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# *Citrus aurantium* L. PEELS AND SEEDS: PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY

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**Abstract.** In this intriguing study, orange peel and seed extracts were tested for their antibacterial effects against pathogens isolated from urinary tract infections and wound swabs. Ethanol and acetone extracts demonstrated potent antibacterial activity against *Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, and Staphylococcus epidermidis*. The ethanol extract showed larger inhibition zones against *P. mirabilis* (27 mm) compared to acetone extract. There was a significant decrease in bacterial growth when acetone extract was used, with the largest inhibition observed *E. coli* (23 mm). The phytochemical analysis revealed the presence of tannins, flavonoids, terpenoids, steroids, alkaloids, and saponin in the extracts, except for acetone extract lacking terpenoids and saponin. This in vitro study demonstrates the significant antibacterial activity of Citrus aurantium peels and seeds against a range of common bacterial pathogens. These findings warrant further investigation to assess the potential real-world applications of these extracts, including toxicity testing and determination of optimal dosage.

***Keywords:*** *Citrus aurantium L., Disk diffusion method, Antibacterial activity, Phytochemical analysis.*

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## 1. Introduction

Fruits have been widely recognized as an important part of a balanced diet, leading to the development of numerous fruit-based food and drink products (Ghasemi *et al.*, 2009). Fruits are a great source of various important bioactive elements, including vitamin C, vitamin B complex, flavonoids, carotenoids, and minerals, which work together to lessen the severity of a number of diseases (Ramful *et al*., 2010).

Among the more than 140 genera and 1,300 species that make up the family Rutaceae are the citrus fruits known as Citrus sinensis (sweet orange) and Citrus aurantium (sour orange) (Mandalari *et al*., 2006). Brazil, the United States, Japan, China, Mexico, Pakistan, and countries in the Mediterranean region are only some of the tropical and subtropical countries where they are grown (Anwar *et al.*, 2008). In the citrus

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processing sectors, where a lot of peel is generated and otherwise goes to waste, the peel is recognized as a valuable by-product. Citrus essential oil, however, is a by-product that adds taste to beverages and dishes. It is used extensively in the manufacturing of pharmaceuticals, soaps, fragrances, and other cosmetics, and household cleaning products (Okwu & Emenike, 2006). There are several phenolic chemicals, including flavonoids, in citrus peels, and these compounds have antibacterial and antioxidant properties (Jayaprakasha *et al*., 2008; Siddique *et al*., 2011; Adnan *et al*., 2014; Parashar *et al*., 2014). Citrus has been used for centuries in traditional medicine in places as diverse as China, India, Japan, and Africa, as shown in a number of studies (Warrier, 1993; Aibinu *et al.*, 2007; Hirota *et al*., 2010; Zhou *et al*., 2013). In addition, antibacterial activity has been observed in plant extracts and phytochemicals that have been employed therapeutically throughout the years (Prabuseenivasan *et al*., 2006; Dhanavade *et al*., 2011; Jeber Jalal *et al*., 2019). *Citrus aurantium* L. (Rutaceae), also known as sour or bitter orange, is widely used in cooking and as a flavoring ingredient (Rouseff & Perez-Cacho, 2007). The essential oils derived from the plant have a wide range of applications, such as analgesic, anti-inflammatory, antibacterial, and antifungal (Caccioni *et al*., 1998; Giamperi *et al*., 2002; de Moraes Pultrini *et al*., 2006; Gruenwald *et al*., 2000; Abdi-Azar & Maleki, 2014; Azadi *et al.*, 2013). In addition, its flavonoids have been linked to a variety of health benefits, including cancer prevention, blood sugar regulation, and cellular reinforcement (Hsouna *et al*., 2013). *Fructus aurantii* is another name for *Citrus aurantium* L., (Bouchard et al., 2005; Haaz *et al.*, 2006). The concentration of the juvenile organic product or strip of *C. aurantium* L. has been widely used in weight loss, dietary supplements, and athletic performance products (Stohs *et al*., 2011). Similarly, *C. aurantium* L. is one of the species that are used in clinical trials due to the different bioactive substances that it contains, such as phenolics, essential oils, minerals, and flavonoids. Since the nineteenth century, most important classes of antibiotics, such as tetracyclines, cephalosporins, aminoglycosides, and macrolides, have suffered a setback, losing viability due to the spread of microbial resistance (Mayers *et al*., 2009). Till now, the impact of antimicrobial resistance is extensive because many bacterial infections no longer respond to treatment due to the emergence of multidrug-resistant bacteria. This growing problem of antimicrobial resistance has become a major global public health issue (Guschin et al., 2015; Martin *et al*., 2015). As a result, the development of novel antibacterial agents has become critical. Natural products continue to be a major source of novel medications in modern research. They are derived from prokaryotic bacteria, eukaryotic microorganisms, plants, and animals of various species. The majority of antibacterial chemicals identified so far come from microbial and plant products (Berdy, 2005). The aim of this study was to evaluate the antimicrobial potential of fruit peel waste from Citrus aurantium L. against pathogenic bacteria, with the goal of identifying potential active ingredients that could serve as sources for the synthesis of new antimicrobial drugs.

