IMPLICATION OF ROSMARINUS OFFICINALIS AS AN ANTIMICROBIAL AGENT AND ITS VALIDATION USING RESPONSE SURFACE METHODOLOGY

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Abstract

The perennial herb rosemary (*Rosmarinus officinalis*), known for its aromatic qualities, belongs to the Lamiaceae mint family. Throughout history, humanity has made use of rosemary, often referred to as the 'memory plant' within the mint family. Research has extensively explored the combination of medicinal plant preparations with antimicrobial agents, demonstrating significant reductions in the minimum inhibitory concentration (MIC) of these agents against resistant strains in vitro. These combined treatments have been characterized by their ability to modify resistance, often termed resistance-modifying activity (RMA). This study aims to investigate the antimicrobial properties of rosemary, utilizing response surface methodology. Natural extracts obtained from the plant using methanol, acetone, and ethanol were subjected to well-cut diffusion assays to assess their efficacy against various bacterial strains. The antimicrobial qualities of rosemary (*Rosmarinus officinalis*) are well known and are ascribed to its rich phytochemical composition. The findings show that extraction variables affect the antimicrobial properties of extract of rosemary and that RSM optimization can be used to determine the perfect circumstances. Through the use of Response Surface Methodology (RSM), the antimicrobial capacity of the extract of rosemary is examined in the present research. The best circumstances for optimizing antimicrobial activity are identified by methodically adjusting extraction parameters

such solvent type, extraction time, and temperature. Additionally, this research assesses how well extract from rosemary works in conjunction with other traditional antimicrobial medicines. The outcomes illustrate possibilities for uses of the extract of rosemary in a variety of industries by demonstrating its effective antibacterial properties under ideal circumstances. Using Response Surface Methodology, the current research examines the antimicrobial properties of the extract of rosemary (RSM). The methanol-based extract demonstrated the greatest effectiveness towards the strain *Staphylococcus aureus* (18.0 mm-32 mm) and moderate effectiveness against *Escherichia coli* (7.0 mm-26.0 mm). Rosemary (*Rosmarinus officinalis*) is well-known for its abundance of bioactive substances, which have strong antimicrobial characteristics. These chemicals include flavonoids, phenolic acids, and essential oils. Applying the context of RSM (Response Surface Methodology) the experiment's design entailed optimizing extraction variables including the temperature, duration, and solvent concentration. An evaluation of the antimicrobial properties was conducted using an assortment of microorganisms that are pathogenic, comprising fungi and bacteria. The antimicrobial property of the extract of rosemary was found to be considerably impacted by the process of extraction variables, according to the findings. Furthermore, the best extraction circumstances for maximizing antimicrobial properties were identified by the RSM (Response Surface Methodology) optimization method. The results demonstrate the potential of the extract of rosemary as an effective antimicrobial and emphasize the use of RSM (Response Surface Methodology) in enhancing biological activity through the method of extraction optimization. The use of it in food preservation, medicines, and cosmetics is made possible by the methodical optimization of extraction variables, which also increases its antimicrobial properties. In order to fully understand the fundamental principles of its action and investigate the possible therapeutic uses of the extract of rosemary as a natural antimicrobial agent, additional study is necessary. This study advances the investigation of natural resources for the production of powerful antimicrobial compounds that may find use in a variety of sectors, such as medicine.

Keywords: Rosemary (*Rosmarinus officinalis L.*); anti-microbial properties; antioxidant properties; antibacterial properties; Response surface methodology, Contour graph

