوراق الزعتر تحوي خاصيه مضاد للاكسده اكثرمن مستخلص اكليل الجبل) مما يفسر استعمالهم في الطب التقليدي.

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1	Abs	Absorbance
2	C.F.U	Colony forming unit
3	DPPH	2.2 Di (4-tert-octylphenyl)-1-picryl-hydrazyl
4	DMSO	Dimethyl sulfoxide
5	*E.coli*	*Escherichia coli*
6	GC/MS	Gas chromatography mass spectrometry
7	MIC	Minimum Inhibitory Concentration
8	MBC	Minimum Bactericidal Concentration
9	NICE	National Institute for Health and Clinical Excellence
10	NIST	The National Institute of Standards and Technology
11	*R. officinalis*	*Rosmarinus officinalis* L.
12	SD	Standard deviation
13	*T. vulgaris*	*Thymus vulgaris* L.
14	UTIs	Urinary tract infections
15	WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

CHAPTER ONE
INTRODUCTION

1.1. Introduction

Infectious diseases according to WHO are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; the diseases can be spread, directly or indirectly, from one person to another. Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span (Tripathi *et al*., 2019).

Escherichia coli considered as major public health problem and predominant cause of UTI (Chittò *et al*., 2019). For centuries, plants have been used for a wide variety of purposes including treating infectious diseases (Kuruppu *et al*., 2019). Aromatic plants and spices have great importance for pharmaceutical industries, cosmetics and food. According to the World Health Organization, approximately 80% of the world population, mostly in developing countries, still relies on medicinal plants and their extracts for primary health care (Sakkas and Papadopoulou ., 2016).Natural extracts of the plants, have been studied because of its bioactive properties. In recent years plant extracts have been developed and used in foods as antioxidants (David *et al*., 2019).

According to WHO medicinal plants would be the best source to obtain a variety of drugs (Al Akeel *et al*., 2014). Pharmacological industries have produced a number of new antibiotics in the last three decades; resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains, which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high

mortality (Talia *et al*., 2011).The interest towards the utilization of crude extracts of herbs and other plant materials rich in phenolics increased in the food industry because they retard oxidative degradation of lipids thereby improving the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers (Kumuda *et al*., 2017). The increased use of antibiotics lead to the incidence of resistant strains (Nieto *et al*., 2018). The world faced with lack of new antimicrobial drugs that have fewer side effects than antibiotics. Resistance in bacteria has created a major problem (Golshani and Sharifzadeh., 2014).

Numerous studies on the pharmacology of medicinal plants have been accomplished, since they constitute a potential source for the production of new medicines and may enhance the effects of conventional antimicrobials, which will probably decrease costs and improve the treatment quality (Barraviera., 2007).

The antimicrobial activity of plants was proven by various examples, in the form of both essential oils and extracts. Thus, this property can be a promising ally in the development of medicines necessary to combat the increasing number of bacterial strains that become resistant to conventional antibiotics (Barraviera., 2007).

R. officinalis which has long been known as a spice and medicinal herb belongs to the Lamiaceae family and receives an increasing attention due to its antimicrobial, anti-inflammatory and antioxidative constituents (Rashid *et al*., 2011) and *T. vulgaris* which belong to the Lamiaceae family, have been widely used for their organoleptic and medicinal properties and they possess a variety of anti-inflammatory, antioxidant, antibacterial, and antifungal activities (Lagouri *et al*., 2011).

1.2. Rationale

Synthetic chemicals are widely used against microorganisms; unfortunately they develop resistance to many antibiotics due to the indiscriminate use of commercial antibiotics. In addition, these antibiotics sometimes cause allergic reactions and immune suppression. Therefore, the use of plant extracts is safer to the human health and environment. *R. officinalis* and *T. vulgaris* are medicinal plants that have many uses in traditional medicine and reported many times for their antimicrobial and antioxidant activity. Both are safe and use in industries in many products also used as food preservatives (Golshani and Sharifzadeh.,2014).

Herbal subjects of the study are commonly used and considered as safe, but only as information, no scientific evidence before, and it wasn't searched locally. Therefore an attempt to conduct such study was considered (Golshani and Sharifzadeh.,2014).

This research was performed to study the *Rosmarinus officinalis* L. Flowers pod and *Thymus vulgaris* L. Leaves ethanolic extract as there was no available information about antimicrobial and antioxidant Activity of *Rosmarinus officinalis* L. Flowers pod and *Thymus vulgaris* L. Leaves ethanolic extract on *Escherichia coli* Urinary isolates in sudan.

1.3. Objectives of the study

1.3.1. General Objective

To study antimicrobial and antioxidant activity of *Rosmarinus officinalis* L. Flowers pod and *Thymus vulgaris* L. Leaves ethanolic extract on *Escherichia coli* urinary isolates.

1.3.2. Specific Objective

1. To reidentify *Escherichia coli* urinary isolates by Gram's stain and conventional biochemical tests.
2. To assess antimicrobial activity of *Rosmarinus officinalis* L. and *Thymus vulgaris* L. Ethanolic extracts against *Escherichia coli* urinary isolates using disc diffusion method.
3. To perform the antibiotic susceptibility testing and to detect Multi drug resistant (MDR) *E.coli* isolates.
4. To measure minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Rosmarinus officinalis* L. and *Thymus vulgaris* L. Ethanolic extracts against *Escherichia coli* urinary isolates using broth dilution method.
5. To measure antioxidant activity of *Rosmarinus officinalis* L. and *Thymus vulgaris* L. using 2.2 Di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical method.
6. To identify the chemical constituents of plants extracts using Gas chromatography mass spectrometry (GC/MS) analysis.

CHAPTER TWO
LITERATURE REVIEW

CHAPTER TWO
LITERATURE REVIEW

2. Literature review

2.1. Medicinal plants

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value and the Lamiacae family, or mint family, is a family of herbs, shrubs and trees comprising about 200 genera and 3200 species, many with a long history of medicinal and food use (Raja., 2012).

One of the prerequisites for the success of primary health care is the availability and use of suitable drugs. Plants have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. It is thus reasonable for decision-makers to identify locally available plants or plant extracts that could usefully be added to the national list of drugs, or that could even replace some pharmaceutical preparations that need to be purchased and imported (Zahra *et al*., 2020).

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and are used for extract as raw drugs and they possess varied medicinal properties. The different parts used include root, stem, flower, fruit, twigs exudates and modified plant organs (Mahesh and Satish., 2008).

2.1.1. *Rosmarinus officinalis* L.

2.1.1.1. Taxonomical classification

Kingdom: Plantae, Order: Lamiales, Family: Lamiacea, Genus: *Rosmarinus* Species: *officinalis* L. (Quattrocchi., 2016).

2.1.1.2. Botanical description

Rosemary is herbal plant with green, picked and fragrant leaves. grows naturally on dry rocky slopes and hillsides or in pine forests and is used fresh, dried or as an essential oil (Tural and Turhan .,2017).

2.1.1.3. Antioxidant activity

There is an increasing interest in phytochemicals as new sources of natural antioxidant and antimicrobial agents. The popular *R. officinalis* plant is a native to the Mediterranean region; however, it has been cultivated throughout world and accepted as one of the spices with highest antioxidant activity (Genena *et al*., 2008).

2.1.1.4. Uses

R. officinalis extracts show biological bioactivities such as hepatoprotective, antifungal, insecticide, antioxidant and antibacterial. It is well known that the biological properties in rosemary are mainly due to phenolic, compounds (Del Campo *et al*., 2010). Sedative, carminative, sudorific, cardiac stimulant, anti-inflammatory, anti-rheumatic, diuretic, digestive, antiseptic, and anti-spasmodic (Quattrocchi.,2016). Also used as employed in perfumes, in scenting soaps, detergents, household sprays. In traditional medicine, rosemary is thought to fortify the brain and refresh the memory (Habtemariam., 2016). Flowering tops and leaves are considered carminative, diaphoretic, diuretic, aperient, emmenagogue, stimulant, stomachic and astringent. *R. officinalis* also serves as a household remedy for headaches, bruises, colds, nervous tension, asthma, baldness and sore throat. In the Philippines, an infusion of the leaves is used as an eye wash for slight catarrhal conjunctivitis, as vapor baths for rheumatism, paralysis and incipient catarrhs, and to bathe women in puerperal state. *R. officinalis* leaves are therapeutically allowed internally for dyspeptic complaints, and externally for rheumatic diseases and circulatory problems. Rosemary is very popular as an ornamental plant used as a ground cover, hedge or shrub and is even transformed by hobbyists into bonsai or planted in hanging baskets. The leaves and flowers can be carefully dried and sold in elegant sachets and potpourris (Uritu *et al*., 2018).

2.1.1.5. Chemical constituents

The polyphenolic profile of these plants is characterized by the presence of carnosic acid, carnosol, rosmarinic acid and hesperidin, as major components among the most effective antioxidant constituents of rosemary, the cyclic diterpenediphenols,

carnosolic acid and carnosol have been identified. In addition, its extract contains carnosic acid, epirosmanol, rosmanol, methylcarnosate and isorosmano. High percent of the antimicrobial activity attributed to carnosic acid and carnosol, also contain several antioxidant components (Nieto *et al*., 2018).

The extract of rosemary also contains other caffeic acid derivates. These compounds react with present metal ions, so chelates are formed; they consequently react with peroxide radicals and in that way, stabilise these free radicals. Essential oil of this herb which exists in 1-2% it consists of borneole, limonene, camphene, camphor and other compounds like phenolic acid including rosmarinic acid, caffeic acid and chlorogenic acid (Nieto *et al*., 2018). The inhibitory effect of *R. officinalis* is the result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol (Jawad *et al*., 2018).

2.1.1.6. Antimicrobial mechanism

They interact with the cell membrane, causing changes in genetic material and nutrients, altering the transport of electrons, leakage of cellular components and production changes in fatty acid. In addition, it also produced an interaction with the membrane of proteins that produced the loss of membrane functionality and its (Vegara *et al*., 2011).

2.1.2. *Thymus vulgaris L*.

