Nature's Antibiotics: A Comparative Study On The Antimicrobial Capacities Of Forest Honey And Extracts From *Punica Granatum*, *Citrus Sinensis* And *Phyllanthus Emblica*

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Abstract
The escalating threat of antimicrobial resistance underscores the urgent need for novel therapeutic approaches. In this study, we investigated the antimicrobial properties of honey and plant extracts derived from pomegranate peel (*Punica granatum*), orange peel (*Citrus sinensis*), and amla seed (*Phyllanthus emblica*) against common bacterial pathogens: *Escherichia coli*, *Staphylococcus sp*, and *Proteus sp*. Well diffusion and disc diffusion assays were employed to assess the inhibitory effects, while minimum inhibitory concentration (MIC) values were determined to quantify potency.

Our findings revealed distinct antimicrobial profiles among the tested substances. Amla seed extract demonstrated potent activity, though lower than pomegranate and orange peel extracts, evidenced by significant zones of inhibition and low MIC values. Honey exhibited broad-spectrum antimicrobial effects, with notable efficacy against *E. coli*. All the plant extracts exhibited a superior activity over honey in case of *Proteus sp*. Interestingly, possible synergistic and antagonistic interactions were observed between honey and certain plant extracts, against particular organisms.

The study highlights the potential of natural antimicrobial agents as alternatives to conventional antibiotics. It also reveals possible synergistic and antagonistic interactions between honey and certain plant extracts against specific organisms. Future research could explore the molecular mechanisms behind these interactions and consider environmental factors and plant sourcing for developing effective antimicrobial formulations. By making use of fruit wastes this research also addresses the management of agro waste towards combating bacterial resistance.

Index Terms: Natural Antimicrobial Agents, Honey, Pomegranate (*Punica granatum)*, Orange (*Citrus sinesis*), Amla (*Emblica officinalis*)

1. INTRODUCTION

The discovery of penicillin revolutionized the treatment of bacterial disease, but the misuse of antibiotics has led to the emergence of resistant strains of bacteria, making the development of new antibiotics crucial. Traditional plant-based medicines, bacteriophage therapies, and combinational therapies are being explored

as alternative treatments. Traditional medicines like traditional Chinese medicine and Ayurveda have utilized natural products for centuries, playing a pivotal role in drug development. Their unique chemical diversity offers significant potential for future discoveries, emphasizing the value of traditional medicine in modern drug research [1].

In an era dominated by advanced pharmaceuticals, the ancient remedy of honey has emerged as a promising frontier in the quest for novel antimicrobial agents. Celebrated for its medicinal properties across cultures, honey has been used for various purposes including wound healing, cough relief, and as a topical antiseptic. Its ability to inhibit fungal growth and toxin production highlights its therapeutic potential [2]. Pomegranate peel has been used in traditional medicine for centuries due to its diverse health benefits. It contains bioactive compounds such as polyphenols and antioxidants that exhibit anti-inflammatory and wound healing properties, prevent cardiovascular issues, treat cancer, arthritis, and inhibit cancer cell growth while improving cardiovascular health [3]. Oranges are nutritious and good for health. They are rich in vitamin C, flavonoids, pectins, and fiber, which offer various health benefits such as reducing cardiovascular risk, managing anxiety and respiratory disorders, and preventing colon cancer [4]. Amla, also known as Indian Gooseberry, is a fruit that has been used for centuries in Ayurvedic medicine. It has many health benefits, including antioxidant, anti-inflammatory, anti-diabetic, and neuroprotective properties. It may also help with cancer management, diarrhea, brain function, collagen production, and reducing lipid levels. Amla extract may even be helpful in treating osteoporosis and rheumatoid arthritis [5].

Combining plant extracts with antibiotics enhances antimicrobial effects and modifies resistance. Synergistic treatment regimens using plant extracts or purified compounds from plants show promise. Non-antibiotic inhibitors alongside antibiotics can safeguard against enzymatic destruction, potentially restoring antibiotic susceptibility and aiding in the fight against resistant bacteria [1]. It's exciting to think about what could potentially be discovered through future research regarding the antimicrobial effects of pomegranate, orange, amla, and honey. While some studies have investigated the antimicrobial properties of these individual ingredients, there has yet to be an exploration of their combined effects with honey.

