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Study of the Inhibitory Activity of Pomegranate Peel Extract Against Pathogenic Bacteria in Food Products

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ABSTRACT: This study aimed to investigate the potential of ethanolic pomegranate peel extract as a natural antimicrobial agent for preserving locally produced cheese. The extracts were prepared at concentrations of 25%, 50%, and 100% and evaluated against Escherichia coli, Pseudomonas aeruginosa, Salmonella spp., and molds & yeasts using the disk diffusion method. The extract showed 100% stronger antibacterial activity, producing an inhibition zone higher than 25% and 50%, which exceeded that of the reference antibiotics amikacin and amoxicillin. The extract showed 25% less antimicrobial activity compared to 100% and was lower than the areas of the reference antibiotics amikacin and amoxicillin. The results also showed an increase in the antioxidant activity and reducing ability of the 100% extract compared to the rest of the extracts and the reference antibiotics amikacin and amoxicillin, indicating a concentration-dependent effect. These results suggest that pomegranate peel extract has great potential as a natural food preservative to enhance microbial safety and extend the shelf life of food products. Further studies are recommended to evaluate its practical application directly or indirectly for food preservation or as an edible coating or preservative treatment for dairy under commercial storage conditions.

1.INTRODUCTION

Medicinal plants have had great importance since ancient times. The ancients used them to treat diseases, either by using them directly or by crushing them and applying them to the site of an injury, whether a wound or any other type of lesion. Medicinal plants are also considered among the most important and oldest plants known and used by humans throughout history for various purposes [1]. including nutrition and medicine. Their use has been renewed in the present time, as medicinal plants in most countries of the world are used to treat many diseases, especially due to the intensive use of synthetic drugs, including antibiotics, for long periods[2].

Plants have the ability to synthesize compounds as secondary metabolic products that are found in seeds, leaves, or roots. Among these compounds, some have medical significance, which is why medicinal plants have attracted the attention of researchers. This is because drugs derived from them tend to be inhibitory yet harmless and free from side effects, unlike antibiotics that are not always inhibitory and often have side effects[3]. Since ancient times, natural plants have been a significant source of many medicinal compounds, and because they contain a variety of bioactive molecules, they are still used today to treat a wide range of illnesses. pomegranate [4]. which comes from the pomegranate tree Punica granatum, is one of

those plants with medicinal importance. Its original homeland is Southwest Asia or Carthage, and its cultivation has spread commercially to most Western countries and Northwestern India [5].

Punica granatum, commonly known as pomegranate, is a fruit-bearing plant native to Southwest Asia and North Africa. Its peel, often discarded as waste, is rich in bioactive compounds with strong antimicrobial properties. Previous studies have highlighted the potential of pomegranate peel extracts in inhibiting foodborne pathogens and enhancing the shelf life of dairy products [6].

This study aims to develop the Inhibitory Activity of Pomegranate Peel Extract Against Pathogenic Bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* and *Salmonella spp* and Molds & Yeasts Found in food products and directly affecting human health.

2. METHODS AND MATERIALS

Name of the laboratory device	Origin	Model
Dry oven	Turkish	LAB
Mixer	SH-SCIENTIFIC	Korean
Sensitive balance	RADWAG-WTB2000	Belgian
GRINDER	China	22-SG
INCUBETOR	Korea	JEMM
HOT PLATE	Turkish	LABTICH
LIMINAR AIR FLOW	LAB	
Autoclave	Thailand	
Centrifuge	Jemme	

2.1 Preparation of Pomegranate Peel Extract

Pomegranate peels were collected, washed, dried at 65°C until constant weight, ground, and sieved (2 mm). Two ethanolic extracts were prepared by soaking 200 g of peel powder in 70% ethanol, stirred for 5 hours, and refrigerated for 24 hours. The extracts were filtiered and concenterated at 40°C to obtain thick dry residues, stored at 4°C until use [7].

