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Combined Effect of Honey, Neem (*Azadirachta Indica),* and Turmeric against *Staphylococcus Aureus* and *E. Coli* Isolated from a Clinical Wound Sample

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Abstract

Antimicrobial resistance has become evident all over the world. Resistance to antibiotics has become a concern in case of a wide variety of bacterial species, both pathogenic and commensal. More recently, *E. coli, pseudomonas, Staphylococcus aureus, Streptococcus,* and *Enterococci* were found to be adversely affecting the healthcare structures of the world, particularly where acute and long-term care facilities are available. Microbial species were identified by Vitek compact-2 and MALDI-TOF, while antibiogram sensitivity was checked using Kirby Bauer disc diffusion and well diffusion method. The study used 20 wound samples from five (5) men and fifteen (15) women. Thirty-four (34) purified colonies of bacteria were created, in which 8 were *E. coli* and 2 were *S. aureus*. The effects of neem, turmeric, and honey with ethanol extracts showed the maximum zone of inhibition against clinically isolated *E. coli,* such as PM33C4 and AM25C4. While, methanol extract also showed the maximum zone of inhibition against PM56C4, AF34C4, and PM57C4, using disc diffusion and well diffusion methods. Correspondingly, the effect of neem, turmeric, and honey with ethanol extracts showed maximum inhibition against *S. aureus*. Whereas, methanol extract showed a sensitive zone of inhibition only against PM54C1 using the disc diffusion method. Hence, it was determined that natural ingredients such as honey, turmeric, and neem are an effective alternative to antibiotics because they manifest excellent antimicrobial activity against clinical bacterial isolates.

1. Introduction

Wound healing can never be considered a genetic term, rather it is a biological process in the human body. Wounds recuperate through different cycles like (coagulation, irritation, grid union and testimony, angiogenesis, fibroplasia, epithelialization, compression, and remodelling). Previous studies have recommended that wound injury, ischemia and various diseases are driving reasons for the pathobiology prompting wound chronicity [1]. Wounds can be punctures (holes), lacerations (tears), incisions (cuts), or burns. Deep ulcers (open sores), large burns, trauma, accident, surgical operation, or animal bite provides

an open door for bacterial infections. Wound infections can also happen in small wounds that are left untreated. When a large number of bacteria get into a wound, it can get infected by the particular bacteria, while causing wound infections. Normal bacteria that lives on one's skin often enter a wound first [2].

Previous studies indicated the usefulness of *honey and neem* for the treatment of various wound infections solving the substantial issue of developing antibiotic resistance in microbes. Excessive use of antibiotics to treat and cure injuries which results in the development of resistance in *Staphylococcus aureus, E. coli, Streptococcus pyogenes, Enterococcus* species, *Proteus* species, *Streptococcus* species, *Klebsiella pneumonia,* and *Pseudomonas aeruginosa* [3].

The expenses involved in treating wound infections are subsequently, intensifying these days. Whereas the use of *honey and neem* as a prevailing healing antiseptic medicine has been used to treat various wound infections that has been perceived as an effective remedy in clinical industry. Honey wound dressings have been used since the Egyptian era and now are again getting attention because of its antiflammatory, antioxidant, and antibacterial nature [4]. The current study has reported *hone, and neem* uses in the treatment of general and microbial wound infections. A good impact of honey was recorded in general in microbial wound infections and its clinical applications. Additionally, more research is required for the contemplation. Furthermore, *honey and neem* would be exploited as a safe antibacterial microbe in general for wound infections to properly assist the infected patients [5].

Antibacterial compounds found in natural products are very important in reducing the global burden of infectious diseases. However, the emergence of Multidrug resistance (MDR) as micro-organisms is the major threat around the world against antibiotics [6].

The unique theoretical and technological characteristics of honey have piqued global interest in the twenty-first century. In vitro and in vivo studies have been conducted on the effects of acute and chronic wound infections and were considered as nanomaterials and nano-medicines against multi-drug resistant micro-organisms. Due to the unknown health effects of honey on humans, more research on toxicity and safety is required in the clinical industry. These nanoparticles presented new challenges in predicting and managing the potential health risks. WHO has considered alternative medicines as a cost-effective alternative for the entire world's population with healthcare [7].

More than 79% of humans use natural therapies for the treatment of wound infections especially, in Asian countries like Pakistan, China, and India [8, 9]. Natural products can play an important role in synthesizing new antimicrobial drugs for the treatment of diseases caused by bacterial infections [10]. The neem tree has been linked to human culture and civilization since the Vedic age, as various advantages of the neem tree are mentioned in the earliest medical writings. The positive impact of turmeric in wound healing can be explained by the curcumin found in turmeric which can decrease the inflammation and oxidation of wounds and repairs the skin quickly [11]. In some cases, these naturally occurring products are also used by combining them with different antibiotics to increase their effectiveness. Many of these substances have been found

with the same inhibitory effect as that antibiotics. These substances have the same mechanism of action as of antibiotics which damage the cell walls of bacteria and also stop the synthesis of protein in bacteria. Honey has been used in traditional medicine for centuries, however, health professionals have just explored it for its effectiveness in both acute and chronic wound dressings.[12]. The current study was designed to check the combined effect of honey, neem, and turmeric against *E. coli* and *S. aureus* isolated from clinically wound infected patients. For many years, antimicrobial drugs have been used to destroy bacteria and other germs. Microbial resistance to antimicrobial substance has developed on a vast scale over time, considerably, lowering its effectiveness. One of the most promising ways of combating microbial resistance is the use of natural products such as *honey and neem* [13, 14].

2. Material and Methods

2.1 Study Area

The current research study was conducted at the head-quarter of the Chughtai Laboratories, Gulberg, Lahore.

2.2 Sample Collection

For the isolation of bacteria, wound samples from various patients were collected from main head-quarter of Chughtai lab, Lahore. The reference (Table 1) contains information about each and every patient. It was necessary to employ selective media, such as blood agar and MacConkey agar, to incubate the plates at temperatures between 35-37℃ for between 24-48 hours, required to isolate and purify *E. coli* and *S. aureus*.

2.3 Identification

All the bacteria selected for the study were purified and identified by gram staining and various biochemical techniques including (citrate, urease, indole, H_2S gas, TSI, and catalase). Vitek-2 compact and MALDI-TOF were also employed to provide additional species confirmation and identification.

2.4 Antibiotic Susceptibility

Kirby Bauer disc diffusion method is a well-known method that researchers and laboratory scientists are using for antibiotic susceptibility. This method was used to employ Muller Hinton Agar (MHA) alongside 11 commercially available antibiotics with defined doses such as Ofloxacin (30µg), Ceftriaxone (30µg), Colistin (10µg), Amikacin (30µg), Gentamicin (10µg), Doxycycline (30ug), Erythromycin (15µg), Chloramphenicol (30µg), Meropenem (10µg), Imipenem (10µg), and Tigecycline (15µg).

