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Research Article

Phytochemical screening and in vitro antimicrobial activity of *Rosmarinus officinalis* leaves

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Abstract. Rosmarinus officinalis is a medicinal plant, which belongs to family Lamiaceae. It is an evergreen bush. It was analyzed for its phytochemical constitution and antibacterial activity. For this purpose, leaves of n-hexane, ethyl acetate and methanolic extracts were used. The tests of phytochemical screening showed that the plant leaves contain anthraquinones, terpenoids, flavonoids, reducing sugars, saponins, cardiac glycosides, coumarins, phenols, carbohydrates, proteins, steroids, alkaloids and quinones, while tannin was absent. The antibacterial activity was determined against *E. coli* and *staphylococcus aureus*; the invitro antibacterial activity was performed by disc diffusion method. The n-hexane and ethyl acetate extracts showed no antibacterial activity against E. coli while ethyl acetate extracts showed antibacterial activity against Staphylococcus sp. The results showed that the highest mean inhibition zone was observed in the methanolic extracts.

Keywords: Rosmarinus officinalis, N-hexane, Ethyl acetate, Methanolic Extract; Phytochemical screening; Antibacterial activity

1. Introduction

Green plants synthesize and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as raw material for various scientific investigations. Many secondary metabolites of plant are commercially important and find use in number of pharmaceutical compounds. Phytomedicine can be derived from barks, leaves, flowers, roots, fruits and seeds that are used for therapeutic purposes. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al, 2008).

Our study was done on plant leaves (*Rosmarinus officinalis*) for determining the main constituents in this plant using phytochemical screening and assess the possible antimicrobial activities of the plant leaves using methanol, ethyl acetate and n-hexane extracts at different concentrations against pathogenic bacteria (*Escherichia coli sp* and *Staphylococcus aureus*) compared to reference antibacterial drugs. *Rosmarinus officinalis* has different phytochemicals that present active against different diseases (Nisar et al, 2006) and different chemical compounds called secondary metabolites are involved in the growth and developments of plants such as alkaloids, terpenes, and phenolic compounds. It contains also different anti-oxidant compounds including phenolic compounds like phenol acid, flavonoids, tannins, etc (Mierici 2009, Parekh and Chanda, 2007). Rosemary is a medicinal plant and used in many ways as a medicinal herb such as it improves the blood circulation (Jalacic et al. 2007). The extract of rosemary relaxes the smooth muscles increasing the therapeutic properties. It helps in treatment of various human diseases such as coronary atherosclerosis, Alzheimer's disease, cancer, as well as in ageing processes.

Rosemary extract has a choleretic, hepatoprotective and anti-tumerogenic, antioxidant activities that reduce the oxidative stress in cells and therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases, and inflammatory diseases. It contains also rosmarinic acid and phenolic diterpenes which are widely used in the food industry.

Rosmarinus officinalis being a medicinal plant, one of them that have been used for centuries as traditional health remedies for human diseases because they contain chemical components of therapeutic value (Nostro, 2000), has an antibacterial activity that was determined against *E. coli* with different concentrations (i.e 0.2 gm) kept for 24 hours duration. The extracts of rosemary contain bioactive properties such as phenolic compound which inhibits the growth of gram negative and gram-positive bacteria. *Escherichia coli* are gram negative bacteria and are a symbiotic bacillus of colon of human beings and vertebrates. *Staphylococcus aureus* are gram positive bacteria and has serious infections on of human body parts. These bacteria are easy to grow in the artificial medium at 37°C under optimal conditions and divide after every 20 minutes (Okwu and Joseph, 2021). *E. coli* has been implicated as one of the major bacteria causing diarrhea.

1.1 Description of The Plant

Rosmarinus officinalis is a medicinal plant belonging to the family Lamiaceae. It is an evergreen, perennial bush, the height is 1-2 meters long (Okwu et al., 2006). It is a much-branched shrub. The branches are cylindrical and the bark of this plant is pale brown fibrous. The leaves of this plant are evergreen opposite, 1-1inch long, blunt at both ends, densely covered with white stellate hairs and flowers are shortly stalked; rather large arranged in opposite pair bracts short acute, calyx is tubular and deeply cut into two lips. Corolla has short tube and opposite one another. The flowers are small and pale blue. The upper lip is cut into two segments. The stamens are filamentous (Ali et al., 1990). It is an evergreen perennial shrub, best known for its strongly aromatic, needle-like evergreen leaves. The flowers are purplish white and strongly two-lipped, and have two long-exserted (protruding) stamens. The fruit consists of four dry nutlets. It is found especially the mediterranean dishes and it has naturally occurred antioxidant and derives its name from its refreshing effects (Mohsenzadeh et al., 2007) and decorative plant gardens or even as a fence, having many culinary and medical uses.