1 Introduction

1.1 Background

The aromatic perennial herb rosemary (*Rosmarinus officinalis*) belongs to the Lamiaceae mint family. Mankind has utilized rosemary, the memory plant of the mint family, from prehistoric days. Cuneiform evidence of rosemary's consumption can be found on Sumerian stone tablets dating back to the fifth millennium BC. Both the Greeks and the Chinese utilized rosemary as a health conditioner; the Greeks also claimed that rosemary fortified the brain and improved memory, and they put garlands made of rosemary around their hair **(Haloui *et al.*, 2000)**. The perennial plant was interred in Egypt alongside the kings of Egypt of Egypt. It is said that Hungary water, a rosemary extract in spirits of wine, was originally made for Queen Izabella of Hungary in 1235 to heal her crippled limbs. The Latin term *"Rosmarinus,"* referring to "sea dew," is where the term rosemary originates. The antique Greeks additionally referred to it "antos," which means "the flower of superiority," or "libanotis," because of the incense-scented air **(Giugnolinini, 1985)**

1.2 Antimicrobial impacts of Rosemary Extract

1.8%-cineol (15–20%), the camphor (15–25%), borneol (16–20%), bornyl acetate (up to 7%), and α-pinene (25%) constitute the majority of the oil. Small quantities of β-pinene, linalool, camphene, subinene, myrcene, α-phellandrene, α-terpinene, limonene, p-cymene, terpinolene, thujene, copaline, terpinen-4-ol, α-terpineol, caryophyllene, methyl chavicol, and thymol are also present in the oil. α-thujene, α-pinene, camphene, β-pinene, and 1,8-cineol make up the majority of the first distillation fraction, whereas bornyl acetate and camphor make up the majority of the second distillation **(Prakasa *et al.*, 1999)**. The profiling and proportion of every constituent in rosemary oil vary based on the developing region and/or additional elements like fertilizers, phenology, and source community **(Wolski *et al.*, 2001 and Guazzi *et al.*, 2001)**. According to research using carbon nuclear magnetic resonance (C-NMR), GC mass spectroscopy (GC-MS), and gas chromatography retention index (GC–RI), **Pintore *et al*. (2002)** revealed 58 constituents from rosemary oil from Sardinia and Corsica (Italy). Three distinct rosemary chemotypes

were found in the research of Moroccan rosemary oil, and 91 chemicals in general were discovered by GC and GC–MS analyses **(Elamrani *et al.*, 2000)**.

Carnosic acid, 12-methoxy carnosic acid, and carnosol, together with antioxidative diterpenes like epirosmarinol, isorosmanol, rosmaridiphenol, rosmariquinone, and rosmarinic acid, constitute the primary antioxidant components of rosemary **(Richheimer *et al.*, 1996)**. The capacity of rosemary to eliminate superoxide radicals, lipid antioxidation, metal chelating, etc., is thought to be responsible for its antioxidant qualities. To prevent oxidation and rancidness, fats, oils, and foods containing fats, such butter, can be stabilized using rosemary isolates along with essential oil **(Zegarska *et al.*, 1996 and Pokorny *et al.*, 1998)** additionally to keep fermented meat-based items stable **(Korimova *et al.*, 1998)**. Various herbal powdered substances, such as rosemary particles, are being sprinkled on preserved grains of wheat and French beans in comparable research in laboratories. The impacts of each of these procedures on the insect pests Sitophilus granarius and Acanthoscelides obtectus were analysed, and it was found that sprinkling grain wheat with rosemary powder may extremely successfully preserve it contrary to *S. granarius*.

Specifically, the relationship amongst the key components, framework, and antimicrobial and anticancer properties of rosemary has been investigated **(Gird *et al.*, 2017)**. Antioxidants (AOXs) have been utilized extensively to prevent food deterioration. While antioxidants stop oxidation by stopping oxidizing chain reactions from starting or spreading, antioxidants also play a significant impact in delaying the ageing processes and a number of disorders. The family of Lamiaceae is thought to constitute a viable supplier of naturally occurring AOXs since it appears to be an abundant producer of species of plants with high phenolic acid contents **(Saini *et al.*, 2020)**. Growing around the globe, *Rosmarinus officinalis L*. is a common household plant that contains several chemicals, primarily polyphenols, that have AOX action. The leaves of this variety most significant AOX ingredients are carnosic acid, caffeic acid, and its related compounds, that include rosmarinic acid and exhibit potent AOX effect. Rosemary extracted from leaves have been studied as possible medicinal products towards a number of ailments and suggested as significant nutritional components for humans **(Abramovic H *et al.*,**

2012). On the other hand, not a lot is now understood about how they are utilized in the treatment of humans.