## 2. Materials and Methods

### *2.1. Plant Materials*

*Citrus aurantium* L. fresh fruit was gathered in February 2023 from trees in the Al-Tarmiyah neighborhood of Baghdad, Iraq. Specimens of the Citrus aurantium plant were recognized by the Botany Department at Baghdad University and placed in the herbarium. The lemons were thoroughly rinsed in clean water, their skins and seeds removed, and

they were then air-dried in the shade before being milled into powder in a Thomas-Willey milling machine.

### *2.2. Preparation of Extracts*

The powdered peels of *Citrus aurantium* L. (20 g) were extracted at room temperature for 6 days using two different solvents which are acetone and ethanol (100 mL each). Filtering the extracts and evaporating off the solvents at a low temperature (35oC) yielded dark yellow solids of 900 mg and 500 mg, respectively. The yield percentage was determined by weighing each dried extract. The fruit peel extracts were all kept at 4 degrees centigrade in a sealed vial. Each fruit peel extract was dissolved in a suitable volume of its own solvent to prepare a working stock.

### *2.3. Phytochemical Screening*

Standard protocols for chemical analysis, which was described by Sofowara (1993), Trease & Evans (1989) and Harborne (1973), were used to identify the contents in the ethanolic and acetonic extracts and the powdered specimens.

#### *2.3.1. Test for Tannins*

The dried powdered peels and seeds of *Citrus aurantium* L., were added to 20 mL of water in a test tube and boiled at 90 ˚C A few drops of 0.5% ferric chloride solution produced a blue-black staining in each extract, confirming the presence of tannins.

#### *2.3.2. Test for Steroids*

A 0.5 g of each extract from *Citrus aurantium* peel and seed was mixed with 2 mL of concentrated $H_2SO_4$ and 2 mL of acetic anhydride and mixed for 1 hr. The presence of steroids was represented by a colour change from violet to green.

#### *2.3.3. Test for Flavonoids*

The presence of flavonoids in the plant sample was ascertained using three different techniques (Gruenwald *et al*., 2000; Abdi-Azar & Maleki, 2014). A 5 mL of 20% ammonia solution was added to a portion of each plant extract's aqueous filtrate, followed by 2 mL concentrated $H_2SO_4$. The presence of flavonoids could be confirmed by the observation of a yellow coloring in each extract. A portion of each filtrate was mixed with a few drops of 1% aluminium solution. The presence of flavonoids was confirmed by the observation of a dark yellow coloring in each extract. Each sample of powdered plant material was treated with 10 mL of ethyl acetate and heated for 3 minutes in a steam bath. After filtering the mixture, 4 mL of the resulting filtrate was mixed with 1 mL of a 20% ammonia solution. All of the samples tested positively for flavonoids, appearing yellow in color.