Table 1. *Scientific classification of the plant* (Miliauskas et al., 2004)

Kingdom	Plantae
Class	Equisetopsida
Subclass	Magnoliidae
Order	Lamiales
Family	Lamiaceae
Genus	Rosmarinus

R. officinalis showed good adaptability in cold and dry areas (Ahmad 2022) cultivated in all countries as an ornamental and medicinal plant, aromatic and seasoning plant used in cosmetic and perfume industries (Jalacic, 2007) and also used as a flavoring agent. Rosemary oil has an effective antimicrobial agent inhibiting the growth of molds and many types of bacteria. Rosemary extract relaxes smooth muscles and has choleretic, hepatoprotective and antitumerogenic activity.

1.2 Composition of the plant

The plant is mainly composed of different components including alkaloids, which are nitrogen organic compounds from vegetable materials which are heterocyclic compounds having potent activity. They are naturally occurring that have a pharmacological effect on humans and other animals (Miliauskas *et al.,* 2004). The name derives from the word alkaline; originally, the term was used to describe any nitrogen containing base (an amine in modern terms). They often have pharmacological effects and used as medications, reduce smooth muscle spasms, recreational drugs, or in entheogenic rituals and have anticholinergic activity. Alkaloids are crystallizable solids, which have rotary capacities. They form salt with acids and solubility depends on their respective pH. When are basic, they are soluble in non-polar solvents but not water. If they are isolated as salts, they are soluble in water and in several polar solvents. They are used in clinical practice, and are still used in medicine usually in form of salts. They act as vasodilating, antihypertensive, anesthetic, analgesic, muscle relaxant. And in treatment for acute pain, such as in severe physical trauma, myocardial infarction, post - surgical pain, and chronic pain, including end - stage cancer and other terminal illnesses in United Kingdom under the name diamorphine (heroin) or morphine (Janga and Tzakos, 2009).

The anthraquinones as component of the plant are quinones which are oxygenized compounds corresponding to the aromatic oxidation. Quinone is a member of cyclic organic compound containing two carbonyl, C=O, either adjacent

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or separated by a vinylene group –CH=CH in a six membered unsaturated ring (Hege et al., 2012). They are crystallized solids colored in yellow, orange or red and less soluble in water and in organic solvents. The free quinones are insoluble in water and extracted in usual organic solvents. They are detected after their hydrolysis using acid. The very useful reaction to characterize them is that of BORTNTRÄGEN (KOH 5% in ethanol) which is positive only for free oxidized quinone, solubilizing it in alkaline solution. They are biologically active compounds, and benzoquinones and naphthoquinones are antimicrobial, antifungal, purgative and can act as vermifuges. Derivatives of quinines are common constituents of biologically relevant molecule, for example vitamin K having a coagulant action (Ufitamahoro, 2011).

The flavonoids are collectively known as vitamin P and Citrin. They are a class of plant of secondary metabolites. They have been identified, many of which occur in fruits, vegetables and beverages like tea, coffee, beer, wine and fruit drinks. They are all ketone containing group with three central ring structures in the form of C6-C3-C6 and recognized dietary antioxidants are vitamin C, vitamin E, selenium, and carotenoids (Bai, N. et al., 2010). Both groups of compounds can occur as glycosides and contain yellow polyphenolic pigments involved in an effect as general antioxidant (Muhizi, 2012). They are soluble in water and alcohol, insoluble in ether, chloroform and benzene. Most genins are soluble in polar organic solvents, when they possess at least free phenol group, they dissolve easily in alkaline medium giving yellowish solution. Flavonoids and proanthocyanidins are all pigments occurring in a long range of plant family (Yang et al., 2001). Flavonoids (both flavonols and flavanols) are most commonly known for their antioxidant activity. They are essentially used to treat vein disorders. Its action is essentially localized on small veins and capillaries decreasing permeability and increase capillaries resistance, so they play the role of vitamin P (Muhizi, 2012). Thus, these compounds are very tolerated by human body and have potential beneficial effects on human health being antiviral, anti-allergic, anti-tumor. They are not very toxics however; their action is very slow.

In addition to these compounds, it has saponins which have a bitter taste, class of many secondary metabolites found in natural sources, some being found in particular abundance in various plants. More specifically, they are amphipathic glycosides classed; in term of phenomenology, by the soap-like foaming obtained when shaken in aqueous solution (Abdulkadir et al., 2007, Quideau. et al., 2011, Harborne, 2015) and in terms of structure, the composition of hydrophilic glycosides moeitites combined with a lipophilic triterpene. They have physiological, bacteriostatic, anti-tumor activity makes these compounds attractive backbone derivatization and screening as novel therapeutic agents and pharmacological properties. They act as natural component of soy and has been intensively investigated as a chemopreventitive agent, mainly against hormonally regulated breast and prostate cancers in animal models being principally oral anticoagulants (Finn et al, 2002, Winkel-Shirley, 2002, Andersen. 2006). They have a P-vitamin action against vein troubles, act as antibiotic against Brucella (Umbelliferone case), have a photosensitive property and used in the treatment of psoriasis (Muhizi , 2012, Boudet, 2007, Yoshida. et al., 2001). This finding supports the traditional use of this plant for the treatment of malaria (Dexeus et al., 2018; Kashiwada, 1992).). They are other compounds found in such as terpenoids, tannins, phenolics, etc.

