
ORIGINAL ARTICLE**Indian Spices Enhance the Activities of Antibiotics against Human Pathogens Synergistically***Hema Shiral^{1*}, R. Swaminathan¹, R. Patil², R. Khandare², N. Desai²**¹Department of Microbiology, D.Y. Patil University, Navi Mumbai- 400706(Maharashtra) India, ²Amity Institute of Biotechnology, Amity University Mumbai, Panvel-410206 (Maharashtra) India*

Abstract:

Background: Bacterial pathogens are considered as predominant cause of human diseases throughout the world. Until recently, antibiotics were considered as promising agents against most bacterial pathogens but recent reports suggest that there is growing resistance to commonly used antibiotics creating a global healthcare problem. **Aim and Objectives:** To investigate the synergistic antibacterial potential of three different antibiotics including Vancomycin, Clindamycin and Cefotaxime with three popular Indian spices namely *Cinnamomum zeylanicum* (Dalchini), *Trachyspermum ammi* (Ova) and *Syzygium aromaticum* (Clove) against human pathogens *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumoniae*. **Material and Methods:** Fourier-transform Infrared (FTIR) spectroscopy analysis was performed to detect molecular changes occurring while synergistic exposure of antibiotics and spices on pathogenic microbes. The addition of spices extracts showed enhanced activity of antibiotics against the pathogens however degree of antibiosis was varied according to bacterial species. Inhibition Zones (IZ) ranged from 0.0-34 mm. The highest IZ of 34.33 mm was found against *S. aureus* where a combination of Cefotaxime and *C. zeylanicum* were applied. The synergy of spice extracts with antibiotics revealed an increase in the bactericidal activity of standard antibiotics against pathogens. FTIR spectral analysis showed that, microorganisms showing resistance to antibiotics (Vancomycin and Clindamycin), alters important functional groups of antibiotics might be resulting in decreased antimicrobial performance. FTIR spectra's revealed common bands in

antibiotics and spices such as nitroamines, aromatic phosphorus, benzene, bromide, carboxylic group, aliphatic esters, sulphonamides, primary alcohols, aliphatic ether, acid anhydride conjugate ring with ketone and azo compounds, aromatic ethers, sulphonil chloride, sulphoamide etc. Interestingly, there was increased antimicrobial response for synergism when decreased concentration of antibiotics and increased concentration of spice extracts were used. **Conclusion:** This investigation suggests that, spice extract could be used independently and in combination to elevate the performance of antibiotics which addresses the issue of drug resistance in human pathogens.

Keywords: Antibiotics, Human Pathogen, FTIR, Multidrug Resistance, Spices

Introduction:

Bacterial pathogens are considered as predominant cause of human diseases throughout the world. Until recently, antibiotics were considered as promising agents against most bacterial pathogens but recent reports suggest that there is growing resistance to commonly used antibiotics creating a global healthcare problem [1]. According to recent reports, there are at least 2 million infections leading to about 23,000 deaths per year in US [2]. The development of resistance against bactericidal and bacteriostatic drugs is because of the inadequate selection of antibiotics along with uncontrolled and excessive use [1]. Among different pathogenic microorganisms,

Staphylococcus aureus, *Enterococcus faecalis*, *Escherichia coli*, and *Klebsiella pneumoniae* are some of the important human pathogens causing the number of dangerous diseases including urinary tract infection, typhoid, diarrhea, septicemia etc [3-5]. These human pathogens have developed resistance to many commonly used antibiotics [6-9]. As antibiotics are having a short life span, it is imperative to search superior alternative agents that can eradicate harmful pathogens efficiently [10].

The fundamental finding of plant extracts aiming to characterize their potential activities at the molecular level is the current trend in research. As a potential replacement of antibiotics, plant-based treatments could be more effective for managing pathogenic infections showing multidrug resistance [10]. Plant spices are widely grown and consumed in India and are well known for their health benefits. Analysis of phyto-metabolites in spices and their further application as antimicrobial agents have also been reported [11-12]. Hence, we had selected three popular spices including *Cinnamomum zeylanicum* (Dalchini), *Syzygium aromaticus* (Clove) and *Trachyspermum ammi* L. (Ova) to study their activity against common human pathogens (*S. aureus*, *E. fecalis*, *E. coli*, and *K. pneumoniae*). These spices have various degree of inhibition activity against pathogenic microbes under study [13].

Bark oil of cinnamon is effective against a diverse array of Gram positive and Gram negative bacteria including *K. pneumoniae*, *Pseudomonas* species, *Bacillus* species, *S. aureus*, *E. coli*, *E. cloaca*, *C. xerosis*, *S. faecalis*, etc [14]. Antibacterial and antibiofilm activities of essential oil extracted from *cinnamomum* were also effective against *S.*

pyogenes, *P. aeruginosa* and *E. coli* [15]. Recently in 2019, antibacterial activity of cinnamon essential oils synergistically with antibiotics is reported [16]. *S. aromaticum* is known for its antimicrobial properties against pathogens like *B. subtilis* and *S. aureus* [17]. Eugenol, a bioactive component of *S. aromaticum* limits the growth of *B. cereus* by inhibiting the growth enzymes [18] and acts as an antifungal agent against *Microsporum canis* and *T. mentagrophytes*. Antibacterial activity of *S. aromaticum* against *E. coli*, *B. cereus* and *S. aureus*, *B. subtilis*, *B. megaterium*, *B. sphaericus* *B. polymixa* have been studied extensively [19-20]. Similarly, ethanolic extract of *T. ammi* seeds showed antibacterial activity against various pathological bacterial strain including *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae* e, *L. acidophilus*, *M. luteus*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and bacillary dysentery-causing microorganisms [21-22]. *T. ammi* extract increases activity of standard antibiotics against various infectious bacterial strains [23]. Principle compounds in *T. ammi* are alkaloids and phenols, glycosides, terpenoids, and tannin [24].

This study was conducted using commonly used antibiotics namely Vancomycin, Clindamycin and Cefotaxime either alone or in combination with spice extract. The selection was based on sensitivity or susceptibility against pathogenic bacteria. Vancomycin is a glycopeptide in nature and exerts antimicrobial activity by inhibiting cell wall synthesis, altering cell membrane permeability and through selective inhibition of ribonucleic acid synthesis [25]. Cefotaxime is third generation cephalosporin antibiotic having a bactericidal activity against Gram positive as well as Gram negative susceptible bacteria. Its action is through inhibition of bacterial cell wall synthesis and is

strongly resistant to the action of Richmond I, III, IV, and V beta-lactamase enzymes [26]. Clindamycin is a synthetic lincosamide antibiotic originally produced from naturally occurring lincomycin. Its antibacterial activity is through bacterial protein synthesis inhibition at the level of 50s ribosome [27].

Fourier-transform Infrared (FTIR) spectroscopy is a highly sensitivity technique used for identifying changes in the functional groups of the compounds. The information about bands position, shift, intensity and width provides crucial information. This technique has been successfully employed for bioremediation studies of textile dyes, hydrocarbons, antibiotics, pesticides, herbicides and many more pollutants [28]. This technique measures the vibration properties of the chemical bonds when excited by the absorption of the IR radiation. It is possible to detect bacterial susceptibility towards different antibiotics in few minutes with the aid of FTIR [1]. There are several reports on synergistic activity of different plant extracts including antibiotics against pathogens like *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*, *Bacillus subtilis*, *E. cloacae*, *K. pneumoniae* and *P. mirabilis* [10, 29-30]. But there is no data available on the validation of combinational antibacterial activity of the spice and antibiotics using the FTIR technique. With this background, ethanolic extracts of *C. zeylanicum*, *T. ammi* and *S. aromaticum* and three antibiotics Cefotaxime, Clindamycin and Vancomycin were exposed to pathogenic bacterial strains (*S. aureus*, *E. fecalis*, *E. col*, and *K. pneumoniae*) to determine bactericidal potential of spices and structural changes in functional groups of antibiotics and spices that confers either susceptibility or resistance.

