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Efficacy of *Vitex negundo* and *Clerodendrum inermae* against bacterial flacherie in mulberry silkworm *Bombyx mori L*

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Abstract

Medicinal and aromatic plant extracts are the potent source, to minimize the flacherie disease as the bacteria develop resistance to conventional antibiotics. Thus the present work is designed to assess the efficacy of crude aqueous extracts of a medicinally important *Vitex negundo* and *Clerodendrum inermae*. *Bacillus* sps and *Enterobacter* sps bacteria were isolated from haemolymph of diseased silkworm. Crude aqueous extracts of *C. inermae* has significant activity against *Enterobacter* sps. And moderate against *Bacillus sps*, similarly *V. negundo* has moderate against both *Enterobacter* and *Bacillus* sps. FTIR analysis showed the presence of phenolic compounds in *C. inermae* and *V. negundo*. The present study recommends, herbal extracts as the effective antibacterial agent utilize sericulture operations.

Keywords: *Bacillus sps; Bombyxmori*; *Clerodendrum inermae; Enterobacter sps; Vitex negundo*

Introduction

The mulberry silkworm, *Bombyx mori* is of great economic importance as a foreign exchange earner in countries like china, Japan, India, Korea, Brazil, Russia, Italy and France. Silkworm diseases are the major problem in sericulture industry. Flacherie is more common in fourth and fifth instar and is a serious problem in mass rearing of silkworm causing 20 to 50 % cocoon crop losses in India. Bacterial infection is managed by antibiotics and the efficacy of antibiotics has been proved by several authors (Manimegalai and Chandramohan, 2005) [16], but the development of resistance to drugs by the bacteria makes the antibiotics become ineffective within a short duration. Thus several attempts have been made to use plant compounds especially the crude aqueous extracts of plants against bacterial pathogens (Priyadharshini *et al*., 2008) [18]. Medicinal and aromatic plants are widely used as a major source of antibiotics. Plant secondary compounds have effects on other organisms rather than its own producer. These secondary metabolites are believed to function as biochemical defence (Jain *et al.*, 2004) [11]. Using plant extract is the possibility of improving silk productivity by reducing the incidence of diseases in silk worms. The medicinal plants are exploited as sources of antibiotics against the bacterial flacherie. The present study was undertaken to find out the possibility of crude extracts of two medicinally important plants *Clerodendrum inermae* (L.) Gaertn and *Vitex negundo* L. for controlling the bacteria causing flacherie disease in the mulberry silkworm, Bombyx mori. The plant *Clerodendrum inermae* (L.) Gaertn and *Vitex negundo* L. belonging to the family Verbenaceae widely distributed in tropical and subtropical regions is used as antimicrobial medicines (Gupta *et al*., 2005) [8] by tribals. This plant is rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids etc which have been found to have antibacterial activities (Alamsher 2009) [2].

Materials and methods

1. Collection and preliminary phytochemical screening

The aerial parts of *Vitex negundo* and *Clerodendrum inermae* were collected and shade dried after washing in tap water. Fifty grams of plant powder was mixed with 250 ml distilled water and vortexed in an orbital shaker (REMI) for about 11 hrs at 30˚C. The extract was filtered and reduced to a paste using rotary evaporator. The extract was stored at 4˚C forfurther analysis. The presence of a variety of phytochemical compounds in the extracts was determined by using the methods of Brindha *et al*. (1977) [5] and Harbone (Table 1).

Table 1: Preliminary Phytochemical analysis

Experiments	Observation
Plant extract + Wagner's reagent	Reddish brown precipitate indicates presence of alkaloid.
Plant extract + Benedict's reagent	Reddish brown precipitate reveals the presence of carbohydrate.
Plant extract + Glacial acetic acid and Ferric chloride solution +Sulphuric acid.	Appearance of violet or a greenish ring indicates the presence of glycosides.
Plant extract + Sodium hydroxide solution	Appearance of intense yellow colour and colourless solution on addition of dilute hydrochloric acid reveales the presence of flavonoids
Plant extract + Ferric chloride	Appearance of deep blue or black colour indicates the presence of phenol.

