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Formulation and chemical characterization of *Clerodendrum infortunatum* leaf extract in relation to anti-fungal activity

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Abstract

The study explored to develop an eco-friendly herbal fungicide from chloroform extract of *Clerodendrum infortunatum* leaves. The extract upon activity guided purification using flash chromatography yielded eight fractions F3 through F10. Total phenol and flavonoid contents in the fractions ranged from 0.12-48.25 mg GAE/g and 0.03−25.29 mg QE/g. LC-MS/MS analysis confirmed the identification of seven phenolic acids across different fractions, the total of which varied between 0 and 2.17 mg/g. Emulsifiable Concentrate (20%) formulation was made with the extract and fractions and tested against *Phomopsis vexans* causing fruit rot disease in brinjal. Of the various fractions, F8 displayed highest

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antifungal activity (ED_{50} = 46.8 μg/ml). Antifungal activity of leaf extract/fractions was correlated with total phenol, total flavonoid and total phenolic acids (r = -0.60 to -0.69). Among the phenolic acids, benzoic acid showed maximum antifungal activity followed by t-cinnamic acid. The relationship between phenolic composition and activity is also reported.

Keywords: Biochemistry, Ecology, Microbiology, Plant biology, Analytical chemistry

1. Introduction

Plant diseases and weeds are reported to adversely affect global agricultural productivity and altogether accounts for economic losses ranging between 20-40% in crop management and post harvest system (Savary et al., 2012). Thus plant diseases and weeds pose a serious threat to food safety and security that can affect the demand of quality food (Strange and Scott, 2005; Tejeswini et al., 2014). The use of synthetic pesticides in agriculture and public health is facing economic and ecological challenges worldwide due to human and environmental contamination caused by majority of the conventional agro-chemicals (Popp et al., 2013; Nicolopoulou-Stamati et al., 2016). Additionally, a major constraint to organic agriculture, which is being encouraged to and practiced by farmers in the country, is lack of suitable formulation that can replace conventional agro-chemicals used for the protection of crops (Silva-Andrade et al., 2016). The demand of such formulation and the knowledge concerning traditional use of plants extract for crop protection have received much scientific attention in the recent past for development of botanicals as an eco-friendly alternative to synthetic plant protection chemicals. Plants are endowed with great diversity of naturally occurring secondary compounds including phenolics, terpenoids, alkaloids and glucosinolates etc. (Chen et al., 2010; Wink, 2015), which are known to protect plants from plant pathogens and insect herbivores. Among the secondary compounds, phenolic compounds are widely distributed in the plant kingdom and occur with extraordinary structural diversity viz. phenolic acids, flavonoids, anthocyanins, coumarins etc. (Nigam, 2009; Martins et al., 2011). In general, all these compounds possess at least one acidic hydroxyl residue attached to a phenyl ring (Ainsworth and Gillespie, 2007). These compounds are either preformed or induced by various types of stresses including pathogens, insects, salinity, drought etc. In addition to their structural and functional role in plant metabolism, phenolic compounds either singly or in combination, are reported to exhibit inhibitory activity in growth and reproduction towards pathogens and predators (Bravo, 1998; Martins et al., 2011; Pusztahelyi et al., 2015; Feng-Xu et al., 2016; Kole et al., 2016).

Thus botanicals as crude extracts and active molecules of some plants have immense potential to act as an alternative to synthetic pesticides for disease management,