The preparation and preservation temperature, storage the environment, partition coefficients, and the relationships among these variables, affect the antimicrobial operations of extracts from plants in food items **(Nieto *et al.*, 2018)**. Food ingredients might affect the anti-microbial elements' solubility and phases distribution properties, interfere with the plant extract, or reduce its antimicrobial efficacy. For instance, the fat in milk that is full of cream shields microorganisms from the effects of essential oils and extracts from plants, but the carbohydrates in food do not seem to do the same. Furthermore, due to the fact that are more nutrients available in food than in laboratory medium and since bacteria might regenerate cells that are wounded more quickly, the antimicrobial impact of the plant extract in food might be lessened. Regarding optimization investigations, the response surface methodology (RSM) is a method of statistical analysis that is widely employed. It concurrently solves multidimensional challenges by using statistical information from a suitable design of experiments. Calculations indicate the cumulative influence of each of the test factors in each reaction, explain how testing parameters affect feedback, and identify how testing components relate to one another. A person who experiments can efficiently explore an arrangement or phenomenon with this method of investigation. As a result, RSM continues to be applied often to food manufacturing optimization **(Yeddes et al., 2022)**.

2. Material and methods

The experimental research was conducted at the laboratory of Lifesciences department of University of Management and technology Lahore.

2.1 Plant Extracts

Rosemary was purchased from local market in Lahore and its extracts were prepared in the lab of the university of Management and Technology. Dried leaves of rosemary have been used to serve as the extraction's source. Because various plant cells have varying water contents, we employed dried plants; plants are typically dried in the air to a consistent weight preceding extraction. The majority of documented studies employed a greater extent of subterranean plant parts such as roots, tubers, rhizomes, bulbs, etc. than above-ground parts in their quest for bioactive substances with antimicrobial potential.

2.2 Choice of Solvents

The sort of solvent utilized in the extraction process has a significant impact on the outcome of the naturally occurring compound's successful identification from the plant material. The ideal solvent for extraction of plants should have minimal toxicity, be easily evaporated at temperatures that are low, promote quick physiological absorption of the extract, have preservation characteristics, and not allow the resulting product to become complex or dissociate. Usually typically, either methanol or ethanol extraction is used for extracting the aromatic substances or saturated organic compounds that make up the majority of the antimicrobial chemicals found in plants. Therefore, methanol, ethanol, and acetone are the most often employed solvents for initially studies of our plant's antimicrobial properties.

2.3 Extraction Method

2.3.1 Stock Solutions

For each sample, a stock solution for antimicrobial plant extract testing was made by dissolving 5 mg of crude extract in 20ml of solvent i.e., acetone, ethanol and methanol. The fundamental idea is to grind the leaves of the rosemary plant more refined, thereby increasing the extraction area and, consequently, the rate of extraction. Eloff (1998b) found that extracting extremely small particles having an average diameter of 10 μm for 5 minutes produced larger proportions than the amounts that were reported after 24 hours in a shaking device using less finely ground material. Previous research revealed that the optimal solvent to sample proportion has been determined found to be 10:1 (v/w) solvent to dry weight ratio. The homogenization of plant tissue in solvent is an approach to extraction that is currently utilized extensively by scientists. The rosemary that has been dried is ground into small pieces in a mortar and pestle, added to 20 milliliters of solvent, and agitated rapidly for five to ten minutes, or for seventy-two hours, until the extract is strained. To find the level of concentration, the filtrate used was dried below low pressure before being reincorporated in the solvent that was used.