1.3 Bacteria (E. coli and S.aureus)

Escherichia coli is the most commonly encountered member of the family Enterobacteriaceae in the normal colonic flora and the most common cause of opportunistic infections. All members of the family Enterobacteriaceae are facultative, all ferment glucose and reduce nitrates to nitrites and all are oxidase negative (Sherris, 1984). Escherichia coli are gram-negative, non-sporing bacilli with most strains being motile and generally possessing both sex pili and adhesive fimbriae (Mahon and Manuselis, 1995). It was initially considered a non-harmful member of the colon flora, but is now associated with a wide range of diseases and infections including meningeal, gastrointestinal, urinary tract, wound and bacteremia infections in all age groups.

Members of the genus *Staphylococcus* (staphylococci) are Gram positive cocci that tend to be arranged in grape-like clusters Staphylococci are spherical cells about 1 m in diameter arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures. Young cocci stain strongly gram-positive; on aging, many cells become gram-negative. Staphylococci are non-motile and do not form spores. Staphylococcus aureus is a facultative anaerobe that grows at an optimum temperature of 37ºC and an optimum pH of 7.5. S. aureus produces white colonies that tend to turn a buff- golden color with time, which is the basis of the species epithet aureus (golden). Most, but not all, strains show a rim of hemolysis clear surrounding β the colony (Ryan and Ray, 2004). On nutrient agar, following aerobic incubation for 24 hours at 37ºC, colonies are 1 – 3mm in diameter, have a smooth glistening surface, an entire edge and an opaque pigmented appearance. S. aureus causes serious infections of the skin, soft tissues, bone, lung, heart, brain or blood. Include pneumonia, bacteremia leading to secondary pneumonia and endocarditis, osteomyelitis secondary to bacteremia and septic arthritis, seen in children and in patients with a history of rheumatoid arthritis. Diseases caused by Staphylococcal toxins include scalded skin syndrome and toxic shock syndrome (Sherris, 1984).

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2. Method

2.1. Plant materials

Fresh leaves of *Rosmarinus officinalis* (Teyi) were collected in TUMBA Sector, HUYE District, Southern Province. The leaves were dried in the Oven and milled into powder with aid of a grinder, and the powder was stored before analysis.

2.2. Laboratory materials

Rotary evaporator, Conical flask, separating funnel, test tubes, wash bottles, funnels, watch glass, spatula, beakers, graduated pipettes, oven, water bath, electrical balance, flat bottom flask, dishes, cotton wool, filter paper, burettes, volumetric flask, decanting glass, gloves, stands, pincers, pipette Pasteur, chromatographic chamber, pencil, ruler, ultraviolet lamp and chromatographic plates.

2.3 *Materials and reagents*

Warner reagent, Dragendoff's reagent, Mayer reagent, Benedict reagent, Millon's reagent, ninhydrin solution, glacial acetic acid, concentrated sulphuric acid, distilled water, aluminium chloride, ferric chloride, potassium chloride, sodium hydroxide, acetic anhydride, chloroform, ammonia, ethyl acetate, water, olive oil, alcohol, hydrochloric acid, n-hexane and methanol.

2.4 Thin Layer Chromotography (TLC)

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved (Vogel and Tatchell, 2009). The mobile phase has different properties from the stationary phase. For example, with silica gel, a very polar substance, non-polar mobile phases such asheptane are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet. To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase (The mobile phase must not be allowed to reach the end of the stationary phase.) This ratio is called the retention factor or Rf. In general, substance whose structure resembles the stationary phase will have low Rf, while one that has a similar structure to the mobile phase will have high retention factor. Retention factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase.

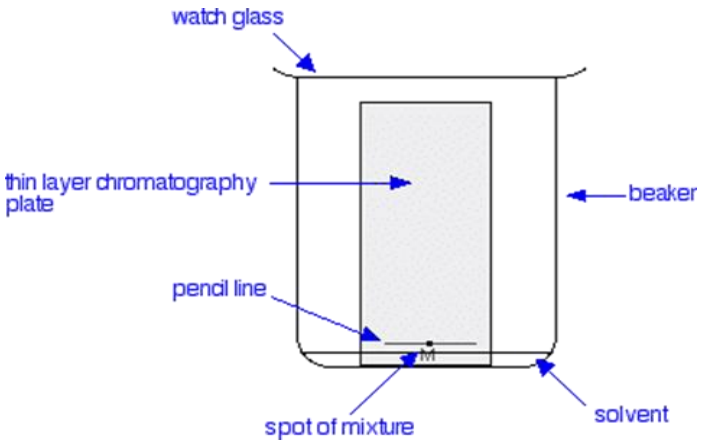


Figure 1. Set up of thin layer chromatography

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For this reason, chemists usually apply a sample of a known compound to the sheet before running the experiment. Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, as saying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents (Reich and Schibli 2007).

2.5 Testing

2.5.1 Preparation of Rosmarinus officinalis leaves extract

Fresh leaves of *Rosmarinus officinalis* (Figure 2) were collected in TUMBA Sector on 6th March, 2017. The leaves were dried at room temperature directly after collection and washing them properly, then they were dried in oven for 24 hours and milled into powder with aid of a grinder, and finally stored it before analysis. In this study, we used maceration method to get extract and three solvents were used for comparing final results at the end of experiments. These solvents are methanol, ethyl acetate and n-hexane.