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which can reduce not only crop losses but also poses little threat to the environment or to human health (El-Wakeil, 2013; Murugan et al., 2016). Many plant species such as *Tagetes erecta L.* (Du et al., 2017); *Cistus ladanifer* leaves (Barros et al., 2013); *Helianthus tuberosus* leaves (Chen et al., 2010); *Terminalia nigrovenulosa* bark (Nguyen et al., 2013), etc. have been reported to possess anti-fungal properties due to the occurrence of natural substances. Thus, the development of botanical pesticides containing diverse chemical compounds with their natural synergism, low mammalian toxicity, high target specificity, as well as biodegradability can be a viable option in the management of plant diseases (Harish et al., 2008). *Clerodendrum*, a representative member of Leguminosae, elaborates a diverse array of chemical compounds including flavonoids and diterpenoids, which are reported to possess antifeeding activity (Pal et al., 1989), antimicrobial activity (Kole and Chowdhury, 1994; Anitha and Kannan, 2006; Tejeswini et al., 2014) and antioxidant activity (Gouthamchandra et al., 2010). We extended our present research work with an aim to explore further the fungicidal bio-efficacy of the formulations of leaf extracts/fractions of *C. infortunatum* in relation to contents of total phenol, total flavonoid and total phenolic acids as well as to establish the relationship between them.

2. Materials and methods

2.1. Collection of plant materials

Fresh leaves of *Clerodendrum infortunatum* were collected from Insrtuctional Farm, Jaguli, Bidhan Chandra Krishi Viswavidyalaya (BCKV), Mohanpur, Nadia, West Bengal. Leaves were washed under running tap water to remove dust particles, insects and plankton, and then dried under the shade at room temperature. The dried plant materials were milled into fine powder using mechanical grinder.

2.2. Extraction of *Clerodendrum* leaf and its fractionation

Powdered *Clerodendrum* leaves (0.6 kg) were extracted with chloroform (Rankem, India) in two batches in a Soxhlet apparatus (2.5 L) for 6 hours. The solvent extract was then evaporated to dryness at 45 °C using Rotavapor (R-3, Buchi) and transferred in a pre-weighed conical flask. One part (10.25 g) of total crude extract (28.56 g) was separated in glass column (diameter: 5 cm, length: 50 cm) fitted with a pre-column cartridge packed with silica gel (60−120 mesh; Merck, India) and florisil (60−100 mesh; Across Organics, USA) in the ratio of 1:3 by Flash Chromatography System (Sepacore, Buchi) equipped with pump manager C-615, UV Photometer C-635, Buchi C-660 fraction collector. The extract was then dissolved in 20 mL chloroform and loaded to the pre-column using a syringe (15 mL). The column was then eluted at the flow rate of 15 mL/min with solvents of increasing polarity such as 100% n-hexane (F1), 10% (F2), 25% (F3), 50% (F4) chloroform

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in n-hexane, 100% chloroform (F5), 2% (F6), 5% (F7), 10% (F8), 20% (F9) and 50% (F10) methanol (Rankem HPLC grade, India) in chloroform. All the chromatographic fractions were concentrated using Rotavapor and were monitored by Thin Layer Chromatography (TLC) and the percent yield of each fraction was determined. Based on the TLC behavior, eight fractions of the chloroform extract (F3-F10) were selected for further study.

2.3. Estimation of total phenol and flavonoid content

A known quantity (0.1 g) of each of crude chloroform extract (M) and its chromatographic fractions (F3 through F10) were mixed with 1.2 M HCl (15 mL) in 50% aqueous methanol and heated at 80−90 °C for 2 hours in a water bath. The extract obtained after centrifugation at 10,000 rpm for 30 minutes was diluted to a known volume and used for analysis of total phenol and flavonoid.

The total phenol content was determined according to Folin-Ciocalteau (FCR; SRL, India) method described by Vinson et al. (1998). Briefly, a suitable aliquot (~0.4 mL) was diluted to 3 mL with distilled water in a test tube. Then 0.5 mL FCR reagent (1:1) was added to it. After 5 minutes, 2 mL of 10% sodium carbonate was added. Then 25 test tubes were warmed at about 60−70 °C for 5 minutes in a water bath. The absorbance of the solution after cooling was read at 650 nm in a UV Spectrophotometer (Systronics, India). Total phenol content was determined from the standard curve of gallic acid (Sigma Aldrich, USA) and expressed as mg of gallic acid equivalent per gram of extract (mg GAE/g).