2.2 Microbial Culture and Media Preparation

Nutrient agar and MacConkey agar were prepared according to manufacturer instructions, sterilized at 121°C for 15 minutes. *Pseudomonas aeruginosa and E. coli* bacterial isolates were cultivated on the appropriate media.

2.3 Antimicrobial Assay

The agar well diffusion method was used to assess the extracts' antibacterial activity. Amoxicillin and Amikacin (10 µg/disc) were employed as positive controls while testing ethanolic extracts at two concentrations (25%, 50%, and 100%) against bacterial strains. On inoculated agar plates, 50 µL of each extract or control solution was applied to agar wells with a diameter of 5 mm. The diameter of the inhibition zones (mm) was used to calculate the antibacterial activity after the plates were incubated for 24 hours at 37°C. Every experiment was carried out in an aseptic environment and repeated as needed to ensure repeatability [8].

2.4 Preparation of the Alcoholic Extract:

Pomegranate peels (Punica granatum L.) were collected from juice-selling locations in Ramaidi, Iraq. The collected peels were waished, dried in a laboratory oven at 65°C until a conestant weight was obtained, and then ground into a fine powder using a laboratory grindir. The powder was passed through a 2 mm sieve, and 200 g of the obetained powder was extracted with 1000

mL of 70% ethanol. The mixture was continuously stirred using a mechanical mixer for 5 h and subsequently stored at 4°C for 24 h to enhance extraction efficiency. The extract was filtered to remove insoluble materials, and the filtrate was concentrated by evaporation at a temperature not exceeding 40°C until a crude dry extract was obtained. The dried extract was collected, powdered, stored in polyethylene containers, and kept refrigerated until further analysis [9].

2.6 Physiological Saline Solution Normal Saline

A normal saline solution85% was prepared by dissolving 0.85 g of sodium chloride (NaCl) in 90 ml of distilled water. Distilled water was used to complete the volume to 100 ml and bring the pH down to 7.0. After sterilization, the mixture was kept at 4°C. During laboratory testing, bacteria isolates were diluted using the produced saline [11].

2.7 Preparation of Culture Media

The culture media were prepared in accordance with the manufacturer's guidelines and autoclaved for 15 minutes at 121°C and 15 psi of pressure. To make sure they weren't contaminated, they were put into sterile plates and incubated for 24 hours at 37°C before being refrigerated at 4°C [11].

2.8 Solid Nutrient Medium Nutrient Agar

Prepared by dissolving 28 grams of solid nutrient medium powder in 1 liter of distilled water (D.W.) according to the manufacturer's instructions. This medium is used for preserving isolates (short-term preservation) [11].

2.9 MacConkey Agar Medium

Prepared by dissolving 51.5 grams of the nutrient agar powder in 1 liter of distilled water (D.W.) according to the manufacturer's instructions. This medium is used for the growth of bacterial isolates as a primary culture for the purpose of bacterial isolation [11].

2.10 Sterilization of Alcoholic Extracts

The alcoholic extract was prepared by weighing 2 grams of the extract made according to steps 2-3, then dissolving it in 10 ml of Dimethyl Sulfoxide (DMSO) diluted with distilled water. The mixture was then sterilized using a pasteurization method at 62°C for 10 minutes. This produced the standard concentration of the extract, which was used for preparing subsequent dilutions [12].

2.11 Evaluation of Antioxidant Activity (FRAP Assay)

The Ferric Reducing Antioxidant Power (FRAP) assay was used to assess the antioxidant activity of the pomegranate peel extract using a slightly modified version of Benzie and Strain's (1996) method. 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution made in 40 mM HCl, and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) were mixed in a 10:1:1 (v/v/v) ratio to create the FRAP reagent.3.0 mL of freshly made FRAP reagent was combined with 100 µL of pomegranate peel extract at concentrations of 25%, 50%, and 100%. For thirty minutes, the reaction mixture was incubated at 37°C in the dark. A UV-visible spectrophotometer was then used to measure the absorbance at 593 nm [11].

