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Determine the Phytochemical Content of *Rosmarinus Officinalis* L. and Estimate its Inhibitory Effectiveness against Some Types of Food Borne Bacteria

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Abstract

Two solvent extracts (Methanol and aqueous) of *Rosmarinus officinalis* were estimated for their antibacterial effectiveness against three types of bacteria that cause food poisoning (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*). The results revealed that the alcoholic extract of Rosemary has significant antibacterial action against bacterial infections compared with aqueous extract. The phytochemical examination revealed the presence of alkaloid, carbohydrates, phenols, flavonoids, tannins, proteins and saponins in the aqueous extract. Additionally, DPPH assay for the antioxidant analysis was also carried out using the two extracts of which alcoholic extract showed exceptional antioxidant activity.

Keywords: Antioxidant activity. Medicinal plant, *Rosmarinus officinalis*, Free radical.

Introduction

In the recent decades, there has been a significant development of novel medicines derived from plants, indicating the crucial role of pharmacological screening in the advancement of new therapeutic agents. Hence, the isolation and characterization of secondary metabolites

derived from medicinal plants hold potential for uncovering noteworthy bioactivities and identifying novel drug candidates for the treatment of various ailments such as diabetes mellitus[1,2].Various studies have illustrated that antioxidant neutraceuticals made from plants rummage free radicals for occasion peroxide, hydroperoxide or lipid peroxyl and hence inhibit the oxidative mechanisms that cause degenerative diseases [3]. Since long times, herbal plants have been used as commendable antioxidant.

In the Mediterranean region, Rosemary (*Rosmarinus officinalis,* L.) is a herb plant that grows wild. These days, this plant is grown all over the world due to its many applications as a typical spice for flavoring. Additionally, due to its naturally high antioxidant activity, Rosemary extracts have been frequently employed as a preservative in the food sector. It has also been utilized as a medicinal herb for centuries, due to noteworthy against a variety of diseases. Because some medications have unfavorable side effects, the use of herbal compounds has become more common due to their hepatoprotective [4], antimicrobial [5,6], antithrombotic [7], diuretic [8], antidiabetic [9], anti-inflammatory [10], antioxidant [11], and anticancer activities [12-15]. They are considered healthier and more environment friendly [16]. They are easy to obtain and well tolerated by the host, with many beneficial pharmacological effects compared to synthetic drugs.

The presence of numerous bioactive compounds in its composition has been attributed to these efficient biological activities. Flavonoids such as genkwanin, cirsimaritin, or homoplantaginin, phenolic diterpenes such as carnosic acid, carnosol, or rosmanol, and triterpenes like ursolic acid are the three major categories of compounds found in Rosemary [17–19].

The objective of this work was to investigate the impact of rosemary plant extracts on the suppression of DPPH-generated free radicals and to qualitatively assess the active chemicals present in the plant using reagents, given their substantial therapeutic significance. Moreover, to assess the antibacterial properties of the plant extract on three distinct bacterial strains, comprising a Gram-positive strain (*S. aureus*) and two Gram-negative strains (*E. coli* and *P. aeruginosa*).

Material and Methods

Collection, Identification and Preparation of the plants extracts:

Rosmarinus officinalis were collected from the local market In Baghdad, the leaves of plant were collected and then leave to dry with clean dish by the help of sun light. After the leaves were dried and grinded. Separately, 10 g of powder samples were dissolved in 100 ml of ethanol 90% and semi hot water, which maintained in conical flasks at room temperature in a shaking bath(BS-31 UK) for 24 hours. After that, the mixture was filtered through muslin and centrifuged at 6,000 rpm for 20 minutes. The supernatant was collected and stored for four hours at 40 °C in the oven. The residual solution was refrigerated for additional phytochemical analysis[20].

Chemical detection for the plant components

Qualitative Phytochemical analysis

Different tests were used to determine the chemical composition of the plant extracts that prepared according to the standard analysis[21]. They included: proteins, carbohydrates, alkaloids, saponins, phenolic compounds, tannins and flavonoids.

1- Determination of Proteins

Biuret test:

The reaction in the biuret test is a colorimetric reaction where the result is indicated by a color change from blue to purple or violet. In an alkaline environment, the cupric (Cu+2) ions in the biuret reagent bind to the nitrogen atoms in the peptide bonds of proteins forming a violet-colored copper coordination complex. The formation of purple color indicates the presence of peptide bonds in the sample.