#### *2.3.4. Test for Alkaloids*

Each extract was mixed with 5 mL of 2*M* hydrochloric acid, and the resulting solutions were heated in a water bath while being constantly stirred for 10 minutes. Dragendorff's reagent was added to a little amount of the filtered, cooled solutions. The presence of reddish-brown precipitates was taken as evidence of the presence of alkaloids in the sample.

*2.3.5. Test for Terpenoids (Salkowski Test)*

Five mL of each extract were mixed with 2.0 mL of chloroform, and then 3.0 mL of concentrated $H_2SO_4$. A reddish-brown interface formed, indicating the presence of terpenoids.

*2.3.6. Test for Saponins*

In a test tube, 20 mL of water was heated at 80 ˚C with 2 g of dried powdered samples of *Citrus aurantium* L. peels and seeds, and the resulting mixture was filtered. Ten mL of the filtrate were added to five mL of distilled water and agitated until a stable and persistent foam formed. Emulsion formation was monitored to confirm the presence of saponins when the foam was combined with three drops of olive oil and aggressively agitated.

### *2.4. Biological Activities*

*2.4.1. Micro-Organism*

The following strains isolates of bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus, and Staphylococcus epidermidis*) were obtained from stock cultures of pathological strains preserved at the Microbiology Laboratory, Department of Medical Laboratory Techniques, Alrasheed University College, Baghdad, Iraq.

*2.4.2. Antibacterial Activity*

The national committee for clinical laboratory standards' disc diffusion method was employed to ascertain how well the plant extract inhibited bacterial growth. Whatmann filter paper No. 1 (6 mm in diameter) was used to prepare discs with various extract concentrations (100 and 150 mg/mL). At 50 °C, the discs were dried. Each of the bacterial isolates' overnight cultures were diluted with sterile normal saline to produce an inoculum size of 106 cfu/mL. After being made, sterilized, cooled, and then placed onto sterile Petri dishes to a depth of 4 mm (about 25 mL per plate), the nutritional agar medium was solidified. The Petri dishes were infused with pure cultures of the test organisms. This was accomplished by applying the inoculate to the prepared nutrient agar plate with sterile cotton swabs that had been dipped in the diluted suspension of the organism. The discs were then aseptically positioned uniformly on the inoculation's surface and gently pressed down to achieve contact with a pair of forceps. Finally, the plates were incubated for 18 to 24 hours at 37 °C. Each of the inoculation plates included 10 mg of amoxicillin as a positive control. As negative controls, plates made following the identical protocols but without extract or antibiotic were also put up. After 24 hours, the plates were checked for a clearly defined zone of inhibition. A pair of calipers were used to measure and record the extract's antibacterial activity, which was then compared to that of the standard antibiotics used in this investigation.

## 3. Results and Discussion

### *3.1. Extraction Using Different Solvents*

The extracts of the *Citrus aurantium* peel and seed using different solvents yielded different results in each of the experiments conducted in this study. The extraction solvent impacted the percentage yield, as extracts from various solvents showed divergent yields.

Table 1 shows the comparison of the percentage yield of extracts obtained from different solvents. For *Citrus aurantium* peel extracts, the ethanol extract showed the highest percentage yield of about 16%, compared with the acetone extract, which showed a percentage yield of about 8.13%. *Citrus aurantium* seed extract showed the highest yield when ethanol was used as a solvent, with a yield percentage of about 12%, followed by acetone extract (7.86%). This variation in yield between ranges of solvents explains the solubility of different plant compounds in different solvents. These crude extracts were then employed for phytochemical analysis and antibacterial activity after being dissolved in pure ethanol and acetone solvents (400 mg/mL).