2.3.2 Crude extract preparation

To create an extremely fine powdered form, the dried rosemary plant leaves have been chopped and processed in a regular crusher. Twenty milliliters each of methanol, acetone,

and ethanol were added to extraction jars along with approximately five grams of dried powder. For six days, these extraction jars were stored at room temperature. The drums were shaking twice a day throughout this time. Whatman filter paper No. 1 was used for filtering the soluble chemicals. The remaining solid material was mixed with fresh methanol, ethanol, and acetone, and the procedure was carried out a total of three times. Methanol, ethanol, and acetone were extracted from the filtered solutions under 45ºC under vacuum pressure, and the resulting solutions were then dried in a rotary evaporator. After extracting the semisolid material, it had been dried in a water bath at roughly 45ºC in a China dish.

2.3.3 Ethanol extract

For the extraction of ethanol extract, a conical flask was filled with 50 ml of ethanol and five grams of dried in the air powdered rosemary. Cotton wool was placed inside the flask, and the mixture was shaken at 190–220 rpm for a whole day. The supernatant was gathered shortly after a day, and the solvent had been evaporated to leave an end product that was a quarter of the initial volume. The mixture was subsequently kept at 4 ℃ in screwcap test tubes or airtight bottles.

2.3.4 Methanol extract

For the methanol extract, 5g of powdered rosemary leaves that had been air dried was combined with 50 ml of methanol in a conical flask. The glass container was subsequently covered with cotton wool and placed on a shaker that was set to 190–220 rpm for a whole day. Following a 24-hour period, the remaining liquid was gathered and the solvent had been evaporated to reduce the resulting quantity to one-fourth of its initial amount. The mixture was subsequently preserved at 4 ℃ in screwcap test tubes or airtight bottles.

2.3.5 Acetone extract

5g of dried in the air powdered rosemary leaves was combined with 50 ml of acetone in a conical flask, sealed with cotton wool, and incubated for 24 hours at 190–220 rpm on a rotating shaker. Following a period of twenty-four hours, the supernatant was gathered and the solvent had been evaporated to reduce the final volume to one-fourth of its initial amount. The mixture was then kept at 4 ℃ in screwcap test tubes or airtight bottles.

2.4 Antimicrobial Activity

2.4.1 The Microorganisms and Culture Conditions

Staphylococcus and *E. Coli* are two types of bacteria. Aureus was received from the UMT Lahore life sciences lab and kept at 4 °C on chocolate agar slants (Oxide). a *staphylococcus* and *E. coli* loop-full culture. Under sterile conditions, Aureus was inoculated into nutritional broth and incubated in a rotary shaker at 37 °C for 24 hours. Throughout the study, the turbidity of the bacterial suspension was adjusted within the range of 1×10^8 bacterial cells/mL using the McFarland standard as a point of reference.

2.4.2 Inoculum preparation and Agar well diffusion method

A *staphylococcus* or a bacteria called *E. coli* colony was used. Using a sterile wire loop, Aureus was taken off of the agar plate and used to inoculate a 10-milliliter aliquot of sterile nutrient broth. It was then incubated at 37 °C for 24 hours. The McFarland 0.5 turbidity method standard was used for checking and adjusting the turbidity. On the surface of the gelled agar plate, an inoculum with a defined volume and standardized concentration is uniformly distributed. Using a sterilized cork borer, four aseptic holes with a diameter ranging from 4 to 8 mm are punched into the middle and sides of the plate of agar. Then, two bored agar wells were filled with varying quantities of rosemary leaves extract; the third well served as a control, containing ethanol, while the other well contained an antibiotic vancomycin. Following that, plates were incubated for the ideal amount of time and temperature based on the test microorganism. The diameter that defines the zone of inhibition has been employed to calculate the rate of growth of bacteria. There were controls for every strain of bacteria in which the solvent extract was replaced with pure solvents. The zone diameter was measured in order to determine the outcome. Response surface approach was used to display the data after the experiment was conducted three times, with the mean values being given using two distinct microorganisms and three different solvents. Minitab software is used for plotting contour graph.

