

EXPLORING THE ANTIMICROBIAL PROPERTIES OF LEMON: A COMPARATIVE ANALYSIS OF PEEL, SEED, AND PULP

ABSTRACT

Aim

Lemons are a treasure trove in nature, belonging to the Rutaceae family and rich in vitamin C, as well as various macro and micronutrients. They are widely known for boosting immunity and can potentially be used as a natural source of medication. D-limonene is one of the main bioactive compounds present in lemons, and it is responsible for the refreshing fragrance of lemons. The study aims to identify whether the waste from lemons can be used as potential nutraceuticals or functional foods

Study design

We took five different species for comparative antibacterial studies from Citrus species those are ***C. aurantifolia***, ***C. limetta***, ***C. sinensis***, ***C. reticulata***, and ***C. maxima***.

Place and Duration of Study:

Biotechnology lab, Techno India University, Kolkata. The duration of this study was 1 year.

Methodology

In this comparative study, the major parts (pulp, seed, and peel) of a fruit were used for the experiments. It is important to reduce the amount of waste in the environment by creating creative and cost-effective, eco-friendly waste management techniques. The antibacterial potential against *E. coli* and *S. aureus* was thoroughly measured using the Kirby-Bauer disc diffusion method.

Result

The results show that in the case of *E. coli* inhibition, the peels of *C. reticulata*(58.33 ±0.4 mm), the seeds of *C. aurantifolia*(58.33 ±0.4 mm), and the pulp of *C. aurantifolia*(55.33 ±2.94 mm) perform well compared to other samples. In the case of *S. aureus* inhibition, the peels of *C. limetta*(51±1.41mm), the seeds of *C. aurantifolia* (58.33 ±0.4 mm), and the pulp of *C. aurantifolia*(49±2.82mm) perform well compared to other samples.

Conclusion

Hence, the results indicate that PEELs can be potential antimicrobial agents and have discovered that various parts of the citrus fruit exhibit a wide range of antimicrobial effects against both gram-positive and gram-negative bacteria.

Keywords: *Citrus peel, seed, pulp, antimicrobial, Citrus fruits,*

1. INTRODUCTION

Citrus fruit belongs to the Rutaceae family. They are rich in citric acid and ascorbic acid, strengthening our immune system. Citrus fruits are one of the most widely grown fruits and are an important source of physiologically active substances and phytochemicals (Suri, Singh, and Nema 2022). They can potentially be employed as a natural medication source or a major component of functional food since they are an excellent source of bioactive compounds and are high in ascorbic and citric acid, which boosts our immune systems. These compounds are mostly recognized in their edible parts. On the other hand, limonoids—typical citrus fruit triterpenoids with an intensely bitter taste and potentially anticarcinogenic and chemo-preventive properties can be extracted from seeds. (Sengupta et al. 2023).

Citrus fruits increase waste generation when consumed raw or juiced. Conventional rubbish disposal techniques contaminate land and waterways, potentially harming aquatic environments. Consequently, to lessen the quantity of waste that builds up in the environment, it is essential to create creative processes and economical, ecologically friendly waste management techniques (Seid et al. 2023).

Bacterial infections worldwide are a leading cause of illnesses, physical impairments, and deaths. Medicinal plants are thought to offer a safer and more affordable alternative for treating bacterial infections because they contain a diverse array of phytochemicals. Natural medicines made from medicinal plants have antibacterial properties that can be used to treat viral, fungus, and bacterial illnesses. Microorganisms are becoming more resistant to antibiotics, despite the pharmaceutical industry having developed several new ones during the past three decades. (Gupta and Sharma 2022) Many years of antibacterial study have been conducted on citrus peels, pulp, and seeds, and it has been demonstrated that seed wastes consist of a substantial number of essential oils and polyphenols with antimicrobial activity. Bioactive chemicals (Roquia et al. 2022) with potential medicinal applications can be found in abundance in citrus trash. Numerous advantageous characteristics of these compounds have been discovered, such as their anti-aging, anti-mutagenic, anti-carcinogenic, and anti-allergenic effects. One particularly interesting source of natural chemicals with potential for therapeutic use is *Citrus sinensis*. New medications may therefore be developed as a result of more research into the extraction and application of these substances from citrus trash. (Baker, Ibrahim, and Salama 2021)

In this current study the antibacterial properties of citrus seed, peel, and pulp extracts from *Citrus reticulata*, *Citrus limetta*, *Citrus aurantifolia*, *Citrus sinensis*, and *Citrus maxima* and d-limonene (essential oil rich in terpenes found in the rind of citrus fruits) were investigated using the agar-well diffusion method against *Staphylococcus aureus* and *Escherichia coli* a gram-positive and a gram-negative bacteria respectively.

LITERATURE STUDY

seeds were found to contain alkaloids, saponins, and other compounds that contribute to their antimicrobial activity. The antimicrobial effects of *Citrus limetta* were also highlighted, attributed to the presence of limonene and other compounds. (Mohammed and Ayoub 2016). The essential oil exhibited dose-dependent activity against *S. aureus*, more so than against *E. coli*, according to the results of the antibacterial activity analysis. In comparison to ampicillin, the positive control, its antibacterial activity is significantly lower. (Valarmathy et al. 2010)

According to the results of the antibacterial activity analysis, the essential oil exhibits dose-dependent activity against *S. aureus* more so than it does against *E. coli*. In contrast to ampicillin, the positive control, its antibacterial activity is significantly lower. (Al-Aamri et al. 2018)

The study revealed that extracts from citrus fruits, specifically *Citrus reticulata* and *Citrus limetta*, have demonstrated antibacterial properties against certain bacterial strains. *Citrus reticulata* peel extract was more effective against gram-positive bacteria, particularly showing a high inhibition zone against *Klebsiella pneumoniae*. On the other hand, *Citrus limetta* peel extract was found to have superior antibacterial effectiveness against specific bacterial strains compared to its juice extract, especially in inhibiting gram-positive bacteria like *Bacillus* sp. However, *Citrus limetta* juice extract was more efficient than its peel extract against gram-negative bacteria. It's worth noting that 10% DMSO did not exhibit any inhibition zone. In the case of *Citrus maxima*, its peel extract showed stronger antibacterial action against *Bacillus* sp among gram-positive bacteria, while its juice extract was more effective against *S. aureus* and had a maximum inhibition zone against *E. coli*. The study also found that the juice extract of *Citrus maxima* was more efficient against gram-negative bacteria than its peel extract. (Shakya et al. 2019)

Citrus aurantifolia is rich in phytochemical components such as flavonoids, alkaloids, tannins, and phenols, all of which have been shown to have antibacterial qualities. (Garba et al. 2016). It was found that ethanolic extract of pomelo (*Citrus maxima*) seeds and pulp exhibited the largest zones of inhibition for *Staphylococcus aureus*. The ethanolic extract of pomelo (*Citrus maxima*) seeds and pulp showed low inhibition zone against *Escherichia coli* and *Bacillus subtilis* compared to ethanolic extract of grapefruit (*Citrus paradisi*) seeds and pulp. Ethanolic extract of grapefruit (*Citrus paradisi*) seeds and pulp showed inhibition zones more for *Staphylococcus aureus* compared to *Bacillus subtilis* and *Escherichia coli*. (Sahlan et al. 2018). The inhibition zones for the *Bacillus subtilis* in the investigated pomelo (*Citrus maxima*) seeds and pulp ethanolic extract is higher than the grapefruit seeds extract sample. (Lahlou 2004)

It was identified that beneficial antibacterial effect has contributed to the antimicrobial activity of citrus flavonoids, such as naringenin and hesperidine. The research also showed clear disparity between

the antimicrobial activity of the pomelo (*Citrus maxima*) seeds and pulp ethanolic extract and the grapefruit (*Citrus paradisi*) seeds and pulp ethanolic extract.(Al-Âni, Tawfik, and Shehab 2011)