2. REVIEW OF LITERATURE

Honey is a highly effective natural antimicrobial agent that can combat various micro-organisms, including both pathogenic and non-pathogenic ones such as yeasts and fungi. One of the most notable benefits of honey is its ability to fight against antibiotic-resistant strains, which makes it a promising alternative to traditional antibiotics [6]. Pomegranate extracts, particularly from the peel, exhibit promise in combating multidrug-resistant bacteria due to compounds like 5-hydroxymethylfurfural, punicic acid, gallic acid, and punicalgin. These compounds disrupt bacterial membranes and target proteins linked to antimicrobial resistance [7]. Citrus peel essential oil exhibits broad-spectrum inhibitory activity, making it a natural alternative to synthetic preservatives in the food industry, thus safeguarding consumer health [8]. Extracts and formulations derived from amla demonstrate diverse therapeutic effects, including antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-aging, nephrotoxicity modulation, antidiabetic, hepatoprotective, insecticidal, anti-cancer, anti-atherogenic, antiproliferative, anti-diarrheal, immunomodulatory, gastroprotective, caddioprotective, neuroprotective and radio-protective activities [9].

3. MATERIALS AND METHODS

3.1. Sample Collection

Kerala forest honey was sourced from Taliparamba, Kerala, while pomegranate, orange, and amla were obtained from the Kannur market. Their peels and seeds were collected and sterilized. Bacterial strains of *Escherichia coli, Staphylococcus sp.,* and *Proteus sp.* were acquired from the Biotechnology lab of Chinmaya Arts and Science College, Kannur. Samples were aseptically subcultured and incubated overnight at 37°C.

3.2. Preparation of Extracts

Peels and seeds were sun-dried for four days, then crushed using a mortar and pestle. Each material was soaked in absolute ethanol for seven days at room temperature, filtered, and air-dried. Extracts were stored in dark, airtight containers.

3.3. Stock Preparation

Stock solutions of fruit extracts were prepared: 1 mg of pomegranate and orange peel extract, and 0.5 mg of amla seed extract, each in 1 ml of absolute ethanol. Separate stock solutions of honey with each extract were made by combining in a 1:1 ratio.

3.4. Well Diffusion Assay

Agar plates were inoculated with microbial cultures, and 20 ul of concentrated honey, individual extract, and combination of honey and extract (1:1 ratio) were added to separate wells. Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured. Tests were performed in triplicates.

3.5. Disc Diffusion Assay

Agar plates were inoculated with microbial cultures, and filter paper discs were impregnated with honey and extracts. Discs were dried and incubated at 37°C for 24 hours. Zones of inhibition were measured. Tests were performed in triplicates.

3.6. Minimum Inhibitory Concentration

Each extract stock solution was mixed with 9 ml of nutrient agar in a test tube labeled 10^0, followed by serial dilution in test tubes labeled sequentially from 10^1 to 10^{-4}. This process was repeated for concentrated honey and combination mixtures of honey and individual extracts (1:1 ratio). Using a sterile pipette, 1 ml of each dilution was applied to separate agar plates inoculated with *E. coli*, *Staphylococcus*, and *Proteus*. Plates were then incubated at 37°C for 24 hours. Triplicate tests were conducted for all extracts of pomegranate peel, orange peel, and amla seeds, along with their combinations with concentrated honey and concentrated honey individually.

4. RESULT AND DISCUSSION

4.1. Antimicrobial Assay of Pomegranate Peel Extract and Honey

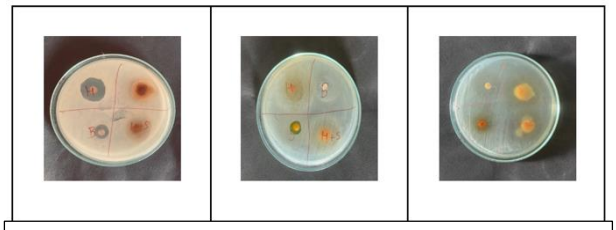


Figure 1: Well Diffusion a) E. coli b) Staphylococcus sp. C) Proteus sp.