**Table 1.** The percentage yield of *Citrus aurantium* peel and seed extracts

| Samples | Solvents | Peels powder weight (g) | Solvent volume (mL) | Dry extract weight (g) | % Yield |
|---|---|---|---|---|---|
| Orange peel | Ethanol | 15 | 100 | 2.41 | 16.06 |
| | Acetone | 15 | 100 | 1.22 | 8.13 |
| Orange seed | Ethanol | 15 | 100 | 1.81 | 12.06 |
| | Acetone | 15 | 100 | 1.18 | 7.86 |

### *3.2. Phytochemical Screening*

Orange peels and seeds extracts were screened for the presence of secondary metabolites like terpenoids, alkaloids, tannins, flavonoids, steroids, and saponins (Table 2).

**Table 2.** The phytochemical screening of *Citrus aurantium* peel and seed extracts

| Phytochemical Test | Orange peel extracts | | Orange seed extracts | |
|---|---|---|---|---|
| | Ethanol extract | Acetone extract | Ethanol extract | Acetone extract |
| Flavonoids | + | + | + | + |
| Tannins | + | + | + | + |
| Steroids | + | + | + | + |
| Alkoloids | + | + | + | + |
| Terpenoids | + | - | + | - |
| Saponin | + | - | + | - |

In this study, the peels and seeds of *C. aurantium* were extracted with ethanol and acetone to reveal the presence of alkaloids, flavonoids, terpenoids, tannins, steroids, and saponin; however, the acetone extracts did not contain saponin and terpenoids. *Citrus aurantium* phytochemicals were more exposed to polar compounds than non-polar compounds. There are several factors that could account for the differences observed in extraction yields between the ethanol and water solvent systems. Ethanol demonstrated superior capability for solvation of the bioactive compounds present, thus facilitating improved recovery during extraction. Additionally, ethanol's higher thermal stability relative to water allowed more complete retention of volatile molecules that may have otherwise been lost during processing such as drying. Water, on the other hand, has lower solvating power for non-polar constituents and its lower boiling point necessitates more cautious handling to avoid loss of heat-labile analytes. Therefore, ethanol's dual advantages of highly effective solvation and greater thermal durability likely enabled greater extraction of target components from the samples. The most common classes of bioactive chemicals that function as main antioxidants or free radical scavengers are flavonoids, tannins, and

polyphenols (Fidrianny & Johan, 2015). Citrus aurantium peels were known to have possible antibacterial effects due to the presence of tannin and phenolic chemicals (Bashir *et al*., 2020). According to literature, flavonoids are an ideal group of secondary metabolites in citrus products that may have natural potential and affect human wellbeing in ways such as antimicrobial, anti-inflammatory, anti-diabetic, anti-cholesterol emic, anti-inflammatory, and anti-carcinogenic (*He et al*., 1997; Vanamala *et al.*, 2006; Huang & Ho, 2010; Chanet *et al.*, 2012; Park *et al.*, 2014).

### *3.3. Antibacterial Activity*

Table 3 shows the results of the antimicrobial activity of the *C. aurantium* peel and seed extracts against the pathogenic bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis*) with the standard positive control antibiotic amoxicillin using the disc diffusion method.

**Table 3**. Results of inhibition of the growth of bacteria by *Citrus aurantium* L. peel and seed extracts using the disc diffusion methods

| **Bacteria species** | **Inhibition of Zone (mm)** | | | | |
|---|---|---|---|---|---|
| | **Orange peel extracts** | | **Orange seed extracts** | | **Control** |
| | Ethanol | Acetone | Ethanol | Acetone | Amoxicillin |
| *Staphylococcus aureus* | 22 | 19 | 19 | 20 | 24 |
| *Klebsiella pneumoniae* | 24 | 21 | 22 | 19 | 24 |
| *Proteus mirabilis* | 22 | 22 | **27** | 22 | 25 |
| *Escherichia coli* | 22 | 23 | 17 | 23 | 26 |
| *Staphylococcus epidermidis* | 00 | 00 | 22 | 22 | 24 |

The ethanol extract of orange seed showed the highest zone of inhibition against *P. mirabilis* (27 mm) and the lowest zone of inhibition against *E. coli* (17 mm) (Table 3). The acetone extract of orange peel and seed inhibited the growth of all tested bacteria and the maximum inhibition zone was recorded against *E.coli* (23 mm). The ethanol extract of orange peel and seed has shown (Table 3) the highest inhibition zone in all the test pathogens compared to the acetone extract. Standard Amoxicillin was found to have a broad spectrum of activity, with a zone of clearance ranging in size from 24 mm to 26 mm in diameter against various bacterial species. Figure 1and 2 shows the setup for this experiment on an LB agar plate.