2.1.2.1. Taxonomical classification

Kingdom: Plantae, Class: Magnoliophyta, Order: Lamiales, Family:Lamiaceae

Genus: *Thymus*, Species: *vulgaris* L. (Quattrocchi., 2016).

2.1.2.2. Distribution

Thymus vulgaris is a member of Lamiaceae family, which distributes in areas of Mediterranean, Asia and is cultivated in all over the world. *Thymus vulgaris* L. was widely used in folk medicine in the treatments of variety of diseases such as gastroenteric and bronchopulmonary disorders (Dauqan and Abdullah., 2017).

2.1.2.3. Chemical constituents

It is packed with minerals and vitamins that are essential for optimum health. Its leaves are one of the richest sources of potassium, iron, calcium, manganese, magnesium and selenium (Sharangi and Guha., 2013).

The herb is also a rich source of many important vitamins such as B-complex, folic acid, beta carotene, vitamin A, K, E and C. Thyme provides 0.35 mg of vitamin B-6 or pyridoxine; furnishing about 27% of daily recommended intake. Pyridoxine keeps up gamma-aminobutyric acid (GABA) (beneficial neurotransmitter in the brain) levels in the brain (Sharangi and Guha., 2013).

2.1.2.4.Antimicrobial activity

Thymol and other phenolic components inhibit microorganisms causing an increase in the permeability of the cell membrane and reduction of vital intracellular substances or disruption bacterial enzyme systems (Khameneh *et al*., 2019).

Its structure disintegrates the external membrane of gram-negative bacteria, releasing lipo polysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP (Silva and Fernandes., 2010).

2.1.2.5. Antioxidant activity

Fresh *T. vulgaris* herb has one of the highest antioxidant levels among herbs Fresh Thyme has the highest level of antioxidants among all herbs attributed to the presence of phenolic monoterpenes, thymol and carvacrol (Dauqan and Abdullah., 2017). Thymol are the main phenolic components that are primarily responsible for its antioxidative activity (Komaki *et al*., 2016)**.**

2.1.2.6. Uses

T. vulgaris present a wide range of functional possibilities in pharmacy, food, and cosmetic industry. The interest in the formulation of pharmaceuticals, nutraceuticals, and cosmeceuticals based on thymol is due to several studies that have evaluated the potential therapeutic uses of this compound for the treatment of disorders affecting the respiratory, nervous, and cardiovascular systems (Salehi *et al.,* 2018).

Moreover, this compound also exhibit antimicrobial, anticarcinogenesis, antioxidant, anti-inflammatory, and antispasmodic activities, as well as a potential as a growth enhancer and immunomodulator (Salehi *et al*., 2018).

2.2. Urinary tract infection

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span (Chittò *et al*., 2019). Urinary tract infection is a term applied to a variety of clinical conditions ranging from asymptomatic presence of bacteria in the urine tosevere infection of the kidney with sepsis (Kumar *et al*., 2016). UTI is also defined as the growth of a known bacterial pathogen more than 10^5cfu/ml in association with a positive dipstick or urinalysis Kline and Lewis., 2012).

2.2.1. Biology of urinary tract

Urinary tract infection affects the parts of the urinary tract which includes the upper and lower urinary tract and the occurrence is high in females due to their reproductive anatomy (Vasudevan., 2014).

The whole urinary system comprising of the parts of the urinary tract is at risk as the infection can affect any part of the urinary tract. Depicts the urinary system comprising of the various parts of the urinary tract including the renal artery and vein, kidneys, bladder, ureter, urethra and provision for urine exit. Kidneys are the organs of at most significance and are known to perform crucial regulatory functions. These acts as innate filters and play a vital role in removing the unwanted water soluble waste from the blood and also enables the re absorption of essential ingredients like water, glucose and amino acids. Kidneys are known for the production of urine which is diverted to the urinary bladder by means of thin tubular structure known as ureter. The urinary bladder is a muscular flexible organ which accumulates the urine collected from the kidneys before they are disposed (Vasudevan , 2014).

The collected water soluble waste in the form of urine is then flushed out from the genitals by means of urethra which connects the urinary bladder and genitals. This

process of production of urine and its disposal is systematic and urinary tract infection greatly influences this process and may result in a variety of symptoms which the patient experiences during the process of contagion. In simple terms urinary tract infection is a consequence of invasion of the urinary tract by infectious organisms resulting in bladder infection and untreated condition leads to renal defects damaging the kidneys. The organized operation of the urinary system relies on the lower and upper tracts which are known to perform diverse regulatory tasks ranging from the production of the urine till their disposal. Entry of an infectious pathogen in the urinary tract causes the infection which usually occurs through the urethra. This is one of the prime reasons for higher incidence among women than men due to the shorter length of urethra in women which makes them vulnerable to such infections. Since the urethra is shorter in women (about 1.5 to 2 inches) when compared to men (8 inches), they are more prone to infections associated with the urinary tract. The shorter length of the urethra in women enhances the scope for the pathogen to invade the bladder resulting in bladder infection (Vasudevan., 2014)

2.2.2. Definition of Urinary tract infection (UTI)

Urinary tract infection (UTI) is a term applied to a variety of clinical conditions ranging from asymptomatic presence of bacteria in the urine to severe infection of the kidney with resultant sepsis (Najar *et al*., 2009).

Urinary tract infection is defined also as the growth of a known bacterial pathogen more than100000 cfu/ml in association with a positive dipstick or urinalysis (Kline and Lewis., 2012).

According to The National Institute for Health and Clinical Excellence (NICE) guidelines, urinary tract infection is defined by a combination of clinical features and the presence of bacteria in urine (NCCLS., 2007).

2.2.3 Classification of UTI

It is understood that the infection targets the different parts of the urinary tract and as a consequence results in the contagion of the lower and the upper urinary tracts. The infection is named based on the site of infection. The infection of urethra and

ureter are referred to as urethritis and ureteritis respectively where as cystitis and phylonephritis corresponds to bladder and kidney infections (Vasudevan., 2014).

Cystitis is a common type of infection where as the infection associated with the renal damage is an issue of serious concern. Therefore the infection of bladder and urethraare referred as the infection of the lower urinary tract whereas the kidney and ureter infection is an indication of upper tract infection. Generally UTIs are classified based on the factors that trigger the infection and the nature of occurrence. Taking these aspects in to consideration, UTIs can be classified to uncomplicated or complicated (based on the factor that triggers the infection) and Primary or recurrent (depending on the nature of occurrence) (Vasudevan., 2014).

2.2.4. Pathogenesis

Uncomplicated urinary tract infections (UTIs) begin when uropathogens that reside in the gut contaminate the periurethral area and are able to colonize the urethra. Subsequent migration to the bladder and expression of pili and adhesins results in colonization and invasion of the superficial umbrella cells. Host inflammatory responses, including neutrophil infiltration, begin to clear extracellular bacteria. Some bacteria evade the immune system, either through host cell invasion or through morphological changes that result in resistance to neutrophils, and these bacteria undergo multiplication and biofilm formation. These bacteria produce toxins and proteases that induce host cell damage, releasing essential nutrients that promote bacterial survival and ascension to the kidneys. Kidney colonization results in bacterial toxin production and host tissue damage. If left untreated, UTIs can ultimately progress to bacteraemia if the pathogen crosses the tubular epithelial barrier in the kidneys (Flores-Mireles *et al*., 2015).

Uropathogens that cause complicated UTIs follow the same initial steps as those described for uncomplicated infections, including periurethral colonization, progression to the urethra and migration to the bladder. However, in order for the pathogens to cause infection, the bladder must be compromised. The most common cause of a compromised bladder is catheterization. Owing to the robust immune

response induced by catheterization, fibrinogen accumulates on the catheter, providing an ideal environment for the attachment of uropathogens that express fibrinogen-binding proteins. Infection induces neutrophil infiltration, but after their initial attachment to the fibrinogen-coated catheters, the bacteria multiply, form biofilms, promote epithelial damage and can seed infection of the kidneys, where toxin production induces tissue damage. If left untreated, uropathogens that cause complicated UTIs can also progress to bacteraemia by crossing the tubular epithelial cell barrier (Flores-Mireles *et al*., 2015).

2.2.5. Risk factors

Both anatomic and physiologic factors put children at risk of developing UTI. Any anatomic or functional abnormalities of the urinary tract that impede urinary flow can increase the host susceptibility to UTI (Najar *et al*., 2009). Anatomic abnormalities include short urethra in females, urinary obstruction, Vesicoureteral reflux, neurogenic bladder which is the improper storage of urine in bladder and improper emptying of urine from bladder, and uncircumcison in boys. Uncircumcised boys have a great tendency to harbor organisms in the foreskin due to warm, moist and mucosal environment as a result bacteria migrate up to the urethra and colonize in the bladder (Heffner and Gorelick., 2008). Another anatomical factors include posterior urethral valves, or bladder diverticulitis (Heffner and Gorelick., 2008). Physiologic factors include dysfunctional voiding, infrequent voiding, incomplete bladder emptying, and constipation. In constipation stool remains in the rectum for a long period of time, and bacteria tends to colonize in the perineum, as a result increasing the risk for UTI (Sampaio *et al*., 2016).

The risk of UTI can be increased by many factors such as urinary retention, urine stasis, and reflux of urine, unstable bladder, frequent UTIs, constipation, sexual intercourse, chronic illness, and prolonged use of 5 antibiotics. Prolonged use of antibiotics can damage periurethral flora allowing uropathogens to colonize and infect the urinary tract (Najar *et al*., 2009).

Various factors make bacteriuria more or less to occur for any individual. These factors are age, gender, race, genetic factors, sexual activity among the teen age girls, and circumcision in boys, nocturnal enuresis and some unhealthy behaviors. UTI is age dependent and bacteriuria is more common at the extremes of life The Incidence of UTI is bimodal; highest during the first year of life and peaking again during adolescence (Heffner and Gorelick., 2008).