The inoculum was taken with the help of sterilized loop from the culture plate. Single and same colonies of *E. coli* and *S. aureus* were picked and transferred into the MacFarlane. MacFarlane is the standardized method that is used to measure the turbidity of the microbial cell against the provided samples which is achieved by calibrating digital MacFarlane meter. The standard unit of the MacFarlane meter is 0.5 as per scientific protocol [15].

By observing the turbidity in the tube lesser than the reference value of 0.5, inoculum was added to tube to maintain the turbidity and reference value of the MacFarlane meter at 0.5. With this regard, the result of the lawn in plates became confluent. The sterilized swab was used to spread 0.5% Macferland evenly on Muller Hinton agar.

Moreover, small light weighted antibiotic discs were used and placed on the inoculated Muller Hinton agar plates by using a sterile antimicrobial disc dispenser and forceps (where required). The 9 cm Muller Hinton agar was used to place 7 antibiotics discs, where six discs were placed a round 14-15 mmfrom the edge of the plate and 1 disc was placed in the mid of the Muller Hinton agar plate. All antibiotics were stacked with the surface of Muller Hinton agar. The plates were kept in incubator overnight at 37°C. After incubation, the diameter for each zone was measured with the help of a ruler in mm

2.5 Phytochemical Analysis

Phytochemical analysis of honey, neem, and turmeric was conducted according to the methods described for the identification of phytochemicals like tannins, alkaloids, steroids, saponins, and flavonoids [16].

2.6 Preparation of Extracts

Collected and weighed leaves of neem plant (*Azadirachta indica*) were washed, dried, and grounded into fine powder. For the extraction purpose three solvents; ethanol, methanol, and distilled water were used. 50 g of powder was mixed separately in 250 ml of each solvent. Turmeric extracts of ethanol, methanol, and distilled water were also prepared by repeating the same procedure. Four dilutions of honey were prepared i.e. 25%, 50%, 75%, and 100%.

2.7 Antibacterial Activity of Extracts

The agar well diffusion and disc diffusion techniques were used to screen the antibacterial activity of honey, neem, and turmeric extracts. The plates were incubated at 37°C for 24 hours and observed for the zone of inhibitions. The antibacterial activity was expressed in this study as the mean diameter of the inhibition zone (mm) produced by the honey, neem, and turmeric.

2.8 Inclusion and Exclusion Criteria

A fresh samples of the wound, already developed resistance against selected antibiotics panel were included and old wound samples (already store samples of patients) were avoided.

3. Results

In the current study 20 wound samples were collected and analysed. There were 5 male subjects and 15 female subjects. Details of patients are given in Table 1.

Table 1. Sample Collection

Sample No.	Age	Gender	Source	Sample ID
1	33	Male	Pus	PM33
2	56	Male	Pus	PM56
3	65	Female	Abscess	ABF65
4	46	Female	Pus	PF46
5	34	Male	Accident	AM34
6	24	Male	Accident	AM24
7	25	Male	Accident	AM25
8	41	Male	Pus	PM41
9	37	Male	Accident	AM37
10	56	Male	Abscess	AM56
11	27	Female	Accident	AF27

Sample No.	Age	Gender	Source	Sample ID
12	34	Male	Accident	AM34
13	57	Male	Pus	PM57
14	31	Male	Pus	PM31
15	54	Male	Pus	PM54
16	47	Male	Pus	PM47
17	34	Female	Accident	AF34
18	59	Male	Abscess	ABM59
19	24	Male	Accident	AM24
20	64	Female	Knee Pus	PF64

Table 2. Biochemical Characterization

ID	Colonies	Citrate	Urease	Indole	Motility	H_2S	TSI	Catalase
PM33	PM33C 4	-	-	+	+	-	A/A	+
	PM33C 5	+	+	-	-	+	K/K	+
	PM33C 7	+	+	-	-	-	A/A	+
PM56	PM56C4	-	-	+	+	-	A/A	+
	PM56C6	+	-	-	-	-	A/A	+
	PM56C7	+	+	-	-	-	A/A	+
ABF65	ABF65C5	+	+	-	+	+	K/K	+
	ABF65C5	+	-	-	-	+	K/K	+
PF46	PF46C4	-	-	+	+	-	A/A	+
	PF46C2	-	-	-	-	-	K/K	-
AM34	AM34C	-	-	-	+	+	K/A	+
	AM34C7	+	+	-	-	-	A/A	+
AM24	AM24C4	-	-	+	+	-	A/A	+
AM25	AM25C2	-	-	-	-	-	K/K	-
	AM25C4	-	-	+	+	-	A/A	+
PM41	PM41C1	+	+	-	-	-	A/A	+
	PM41C7	+	+	-	-	-	A/A	+
AM37	AM37C5	+	+	-	+	+	K/K	+
AM56	AM56C4	-	-	+	+	-	A/A	+
	PM57C7	+	+	-	-	-	A/A	+
AF27	AF27C5	-	+	+	+	-	K/A	+

ID	Colonies	Citrate	Urease	Indole	Motility	H_2S	TSI	Catalase
AM34	AM34C6	+	-	-	-	-	A/A	+
	AM34C7	+	+	-	-	-	A/A	+
PM57	PM57C4	-	-	+	+	-	A/A	+
PM31	PM31C4	-	-	+	+	-	A/A	+
	PM31C7	+	+	-	-	-	A/A	+
PM54	PM54C2	-	-	-	-	-	A/A	-
PM47	PM47C1	+	+	-	-	-	A/A	+
AF34	AF34C5	-	-	+	-	+	K/A	+
ABM59	ABM59C4	-	-	+	+	-	A/A	+
AM24	AM24C3	+	+	-	-	-	A/A	+
	AM24C7	+	+	-	-	-	A/A	+
PF64	PF64C6	+	-	-	+	-	A/A	+
	PF64C7	+	+	-	-	-	A/A	+

Positive Biochemical Test (+), Negative Biochemical Test (-)

From the above 20 samples in Table 1, 34 obtained colonies were characterized biochemically (Table 2). Their further identification was done by using Vitek 2 compact and the MALDI-TOF methods. On the basis of all the above-selected methods, 8 were identified out of 34 colonies as *E. coli* and 2 were identified as *S. aureus* (Table 3).