2.12 Antibiotic susceptibility testing of bacterial isolates:

The bacterial isolates were subjected to susceptibility testing for two antibiotics, and the results were recorded by describing the bacteria as resistant I, intermediate (I), or sensitive (S) based on the measurement of the inhibition zone diameter, according to the [13].

2.13 Reduction ability test:

The FRAP reagent was newly prepared by mixing the regulated acetate solution (acetate buffer, pH 3.6) with the prepared TPTZ solution in hydrochloric acid and the ferric chloride solution ($FeCl_3 \cdot 6H_2O$) in a ratio of 10:1:1 respectively. Next, an appropriate volume of pomegranate peel extract was added to the FRAP reagent, and the mixtures were incubated for 30 min at 37°m. Optical absorption was measured at 593 nm by spectrophotometer after the incubation period was finished and reducing power was calculated from a standard curve of ferrous sulfate (FeSO4) solution and expressed as µmol Fe2+/g extract. [13].

2.14 Method:

After preparing the culture media Nutrient agar and MacConkey agar and sterilizing them using an autoclave, the Nutrient agar was poured into Petri dishes and left to solidify. As for the MacConkey agar, it was also poured into Petri dishes, and after solidification, bacteria including E. coli and various infection sources of Pseudomonas aeruginosa were streaked on it and placed in an incubator for 24 hours.White test tubes filled with pre-prepared saline solution were sterilized for 30 minutes, then placed and transferred to a work cabinet sterilized with alcohol and heat using a Bunsen burner. The inoculator loop was used to take swabs from the activating bacteria and swabs from different sources of Pseudomonas aeruginosa infections [14]. The tubes were shaken using a shaker to distribute all the bacteria in the saline solution. Then, quantities of alcoholic and aqueous extracts were taken and added to a sterile test tube, which was then shaken. The well diffusion assay method was used to test the antibacterial activity of the alcoholic extract previously mentioned by [15]. The plates containing the medium with the test bacteria were inoculated by spreading them on the surface of the nutrient medium using a swab, then left to dry. Three wells of 5 mm diameter were made on the surface of the medium in each plate using a sterile Falini borer. Extracts at concentrations of 50% and 100% were transferred into two wells, 50 microliters per well, while distilled water was placed in the third well as a control solution. Antibiotics (Amoxicillin and Amikacin) were placed in the fourth area[16].

2.15 Statistical analysis:

All data were analyzed using the least significant difference (LSD) test with the SAS statistical analysis system [17].

3. RESULTS AND OBSERVATIONS

3.1 Inhibitory effectiveness of pomegranate peel extract against *Escherichia coli* and *Psydomonas aeruginosa* bacteria

Table: Inhibition Zones of Antibiotics and Pomegranate Peel Extracts Against Target Bacteria

Substance	Code	Concentration (µg/disc)	Inhibition Zone against *E.coli* bacteria (mm)	Inhibition Zone against *Pseudomonas aeruginosa* (mm)
Amoxicillin	AMO	10	15	14
Amikacin	AMK	10	16	18
Ethanolic Extract 25%	—	—	12	13
Ethanolic Extract 50%	—	—	14	15
Ethanolic Extract 100%	—	—	17	19
Value of L.S.D	—	—	0.031	0.037

In this study, we evaluated the antimicrobial activity of pomegranate peel extracts compared to conventional antibiotics such as amoxicillin and amikacin against E.coli and Pseudomonas aeruginosa. Ethanol extracts of pomegranate peel (in concentrations of 25%, 50% and 100%) were prepared and their antibacterial activity was tested with the method of disc diffusion. The outcomes in Table Number. (1)showed that the ethanolic extract at a concentration of 100% showed the largest inhibition zone, as it was for E. coli and Pseudomonas aeruginosa bacteria (17, 19 mm), respectively. It is a larger zone