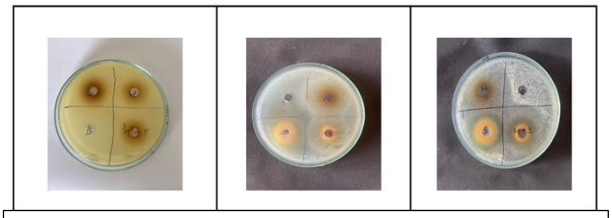


Figure 2: Disc Diffusion a) E. coli b) Staphylococcus sp. C) Proteus sp.

Organism	Zone of Inhibition (in mm)		
	S	H	H+S
E. coli	19	19	13.5
	19	20	20
	18.5	19	22.5
Average	18.8	19.3	18.7
Staphylococcus sp.	11	15	9
	11	13	7
	10	16	8.5
Average	10.7	14.7	8.2
Proteus sp.	20	9	19
	23	10	18
	22	7	17
Average	21.7	8.7	18

Table 1: Well Diffusion Assay

Organism	Zone of Inhibition (in mm)		
	S	H	H+S
E. coli	18	21.5	21
	14.5	24	16
	13	23	15.5
Average	15.2	22.8	17.5
Staphylococcus sp.	9	13.5	8
	10.5	13	7.5
	10	12	10
Average	9.8	12.8	8.5
	20	10.5	16.5
	25	12	22.5
Average	23.2	12.2	20.8

Table 2: Disc Diffusion Assay

The antimicrobial efficacy of honey (H), pomegranate peel extract (S), and their combination (H+S) varies across bacterial strains. Pomegranate peel extract tends to exhibit higher effectiveness against *Proteus*, while honey demonstrates stronger efficacy against *Staphylococcus*. In case of *E. coli*, both have comparable effects. Also, the combination's impact is variable and depends on the specific bacteria tested. Notability, an antagonistic effect is observed in case of *Staphylococcus*.