2- Determination of total carbohydrates by Molisch method

Molisch reagent was prepared, 3.75g of alphanaphthol reagent was dissolved in a 25 ml solution of 99% ethanol. The mixture must always be prepared fresh and concentrated sulphuric acid; each distilled water and test plant extracts is taken about 2 ml in test tubes separately. Two drops of Molisch reagent were added to each tube, the tube hold in an inclined position and gently 1 ml of, concentrated H2SO4 was added along the side of the

test tube, the acid must not be mixed with the solution. A black ring is formed if the concentrated acid is not added slowly, which generates heat from the reaction that can char the carbohydrates. The test tube is observed for the formation of a purple-coloured ring at a layer between the solution and the acid

3- Detection of Alkaloids by Mayer's method

According to this test procedure, 2 ml of concentrated HCl was added to 2 ml of the respective plant extract samples followed by an addition of few drops of Mayer's reagent. Either formation of white precipitate or green color confirmed the existence of alkaloids in that tested sample.

4- Foam test for detection of Saponins

A little amount of plant extracts were shaken vigorously by vortex and formation of permanent foam indicated to saponins refer.

5- Detection of Phenolic compounds

Ferric chloride test was used by taking small quantities of various extracts separaetely in water. Few drops of 5% FeCl3 solution were added to 1 ml of each extracts. The appearance of a green blue or deep blue (black) colour indicates the presence of phenolic compounds.

6- Detection of Tannins:

About 0.1 g of the various extracts was taken separately in 5 ml water and test for the presence of tannins, which was carried out with the following reagents. Braymer's test was used by treating 2 ml of extracts with 10% alcoholic FeCl3 solution, the formation of blue or greenish colour solution indicates the presence of tannins. Lead acetate test was used by adding 1 ml of 10% Pb(CH3COO)2 solution to 1 ml of each extract, the appearance of white precipitate indicate the presence of tannins.

Antioxidant Activity

1- Qualitative determination of free radical scavenging activity (TLC method)

This test carried out according to AOAC [22], The initial analysis of antioxidant components involved the utilization of thin-layer chromatography (TLC) followed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Approximately 100 ml of extract, specifically Standard Gallic acid, together with plant extracts, were introduced onto thin-layer chromatography (TLC) plates measuring 10×10 cm^2, manufactured by Merck (The plates were developed in 10% chloroform in methanol and Methanol:Chloroform:Hexane(7:2:1) to separate the various constituents of the extracts). The plates were subjected to air drying and subsequently exposed to visible and UV-VIS light at wavelengths of 240 nm and 300 nm. Various distinct locations were identified and their retention factor (Rf) values were measured. Following the completion of the analysis, a 0.05% solution of DPPH in methanol was applied over the surface of the thin-layer chromatography (TLC) plates. The plates were subjected to incubation at ambient temperature for a duration of 30 minutes. Yellow dots were seen in the antioxidant active component and plant extracts. The activity of the selected chemicals was assessed by examining the observed changes in the color.

2- Quantitative determination of free radical scavenging activity assay (DPPH method).

The free radical scavenging assay used the procedure outlined by Ahmed *et al*.[23] to measure free radical activity. A total of 24 milligrams of DPPH were dissolved in 100 ml of methanol for making the stock solution. Filtration of DPPH stock solution using methanol yielded a usable mixture with an absorbance of around 0.973 at 517 nm. In a test tube, 3 ml DPPH workable solutions were combined with 100 µl of plant extract. 3 µl of solution containing DPPH in 100 µl of methanol is often given as a standard. After that, the tubes were kept in complete darkness for 30 min. The absorbance was therefore determined at 517 nm. The following formula was used to compute the percentage of antioxidants:

DPPH inhibition (%) = A control- A test/ A control * 100

Standards and samples' percentages of inhibition at various concentrations and standard curve were done showing the correlation between the two extracts. By examining the data, we were able to calculate the IC50 value, which is the concentration of a chemical (in g/ml) that causes a 50% decrease in DPPH absorbance.

Preparation of the standard solution:

Using the method by Braca *et al.* [24]. Stock solutions was prepared by dissolve 1mg of standard Gallic acid in 10ml of methanol to prepare a stock solution of 100 µg/ml. Different concentrations from Gallic acid (25,50,100,200 and 400µg/ml) was prepared from the stock solution 100µg/ml and used as standard solutions.

- **Preparation of sample:**

To prepare a sample stock solution, 10 mg plant extracts were dissolved in 10 ml methanol to prepare a 1 mg/ml stock solution.