2.2.6. Symptoms

Lower urinary tract infection is also referred to as a bladder infection. The most common symptoms are burning with urination and having to urinate frequently (or an urge to urinate) in the absence of vaginal discharge and significant pain (Nicolle., 2008). These symptoms may vary from mild to severe and in healthy women last an average of six days (Colgan and Williams., 2011). Some pain above the pubic bone or in the lower back may be present. People experiencing an upper urinary tract infection, or pyelonephritis, may experience flank pain, fever, or nausea and vomiting in addition to the classic symptoms of a lower urinary tract infection(Lane and Takhar., 2011).

2.2.7. Causative agents

The Gram-negative rods *Escherichia coli* is commonest cause of ascending UTIs about 60-90%; this is probably because they are often present in the colon and virulence factors which include: the possession of K antigens and specialized fimbriae (Cheesbrough., 2009).

Proteus mirabilis and *klebsiella* species are often multiply antibiotic-resistant. *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *staphylococcus aureus* are cause infection especially after catheterization or instrumentation. Fastidious Gram-positive bacteria (e.g. lactobacilli, streptococci, corynebacteria), which require incubation for 24–48 hour in the presence of CO_2 for isolation, acute uncomplicated UTI is usually due to one type of organism and Chronic infection is often associated with more than one type of organism (Sleigh and Timbury., 1998). Obligate anaerobes are very rarely involved, other species may be found like*Salmonella*

typhi, Staphylococcus aureus and *Mycobacterium tuberculosis* (Cheesbrough., 2009).

2.2.8 *Escherichia coli*

Escherichia coli is belong to the large group of Gram negative rods referred to as enterobacteria, they are cause primary and opportunistic infections in humans belong mainly to lactose fermenting, often referred to as coliforms, they are aerobes and facultative anaerobes, non sporing and motile (Cheesbrough.,2009).

E. coli is the cause of 60-90% of urinary tract infection. Certain serotypes of *E .coli* are particularly common in urinary infection (e.g. 02, 04, 06, 07, 018, 075); this is probably because they are often present in the colon, rather than because of inherently high pathogenicity for the urinary tract. Some strains are reputed to more invasive than other. Factor associated with virulence include: the possession of K (capsular) antigen, which inhibit phagocytosis and bactericidal effect of normal human serum, the ability to adhere to uro–epithelium due to specialize fimbriae (Chittò *et al*., 2019).

2.3. Previous studies

Saeed and his colleagues found that *E. coli* urinary isolates was highly resistant to the most utilized antibiotics and it showed highly resistant rates against ampicillin, amikacin, amoxicillin, nitrofurantoin, co-trimoxazole and tetracycline and cephalosporins has continued to increase in the past decade and now approaches 40 % resistance. It also showed high susceptible rates to gentamicin(71%) (Saeed *et al.,* 2017).

Rashid and his colleagues found that 320 ul/ml of *Rosmarinus officinalis* L. extract was enough to inhibit *Escherishia coli* by disc diffusion method they used Sterile filter paper disks of uniform size (6 mm) were soaked with different concentrations(10, 20, 40, 80, 160, 320 and 640 mg/ml)(Rashid *et al*., 2011).

Golshani and Sharifzadeh found that MIC of the *Rosmarinus officinalis* L. extract that inhibit *Escherichia coli* growth was 200 mg/ml with inhibition zone 13mm

they used microplate method concentrations 400, 200, 100 and 50 mg/l of plant were prepared (Golshani and Sharifzadeh.,2014).

Al-Jiffri and his colleagues detected MIC of *Rosmarinus officinalis* L. against *Escherishia coli* by disc diffusion method, they found that 250mg/ml to 500mg/ml of *R. officinalis* L. extract was enough to inhibit 2 isolates *Escherishia coli* and MDIZ 5.3mm (Al-Jiffri *et al*., 2011).

In Morocco a study conducted by Bayoub and others found that the MIC of *Rosmarinus officinalis* L. against *Escherishia coli* by agar well diffusion method was 5.25mg/ml (Bayoub *et al*., 2010).

In France Campo and his colleagues found that 150 mg/ml of *R.officinalis* L. was necessary to inhibit *E. coli* (Campo *et al*., 2000).

In India a study included antibacterial test for *R.officinalis* L. at a concentration of 5 mg /ml against *E. coli* by the agar disc diffusion method and the result showed 10.37mm inhibition zone (Kumuda *et al*., 2017).

The study applied by Al-Jiffri and his colleagues at 4 isolate of *E. coli* conclude that *Thymus vulgaris* L more effective than *R.officinalis* against *E. coli*. It showed MDIZ 8.5 at concentration 500mg/ml for 3 isolates (Al-Jiffri *et al*., 2011).

In Morocco research examined by Bayoub and others also showed the MIC of *Thymus vulgaris* L. against *Escherishia coli* by agar well diffusion method and MIC was 1.56mg/ml and the study illustrated that *Thymus vulgaris* L.is more effective than *R.officinalis* L. (MIC was 5.25mg/ml) (Bayoub *et al*., 2010).

Elastal and his colleagues Detected the MIC of *Thymus vulgaris* L. against *E. coli* at concentration from 2.5 to 40mg/ml by whole plate diffusion method and all showed no zone (Elastal *et al*., 2005).

Zarringhalam and his colleagues found that *Thymus vulgaris* L. at concentration 200mg/ml showed a 15mm inhibition zone against *E. coli* by agar diffusion method.

Kumuda and his colleagues found that *Rosmarinus officinalis* L. has high antioxidant activity and the DPPH radical scavenging activity was 77.37% (Zarringhalam *et al*., 2013).

Martínez and his colleagues found that *R. officinalis* has high antioxidant activity the DPPH radical scavenging activity was 81.26% (Martínez *et al*., 2019).

Wisam and his colleagues found that DPPH radical scavenging activity of *Thymus vulgaris* L. was 94.51% (Wisam *et al*., 2018).

Iuliana and his colleagues found that ethanolic extracts of *Thymus vulgaris* L. presents significant antioxidant activity (Iuliana *et al*., 2014).

Rashid and his colleagues found that rosemary extracts contain endo-borneol, camphor and eucalyptol but at different concentrations. The differences may be due to seasonal variation, plant status and the extraction method (Rashid *et al.,* 2011)

costescu *et al.,* (2014)who found *T. vulgaris* extract contains thymol, camphor, fatty oil, palmitic, stearic, oleic, linoleic and linolenic but at different concentrations. The differences may be due to seasonal variation, plant status and the extraction method (costescu *et al.,* 2014)

Al-Jiffri and his colleagues found that ethanolic extracts of *R. officinalis* against *E. coli* give zone with mean (10.5mm) at concentration 500mg/ml(Al-Jiffri *et al*., 2011).

Al-Jiffri and his colleagues also found that ethanolic extracts of *Thymus vulgaris* against *E. coli* give zone with mean (11mm) at concentration 500mg/ml(Al-Jiffri *et al*., 2011).

CHAPTER THREE
MATERIALS and METHODS

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MATERIALS and METHODS

3. Materials and methods

3.1. Study design

This was a cross sectional study.

3.2. Study area and duration

This study was carried out at Police's Hospital, in Khartoum State in Sudan, during the period from February to April 2019.

3.3. Study sample

The current study was conducted in 53*Escherichia coli* urinary isolates.

3.4. Sample size

A total of fifty three (n=53) *E.coli* were collected.

3.5. Data collection

Data were collected from hospital check list.

3.6. Ethical consideration

The study received ethical clearance from the research board at the faculty of Medical Laboratory Science, Sudan University of Science and Technology.

3.7. Sampling technique

The study based on non probability convenience sampling technique.

3.8. Specimen collection

Isolates obtained were sub cultured on Nutrient slope and incubated aerobically at 37^{o}C for 24 hours and preserved at-20^{o}C and reidentified later by Gram's stain, cultivation on MacConkey and blood agar and conventional biochemical tests were used.

3.9. Sample processing

3.9.1. Cultivation of the isolates

E.coli isolates were subcultured under aseptic condition on Blood agar and MacConkey agar. The inoculated culture media were incubated aerobically at 37^{o}C for 24 hours.

Colonies on MacConkey agar was medium, pink and lactose fermenting colonies. On Blood agar colonies were medium, greyish white and beta haemolytic.

3.9.2. Gram's stain

First smear was prepared and air dried then fixed by heat then covered by crystal violet for 1 minute, washed by water, stained by iodine for 1 min, rinsed by alcohol and washed immediately then stained by safranin for 1min, washed by water and dried then examined microscopically by oil immersion lens ×100 and to detected the Gram reaction (Cheesbrough., 2009).

3.9.3. Biochemical tests

Biochemical tests included were sugar fermentation, gas and H_2S production test, indole production test, citrate utilization test, urease test, methyl red test and motility test.

3.9.3.1. Indole productions

It's used in the identification of *Enterobacteriaceae***,** indole production detected by Kovac's reagent and the reaction gave pink ring (Cheesbrough., 2009). Tryptophane broth inoculated by 3-4 colonies and incubated at 37^{o}c for 24hours, 0.5ml of Kovac's reagent added to the broth culture. *E.coli* gave pink ring.

3.9.3.2. Sugar fermentation, gas and H_2S

It is used for the differentiation of members of the Enterobacteriaceae on the basis of their ability to ferment glucose, lactose and production of gas and hydrogen sulfides (Cheesbrough., 2009). The kliglar iron agar inoculated by a single colony using straight wire and incubated at 37^{o}c for 24hours. *E.coli* on kliglar iron agar gave yellow butt and slope , produce gas and no production of hydrogen sulphide.

3.9.3.3. Citrate utilization test

It's used in the identification of *Enterobacteriaceae*, based on the utilization of sodium citrate as a sole carbon source and give blue colour (Cheesbrough., 2009). Simmons citrate agar inoculated by a single colony using straight wire and incubated at 37^{o}c for 24hours. *E.coli* gave no change in colour (green).

3.9.3.4. Urease test

Testing for urease enzyme activity is important to differentiate *Enterobacteriaceae* if urea breaks to ammonia and carbon dioxide media will change to pink (Cheesbrough., 2009). A colony inoculated on surface of urea agar by straight wire and incubated at 37^{o}c for 24hours. *E.coli* gave no change in colour (yellow).