There may be a wide variety of antibacterial compounds in the ethanol extract, each with their own unique mechanism of action, or the bacteria may have evolved a unique metabolic strategy to counteract or adapt to its activity. Because of its high antibacterial activity and high yield, ethyl alcohol is an excellent solvent for extracting antibacterial compounds from lemon peel. Acetone extract was able to limit the growth of all of the pathogens examined, but with a somewhat reduced sensitivity. According to Ashokumar et al. (2012), the variation could be attributable to the extraction technique, environmental conditions, or a distinction in the citrus plant genotypes employed, all of which affect the phytochemical makeup of different portions of the plant. The antibacterial effectiveness of the orange peel extract was comparable or higher.

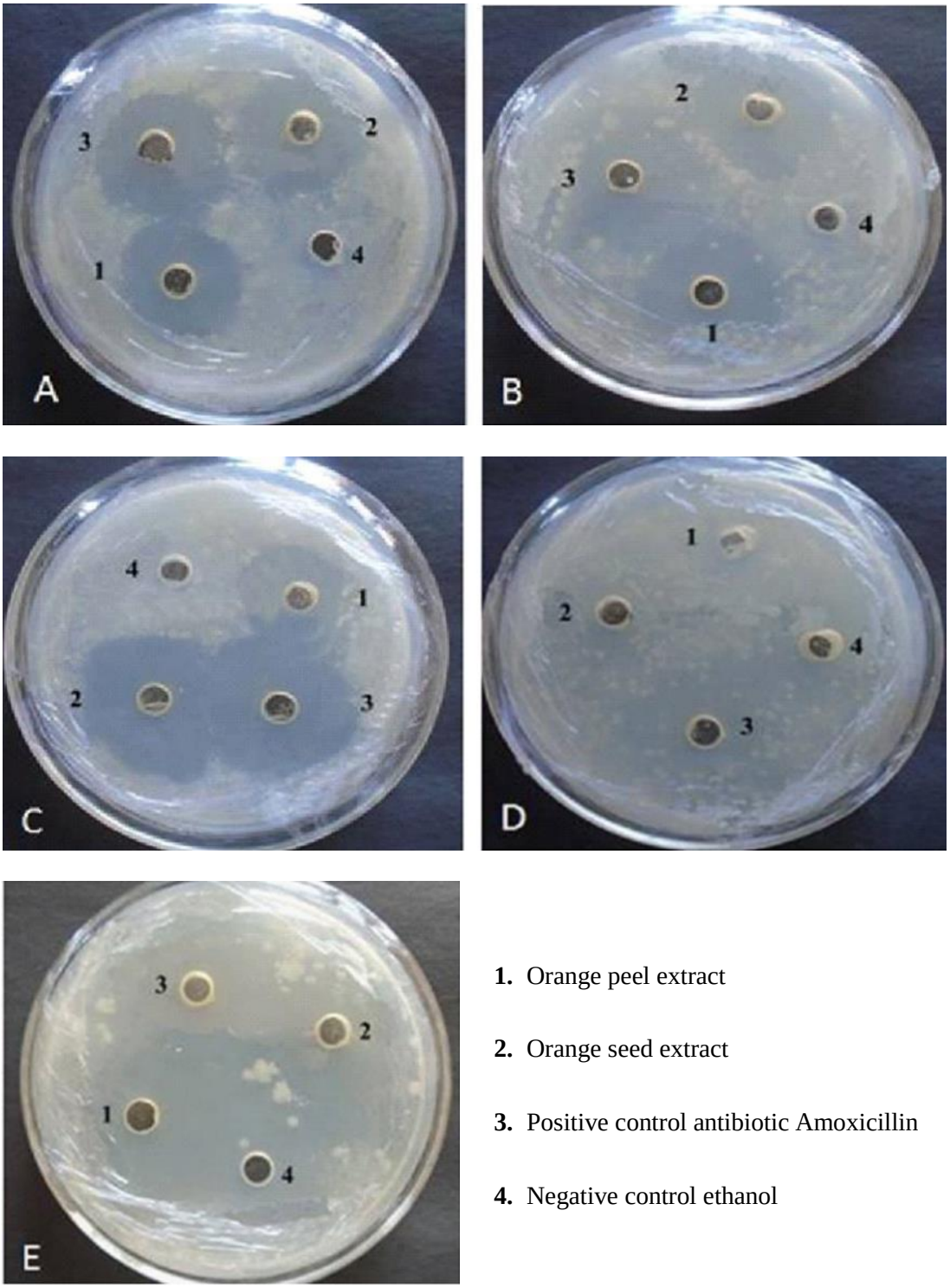


**Figure 1.** Antibacterial activity of ethanol extracts of *C. aurantium* peel and seed against (**A**) *S. aureus*, (**B**) *K. pneumoniae*, (**C**) *P. mirabilis*, (**D**) *E. coli*, and (**E**) *S. epidermidis*