The total flavonoid content of the extract/fraction was determined following the method adopted by Marinova et al. (2005). The supernatant aliquot (5 mL) was diluted to 25 mL with 1.2 N HCl in 50% aqueous methanol. At the onset of the experiment, 0.3 mL of 5% $NaNO_2$ (Merck, India) was added to 1 mL of aliquot solution. After 5 minutes, 3 mL of 10% $AlCl_3$ (Merck, India) was added. At 6 min, 2 mL of 1 M NaOH (Merck, India) was added to the mixture. Immediately, the solution was diluted to a final volume of 10 mL with water and mixed thoroughly. The absorbance of the solution was read at 510 nm in a UV Spectrophotometer against the prepared blanks. The total flavonoid content was determined from the standard curve prepared using quercetin (Sigma Aldrich, USA) and expressed as mg quercetin equivalent per gram extract (mg QE/g).

2.4. LC-MS/MS analysis

An aliquot (2 mL) of chloroform extract each of *Clerodendrum* leaf and its chromatographic fractions (100 ppm in methanol) was taken in a nitrogen tube and evaporated to dryness in a nitrogen evaporator (Turbo Vap, Caliper Life Science, USA). The residue was dissolved in acetonitrile-water (1:1) and vortexed for 2 min and

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were filtered with the help of syringe filter (SGE Int. Pvt. Ltd. with 13 mm, 0.22 μ Nylon filter paper) and transferred into the vials for identification and quantification of phenolic acids by LC-MS/MS (Agilent Technologies Infinity 1260, 6420 Triple Quad).

The chromatographic system was equipped with Eclipse XDB C8 (5 μm × 2.1 mm × 150 mm) column. Separation was performed with a gradient consisting of solvent A (0.25% acetic acid in water) and solvent B (acetonitrile) applied at a flow rate of 0.3 mL/min as follows: 90% A linear from 0 to 10 minutes, 70% A linear from 10 to 30 min and 90% A linear from 30 to 42 minutes. The samples were injected (5 μL) in duplicate. The mass spectrometric detection system (6420 Triple Quad) was equipped with electrospray ionization source (ESI) using nebulizer gas (N_2): flow (11.0 L/min), temperature (300 °C), pressure (50 psi), capillary voltage (4000 V), dwell time (100 μs) with mass temperature, 100 °C. Chromatographic peaks were identified by comparison of retention times with those of standards and confirmed by analysis of mass spectra recorded for each peak. The ESI was operated in negative mode with MRM scanning and variable fragmentation voltage of 15, 35 and 35V each for chlorogenic and *p*-coumaric; 10 and 30V for gallic; 13 and 40V for caffeic; 9, 9 and 21V for vanillic; 10, 10 and 10V for *t*-ferulic; 5 and 10V for benzoic; and 10 and 20V for *t*-cinnamic acid, which were used as standards.

Analytical standards of phenolic acid were procured from Sigma Aldrich, USA. Standard solution of respective phenolic acids (ranging between 0.01 and 0.50 μg/mL) were prepared in 50% aqueous acetonitrile (J.T. Baker, Phillipsburg, NJ) from the stock solution of representative phenolic acids (1000 μg/mL) in methanol (J.T. Baker, Phillipsburg, NJ). A mixture of the standard phenolic acids (10 μg/mL) each was also prepared. The concentration of different phenolic acids in the mother extract and its fractions were quantified based on calibration curve prepared by Mass Hunter Software (B 7.0) using a linear regression for the relationship of peak area versus phenolic acids concentration.