Material and Methods:

Tested organisms, antibiotics and spices:

Human pathogens, *Staphylococcus aureus* (ATCC3750), *Enterococcus faecalis* (ATCC29212), *Escherichia coli* (ATCC35218) and *Klebsiella pneumoniae* (ATCC700603) were procured from Haffkine Institute, Mumbai (M.S.) India. The cultures were freshly grown into laboratory and further stored using 20% glycerol in cryogenic vials at -80°C. Respective cultures were maintained regularly on the Nutrient Agar medium for experimental purpose. Ten different antibiotics including Gentamicin, Ampicillin, Levofloxacin, Clindamycin, Vancomycin, Tetracycline, Meropenem, Co-trimaxazole, Cefotaxime, Ceftriaxone were purchased from Medical store, Nerul, Navi Mumbai. Ten routinely used Indian spices including *Cinnamomum zeylanicum* (Dalchini), *Cuminum cyminum* (Cumin seeds- Jeere), *Coriander sativum* (Coriander seeds – Dhane), *Curcuma longa* (Turmeric – Haldi), *Myristica fragrans* (Nutmeg- Jayfal), *Piper nigrum* (Black pepper- kali miri), *Syzygium aromaticum* (Clove), *Trigonella foenum* (Fenugreek- Methi), *Trachyspermum ammi* (Ajwain- Ova) and *Zingiber officinale* (Dried Ginger) were purchased from local spice market of Nerul, Navi Mumbai.

Preparation of ethanolic extracts:

All the above species were procured from local market of Navi Mumbai for research purpose. Ethanol was used as an extraction solvent according to earlier reported method [31] with some minor modifications. In brief, spices were dried at 45°C using incubator and were ground using a grinder into a fine powder. The respective powders were soaked in ethanol (1:10 w/v) in sterilized jam bottles for 48 hrs at 25°C on a rotator

shaker at 120 rpm. The samples were filtered through Whatman filter paper No. 2 and kept for solvent evaporation in an incubator at 37°C. The dried extracts were dissolved in ethanol to achieve the final concentration of stock to 2mg/ml and stored at 4°C.

Antimicrobial screening by well diffusion method:

Müller-Hinton (MH) agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing. From the exponentially grown bacterial culture, 100 µl were uniformly spread using sterile glass spreader on MH agar plates in a sterile condition. Eight mm diameter holes were cut in the agar using a cork borer. The desired volume of antibiotics and spice extracts were loaded in the well. The plates were incubated at 37°C for 24 hrs. The zone of inhibition of the bacterial growth was measured in mm.

FTIR analysis:

Three different spices extracts, three standard antibiotics and their individual, as well as combinatorial effect on pathogenic strains were studied using FTIR analysis at Chemostat Laboratory, Wadala Mumbai. FTIR analysis was carried to determine the functional group using SHIMADZU 8400S FTIR model in the scanning range 4000 to 500 cm⁻¹ with a resolution of 8 cm⁻¹ and a mirror velocity of 0.48 cm⁻¹.

Results:

Antibacterial activity:

Primarily, ten different spices and ten different antibiotics were screened among which spices viz. *C. zeylanicum*, *T. ammi*, *S. aromaticum* and antibiotics viz. Vancomycin, Cefotaxime, Clindamycin were selected for further investigation (Data not shown). Antibacterial

activity of sensitive antibiotic Cefotaxime was determined against four human pathogens under study. From the Table 1 it is clear that, Cefotaxime and spice extracts alone or in combination have strong antimicrobial potential. The combination of minimum Cefotaxime concentration (15 µl) with *C. zeylanicum*, *T. ammi* and *S. aromaticum* extract (90 µl) showed highest zone of inhibition for *S. aureus* (34.33 ± 2.89, 33.67 ± 2.31, 28.67 ± 2.31 respectively). The promising inhibition of *E. fecalis* (31.67 ± 1.53 mm) and *K. pneumoniae* (31.00 ± 2.65 mm) were observed for Cefotaxime and *T. ammi* (15 + 90 µl). The maximum growth suppression of *E. coli* (32.67 ± 6.11 mm) was detected for the combination of Cefotaxime and *S. aromaticum* extract (15 + 90 µl). Interestingly, all the pathogens were susceptible to the decreased concentration of antibiotics with an increased concentration of respective spices extract. Antibacterial activity on *S. aureus* was carried out using Vancomycin (resistant antibiotic) in combination with different spices extract (*C. zeylanicum*, *T. ammi* and *S. aromaticum*) at various concentrations (Fig. 1 and Table 2). Though, *S. aureus* showed little growth in the presence of Vancomycin, the minimum zone of inhibition suggests its resistant nature. Spice extracts exhibited antimicrobial activity but susceptibility of *S. aureus* was increased when extract and Vancomycin used synergistically. Moreover, zone of inhibition was increased with decreased concentration of antibiotic and increased concentration of spice extract. The highest zone of inhibition (25.33 ± 2.31 mm) was observed with Vancomycin (1 µl) plus *T. ammi* extract (45 µl). Susceptibility of remaining three pathogens was assessed using Clindamycin as a resistant antibiotic (Table 3). Clindamycin was bactericidal

to a lesser extent for *E. fecalis* (zone of inhibition up to 12.00 ± 0.00 mm). But the highest zone of inhibition for *E. fecalis* was observed for 1 μ l of Clindamycin and 45 μ l of *T. ammi* extract (19.67 ± 0.47 mm). *E. coli* showed better susceptibility for 1 μ l of Clindamycin with 45 μ l of *C. zeylanicum* extract (16.00 ± 1.41 mm). The superior bactericidal activity on *K. pneumoniae* was found

at 1 μ l concentration of Clindamycin with 45 μ l of *T. ammi* extract (29.33 ± 0.94 mm). It is notable that, though Clindamycin alone was ineffective against *E. coli* and *K. pneumoniae*, its combination with spice extract showed enhanced bactericidal activity. This could be attributed to certain functional group modifying properties present in secondary metabolites of spice extract.

Table 1: Effect of combinations of Antibiotic (Cefotaxime) with Different Spices Extracts (*Cinnamomum zeylanicum* (Dalchini), *Trachyspermum ammi* (Ova) and *Syzygium aromaticum* (Clove))

Cefotaxime (Cefo)+ Dalchini (Dal)						
Organism	Cefo (30 μ l)	Dal (60 μ l)	Cefo +Dal (30+60 μ l)	Cefo +Dal (25+70 μ l)	Cefo +Dal (20+80 μ l)	Cefo +Dal (15+90 μ l)
<i>S. aureus</i>	25.33 ± 2.89	26.67 ± 2.31	30.00 ± 2.00	30.33 ± 2.52	33.33 ± 2.89	34.33 ± 2.89
<i>E. fecalis</i>	20.67 ± 0.58	21.67 ± 0.58	20.00 ± 1.73	21.33 ± 2.31	22.33 ± 2.31	23.33 ± 2.31
<i>E. coli</i>	28.67 ± 2.31	18.33 ± 0.58	26.67 ± 0.58	26.67 ± 0.58	28.00 ± 1.00	30.00 ± 0.00
<i>K. pneumoniae</i>	17.67 ± 0.58	18.00 ± 0.00	21.67 ± 1.53	21.00 ± 2.65	21.67 ± 3.06	23.00 ± 2.65
Cefotaxime (Cefo) + Ova						
Organism	Cefo (30 μ l)	Ova (60 μ l)	Cefo +Ova (30+60 μ l)	Cefo +Ova (25+70 μ l)	Cefo +Ova (20+80 μ l)	Cefo +Ova (15+90 μ l)
<i>S. aureus</i>	20.67 ± 1.15	22.00 ± 5.20	28.67 ± 2.31	30.67 ± 2.31	32.00 ± 1.73	33.67 ± 2.31
<i>E. fecalis</i>	18.67 ± 1.15	20.33 ± 0.58	21.33 ± 1.53	25.33 ± 2.52	27.67 ± 2.08	31.67 ± 1.53
<i>E. coli</i>	25.00 ± 1.73	23.33 ± 4.62	24.67 ± 1.53	26.33 ± 1.15	29.33 ± 1.15	32.00 ± 0.00
<i>K. pneumoniae</i>	17.67 ± 0.58	24.33 ± 1.15	21.67 ± 0.58	24.33 ± 2.08	28.00 ± 3.46	31.00 ± 2.65
Cefotaxime (Cefo) + Clove (Cl)						
Organism	Cefo (30 μ l)	Clove (60 μ l)	Cefo +Cl (30+60 μ l)	Cefo +Cl (25+70 μ l)	Cefo +Cl (20+80 μ l)	Cefo +Cl (15+90 μ l)
<i>S. aureus</i>	23.33 ± 1.15	18.00 ± 1.73	28.00 ± 2.65	28.67 ± 2.31	28.00 ± 2.00	28.67 ± 2.31
<i>E. fecalis</i>	20.67 ± 0.58	19.67 ± 0.58	17.00 ± 1.00	19.67 ± 0.58	25.33 ± 1.53	28.33 ± 2.89
<i>E. coli</i>	28.67 ± 2.31	24.00 ± 1.73	28.00 ± 3.46	30.67 ± 4.93	32.67 ± 6.11	32.67 ± 6.11
<i>K. pneumoniae</i>	17.67 ± 0.58	15.67 ± 0.58	15.67 ± 0.58	18.33 ± 2.08	22.67 ± 2.31	27.33 ± 4.62