In the study, it was found that pulp has more antioxidant activity and antimicrobial activity as compare with the orange peel.(Arora and Kaur 2013).It was also observed that limonene showed a significant antibacterial activity in the growth and reproduction of *S. aureus*. The number of colonies lowered gradually with the increased concentration of limonene(Han, Chen, and Sun 2021).It was determined that the antibiotic potential and antibacterial activities of lemon is significantly higher against drug-resistant phenotypes compared to other fruits.(Sengupta, Banerjee, et al. 2023)

2. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Collection of samples

Sample collection was done from local vendors in Kolkata, West Bengal between August 2023 and October 2023 throughout the year. The collected sample of seeds, peels, and pulps were then powdered in a hot air oven. According to Sulaiman et al., It is best to limit the interval between sample harvest and experimental work, as dried samples are easier to work with than fresh samples, which are difficult to handle and tend to disintegrate easily. (Sulaiman et al. 2011). Oven drying that uses thermal energy to eradicate moisture from samples is considered an easy and rapid process that preserves phytochemicals and essential antioxidants as mentioned by(Mediani et al. 2013)_D-limonene source from Sigma-Aldrich

2.1.2 Microorganisms

Staphylococcus aureus and *Escherichia coli* bacterial strains were obtained from the Department of Biotechnology, Techno India University, West Bengal, Salt Lake, Kolkata-700091. The bacteria strains were grown in Himedia M002-100G Nutrient broth at 37⁰ Celsius or 98.6 Fahrenheit in a shaker incubator overnight.

2.1.3 Sample preparation

The dehydrated samples were then powdered finely using a household blender and 1 g of every sample was then added to 20 ml of double distilled water to make a final concentration of 50 mg/ml. After thoroughly mixing the mixture, it was left in the dark for an entire night on a vibration table set to a low setting. After passing the mixture through the Whatman No. 1 filter paper, the aqueous extract was obtained and used without any additives.

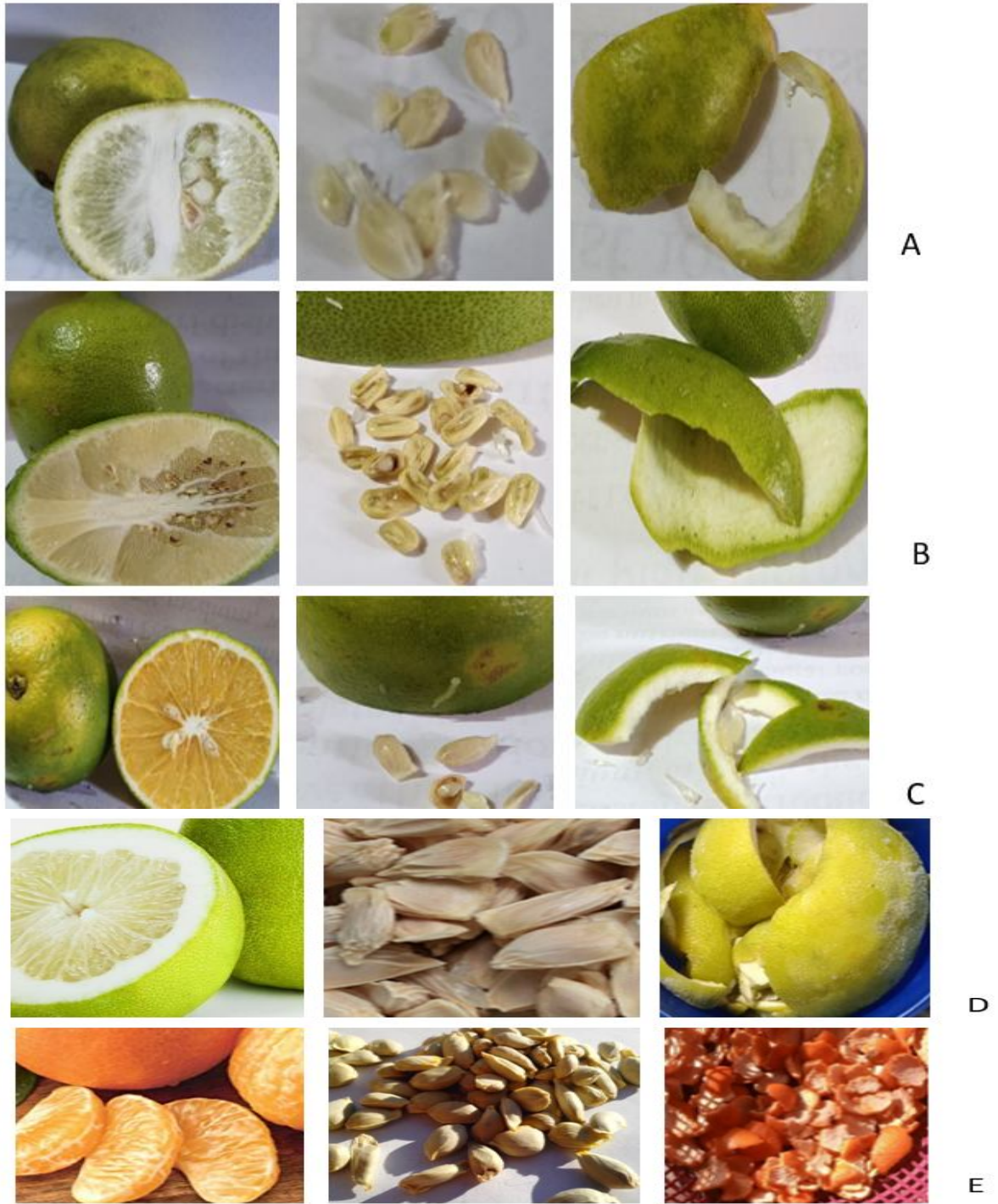


Image 1: The samples are A – *Citrus aurantifolia*, B- *Citrus reticulata*, C – *Citrus limetta*, D- *Citrus maxima*, E- *Citrus sinensis*. 1 denotes pulp, 2 denotes seed and 3 denotes peel of the above-mentioned samples.

2.2 Methodology

The Kirby-Bauer test was used to assess antibiotic susceptibility. Extracts and d-limonene were tested using the disc diffusion method on agar plates. Samples were added to filter paper discs and placed on bacterial plates, which were then incubated to observe the zones of inhibition.

3. RESULTS AND DISCUSSION

The seeds, pulp, and peel of a total of 5 commonly found citrus species samples were taken namely *Citrus reticulata*, *Citrus limetta*, *Citrus aurantifolia*, *Citrus sinensis*, and *Citrus maxima* along with d-limonene, a vital essential oil found in every citrus species. Water extracts of the samples were subjected to the Kirby-Bauer test for antibiotic susceptibility. We chose two different bacteria, *Staphylococcus aureus*, and *Escherichia coli* which are gram-positive and gram-negative respectively. Different concentrations of ampicillin were also subjected to the test to compare the efficiency of the samples.