Table 3. Identification of *E. coli* and *S. aureus* on the Basis of Vitek 2 Compact and the MALDI-TOF

Sr. No	ID	Isolated microbe
1	PM33C4	*E. coli*
2	PM56C4	*E. coli*
3	AM24C4	*E. coli*
4	AF34C4	*E. coli*
5	PM31C4	*E. coli*
6	PM57C4	*E. coli*
7	AM56C4	*E. coli*
8	AM25C4	*E. coli*
09	PM41C1	*S. aureus*
10	PM54C1	*S. aureus*

Antibiotic sensitivity of *E. coli* and *S. aureus* were carried out. *E. coli* showed a maximum sensitivity against imepenem and meropenem and only one sample PM56C4 showed a maximum 26mm zone of inhibition for ceftriaxone. Whereas the rest of the samples showed zero zones of inhibition size, only PM33C4 showed a minimum (zero) zone of inhibition, whereas the remaining samples showed a maximum zone of inhibition for Amikacin with 25mm as a maximum zone of inhibition. Gentamicin has similar antibiotic sensitivity as that of Amikacin because both are aminoglycosides. Doxycycline (Tetracycline) was resistant in PM33C4, AM23C4, PM41C1, and PM54C1 and showed a 22mm zone of inhibition in PM56C4. Erythromycin was used only in two samples that were gram-positive; PM41C1 and PM54C1 which showed a zero zone of inhibition. Similarly, oxacillin was also used in gram-positive

samples and showed a maximum zone of inhibition of 30mm. Chloramphenicol and Tigecycline (3rd line of defence) were used only in gram-negative organisms and showed a sensitive zone of inhibition in all of them with 26mm and 27mm maximum zone of inhibitions, respectively (Table 4).

Table 4. Antimicrobial Activity of *E. coli & S. aureus* against Different Antibiotics

Microorganisms	Sample ID'S									
	E. coli								*S. aureus*	
	PM33 C4	PM56 C4	AM24 C4	AF34 C4	PM31 C4	PM57 C4	AM56 C4	AM25 C4	PM41 C1	PM54 C1
Antibiotic discs	**Inhibition zone (mm)**									
Ofloxacin (30µg)	*	*	*	*	*	*	*	*	28	30
Ceftriaxone (30µg)	10	26	0	12	0	0	0	0	*	*
Colistin (10µg)	<0.5	<0.5	<0.5	1	<0.5	<0.5	2	1	*	*
Amikacin (30µg)	0	19	22	24	23	24	25	20	25	25
Gentamicin (10µg)	16	17	19	20	22	21	23	20	10	25
Doxycyclin (30µg)	15	22	0	19	11	0	10	0	20	24
Erythromycin(15µg)	*	*	*	*	*	*	*	*	0	10
Chloramphenicol (30µg)	24	24	23	24	25	26	24	25	24	23
Meropenem (10µg)	15	30	28	30	30	28	29	30	*	*
Imepenem (10µg)	18	24	26	25	25	28	29	28	*	*
Tigecycline (15µg)	24	25	25	26	26	27	26	25	*	*

* Drug not recommended for *S. aureus* as per CLSI panel

Table 5. Presence of Phytochemicals in Neem, Honey, and Turmeric

Phytochemical constituents	Honey	Extracts of Neem and Turmeric			
		Extracts	Ethanol	Methanol	Distilled water
Saponins	+	Neem	+	+	-
		Turmeric	+	+	+
Tannins	–	Neem	+	+	-
		Turmeric	+	+	+

Phytochemical constituents	Honey	Extracts of Neem and Turmeric			
		Extracts	Ethanol	Methanol	Distilled water
Reducing Sugars	+	Neem	+	+	+
		Turmeric	+	+	+
Glycosides	+	Neem	-	+	-
		Turmeric	+	+	+
Alkaloids	+	Neem	+	+	+
		Turmeric	+	+	+
Flavonoids	+	Neem	+	+	+
		Turmeric	+	+	+
Volatile oils	+	Neem	-	-	-
		Turmeric	+	+	+
Terpenoids	–	Neem	+	+	-
		Turmeric	+	+	+

The neem extract showed a maximum zone of inhibition as sensitive in most of the ethanol extracts for instance, PM56C4, AM24C4, AM34C4, PM31C4, PM57C4, and AM56C4. Whereas the methanol and distilled water extracts of neem in all these isolated bacteria showed no zone of inhibition. The results of the well-diffusion method and disc diffusion method are the same (Figure 1 and 2). The turmeric showed the minimum zone of inhibition and only showed a zone of inhibition in ethanol extracts of AM24C4, AF34C4,.PM57C4, and AM56C4, was resistant in methanol ethanol and distilled water extracts in all other isolated bacteria except the methanol extract of PM31C4 (Figure 3 and 4).

Table 6. Antimicrobial Activity of Plant Extracts on *E. coli* by Well Diffusion Method and Disc Diffusion Method

Antimicrobial Activity Assay		Well Diffusion Method			Disc Diffusion Method			
Sample ID	Plant Extracts	E	M	W	E	M	W	Control Ethanol
PM33C4	Turmeric	0mm	0mm	0mm	0mm	0mm	0mm	0mm
	Neem	0mm	0mm	0mm	0mm	0mm	0mm	0mm
PM56C4	Turmeric	0mm	0mm	0mm	0mm	0mm	0mm	0mm
	Neem	9mm	0mm	0mm	10mm	0mm	0mm	0mm

Antimicrobial Activity Assay		Well Diffusion Method			Disc Diffusion Method			
Sample ID	**Plant Extracts**	**E**	**M**	**W**	**E**	**M**	**W**	**Control Ethanol**
AM24C4	Turmeric	7mm	0mm	0mm	6mm	0mm	0mm	0mm
	Neem	8mm	0mm	0mm	6mm	0mm	0mm	0mm
AF34C4	Turmeric	10mm	0mm	0mm	0mm	0mm	0mm	0mm
	Neem	7mm	0mm	0mm	5mm	0mm	0mm	0mm
PM31C4	Turmeric	8mm	8mm	0mm	0mm	4mm	0mm	0mm
	Neem	6mm	0mm	0mm	6mm	0mm	0mm	0mm
PM57C4	Turmeric	10mm	0mm	0mm	0mm	0mm	0mm	0mm
	Neem	8mm	0mm	0mm	5mm	0mm	0mm	0mm
AM56C4	Turmeric	9mm	0mm	0mm	0mm	0mm	0mm	0mm
	Neem	10mm	0mm	0mm	7mm	0mm	0mm	0mm
AM25C4	Turmeric	0mm	0mm	0mm	0mm	0mm	0mm	0mm
	Neem	0mm	0mm	0mm	0mm	0mm	0mm	0mm