*Concentration of antibiotic stock- 1 μ g/ μ l and concentration of spice extract- 2 μ g/ μ l

Table 2: Effect of Combinations of Antibiotic (Vancomycin) with Different Spices Extracts (*Cinnamomum zeylanicum* (Dalchini), *Trachyspermum ammi* (Ova) and *Syzygium aromaticum* (Clove) against *S. aureus*

Vancomycin (Vanco) + Dalchini (DI)						
<i>S. aureus</i>	Vanco (30 µl)	Dal (30 µl)	Vanco+Dal (30+ 30) µl	Vanco+Dal (25+ 35) µl	Vanco+Dal (20+ 40) µl	Vanco+Dal (15+45) µl
	16.67 ±1.15	18.00 ±5.20	17.67 ± 3.06	19.00 ± 3.00	21.00 ± 2.65	23.33 ± 4.16
Vancomycin (Vanco) + Ova						
<i>S. aureus</i>	Vanco (30 µl)	Ova (30 µl)	Vanco+ Ova (30+30) µl	Vanco+ Ova (25+35) µl	Vanco+ Ova (20+40) µl	Vanco+ Ova (15+45) µl
	16.67 ± 1.15	16.67 ± 2.89	19.33 ± 2.31	21.33 ± 2.31	23.33 ± 2.31	25.33 ± 2.31
Vancomycin (Vanco) + Clove(CI)						
<i>S. aureus</i>	Vanco (30 µl)	CI (30 µl)	Vanco+ CI (30+ 30) µl	Vanco+ CI (25+ 35) µl	Vanco+ CI (20+ 40) µl	Vanco+ CI (15+45) µl
	16.00 ± 0.00	11.67 ± 1.15	16.00 ± 0.00	16.00 ± 1.00	16.67 ± 1.15	17.00 ± 1,00

*Concentration of antibiotic stock-1 µg/ µl and concentration of spice extract-2 µg/ µl

Table 3: Effect of Combinations of Antibiotic (Clindamycin) with Different Spices Extracts (*Cinnamomum zeylanicum* (Dalchini), *Trachyspermum ammi* (Ova) and *Syzygium aromaticum* (Clove))

Clindamycin (Clinda) + Dalchini (Dal)				
Organism	Clinda (2 µl)	Dal (30µl)	Clinda +Dal (2+30 µl)	Clinda +Dal (1+45 µl)
<i>E. fecalis</i>	12.00 ± 0.00	15.67 ± 0.58	14.00 ± 1.00	17.33 ± 0.94
<i>E. coli</i>	R ± 0.00	15.00 ± 1.73	14.00 ± 2.65	16.00 ± 1.41
<i>K. pneumoniae</i>	R ± 0.00	13.67 ± 0.58	14.33 ± 0.58	17.33 ± 0.94
Clindamycin (Clinda) + Ova				
Organism	Clinda (2 µl)	Ova (30µl)	Clinda +Ova (2+30 µl)	Clinda +Ova (1+45 µl)
<i>E. fecalis</i>	12.00 ± 0.00	18.67 ± 1.15	18.33 ± 0.58	19.67 ± 0.47
<i>E. coli</i>	R ± 0.00	14.00 ± 1.73	13.00 ± 1.00	14.67 ± 0.47
<i>K. pneumoniae</i>	R ± 0.00	24.67 ± 4.04	24.33 ± 1.15	29.33 ± 0.94

Continued...

Clindamycin (Clinda) + Clove (CI)				
Organism	Clinda (2 µl)	CI (30µl)	Clinda +CI (2+30 µl)	Clinda +CI (1+45 µl)
<i>E. fecalis</i>	11.00 ± 0.00	15.67 ± 0.58	15.00 ± 0.00	16.67 ± 0.47
<i>E. coli</i>	R ± 0.00	14.33 ± 1.15	14.33 ± 3.06	15.67 ± 1.89
<i>K. pneumoniae</i>	R ± 0.00	15.67 ± 0.58	14.67 ± 1.53	17.33 ± 2.49

*Concentration of antibiotic stock-1 µg/ µl and concentration of spice extract-2 µg/ µl

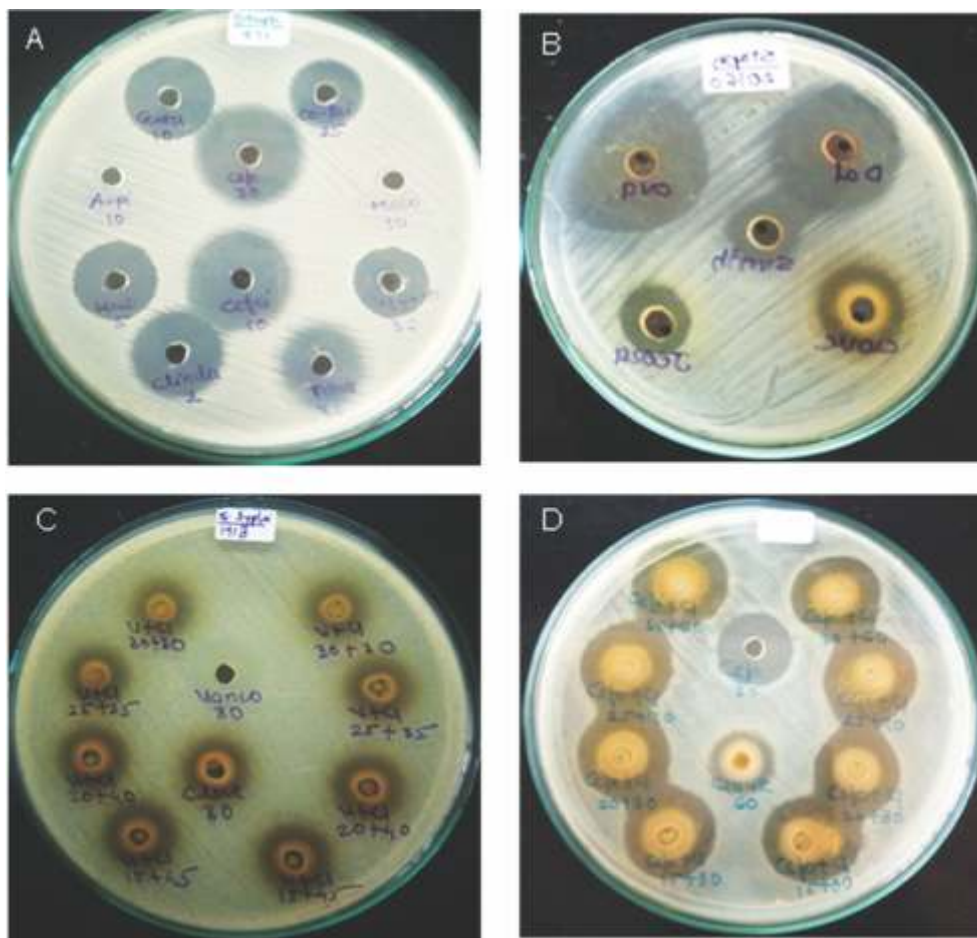


Fig. 1: Effect of Antibiotics and Spice Extract on Growth of *S. aureus*

- A) Screening with Antibiotics
- B) Screening with Spice Extracts
- C) Screening with Vancomycin + Clove Extract
- D) Screening with Cefotaxim + Clove Extract