3.1 Antimicrobial efficacy of samples against gram-negative bacteria *Escherichia coli*

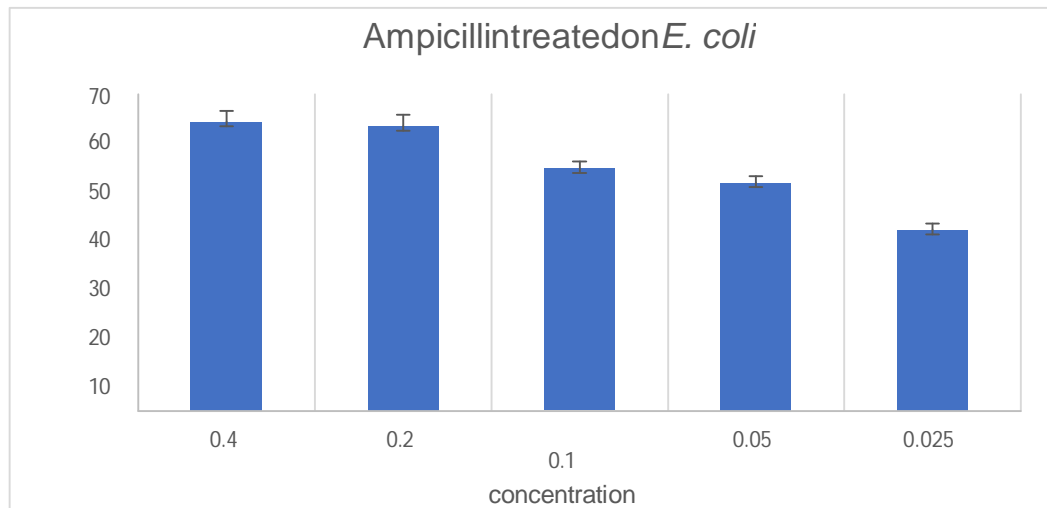


Fig 1: Diagrammatic representation of the antimicrobial activity of Ampicillin on *E. coli* bacteria

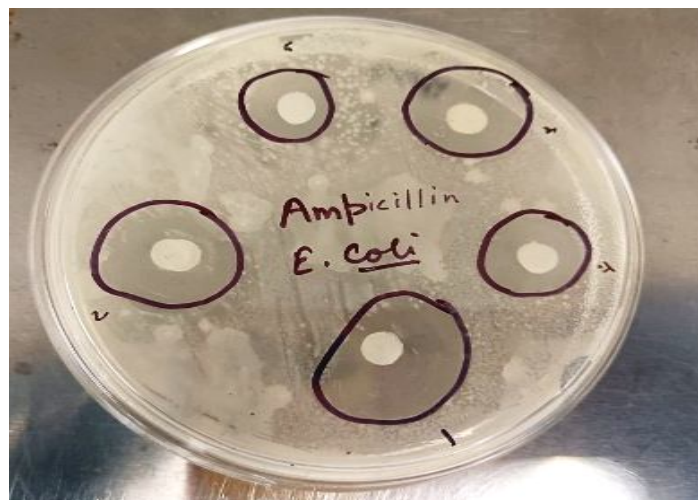


Fig 2: a pictorial representation of the inhibitory zones of ampicillin

Different concentrations of **Ampicillin** have shown different inhibitory zones on *E. coli*. Every time 40 μ l of the sample was used for the treatment 0.4 mg 64 ± 2.44 mm, 0.2 mg - 63 ± 2.5 mm, 0.1 – 53.66 ± 1.6 mm, 0.05 mg - 50 ± 1.5 mm, 0.025mg – 40 ± 1.5 mm [fig 2]

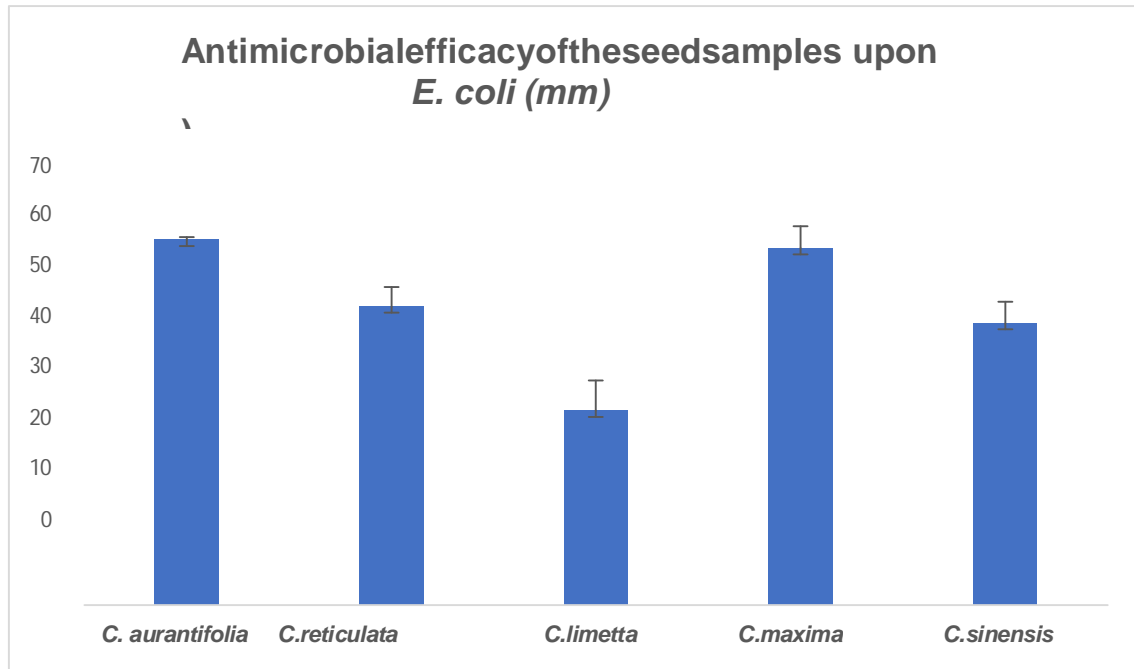


Fig. 3: Diagrammatic representation of the antimicrobial activity of the seed samples on *E. coli* bacteria.

The result shows *C. aurantiifolia* and *C. maxima* have the highest antimicrobial activity among other samples. Sd measured (n=3) for each sample [fig 3] Pictures show the zones in which the samples have inhibited bacterial growth. Seed extracts of *C. aurantiifolia* (58.33 ± 0.4 mm) and *C. maxima* (57 ± 3.5 mm) showed good antibacterial efficacy [fig 6]