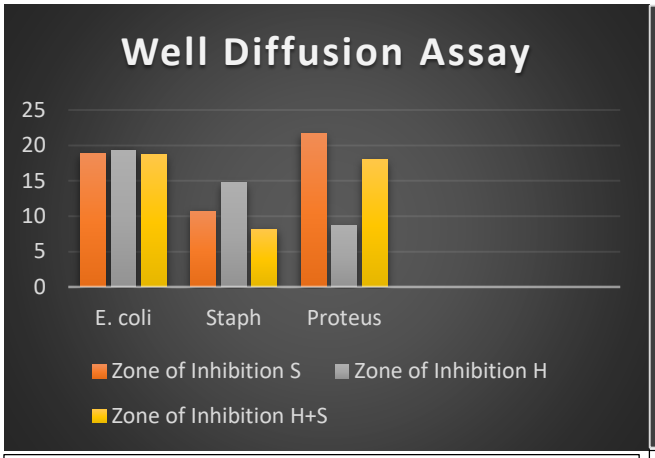


Figure 3: Well Diffusion Assay

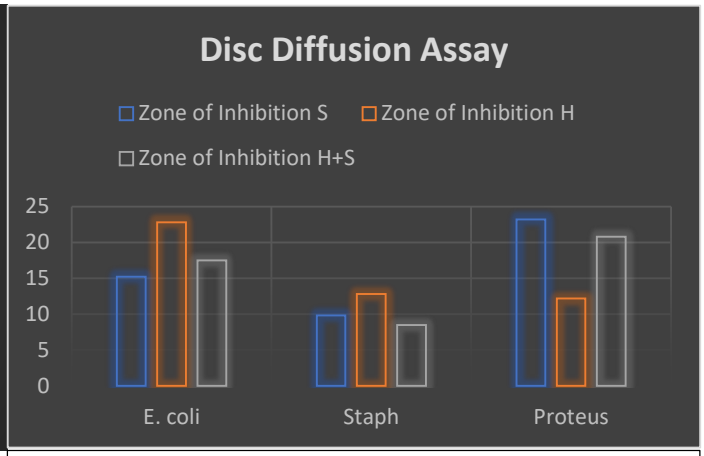


Figure 4: Disc Diffusion Assay

4.2. Antimicrobial Assay of Orange Peel Extract and Honey

Figure 5: Well Diffusion a) *E. coli* b) *Staphylococcus sp.* C) *Proteus sp.*

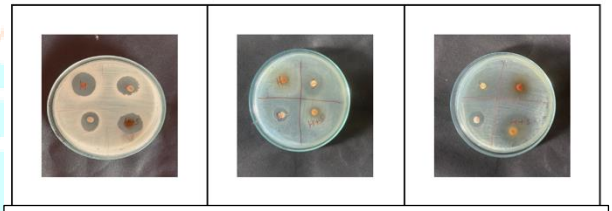


Figure 6: Disc Diffusion a) *E. coli* b) *Staphylococcus sp.* C) *Proteus sp.*

Organism	Zone of Inhibition (in mm)		
	S	H	H+S
E. coli	2	12	13
	2	16	14
	1	14	13
Average	1.7	14	13.3
Staphylococcus sp.	7.5	10	8
	8	10.5	9.5
	8	8	7.5
Average	7.8	9.5	8.3
Proteus sp.	17	7.5	9
	19	12	15.5
	20.5	11	19
Average	18.8	10.2	14.5

Table 3: Well Diffusion Assay

Organism	Zone of Inhibition (in mm)		
	S	H	H+S
E. coli	12.5	19	16
	11.5	14	24.5
	9.5	11	17
Average	11.2	14.7	19.2
Staphylococcus sp.	17.5	9	7.5
	16	13	10
	17	12	10
Average	16.8	11.3	9.2
Proteus sp.	21	10.5	18
	19	10	17.5
	23	11.5	24
Average	21	10.7	19.8

Table 4: Disc Diffusion Assay

The effectiveness of honey (H), orange peel extract (S), and their combination (H+S) varies across bacterial strains and depends on the assay method used. For *E. coli,* honey and the combination exhibit comparable effectiveness, while orange peel extract shows lower effectiveness. The results hint at potential synergistic effects between honey and orange peel extract, particularly against E. coli, but further analysis is recommended for confirmation. Against *Proteus*, orange peel extract is the most effective, with the combination showing intermediate effectiveness and honey being the least effective. Inconsistencies between the methods, as in case of *Staphylococcus*, suggest the importance of considering the specific bacteria and the characteristics of the substances being tested, highlighting the need for method-specific considerations in antimicrobial testing.