3. Results and Discussion

Staphylococcus aureus

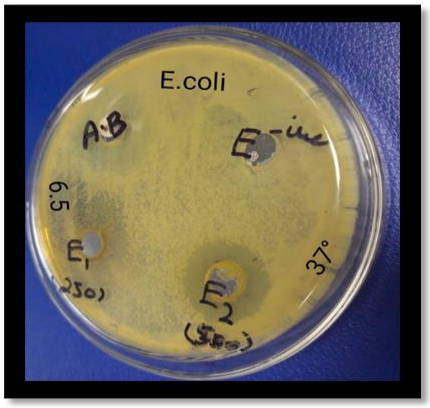
Ethanol, PH 5
Temp 37
Figure 3.1

Staphylococcus aureus

Methanol, PH 8
Temp 35
Figure 3.2

Maximum zone of inhibition shown by acetone and methanol extract against *Staphylococcus aureus* at respective temperature, PH and concentration in fig1 and fig2.

E. coli



Acetone, PH 6.5
Temp 37° C
Figure 3.3

E. coli

Methanol, PH 6.5
Temp 37° C
Figure 3.4

Maximum zone of inhibition shown by ethanol and methanol extract against *E. coli* at respective temperature, PH and concentration in fig3 and fig4

3.1 Contour Graphs

Figure 3.5: Effects of Concentration, Temperature on antimicrobial activity of *Rosmarinus officinalis* extract of acetone against *E. coli.*

The graphs indicate the highest zone of inhibition of rosemary acetone extract against *E. coli*. On X-axis there is temp while on Y-axis it will be Conc, Zones of inhibition are in between 5-20. Darker the color, highest the zone of inhibition (ZOI). This contour plot illustrates the antimicrobial activity of rosemary acetone extract against *E. coli* on different concentration and temperature (Fig 4.5).

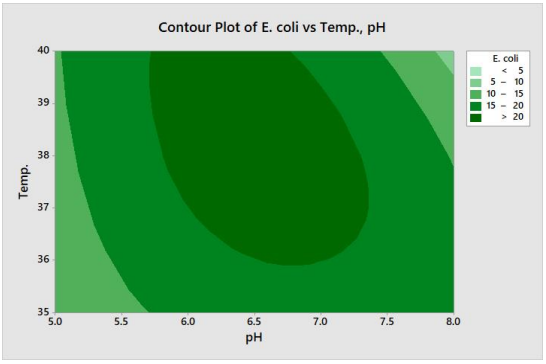


Figure 3.6: Effects of Concentration, pH on antimicrobial activity of *Rosmarinus officinalis* extract of acetone against *E. coli.*

The graphs indicate the highest zone of inhibition of rosemary acetone extract against *E. coli*. On X-axis there is Ph while on Y-axis it will be Conc, Zones of inhibition are in between 5-20. Darker the color, highest the zone of inhibition (ZOI). This contour plot illustrates the antimicrobial activity of rosemary acetone extract against *E. coli* on different concentration and temperature (Fig 4.6).

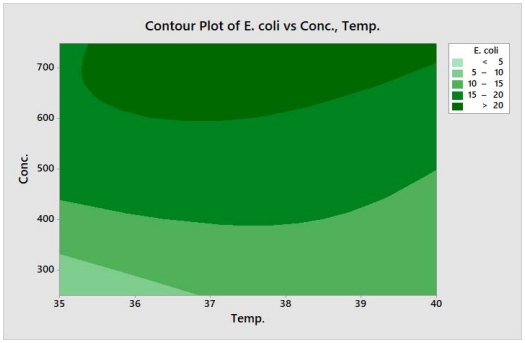
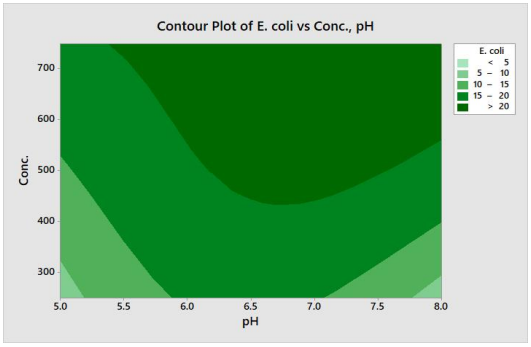


