RESEARCH ARTICLE

# INHIBITORY MECHANISM OF N-HEXANE AND DICHLOROMETHANE LEAF EXTRACTS OF *CLERODENDRUM PHLOMIDIS* LINN. ON FOOD BORN PATHOGEN *BACILLUS LICHENIFORMIS*

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**Abstract:** Bacillus related microbes are mainly found in the severe cases of foodborne infection and many other lethal diseases. Few herbal medicines are effective against *Bacillus licheniformis*, but their mechanism of action is not reported. The antimicrobial potential of herbal extract from *Clerodendrum phlomidis* (n-hexane and dichloromethane extract) was assessed by well diffusion assay which showed a zone of inhibition of 19 and 15.5 mm respectively. Underlying mechanism of action behind the n-hexane and dichloromethane extract of *C. phlomidis* is reported by an *in vitro* study over Bacillus licheniformis through FACS, SEM, and DNA fragmentation analysis. Fluorescent activated cell sorting was done to determine membrane potential disruption caused by extracts that revealed dichloromethane has more activity for membrane potential disruption. The SEM analysis of bacteria showed irregular structure in cell wall, blebbing and leakage of cellular contents and disruption of membrane. DNA fragmentation analysis showed a precise smear formation of bacterial cellular DNA providing evidence of its apoptosis. The FTIR spectroscopic analysis of herbal extract was done to determine the dominance of functional groups present within purified antimicrobial extracts and which had shown the dominance of Alcoholic (OH-) group, halogen groups(C-X), Aldehydic (CHO-) groups within them. The antibacterial action is mainly contributed by disruption of membrane potential, degradation of bacterial genomic DNA, damage of bacterial cell membrane and leakage of cellular content. The result provides a significant contribution towards understanding the antibacterial mechanism of n-hexane and dichloromethane extract of *C. phlomidis*.

**Keywords:** *Clerodendrum phlomidis, Bacillus licheniformis,* Herbal extract, FACS, SEM, DNA fragmentation

## INTRODUCTION

Foodborne pathogenic bacteria are an alarming challenge in both developed and developing nations (Lu *et al.* 2012). The overuse of synthetic antimicrobials regarding control of foodborne infection leads to development of resistant strain and severe side effects. To overcome such problems related to foodborne diseases people are tending towards the use of natural antimicrobials (Yang *et al.* 2013).

Many natural herbal phytochemicals such as 8-hydroxyquinoline a common phytochemical isolated from *Sebastianiacorniculata* has antimicrobial property against foodborne bacteria (Yang *et al.* 2013). Apart from 8-hydroxyquinoline many other naturally derived phytochemicals such as 6-(4, 7-dihydroxy-heptyl) Quinone's derived from *Pergulariadaemia* is effective against some food causing bacteria such as *Bacillus subtilis, Staphylococcus aureus,* and *Escherichia coli* (Ignacimuthu *et al.* 2009). Besides these herbal phytochemicals many essential oils from *Cinnamomum cassia* bark also found active against food pathogenic bacteria such as *Staphylococcus aureus,Bacillus subtilis, Salmonella typhimurium* and *E.coli*(Huang *et al.* 2014). *Bacillus licheniformis* which is also found in many severe clinical cases such as Septicemia, Peritonitis, Ophthalmitis, and food poisoning in humans, as well as with bovine toxemia and abortions(Johnson *et al.* 1994). *B. licheniformis* is a common contaminant of dairy products and Bakery products (Frank *et al.* 1997, Salonen *et al.* 1999, Pepe *et al.* 2003).

*C.phlomidis* Linn. is cultivated and grown in arid area for its medicinal property fall under the family Verbenaceae. In various vernacular languages it is known as Glory Bower, Arni and Aginmentha. Today this is one of the most highly traded medicinal plants in the tropical forest due to its various medicinal properties. The popular medicinal property of *C.phlomidis* Linn. includes its anti-inflammatory action(Pandey *et al.* 2005), anti-diabetic(Dhanabal *et al.* 2008) and in treatment of nervous disorders (Khare 2007). Apart from this it is also used for the veterinary and agricultural application for treating the problems related to skin infection, hypothermia of cattle's (Keshabhai 1992, Vaidyar 1997) and controlling the insect pests like aphids in agricultural fields (Bharvad 2005).

## MATERIALS AND METHODS

### Collection of plant material and Extraction

The whole plant of *C.phlomidis* Linn. was collected from the bank of Phaphamau, Allahabad, India. The

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collected plant material was pressed underweight in paper and dried well for herbarium preparation. Plant was taxonomically characterized by Botanical Survey of India, Central Circle, Allahabad. Dry leaves of plants were grounded into fine powder and 15 gm of it was thimble in soxhlet apparatus for the extraction using various polar and non-polar solvents such as n-hexane, dichloromethane, methanol and milli-Q water. After extraction the content in solvent was filtered using Whatman filter paper no. 42. Herbal phytochemicals was isolated in crude form using rotary evaporator.