Figure 2. Fresh leaves (left) and dried leaves of of *R. officinalis* (right)

2.5.2 *Extraction and crude extract preparation*

20g of *R. officinalis* powder were soaked in 100 ml of deionized water for 30 minutes in a beaker and voltex for 5 minutes to obtain a suspension. The suspension was filtered with whatman filter paper. The results are recorded in Table 1.

The extraction is done in 4 steps by weighting 50 g of powder (of plant material), macerate in 300 ml of the solvent (Methanol, n-hexane and ethyl acetate respectively) in decanting glass during 48 hours, filtrate using a cotton wool in the bottom of decanting glass and finally, concentrate the filtrate using rotator evaporator at 40 0C. Perform these 4 steps for Methanol, N-hexane and Ethyl acetate extraction respectively.



Figure 3. (a) Maceration of *R. officinalis* powder in decanting glass with n-hexane, ethyl acetate and methanol solvents, **(b)** Macerate of *R. officinalis* powder with n-hexane, ethyl acetate and methanol solvents and **(c)** Concentration of extract of *R. officinalis* leaves on rotavapor respectively.

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2.5.3 *Phytochemical Screening*

The test for anthraquinones is done by taking a sample (0.5g) of the aqueous extract, boiled with 10 ml of sulphuric acid, H_2SO_4, and filtered while hot. The filtrate was shaken with 5 ml of chloroform and the chloroform layer pipetted into another test tube after which 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes. The presence of anthraquinones is indicated by the yellow colour, while the test for terpenoids (Salkowski test) is done by taking 2 ml of chloroform and added to 0.5g sample of the extract. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. The presence of terpenoids is indicated by a reddish brown coloration at the interface.

The test for flavonoids is done by taking diluted ammonia (5 ml) and add a portion of an aqueous extract. Concentrated sulphuric acid (1 ml) was also added. The presence of flavonoids is indicated by a yellow color that disappear on standing. A second method entailed addition of a few drops of 1% Aluminum a portion of the filtrate. The presence of flavonoids is indicated by a yellow color. For the third method, the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. The presence of flavonoids is indicated by a yellow color, while the test for reducing sugars (Fehling's test) is basically done by using an equal volume of Fehling A and Fehling B reagents which were mixed together to make the mixture. 2 ml portion of the mixture were added to 1ml of crude extract of the plant and boiled gently. The presence of reducing sugars is indicated by a brick red precipitate that appears at the bottom of the test tube.

The test for saponins consists of taking 5 ml of distilled water and added to 0.5 g of extract in a test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously. The presence of saponins is indicated by the formation of emulsion, while the one for tannins consits of using 0.5 g of the extract and boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added. The presence of tannins is indicated by a brownish green or a blue-black coloration positively observed. The result is indicated in Table 2 .

The test for alkaloids is done by using 0.5 g of extract which was diluted to 10 ml with alcohol (ethanol), boiled and filtered. To 5 ml of the filtrate 2 ml of dilute ammonia was added. Chloroform (5ml) will be then added followed by shaking to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions: Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The presence of alkaloids is indicated by the formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent), while the test for cardiac glycosides (Keller-Killiani test) consists of taking a sample of the extract (0.5g); mixed with 5 ml distilled water and 2 ml of glacial acetic acid containing one drop of ferric chloride solution was also added. This was underplayed with 1 ml of concentrated sulphuric acid. The presence of a deoxysugar of cardenolides is indicated by a brown ring at the interface. A violet ring appeared below the brown ring, while in the acetic acid layer a greenish ring formed just above the brown ring and gradually spreaded throughout these layers. The presence of cardiac glycosides is indicated by a greenish ring above the brown ring.

The test for Phenols is done by taking a quantity of 1 ml of the extract, 3 ml of distilled water followed by few drops of 10% aqueous Ferric chloride solution added into the mixture. The presence of phenols is indicated by the formation of blue or green color, while the test for carbohydrates (Benedict's test) consits of using 1 ml of crude extract which was mixed with 2ml of Benedict's reagent and boiled. The presence of the carbohydrates is indicated by a brown precipitate. The test for proteins (Ninhydrin test) is performed by using 1 ml of crude extract and mixed with 2ml of 0.2% solution of Ninhydrin and boiled. The presence of proteins is indicated by a violet colour precipitate, while the one for Coumarin is done by using 10 % Sodium hydroxide chich was added to the extract and chloroform was also be added. The presence of Coumarins is indicated by the formation of yellow color. The test for Steroids consists of taking 2 ml of acetic anhydride which were added to 0.5 ml of crude extract containing 2 ml of sulphuric acid. The presence of steroids is indicated by the colour change from violet to blue or green in samples, while the test for Quinones is done by using diluted sodium hydroxide chich was added to the 1 ml of crude extract. The presence of quinones is indicated by a blue green or red coloration. Note that the results of these procedures are indicated below in Table 2.