Table 7. Antimicrobial Activity of Extracts on *S. aureus* by Well Diffusion Method and Disc Diffusion Method

Antimicrobial Assay		Well Diffusion Method			Disc Diffusion Method			
Sample ID	**Plant Extracts**	**E**	**M**	**W**	**E**	**M**	**W**	**Control**
PM41C1	Turmeric	8mm	0mm	2mm	0mm	0mm	0mm	0mm
	Neem	10mm	0mm	0mm	8mm	0mm	0mm	0mm
PM54C1	Turmeric	9mm	0mm	0mm	7mm	0mm	0mm	0mm
	Neem	0mm	2mm	0mm	0mm	0mm	0mm	0mm

Different dilutions of honey showed different zones of inhibition. The dilution showed a minimum zone of 25% inhibition ranging from 5mm (minimum) to 10 mm (maximum). Only PM54C1 showed zero zones of inhibition. In a likewise manner, 50% showed a higher zone of inhibition in all samples as compared to the 25%. The minimum zone of inhibition showed by 50% dilution of honey was in PM54C1. However, 75% dilution showed a higher zone of inhibition for instance, 12mm in PM56C, PM33C4, AM56C4, and PM41C1. In addition, 100 % dilution (pure honey) showed a maximum zone of inhibition (Figure 5). A maximum zone of inhibition, showed by 100% was 15mm an indicated in Table 8.

Table 8. Antimicrobial Effect of Different Dilutions of Honey

Sample ID	25%	50%	75%	100%
PM33C4	00mm	02mm	10mm	15mm
PM56C4	00mm	10mm	10mm	14mm
AM24C4	08mm	08mm	10mm	13mm
AF34C4	05mm	08mm	11mm	13mm
PM31C4	10mm	11mm	13mm	15mm
PM57C4	08mm	10mm	13mm	15mm
AM56C4	07mm	09mm	12mm	15mm
AM25C4	06mm	09mm	11mm	14mm
PM41C1	08mm	09mm	12mm	13mm
PM54C1	0mm	05mm	07mm	10mm

Combined Effect of Neem, Turmeric, and Honey against *E. coli*

The ethanol extracts showed a maximum zone of inhibition against most of the isolated bacteria. Ethanol extracts showed zero zones of inhibition only in PM33C4 and AM25C4 in both disc and well-diffusion methods. The methanol extracts showed a maximum zone of inhibition only in PM56C4, AF34C4, and PM57C4 in both disc and well diffusion methods (Figures 6 and 7). Distilled water extracts showed no or zero zones of inhibition in all isolated bacteria in disc diffusion and well-diffusion methods as shown in Table 9.

Table 9. Combined Effect of Neem, Turmeric, and Honey against *E. coli.*

Methods	Well Diffusion Method			Disc Diffusion Method		
Sample ID	E	M	W	E	M	W
PM33C4	0mm	2mm	0mm	0mm	0mm	0mm
PM56C4	14mm	10mm	0mm	10mm	08mm	0mm
AM24C4	14mm	0mm	0mm	12mm	0mm	0mm
AF34C4	10mm	10mm	0mm	10mm	0mm	0mm
PM31C4	8mm	0mm	0mm	11mm	0mm	0mm
PM57C4	10mm	9mm	0mm	10mm	0mm	0mm
AM56C4	9mm	0mm	0mm	8mm	0mm	0mm
AM25C4	0mm	0mm	0mm	0mm	0mm	0mm

The ethanol extracts showed a maximum zone of inhibition in both well-diffusion and disc diffusion methods against both isolated *S. aureus*. The methanol extracts showed a sensitive zone of inhibition only against PM54C1 in the disc diffusion method as shown in Table 4.10. All other extracts showed zero zones of inhibition.

Table 4.10. Combined Effect of Neem, Turmeric, and Honey against *S. aureus*

Antimicrobial Activity Assay	**Well Diffusion Method**			**Disc Diffusion Method**		
Sample ID	E	M	W	E	M	W
PM41C1	8mm	0mm	0mm	8mm	0mm	0mm
PM54C1	10m	0mm	0mm	10mm	8mm	0mm

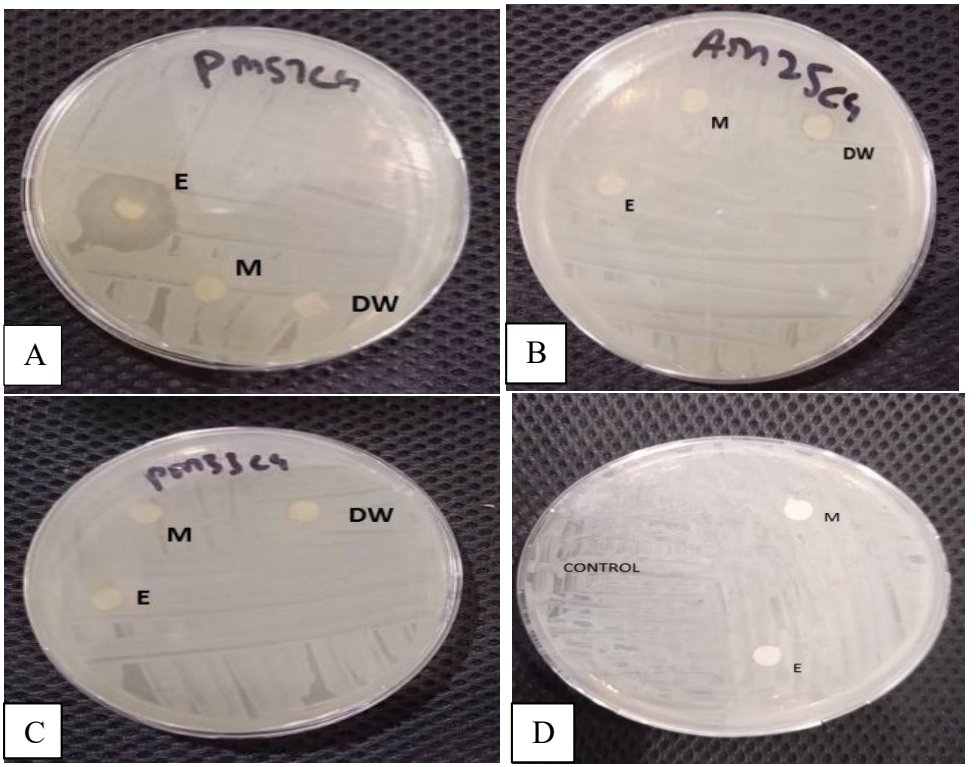


Figure 1. Effect of Neem on *E. coli* by Disc Diffusion method (a) Effect of Neem on PM57C4 (b) Effect of Neem on AM25C4 (c) Effect of Neem on PM33C4 (d) Control