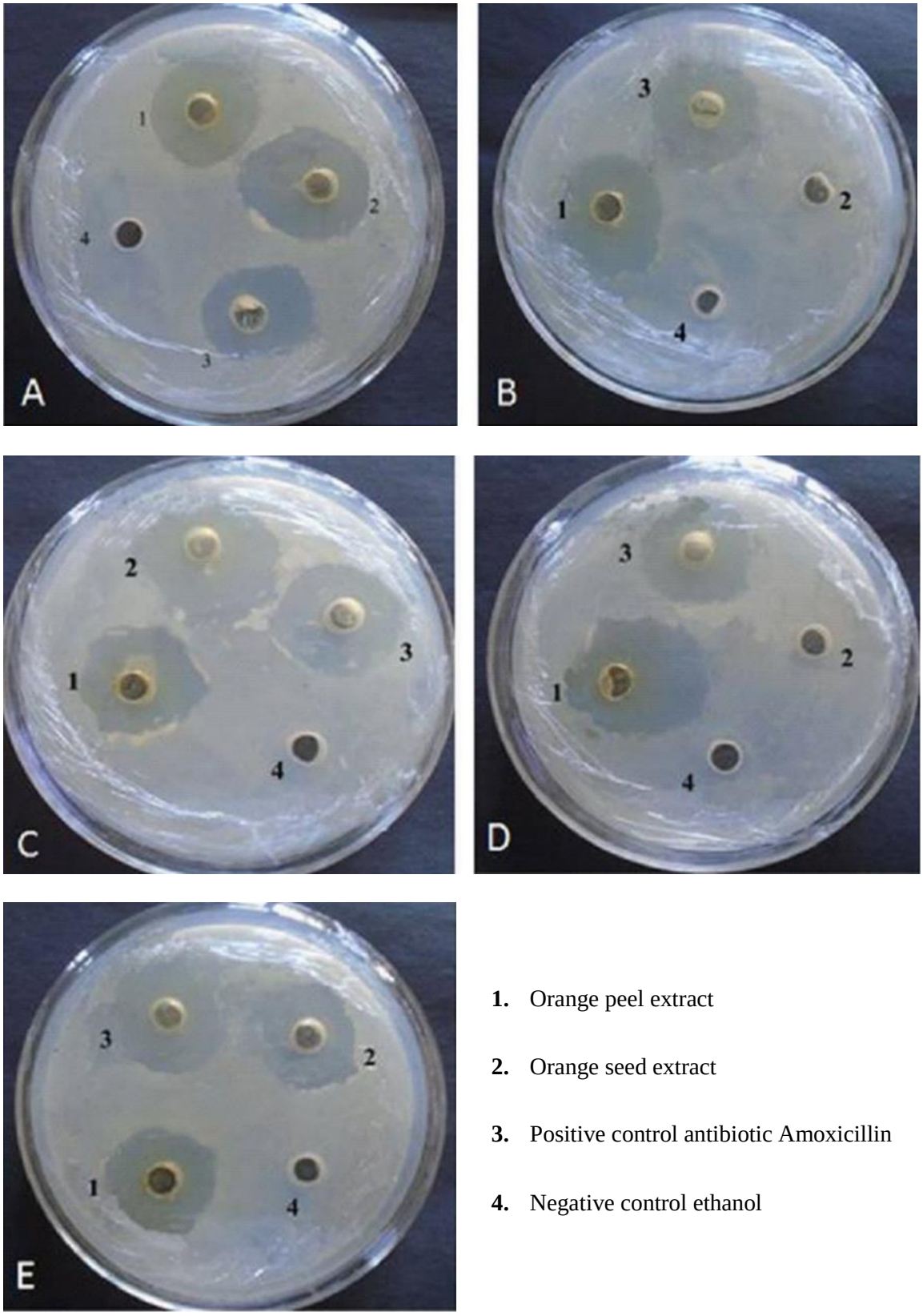


**Figure 2.** Antibacterial activity of acetone extracts of *C. aurantium* peel and seed against (**A**) *S. aureus*, (**B**) *K. pneumoniae*, (**C**) *P. mirabilis*, (**D**) *E. coli*, and (**E**) *S. epidermidis*

The antimicrobial properties of orange and pineapple peel extracts can be attributed to the presence of a variety of active phytochemicals, including flavonoids, terpenoids, vitamins, carotenoids, coumarins, lignin, sterols, and saponins (Al-Ani *et al*., 2021). Ascorbic acid, flavonoids, phenolic compounds, pectins, and other bioactive chemicals and antioxidants that are crucial for human nutrition can be found in abundance in citrus fruits and juices (Fernandez-Lopez *et al*., 2005). Additionally, essential oils from pineapple and orange peel extracts showed antibacterial action against a variety of pathogenic microorganisms (Samy, 2005). The main bioactive components for the sample's antibacterial activity have been identified as phenolic chemicals. Since the vast majority of plant phenolic compounds are safe for human consumption, they have the potential to be used as natural antioxidants in food and beverages, they could be utilized to stop the development of numerous food-borne and food-spoiling microbes in foods. Additionally, it has been claimed that citrus essential oils extend the shelf life and safety of little processed fruits (Lanciotti *et al.