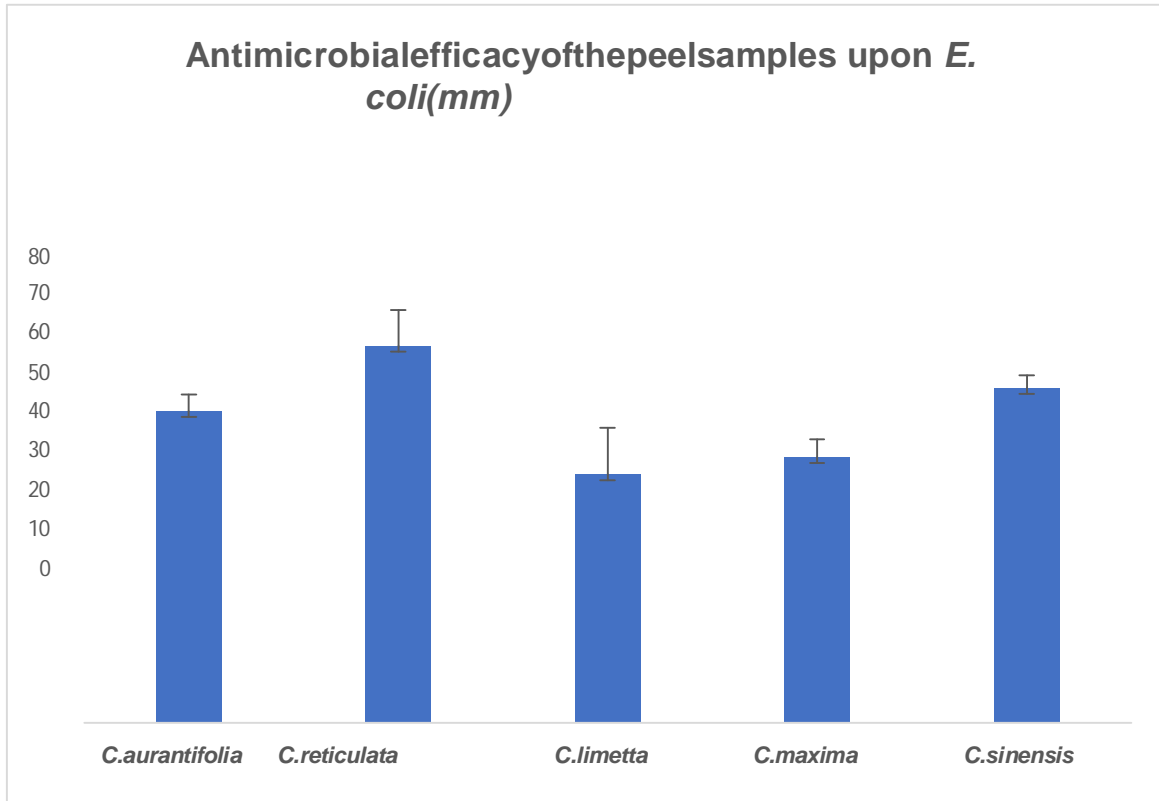


Fig. 4: Diagrammatic representation of the antimicrobial activity of the peel samples on *E. coli* bacteria

C. reticulata and *C. sinensis* have the highest antimicrobial activity among other samples. Sd measured (n=3) for each sample [fig 4] Pictures show the zones in which the samples have inhibited bacterial growth. peel extracts of *C. reticulata* (58.33 ± 0.4 mm) and *C. sinensis* (57 ± 3.5 mm) showed good antibacterial efficacy

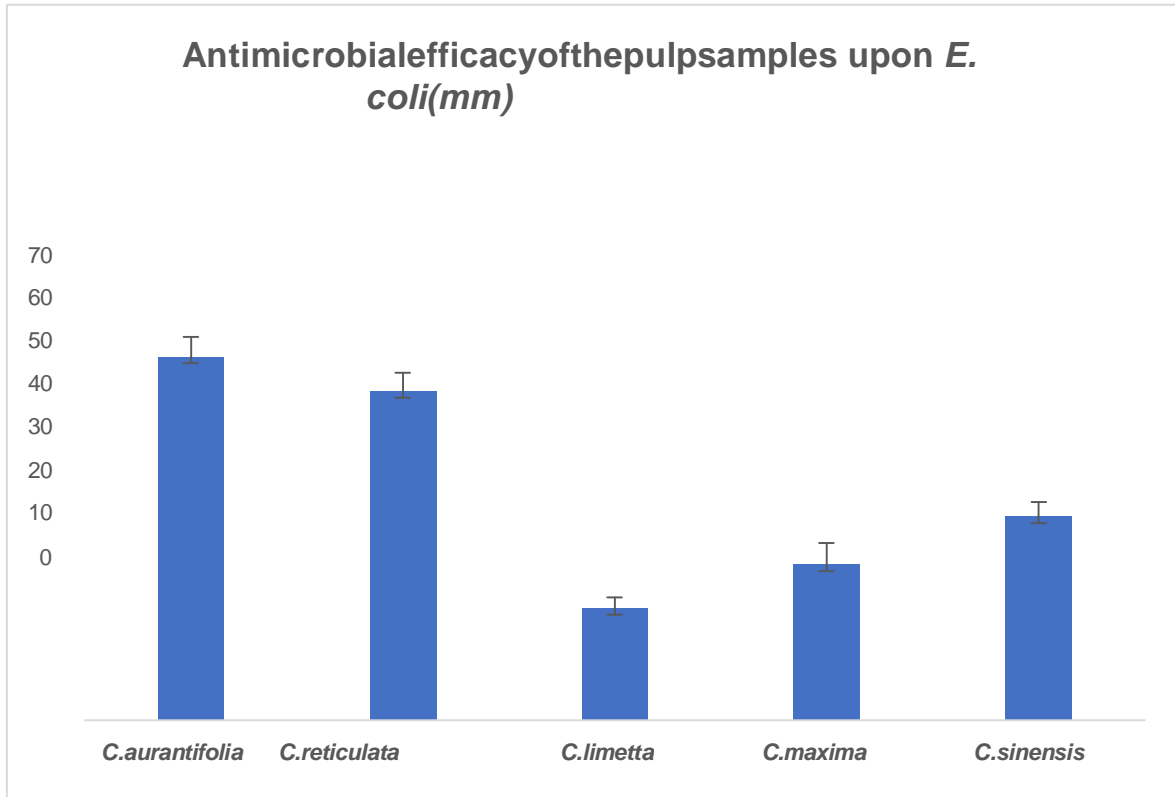


Fig. 5: Diagrammatic representation of the antimicrobial activity of the pulp samples on *E. coli* bacteria

The result shows *C. aurantiifolia* and *C. maxima* have the highest antimicrobial activity among other samples. Sd measured (n=3) for each sample [fig 5] Pictures show the zones in which the samples have inhibited bacterial growth. Pulp extracts of *C. aurantiifolia* (55.33 ±2.94 mm) and *C. reticulata* (50±2.6 mm) showed good antibacterial efficacy [fig 6]