**Bacterial isolation and growth**

The bacterial sample was isolated from cheese water sample and streaked on Luria Bertani Agar petri plate and incubated overnight at 37ºC for growth. After bacterial growth on LB agar it again grown in Luria Bertani Broth for the serial dilution of bacteria aimed for CFU measurement. The colony forming unit of bacteria was also measured using 10-6 serial dilution of bacteria. The bacteria grown on LB agar plate were streaked on autoclaved test tubes.

**Antibacterial assay**

**Well diffusion assay**

The agar well diffusion method was employed to determine the antimicrobial activity of plant extract. The fresh overnight cultured bacterial inoculum (100 µL) was spread on sterile petri dish of LB agar. Wells were made after drying the petri dishes and 300 µL of 5% DMSO extract solution of n-hexane and dichloromethane extracts having concentration 20 mg/ml was poured in them and kept it overnight in incubator at 37 ºC and 5% DMSO were used as control. Antimicrobial activity of isolated extract was evaluated by measuring the diameter zone of inhibition on well diffusion agar plates (Valgas et al 2007).

**MIC determination**

The minimal inhibitory concentration (MIC) of n-hexane and dichloromethane extract of *C.phlomidis* was determined as per Clinical and Laboratory Standards Institute-(CLSI) recommended broth micro dilution assay with some modification. Gradual dilution of plantextract was prepared with LB broth at a total volume of 100 µL in 96 well plates. The final concentration of *C.phlomidis* n-hexane and dichloromethane extract was madein between 0.01 to 2 mg/mL. The microtiter plates were inoculated with 100 µL of n-hexane and dichloromethane extract and then incubated at 37 °C for an overnight. After overnight incubation the absorbance was measured at 600 nm using the microtiter plate reader to access the cell growth. Thebacterial cell with LB broth was taken as negative control and LB broth with extract without cell suspension as positive control. The MIC of the plant extract was determined by lowest concentration of the plant extract that inhibit the growth as compared to the negative control. All the experiment was performed in triplicate and their average value was taken as MIC.

**Investigation of bacterial cell membrane potential by Flow Cytometery**

Bacterial cell having an intact cytoplasmic membrane, there exist a membrane potential across the membrane having negative charge inside the membrane and positive charge on the bacterial surface membrane. The membrane potential of *Bacillus licheniformis* through the exposure of n-hexane and dichloromethane extract *of C.phlomidis* was determined by Flow cytometry. The effect of plant extract over the bacterial membrane potential was done using the propidium iodide (PI) dye with few modifications (Shi *et al*. 2007). Bacterial cells were grown overnight at 37 ºC and treated with plant extract and again incubated 72 hours at 37 ºC. Cells were then harvested, washed twice with cold Phosphate Buffer Saline (PBS). After washing with PBS twice, cells were then dissolved in PBS and then stored at –20 ºC for few minutes before staining and analysis. Fixed cells were then stained with 10µL/mL propidium iodide and then incubated for 10 minutes in dark. Fluorescence intensity was measured with the help of flow cytometry (FAC Scan, Becton Dickinson). With a subsequent decrease in fluorescence intensity gives the measure of altered membrane potential.

**DNA fragmentation analysis**

The DNA fragmentation analysis is considered as an evidence for the apoptosis. The fragmentation analysis of *Bacillus licheniformis* genomic DNA was carried out after 72 hour incubation with plant extract. The DNA of treated and untreated bacterial cells was isolated by Phenol: Chloroform: Isoamyl (25:24:1) and precipitated with equal volume of isopropanol. The DNA sample after isolating from bacterial cells was allowed for electrophoresis on 1% agarose gel for 2 hour at 65 volt. Finally, the apoptotic DNA fragments were visualized using Gel Documentation system (De *et al.* 1994).

**Scanning Electron Microscopic study**

Scanning electron microscopic study was done to examine the morphological changes in the surface structure of bacterial cells after treating 5% DMSO stock of n-hexane and dichloromethane extract of *C.phlomidis* extract for both treated and untreated bacteria (as control) respectively. Bacteria was grown overnight at 37ºCand at constant agitation (80 rpm). After getting complete growth of bacterial cells, they were treated with plant extract and kept inside the incubator for 24 hours at constant temperature and agitation. After 24 hours incubation, bacterial cells were centrifuged at 8000 rpm for 15 minutes to get pellet of bacterial cells. Bacterial pellets were washed (2 times) with cold phosphate buffer saline (PBS) at above condition. These washed bacterial pellets were fixed with gluteraldehyde (2.5%) for 2 hours and then centrifuged at 8000 rpm for 15 minutes. After

centrifugation cells were fixed with mercuric chloride ($HgCl_2$) for 30 minutes. Fixing with $HgCl_2$ for 30 min again centrifuged at 8000 rpm for 15 min and samples were dehydrated in 10, 20, 30, 40, 50, 70,80, 90% and absolute grade ethanol solution with 2 wash at each time. Samples were then dried in an incubator at 55ºC for 4 days and coated with Gold and Pallidum (80:20). The morphological change in bacterial surface was observed with the aid of scanning electron microscope (Teanpaisan *et al.* 2014).