FTIR Spectra of antibiotics and spices

All the three antibiotics Cefotaxime, Clindamycin and Vancomycin were studied for their FTIR spectra. The representative FTIR spectra are shown in the Fig. 2. The FTIR spectra of Vancomycin reveal the presence of nitroamines at 1496.66 cm^{-1} . An aromatic phosphorus compound was at 1226.64 cm^{-1} . Similar aromatic phosphorus compounds were also observed in Cefotaxime at 1242.07 cm^{-1} . All the three antibiotics showed the presence of benzene with allieids from 725 to 756 cm^{-1} regions of spectra and bromides from 586.32 cm^{-1} to 593.03 cm^{-1} . Further detail account of spectral analysis is included with flow of discussion.

The FTIR spectra for ethanolic extracts of *C. zeylanicum*, *T. ammi* and *S. aromaticum* were recorded. The *C. zeylanicum* extract showed predominance of compounds containing carboxylic group (C=C) at 1650.95 cm^{-1} and other molecules like aromatic compounds, aliphatic esters and sulphonamides at 1319.22 cm^{-1} and primary alcohols with aliphatic ether compounds at 1072.35 cm^{-1} . While *T. ammi* extract showed acid anhydride conjugate ring at 1743.53 cm^{-1} along with ketone and azo compounds (N=N) at 1620.29 cm^{-1} and alkane aromatic prime amines, aromatic ethers at 1226.64 cm^{-1} to 1373.22 cm^{-1} with sulphonil chloride, sulphoamide, benzene containing compounds at 725.18 cm^{-1} to 1157.21 cm^{-1} . The *S. aromaticum* extract showed vast array of aldehydes, urea, quinines, acidic amino acids from 1604.66 cm^{-1} to 1766.67 cm^{-1} . Presence of aromatic ether and aromatic phosphorus compounds at 1234.36 cm^{-1} and benzene containing alkanes, halides, bromides from 473.31 cm^{-1} to 910.34 cm^{-1} .

FTIR Spectra of antibiotics and spice extract after exposure to pathogens***Staphylococcus aureus***

S. aureus was found to be sensitive to Cefotaxime and slightly resistant towards Vancomycin as shown by the zone of inhibition (Table 1 and 2). As earlier discussed, Cefotaxime had sulfonamide and azide functional groups as evident from the band at 1303.79 cm^{-1} , similar groups were also observed in *T. ammi* extract at 1326.96 cm^{-1} . Such active functional groups were absent in Vancomycin. There was increased number of aromatic phosphorus compounds in *S. aromaticum* which were evident from bands at 1234.36 cm^{-1} , 1203.50 cm^{-1} and 1041.49 cm^{-1} . *C. zeylanicum* however did not have these bands. Additionally, *T. ammi* extract has a greater number of benzene containing compounds as revealed by bands at 848.62 cm^{-1} , 794.63 cm^{-1} and 725.13 cm^{-1} . In *S. aromaticum* benzene was also prominent and observed with bands at 856.26 cm^{-1} , 817.76 cm^{-1} , 786.90 cm^{-1} and 748 cm^{-1} .

Enterococcus faecalis

E. faecalis was found to be sensitive to Cefotaxime and resistance to Clindamycin (Table 1 and 2). Clindamycin after exposure to *E. faecalis* did not show the band at 1450.37 cm^{-1} which represents aromatic compound band. Further, another band at 1427.23 cm^{-1} , 1311.50 cm^{-1} , 1211.21 cm^{-1} and 1041.49 cm^{-1} representing S=O stretching (sulfonic acids) were also found to be absent after exposure to organism. In addition to bands found in Clindamycin, Cefotaxime had showed β -lactum band at 1627.81 cm^{-1} .

C. zeylanicum extract showed a band at 1404.08 cm^{-1} as found in Ketones. Further, bands were at 1650.95 cm^{-1} , 1450.37 cm^{-1} for C=C stretching as

in aromatic multiple bands. 1242.09 cm^{-1} and 1087 cm^{-1} were observed in *C. zeylanicum* extract which stands for oximes and quinones, sulfites, aromatic ethers and aliphatic ethers. *T. ammi* extract exhibited bands at 1666.38 cm^{-1} for C-N stretching as in oximes and a band at 1643.24 cm^{-1} for presence of azo compound. Further, it has unique N_2 add to stretching which is represented by a band at 1326.93 cm^{-1} showing azide like compound. Other bands at 1380.94 cm^{-1} , 1226.64 cm^{-1} , 1183.07 cm^{-1} and 1056.92 cm^{-1} stands for S=O stretching in sulfur containing compounds. A number of other phenolic compounds may be present in the extract as shown by bands at 1512.09 cm^{-1} , 879.48 cm^{-1} & 810.05 cm^{-1} . In *S. aromaticum*, azo and ketone representing bands were not observed. However, bands at 1195.78 cm^{-1} for P-O stretching were observed which represent aromatic phosphorus compounds. Further, sulfur containing compounds were observed as evident from bands at 1149.50 cm^{-1} and 1033.77 cm^{-1} . Aromatic benzene containing compound were also represented by bands at 910.34 cm^{-1} , 856.34 cm^{-1} , 817.76 cm^{-1} , 786.90 cm^{-1} , 748.33 cm^{-1} .

Escherichia coli

The structure of Cefotaxime even after exposing to *E. coli* remains intact along with its benzene ring and phosphorous group. Additionally, Cefotaxime showed presence of ketone and azo group at 1650.95 cm^{-1} . It was further observed that the spectra in the range of 1400 to 1200 in case of Cefotaxime showed presence of an alcoholic group at 1373.22 cm^{-1} and aromatic ether at 1218.93 cm^{-1} . While in region 1200 to 1000 showed presence of sulphonyl chlorides at 1172.64 cm^{-1} and benzene and aromatic like compounds at 1095.49 cm^{-1} and 1026.06 cm^{-1} . It also showed presence of benzene, halides and bromides from 1000 to 400 ranges.

However, in case of Clindamycin, all such groups were completely degraded in presence of *E. coli*.

When *E. coli* was exposed to ethanolic extracts of *C. zeylanicum*, *T. ammi* and *S. aromaticum* showed varied response. In presence of *C. zeylanicum* extract strong growth inhibition of *E. coli* was observed. *C. zeylanicum* extract had showed presence of sulphonyl chlorides at 1157.21 cm^{-1} and aliphatic ether 1080.06 cm^{-1} . The *T. ammi* extract showed presence of sulphonic acid, phosphorus compounds and sulfonyl chlorides at 1177.64 cm^{-1} to 1026.06 cm^{-1} and benzene halides and bromides from 1000 to 400. Similarly, *S. aromaticum* extract showed presence of sulphides, benzene, primary alcohols in the range of 1195.78 cm^{-1} to 1033.77 cm^{-1} . Sulfur containing compounds were seen at 1157.21 cm^{-1} , after exposure of *E. coli* to *T. ammi* extract. It was found that band at 1026.06 cm^{-1} representing phosphorus compounds. Benzene ring containing compounds were also visible as found with the band at 879.48 cm^{-1} and 794.62 cm^{-1} . Further, oximes, quinones and nitroamines were present at 1650.95 cm^{-1} , 1635.52 cm^{-1} , 1458.08 cm^{-1} respectively. Sulfur containing compounds were also present along with phosphorus compounds at 1172.64 cm^{-1} and 1026.06 cm^{-1} respectively.