Plant extract + Ninhydrin solution	Purple colour reveals the presence of protein and amino acid.
Plant extract + Ferric chloride solution.	Appearance of Blue or greenish color indicates the presence of Tannins.
Plant extract + Water. The mixture was shaken vigorously	Formation of persistent foam indicates the presence of saponins
Plant extract + Hydrochloric acid.	Appearance of Red precipitate indicates the presence of phloba tannins.
Plant extracts + Chloroform + Sulphuric acid	Appearance of Reddish brown precipitate indicates the presence of terpenoids.

2. Collection and identification of microbial isolates

Flacherie infected silkworms were collected from Government Silkworm Rearing Station, Konam, Nagercoil, Tamilnadu. Haemolymph was collected by after surface sterilization with methanol choked cotton. The hemolymph was diluted, 10^{-7} and10^{-8} dilutions were plated on Nutrient agar medium and were incubated at 37°C (Govindhan *et al*. 1998) after 72 hrs the microbes were isolated, sub cultured. The morphology of isolated colonies were observed and identified according to Bergey's Manual of Systematic Bacteriology (1989).

3. Preparation of inoculums and Determination of Minimum Inhibitory Concentration (MIC)

Inoculums were standardized to give a density of 10^6 colony-forming units (CFU)/ml. A loopful of the test organism was inoculated into 5.0 ml of nutrient broth and incubated at 3°C for 24 h. 0.2 ml from the 24-h culture of the organism was dispensed into 20˚ml sterile nutrient broth and incubated for 3–5 h to standardize the culture to 10^6 CFU/ml. Plates were inoculated within 15 min of standardizing the inoculum, to avoid changes in inoculum density (Abalaka *et al*, 2012) [1].

The Minimum Inhibitory Concentration (MIC) of was determined. One ml of selected plant extract at different concentrations (1000, 100, 10, 1 0.1 µg/ml) were taken in separate test tubes, 1 ml of nutrient broth and a loopful of the test organism were added. Same procedure was repeated using standard antibiotics amoxicillin as positive control and a negative control without amoxicillin. The inoculated test tubes were then incubated at 37°C. After 24 hours OD was taken. The decrease in the optical density of the culture was taken as an indication of the effectiveness of the herbal extract against the growth of the microbial pathogen (Isaiarasu *et al.*, 2011) [9].

4. Microbial sensitivity test

The sensitivities of the isolated bacterial species against different concentration (100, 200, 300, 400 and 500µg) of plant extracts and antibiotics were tested based on the disc diffusion (Kirby–Bauer) technique (Bauer *et al*., (1966) [4] as described by Saif *et al*. (2017) [19].

5. Fourier Transform Infrared Spectrophotometer (FTIR)

Crude plant extract of both plant materials were used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FTIR spectroscope with a Scan range from 400 to 4000 cm 1 with a resolution of 4 cm^{-1}.

Results and Discussion

The preliminary phytochemical analysis of *Clerodendrum inermae* and *Vitex negundo* aqueous extracts (table 2) showed the presence of carbohydrates, glycosides, phenol, tannins, saponins and terpenoids. Secondary metabolites of plants compounds exhibit many biological activities and are produced by the plant cell through metabolic pathways shown to possess various biological effects. Phenols are known for its antimicrobial effect due to their ability of binding with extracellular proteins of bacterial cell wall. Another metabolite, saponins a bitter compound known to produce inhibitory effect through the formation of foams in aqueous solutions precipitating and coagulating proteins (Praveen and Rajesh, 2019) [17]. Deb *et al*. (2016) [6] also reported the presence of alkaloids, flavanoids, phenols, tannins, saponins, phlobatannins and terpenoids in *Vitex negundo* and *Clerodenrum inermae* aqueous extracts.

Table 2: Result of preliminary analysis of aqueous extracts of selected plants

	V. negundo	***C. inermae***
Alkaloid	+	-
Carbohydrates	+	+
Glycosides	+	+
Flavanoids	+	+
Phenol	+	+
Protein	+	-
Aminoacid	-	-
Tannins	-	+
Saponins	+	+
Phlobatannins	+	+
Terpenoids	-	-

'+'present; '-' absent

The morphological and biochemical analysis of subculture developed from the haemolymph of the infected silkworm contains gram negative bacteria such as *Bacillus sps*. and *Enterobacter* sps. (table 3).