3.9.3.5. Methyl red test

To identify bacteria based on their pattern of glucose metabolism. Glucose phosphate peptone water was inoculated by 3-4 colonies and incubated at 37^{o}c for 24hours, 0.5ml of Methyl red reagent added to the broth culture. *E.coli* gave pink ring. *E.coli* gave red colour after turning methyl red to red by acid pH formed (Cheesbrough., 2009).

3.9.3.6. Motility test

By using semi solid media that inoculated by a single colony using straight wire and incubated at 37^{o}c for 24hours. *E.coli* is motile bacteria.

3.9.4. Susceptibility test of isolates against selected antibiotics discs

Antibiotic susceptibility pattern of the isolates was studied by Kirby Bauers disc diffusion method. Both broad spectrum and narrow spectrum antibiotics were used. The antibiotics tested were Chloramphenicol (30 mcg), Gentamicin (10 mcg), Amoxicillin (25mcg) , Ceftriaxone(30 mcg) and Ciprofloxacin (50 mcg). The plates were left at room temperature then incubated at 37ºC for 18-24 hours and the inhibition zones were measured, results interpreted according to CLSI chart and recorded in chapter four (Bauer, *et al*., 1966).

3.10. Plant material

The flowers of *R.officinalis* and leaves of *Thymus vulgaris* were used as basic materials in this study. Commercial samples were obtained from local herbalist shop on Omdurman- Khartoum, Sudan. The collected materials were authenticated and specimens in Aromatic and Medicinal Plant Institute, Research centre.

3.10.1. Pre plant extraction

The plant materials were purchased and washed by water and air dried at room temperature (about 30 ˚C) for approximately 5 days. After that each material was ground to a course powder using a pestle and mortar and stored in a clean container ready for analysis (Zeynab and Ali., 2009).

3.10.2. Plant extraction

Seventy grams of plants weighted and dissolved in 300ml of ethanol and left at room temperature for 3 days. Extracts were filtered through filter paper. After filtration then left to evaporate and dry for 4 days, the extracts were vacuum concentrated to dryness (Golshani and Sharifzadeh., 2014).

3.10. *In vitro* testing of extracts for antibacterial activity and determination of MIC

3.11.1. Preparation of bacterial suspension

Stock cultures were maintained at 4°C on nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to tube contained 1 ml of normal saline for bacteria that was incubated for 24 hrs at 37°C. The cultures were then diluted with normal saline to achieve McFarland 0.5 turbidity standard (Hecht *et al*., 2007).

3.11.2. Disc diffusion method

Twenty ml aliquots of Mueller Hinton agar were distributed into sterile Petri-dishes. The isolates and standardized bacterial stock suspension adjusted to 0.5 McFarland streaked on Mueller Hinton agar medium plates using sterile cotton swab. Sterile filter paper discs of uniform size (6 mm Whatman NO1) were soaked with different concentrations (12.5, 25, 50 and 100 mg/ml) of ethanol extracts, then were placed

on surface of the test bacteria plates. The plates were incubated for 24 hrs at 37˚C and the diameters of the inhibition zones were measured in mm using ruler (Rashid *et al*., 2011).

3.11.3. Reading of the results

The plates were examined for the presence of inhibition zone of bacterial growth (clear hallows) around the disks and zone measured by mm also MIC determined by lowest concentration among 12.5, 25, 50 and 100 mg/ml that was enough to inhibit *E.coli.* (Bauer *et al*., 1996 and NCCLS., 2009).

3.11.4. Broth dilution technique for detection of MIC and MBC

Amount of 100 mg of extract was dissolved in 1 ml of Dimethyl sulfoxide (DMSO) and so the initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 1ml of the sterile plant extract (stock solution) into 1ml of sterile Mueller Hinton broth to obtain 50 mg/ml concentration. The above process was repeated several times to obtain other dilutions: 25 mg/ml and 12.5 mg/ml**,** bacterial concentration was adjusted to a 0.5 McFarland standard. The bacteria was added to the diluted extract (Jorgensen and Turnidge., 2009).

3.11.5. Reading of the results

In order to find out if there was any bacterial growth, the turbidity of the solution in each tube was observed on the next day. The tubes that showed no turbidity were recorded as the MIC value . To ensure the presence or absence of bacterial growth in the tubes, a standard loop of the suspensions in each tube was inoculated on Mueller hinton agar and incubated overnight at 37 ºC. The plates were observed following incubation to confirm absence or growth of bacteria. The lowest concentration of extract dilution showing no visible growth was recorded as the MBC value (Jorgensen and Turnidge., 2015).

3.12. Determination of antioxidant activity

3.12.1. DPPH method

The DPPH radical scavenging was determined according to the method of (Hilmi *et al*., 2014) with some modification. In 96-wells plate, the test samples were allowed

to react with 2.2 Di (4-tetra-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37°C. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiplate reader Spectrophotometer (Fig 13.a). Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. Ascorbic acid was used as standard and all tests and analyses were run in triplicates (kumuda *et al*., 2011).

3.12.2. Reading of the result

DPPH % =[($Abs_{control}$ - Abs_{sample})] /($Abs_{control}$)] x 100

Where,

$A_{control}$ is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference.

3.13. Gas chromatography mass spectrometry

The qualitative and quantitative analysis of the sample was carried out by using GC-MS technique model (GC-MS-QP2010-Ultra) from Japans 'Simadzu Company, with capillary column (Rtx-5ms-30 m×0.25 mm×0.25 μm).The sample was injected by using split mode, helium as the carrier gas passed with flow rate 1.61 ml/min. The temperature program was started from 60°C with rate 10°C /min to300°C as final temperature degree. The sample was analyzed by using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 26 min. Identification of components for the sample was achieved by comparing their retention times and mass fragmentation patterns with those available in the library from the NIST.

3.14. Data analysis

The data analyzed by using Statistical Package for Social Sciences (SPSS v.20) program to get the mean and the standard deviation.

CHAPTER FOUR
RESULTS

CHAPTER FOUR
RESULTS

A total of 53*E.coli* urinary isolates were included in this study. The antibacterial activity at concentration 12.5, 25, 50 and 100 mg/ml of the ethanol extract of *R. officinalis* flowers pod and *Thymus vulgaris* leaves was determined against 53 Gram negative *E.coli* isolated bacteria using disc diffusion method. Different extracts showed variable activity against the tested bacteria. The *in vitro* antioxidant activity of the ethanolic extracts of *R. officinalis* and *T. vulgaris* extracts was evaluated using DPPH scavenging activity and the chemical analysis of *R. officinalis* and *T. vulgaris* extracts was carried out using gas chromatography-mass spectrometry (GC-MS).

Antibiotic susceptibility test was applied against *E.coli* urinary isolates.47isolates (88.7%) were sensitive to Chloramphenicol, 21isolates (39.6%) were sensitive to Ceftriaxone, 46 isolates (87%) were sensitive to Gentamycin , none of isolates (0%) was sensitive to Amoxycillin and 38isolates (71.7%) were sensitive to Ciprofloxacin and there was 7 (13%) *E.coli* isolates which were multi drug resistant as shown in table 4.1.

The inhibitory zone of *R. officinalis* against *E.coli* was between (8-23mm) and for *T. vulgaris* was between (8-20mm).

R. officinalis extract 100mg/ml inhibition zone was between (9-23mm) for 91% with mean (16±7 mm), 50mg/ml inhibition zone was between (8-19mm) for 64% with mean (13.5±6 mm), 25mg/ml inhibition zone was between (8-12mm) for 34% with mean (10± 2 mm) and 12.5mg/ml inhibition zone was between (0-8mm) for 2% with mean (4±4 mm) as shown in table 4.2. For *T. vulgaris* extract 100mg/ml inhibition zone was between (8-20mm) for 94% with mean (14±6 mm), 50mg/ml inhibition zone was between (8-15mm) for 77% with mean (11.5±3.5 mm), 25mg/ml inhibition zone was between (8-12mm) for 54% with mean (10± 2 mm) and 12.5mg/ml

inhibition zone was between (8-12mm) for 28% with mean (10±2 mm) as shown in table 4.3.

The MIC of of *R. officinalis* and *T. vulgaris* against isolates between 12.5 to 50 mg/ml and the MBC between 50to 100 mg/ml as shown in table 4.4.

The DPPH radical scavenging potential of *T. vulgaris* was 83.09% followed by *R. officinalis* (81.26 %) as shown in table 4.6.

The GC/MS analysis of both plants revealed presence of different chemical constituents which have different biological activities. The chemical analysis of *R. officinalis* showed the most abundant compounds were, Eeucalyptol, Bicycloheptan and Octahydrodibenz anthracene, for *T. vulgaris* the most abundant compounds were Thymol**,** phytol and Hexadecanoic acid as shown in table 4.7and 4.8.