than amoxicillin, as it was (15, 14 mm), respectively, and close to the amikacin zone, as it was (16, 18 mm), respectively. These results were close to what [18]. The extract at a concentration of 50% also showed remarkable activity against bacteria, as the area was (14 and 15 mm) respectively, which is an area very close to amoxicillin, as it was (15 and 14 mm) respectively, and less than the amikacin area, as it was (16 and 18 mm) respectively, and these results were less than what was found [19]. while the results showed that the lowest inhibition zone was 25% compared to the higher levels, as in Figure (1), which were (12,13 mm), respectively, and were lower than the amoxicillin and amikacin zones, which indicates that the effect depends on the dose. These results suggest that pomegranate peel extract, especially in high concentrations, can be used as a natural alternative to preservatives to further inhibit bacterial growth in food products.This is consistent with[20]. The statistical differences in the results between the different concentrations ($P \leq 0.05$) indicate that increasing the concentration of the extract had a significant effect in improving the ability to resist bacteria, as the concentration of 100% achieved the highest values compared to the concentrations of 50% and 25%. This indicates a direct relationship between the concentration of the extract and its antibacterial efficiency.

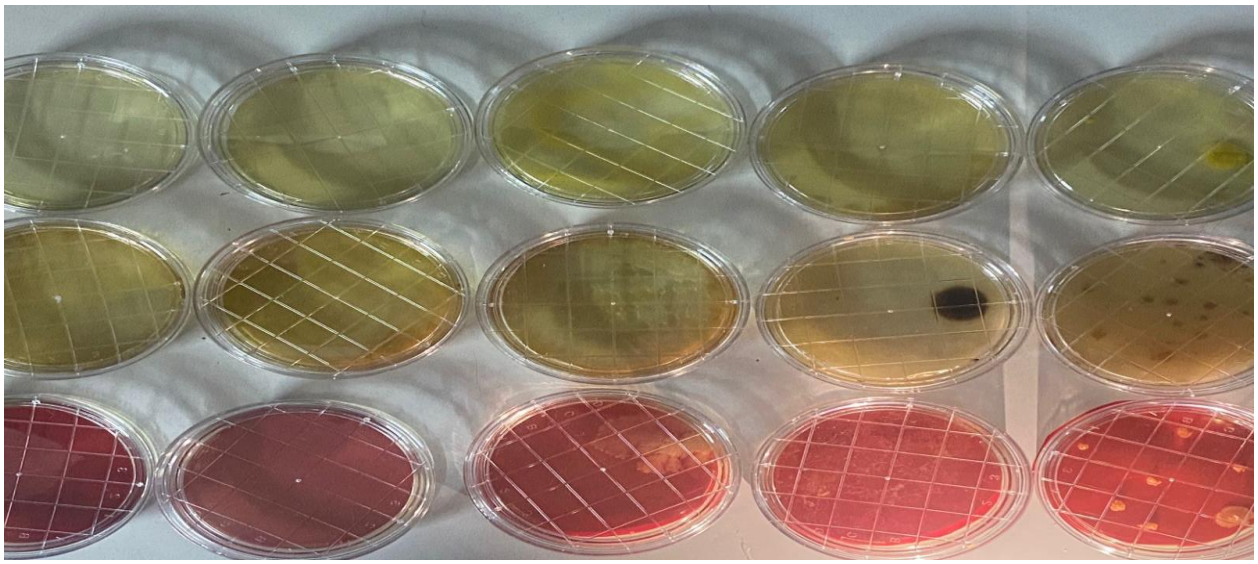

Figure (1) Bacterial inhibition zones at concentrations of 25%, 50%, and 100% for pomegranate peel extract.

The strong inhibition zone observed with the 100% extract may be attribuited to its higher concentration of phenolic compounds, which are known to exhibit bactericiedal propeirties through mechaenisms such as enzyme inhibition, protein precipitation, and interaiction with microbial DNA. Moreover, the efficacy of the extract against the tested bacteria is equal to or superior to that of standard antibiotics [21]. that natural substances, particularly in food preservation applications, may be useful substitutes or supplements to manufactured antibacterial agents [22].