- **Preparing the solution of DPPH:**

A weight of 3.9 mg of DPPH was dissolve in 3 ml of methanol and covered from lights by using a dark can covering with aluminum foil.

- **Schedule for estimate of DPPH scavenging capacity:**

1. A volume of 150µl DPPH solution was added to 3ml methanol and absorbance was occupied directly at 517nm for control reading.

2. Various concentrations of extract samples (10, 20, 25, 50 and 60µg/ml) was prepared from 1 ml stock solution and 250µl of each was withdrawn and diluted with methanol up to 1.75ml. A volume of 250µl DPPH solution was added to all of the test tubes.

3. Absorbance was measured at 517 nm in UV- Visible spectrophotometer after 30 minutes by the use of methanol sol as a blank.

4. A volume of 250µl of all the concentrations of Gallic acid (25, 50, 100, 200 and 400µg/ml) was withdrawn and diluted with methanol to the value 1.75ml. A volume of 250µl DPPH sol was added to all of the test tubes.

5. Absorbance was used at 517nm in UV- Visible spectrophotometer after 30 minutes using methanol sol as a blank.

6. Free radical antiradical capacity (%scavenging capacity) was calculated by using the equation below:

Antiradical activity (%) = A control- A test/ A control * 100

Where:

$A_{control}$: the absorbance of reaction for control (containing all indicators except the extract of the sample)

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A_{test}: the absorbance of the plant extracts.

Antibacterial activity of the plant extracts

Bacterial isolates

To test the antibacterial properties of plant extracts, we used three isolates of food poisoning bacteria: *S. aureus* (Gram-positive), and *E. coli and P. aeruginosa* (Gram-negative). The bacteria were obtained from the Department of Biology at Al-Mustansiriya University Baghdad, Iraq. The bacteria were left overnight to grow on agar medium (Mueller–Hinton) at 35°C using a spectro-photometer. We collected 5 ml of sterile saline water and diluted the growth of the bacteria to a concentration of 10^7 CFU/ ml at 580 nm.

Antibacterial activity of plant extracts

The antibacterial activity of the plant extracts was assessed against the test isolates using the Agar-well diffusion assay, following the methodology outlined by Nwinyi *et al*. (2009). The McFarland's standardized isolates (1.5×10^8 CFU/ml) were streaked over the surface of sterile Mueller Hinton Agar (MHA) plates using a sterilized swab stick. Aseptic techniques were employed to hole and label wells on agar plates using a sterile cork borer with a diameter of 6mm. Subsequently, varying quantities (200, 100, and 50 mg/ml) of the extracts were introduced in a consistent volume (0.1 ml) into the respective wells of the plates. The plates were placed on the bench for a duration of 40 minutes to facilitate pre-diffusion of the extract. Subsequently, they were subjected to incubation at a temperature of 37°C for a period of 24 hours. The diameter of inhibitory zones was measured using a transparent ruler calibrated in millimetres. The measurements were analysed to determine the zone width of inhibition shown by the bacterial isolate under investigation at the given concentration. Ampicillin was utilized as the positive control, whereas DMSO was employed as the negative control.

Statistical analysis

The statistical analysis of this study was conducted using an analysis of variables (ANOVA) in line with the SPSS program (2010). The analysis of variance (ANOVA) was employed to assess the differences among many groups, with a minimum statistically significant moral

difference of 0.05 or its equivalent. The experiment involving the suppression of artificially generated free radicals was conducted using three repetitions.

Results and Discussion

Qualitative phytochemical analysis

The present investigation employed chemical reagents to ascertain the presence of distinct chemical components within the plant extracts. Table 1 presents empirical evidence indicating that the plant extracts encompass numerous noteworthy active chemicals that provide considerable therapeutic potential for a wide range of ailments. Alkaloids exhibit solubility in organic solvents, such as ether or alcohol, whereas their corresponding salts demonstrate limited solubility in aqueous solutions. Consequently, alkaloids can be found in both aqueous and alcoholic extracts. The inhibitory effect against various microorganisms exhibited by these groups of chemicals has been documented by Piyawan *et al*.,[25]. Hence elucidating their utilization in traditional medicine. The prior literature has established the antimicrobial efficacy of plant extracts that contain flavonoids [26, 27]. The findings derived from this investigation indicate that the phytochemical components found in the study may possess bioactive properties that contribute to the antibacterial effectiveness of the plant extract.

Table 1: The phytochemical screening in the rosemary extract.