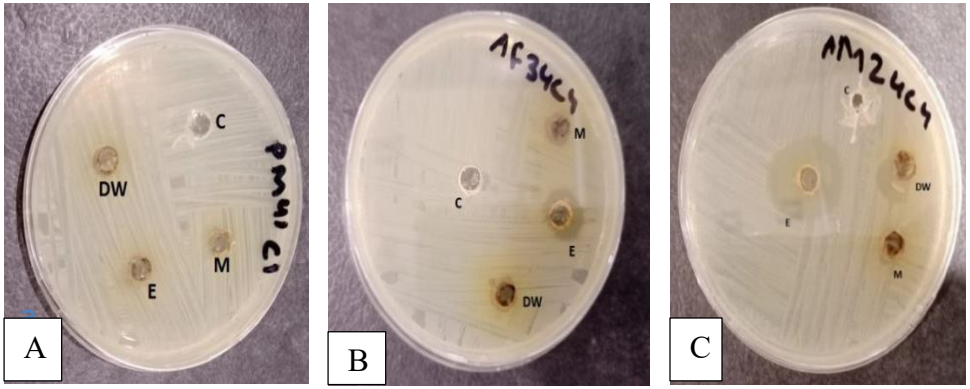


Figure 2. Effect of Neem by Well Diffusion Method (a) Effect of Neem Extracts on PM41C1 (b)Effect of Neem Extracts on AF34C4 (c) Effect of Neem Extracts on AM24C4.

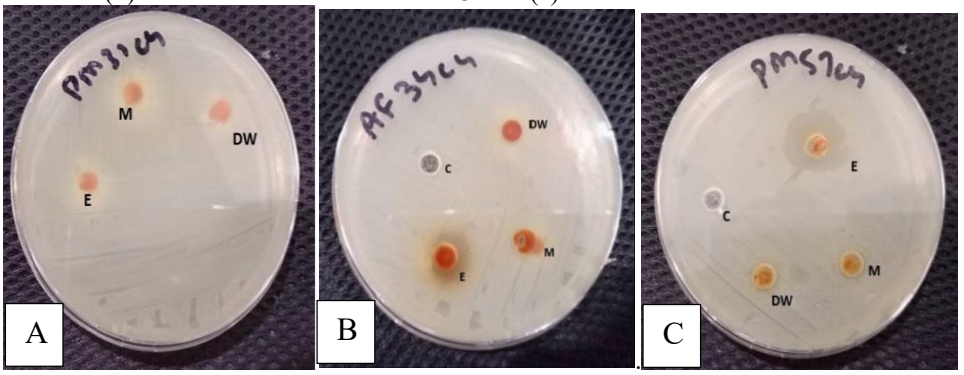
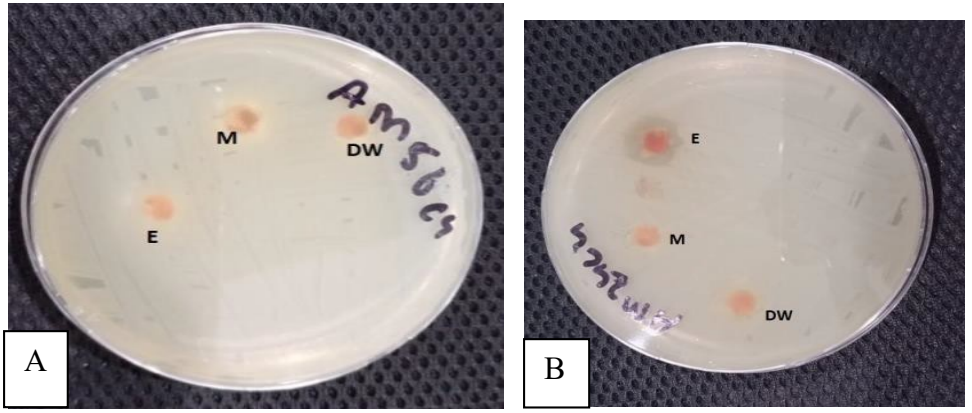


Figure 3. Effect of Turmeric by Well Diffusion Method (a) Effect of Turmeric Extract on PM31C4 (b) Effect of Turmeric Extract on AF34C4 (c) Effect of Turmeric Extract on PM57C4



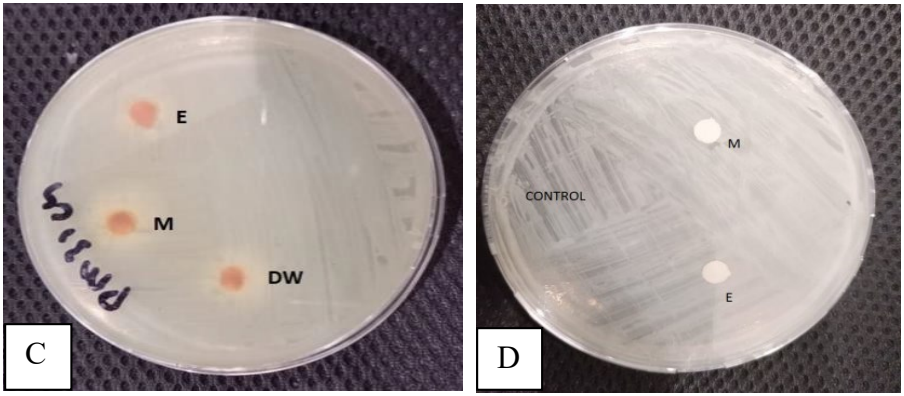


Figure 4. Effect of Turmeric by Disc Diffusion Method (a) Effect of Turmeric Extract on AM56C4 (b) Effect of Turmeric Extract on AM24C4 (c) Effect of Turmeric Extract on PM31C4 (d) Control