Furthermore, the findings align with studies on edible films and coatings. For example [23]. Higher oxidative stability and antibacterial activity in cheese products were demonstrated by a study on chitosan-based edible films enhanced with pomegranate peel extract. This supports the notion that compounds produced from pomegranates are advantageous both in vitro and in real food systems [24].

3.2 Inhibitory effectiveness of pomegranate peel extract against *Salmonella spp*

Table 2: Inhibition Zones of Antibiotics and Pomegranate Peel Extracts *Salmonella spp*

Substance	Code	Concentration (µg/disc)	Inhibition Zone against *Salmonella spp* (mm)
Amoxicillin	AMO	10	15.5
Amikacin	AMK	10	16
Ethanolic Extract 25%	—	—	15

Substance	Code	Concentration (µg/disc)	Inhibition Zone against *Salmonella spp* (mm)
Ethanolic Extract 50%	—	—	19
Ethanolic Extract 100%	—	—	21
Value of L.S.D	—	—	0.013

sing the disc diffusion method, the antibacterial efficacy of ethanol pomegranate peel extracts against salmonella was assessed and contrasted with two common antibiotics, amoxicillin and amikacin. all pomegranate peel extract doses demonstrated strong anti-salmonyl action, as seen in Table 2. Amikacin (16 mm) and amoxicillin (15.5 mm) were outperformed by the 100% ethanol extract, which had the biggest inhibition zone (21 mm).These outcomes closely matched his findings [25]. Strong inhibition (19 mM) was also demonstrated by the 50% extract, which outperformed amikacin and amoxicillin.The 25% concentration produced findings that were higher than those reported [26] and were (15 mm) below the amikacin inhibition zone and near the amoxicillin inhibition zone. who made the observation that the concentration obviously affects the antibacterial effect. These findings imply that pomegranate peel extract, particularly at maximum concentration, may be a potent natural antibacterial agent that, in some circumstances, is on par with or even better than traditional antibiotics. These findings imply that bioactive substances found in pomegranate peel, particularly tannins and polyphenols, are essential for rupturing bacterial cell membranes and preventing microbial development [27]. as depicted in Figure (2).

These conclusions are supported by a number of recent investigations. Take the study conducted by [28], for instance. demonstrated how adding pomegranate peel powder to soft white cheese improved its antibacterial qualities and decreased the total amount of bacteria and fungi while it was being stored. Likewise, a research by [29]. A study revealed that adding pomegranate peel extracts to cheese matrices reduced Staphylococcus aureus levels, demonstrating the extracts' broad antibacterial activity.

The growth in inhibitory zones seen in Figure (2) suggests that these natural extracts could be used to preserve local cheeses, particularly in areas where synthetic preservatives are unavailable or undesirable. Pomegranate peel extracts have the potential to be multipurpose food additives due to their antibacterial, anti-inflammatory, and antioxidant qualities [21].