Table (4.1): Antibiotics susceptibility testing against *E.coli* isolates

Antibiotic	**Antimicrobial susceptibility pattern**		
	Sensitive	Intermediately sensitive	Resistant
Chloramphenicol(30mcg)	88.7%)(47	0%) (0	11.3%) (6
Ceftriaxone (30mcg)	39.6%)(21	18.9%)(10	41.5%)(22
Gentamicin (10mcg)	87%) (46	0%) (0	13%)(7
Amoxycillin (25mcg)	0%) (0	0%) (0	100%) (53
Ciprofloxacin(50mcg)	71.7) (38	0%) (0	28.3%) (15

Table (4.2): Antimicrobial activity of different concentrations of Ethanolic extract of *R. officinalis* against *E.coli* urinary isolates

Concentration	Inhibition zone Minimum-Maximum(mm)	Mean ± SD (mm)	Isolates showed Inhibition zone
12.5 mg/ml	0-8	0.15±1	1 (2%)
25 mg/ml	8-12	3±4	18 (34%)
50 mg/ml	8-19	7±5	34 (64.2%)
100 mg/ml	9-23	12±5	48 (90.6%)

Table (4.3): Antimicrobial activity of different concentrations of Ethanolic extract of *T. vulgaris* against *E.coli* urinary isolates

Concentration	Inhibition zone Minimum-Maximum(mm)	Mean ± SD (mm)	Isolates showed Inhibition zone
12.5 mg/ml	8-12	3±4	15 (28.3%)
25 mg/ml	8-12	5±4	29 (54.7%)
50 mg/ml	8-15	8±4	41 (77.4%)
100 mg/ml	8-20	12±3	50 (94.3%)

Table (4.4): Correlation between MIC of ethanolic extract of *R. officinalis* against the 7 multidrug resistant *E.coli* isolates

Isolate number	MIC	MBC
1	50 mg/ml	100 mg/ml
2	50 mg/ml	100 mg/ml
3	50 mg/ml	100 mg/ml
4	25 mg/ml	50 mg/ml
5	12.5 mg/ml	25 mg/ml
6	25 mg/ml	50 mg/ml
7	50 mg/ml	100 mg/ml

Table (4.5): The MIC and MBC of ethanolic extract of *T. vulgaris* against the 7 multidrug resistant *E.coli* isolates

Isolate number	MIC	MBC
1	25 mg/ml	50 mg/ml
2	50 mg/ml	100 mg/ml
3	12.5 mg/ml	25 mg/ml
4	25 mg/ml	50 mg/ml
5	50 mg/ml	100 mg/ml
6	50 mg/ml	100 mg/ml
7	25 mg/ml	50 mg/ml

Table (4.6): DPPH Radical scavenging potential of the ethanolic extract of *R. officinalis* and *T. vulgaris*

Sample	Rosemary	Thyme	Ascorbic acid (Control +ve)
DPPH%	81.26 %	83.09 %	92%

Table (4.7):, **Compounds ,peak area%, compound nature and bioactivity of *R. Officinalis* ethanol extract**

Name of compound	Peak area%	Biological activities
Eeucalyptol	18.57%	Insecticide, mosquito larvicide, insect repellent and gastroprotective activities(Seol and Kim., 2016) .
Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	10.01%	Antibacterial, Antiallergic agents suppress various allergic inflammatory responses such as increased vascular permeability in allergic rhinitis, conjunctivitis, and asthma models(Mitsumori *et al*., 2003)
endo-Borneol	4.85%	Relieving symptoms of anxiety, fatigue and insomnia, anesthesia and analgesia to alleviate abdominal pain, wounds and burns; relieving rheumatic pain, hemorrhoids, skin diseases and ulcers ,treat cardiovascular and cerebrovascular diseases and has a significant therapeutic effect on neuralgia (Yu *et al*., 2003)
1,2,3,4,4a,5,6,14b-Octahydrodibenz[a,h]anthracene	7.44%	Antibacterial activity (ElLakany., 1996).
Flavone, 5,7-dihydroxy-8-methoxy	5.85%	Anti-inflammatory activity and antihypertensive activity(Wu *et al*., 1992)
4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a ,14,14a,14b-octadecahydro-2H-picen-3-one	5.25%	Antimicrobial activity(Keskın *et al*., 2012)
beta.-Amyrin	3.13%	Antimicrobial activity(Vázquez *et al*., 2012)

Table (4.8):, Compounds ,peak area%, compound Nature and bioactivity of *T. Vulgaris* ethanolic extract:

Compound	Peak area%	Biological activities
Thymol	5.7%	antiseptic, antibacterial, and antifungal actions (Bayoub *et al*., 2010)
phytol	7.92%	strong antioxidant effect and anti nociceptive activity, antimicrobial and anticancer activities (Santos *et al*., 2013)
n-Hexadecanoic acid	10.27%	Antibacterial activity (Aparna *et al*., 2012)
Hexadecanoic acid, ethyl ester	18.51%	Antioxidant, Hemolytic,Hypocholesterolemic, Flavor,Nematicide, Anti-androgenic (Tyagi and Agarwal., 2017)
Stigmasterol	2.02%	Antioxidant, antimicrobial, anticancer,anti-arthritic, anti-asthama, anti-inflammatory, diuretic activities (Aparna *et al*., 2012)
Linolelaidic acid ethyl ester	17.68%	Hypocholesterolemic, nematicide, antiarthritic, hepatoprotective, antiandrogenic, 5-α reductase inhibitor, antihistaminic, anticoronary, insectifuge, antieczemic, antiacne, antimicrobial (Adeoye *et al*., 2012)
Ethyl 9,12,15-octadecatrienoate	14.31%	Antimicrobial, Anticancer Hepatoprotective, Anti-arthritic, anti-asthama, diuretic (Aparna *et al*., 2012) .
gamma-sitosterol	2.59%	reduces hyperglycemia increased insulin secretion and inhibition of glucogenesis. It can be used in the treatment of Diabetes mellitus (Tripathi *et al*., 2013)

CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATION

CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Nostro *et al*., 2000).Urinary tract infection (UTI) is one of the most important causes of morbidity in the general population and is the second most common cause of hospital visits (Ronald and Pattullo., 1991).

Urinary tract infections were the most common pathogenic infections, these infections lead to many difficult problems, especially increasing resistance to antibiotic drugs (Al-Jiffri and Al-Sharif., 2010). *E.coli* is one of the most common bacteria capable of causing infection in humans, particularly urinary tract infection (Iroha *et al*., 2009).Fifty three *E. coli* urinary isolates were reidentified by Gram's stain, colonial morphology and conventional biochemical tests.

In this study 7(13%) *E.coli* urinary isolates showed multidrug resistance which is in an agreement with Saeed *et al.,* (2017) who found that *E. coli* urinary isolates in sudan was highly resistant to the most utilized antibiotics and it showed highly resistant rates against ampicillin, amikacin, amoxicillin, nitrofurantoin, co-trimoxazole and tetracycline and cephalosporins that has continued to increase in the past decade and now approaches 40 % also showed high susceptible rates to gentamicin.

In this study *R.officinalis* extract showed zone with mean of (12mm) against *E.coli* at 100 mg/ml and above. This result is in disagreement with Al-Jiffri and Al-Sharif*.,* (2011) who stated that zone with mean of (10.5mm) obtained at 500mg/ml The differences may be due to resistance of *E. coli*.

Antimicrobial activity with MIC of *R.officinalis* L. ethanolic extract against *E.coli* was examined using the concentrations 100, 50, 25 and 12.5 mg/ml and it was found that MIC for one isolate was12.5mg/dl and for other isolates ranged from 25mg/dl

and above. These results are in a disagreement with Genena *et al*., (2008) who stated that. higher concentration of the extract was needed to inhibit *E.coli* and it was 320mg/ml also disagree with Golshani *and* Shaifzadeh., (2014), whom found MIC of the *R.officinalis* L. extract on that inhibit *Escherichia coli* growth was 200 mg/ml. The differences may be because of resistance of *E. coli* due to cell membrane permeability or due to other genetic factors.

T. vulgaris extract showed zone with mean of 11mm against *E.coli* at 100 mg/ml and above. This result is in disagreement with Al-Jiffri and Al-Sharif*.,* (2011) who stated that zone with mean of (11mm) obtained at 500mg/ml. The differences may be due to resistance of *E. coli*.

Antimicrobial activity with MIC of *T. vulgaris* ethanolic extract against *E.coli* was examined using the concentrations 100, 50, 25 and 12.5 mg/ml. It was found that MIC ranged from 12.5 mg/dl and above. These results are in disagreement with Al-Jiffri and Al-Sharif*.,* (2011) who stated that, higher concentration of the extract was needed to inhibit *E.coli* and it was 500mg/ml also disagree with Bayoub *et al.,* (2010) who found MIC of the *T. vulgaris* extract on that inhibit *Escherichia coli* growth was 1.56 mg/ml The differences may be because of use of other method or resistance of *E. coli* due to cell membrane permeability or due to other genetic factors.

The present result showed the DPPH radical scavenging activity of *R.officinalis* L. was 81.26 % and it was in agreement with the findings of Martínez *et al.,* (2019) who reported that rosemary exhibited a high radical scavenging activity and Kumuda *et al.,* (2017) who reported DPPH radical scavenging activity of *R.officinalis* L. was 77.37%.

The present result showed the DPPH radical scavenging activity of *Thymus vulgaris* L. was 94.51% and it was in agreement with the findings of Wisam *et al.,*(2018) who reported that rosemary exhibited a high radical scavenging activity Iuliana *et al.,* (2014) and who found that *Thymus vulgaris* L. presents significant antioxidant activity In this study analysis of *Rosmarinus officinalis* L. extract by GC-MS led to

the identification of 77compounds by comparison of recorded mass spectra with those of a computer library but the most active compounds were Eeucalyptol, camphor, endo-borneol, beta.-Amyrin, Wogonin, beta-Amyrone and Octahydrodibenz [a,h] anthracene. Eeucalyptol was obtained at a highest percent was considered and camphor which was found in the extract has a great antioxidant and antimicrobial activity. These results are in harmony with Rashid *et al.,* (2011) who found that rosemary extracts contain endo-borneol, camphor and eucalyptol but at different concentrations. The differences may be due to seasonal variation, plant status and the extraction method.

The analysis of *T. vulgaris* extract by GC-MS led to the identification of 24compounds by comparison of recorded mass spectra with those of a computer library but the most active compounds were Thymol, phytol, Hexadecanoic acid, Hexadecanoic acid- ethyl ester, Stigmasterol, Linolelaidic acid ethyl ester, octadecatrienoate and gamma-sitosterol. Hexadecanoic acid, ethyl ester was obtained at a highest percent was considered as Antioxidant, Hexadecanoic acid and Thymol which were found in the extract has an antimicrobial activity. These results are in harmony with Costescu *et al.,* (2014) who found *T. vulgaris* extract contains thymol, camphor, fatty oil, palmitic, stearic, oleic, linoleic and linolenic but at different concentrations. The differences may be due to seasonal variation, plant status and the extraction method.

5.2 Conclusion

Rosmarinus officinalis L. and *Thymus vulgaris* L. plants extracts have antimicrobial properties which might justify the use of those herbs in pharmaceutical industries for the production of new synthetic agents against *E.coli*. Screening of *R. officinalis* and *T. vulgaris* against *Escherichia coli* as well as the radical scavenging potential showed that the ethanolic extracts of *R. officinalis* and *T. vulgaris* have broad antimicrobial and antioxidant activity. This study demonstrated support for the claimed uses of the plants in the traditional medicine.