S. aromaticum extract was found to be active against *E. coli* as it had phosphorus compound represented by a band at 1195.78 cm^{-1} and 1033.77 cm^{-1} , and benzene ring represented by bands at 794.62 cm^{-1} , 748.33 cm^{-1} . Additionally, oximes and quinones are presented by a band at 1650.95 cm^{-1} . Sulfur containing compounds were also observed as showed by bands at 1319.22 cm^{-1} , 1149.50 cm^{-1} . Nitroamines and aromatic nitro compounds were evident by bands at 1458.09 cm^{-1} and 1319.22 cm^{-1} .

Klebsiella pneumoniae

K. pneumoniae was sensitive to Cefotaxime and

resistance to Clindamycin. Cefotaxime showed greater number of sulfur containing compounds and azides as revealed by the band at 1311.50 cm^{-1} and 1234.36 cm^{-1} . Whereas, active groups evident in Cefotaxime were absent in Clindamycin. As discussed earlier, spice extracts showed quinones, oxime, C=N stretching like groups. Moreover, *S. aromaticum* and *C. zeylanicum* revealed aromatic

amines as apparent from bands at 1265.22 cm^{-1} and 1249.79 cm^{-1} . In *T. ammi* extract bands representing sulfur and azide groups were dominant at 1311.50 cm^{-1} and 1234.36 cm^{-1} . The *S. aromaticum*, *T. ammi* and *C. zeylanicum* commonly showed aliphatic ether like compounds as were observed by the presence of bands at a common band at 1080.06 cm^{-1} .

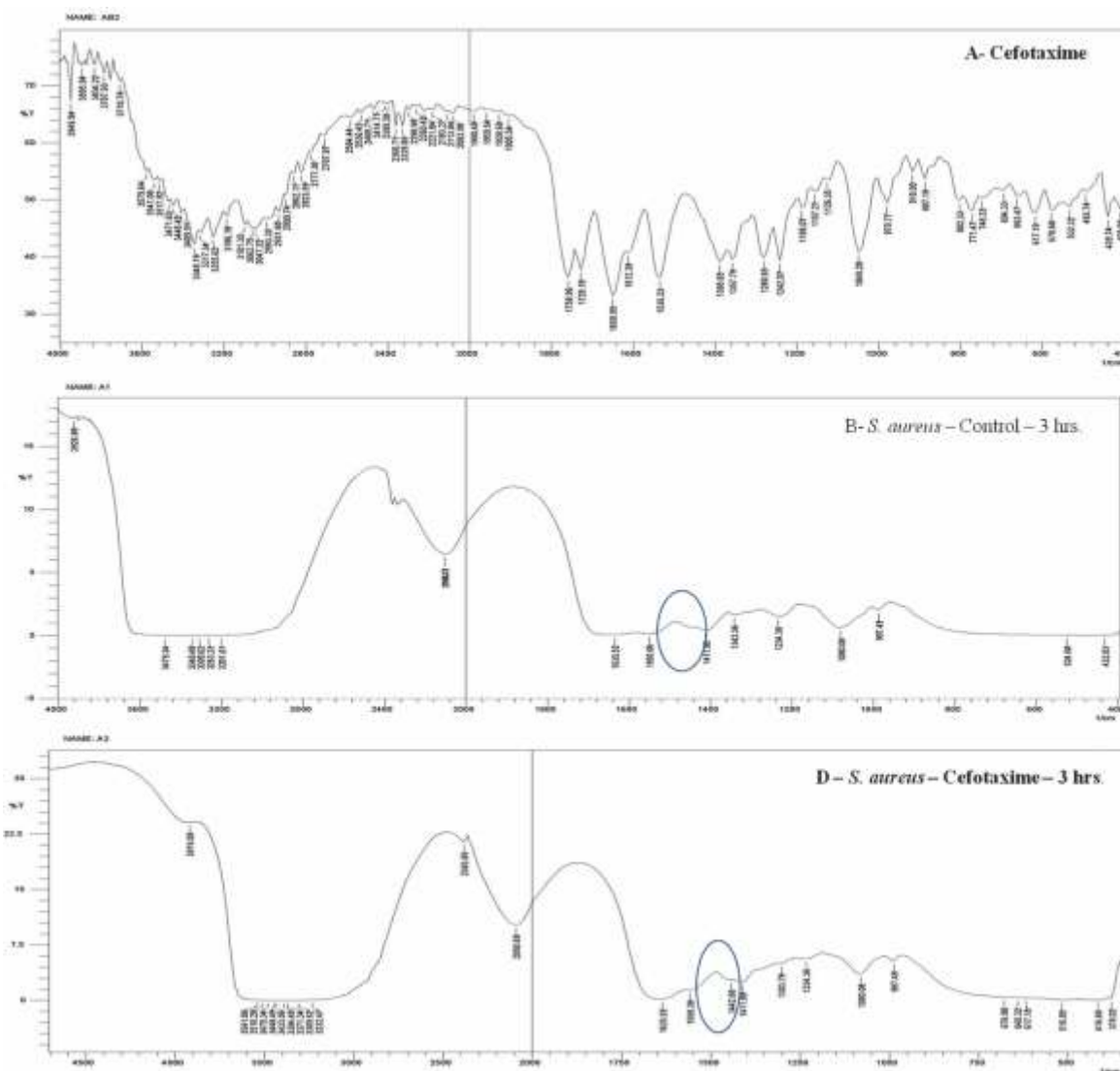


Fig. 2: FTIR Spectrum of *S. aureus* - A) Cefotaxime, B) Control- *S. aureus*, C) *S. aureus* with Cefotaxime