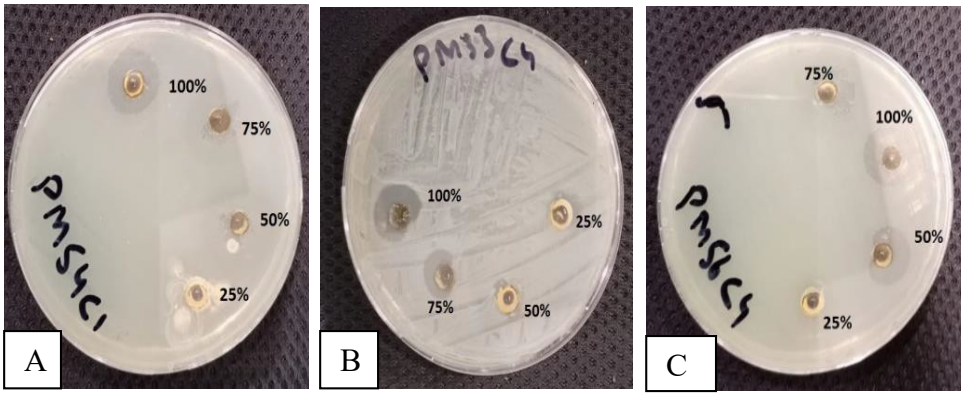
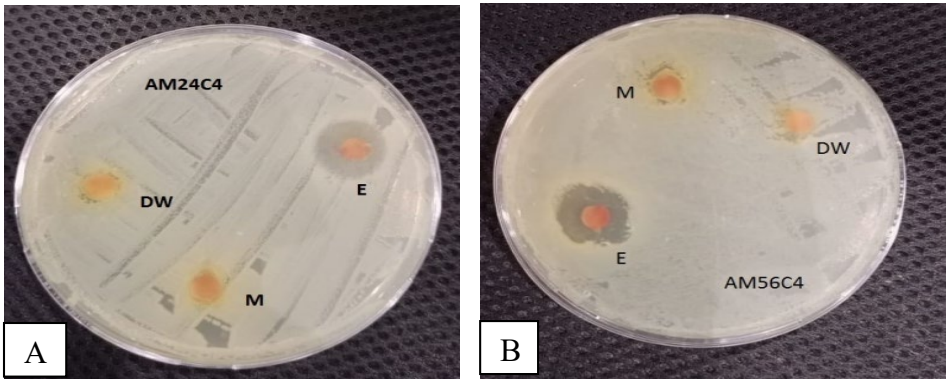


Figure 5. Effect of different dilutions of honey by Well Diffusion Method (a) Effect of Honeyon PM54C1 (b) Effect of Turmeric Extract on PM33C4 (c) Effect of Turmeric Extract on PM56C4



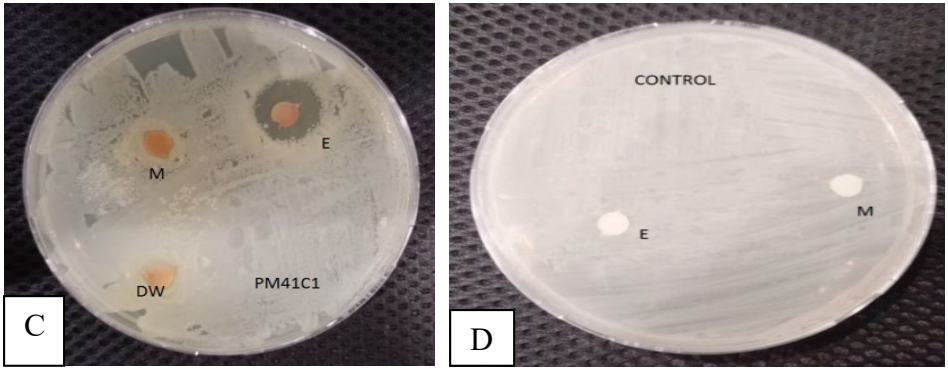


Figure 6. Combine Effect by Disc Diffusion Method (a) Effect on AM24C4 (b) Effect on AM56C4 (c) Effect of Extract on PM41C1 (d) Control

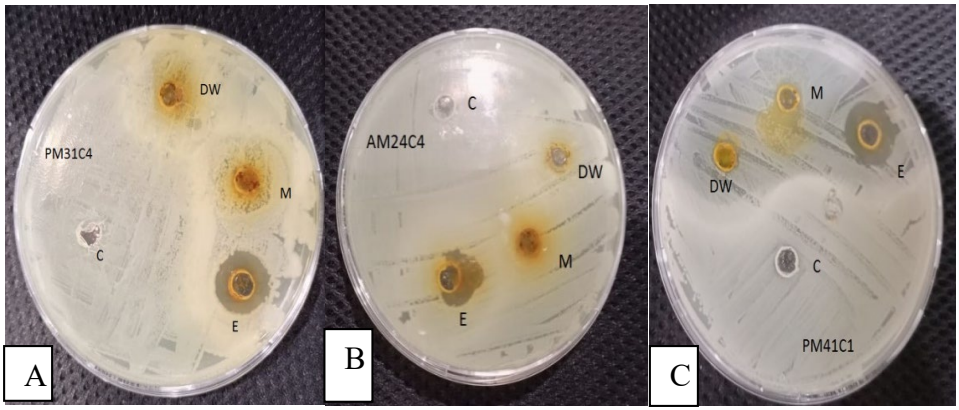


Figure 7. Combine Effect by well Diffusion Method (a) Effect on PM31C4 (b) Effect onAM24C4 (c) Effect on PM41C1

4. Discussion

In recent years, there have not been number of microbes reported having antibiotics resistance. They are turning into the predominant strains of the bacterial species that colonize the human body. The turn of events and spread of resistance is a complicated cycle that is impacted by particular pressures for instance, the pre-existence of resistive genes and the utilization of disease control measures. A sum of 20 samples were processed in the current study in which 34 colonies were isolated out which 8 were *E. coli* and 2 were *S. aureus.* The antibiotic sensitivity pattern of E. coli and S. aureus was like that E. coli showed maximum sensitivity against imepenem and meropenem and only one sample PM56C4 showed a maximum 26mm zone against ceftriaxone, while the rest of the samples developed 0mm zones. In a likewise manner, PM33C4 also developed a 0mm zone, whereas other samples developed a maximum zone against Amikacin such as 25mm as an observed and impressive result. Gentamicin has developed a similar antibiotic sensitivity pattern as Amikacin.

Doxycycline (Tetracycline) was resistance in PM33C4, AM23C4, PM41C1, and PM54C1, and developed 22mm PM56C4. Erythromycin was used for two samples which were positive for gram-positive such as (PM41C1 and PM54C1) with a 0mm zone.

Similarly, oxacillin was used in gram-positive samples and developed a maximum zone of 30mm. Chloramphenicol and Tigecycline were used only in gram-negative organisms and developed a sensitive zone with 26mm and 27mm so, the zones were reported as the maximum zone of inhibitions.