2.5.4 Separation Methods By Thin Layer Chromotography (TLC)

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures and different solvents have been used as eluent to detect the presence of active principles. The materials to use areTLC plates: a silica gel plate with no added dyes; 10 x 20 or 20 x 20 cm, TLC tank and lid, organic solvents for running plate, pencil, UV lamp, ruler, watch glass, gloves and pipette Pasteur, and solvent used are Methanol and ethyl acetate.

The procedures used for consists of firstly, preparing of the solution in container by pouring the eluent in the chromatography tank. To aid in saturation of TLC chamber with solvent vapors, we covered the chromatography tank with a watch glass swirl it gently. Secondly, preparation of the TLC plate by measuring 1cm from the bottom of the plate using the pencil, and we drawed a line across the plate at 1cm mark on which the samples were applied on the

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plate. Thirdly, spot the TLC plate by dissolving each extract in its solvent, and pipettes pasteur were used to take a spot from dissolved extract to the plate.

Forthly, develop the plate by taking those TLC plate into the chromatography tank containing eluent and covered the chamber with the watch glass, and left it undisturbed on our bench top. The solvent rose up to the plate, we removed the plate from the chromatography tank and immediately marked the solvent front with a pencil. Lastly, visualize the spots by circling the colored spots lightly with a pencil and those which appeared confused were visualized using a UV light. To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase, retention factor (Rf) value and is always smaller than 1.

Rf= distance traveled by the substance (Y)/ distance traveled by the solvent(X).

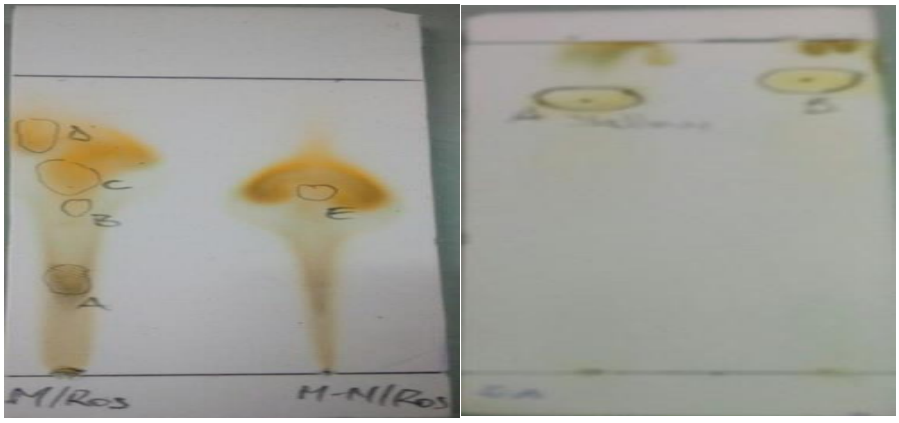


Figure 4. TLC for methanol and n-hexane (left) and ethyl acetate extract (right) of *Rosmarinus officinalis* leaves in methanol as eluent

2.5.5 Antimicrobial activity of R. officinalis against Escherichia coli extract

The antimicrobial activity was determined using *Rosmarinus officinalis* leaves extracted already with methanol, ethyl acetate and n-hexane solvents against *E.coli* pathogenic human colon bacteria. The experiment conducted to determine the antimicrobial activity of the extracts of *Rosemarinus officianalis* against *E. coli* basing on key step for the successful determination of an organism is sterilization. Sterilization was followed by two methods: dry and wet sterilization. All The glassware (Petri-plates) were covered with aluminium foil and then kept into the oven at 37°C for two hours.

- ✓ **Materials and Methods:** Antimicrobial activity was assessed by the agar well diffusion method (Kinsbury and Wagner, 1990). The inoculums suspension was spread over lurial agar medium plates to achieve confluent growth, and allowed to dry. Petri-plates, laminar flow, beakers, spatula, test tubes, wax matches, spreaders, loops, balance, aluminium foils, incubator, autoclave, toothpicks, cotton wool, ethanol, distilled water, ampicillin and bacterial strain.
- ✓ **Preparation of extracts:** Methanol, ethyl acetate and n-hexane extracts from *Rosemarinus officianalis* leaves were already prepared and packed in bottles. 0.2g of extract were weighted on each type and put them in test tubes separately, and few drops distilled water in to allow them become more liquid, shook and left them well for 24 hrs. After the completion of time filter the solution and soak the Watmann's filter paper discs into the filtrate.
- ✓ **Preparation of sterile disc:** Whatman's filter paper was punched into 5mm disc form and sterilized. Each sterile disc was incorporated individually with 10 ml of extracts using micropipette with precautions to prevent the flow of the solvent extract from the discs to the outer surface. The condensed extracts were applied in small quantities on discs, while the preparation of Lurial agar is done by taking 5.504g of lurial broth together with 4.8g of agar were suspended in 320ml of distilled water to prepare of lurial agar (L.A) medium in a 1000ml conical flask. This medium was autoclaved at 121 ^{0}C C for 15 minutes.
- ✓ **Preparation of Manitol Salt Agar (MSA):** 35.6g of Manitol Salt Agar were suspended in 320ml of distilled water to prepare of MSA medium in a 1000ml conical flask. This medium was autoclaved at 121 ^{0}C for 15 minutes, while the preparation of peptone broth is done by taking 2.4g of peptone powder were suspended in 160ml of distilled water to prepare peptone broth. This was autoclaved at 121 ^{0}C for 15 minutes.
- ✓ **Serial dilution:** For better quantification of bacterial strain, aliquots of serial diluted samples were spread on the nutrient agar that allowed the growth of all bacterial strains. Serial dilution was done by taking 1ml of