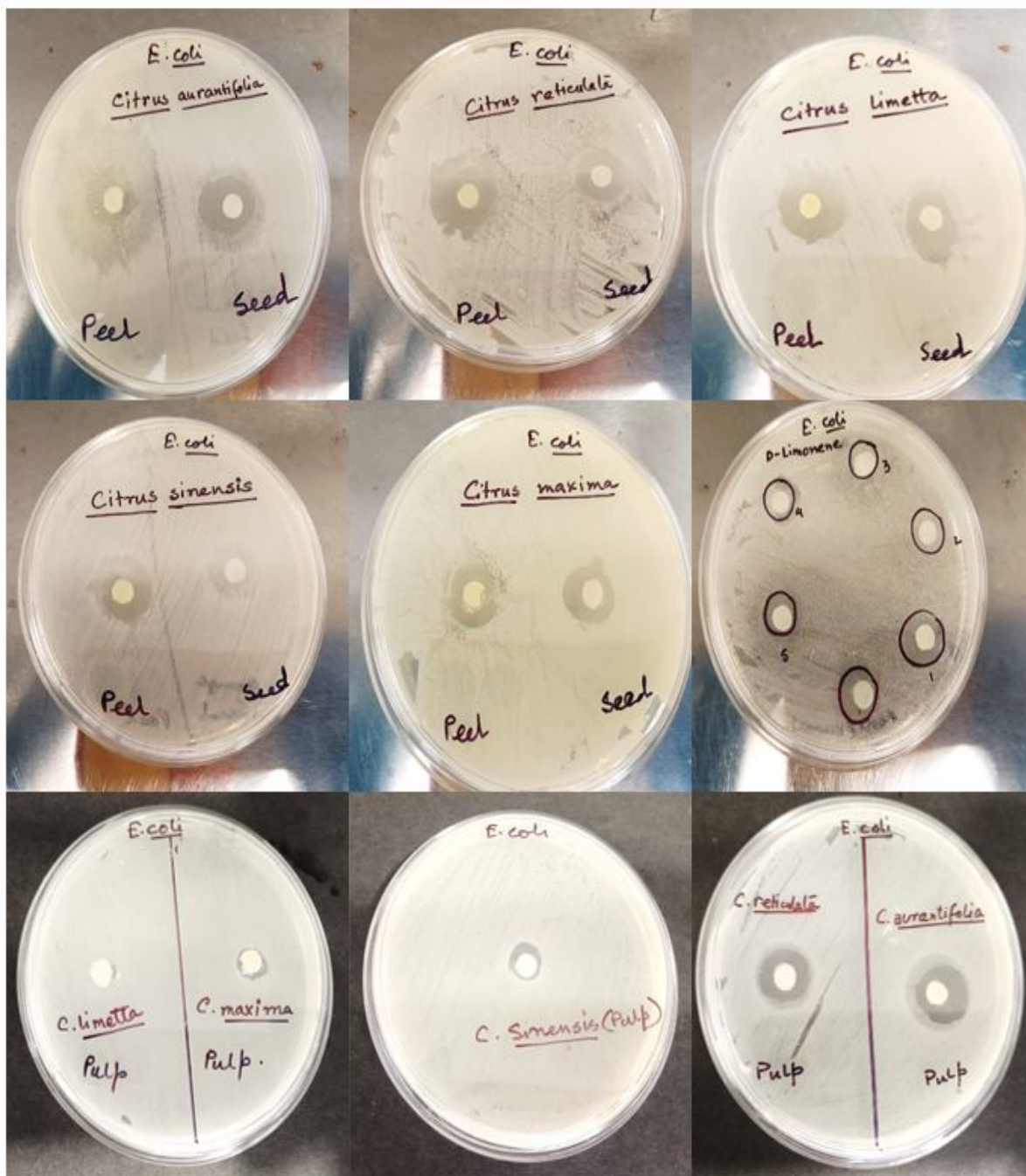


Fig. 6: Pictorial representation of the antimicrobial activity [zone of inhibition] of the seed peel and pulp samples on *E. coli* bacteria.

Part	Samples	Zone of Inhibition(mm)
Seed	<i>C.aurantifolia</i>	58.33±0.471
	<i>C.reticulata</i>	47.66±3.09
	<i>C.limetta</i>	31±4.89
	<i>C.maxima</i>	57±3.55
	<i>C.sinensis</i>	45±3.45
Peel	<i>C.aurantifolia</i>	54±2.82
	<i>C.reticulata</i>	65.33±6.16
	<i>C.limetta</i>	43±8.06
	<i>C.maxima</i>	46±3.09
	<i>C.sinensis</i>	58±2.16
Pulp	<i>C.aurantifolia</i>	55.33±2.94
	<i>C.reticulata</i>	50±2.82
	<i>C.limetta</i>	17±1.63
	<i>C.maxima</i>	23.66±3.29
	<i>C.sinensis</i>	31±2.16

Table 1. Inhibitory zones(mm) of three different parts of citrus fruits of five different species.

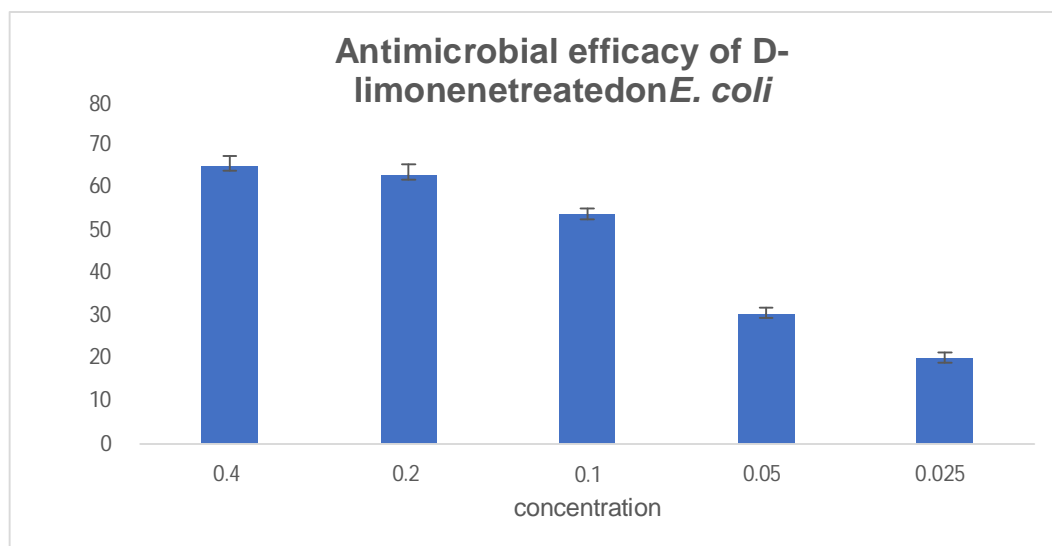


Fig 7: Different concentrations of D limonene have shown different inhibitory zones on *E.coli*.

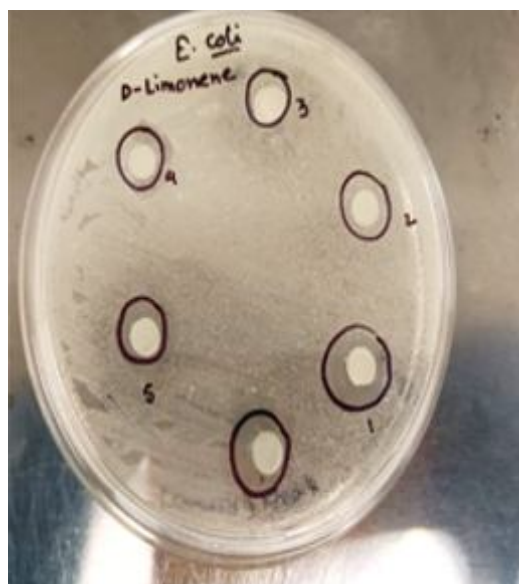


Fig 8: Pictorial representation of the antimicrobial activity of the bioactive compounds D-Limonene

Treatment	Inhibitory zones (mm)
0.4	65±2.44
0.2	63±2.5
0.1	53.66±1.6
0.05	30.5±1.5
0.025	20±1.5

Table 2. Range of concentration of d-limonene treatment along with their Inhibitory zones(mm)

Every time 40 µl of the sample has been used for the treatment 0.4 mg 65± 2.44 mm, 0.2 mg - 63 ± 2.5 mm, 0.1 – 53.66 ±1.6 mm, 0.05 mg - 30.5 ±1.5 mm, 0.025mg – 20 ±1.5 mm [fig 7, 8]

3.2 Antimicrobial efficacy of samples against gram-positive bacteria *Staphylococcus aureus*

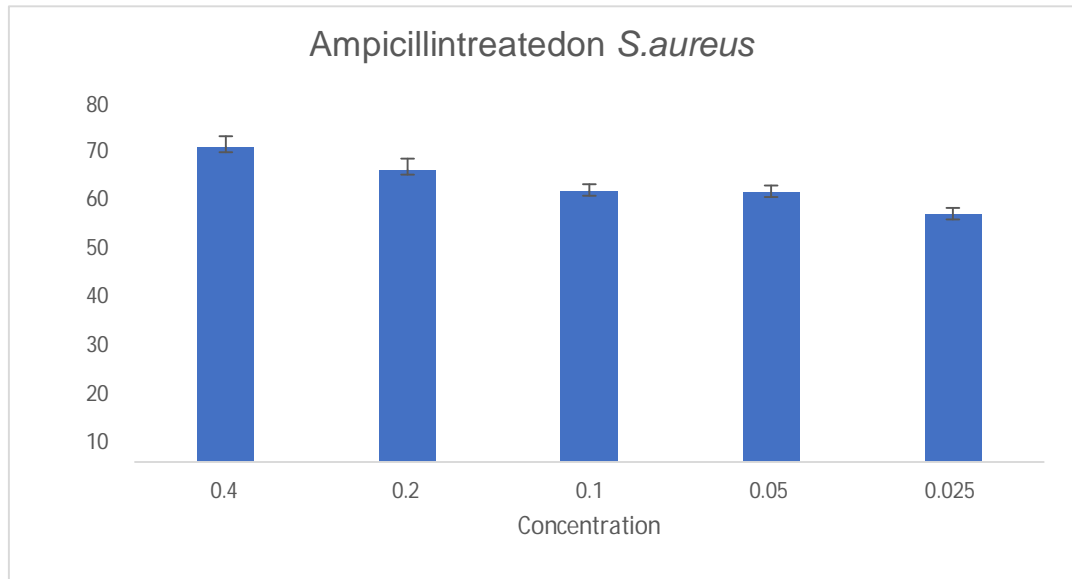


Fig. 9: Diagrammatic representation of the antimicrobial activity of Ampicillin on *S.aureus* bacteria