Compounds	Detection method	Colour reaction	Aqueous extract	Alcohol extract
Proteins	Biuret test	Purple blue	+ve	+ve
Carbohydrates	Molish test	Violet ring	+ve	+ve
Alkaloids	Mayer's reagent	White ppt	+ve	+ve
Phenolic compounds	Ferric chloride test	Green ppt	+ve	+ve
Tannins	Lead acetate	Yellow white ppt	+ve	+ve
Saponins	Fast stirring	Dense foam for long time	+ve	-ve

Antioxidant activity

1-Qualitative determination of free radical scavenging activity.

Thin layer chromatography technique was applied for detection antioxidant activity of the extracts of rosemary.

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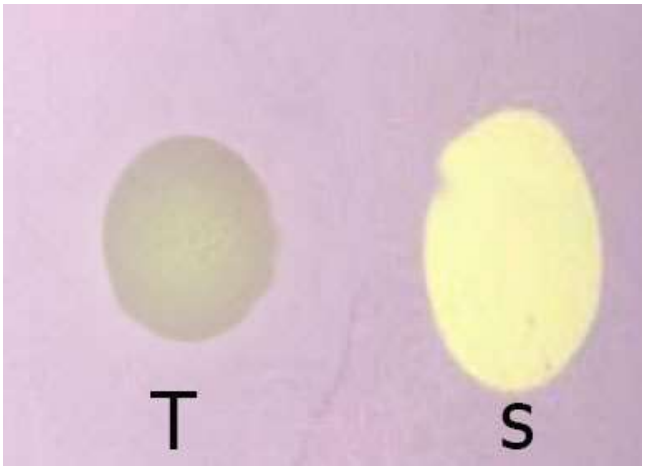


Figure 1: TLC autography result standard Gallic acid and aqueous plant extract

T: Aqueous plant extract S: standard Gallic acid

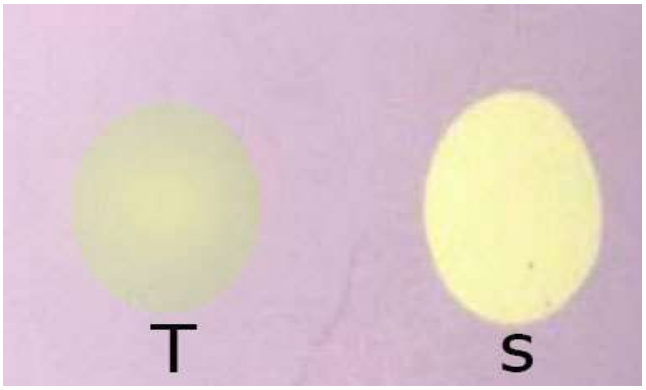


Figure 2: TLC autography result standard Gallic acid and alcohol plant extract

T: Alcohol plant extract S: standard Gallic acid

2- Quantitative determination of free radical scavenging activity assay

The DPPH used to determine the scavenging activity for the plant extract quantitatively which represent in table (2) showed that two extracts have a potent scavenging activity and IC^{50} as competed with Gallic acid, the results which appeared in figures 2,3,4 showed that the scavenging activity for an alcoholic extract is more effective than aqueous extract, the IC^{50} for alcoholic extract was 79.4 µg\ml, while IC^{50} for aqueous extract was 105 µg\ml and the IC^{50} for standard Gallic acid was 38.5 µg\ml. The free radical scavenging activities of the

extracts increased with increasing concentration. Extracts with high free radical scavenging activity also revealed high levels of phenolic compounds (28,29). Rosemary may be a natural source of antioxidants since the latter is metabolically active in which the plant contains rosmarinic acid and rosmarol as antioxidants [30] and its essential oils are used to food preservation, medicine, alternative therapies and natural treatments. The antioxidant capacity of plant extracts for this action, as demonstrated in many investigations, is not due to a single phenolic compound but to a wide distribution among chemical constituents. Phytochemicals, especially anthocyanins.

Table 2: The quantitative antioxidant activity for the rosemary extracts. The standard Gallic acid and their IC^{50}.