**Purification of compound for antimicrobial activity and FTIR analysis**

The separation of organic compound from the extract of dichloromethane was done by usingthe column chromatography. The dichloromethane extract was purified through silica gel chromatography on the basis of molecular weight. The extract of dichloromethane was dissolved in the diethyl ether and butanol in the ratio of 3:2 and 15 active antimicrobial fractions were collected. Collected antimicrobial fractions were tested for antibacterial activity through well diffusion assay. The isolated antimicrobial compound were first dissolved in potassium bromide solution and then further processed for the FTIR analysis. The sample were scanned from 400 to 4000 cm-1 and data obtained after the scanning of antimicrobial compound were analyzed and graph was made by using Spekwin software (Al-Bayati 2009).

## RESULTS AND DISCUSSION

**Antimicrobial activity of n-hexane and dichloromethane extract of *C.phlomidis***

The n –hexane and dichloromethane extract of *C.phlomidis* was evaluated for the antimicrobial activity against common food poisoning bacteria Bacillus *licheniformis* by an agar well diffusion assay. The *Bacillus licheniformis* were found susceptible against n hexane and dichloromethane extract of *C.phlomidis*. The solvent 5% DMSO (positive control) and n-hexane and dichloromethane (negative control) both didn't showed antimicrobial activity on them. The zone of inhibition of n- hexane and dichloromethane extract of *C.phlomidis* was found to be 19 mm and 15.5 mm respectively. Various researchers have also reported that ethanolic extract has also quite effective antimicrobial activity against *Pseudomonas aeruginosa*(Sathish *et al.* 2013). The potential of 50 antimicrobial activity of crude extract of *C.phlomidis* was mainly due to the presence of phenolic and flavonoid content. The various isolated compounds from ethanolic extract through column chromatography such as ethyl- 2-hydroxyl -4- methyl benzoate also has very excellent antimicrobial activity. The potential of flavonoid content of *C.phlomidis* is reported by over various gram positive and gram negative bacterial strains (Kaur &Renu, 2012). Moreover they also reported that flavonoid content is also effective on fungus such as *Aspergillus niger, Aspergillus flavus, Candida albicans* and *Candida glabrata.*

**Minimal inhibitory concentration**

The MIC of the plant extract was determined by lowest concentration that inhibits the growth in comparison with the negative control. The MIC value was found to be 0.4 and 0.6 mg/mLrespectively for n-hexane and dichloromethane extracts of *C.phlomidis.*

**Flow cytometry analysis of bacterial cells membranepotential**

It revealed that mean fluorescence intensity of n-hexane and dichloromethane is lower as compared to the control. Treatment of n-hexane and dichloromethane extract of *C.phlomidis* resulted in decrease in mean fluorescence intensity that gives significant evidence for reduction of membrane potential and hence suggesting the mechanism of antibacterial activity involves disruption of membrane potential. Among the n-hexane and dichloromethane extract of *C.phlomidis* tested over *Bacillus licheniformis* dichloromethane extract was found more potent in disrupting the membrane potential as shown in fig. 1 (a and b).

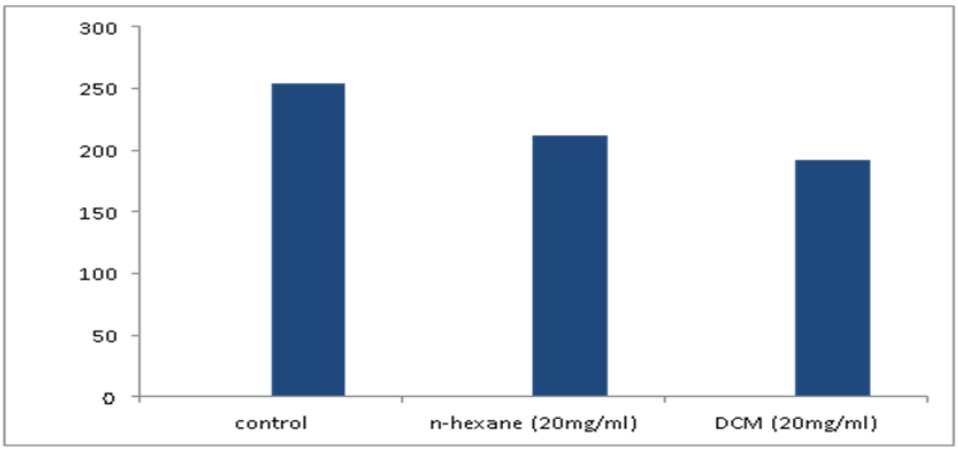

**Fig. 1(a).** The membrane potential disruption by n-hexane and DCM extract: *Bacillus licheniformis* treated with 20 mg/ml of n –hexane and dichloromethane extract of *C. phlomidis*extract. Bar graph shows membrane potential of *Bacillus licheniformis* without treatment (Control) or with treatment (n-hexane and DCM extract).