Figure3.7: Effects of pH, Temperature on antimicrobial activity of *Rosmarinus officinalis* extract of acetone against *E. coli.*

The graphs indicate the highest zone of inhibition of rosemary acetone extract against *E. coli*. On X-axis there is pH while on Y-axis it will be temp, Zones of inhibition are in between 5-20. Darker the color, highest the zone of inhibition (ZOI). This contour plot illustrates the antimicrobial activity of rosemary acetone extract against *E. coli* on different concentration and temperature (Fig 4.



S.no	Temp. (° C)	pH	Conc.(µg/ml)	*Staphylococcus aureus*	*E. coli*
1	35	6.5	750	27	20
2	40	6.5	750	29	25
3	37.5	6.5	750	32	27
4	37.5	6.5	750	32	27
5	35	6.5	750	27	20
6	35	6.5	750	27	20
7	35	6.5	750	27	20
8	37.5	6.5	750	32	27
9	37.5	6.5	750	32	27
10	37.5	6.5	750	32	27
11	40	6.5	750	29	25
12	37.5	6.5	750	32	27
13	40	6.5	750	29	25
14	40	6.5	750	29	25
15	40	6.5	750	29	25
16	40	6.5	750	29	25
17	40	6.5	750	29	25
18	35	6.5	750	27	20
19	37.5	8	750	28	24
20	35	8	750	27	23
21	35	8	750	26	23
22	37.5	5	750	27	22
23	40	5	750	29	19
24	35	5	750	26	21
25	37.5	6.5	250	21	19
26	40	6.5	250	19	16
27	37.5	6.5	250	21	19
28	37.5	6.5	500	25	22
29	37.5	6.5	500	25	22

30	37.5	6.5	500	25	22
31	40	5	250	19	13
32	37.5	5	250	21	12
33	37.5	5	250	21	12
34	40	5	500	20	17
35	37.5	5	500	22	23
36	40	6.5	750	29	22
37	37.5	6.5	750	32	27
38	40	6.5	750	29	22
39	35	8	500	25	19
40	37.5	8	500	24	22
41	35	8	500	25	19
42	40	8	250	19	11
43	37.5	8	250	21	12
44	37.5	8	250	21	9
45	37.5	5	500	24	13
46	35	5	250	18	7
47	35	5	250	18	7
48	37.5	5	250	20	8
49	37.5	6.5	750	32	27
50	37.5	6.5	750	32	27
51	37.5	6.5	750	32	27
52	40	5	500	22	17
53	37.5	5	500	24	13
54	37.5	5	500	24	13
55	35	8	500	22	19
56	37.5	8	500	25	22
57	37.5	8	500	25	22
58	35	8	250	17	7
59	37.5	8	250	18	12
60	35	8	250	17	7