Various strains of bacteria such as *Bacillus subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *E.coli*, *Pseudomonas fluorescence*, *Bacillus cereus* and *Klebsiella cloacae (*Sakthivel *et al*., 2012) were isolated from flacherie infected silkworms. Different researchers (Sengupta *et al*., 1990; Yungen and Bharthi, 2001) [25] have already reported that bacteria such as *Bacillus thuringiensis*, *Streptococcus faecalis*, *Staphylococccus* and *Serratia marcescens* associated with silkworm diseases either singly or in combination. *Bacillus thrungiensis* a facultative entomogenous bacterium also reported as pathogenic to the silkworm by Selvakumar *et al*. (1999) [21].

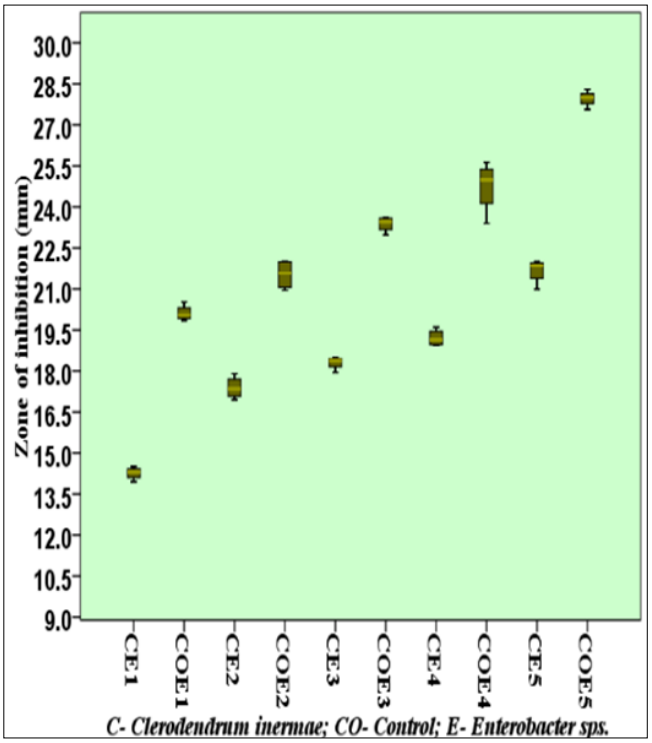
Table 3: Morphological and biochemical characters of isolated bacterial species.

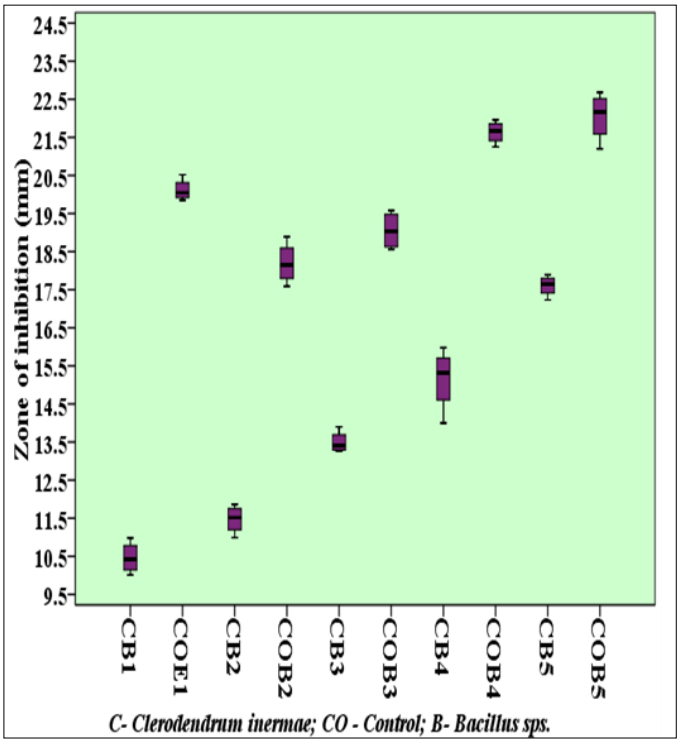
Physical/ chemical characters	***Enterobacter***	***Bacillus***
Cultural characters	Mucoid colonies	Mucoid with central portion raised colonies
Shape	Rod	Rod
Grams stain	-'ive	-'ive