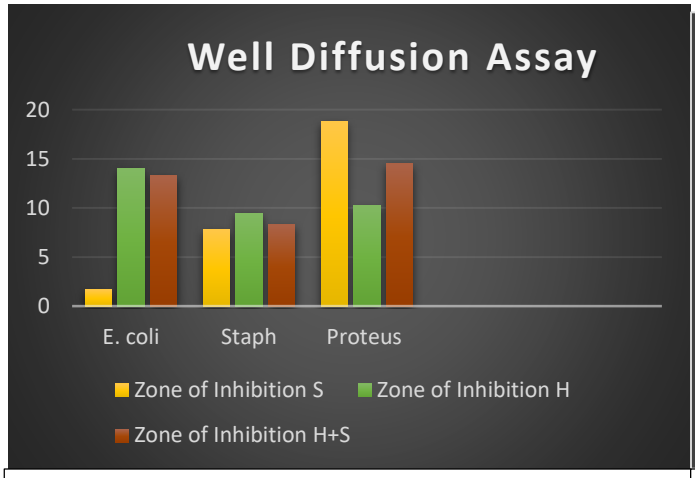


Figure 7: Well Diffusion Assay

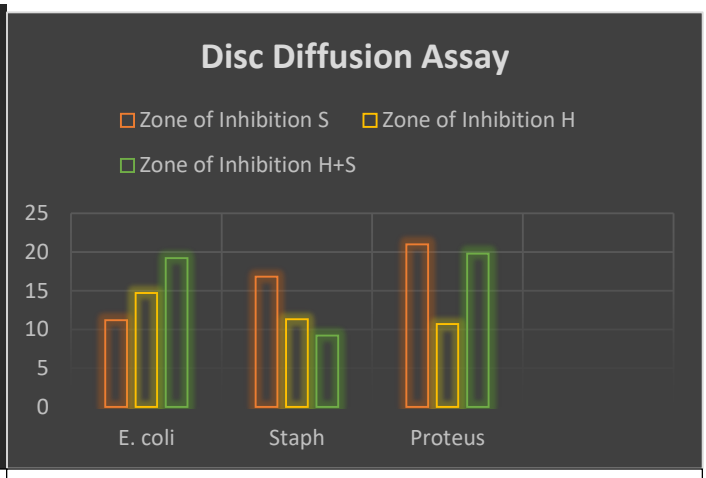


Figure 8: Disc Diffusion Assay

4.3. Antimicrobial Assay of Amla Seed Extract and Honey

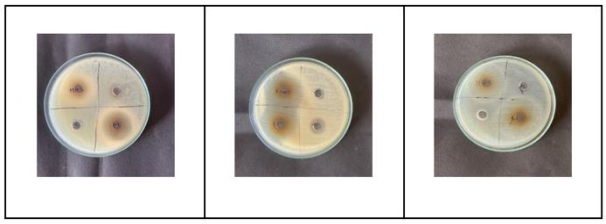


Figure 9: Well Diffusion a) *E. coli* b) *Staphylococcus sp.* C) *Proteus sp.*

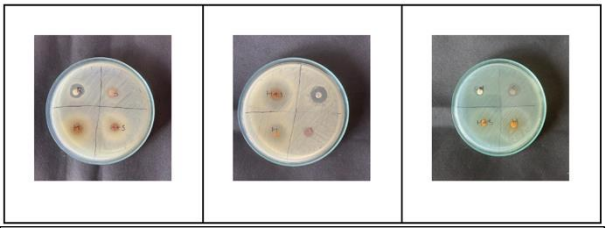


Figure 10: Disc Diffusion a) *E. coli* b) *Staphylococcus sp.* C) *Proteus sp.*

Honey (H) consistently shows the highest effectiveness against *E. coli*. The combination (H+S) performs well, and amla seed extract (S) is the least effective. The combination of honey and amla seed extract seemingly exhibits a synergistic effect against *Staphylococcus*.

Organism	Zone of Inhibition (in mm)		
	S	H	H+S
E. coli	3.5	18	16.5
	5	19	17
	4	17	15
Average	4.2	18	16.2
Staphylococcus sp	7.5	27	29.5
	6.5	28	29
	8	27	28.5
Average	7.3	27.3	29
Proteus sp	2.5	7	4.5
	1	5.5	5
	3	6	3.5
Average	2.2	6.2	4.3

Table 5: Well Diffusion Assay

Organism	Zone of Inhibition (in mm)		
	S	H	H+S
E. coli	9	20	17
	9	19	17.5
	8	18.5	17.5
Average	8.7	19.2	17.3
Staphylococcus sp.	6	12	21
	7	13.5	14
	6	14	16.5
Average	6.3	13.2	17.2
Proteus sp.	4.5	4	3.5
	6	4.5	3
	8	6	4.5
Average	6.2	4.8	3.7