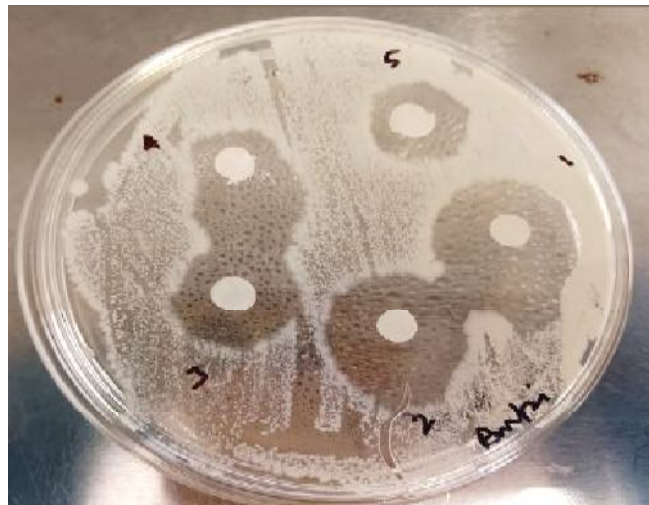


Fig. 10: Pictorial representation of the antimicrobial activity of Ampicillin on *S. aureus* bacteria

Different concentrations of **Ampicillin** have shown different inhibitory zones on *S. aureus*. Every time 40 μ l of the sample was used for the treatment 0.4 mg 70 \pm 2.44 mm, 0.2 mg - 65 \pm 2.5 mm, 0.1 - 60.2 \pm 1.6 mm, 0.05 mg - 60 \pm 1.5 mm, 0.025mg - 55 \pm 1.5 mm [fig 9 and 10]

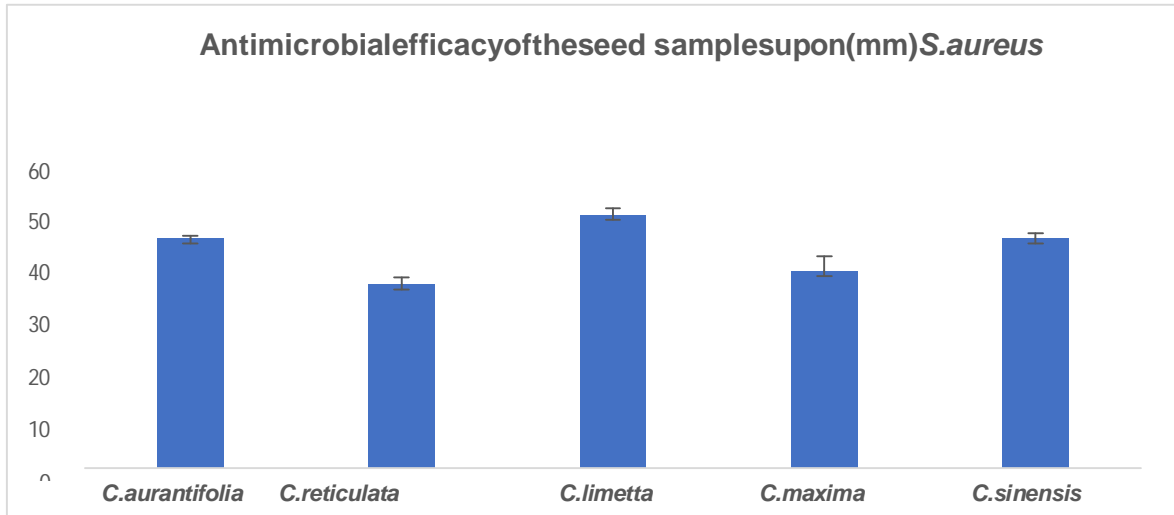


Fig 11 : Diagrammatic representation of the antimicrobial activity of the seed samples on *E. coli* bacteria.

The result shows *C. aurantiifolia* and *C. maxima* have the highest antimicrobial activity among other samples. SD measured (n=3) for each of the samples. Pictorial representations show the zones in which the samples have inhibited bacterial growth. Seed extracts of *C. aurantiifolia* (58.33 ± 0.4 mm) and *C. maxima* (57 ± 3.5 mm) showed good antibacterial efficacy.

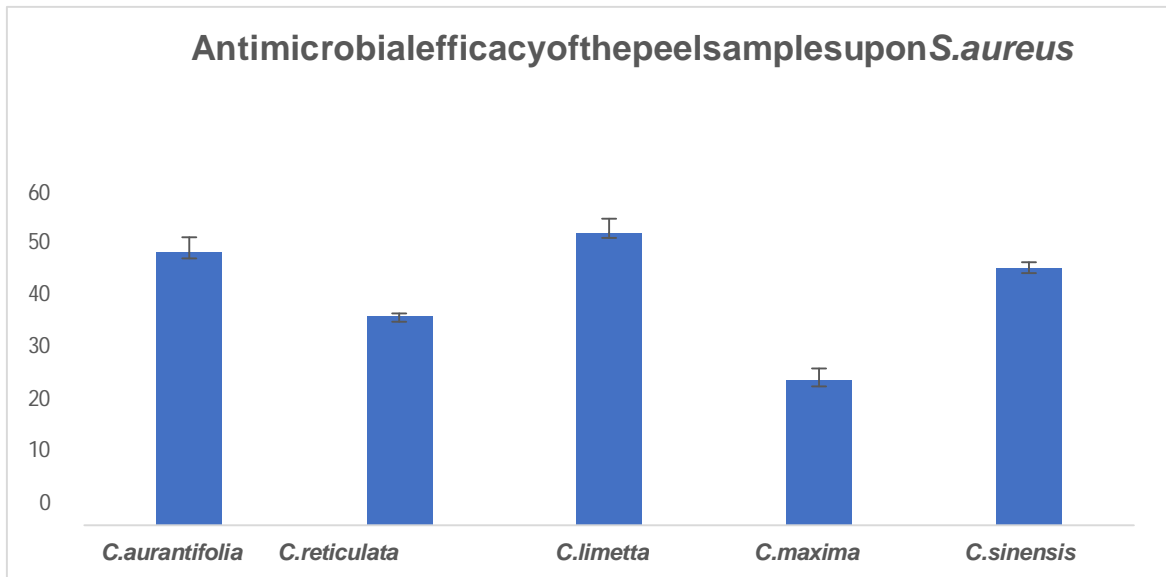


Fig 12: Diagrammatic representation of the antimicrobial activity of the peel samples on *E. coli* bacteria.