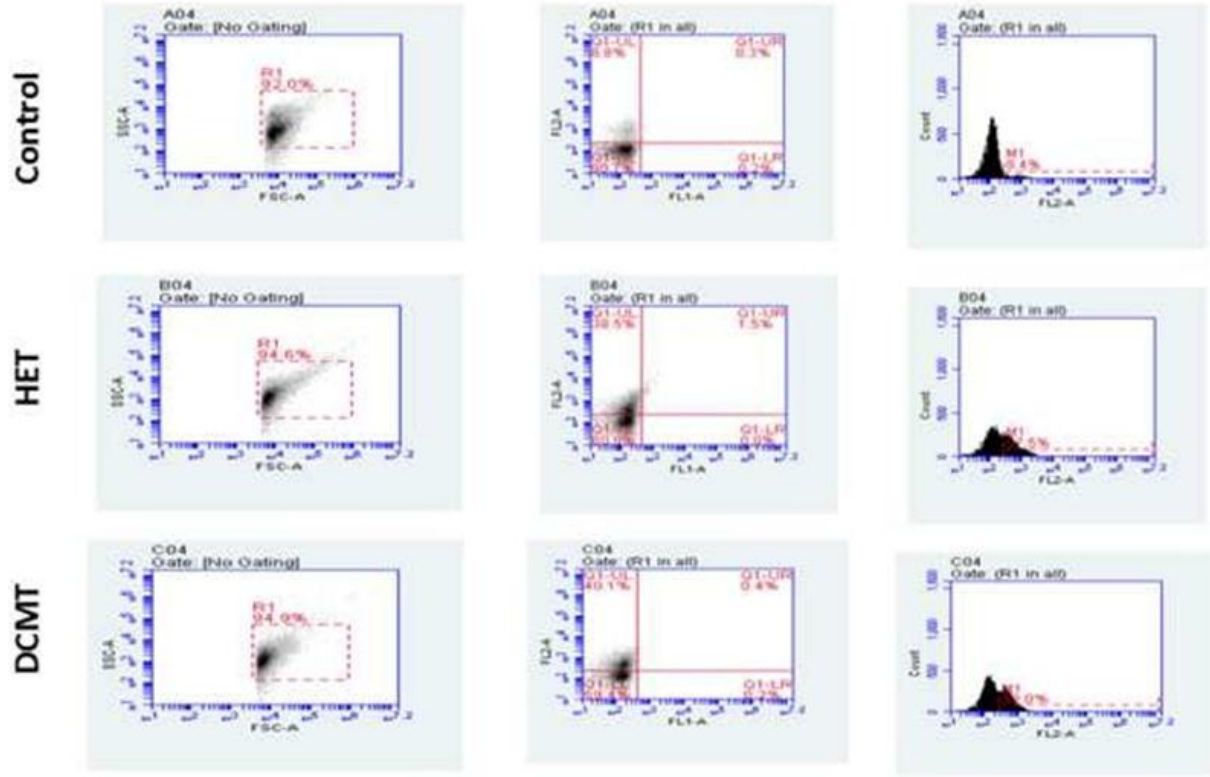


**Fig. 1(b).** Membrane potential disruption by plant extract: *Bacillus licheniformis* treated with 20mg/ml of n-hexane and dichloromethane extract for 72 hours.

Here in this study we observed that n-hexane and dichloromethane extract cause the disruption of membrane integrity of *Bacillus licheniformis* which subsequently lead to disruption of membrane potential. Various studies have reported membrane potential disrupting capability of some medicinally important plants other than bacterial cell. For example, *Gracilariatenuistipitata* methanolic extract had shown reduction of mitochondrial membrane potential of Ca9-22 cancer treated cells (Yeh *et al.* 2012). The ethanolic extract of *Annona muricata* had shown its capability for disruption of mitochondrial membrane potential for the induction of apoptosis in HL-60 cells (Pieme *et al.* 2014). Furthermore, Bupleurum Chinese ethanolic root extract had shown its apoptotic effect on HO-8910 ovarian cancer cells by disruption of mitochondrial membrane potential (Gu *et al.* 2015).