Table 6: Disc Diffusion Assay

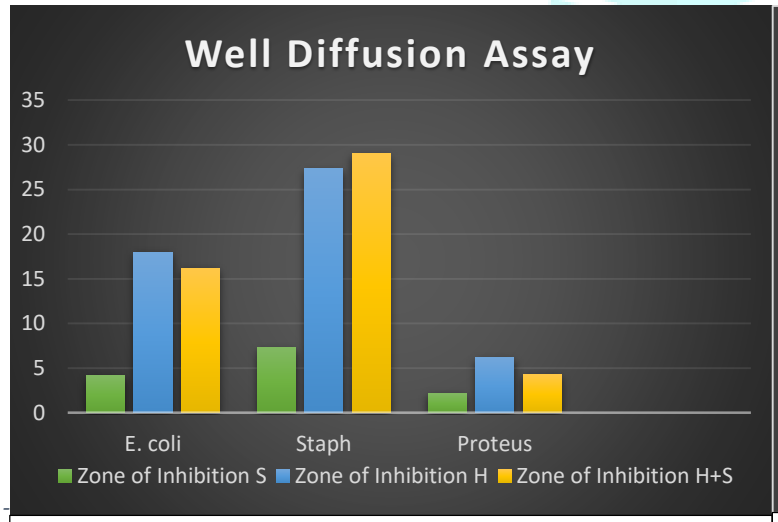


Figure 11: Well Diffusion Assay

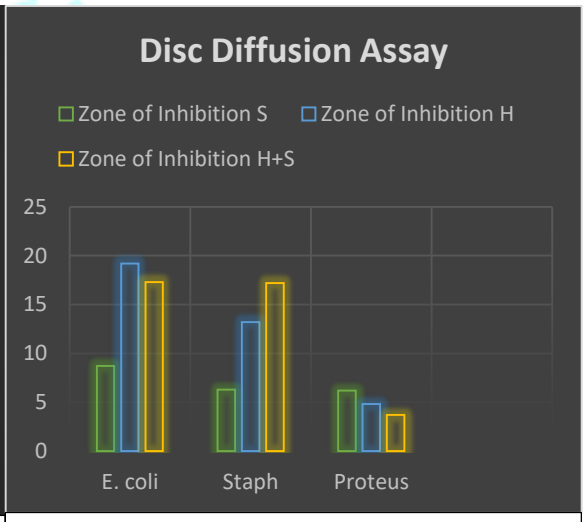
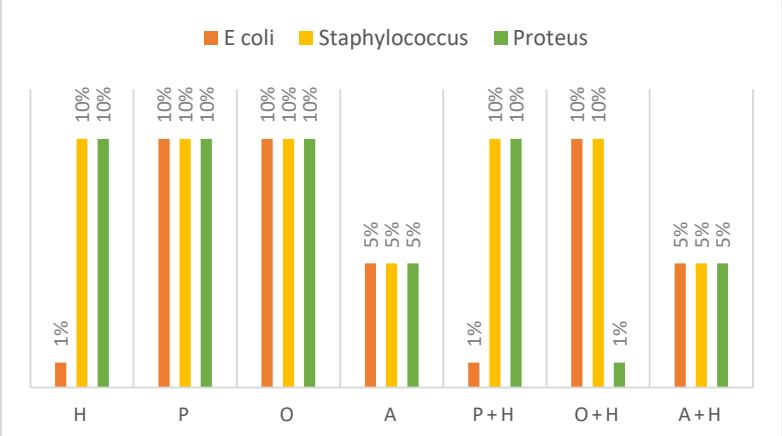


Figure 12: Disc Diffusion Assay

- Pomegranate peel and orange peel exhibit moderate effectiveness against *E. coli*, while amla seed shows lower efficacy. Honey consistently performs well, demonstrating the highest effectiveness.
- For *Staphylococcus*, pomegranate peel is moderately effective, orange peel shows variable effectiveness with synergistic effects when combined with honey, and amla seed exhibits the highest effectiveness when combined with honey. Honey alone is effective and enhances the effectiveness of certain extracts.
- Pomegranate peel and orange peel have high efficacy in case of *Proteus*, while the effectiveness of amla seed is comparatively lower. Unlike in the other two cases, honey does not appear much effective against *Proteus*.