2.5. Preparation of formulation of leaf extract and its fractions

A 20% (w/v) emulsifiable concentrate (EC) formulation of mother extract together with each of its chromatographic fractions was prepared using liquid solvent naptha (LSN) as base solvent (75%) followed by addition of 5% mixture of two surfactants (N-Alkaline Sulfonate (A): K-Alkaline Sulfonate (B) at different ratio (A:B = 5:0, 4:1, 3:2, 2:3, 2.5:2.5 and 0:5) and stirring in a homogenizer (IKA, Japan) at 20,000 rpm (Knowles, 2008). The physico-chemical characteristics of different EC formulations such as identity, pH, emulsion stability and re-emulsification, persistence of foam and storage stability (at 10 ± 2 °C, 25 ± 2 °C, 54 ± 2 °C) were tested using the standard protocol adopted by Food and Agricultural Organization and World Health Organization (WHO and FAO, 2016). The active ingredient

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(a.i.) content of the formulations was ascertained in terms of determination of total phenol content using Folin-Ciocalteau reagent as described in the above sections.

2.6. Bio-assay for anti-fungal activity

2.6.1. Testing by poisoned food technique

The bioassay of different formulations of *Clerodendrum* was tested following poisoned food technique against *Phomopsis vexans,* a plant pathogenic test organism. The formulation (20% EC) at four different concentrations (50, 100, 200, and 400 μg/mL) was spiked into 20 mL sterile molten potato dextrose agar (PDA: 200 g potato decoction, 20 g dextrose and 20 g agar powders) medium. The formulation treated medium was poured on to the sterile Petri plates (9 mm diameter) under aseptic conditions and allowed to solidify at room temperature for thirty minutes in laminar flow cabinet. A control was set only with the additives of the formulation. Pre-grown fungal cultures discs (5 mm diameter) of the test pathogens were aseptically inoculated at the center of the sterile petri plates. The inoculated plates were allowed to grow following incubation at 28 ± 1 °C. Mancozeb 75% WP, a commercial fungicide at four different doses (5, 10, 25 and 50 μg/mL) was used as a control. Bioassay study was also conducted using the standard phenolic acids at 5 and 10 ppm and compared with Mancozeb 75 WP at 25 and 50 ppm with an untreated control, where the pathogen was grown in PDA media without application of extract or phenolic acids.

2.6.2. Effect on fungal growth inhibition

The effect on fungal growth was determined by measuring the diameter of the colony of the pathogen relative to control plates that showed sufficient growth 7 days after application. The percent inhibition of the fungal growth was calculated using the formula:

$$\text{Growth inhibition (GI, \%)} = \frac{(X - Y)}{X} \times 100 \quad \text{(i)}$$

Where, X = Radial growth (cm) in control and Y = Radial growth (cm) in treatment.

2.7. Statistical analysis

The ED_{50} value of different formulation was calculated from the logarithm of each concentration and corresponding probit value to each inhibition percentage (Gomez and Gomez, 1984). The ED_{50} values and phenolic constituents present in the plant extract/fractions were subjected to correlation study. Correlation co-efficient (r) was calculated at 5% and 1% level of significance (Gomez and Gomez, 1984).

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3. Results and discussion

3.1. Total phenol and total flavonoid content

Table 1 shows the percent recovery of the chloroform extract of *Clerodendrum* leaf by different chromatographic fractions and total phenol and flavonoid content of the extract and its different fractions. Elution of the extract with solvents of increasing polarity involving Flash chromatography yielded 8 fractions, which together constitute 74.09% of the extract, while retaining 23.96% in the column. The highest yield was recorded in F4 (14.05%) followed by F7 (12.39%), F5 (11.61%) and F9 (11.02%). The total phenol and flavonoid content in different fractions varied from 0.12 to 48.25 mg GAE/g and 0.03 to 25.29 mg QE/g respectively. The mean total phenol and flavonoid content across different fractions form 89% and 92% total phenol (22.52 mg GAE/g) and flavonoid (11.38 mg QE/g) content of the extract respectively. Interestingly, F8 with lowest yield (4.19%) registered maximum total phenol and flavonoid. F6 and F9 with total phenol content of 22.89 and 21.47 mg GAE/g ranked distinct second and third respectively while the inverse order is found in terms of flavonoid content.