**DNA fragmentation assay**

DNA fragmentation is considered as an evidence of apoptosis. DNA fragmentation assay was carried out in *Bacillus licheniformis* cells after treating them with n–hexane and dichloromethane extract of *C.phlomidis*after 72 hours treatment. No any DNA fragmentation observed in 24 hours of treatment and concluded that it initiated after 48 hours. The outcome of experiment showed shearing of bacterial genomic DNA in treated cells as compared to the control as shown in fig. 2(a and b). Several other studies have shown that herbal flavonoids have potential to inhibit DNA and RNA formation potential (Ulanowska *et al.* 2006). The flavonoid content of *C.coriaria*has antimicrobial action shown through DNA fragmentation activity of*E.coli*, *S.aureus* and *K.pnuemoniae*(Anandhi *et al.* 2014). The *Syzygiumcumini* leaf extract has also reported DNA fragmentation potential on *Vibrio cholera* (Ahsan *et al.* 2012) and *Bacillus subtilis* (Yadav *et al.* 2017). The methanolic *Syzygiumcumini* extract alongwith phytic acid and sodium chlorideexhibit better DNA fragmentation results (Yadav *et al.* 2018).Several other reports had concluded that flavonoids significantly inhibits the DNA synthesis in case of *Proteus vulgaris*(Mori *et al.* 1987).

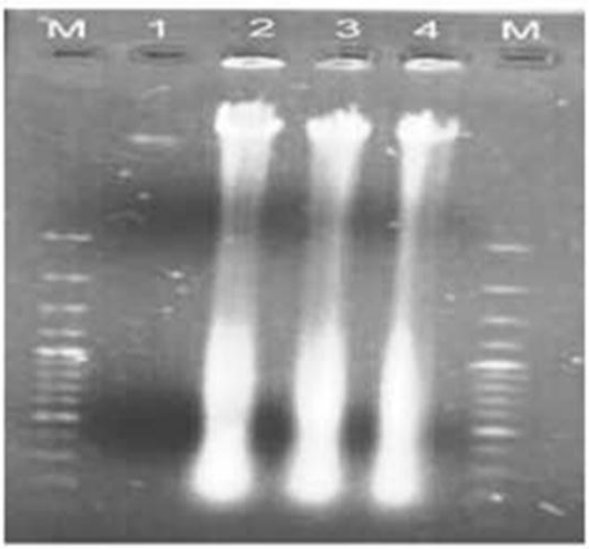


Fig. 2(a): DNA fragmentation by n-hexane extract of *C. phlomidis*

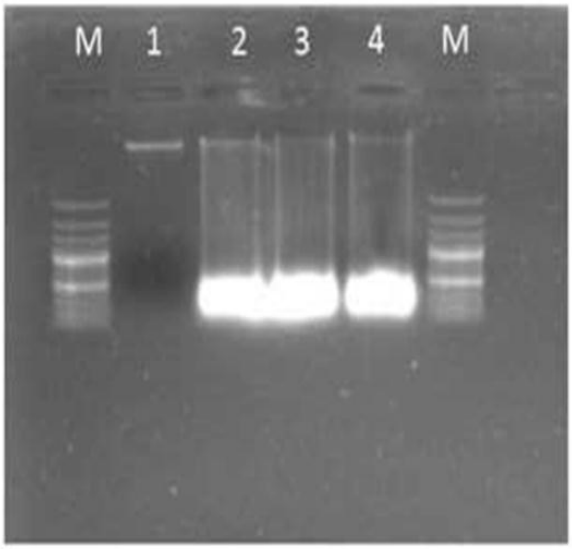


Fig. 2(b): DNA fragmentation by dichloromethane extract of *C. phlomidis*

**Fig. 2.** Induction of bacterial DNA fragmentation by **(a)** n-hexane extract of *Clerodendrumphlomidis*, **(b)** dichloromethane extract of *Clerodendrumphlomidis.* M= DNA ladder (1kb), 1= Control (Bacterial genomic DNA), 2, 3, 4= Treated bacterial DNA for 72 hours.

**Scanning electron microscopic study of *Bacillus licheniformis***

The scanning electron microscope analysis was done to determine the change in surface morphology of bacterial cells as compared to the control of *Bacillus licheniformis*. It is quite well reported the mechanism behind antimicrobial action of various small molecules causes the leakage of cellular content and formation of pores in bacterial membrane (Yenugu *et al.* 2006). As shown in fig. 3 (a to c) the change in surface morphology of bacterial cells of *Bacillus licheniformis* was observed after treating them with concentration of 20mg/mLusing SEM analysis as compared to the control. Bacterial cells of control showed smooth surface and regular morphology as treated cells has degraded surface morphology shown in fig.3 (a to c). It concludes that after treating bacterial cells with plant extract of *C.phlomidis* has an effect on bacterial cell wall is distorted, irregular cell wall structure,bleebing and leakage of cellular content as clearly depicted in SEM image. Similarly the antibacterial mechanisms of alcoholic extract of *Hemidesmus indicus* (L.) R. Br. Ex Schult, *Leucas aspera* (Wild.), *Plumbago zeylanica* L., and *Tridaxprocumbens* (L.), Schult has shown that except Tridaxprocumbens (L.) these extract has also has shown blebbing and leakage of cellular contents and disruption of membrane (Saritha *et al.* 2015). The antibacterial mechanism of *Curcuma longa* rhizome extract against *Staphylococcus aureus* had corresponded cell disruption, plasmolysis, and partial disappearance of cytoplasmic membrane are observed (Gupta *et al.* 2015).