Some isolates of *E.coli* were MDR.

Some compounds extracted from the two plants were found to be antimicrobial or antibacterial.

5.3 Recommendations

This study recommended that:

Screening others parts of both plants.

Use other solvents rather than ethanol for the extraction.

More studies on the plant's active constituents so as to obtain a more natural drugs with less side effect for the patient.

Further investigations to determine the other medicinal active compounds of the plant and experimental as well as clinical studies are warranted.

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Appendices

Appendix 1

Nutrient agar

Ingredients g/L

Peptic digest of animal tissue 5.00

Beef extract/yeast extract 3.00

Agar 15.00

NaCl 5.00

pH is adjusted to neutral (7.4) ± at 25 °C.

Preparation

Suspend 23 g of the powder in 1 litre of Distilled water. Heat with Frequent agitation and boil for 1 minute to completely dissolve the powder.Autoclave at 121˚C for 15 minutes.

Mueller Hinton Agar

Ingredients g/L

Beef infusion 300.0

Casein acid Hydrolysate 17.50

Starch 1.50

Agar 17.00

Preparation

Suspend 38g of the powder in 1 litre of Distilled water Mix thoroughly. Heat to completely dissolve the powder. Autoclave at 121^{O}C for 15 minutes.

Blood agar

Ingredients g/L

Agar = 15.0 gm

Beef extract = 10.0 gm

Peptone = 10.0 gm

Sodium Chloride (NaCl) = 5.0 gm

Sheep blood, defibrinated = 50.0 mL

pH 7.3 ± 0.2 at 25°C

Preparation:

Add above components (40 gm), except sheep blood, to distilled/deionized water and bring volume to 950.0 mL. Mix thoroughly. Heat with frequent agitation and boil for 1 min to completely dissolve Autoclave for 15 min at 15 psi pressure at 121°C. Cool to 45°- 50°C. Aseptically add 50.0 mL of sterile, defibrinated sheep blood. Mix thoroughly and pour into sterile Petri dishes.

MacConkey agar:

Ingredients

Peptone (Pancreatic digest of gelatin) 17 gm

Proteose peptone (meat and casein) 3 gm

Lactose monohydrate10 gm

Bile salts1.5 gm

Sodium chloride5 gm

Neutral red0.03 gm

Crystal Violet0.001 g

Agar13.5 gm

Distilled Water Add to make 1 Liter

Final pH 7.1 +/- 0.2 at 25 degrees C

Preparation of MacConkey Agar

Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by

autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well before pouring.

Christensen's Urea Agar

Ingredients g/L

Sodium Chloride 5.00

Monopotassium Phosphate 0.8

Dipotassium Phosphate 1.2

Peptone 1.00

Dextrose 1.00

Phenol Red 0.012

Agar 15.0

Final pH 7.4 ± 0.2 at 25 ° C.

Preparation

Suspend 24 grams in 950 ml of distilled water heat boil to dissolve

Completely Autoclave at 121 ° C for 15 minutes, then cool to 50° C

Add Aseptically 40% urea solution and mix well , dispense in sterile tube and allow to set at slant position

Simmon's Citrate Agar

Ingredients g/L

Sodium Chloride 5.0

Sodium Citrate 2.0

Ammonium Dihydrogen Phosphate 1.0

Dipotassium Phosphate 1.0

Magnesium Sulfate 0.2

Bromothymol Blue 0.08

Agar 15.0

Final pH 7.4 ± 0.2 at 25 ° C

Preparation

Suspend 24.28 grams in 100 ml of distilled water heat boil to dissolve completely Autoclave at 15 Ibs pressure (121° C) for 15 minutes.
Then cool to50° C aseptically dispenses in sterile tube and allow to set.

Peptone Water

Ingredients g/L

Sodium Chloride 5.0

Peptic digest of animal tissue 10.0

Final pH 7.4 ± 0.2 at 25 ° C

Preparation

Suspend 15 grams in 100 ml of distilled water heat boil to dissolve completely Autoclave at 15 Ibs pressure (121° C)for 15 minutes. Then cool aseptically, dispense in sterile tube and allow to set

Kligler Iron Agar (KIA)

Ingredients g/L

Peptic digest of animal tissue 15

Lactose 10.0

Proteose Peptone 10.

Sodium Chloride 5.0

Beef Extract 3.0

Yeast Extract 3.0

Dextrose 1.0

Sodium Trisulphate 0.3

Ferrous Sulfate 0.2

Phenol Red 0.024

Agar 15.0

Preparation

Suspend 57.52 grams in 1000 ml of distilled water heat boil to dissolve completely Autoclave at 15 Ibs pressure (121° C) for 15 minutes. Then coolaseptically, dispense in sterile tube and allow setting slant position

Glucose phosphate peptone water:

Ingredients g / Litre

Buffered peptone 7

Dextrose

Dipotassium phosphate 5

Final pH (at 25°C)

Preparation:

22.3 grams in every 100 ml of distilled water. Sterilize by autocalving (with cap loosened) at 121°C for 15 minutes.

Semisolid media for motility:

Tryptose 10

Sodium chloride Agar 5

Final pH (at 25°C)

Preparation:

Suspend 20 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow tubed medium to cool in an upright position.

3.1.2 Chemicals

Distilled water

Normal saline

Alcohol 70%

Kovac´s reagent

Crystal violet

Lugol´s iodine

Safranine

3.1.3 Chemotherapeutic agents

1,1-diphenyl-2-picryl hydrazyl (DPPH)

Ascorbic acid

3.1.4 Apparatus

Sterile containers

Slides

Petri dishes

Benzene burner

Loops (straight + calibrated wire loop)

Racks

Forceps

Pasteur pipette

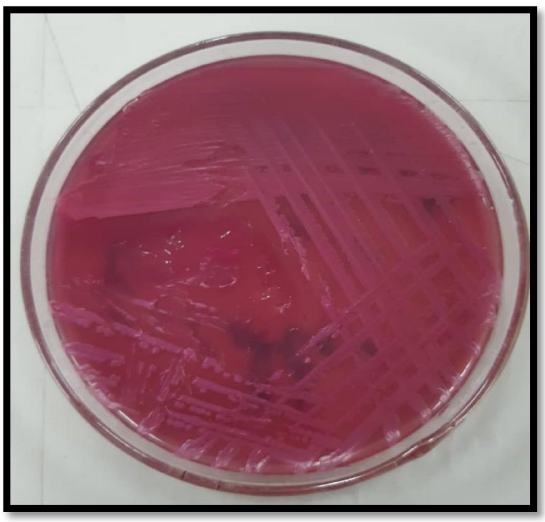
Oil immersion

Test tubes

Glass bottle

Appendix 2

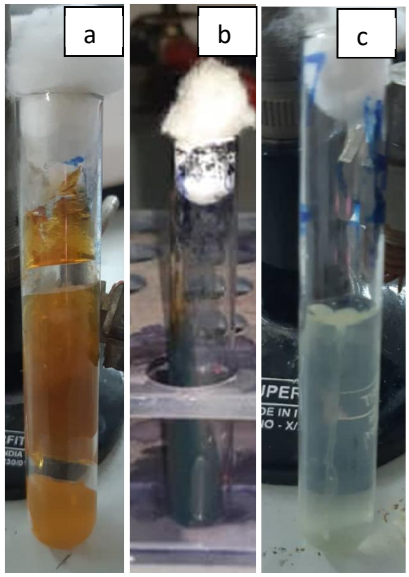
Color plate (1): Sterile Blood agar



Color plate (2) *E.coli* colonies on MacConkey agar

Appendix(3)

Color plate (3): Positive indole test, methyl red test and negative citrate and urease test and yellow butt and slope on kligler iron agar

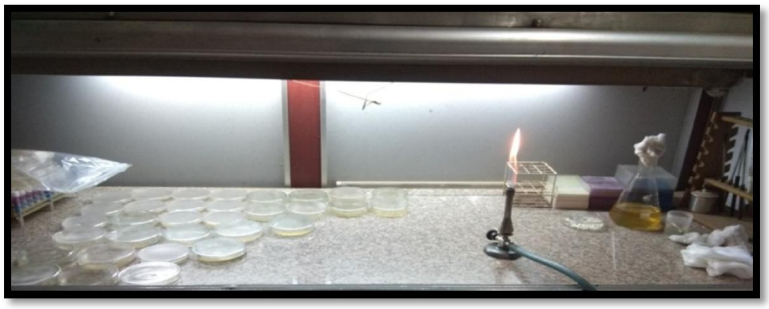


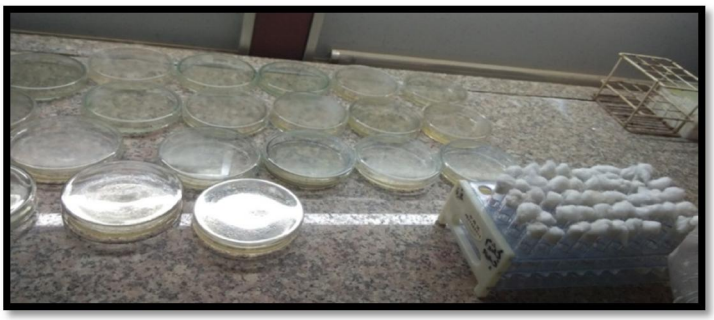
Color plate (4) a)***E.coli*** **colonies on kligler iron agar**

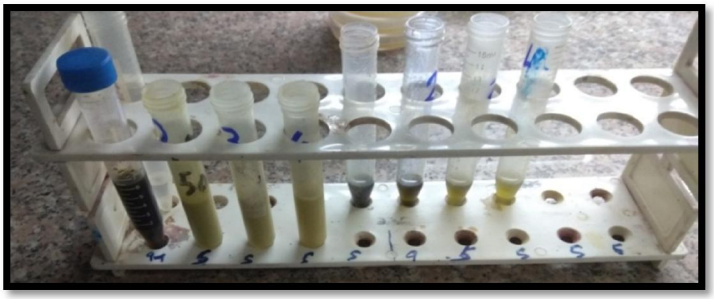
b)***E.coli*** **colonies on citrate agar**

c)***E.coli*** **colonies on motility media**

Appendix(4)