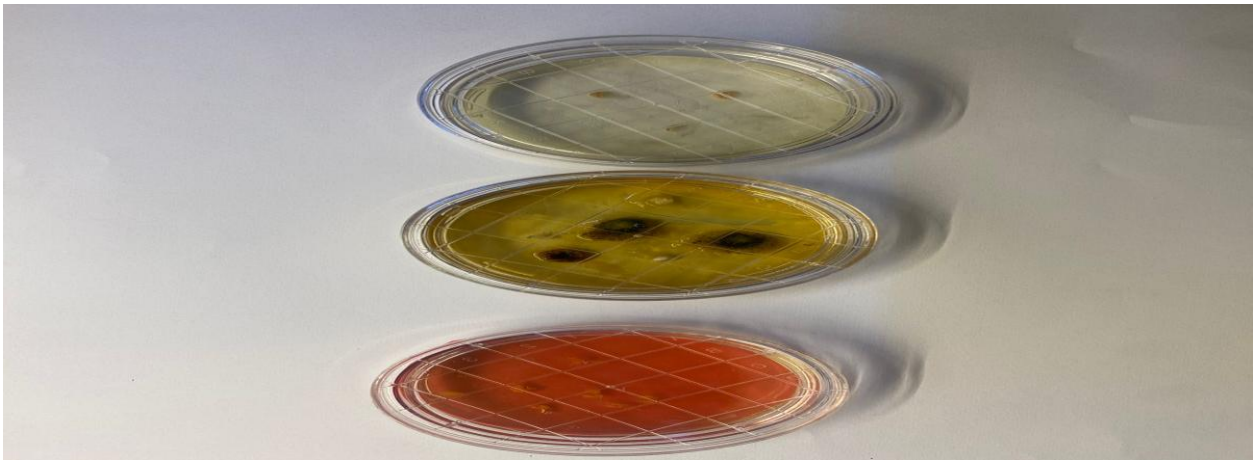

Figure (2): Effect of pomegranate peel extract concentrations (25%, 50%, and 100%) on areas of bacterial growth inhibition.

3.3 Evaluation of the antioxidant activity of pomegranate peel extract

Table 2: Results of FRAP and DPPH tests for pomegranate peel extract at different concentrations.

Pomegranate peel extract concentrations	Reducing Power (FRAP) (µmol Fe^{2+}/g extract)	Antioxidant activity (DPPH %)
25 %	285	42.8
50 %	517	68.5
100 %	894	91.7
Value of L.S.D	0.57	0.21

The results in Table (3) showed that the antioxidant activity of pomegranate peel extract gradually increased with increasing extract concentration. The FRAP value increased from 285 µmol Fe^{2+}/g extract at 25% concentration to 517 µmol Fe^{2+}/g extract at 50% concentration, then reached 894 µmol Fe^{2+}/g extract at 100% concentration. This indicates an increase in the reducing power of the extract as its concentration increases. reflecting the presence of larger amounts of compounds capable of giving electrons and reducing ternary iron (Fe^{3+}) ions to binary iron (Fe^{2+})[30].

The results also showed an increase in antioxidant activity, as measured by the DPPH test, with the rate of free radical inhibition increasing from 42.8% at a concentration of 25% to 68.5% at a concentration of 50%, and reaching 91.7% at a concentration of 100%. This increase indicates an improvement in the extract's ability to neutralize free radicals with increasing concentration, which suggests an increase in the efficiency of the biologically active compounds present in pomegranate peel extract [31].

These results can be explained by the high content of phenolic compounds and flavonoids in pomegranate peels, such as gallic acid, ellagic acid, and ellagitanins, which possess a high ability to donate electrons or hydrogen atoms, contributing to the inhibition of oxidation processes and reducing the formation of free radicals. Therefore, increasing the concentration of the extract typically results in an increase in the concentration of these compounds, which is reflected in higher values of both FRAP and DPPH.

Statistical differences in the results between the different concentrations ($P \leq 0.05$) indicate that increasing the extract concentration had a significant effect in improving the reducing ability and antioxidant activity, as the 100% concentration achieved the highest values compared to the 50% and 25% concentrations. This indicates a direct relationship between the concentration of the extract and its antioxidant efficiency within the range of concentrations studied [32].

Overall, these findings show that pomegranate peel extract may have concentration-dependent antioxidant activity and that, when compared to lesser concentrations, the undiluted extract (100%) offers the best efficiency. Since the phenolic compounds responsible for antioxidant activity are also among the most important molecules that contribute to limiting the growth of many harmful bacteria linked with food spoilage, [33]. it is expected that this favorable activity will be reflected in its antibacterial efficiency.