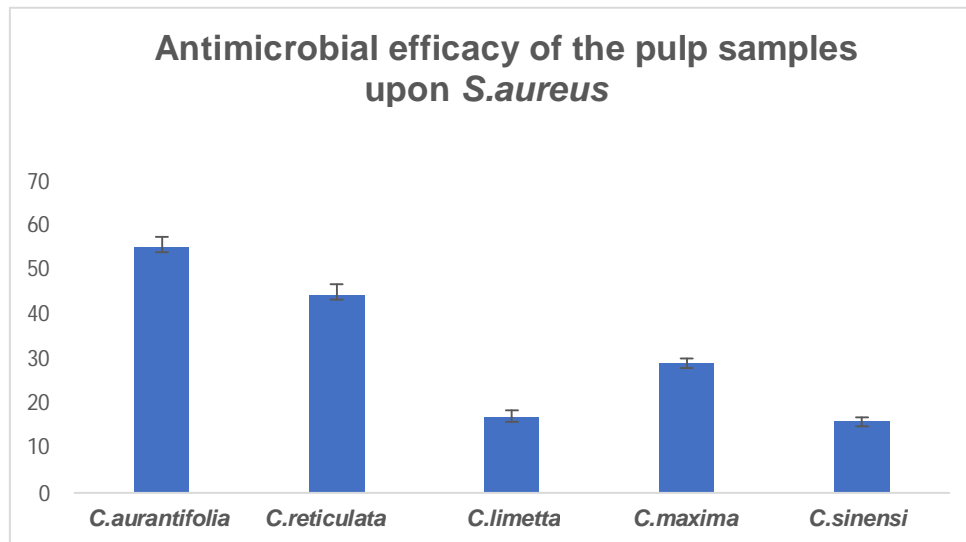


Fig 13: Diagrammatic representation of the antimicrobial activity of the pulp samples on *E. coli* bacteria.

The seeds of citrus species *Citrus limetta* and *Citrus sinensis* both had a high zone of inhibition of 51 ± 1.41 mm and 46.33 ± 0.94 mm respectively against *Staphylococcus aureus* followed by *Citrus aurantifolia* with a zone of 46.33 ± 0.47 mm. In the case of peels, *Citrus limetta* gave the highest zone of inhibition of 52.66 ± 2.49 mm followed by *Citrus aurantifolia* with a zone of 49 ± 2.82 mm. In the case of the pulp portion of the fruit, *Citrus aurantifolia* gave the highest zone of inhibition of 55 ± 2.44 mm followed by *Citrus reticulata* with an inhibition zone of 44.33 ± 2.49 mm. [fig 14]

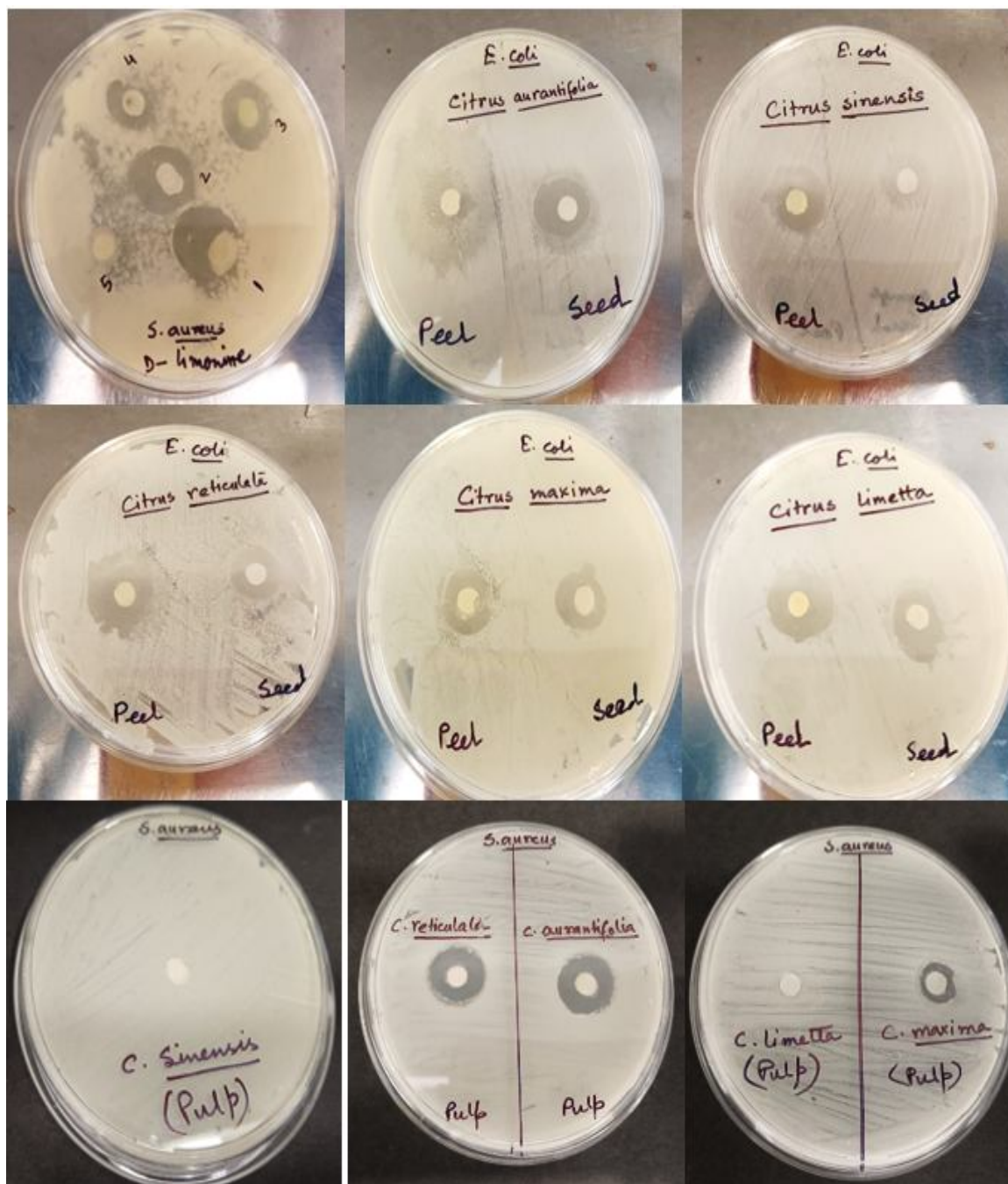


Fig. 14. Zone of inhibition of seeds, peels, and pulps of all five citrus species along with d-limonene against gram-positive bacteria *Staphylococcus aureus*

Part	Samples	Zone of Inhibition(mm)
Seed	<i>C.aurantifolia</i>	46.33±0.47
	<i>C.reticulata</i>	37±1.41

	<i>C.limetta</i>	51±1.41
	<i>C.maxima</i>	39.66±3.09
	<i>C.sinensis</i>	46.33±0.94
Peel	<i>C.aurantifolia</i>	49±2.82
	<i>C.reticulata</i>	37.66±0.4
	<i>C.limetta</i>	52.66±2.49
	<i>C.maxima</i>	26±2.16
	<i>C.sinensis</i>	46.33±0.943
Pulp	<i>C.aurantifolia</i>	55±2.44
	<i>C.reticulata</i>	44.33±2.49
	<i>C.limetta</i>	17±1.63
	<i>C.maxima</i>	29±1.24
	<i>C.sinensis</i>	16±0.94

Table 3. Inhibitory zones(mm) of three different parts of citrus fruits of five different species.