4. Discussion

The impact of isolates from rosemary leaves on the foodborne pathogenic microorganisms such as *Staphylococcus aureus* and *E. coli*, respectively, has been studied. Rosemary extracts significantly increased the sensitivity of bacterial cultures that were gram-positive. For instance, the MICdil for *Staphylococcus aureus* measured by using the agar dilution technique ranged from 0.078 to 5.0 mg/ml. In keeping alongside previous observations, *Staphylococcus aureus* appeared to be extremely responsive to the several isolates that were examined. One of the variety of microorganisms that is reportedly extremely susceptible to the extract of rosemary is *Staphylococcus aureus*, or *S. aureus*. bacteria like Staph had been nevertheless, considerably more vulnerable than *Escherichia coli* when compared with MICs as well. The gram-negative bacteria *Escherichia* showed significant antimicrobial activity against each of the extract preparations evaluated, as reported by A **(Klancnik *et al.,* 2009)**. The ability to resist could have been caused by the Gram-negative bacteria's outer membrane, that limits the flow of substances by means of its lipopolysaccharide coating. Gram-positive bacteria's single cell membrane makes it far more vulnerable to antibiotic permeability. The polyphenol composition and bacterium type having a bearing on how sensitive microorganisms are to polyphenols. The alcohol-based tea grass infusion had the smallest impact, but in the course of the third hour, these modifications reverted and stopped bacteria such as *E. coli* from growing. The most efficient extracts on bacteria like *S. aureus* have been determined to be the methanolic extract of olive and ethanol-based extracts of rosemary, in accordance to a study by **(Seedpanah *et al.,* 2022)**. These findings were consistent with earlier research by (**Mashreghi *et al.,* 2012)**. According to the research conducted by (**Golshani *et al.,* 2014)** the growth inhibiting zone of rosemary leaves extract made with methanol on *Staphylococcus aureus* had a measurement of 20 to 25 mm, while the growing inhibitory zone of *P. aeruginosa* treated with methanol had a diameter of 15 to 18 mm. A further investigation that looked into the antimicrobial properties of rosemary essential oil discovered showed the essential oil had an expanding circle of 28 mm on *Staphylococcus aureus* and additionally that it had beneficial effects on *Bacillus cereus*, as well, and other Gram-positive organisms. The particular kind of plant and solvent used in this investigation showed different impacts

on the growth of *Escherichia coli* and the bacteria *Staphylococcus aureus*. Antibiotics serve as effective medications for treating a wide range of illnesses in humans, nevertheless abuse of these therapies leads to increases in germ resilience. Consequently, in an effort to find novel medications derived from plants, researchers decided to concentrate their investigations on various sections of plants used for medicinal purposes.

Table 4.1 Comparative study of antimicrobial activity against different bacterial species

Gram-positive/negative bacteria	**Zone of inhibition (mm)**	**References**	**Extract of plant**
S. aureus	29-33	(Bahman *et al.* 2018)	Rosemary acetone extract
Streptococci	12-24	(Mashreghi *et al*.2017)	Rosemary methanol extract
Enterococci.	21-28	(Ahmadyasbchin *et al*., 2016)	Rosemary methanol extract
P. aerogenes	3-12	(Fu *et al.*, 2007)	Rosemary ethanol extract
Vibrio aerogenes	7-22	(Santoyo *et al*., 2005)	Rosemary methanol extract
E. coli	12-28	Tavassoli *et al.* (2011)	Rosemary acetone extract
S. aureus	24-32	Present Study	Rosemary methanol extract
E. coli	12-25	Present Study	Rosemary acetone extract

5. Conclusion

Employing well-cut diffusion procedures, natural extracts of methanol, acetone, and ethanol from the aforementioned plant were produced and tested towards several types of bacteria. As observed by Table 1, the raw extracts frequently produced positive results towards the majority of microbiological pathogens. The findings presented in Table 1 verified the fact that the methanol extract's absolute activity units

(AU) varied between 18.0 and 32.0, while the ethanol extract's AU from 17.0 to 31.0, and the acetone extract's AU from 16.0 to 30.0. The maximum values (32.0 mm) in the extract of methanol demonstrated effectiveness towards *Staphylococcus aureus*, then the extract made from ethanol exhibited the strongest antimicrobial activity towards the bacteria *Staphylococcus aureus*. The technique known as response surface methodology (RSM) was used to optimize extraction. Despite a very modest cytotoxic profile, research demonstrated that the synthetically produced extract of rosemary had significant antimicrobial and antibacterial activity. Prior to starting the encapsulating procedure, the optimized extract's antimicrobial properties and acute toxic effects had been evaluated. subsequently turned out that the optimized essential oil exhibited significant antimicrobial efficacy towards Gram-negative organisms such as pneumonia aeruginosa and *E. coli*, respectively.

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