3.4 Effect of pomegranate peel extract against Molds & Yeasts

Table 3: Inhibition Zones of Antibiotics and Pomegranate Peel Extracts Molds & Yeasts

Substance	Code	Concentration (µg/disc)	Inhibition Zone against Molds & Yeasts (CFU / gm)
Amoxicillin	**AMO**	**10**	**15.5**
Amikacin	**AMK**	**10**	**16**
Ethanolic Extract 25%	**—**	**—**	**15**
Ethanolic Extract 50%	**—**	**—**	**19**

Substance	Code	Concentration (µg/disc)	Inhibition Zone against Molds & Yeasts (CFU / gm)
Ethanolic Extract 100%	—	—	21
Value of L.S.D	—	—	0.021

The results shown in Table (3) indicate that ethanol extracts of pomegranate peel possess strong antibacterial properties against yeast and mold, with effectiveness exceeding that of antibiotics (amoxicillin and amikacin). The inhibition zone for the concentrations of pomegranate peel extract (25%, 50% and 100%) was (15, 19, 21) respectively, and for the antibiotics (amoxicillin and amikacin) it was (15.5, 16) respectively. This is consistent with what [34].stated that the anti-yeast and anti-rot activities attributed to the phenolic compounds and tannins present in the peel increase with increasing concentrations of phenolic compounds, as the highest inhibition zone was for a concentration of 100% It was higher than the amikacin inhibition zone, and the lowest inhibition zone for yeasts and molds was 25% compared to the rest of the concentrations, as shown in Figure (3).

These results are in line with recent research [35].demonstrated that adding pomegranate peel powder to soft cheese inhibits the growth of mold and yeast while delaying food deterioration. revealed that pomegranate peel extracts successfully decreased Staphylococcus aureus infection in cheese [36], highlighting the wide range of antibacterial activity of these organic substances [37].

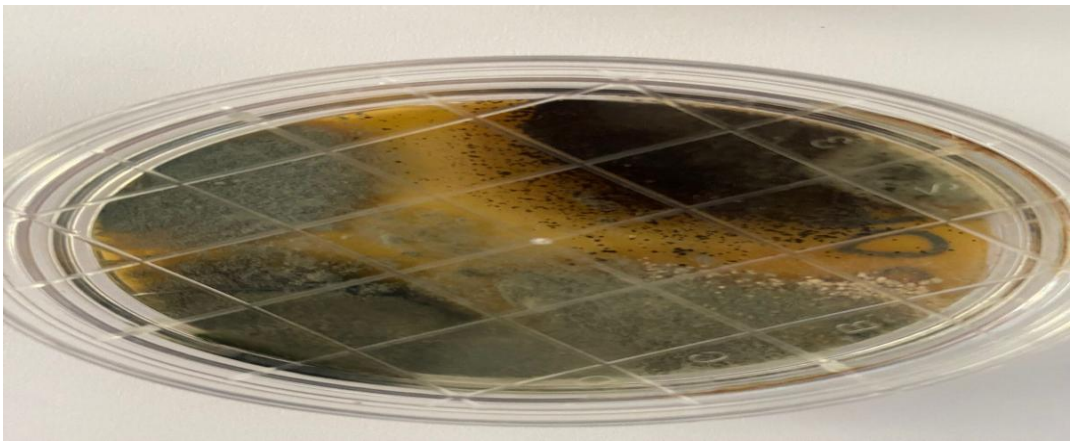

Figure (3) Yeast and mold inhibition zone at 25% concentration of pomegranate peel extract

4. CONCLUSION

In this investigation, ethanolic pomegranate peel extracts showed strong antibacterial activity against a few foodborne pathogens. According to the results, pomegranate peel extract may be used directly or as an active coating to increase food safety and prolong shelf life as a natural antibacterial agent for food preservation applications. Nevertheless, the complex circumstances of actual food systems are not adequately represented by the antibacterial activity found using the disc diffusion approach. A number of variables, including as pH, fat content, and interactions with food matrix elements like proteins, may affect how effective bioactive chemicals are. In order to establish their industrial usefulness, more research is advised to optimize extract formulations and assess their efficacy through direct application in food products under realistic storage circumstances.

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