Colour	White	Slight pink
Mannitol	+	-
Motility	+	+
Glucose	+	-
Lactose	+	-
Sucrose	+	-
H_2S production	-	-
Gas production	-	-
Peptone water	-	-
Simmon water	+	-
Oxidase	+	+
Catalase	+	+

A bacterium is the etiological agent of flacherie in silkworms (Anitha *et al*.. 1994) [3]. A change in the optical density of bacteria inoculated in the nutrient broth of experimental categories compared to the control positive and negative shows the inhibitory effect of plant extracts. Among the two selected plants minimum of 62.5µg/ml of *C. inermae* and 125µg/ml of *V. negundo* is required to inhibit the growth of *Enterobacter sps* where as the Minimum inhibitory concentration of both selected plant extracts for Bacillus *sps* is 125µg /ml. However in control (amoxicillin), very low amount of about 10µg/ml for *Enterobacter* and 100µg/ml for *Bacillus sps.*

The aqueous extracts of *C. inermae* and *V. negundo* when tested for their efficacy of antimicrobial activity showed different zone of inhibition with flacherie causing *Enterobactor sps.* and *Bacillus sps. C. inermae* showed maximum zone of inhibition for *Enterobacter* (21.7 ± 0.46 mm*)* and *Bacillus sps.* (17.60 ± 0.28 mm) followed by *V. negundo* (16.29 ± 0.23mm) (Figure B) and14.74 ± 0.69 (Figure D). Similar growth inhibition was observed by Karthikairaj *et al*. (2014) [13] and Sivakumar *et al*. (2012) [23]. When compared to the control *(Enterobacter* - 28 ± 0.30 mm and *Bacillus sps* - 22.05 ± 0.64mm) group, the experimental categories exhibit less antimicrobial activity. Kuete (1999) [14] considered the plant extracts as to possess significant activity when they have MIC below 100 µg/mL, moderate activity when their MICs vary between 100 and 625 µg/mL and weak activity when MICs above 625 µg/mL. Thus in the present study *C. inermae* has significant activity against *Enterobacter sps* and moderate against bacillus, similarly *V. negundo* has moderate against both *Enterobacter* and *Bacillus sps* of bacteria. Plants with good source of anti-infective agents were found to be effective against microbial infections. A number of secondary metabolites derived from plants such alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, saponins, tannins, steroids and triterpenes have previously showed antibacterial activities (Iwu *et al*., 2010) [10].