Currently, no previous research has been reported in which the researcher has investigated honey, turmeric, and neem extracts for their antibacterial effects in a single experiment. *E. coli* and *S. aureus* isolated in previous studies were also identified [17, 18]. In the current study, samples collected from males and females of different age groups were subjected to biochemical characterization for identification purposes. Biochemical identification test includes citrate, urease, indole, motility, H_2S gas, TSI, and catalase which confirmed the presence of *E. coli* and *S. aureus* in the collected samples. In addition, gram staining helped to differentiate between gram-positive and gram-negative bacterium. *E. coli* was observed as gram-negative bacteria, whereas *S. aureus* was identified as gram-positive bacteria [19]. Furthermore, the bacteria was isolated and identified by using the Vitek 2 compact technique. All the findings were compared with American-type cell culture which confirmed *E. coli* presence in 8 samples and *S. aureus* in the other 2 samples [20].

One of the major threats to medicine is the development of antibiotic resistance in microbes. The reason behind the resistance against commonly used antibiotics is their indiscriminate use, hygiene/faecal colonization, their use in livestock, and some multifactorial elements like intrinsic and extrinsic resistance in bacteria [21, 22]. The highest resistance in *E. coli* and *Staphylococcus aureus* against several antibiotics like amoxicillin, tetracycline, and cefotaxime was reported previously [23]. The main categories in the mechanism of antimicrobial resistance include modifying a drug target, limiting the uptake of a drug, active drug efflux, and inactivating a drug. Gram-positive and gram-negative bacteria differ in their resistance mechanisms because of the different variations in their structures. The gram-negative bacteria make use of all mentioned mechanisms, whereas gram-positive bacteria cannot limit the drug uptake and don't have the potential for certain kinds of drug efflux mechanisms because they lack lipopolysaccharide outer membrane [24].

Previously, it was observed that *E. coli* was resistant to Ceftriaxone and ofloxacin. However, *E. coli* was sensitive to Imipenem, Meropenem, Tigecycline, Colistin, and Gentamicin. In the current study, similar results were observed, isolated samples PM33C4, AM24C4, AF24C4, PM31C4, PM57C4, and AM25C4 were resistant against 30 ugs Ceftriaxone. Conversely, these samples were sensitive to antibiotics Imipenem, Meropenem, Tigecycline, Colistin, and Gentamicin. *E. coli* resistant strains have the potential to pass on antibiotic resistance determinants to more *E. coli* strains and to other bacteria residing in the gastrointestinal tracts [25]. *E. coli* isolated from patient of the urinary tract infection also showed increased resistance against

different commercially reported antibiotics [26].

Gurung et al. [27] used the Kirby–Bauer disc diffusion method and examined the antibiotic susceptibility of *S. aureus* against thirteen antibiotics including ofloxacin (5 µg) and gentamicin (10 µg). It was noted that *S. aureus* was less resistant to gentamicin. In the current study tested samples of *S. aureus* PM41C1 and PM54C1 were sensitive to 30 ug ofloxacin. However, the sample PM41C1 showed resistance against 10 ug gentamicin and sample PM54C1 was sensitive toward it. Pandey et al. [28] in their studies demonstrated that *S. aureus*-associated infection was more common in children, because of their low immunity. Furthermore, they added that 78.37% *S. aureus* was isolated from wounds and pus, 4.50% from urine, and 17.11% from the blood. This bacterium normally inhabits the skin, therefore, it causes pyogenic infections for instance soft tissue and skin disease.

Natural ingredients like honey, turmeric, and neem are effective alternates to antibiotics because they possess excellent antimicrobial activity against clinical bacterial isolates. Mandal et al. [29] studied the antimicrobial activity of honey against *Salmonella enterica serovar Typhi, E. coli,* and *Pseudomonas aeruginosa* using the agar dilution method. Minimum bactericidal concentration, minimum inhibitory concentration, and partial inhibitory concentration of autoclaved honey were also determined for test strains. Honey showed a bacteriostatic effect at 0.50-1.25% (v/v) concentration. Moreover, at a concentration of 3-4% (v/v) bactericidal effect of honey was observed for *E. coli*. Mulu *et al*., reported that for full prevention of the growth of *E. coli* 6.5% (v/v) of honey concentration was very effective [30]. In an experiment conducted by French *et al*., manuka honey at a concentration of 2.7-5% (v/v) inhibited the growth of Staphylococcus isolates. However, simulated honey was effective in inhibiting bacterial growth at 27.5-31.7% (v/v) concentration. Thereby, it was concluded that natural honey had 5.5-11.7 times greater antimicrobial activity because of the high osmotic effect of honey's sugar content [31]. The difference in the antibacterial potential of honey against various test isolates occurred because of differences in inoculum size, source of microorganism, the growth rate of the pathogen, and test method itself. Moreover, it also depends upon the processing, the origin of honey, season, and place in which the vegetative flower is blooming, and from which the nectar has been gathered up.

In the current study, honey was used in different forms for instance, ethanolic extract, methanolic extract, and honey in water. The agar disk diffusion method and agar well diffusion methods were used to evaluate the antibacterial activity. The *E. coli* sample PM33C4 was resistant to all three forms of honey. Whereas the remaining 7 samples PM56C4, AM24C4, AF34C4, PM31C4, PM57C4, AM56C4, and AM25C4 showed resistance and sensitivity towards different forms of honey as indicated in Table 8. On the other hand, *S. aureus* samples PM41C1 and PM54C1 exhibited sensitivity, resistance, and intermediate response toward different honey forms as illustrated in Table 9. This was because of the difference in osmotic effect and pH of honey in presence of ethanol, methanol, and water. Some earlier authors highlighted that the antimicrobial potential of honey has been ascribed to its hydrogen peroxide concentration, methylglyoxal, phytochemical nature, high osmotic effect, and high acidic nature (pH

being 3.2-4.5) [32]. Maghsoudi et al. [33] also evaluated the antibacterial activity of ethanolic, methanolic, and ethyl acetate extracts of raw and processed honey against gram-negative bacteria including *E. coli,* and gram-positive bacteria including *S. aureus*. Both types of honey showed an antibacterial effect with 6.94-37.94 mm zones of inhibition. Among all extracts methanolic extract was the most potent, while in the current study, ethanolic extracts gave more promising results. Moreover, gram-negative bacteria were more prone to the antibacterial activity of honey. Our findings were similar with to the study of Maghsoudi et al., but with ethanolic extracts being more efficient against *E. coli*.

Afrose *et al*., conducted a study on the antibacterial effect of an aqueous paste of crude turmeric against *E. coli* and *S. aureus*. Nutrient agar media was selected to incorporate turmeric paste. About 30% and 10% growth of *E. coli* and *S. aureus* were inhibited, respectively. The minimum inhibitory concentration of 2000 µg/ml was noted against *E. coli* and 800 µg/ml against *S. aureus*. Their study concluded that turmeric has good potential to inhibit antibiotic-resistant bacteria growth [34]. Gul et al. [35] prepared turmeric extract in water, ethanol, n-hexane, methanol, and chloroform at 1% or 2% concentration and examined their antibacterial effect using the disk diffusion method against *S. aureus*, *Candida albicans*, *E. coli,* and *Salmonella typhi*. Turmeric extract in water and methanol successfully inhibited the growth of *S. aureus* and *E. coli*. It was clear that in combination with any preservative, turmeric extract can be used effectively to store food. In contrast to his findings, this study indicated that ethanolic extracts of turmeric showed good antibacterial activity against test isolates.