Extract Concentration µg\ml	Aqueous extract activity %	Alcoholic extract activity %	Standard Gallic acid activity %
25	11.8	15.2	43.6
50	34.9	41.3	54.8
100	55.3	63.7	63.1
200	67.4	72.6	85
400	73.9	85.7	94.6
IC^{50}	105 µg\ml	79.4 µg\ml	38.5 µg\ml

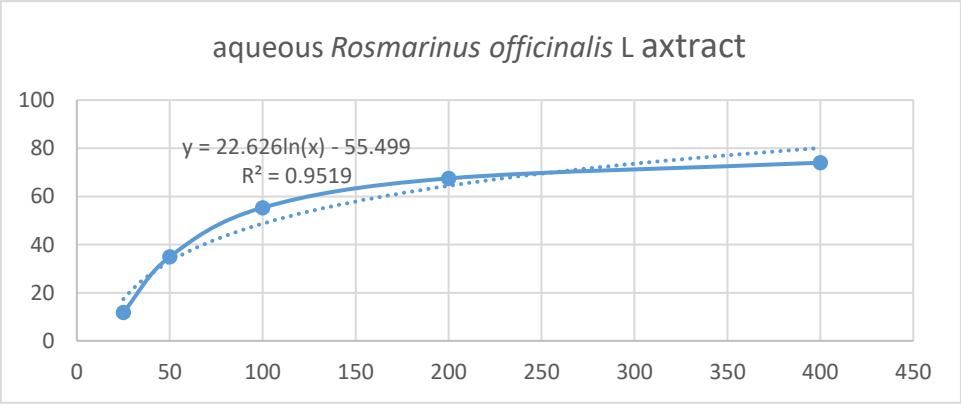


Figure 3: curve for determine IC^{50} for aqueous plant extract.

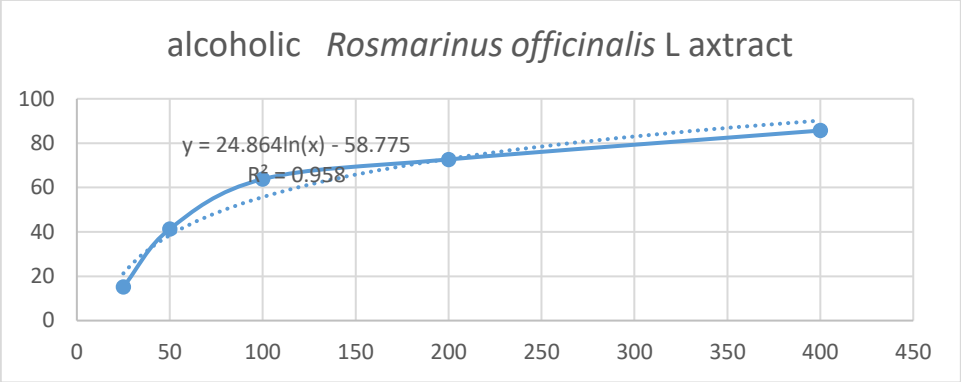


Figure 4: curve for determine IC^{50} for alcoholic plant extract.