“○” shows structural changes due to bacterial action of the molecule

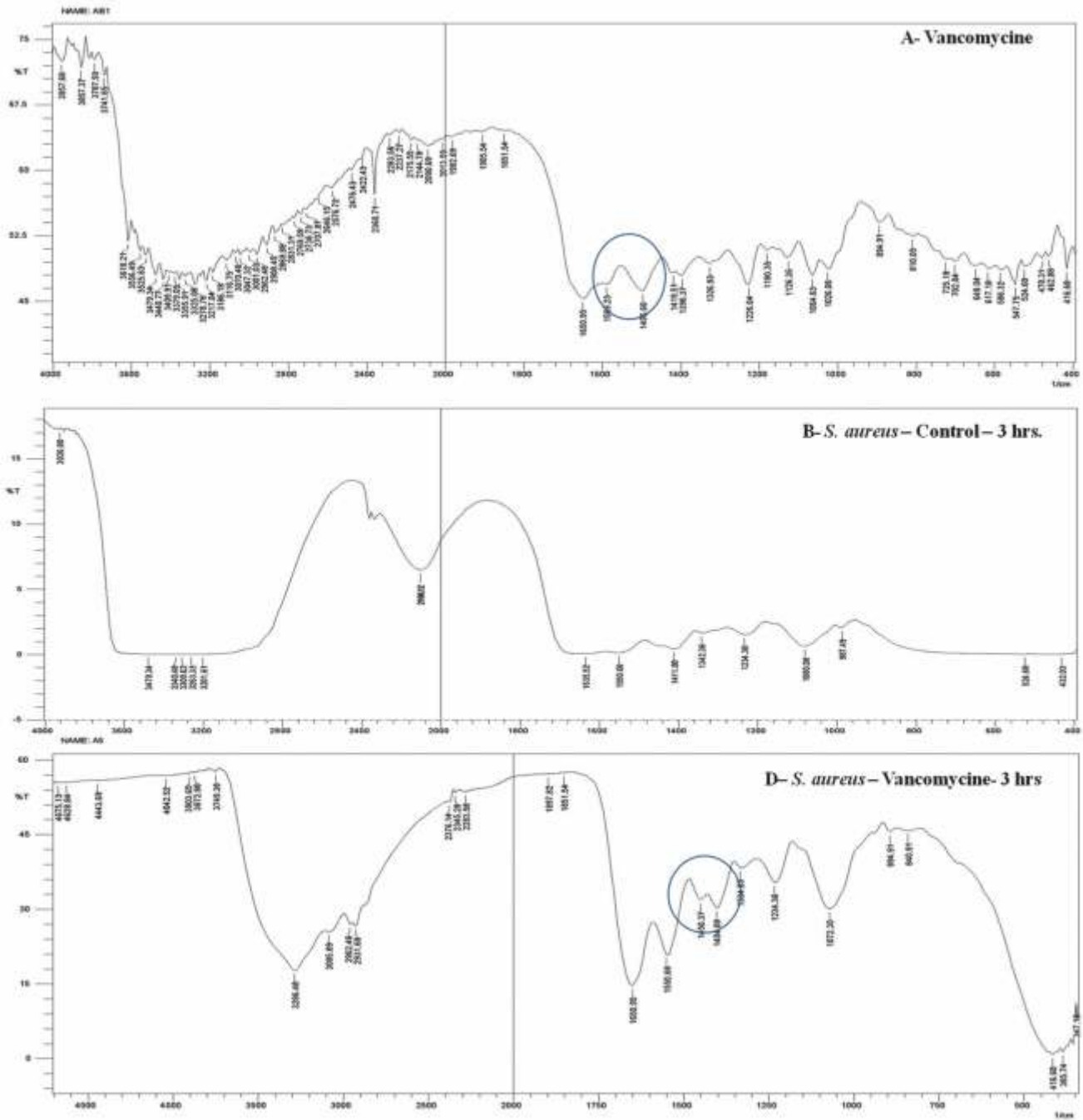


Fig.3: FTIR Spectrum of *S. aureus* -A) Vancomycin, B) Control- *S. aureus*, C) *S. aureus* with Vancomycin

“○” shows structural changes due to bacterial action of the molecule

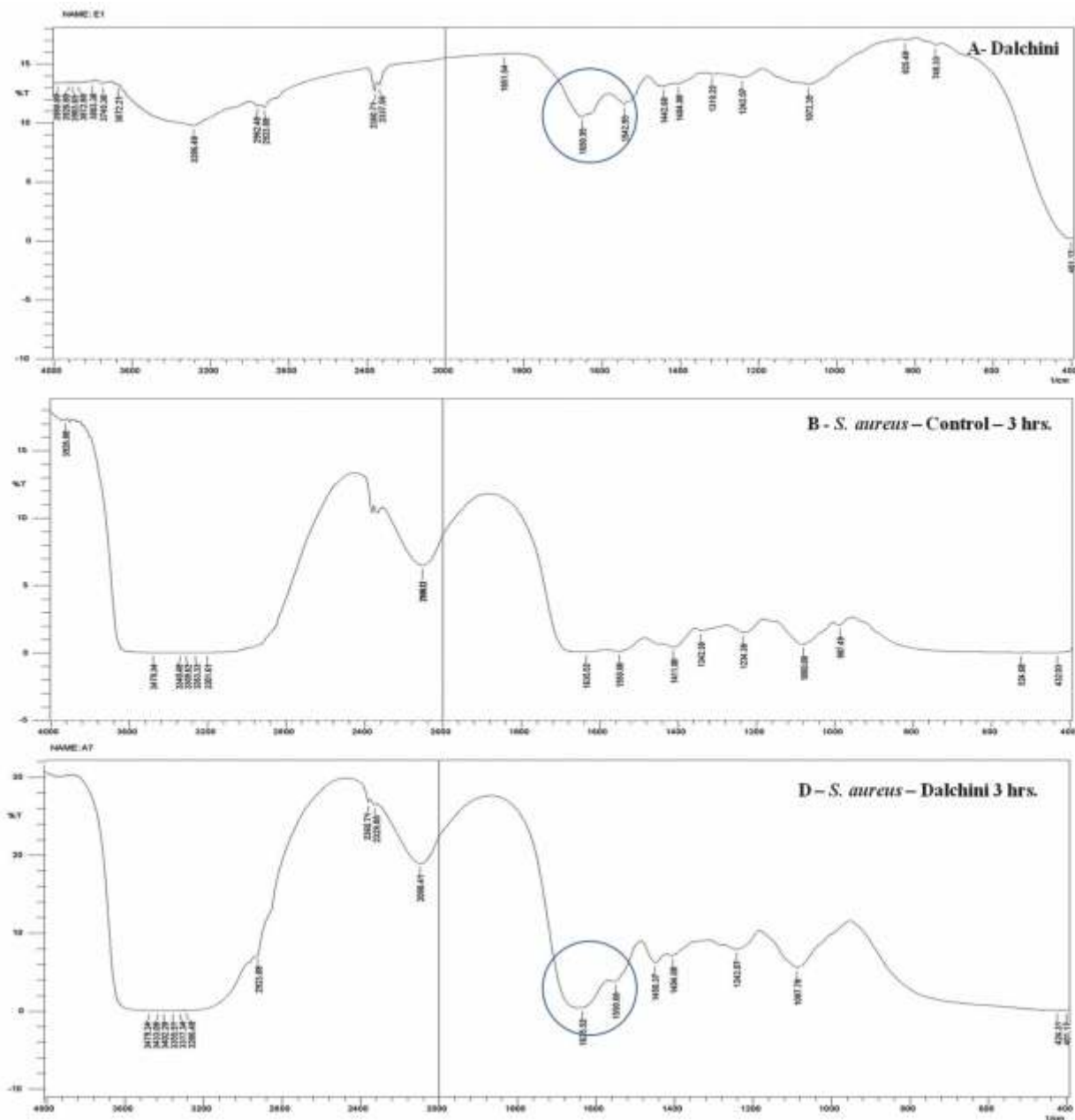


Fig. 4: FTIR Spectrum of *S. aureus* - A) *Cinnamomum zeylanicum* (Dalchini), B) Control-*S. aureus*, C) *S. aureus* with *Cinnamomum zeylanicum* (Dalchini)