Lim et al. [36] highlighted the free radical scavenging and antioxidant properties of turmeric leaves. Similar results were also observed by [36-38]. Kim *et al*., reported that the antibacterial effect of turmeric against *S. aureus* and *E. coli* was because of the presence of a phenolic compound called curcuminoid, curlone and turmerone components, essential oil presence, turmeric oil, curcumins, and valeric acid. The possible mechanisms for the antimicrobial effect of turmeric include inhibition or inactivation of extracellular and intracellular enzymes and rupturing of cytoplasmic membranes [39]. In the current study autoclaved ethanolic, methanolic, and aqueous extracts of turmeric were prepared and examined for their antibacterial effect against *E. coli* and *S. aureus* using well diffusion and disk diffusion methods. Both methods are used by many researchers to evaluate antibacterial activity.

Agar disk diffusion method was used to examine drugs, essential oils, and plant extracts. It is commonly used in diagnostic labs for routine testing and antimicrobial screening. It is advantageous to use because of its low cost and simplicity. However, the agar well diffusion method is specially designed to accurately screen plant extracts possessing antimicrobial activity. It provide qualitative results by ranking bacteria as resistant, intermediate, and sensitive. Therefore, it is beneficial to use agar well diffusion for screening plant extracts [30]. Moreover, in our case agar, the well diffusion method gave more promising results as compared to the agar disk diffusion method. Tables 8 and 9 illustrated that among the prepared extracts, the ethanolic extract was most effective, and bacterial isolates were sensitive toward it. However, the methanolic and aqueous extracts were not as effective.

Francine et al. [40] prepared aqueous and ethanolic extracts of neem leaves and evaluated their antimicrobial effect against *E. coli* and *S. aureus* using the disk diffusion method. It was noted that fresh neem leaves effectively inhibited bacterial growth and the inhibition zone increased with an increase in the concentration of extract. The effectiveness of neem leaves depends upon the concentration of extract used. Similarly, in the current study the ethanolic, methanolic, and aqueous extracts were prepared and tested against eight samples of *E. coli* and two samples of *S. aureus* as shown in Tables 5 and 6. The samples PM33C4, AM25C4, and PM54C1 were completely resistant to all three extracts. However, the samples PM56C4, AM24C4, AF34C4, PM31C4, PM57C4, AM56C4, and PM41C1 were sensitive toward the ethanolic extract of neem. Among the three extracts, the ethanolic extract was most effective in comparison with methanolic and aqueous extracts. Maleki et al. [41] also evaluated the antibacterial activity of neem leaves in form of ethyl acetate, ethanolic, and methanolic extracts against the pathogenic bacterial isolates *E. coli* ATCC 25922 and *S. aureus* ATCC 6538. Microdilution and agar well diffusion method was employed to evaluate the antibacterial activity. It was noted that methanolic extract showed the strongest antibacterial effect as compared to ethanol and ethyl acetate. Conversely, in current study of *E. coli* samples and *S. aureus* samples were sensitive toward ethanolic extracts of neem. The difference in results may occur because of the different concentrations and the purity of ethanol and methanol used.

Banna et al. [42] also investigated the antibacterial effect of neem seeds, flowers, and leaves against S. aureus, *Salmonella, and Klebsiella.* The growth inhibitory effects were found at a concentration of 6.2 mg/ml and 12.5mg/ml. At higher concentrations, growth inhibition started to decrease. In other studies, it was noted that the most essential active components present in neem are nimbidol, polyphenolic flavonoids, nimbolinin, ascorbic acid, salannin, gedunin, and quercetin. Neem also possesses a significant anti-oxidant potential that made it an effective agent to inhibit bacterial growth [10, 43, 44]. Yerima et al. [45] reported that seed, fruit extracts, bark, and a leaf of neem showed antibacterial effects against bacteria isolated from the adult mouth. The strong antibacterial activity was shown because of the presence of an active phytochemical compound. Okemo et al. [46] examined that crude extract of the neem plant was very effective against *E. coli* and *S. aureus*. It showed the bacteriostatic and bactericidal effects [46]. Our findings suggested that among neem, honey, and turmeric, neem showed the strongest antibacterial effect. Moreover, the most potent neem extract was ethanolic extract as indicated in Tables 8 and 9.

Later, the combined effect of neem, turmeric, and honey against 8 samples of *E. coli* and 2 samples of *S. aureus* were investigated. It was noted that sample PM33C4 was resistant against all the preparations of ethanolic, methanolic, and aqueous. However, the remaining samples of *E. coli* also showed sensitivity along with resistance as illustrated in Table 10. In the case of *S. aureus,* both samples showed sensitivity and resistance against different preparations, as indicated in Table 11. Sinha *et al*., used the agar diffusion method to illustrate the combined antibacterial effect of neem and turmeric in comparison with sodium hypochlorite and chlorohexidine. The test organism was *Enterococcus faecalis*. It was noted that the

combination of natural ingredients alters the osmotic equilibrium of bacterial cell membranes. As a result, bacterial growth is inhibited. This antibacterial effect was similar to sodium hypochlorite and chlorohexidine [47]. It is evident from previous studies, that ethanolic preparation of neem, turmeric, and honey have more potential as compared to aqueous plant extracts. The findings of other researchers are in accordance with the current study results. This study observed that ethanolic plant extract exhibited larger zones of inhibition and potential antimicrobial activity. This is because ethanolic extracts activate plant components such as, nimbidol, polyphenolic flavonoids, nimbolinin, ascorbic acid, salannin, gedunin, quercetin, curcuminoid, curlone, turmerone components, essential oil presence, turmeric oil, curcumins, and valeric acid that play a vital role in antimicrobial activity against multiple drug-resistant pathogens [48].

4.1 Conclusion

The current research covered the antimicrobial activity of honey, neem, and turmeric. Natural ingredients like honey, turmeric, and neem acclaimed to be effective alternatives to antibiotics because they possess excellent antimicrobial activity against clinical bacterial isolates. Honey, neem, and turmeric have patent activities against clinically isolated bacteria such as (gram-negative). Natural remedies like honey, neem, and turmeric require further refinement and implementation in the pharmaceutical industries to combat the antimicrobial resistance.

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