inoculums into 9 ml of peptone broth; after mixing to homogeneous for 30 minutes, 1 ml of the suspension was transferred to a sterile test tube to make 10-1 of the dilution; after agitation 1 ml of the suspension from 10-1 was transferred to another 9 ml test tube of peptone broth to make 10-2 of the dilution, and the dilution was made up to 10-3. A 100 µl of each aliquot of the bacterial strain were spread on the petri-plates (nutrient agar plate) by the help of the spreader and incubated at 37 °C for 24 hours.

- ✓ **Assay of antimicrobial activity using Disc diffusion method:** The 25 ml of sterilized lurial and manitol salt agars were poured into sterile petriplates. After solidification of the medium on petriplates , 50 µl of inoculed bacterial strains were spread on the respective plates. The sterilized discs containing extracts were put over the agar plates using sterile forceps at different concentrations (10-1, 10-2 and 10-3 M) from serial dilution. The plates were incubated for 24 hours at 370C in oven. After incubation, the diameter of inhibitory zones formed around each discs were measured in cm and recorded (Mahalngam.R. et al., 2011).
- ✓ **Antimicrobial activity of commercially available antibiotics:** The antimicrobial activities of plant extracts on human pathogenic bacteria were compared with the commercially available antibiotic. The sterilized discs containing antibiotic (ampicillin) were placed on the surface of the plates and incubated at 37 ^{0}C for 24 hours. After incubation, the diameter of the inhibition zones was measured in cm and recorded (Daniyan, S.Y. and H.B. Mahammad., 2008).

3. Result and Discussion

3.1. preparation of crude extract

During extraction process of *Rosmarinus officinalis* leaves, we used maceration method with solvents such as methanol, n-hexane and ethyl acetate respectively.

Table 2, Extract of *Rosmarinus officinalis* leaves

Solvent used	Mass of powder extracted (g)	Mass of extract obtained (g)	Yield (%)
Methanol	50	8.2	16.3
N-hexane	50	2.9	5.9
Ethyl acetate	50	5.2	10.4
Water	20	6.2	31

From Table 2, by extracting 50 g of powder, 8.2 g was obtained as mass of the extract, leading to the yield of 16.3% using methanol extraction, 50 g of powder; 2.9 g was obtained as mass of the extract, leading to the yield to 5.9% by using n-hexane extraction while, by using 50 g of powder, 5.2 g was obtained as mass of the extract, leading to the yield 10.4% by using ethyl acetate extraction method. Lastly, using 20 g of powder, 6.2 g was obtained as mass of extract leading to the yield of 31% (Herrero et al, 2010). After all, we saw that n-hexane extract has a small yield compared to other solvents.

3.2. Phytochemical screening of Rosmarinus officinalis leaves

Based on phytochemical screening method discussed in 3.1, the results showed that *Rosmarinus officinalis* leaves contains anthraquinones, terpenoids, flavonoids, reducing sugars, saponins, cardiac glycosides, coumarins, phenols, carbohydrates, proteins, steroids, alkaloids and quinones. All results obtained from phytochemical screening on the *Rosmarinus officinalis* leaves with methanol, n-hexane an ethyl acetate are recorded in the Table 3.

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Table 3 Results of Phytochemical screening of Rosmarinus officinalis leaves

Active compound	Reagents used	Expected result	Obtained result	Conclusion
Anthraquinones	H_2SO_4, chloroform, dilute NH_3	Yellow colour	Yellow colour	++
Alkaloids	Chloroform, acetic acid, Mayer's reagent and Draggendorff's reagent	Formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent	Formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent	++
Terpenoids	**C**hloroform, Conc H_2SO_4	Reddish brown colour	Reddish brown colour	++
Flavonoids	Dilute NH_3, Conc H_2SO_4	Yellow color	Yellow color	++
Tannins	Water, $FeCl_3$	brownish green or a blue-black coloration	No colour change	-
Phenolics	Distilled water, aqueous $FeCl_3$	Formation of blue or green color	Formation of blue or green color	+
Saponins	Distilled H_2O, olive oil	Formation of emulsion	Formation of emulsion	+
Reducing sugar (Fehling's test)	Fehling A, Fehling B	Brick red precipitate	Brick red precipitate	+
Cardiac glycosides (Keller-Killiani test)	Distilled water, glacial acetic, ferric chloride, conc H_2SO_4. And gradually spreaded throughout these layers.	Brown ring at the interface, violet ring below the brown ring, With acetic acid layer, greenish ring just above the brown ring	Greenish ring above the brown ring	++
Coumarins	Chloroform	Formation of yellow color	Formation of yellow color	++
Carbohydrates (Benedict's test)	Benedict's reagent The presence of the carbohydrates is indicated	Brown precipitate	Brown precipitate	++
Proteins (Ninhydrin test)	Ninhydrin solution	Violet colour precipitate	Violet colour precipitate	++
Steroids	Acetic anhydride, H_2S0_4	Colour change from violet to blue or green	Colour change from violet to blue or green	++
Quinones	Dilute NaCl	Blue green or red colour	Blue green or red colour	++

++: High concentration, +: Low concentration, -: Absent.