“○” shows structural changes due to bacterial action of the molecule

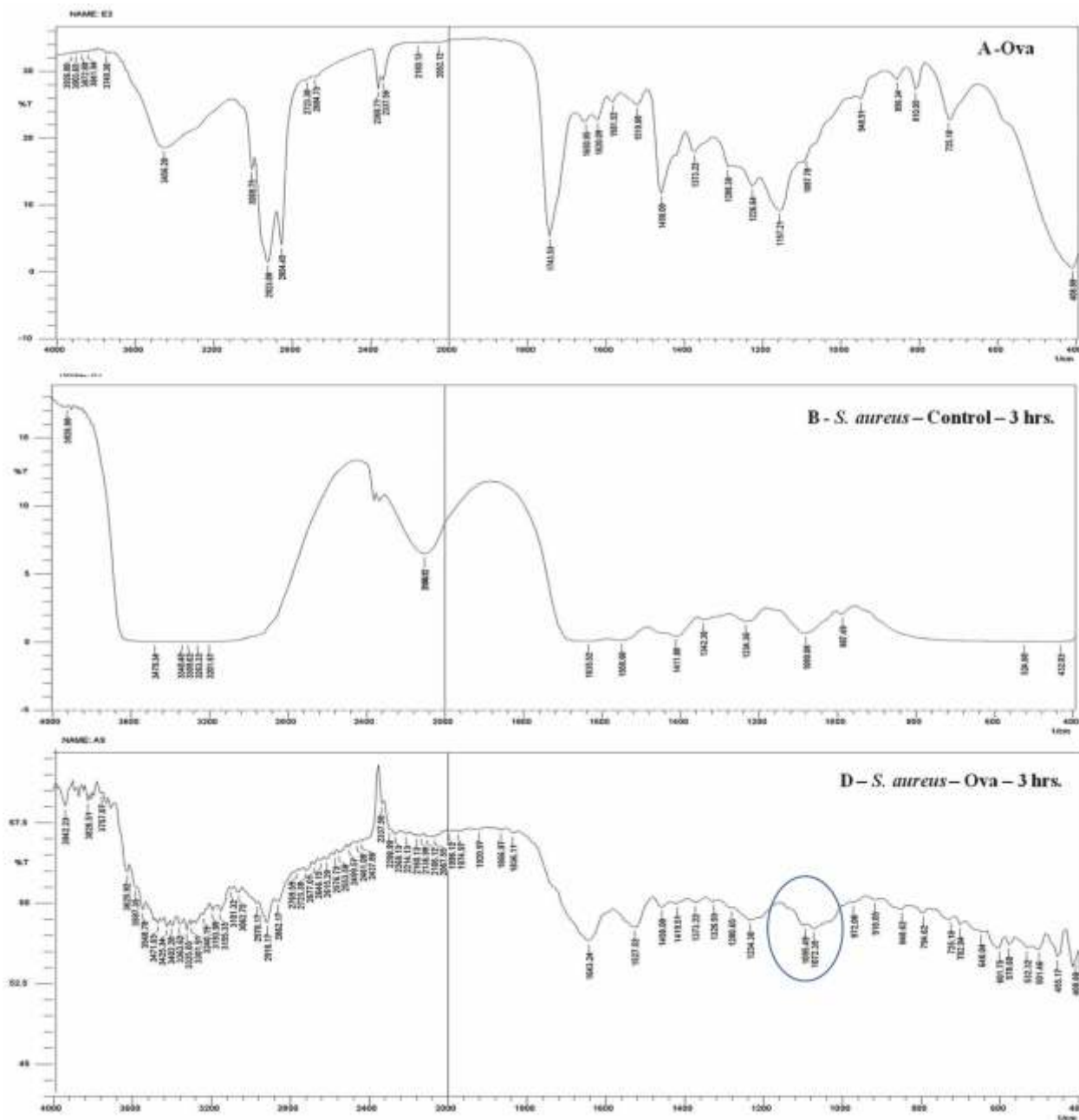


Fig. 5: FTIR Spectrum of *S. aureus* – A) *Trachysparnum ammi* (Ova), B) Control-*S. aureus*, C) *S. aureus* with *Trachysparnum ammi* (Ova)

“○” shows structural changes due to bacterial action of the molecule

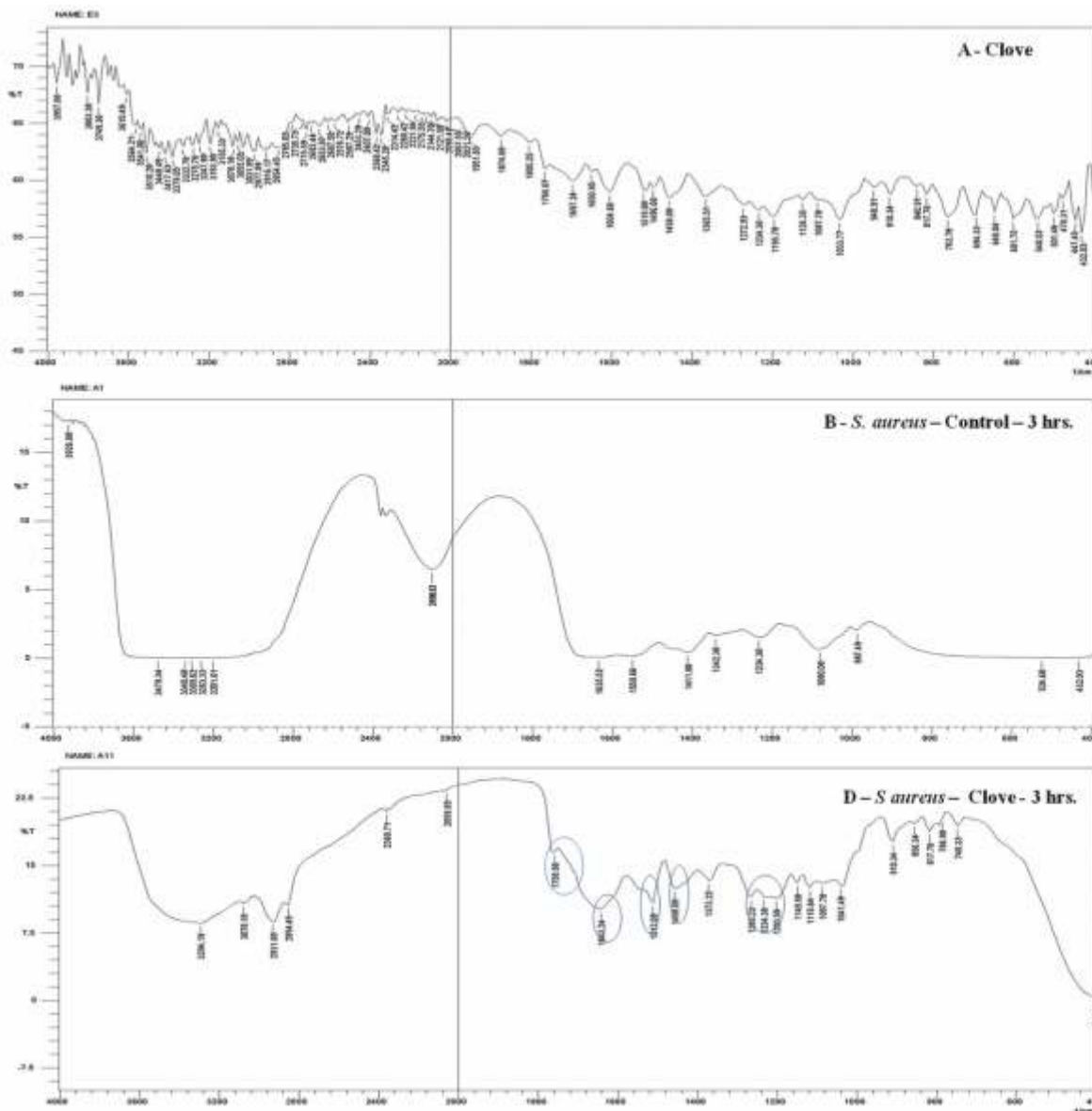


Fig. 6: FTIR Spectrum of *S. aureus* A) *Syzygium aromaticum* (Clove), B) Control-*S. aureus*, C) *S. aureus* with *Syzygium aromaticum* (Clove)