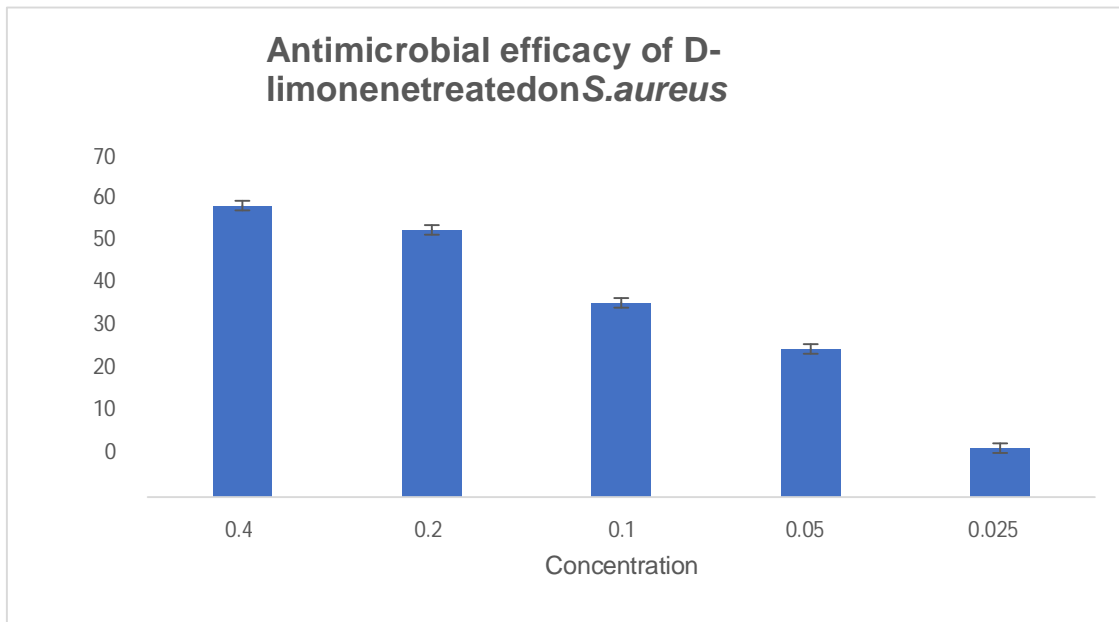


Fig 15: Diagrammatic representation of the antimicrobial activity of the bioactive compound D-Limonene on *S. aureus* bacteria



Fig 16: A pictorial representation of the antimicrobial activity of the bioactive compound D-Limonene on *S. aureus* bacteria

Treatment	Inhibitory zones (mm)
0.4	60±2.44
0.2	55±2.5
0.1	40±1.6
0.05	30.5±1.5
0.025	10±1.5

Table 4. Range of concentration of d-limonene treatment along with their Inhibitory zones(mm)

Different concentrations of **D limonene** have shown different inhibitory zones on *S. aureus*. Every time 40 µl of the sample has been used for the treatment 0.4 mg 60 ± 2.44 mm, 0.2 mg - 55± 2.5 mm, 0.1 – 40 ±1.6 mm, 0.05 mg - 30.5 ±1.5 mm, 0.025mg – 10 ±1.5 mm [fig 15 16]

Discussion

The significant antimicrobial activities observed after treating three different parts of lemons can be attributed to the bioactive compounds present, particularly limonene. Limonene is a monocyclic monoterpene with strong antioxidant qualities (Gonçalves, S., Monteiro, M., Gaivão, I., & Matos, R. S. 2024) that can scavenge reactive oxygen species and react with bacterial proteins due to its basic characteristics. The natural compounds present in our samples demonstrate therapeutic behavior and can exhibit antimicrobial activities. Ampicillin is a common β-lactam antibiotic in clinical settings and used as a control in microbiological research. It has been validated for quantitative analysis in injectable solutions using turbidimetric tests and *Staphylococcus aureus* as the test microorganism (Tótolí & Salgado, 2013). *Escherichia coli* is a versatile bacterium found in various environments, including the gastrointestinal tracts of warm-blooded animals (Jang et al., 2017; Blount, 2015). While *E. coli* has traditionally been used as an indicator for fecal contamination, recent research has shown that certain strains of the bacteria can survive and thrive outside the intestines, calling into question its accuracy as an indicator (Jang et al., 2017). *E. coli* exhibits genetic diversity

and subspecies structure, with different groups inhabiting various ecological niches and following different life history strategies (Gordon, 2004). Some strains have the potential to cause enteritis and other illnesses in humans and animals (Vila Estapé&Zboromyrska, 2012; Gordon, 2004). Environmental factors affect the genetic makeup and long-term survival of *E. coli* populations (Jang et al., 2017; Gordon, 2004). Understanding the life history and ecology of *E. coli* can improve its usefulness as a model organism and has implications for assessing water quality and controlling the spread of disease (Blount, 2015; Gordon, 2004)Seeds of *C.aurantifolia*(58.33±0.471) and *C. maxima* (57 ±3.5 mm)showed good antibacterial efficacy [fig 6]peel extracts of *C. reticulata* (58.33 ±0.4 mm) and *C. sinensis* (57 ±3.5 mm) showed good antibacterial efficacy. Pulp extracts of *C. aurantiifolia*(55.33 ±2.94 mm) and *C. reticulata* (50±2.6 mm)showed good antibacterial efficacy [fig 6]Every time 40 µl of the limonene sample has been used for the treatment 0.4 mg 65± 2.44 mm, 0.2 mg - 63 ± 2.5 mm, 0.1 – 53.66 ±1.6 mm, 0.05 mg - 30.5 ±1.5 mm, 0.025mg – 20 ±1.5 mm [fig 7, 8]and the *S.aureusbaceteria*the result shows Seed extracts of *C. aurantiifolia* (58.33 ±0.4 mm) and *C. maxima* (57 ±3.5 mm)showed good antibacterial efficacy. *Staphylococcus aureus*, a common bacterium, is responsible for many infections acquired in the community and in hospitals (Al-Mebairik et al., 2016; Choudhuri, 2022). It can cause a wide range of diseases, from minor skin infections to more serious conditions such as endocarditis, sepsis, and toxic shock syndrome, as it possesses multiple characteristics that allow it to evade the body's defenses (Al-Mebairik et al., 2016; Choudhuri, 2022; Pal et al., 2020). *S. aureus* can colonize human skin and mucous membranes, with about 30% of people being chronic carriers (Choudhuri, 2022). The bacterium's ability to form biofilms on medical devices facilitates healthcare-associated infections (Al-Mebairik et al., 2016). Methicillin-resistant *S. aureus* (MRSA) is becoming increasingly common in hospitals and communities due to its resistance to several antibiotics, posing a serious risk to public health (Al-Mebairik et al., 2016; Archer, 1998; Pal et al., 2020). *S. aureus* infections have a significant financial cost and impact both human and animal health (Pal et al., 2020).In the case ofpeel extracts, *Citrus limetta* gave the highest zone of inhibition of 52.66±2.49mm followed by *Citrus aurantifolia* with a zone of 49±2.82mm. In the case of the pulp portion of the fruit,*Citrus aurantifolia* gave the highest zone of inhibition of 55±2.44mm followed by *Citrus reticulata* with an inhibition zone of 44.33±2.49mm. [fig 14]Different concentrations of **D limonene** have shown different inhibitory zones on *S. aureus*. Every time 40 µl of the sample has been used for the treatment 0.4 mg 60 ± 2.44 mm, 0.2 mg - 55± 2.5 mm, 0.1 – 40 ±1.6 mm, 0.05 mg - 30.5 ±1.5 mm, 0.025mg – 10 ±1.5 mm [fig 15, 16]

4. CONCLUSION

In our research, we discovered that various parts of the citrus fruit exhibit a wide range of antimicrobial effects against both gram-positive and gram-negative bacteria. Our findings indicate that citrus *reticulata* peels demonstrated the most significant zone of inhibition at 65.33±6.16mm against *E. coli*, while the pulps of citrus *aurantifolia* displayed the highest zone of inhibition at 55±2.44mm

against *S. aureus*. Furthermore, d-limonene, an essential bioactive compound present in different citrus species, exhibited the highest zone of inhibition.

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Competing interests

Authors have declared that no competing interests exist.

Authors' Contributions

'Author A' designed the study, performed the statistical analysis, wrote the protocol, and Author wrote the first draft of the manuscript. 'Author B' and 'Author C' managed the analyses of the study. 'Author C' managed the literature searches..... All authors read and approved the final manuscript.

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