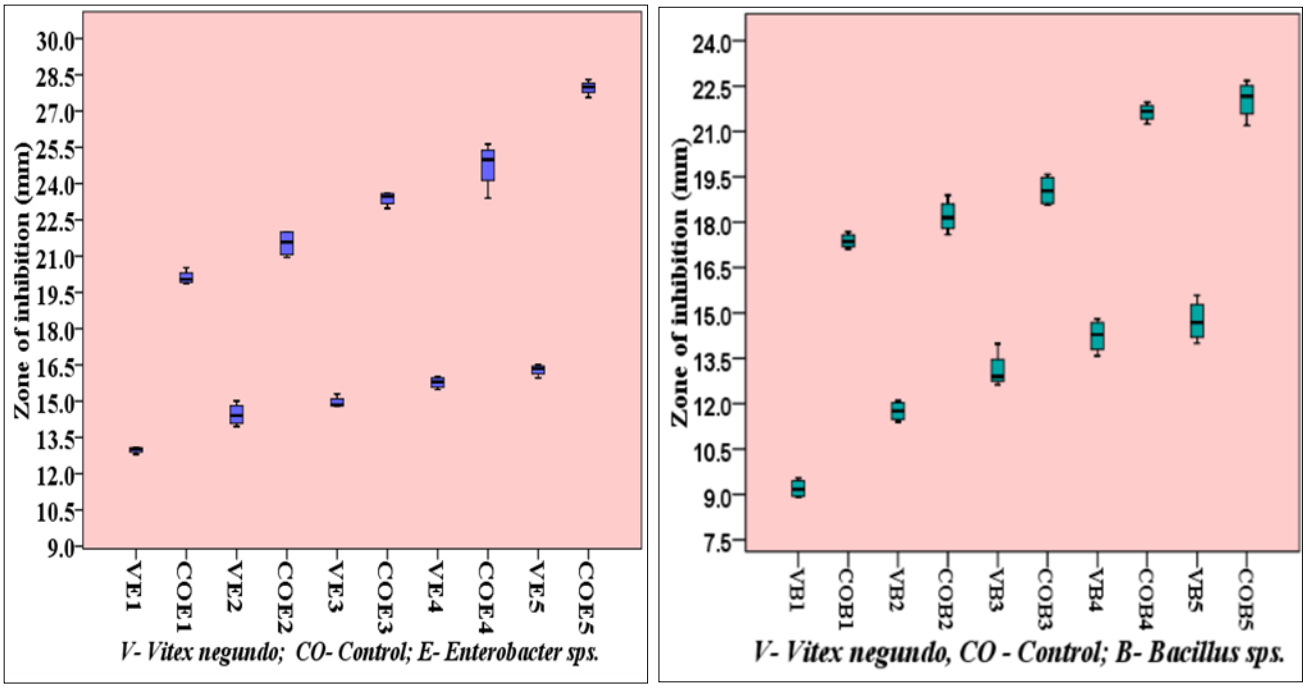


Fig 1: Antibacterial activity of aqueous extracts of *V.negundo* (VN), *C.inermae* (CI) at different concentration (100,200, 300,400 and 500µg) on *Bacillus* (B) and *Enterobacter* (E) species of flacherie causing bacteria.

2. Fourier Transform Infrared Spectrophotometer (FTIR)

The results of FTIR analysis of *C.inermae* and *V. negundo* leaves extracts have been recorded in table 3a & 3b. Characteristic peaks for hydroxyl compounds O-H (stretch) were obtained in *C.inermae* (3639.68, 3392.79 cm-1) *V. Negundo* (3479.58, 3419.79, 3244.27 cm-1) hinting the presence of alcohol / phenol. C-H stretching has been found in *C. Inermae* (1625.99 cm-1) and *V. negundo* (2862.36 cm-1) mainly for alkane. Phosphorus function (P-H) stretch was recorded only in *C.inermae* at (2362.8 cm-1). Aromtic compounds C=C stretch and Primary amine N-H stretch have been recorded in *V. negundo* at 1635.64, 2193.06 and 3361.93 cm-1) hinting the presence of amine. Alkenes Ar-CH-CHR was found in *C.inermae.* Fourier Transform Infrared Spectrophotometer (FTIR) is the most powerful tool for identifying the types of functional groups present in compounds. The wavelength of light absorbed is characteristic of the chemical bond. The chemical bonds in a molecule can be determined by interpreting the infrared absorption spectrum. By using FTIR spectrum, we can confirm the functional constituent's presence in the leaf extract and even evaluate the qualities of medicinal materials.