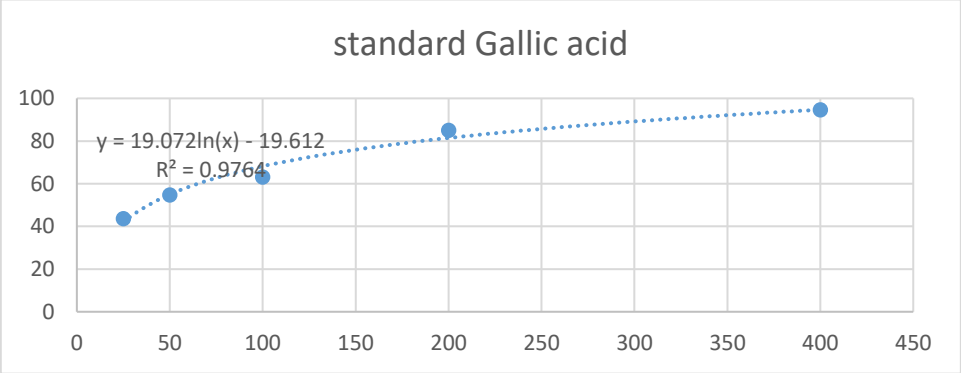


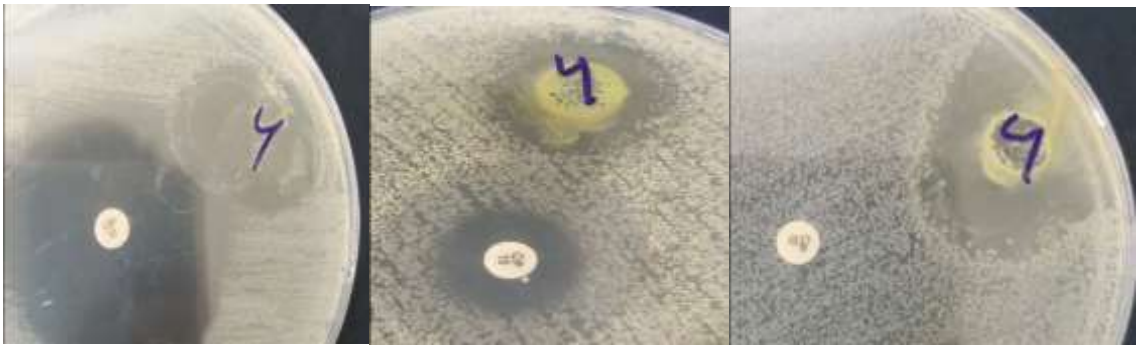
Figure 5: curve for determine IC^{50} for standard Gallic acid.

Antibacterial activity of plants extract

Two plant extracts (water and alcohol) were tested against two isolates of Gram-negative bacteria (*P.* aeruginosa and *E.coli*) and Gram-positive isolates bacteria (*S. aureus*) in order to investigate the antibacterial activity of the plant against pathogenic bacteria . Bacteria were analyzed to determine their susceptibility using the disc diffusion method. On the other hand, ethanol extract was found to be effective against *P. aeruginosa*, *S. aureus* and *E. coli* suppressing their growth with inhibition zones of 14.6, 15.4, 11.8 and 13.4 mm respectively.

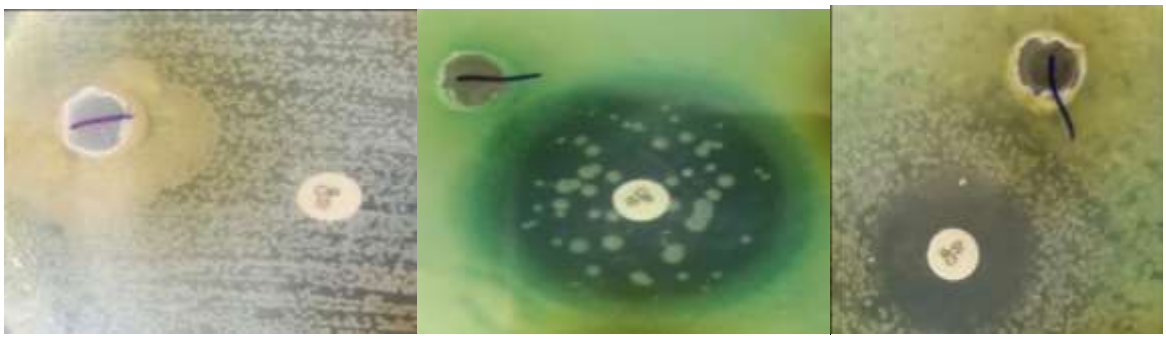
According to Zankana [31] noticed that the inhibitory effects of the alcohol extract is due to the active components' good solubility in organic solvents. The alcohol content of the extract is also quite high. The solubilizing impact can stifle bacterial growth by disrupting the cytoplasm, ribosomes, or DNA of bacteria [32] or by permeating the bacterial cell wall. According to Ashneel *et al*., [33], the typical mechanism of action involves the disruption of protein structures within bacterial cells through the formation of hydrogen bonds. This

disruption ultimately inhibits bacterial action. Based on the findings of this study, it has been observed that certain plant extracts exhibit potential activity and can be effectively employed as natural preservatives to mitigate the occurrence of foodborne illnesses and prolong the shelf life of preserved food items, all while ensuring the absence of any associated health hazards.



(A) (B) (C)

Figure 6: Alcoholic plant extract used to inhibit growth of some food poisoning bacterial strains(A= *P. aeruginosa* B= *S. aureus* C= *E. coli)*



(A) (B) (C)

Figure 7: Aqueous plant extract used to inhibit growth of some food poisoning bacterial strains(A= *E. coli* B= *P. aeruginosa* C= *S. aureus)*

Conclusions

The utilization of chemical preservatives in the food business can serve as a method to prevent this issue. However, it is important to acknowledge that these measures might have adverse effects on human health. Additionally, the introduction of these chemicals into various food chains can potentially lead to toxicity and give rise to long-term complications. In response to the adverse effects caused by conventional preservatives, there is a need to

develop natural preservatives that are both effective and safe, while also being less complex in their use. In order to mitigate the risk of foodborne illnesses, it is possible to utilize plant extracts that have exhibited promising efficacy as a viable alternative to synthetic antibacterial agents.

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