Rosemarinus officinalis underwent analysis to determine its chemical composition. It tested positively for Anthraquinones, Alkaloids, Terpenoids, Flavonoids, Phenolics, Saponins, reducing sugar (Fehling's test), Cardiac glycosides (Keller-Killiani test), Coumarins, Carbohydrates (Benedict's test), Proteins (Ninhydrin test), Steroids, and Quinones, indicating their presence in the plant. Conversely, Tannins yielded negative results, indicating their absence.

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3.3. Splitting Of Crude Extract using thin-layer chromatography

The crude extracts from Rosmarinus officinalis leaves were analyzed by TLC. The previous figures show the component groups of methanol, ethyl acetate and n-hexane extracts. Methanol extract in Table 4 showed four constituents A, B, C and D while n-hexane in Table 4 showed the constituent E and F. Ethyl acetate extract in Table 4 showed the constituents A and B. All results of retention factors are summarized in the following table. The TLC analysis of the methanol extract, depicted in Table 4, revealed retention factors of 0.3 for component A, 0.542 for B, 0.657 for C, and 0.814 for D. In the ethyl acetate extract, components A and B displayed retention factors of 0.812 and 0.875, respectively. The n-hexane extract showed retention factors of 0.144 for component E and 0.4605 for F.

Table 4 Retention factors of Rosmarinus officinalis leaves on TLC

Extract	**Result**
Methanol extract in methanol solvent	A: R_f=0.3
	B: R_f=0.542
	C: R_f=0.657 and D: R_f=0.814
Ethyl acetate extract	A: R_f=0.812
	B: R_f=0.875
N-hexane extract	E: R_f=0.144
	F: R_f=0.4605

3.4. Antimicrobial activity of Rosmarinus officinalis

The antimicrobial activity of methanol, ethyl acetate and n-hexane extracts of *Rosmarinus officinalis* plant were investigated using disc diffusion method against *Escherichia coli sp*. This pathogen has also tested with commercially available antibiotic. All plant extracts used against the pathogenic organism have showed varied degree of antimicrobial activity against the pathogen.

- Antimicrobial activity of ethyl acetate extracts: The antimicrobial activity of ethyl acetate extracts of *Rosmarinus officinalis* on *Escherichia coli sp* and *Staphylococcus aureus* using disc diffusion method has showed maximum zone of inhibition 1.1 cm at 10^{-1} M, 0.9 cm at 10^{-2} M, 0.7 cm at 10^{-3} M and 0.7 cm at 10^{-1} M, 0.8 cm at 10^{-2} M concentrations of bacterial strains respectively.
- Antimicrobial activity of n-hexane extracts: The antimicrobial activity of n-hexane extracts of *Rosmarinus officinalis* on *Escherichia coli sp* and *Staphylococcus aureus* using disc diffusion method has showed no inhibition zone.
- Antimicrobial activity of commercially available antibiotics: Ampicillin has wide range of impact on *Rosmarinus officinalis* leaves extracts. The maximum zone of inhibition was observed against *Escherichia coli sp* on methanol extract was 1.7 cm at 10^{-1} M, 1.6 cm at 10^{-2} m and 1.5 cm at 10^{-3} M concentrations. On ethyl acetate extract, it had an inhibitory zone of 1.3 cm at 10^{-1} M, 1.3 cm at 10^{-2} M and 1.2 cm at 10^{-3} M concentrations and n-hexane 0.7 cm at 10^{-1} M, 1.5 cm at 10^{-2} M and 1.8 cm at 10^{-3} M concentrations of bacterial strain. From this, it showed that Ampicillin showed a high inhibition zone on methanol extract.

The maximum zone of inhibition observed against *Staphylococcus aureus* on methanolic extract was 1.5 cm at 10^{-1} M and 10^{-2} M concentrations. On ethyl acetate extract, it had an inhibitory zone of 1.6 cm at 10^{-1} M, 1.7 cm at 10^{-2} M concentrations of bacterial strain. From this, it showed that Ampicillin showed a high inhibition zone on ethyl acetate extract.