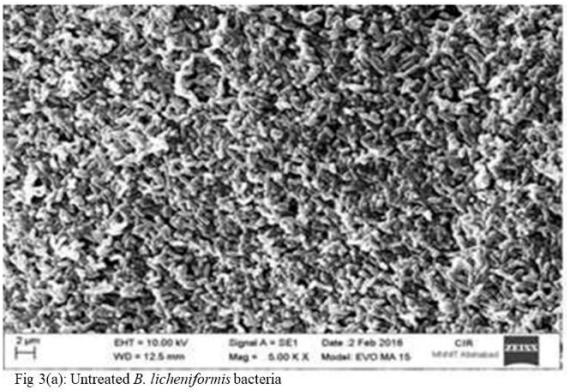

Fig 3(a): Untreated *B. licheniformis* bacteria

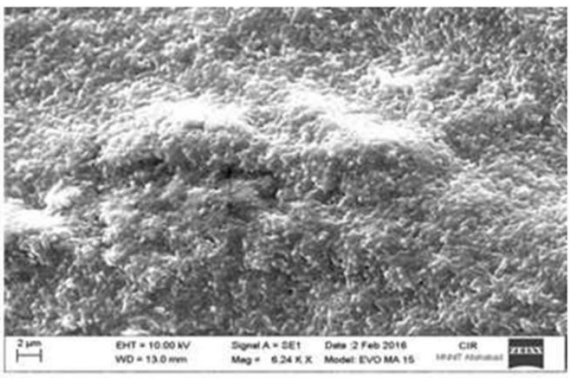

Fig 3(b): Treated *B. licheniformis* bacteria with dichloroethane extract of *C. phlomidis*

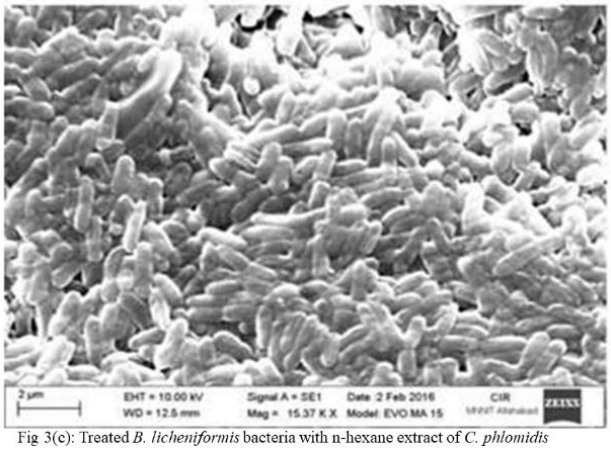

Fig 3(c): Treated *B. licheniformis* bacteria with n-hexane extract of *C. phlomidis*

**Fig. 3.** Images of SEM analysis for changes in cell surface morphology (a) Untreated *Bacillus licheniformis* bacteria, **(b)** Treated *Bacillus licheniformis* bacteria with Dichloromethane extract of *C. phlomidis.* **(c)** Treated *Bacillus licheniformis* bacteria with n-hexane extract of *C. phlomidis.* All these images are taken at 2 µm

**Separation and purification of compound for antimicrobial activity and FTIR analysis**

Dichloromethane extract of *C.phlomidis* was dissolved in the diethyl ether and butanol (3:2) ratio and 15 fractions were collected and applied for their antimicrobial potential. Out of those 15 fractions only 4 showed the antimicrobial activity against the *Bacillus licheniformis.* For the determination of functional group present within these antimicrobial fraction was done by using FTIR (Fourier Transform Infrared Spectroscopy).