3.2. Identification and quantification of phenolic acids

The presence of each of the different phenolic acids in different chromatographic fractions was identified based on comparison of retention times in LC-MS with those of the corresponding standards (Fig. 1) and confirmed with the MS-MS analysis. Gallic acid eluted at a retention time of 7.60 min and presented a molecular ion

Table 1. Total phenol and total flavonoid content in *Clerodendrum* leaf extract/ fraction.

Sample ID	Fraction Yield (%)	Total phenol* (mg GAE/g extract) ± RSD≠	Total flavonoid* (mg QE/g extract) ± RSD≠
M	100	18.59 ± 7.59	5.17 ± 17.87
F3	8.10	0.12 ± 16.67	0.03 ± 17.32
F4	14.05	17.85 ± 11.24	2.51 ± 18.30
F5	11.61	19.61 ± 2.67	5.69 ± 11.57
F6	8.49	22.89 ± 8.30	11.41 ± 13.47
F7	12.39	12.83 ± 8.37	8.58 ± 13.88
F8	4.19	48.25 ± 4.06	25.29 ± 9.21
F9	11.02	21.47 ± 7.11	17.39 ± 7.64
F10	4.24	16.88 ± 5.65	12.71 ± 7.40

M = Mother extract; F3 − F10 = Fractions.

* Data are average of three replications.

≠ values in parentheses indicate relative standard deviation (RSD)

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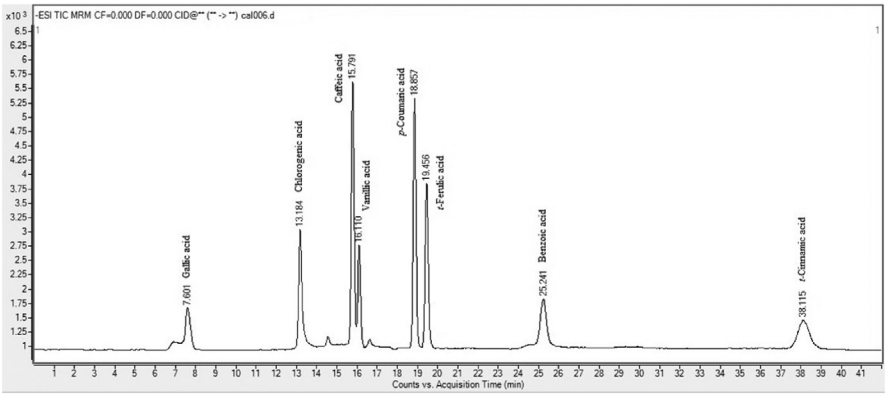

Fig. 1. LC-MS/MS chromatogram of mixture of 8 phenolic acids.

[M-H] at m/z 168.8. The MS-MS analysis of [M-H] gave one fragment ion at m/z 124.9 [M-H-CO_2] and the other fragment ion at m/z 77, which corresponds to benzyl cation, [$C_6H_5^+$]. Chlorogenic acid with a retention time of 13.18 min presented a molecular ion [M-H] at m/z 352.7 and subsequent MS/MS analysis of [M-H] resulted fragment ions at m/z 190.8 [M-H-163 + H], loss of caffeoyl moiety and at m/z 92.8 corresponding to phenoxyl cation, [$C_6H_5O^+$]. Caffeic acid gave a retention time of 15.17 min and molecular ion [M-H] at m/z 178.8. The MS-MS analysis of this molecular ion produced fragment ions at m/z 134.8 [M-H-CO_2] and at m/z 88.9 [134.8- C_2H_4-H_2O]. Vanillic acid with a retention time of 16.11 min showed molecular ion [M-H] at m/z 166.8 and fragment ions from MS-MS analysis of [M-H] at m/z 122.9 [M-H-CO_2] and 107.8 [122.9-CH_3]. *p*-Coumaric acid showed a retention time of 18.86 min and molecular ion [M-H] at m/z 162.8. MS-MS analysis of [M-H] gave fragment ion at m/z 118.9 [M-H-44], at m/z 93 corresponding to [$C_6H_5O^+$]. t-Ferulic acid gave a retention time of 19.46 min and molecular ion [M-H] at m/z 192.8. MS-MS analysis produced fragment ions at m/z 177.8 [M-H-CH_3], at m/z 148.9 [M-H-CO_2]. Benzoic acid eluted at a retention time of 25.25 min and presented molecular ion [M-H] at m/z 120.9. The MS-MS analysis of [M-H] gave fragment ions at m/z 76.9 [M-H-CO_2] and at m/z 93 [M-H-CO]. t-Cinnamic acid with a retention time of 38.12 min registered molecular ion [M- H] at m/z 146.8 by LC-MS/MS and gave fragment ions at m/z 102.9 [M-H-CO_2] and at m/z 76.9, which corresponds to benzyl cation, [$C_6H_5^+$]. These transitions are presented in Fig. 2.