*, 2004). This study's findings show some very encouraging trends. According to the present literature, there are a number of restrictions on how well orange peel and seed can be used as a multipurpose antibacterial therapeutic agent across all organisms. Finally, it is concluded that Citrus sinensis and Ananas comosus, based on their characterization, could be helpful sources for the synthesis of antibiotics and have the potential for medical and industrial applications. Since there has been a growing trend to replace synthetic antioxidants and antimicrobials with natural ones, research into the antioxidant and antimicrobial properties of various extracts from many plants has received a lot of attention (Deab *et al.*, 2008). This is because these extracts may be used as natural additives in food products. Tests for phytochemical analysis are preliminary qualitative tests that are helpful in identifying bioactive substances and may subsequently be helpful for the development of new drugs (Mallikharjuna *et al*., 2007).

## 4. Conclusion

The experimental findings demonstrated that Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, and Staphylococcus epidermidis exhibited susceptibility to peel and seed extracts of Citrus aurantium obtained using ethanol and acetone solvents.. The findings of this study indicate that *C. aurantium* peel and seed extracts in ethanol and acetone have antibacterial activities that could be investigated as a potential replacement for commercially available antibiotic medications. It has to be tested on other microbes and against different illnesses, and the results would be solid support for the plant's effectiveness as an antibacterial agent.

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