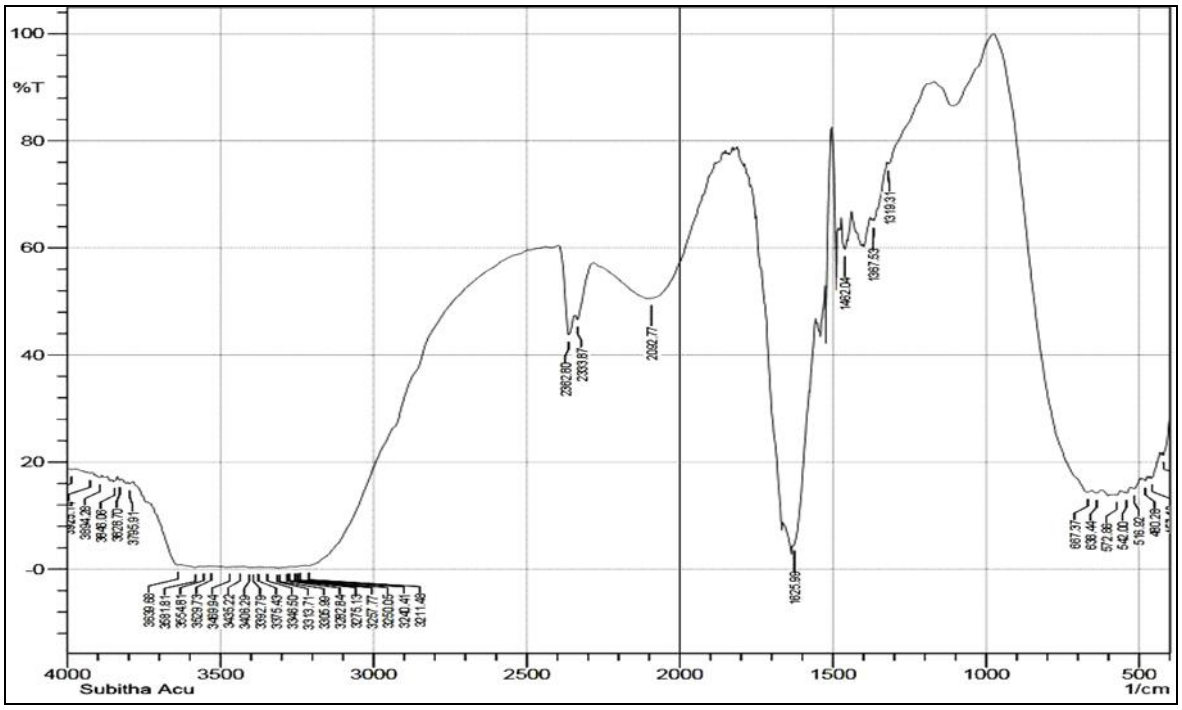
Fourier Transform Infrared Spectrophotometer (FTIR) analysis revealed that phenol, alkane, alkene, carboxylic acid, aromatic compound, nitro compound, alcohol and benzene compounds are the functional constituents present in aqueous leaf extracts of *C. inermae* and *V. negundo.* The broad band in *C. inermae* and *V. negundo* corresponding to OH- group confirms the presence of phenolic compounds in leaf extract. The efficiency of the aqueous extracts of *C. inermae* and *V. negundo* against the bacteria could be attributed to the phenolic composition. Phenolic compounds were also observed in *V. negundo* by Liu *et al* (2006) [15]; Janakiraman and Jeyaprakash (2015) [12]. Phenolic compounds are resulting in bacterial death since these compounds disrupting the bacterial cell wall, interfering with the ATP pool and altering its membrane potential (Tiwari *et al*., 2015) [24].