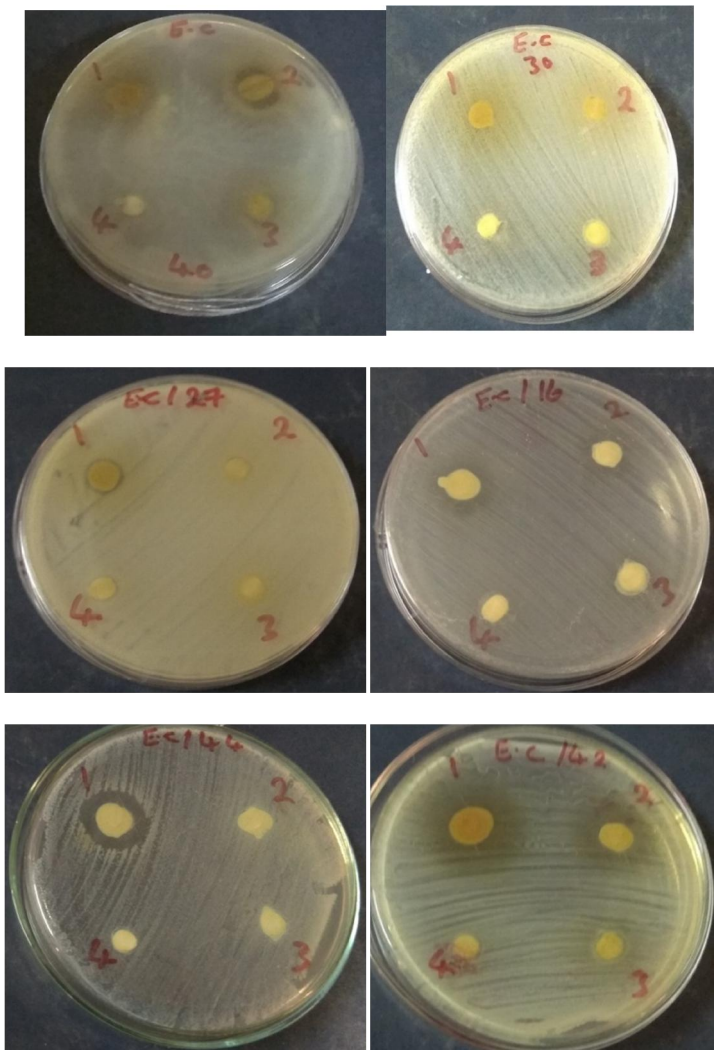






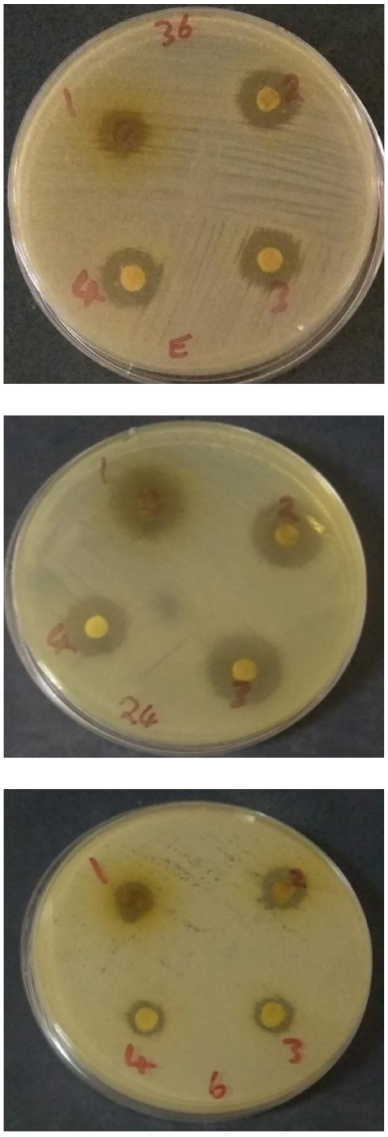
Color plate (5) Antimicrobial activity of plant extracts against *E.coli* isolates

Appendix(5)



Color plate (6) Antibacterial activity of *R. officinalis* against *E.coli* bacteria

Appendix(6)



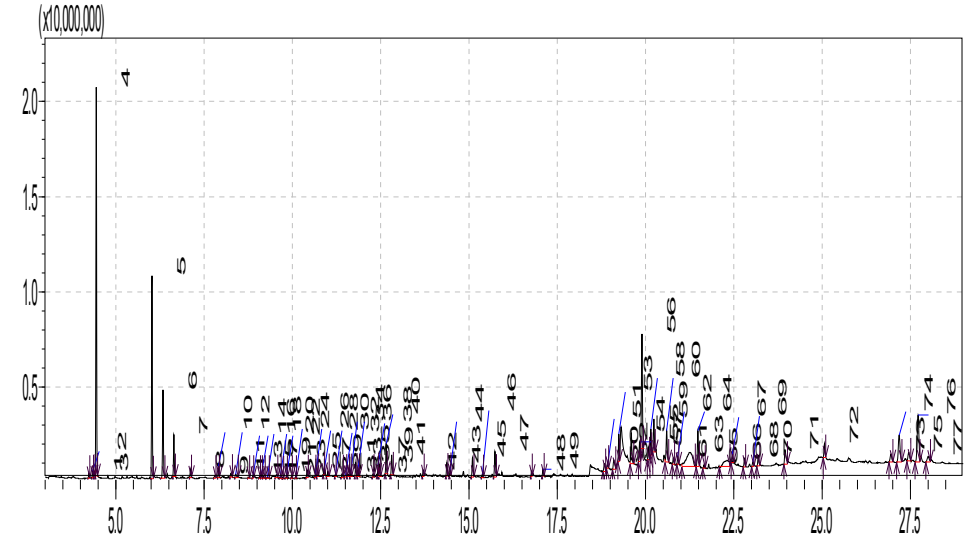
Color plate (7) Antibacterial activity of *T. vulgaris* against *E.coli* bacteria

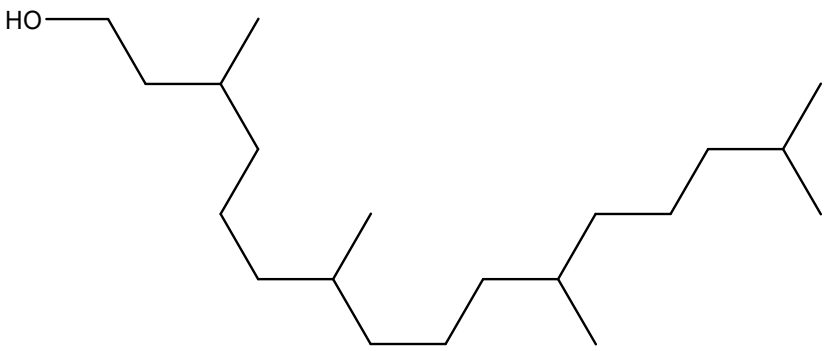
Appendix(7)

Color plate (8) The MIC of Ethanolic extract of *T. vulgaris* against Multidrug resistant *E.coli*

Color plate (9) The MIC of Ethanolic extract of *R. officinalis* against Multidrug resistant *E.coli*

Appendix(8)

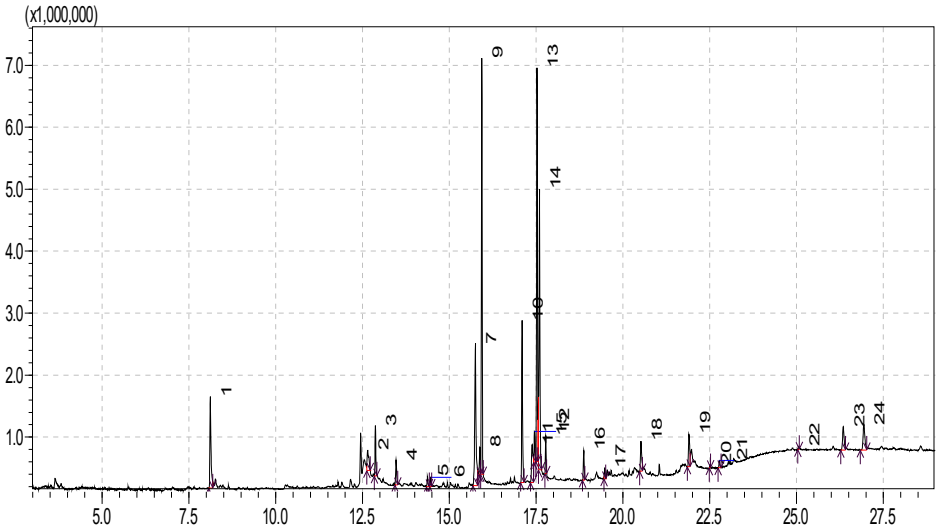


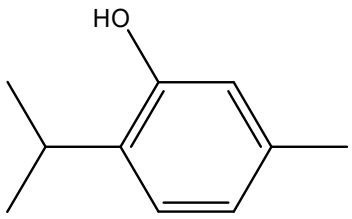


phytol

Figure (9) GC/MS Chromatogram of ethanolic extracts of *R. officinalis* and Chemical structure of Phytol

Appendix(9)





Thymol

Figure (9) GC/MS Chromatogram of ethanolic extracts of *R. officinalis* and Chemical structure of Thymol

Appendix(10)