- Both orange peel and amla seed extracts exhibit synergistic effects when combined with honey, resulting in significantly enhanced effectiveness against *E. coli* and *Staphylococcus* respectively. This suggests a cooperative interaction that enhances their antimicrobial effectiveness, particularly against these organisms.
- A potential antagonistic effect is observed when honey and pomegranate peel extract are combined against *Staphylococcus*, indicating the presence of compounds that may counteract each other or interfere with their respective antimicrobial activities. Detailed concentration studies and further experimentation would be required to confirm the same.
- The variations in antimicrobial activity observed across different extracts and bacterial strains can be attributed to various factors. Each extract (Pomegranate Peel, Orange Peel, Amla Seed) contains unique chemical compositions with different bioactive compounds, influencing their effectiveness. Additionally, different bacterial strains (*E. coli, Staphylococcus sp., Proteus sp.*) have varying sensitivities to specific antimicrobial agents due to their inherent characteristics like cell wall structure. When extracts are combined with honey, complex interactions between compounds occur, resulting in synergistic, antagonistic, or neutral effects. Differences in concentration of bioactive compounds and optimal concentrations for inhibitory effects can also impact effectiveness. Moreover, variations in diffusion rates and sensitivity of methods like well diffusion and disc diffusion contribute to differences in results. Bacteria can also develop resistance mechanisms over time, further affecting effectiveness. Additionally, factors such as experimental conditions, sample preparation, and biological variability can introduce inconsistencies in results.

4.4. Minimum Inhibitory Concentration



MIC of Honey (H), Pomegranate Peel Extract (P), Orange Peel Extract (O), Amla Seed Extract (A), and Their Combinations with Honey, against *E. coli*, *Staphylococcus sp*. and *Proteus sp*.

These MIC values indicate the minimum concentration at which each substance inhibits bacterial growth. Honey demonstrates a Minimum Inhibitory Concentration (MIC) at 10% v/v for *Staphylococcus* and *Proteus*. However, for *E. coli*, 1% v/v is effective. Amla seed extract and its combination with honey exhibit minimum inhibition at 5% v/v. Pomegranate peel extract and orange peel extract alone are effective at 10%. Notably, when combined with honey, only 1% of pomegranate peel extract and 1% of orange peel extract are effective against *E. coli* and *Proteus*, respectively.

The Minimum Inhibitory Concentration (MIC) represents the lowest concentration of an antimicrobial agent (in this case, extracts and honey) required to inhibit the visible growth of a microorganism. MIC is a specific concentration at which growth is inhibited, and it doesn't directly correlate with the size or range of inhibition zones obtained from other methods. While the MIC gives a quantitative measure of the effectiveness of an antimicrobial agent, the zone of inhibition obtained from diffusion methods reflects the overall inhibitory activity, including factors like diffusion rates, concentrations used, and interactions with the agar medium. Both MIC and diffusion methods provide complementary information about the antimicrobial activity of substances against specific microorganisms.

5. CONCLUSION

Honey and plant extracts have been used for centuries due to their antimicrobial properties. Honey has been used for wound healing, while pomegranate, orange peel, and amla seed extracts have been recognized for their antibacterial attributes in various cultures. This study delved into the antimicrobial properties of honey, pomegranate peel extract, orange peel extract, and amla seed extract, shedding light on their individual efficacy and potential synergistic effects with honey. The results showcase the remarkable antimicrobial potential of these natural agents. Honey demonstrated notable inhibitory effects, particularly at lower concentrations against *E. coli*, emphasizing its broad-spectrum activity. Pomegranate peel and orange peel extracts exhibited effectiveness at higher concentrations, with intriguingly lower concentrations proving effective when combined with honey. The comparison of results obtained from well and disc diffusion methods, along with Minimum Inhibitory Concentration (MIC) values, provided a comprehensive understanding of their inhibitory capacities. The antimicrobial potential of plant-derived extracts and honey offers a promising avenue for integrating traditional remedies with scientific advancements. Further research is needed to understand the complex interactions between these natural agents and bacteria. This research contributes to bridging the gap between traditional knowledge and contemporary scientific approaches, offering valuable insights for the development of effective, nature-inspired antimicrobial strategies.

6. ACKNOWLEDGEMENT

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