The possible function groups present within the antimicrobial compound were analyzed and tabulated below in Table 1 and fig. 4 (a to d). The various antimicrobial compound isolated from the dichloromethane purified extract showed the presence of - OH group, -CHO, halogens (-X-), etc. The Peak value of various fractions obtained from FTIR analysis antimicrobial compounds of fraction five F (5) showing presence of Halogen group at 475.92 $cm^{-1}$and 611.94 with the stretching of C-Br and C-Cl. The peak value at 1629.68 and 3415.57 showing presence of Aldehyde and Alcohol group with C-H stretching. Peak value obtained after the FTIR analysis of compound fraction eight F(8) of dichloromethane purified extract showing the presence of halogen group with O-H stretching at 524.06 $cm^{-1}$. However at 1636.6 and 3436.81 peak values are showing the presence of aldehyde and alcohol group with C-H and O-H Stretching. Likewise peak value obtained by the FTIR analysis of antimicrobial compound of fraction nine F(9) showing the presence of halogen group at 477.79 and 611.94 $cm^{-1}$with the stretching of C-Cl and C-Br. However at 3714.43 $cm^{-1}$peak value showing alcoholic group with C-H stretching. The FTIR analysis of fractionated compound (F15) obtained after the purification are showing the presence of halogen group at peak value of 607.27 $cm^{-1}$. From peak value of 950.45, 1038.76, 1062.31, 1124.12 $cm^{-1}$are showing presence of ether group having C-H stretching. Peak values from 1383.16 to 1462.13 are showing the presence of alkane group with C-H stretching. At 1637.3 $cm^{-1}$peak value presence of aldehyde is noticed. Again from 2872.16, 2962.31 and 3447.16 $cm^{-1}$is showing presence of alkane and alcohol with C-H and O-H stretching.

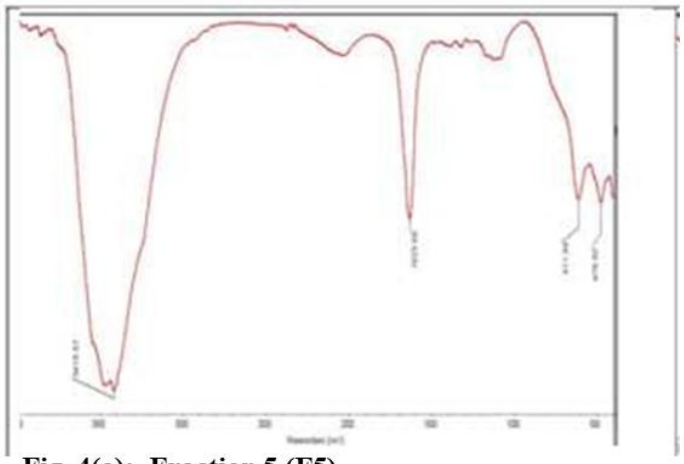

**Fig. 4(a): Fraction 5 (F5)**

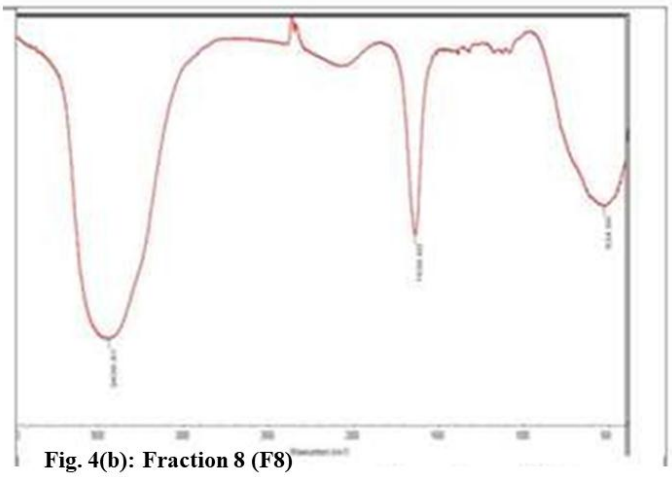

Fig. 4(b): Fraction 8 (F8)

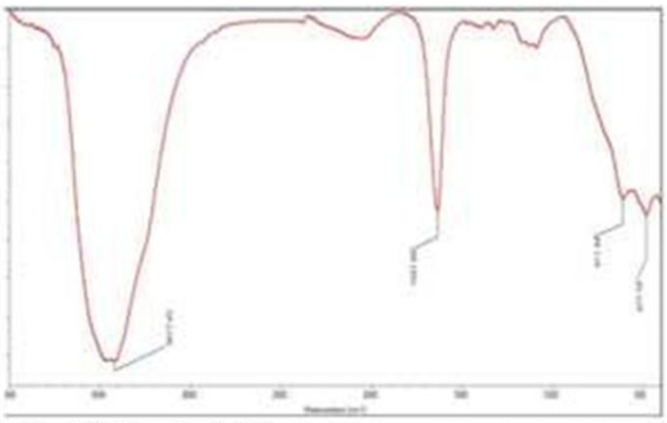

Fig. 4(c): Fraction 9 (F9)

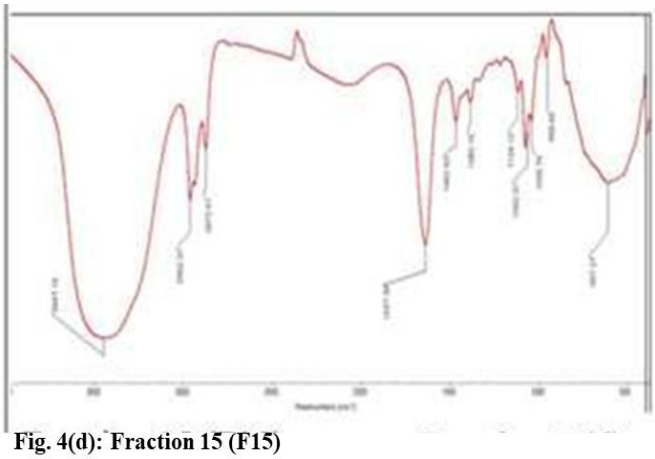

Fig. 4(d): Fraction 15 (F15)