Diameter inhibition zone (mm) of *R.officinalis* against *E. coli*

Sample	Diameter of Inhibition Zone (mm)			
	Con. 100 mg/ml	Con. 50 mg/ml	Con. 25 mg/ml	Con. 12.5 mg/ml
1	9	8	-	-
2	11	9	8	-
3	14	8	-	-
4	11	10	8	-
5	10	8	-	-
6	16	12	8	-
7	15	14	8	-
8	12	8	-	-
9	11	9	-	-
10	17	13	10	-
11	10	9	8	-
12	10	9	-	-
13	10	8	-	-
14	10	9	-	-
15	9	-	-	-
16	14	11	8	-
17	11	8	-	-
18	9	-	-	-
19	10	8	-	-
20	9	-	-	-
21	15	13	10	8
22	11	-	-	-
23	12	8	-	-
24	-	-	-	-
25	20	19	11	-

Continue:

Sample	Diameter of Inhibition Zone (mm)			
	Con. 100 mg/ml	Con. 50 mg/ml	Con. 25 mg/ml	Con. 12.5 mg/ml
26	11	10	8	-
27	10	8	-	-
28	21	19	12	-
29	23	15	8	-
30	-	-	-	-
31	10	-	-	-
32	-	-	-	-
33	-	-	-	-
34	12	-	-	-
35	13	-	-	-
36	15	-	-	-
37	11	-	-	-
38	20	13	-	-
39	-	-	-	-
40	12	10	8	-
41	11	10	-	-
42	12	11	-	-
43	14	-	-	-
44	20	15	8	-
45	9	-	-	-
46	11	-	-	-
47	11	-	-	-
48	18	13	9	-
49	10	8	-	-
50	20	17	10	-
51	12	10	9	-
52	11	9	8	-
53	9	-	-	-

Appendix(11)

Diameter inhibition zone (mm) of *Thymus vulgaris* against *Escherichia coli*:

Sample	Diameter of Inhibition Zone (mm)			
	Con. 100 mg/ml	**Con. 50 mg/ml**	**Con. 25 mg/ml**	**Con. 12.5 mg/ml**
1	9	-	-	-
2	12	8	-	-
3	15	13	12	10
4	8	-	-	-
5	11	8	8	-
6	15	11	10	8
7	13	10	8	-
8	12	9	-	-
9	11	-	-	-
10	14	11	10	-
11	-	-	-	-
12	12	10	8	8
13	8	-	-	-
14	-	-	-	-
15	12	8	-	-
16	15	12	9	-
17	9	-	-	-
18	12	8	8	-
19	13	12	8	-
20	10	9	8	-
21	8	8	-	-
22	15	13	12	12
23	8	-	-	-
24	16	15	10	12
25	13	10	9	-

Continue:

Sample	Diameter of Inhibition Zone (mm)			
	Con. 100 mg/ml	**Con. 50 mg/ml**	**Con. 25 mg/ml**	**Con. 12.5 mg/ml**
26	15	14	11	10
27	9	-	-	-
28	11	9	9	-
29	14	10	-	-
30	11	9	8	8
31	13	10	8	-
32	9	10	9	9
33	8	8	-	-
34	13	10	9	8
35	11	9	9	-
36	14	11	12	10
37	-	-	-	-
38	12	10	9	8
39	8	8	-	-
40	13	8	-	-
41	8	-	-	-
42	13	10	8	-
43	17	11	8	-
44	13	11	-	-
45	15	12	-	-
46	12	10	8	8
47	15	10	8	-
48	10	-	-	-
49	13	10	-	-
50	18	14	10	9
51	20	13	12	10
52	14	9	-	-
53	16	14	9	9

Appendix(12)

Chemical composition by GC/MS OF *R. officinalis*

NO.	Name	Ret.Time	Area %
1.	(+)-4-Carene	4.239	0.25
2.	o-Cymene	4.344	0.31
3.	D-Limonene	4.396	0.23
4.	Eucalyptol	4.447	18.55
5.	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	6.024	10.01
6.	endo-Borneol	6.335	4.85
7.	.alpha.-Terpineol	6.647	2.14
8.	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-	7.114	0.42
9.	1,7-Octadiene-3,6-diol, 2,6-dimethyl-	7.803	0.21
10.	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	7.905	0.53
11.	Phenol, 2-methyl-5-(1-methylethyl)-	8.263	0.30
12.	2-Methoxy-4-vinylphenol	8.396	0.30
13.	1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-	8.754	0.08
14.	.alpha.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranemethanol	8.886	0.13
15.	Ylangene	9.065	0.07
16.	Copaene	9.125	0.21
17.	1-Dodecanol	9.179	0.12
18.	Hydroxy-.alpha.-terpenyl acetate	9.291	0.10
19.	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha.,3.alpha.,4.beta.)]-	9.551	0.23
20.	Caryophyllene	9.652	0.07
21.	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-	9.727	0.09
22.	Bicyclo[7.2.0]undecane, 10,10-dimethyl-2,6-bis(methylene)-, [1S-(1R*,9S*)]-	9.830	0.09
23.	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	9.976	0.09
24.	1R,3Z,9s-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-3-ene	10.094	0.09
25.	.gamma.-Muurolene	10.415	0.42
26.	1,4-dihydroxy-p-menth-2-ene	10.645	0.14
27.	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	10.704	0.09
28.	.beta.-copaene	10.896	0.21

29.	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	11.006	0.26
30.	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	11.217	0.05
31.	Caryophyllene oxide	11.408	0.26
32.	Dodecanoic acid	11.505	0.13
33.	4-Methyl-2,5-dimethoxybenzaldehyde	11.570	0.19
34.	13-Octadecenal, (Z)-	11.649	0.18
35.	3-Heptadecen-5-yne, (Z)-	11.795	0.66
36.	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	11.857	0.39
37.	Murolan-3,9(11)-diene-10-peroxy	12.283	0.18
38.	Isoaromadendrene epoxide	12.430	0.58
39.	Methyl jasmonate	12.459	0.61
40.	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,4.beta.,4a.beta.,7.alpha.,7a.beta.,7b.alpha.)]-	12.657	1.57
41.	1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)-	12.813	1.17
42.	Eicosanoic acid	13.703	0.46
43.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	14.375	0.79
44.	2-Pentadecanone, 6,10,14-trimethyl-	14.465	0.23
45.	4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	15.099	1.37
46.	Phenanthrene, 7-ethenyl-1,2,3,4,4a,5,6,7,8,9,10,10a-dodecahydro-1,1,4a,7-tetramethyl-	15.380	0.17
47.	n-Hexadecanoic acid	15.737	1.86
48.	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	16.775	0.28
49.	Phytol	17.096	0.38
50.	Humulane-1,6-dien-3-ol	18.837	0.68
51.	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methylene-4-pentenyl)-, [4aS-(4a.alpha.,5.alpha.,8a.beta.)]-	18.923	0.23
52.	Estra-1,3,5(10)-trien-16-one, 3-[(trimethylsilyl)oxy]-	19.100	0.92
53.	Ferruginol	19.241	1.45
54.	9(1H)-Phenanthrenone, 2,3,4,4a,10,10a-hexahydro-6-hydroxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	19.593	2.18
55.	Isocarnosol	19.836	1.50
56.	1,2,3,4,4a,5,6,14b-Octahydrodibenz[a,h]anthracene	19.899	7.44
57.	Isosteviol acetate	20.079	0.82
58.	Dibenz[d,f]cycloheptanone, 2,3,9-trimethoxy-	20.150	1.35
59.	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-	20.236	1.98

	trimethyl-1-(1-methylethyl)-, acetate, (4bS-trans)-		
60.	(-)-20-Deoxocarnosol	20.597	2.22
61.	p-Benzoquinone, 2,5-bis(1,1,3,3-tetramethylbutyl)-	20.785	0.37
62.	13-Isopropylpodocarpen-12-ol-20-al	20.911	0.45
63.	Flavone, 5,7-dihydroxy-8-methoxy-	21.253	5.85
64.	Buphanamin	21.482	2.55
65.	Dibenz[c,E]cycloheptanone, 3,4,7-trimethoxy-	21.654	0.19
66.	.alpha.-Lumicolchicine	22.320	2.26
67.	4-Acetylphenyl 5-acetyl-2-methoxyphenyl ether	22.454	0.55
68.	Phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-6-methoxy-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	22.803	0.53
69.	2-Piperidinone, N-[4-bromo-n-butyl]-	23.040	0.37
70.	Tetratetracontane	23.191	0.73
71.	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-	23.948	1.61
72.	Vitamin E	25.064	0.87
73.	.gamma.-Sitosterol	26.949	0.81
74.	.beta.-Amyrin	27.182	3.13
75.	.alpha.-Amyrin	27.451	0.90
76.	4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	27.716	5.25
77.	Urs-12-en-3-ol, acetate, (3.beta.)-	28.001	0.71

Appendix(13)

Chemical composition of *T. vulgaris* leave by GC/MS

NO.	Name	Ret.Time	Area%
1.	Thymol	8.115	5.74
2.	Naphthalene, 1,2,3,4-tetrahydro-1-methoxy-	12.647	1.89
3.	Apiol	12.870	2.30
4.	2-Pyridinamine, 6-methyl-	13.465	1.44
5.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	14.370	0.48
6.	2-Pentadecanone, 6,10,14-trimethyl-	14.463	0.40
7.	n-Hexadecanoic acid	15.749	10.27
8.	Ethyl 9-hexadecenoate	15.877	1.16
9.	Hexadecanoic acid, ethyl ester	15.935	18.50
10.	Phytol	17.095	7.92
11.	9,12-Octadecadienoic acid (Z,Z)-	17.383	3.38
12.	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	17.452	1.45
13.	Linoleic acid ethyl ester	17.525	17.68
14.	Ethyl 9,12,15-octadecatrienoate	17.596	14.31
15.	Octadecanoic acid, ethyl ester	17.777	1.57
16.	2-Butenoic acid, 2-methyl-, 2-(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-7,10-dihydroxy-1,1,3,6,9-pentamethyl-4a,7a-epoxy-	18.871	1.46
17.	Eicosanoic acid, ethyl ester	19.475	0.46
18.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	20.518	2.03
19.	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	21.901	2.27
20.	Ethyl tetracosanoate	22.508	0.14
21.	Squalene	22.778	0.37
22.	Vitamin E	25.062	0.17
23.	Stigmasterol	26.344	2.02
24.	.gamma.-Sitosterol	26.937	2.59