“○” shows structural changes due to bacterial action of the molecule

Discussion:**Antibacterial activity**

Initially, screening of antibacterial activity was performed using ten different antibiotics and ten widely used Indian spices. Among ten antibiotics, one sensitive and one resistant antibiotic was taken for further study. Except *S. aureus* which showed resistance with minimum zone of inhibition to Vancomycin, all the other pathogens were resistant to Clindamycin. All the pathogens were sensitive (giving highest zone of inhibition) to Cefotaxime. Among ten different Indian spices, *C. zeylanicum*, *T. ammi* and *S. aromaticum* proved better with respect to bacterial growth inhibition (Fig 1). Further, potency matching of selected standard antibiotics with different concentrations of spice extract was performed and 30 µl of *C. zeylanicum*, 30 µl of *T. ammi* and 60 µl of Clove extract showed promising results when compared with antibiotics under study. On the basis of this preliminary data, we further studied combinatorial effect of selected antibiotics and spices and FTIR spectra for the same were determined. Moreover, the study was aimed to minimize the concentration of antibiotic with the increase of natural spice extract. The combinatorial effect of selected antibiotics and spices and their FTIR spectra were considered. *S. aureus* showed increased susceptibility for the combination of Vancomycin and spice extract. The highest zone of inhibition was obtained for *T. ammi* extract which was used 90 mg synergistically with 15 µg Vancomycin. Other three bacterial pathogens were tested using Clindamycin as a resistant antibiotic. The lowest concentration of Clindamycin combined with spice extract was 1 µg. It is notable that, though Clindamycin alone was ineffective against *E. coli*

and *K. pneumoniae*, its combination with spice extract showed enhanced inhibition activity. As discussed earlier, these antibiotics show bacteriostatic or bactericidal activity either through altering cell membrane permeability, inhibiting cell wall synthesis and/or protein synthesis inhibition at the level of 50S ribosome. Enhanced antibacterial activity of antibiotics synergist with spice extract strongly suggest that secondary metabolites present in extract have direct bactericidal properties or it enhance the efficiency of present antibiotics. Efficiency enhancing properties could be attributed to certain functional group modifying compounds in spice extract. Further exploration of the mechanism is and further studies at the molecular level are required. Notably, all the pathogens were susceptible to the decreased concentration of standard antibiotics with increased concentration of respective spices extract. The highest cost of synthetic antibiotics and their further harmful effects on health are the major concern. Hence, minimizing antibiotic concentration with natural plant extract might be beneficial for future drug development.

FTIR spectra of antibiotic and spices

FTIR technique measures the adsorption of infrared radiation by the sample versus wavelength. Particular band refers to the compounds present in the sample. The FTIR spectrum has to be measured from 0 path difference to a maximum length that depends on the resolution required. FTIR technique has been successfully employed for phytochemical analysis from plant extract such as medicinal plants such as *Phyllanthus amarus*, *Senna auriculata*,

Phyllanthus maderaspatensis and *Solanum torvum* [32]. In the present research attempt, FTIR has provided molecular evidence in structural changes during the antimicrobial action of antibiotics and spices which is discussed herewith. The bromides and benzene group found in antibiotics may be conferring the major antimicrobial activity to the compounds. Interestingly, *S. aromaticum* extract showed the presence of bromides in the same range and benzene compounds along with sulfur compounds, and aromatic ether. *C. zeylanicum* extract exhibited majority of compounds comprising carboxylic group, aromatic compounds, aliphatic esters, primary alcohols with aliphatic ether compounds. While *T. ammi* extract reveals the presence of sulfonic amides and aliphatic ether compounds along with benzene and halides. All these functional groups are predominantly found in plant phenolics, flavonoid, tannins, proanthocyanidins, glycosides etc. which are known for antimicrobial properties.

FTIR after exposure to pathogens

The overall response of *S. aureus* was in the range of 1800 to 1600 represents organism's antibiotic resistance through over expression of molecules responsible for membrane stability. Further under any stress condition, the reactive oxygen species generated causes cell death by oxidizing biomolecules viz proteins, nucleic acids and lipids [33].

E. fecalis was found resistant to Clindamycin and sensitive to Cefotaxime. Clindamycin, after exposure to *E. fecalis* showed certain changes in their functional groups. Aromatic compound band, S=O (sulfonic acid) stretching was absent which could be attributed to enzymatic action by organism to lessen the toxicity of Clindamycin

resulting in resistance. The Clindamycin band at 1380.94 cm^{-1} which represents CH_3 deformation was not detected when the organism was exposed. The β -lactam band present in Cefotaxime might be responsible for antibiosis against *E. fecalis*. Different compounds present in *C. zeylanicum* extract were ketone, aromatic, oximes, quinine's, sulfites, aliphatic, and aromatic ethers which could be active against microorganism. Similarly, *T. ammi* extract presented antibiotic like properties might be contributed by metabolites with oximes, azo, azide, sulfur and phenolic functional groups. There was slightly lowered antibiotic activity in *S. aromaticum* as azo and ketone representing bands were not observed. However, it had aromatic phosphorus, sulfur containing compounds and benzene presenting a mixture desire to have antibiotic properties.

FTIR spectra were recorded for *E. coli* before and after exposure to Cefotaxime and Clindamycin. The active functional groups like benzene ketone, azo group and phosphorus group present in Cefotaxime remains intact which confirms susceptibility of *E. coli* towards Cefotaxime. Clindamycin was ineffective against *E. coli* which was confirmed by degradation of functional group including aliphatic ether present in it. Strong growth inhibition in presence of *C. zeylanicum* extract may be attributed to sulphonyl chlorides and aliphatic ether group. *T. ammi* extract had some additional functional groups such as sulphonic acid, phosphorus compounds, benzene halides, sulphides, benzene, primary alcohols and bromides which were absent in antibiotics. From these spectra it is evident that the natural complex compounds when compared with pure antibiotics showed presence of additional molecules re-

ling bactericidal properties. Sulfur containing compounds in *T. ammi* extract after exposure to *E. coli* were seen suggesting some molecular alteration in cellular structure with metabolites present in the extract has taken place. *S. aromaticum* extract exhibited strong antibacterial activity against *E. coli* might be because of phosphorus compound, oximes and quinones, sulfur containing compounds, nitroamines and aromatic nitro compounds.

K. pneumoniae was observed to be completely resistant to Clindamycin and sensitive towards Cefotaxime. Several important functional groups were absent in Clindamycin suggesting possible degradation by microorganism. The *C. zeylanicum*, *S. aromaticum*, and *T. ammi* had promising bactericidal activity might be because of the presence of aromatic amine, aliphatic ether, quinone, oximes, and azide groups etc.

In presence of antibiotic or in presence of natural spice extracts, all the bacterial species under study showed common molecules in the range of 1800 to 1600 were observed containing oximes, quinines and amino acid with NH_2 group. Interestingly the proportion of these molecules reduced drastically in presence of *C. zeylanicum*, *T. ammi*, and *S. aromaticum* extracts. The molecules in this range may be responsible for membrane stability of the organisms conferring resistance to antibiotic and susceptibility to natural compounds like spices [33]. Menaquinones and other isoprenoid quinines are components of the cytoplasmic membrane of Gram positive and Gram negative organisms [34]. Their role within the cytoplasmic membrane includes acting as an obligatory component of electrons transport chain promoting endospore formation and participating in active transport. The

instability of menaquinon in presence of antibiotics or natural compounds leads to a decrease in oxygen consumption and disruption of electron transport within the bacterial membrane [35]. There were some of common function groups such as oximes, quinones and nitroamines in spice extract and sensitive antibiotics Cefotaxime signifying their particular role in toxicity against pathogens. Notably, alteration in functional groups was found in case of resistance antibiotics when exposed to microorganism.

In our earlier study [11] we proved enhanced activities of antioxidant enzymes like catalase, superoxide dismutase, manganese peroxidase, and laccase particularly in the multidrug resistance bacterial strains when exposed to antibiotics. Present FTIR studies provide evidences about how pathogens are gaining resistance by either secreting membrane stability components or through the initiation of antibiotic degradation pathways.

Conclusion:

In the current research attempt, human pathogens showed resistance to antibiotics such as Vancomycin (in case of *S. aureus* only) and Clindamycin while sensitivity for Cefotaxime. *C. zeylanicum*, *S. aromaticum* and *T. ammi* extracts presented considerable antimicrobial activity. FTIR spectral data of spices, synthetic antibiotics and pathogenic microorganism showed different active chemical moieties signifying their possible functional role in vulnerability or resistance. The microorganism susceptible to spice extract or antibiotic showed a change in spectra after exposure which directly relates to altered membrane integrity as well as internal metabolic and enzymatic activities. Further, FTIR analysis of

spices proved numerous metabolic entities present in it which might have beneficial effects against multidrug resistance strains which could be future promising drug strategy. Hence, the spectral analysis has provided simple, fast, cost effective and precise tool for analyzing antibacterial

activity of natural as well as synthetic drugs. The present study suggests future scope for novel drug development using a combination of antibiotics with purified active compounds of spices under study.

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