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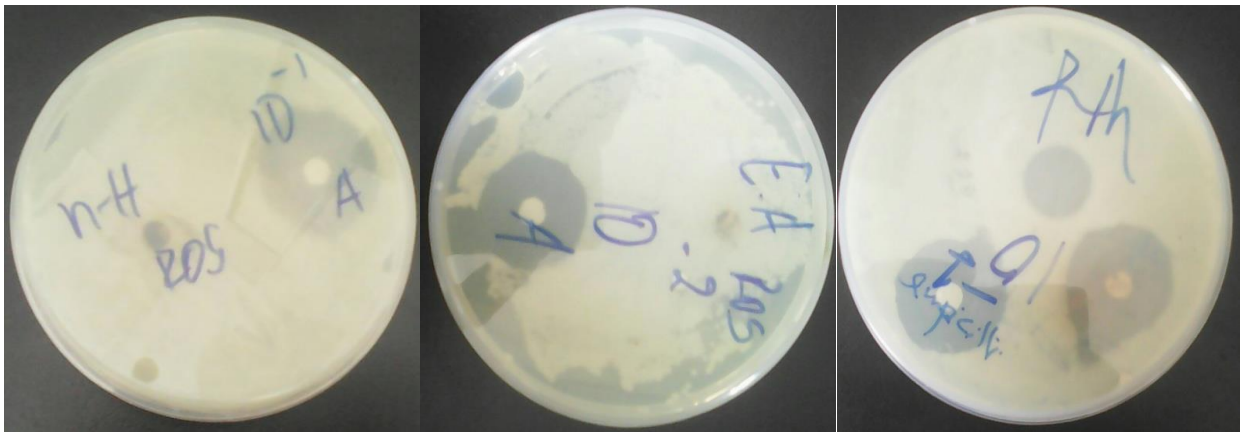


Figure 5. Antimicrobial activity of *Escherichia coli* on n-hexane, ethyl acetate and methanol extract respectively.

3.5 Discussion

The plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being. The use of plant extracts with known antimicrobial properties can be of great significance for therapeutic treatment (Wu et al., 2021).

The *Rosemarinus officinalis* was investigated for its phytochemical constitution according to the compounds making them, positive when tested against Anthraquinones, Alkaloids, Terpenoids, Flavonoids, Phenolics, Saponins, Reducing sugar (Fehling's test), Cardiac glycosides (Keller-Killiani test), Coumarins, Carbohydrates (Benedict's test), Proteins (Ninhydrin test), Steroids and Quinones, meaning that they present in the *R. officinalis* and show a negative test when tested against Tannins, meaning that they are absent. The tests against the anti-microbial activity resulted on ethyl acetate extracts of *Rosmarinus officinalis* on *Escherichia coli sp* and *Staphylococcus aureus* using disc diffusion method has showed maximum zone of inhibition of 1.1 cm at 10^{-1} M, 0.9cm at 10^{-2} M, 0.7cm at 10^{-3} M and 0.7 cm at 10^{-1} M, 0.8 cm at 10^{-2} M concentrations of bacterial strains respectively. On n-hexane extracts, the antimicrobial activity on *Escherichia coli sp* and *Staphylococcus aureus* using disc diffusion method has showed no inhibition zone. And on commercially available antibiotics: Ampicillin showed a wide range of impact on *Rosmarinus officinalis* leaves extracts with an inhibition zone against *Escherichia coli sp* on methanol extract of 1.7 cm at 10^{-1} M, 1.6 cm at 10^{-2} M and 1.5 cm at 10^{-3} M concentrations, 1.3 cm at 10^{-1} M, 1.3 cm at 10^{-2} M and 1.2 cm at 10^{-3} M concentrations on ethyl acetate extract, and 0.7 cm at 10^{-1} M, 1.5 cm at 10^{-2} M and 1.8 cm at 10^{-3} M concentrations of bacterial strain n-hexane extract. From this, it showed that Ampicillin showed a high inhibition zone on methanol extract. The result of on TLC showed that on methanol extract as shown in Figure 5, the retention factor is of 0.3 on A, 0.542 on B, 0.657 on C and 0.814 on D constituents respectively, 0.812 on A and 0.875 on B constituents when using the ethyl acetate extract and 0.144 on E and 0.4605 on F constituents when using n-hexane extract.

In this present study, preliminary screening for antimicrobial activity showed, that the methanolic extract of *R. officinalis* exhibited maximum inhibitory zone against *E. coli* than *S. aureus*. Results obtained from this study, indicated that, the plant extracts showed the least antimicrobial activity than the commercially available antibiotics. For instance, ampicillin showed the maximum zone of inhibition against *E. coli sp*.

4. Conclusion

From this, *Rosemarinus officinalis* was analyzed for its antimicrobial activity against *E. coli* and *S. aureus* and phytochemical composition. The plant material was collected from TUMBA Sector. To investigate the antimicrobial activity of *Rosemarinus officinalis* plant, leaves were collected and kept for 24 hours for drying before extracting with methanol, ethyl acetate and n-hexane solvents.

The highest inhibition zone was observed in methanolic extracts (i-e. 1.7 ± 1.5 cm). The extracts of n-hexane solvent showed no inhibition zone while ethyl acetate showed mean inhibition zone. Different tests including reducing sugar, flavonoids, terpenoids, tannins, saponins and others were practiced for phytochemical screening. All the tests were positive except tannins. Our results showed best inhibition against *E. coli*. Thus, the researcher suggests future researchers to do an advanced phytochemical study on other parts of these plants, to perform test on their toxicity, biological activities and pharmacological properties.

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