Table 4: FTIR analysis of *C. inermae*

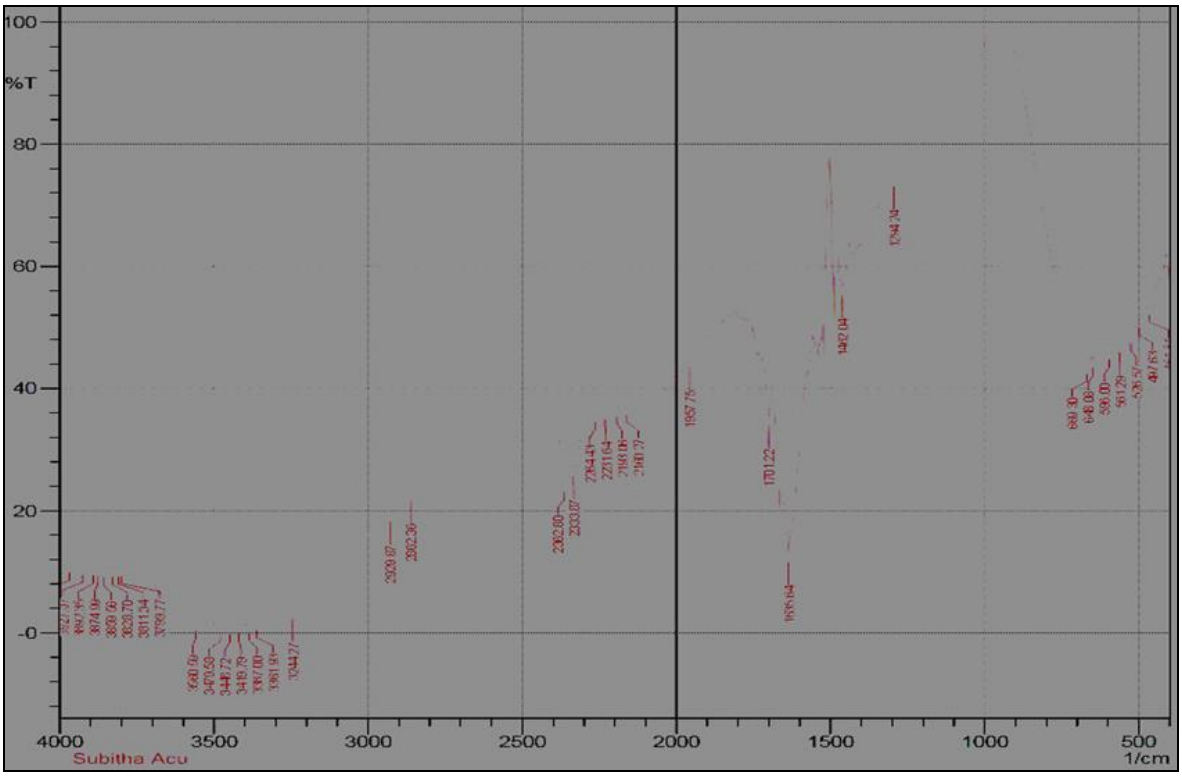
S. No	Characteristic absorptions (cm-1)	Type of bonds	Functional group	Type of vibration	Intensity
1	3795.91, 3639.68, 3581.81, 3554.73, 3529.73, 3469.94 3435.22, 3406.29, 3392.79, 3375.43, 3346.50, 3313.71 3305.99.	O-H Hydrogen bonded	Alcohol/ Phenol	Stretching	Strong
2	3282.84, 3275.13, 3257.77, 3250.05, 3240.41 3211.48	O-H	Carboxylic acid	Stretching	Strong
3	2362.8	O=C=O	CO2	Stretching	Medium
4	2333.87	O=C=O	CO2	Stretching	Medium
5	2092.77	C ≡ C	Alkyne	Stretching	Medium
6	1625.99	N-H	Amines	Bending	Strong
7	1462.04	C-H	Alkane	Bending	Medium
8	1367.53	C-H	Alkane	Bending	Medium
9	1319.31	O-H	Phenol	Bending	Weak

Table 5: FTIR analysis of *V. negundo*

3b	Characteristic absorptions (cm-1)	Type of bonds	Functional group	Type of vibration	Intensity
1	3560.59, 3479.58, 3448.72, 3419.7, 3387.0, 3361.93, 3244.27	O- H	Alcohol/ Phenol	Stretching	Strong
2	2929.87	C-H	Alkanes	Stretching	Strong
3	2862.36	C-H	Alkanes	Stretching	Strong
4	2362.8	O=C=O	CO2	Stretching	Medium
5	2333.87	O=C=O	CO2	Stretching	Medium
6	1701.22	C= O	Conjugated aldehyde	Stretching	Medium
7	1635.64	C=C	Alkane	Stretching	Strong
8	1462.04	C-H	Alkane	Bending	Medium



A



B

Fig 2: FTIR analysis of *C. inermae* (A) and *V. negundo* (B)

Conclusion

The aqueous extract of two medicinally important plants *Clerodentrum inermae* and *Vitex negundo* were tested to find its efficacy against the silk worm flacherie pathogen ie *Enterobacter sps.* and *Bacillus sps.* The experiment showed that the aqueous extracts of *C. inermae* has significant activity against *Enterobacter sps* and moderate against bacillus, similarly *V. negundo* has moderate against both *Enterobacter* and *Bacillus sps* of bacteria. The phytochemical analysis of FTIR showed the presence phenolic compounds. Thus it was confirmed that the reduction in growth efficiency of *Enterobacter and Bacillus* sps. could be attributed to the phenolic composition.

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