**Fig. 4.** The FTIR spectroscopic analysis of *Clerdodendrumphlomidis* column compounds. (a) Fraction 5 (F5), (b) Fraction 8 (F8), (c) Fraction 9 (F9), (d) Fraction 15 (F15).

**Table 1.** FTIR spectral analysis of various fractions along with the presence of functional groups.

| | Fraction 5 | | |
|---|---|---|---|
| S. No | Peak Value | Stretching | Interpretation |
| 1 | 475.92 | C-Br | Halogen |
| 2 | 611.94 | C-Cl | Halogen |
| 3 | 1629.68 | C-H | Aldehyde |
| 4 | 3415.57 | C-H | Alcohol |
| | Fraction 8 | | |

| | | | |
|---|---|---|---|
| 1 | 524.06 | O-H | Halogen |
| 2 | 1636.6 | C-H | Aldehyde |
| 3 | 3436.81 | O-H | Alcohol |
| | Fraction 9 | | |
| 1 | 477.79 | C-Br | Halogen |
| 2 | 611.94 | C-Cl | Halogen |
| 3 | 3714.43 | C-H | Alcohol |
| | Fraction 15 | | |
| 1 | 607.27 | O-H | Halogen |
| 2 | 950.45 | C-H | Ethers |
| 3 | 1038.76 | C-H | Ethers |
| 4 | 1062.31 | C-H | Ethers |
| 5 | 1124.12 | C-H | Ethers |
| 6 | 1383.16 | C-H | Alkane |
| 7 | 1462.13 | C-H | Alkane |
| 8 | 1637.3 | C-H | Aldehyde |
| 9 | 2872.61 | C-H | Alkane |
| 10 | 2962.31 | C-H | Alkane |
| 11 | 3447.16 | O-H | Alcohol |

Some studies reported the presence of dominant functional group within the potent herbal extract through FTIR spectroscopy. Spectroscopic analysis of *Aervalanata* (L.) extracts showed the presence of C=O, C-H, C=C, O-H, C-CHO, C-N and C-Cl at 1735.81 $cm^{-1}$, 2921.96 $cm^{-1}$,1612.38 $cm^{-1}$,3319.26 $cm^{-1}$,1400.22 $cm^{-1}$,1319.22 $cm^{-1}$, 752.19 and 696.25 $cm^{-1}$ respectively(Murugan& Mohan 2014).

## CONCLUSION

Food poisoning is an alarming concern for all the developed and developing countries. *Bacillus licheniformis* strain mainly involved in the food poisoning in many industrially processed food such as cheese, yogurt and many other items (Pepe *et al.* 2003). The *Bacilluslicheniformis* is also found in the case of some severe sepsis (Haydushka *et al.* 2002). There are many kinds of pharmaceutical drugs are being available in market but having some severe side effect. Day by day the antimicrobial agents against *Bacillus licheniformis* are increasing but very few studies is done over understanding the common mechanism of their action for inhibiting them. To conclude, it clearly revealing that n-hexane and dichloromethane extract of *C.phlomidis* has potent antibacterial property. The results of well diffusion, FACS, DNA fragmentation analysis and scanning electron microscope are providing sufficient informationregarding it's mode of action as antibacterial property. Furthermore the antibacterial activity of this herbal extract can be exploited very effectively in the formulation of herbal drugs for treatment and inhibition of Bacillus related species of food born infection without any side effect. Moreover,*C.philomidis* Linn. extract can be applied for veterinary and agricultural application as described earlier.

As per our present knowledge, this is the first report for understanding the underlying mechanism of action of n-hexane and dichloromethane extracts of *C.phlomidis* for antimicrobial action against *Bacillus licheniformis.* The result of FTIR analysis derived shows the presence of functional groups /chemical bond present within them. Moreover, the FTIR analysis provided the presence of dominant functional group within the fraction. Here the present FTIR analysis shows dominance of Alcohol (-OH) group, Aldehydes(-CHO), halogens (-X-) etc. The present result shows that n-hexane extract has more potent antimicrobial activity than the dichloromethane extracts on the basis of zone of inhibition activity. The mechanism involves behind the antibacterial action of these extracts are mainly attributed to the disrupting membrane potential, leakage of cellular content and degradation of bacterial genomic DNA.

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**Conflict of interest:** The authors declare that they have no conflict of interest.

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