The results pertaining to contents of different phenolic acids (Table 2) in mother extract and its different chromatographic fractions indicated that different extract/fractions varied considerably in the composition and contents of individual phenolic acids. None of the phenolic acids examined in the present study could be detected in F3 and F4. On the other hand, F5 and F7 fractions showed the presence of only caffeic acid (30.21 μg/g) and p-coumaric acid (128.98 μg/g) respectively. F6 and F9 fractions documented three phenolic acids each and caffeic acid (934.74 μg/g) and

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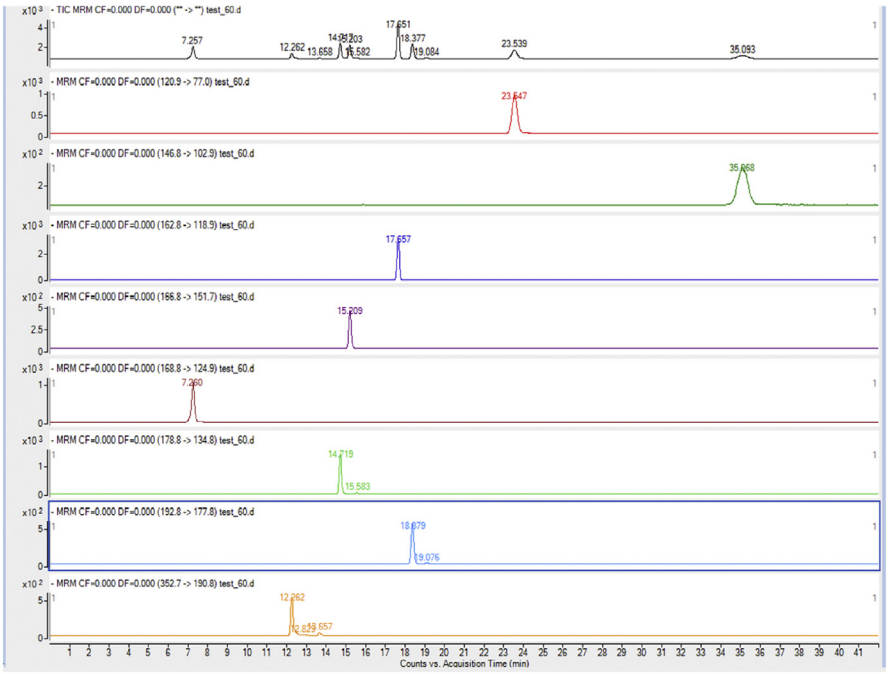

Fig. 2. Multiple reactions monitoring transition (Quantifier) of eight standard phenolic acids.

p-coumaric acid (947.15 μg/g) form the major component respectively. F10 registered two phenolic acids with p-coumaric acid (316.19 μg/g) as major component. All the phenolic acids but t-cinnamic acid could be detected in F8 fraction and benzoic acid (1036.95 μg/g) occurred in highest amount followed by gallic acid (393.40 μg/g) and p-coumaric acid (341.72 μg/g), which together presented a total of 2166.46 μg/g phenolic acids. F6 with total phenolic acid content of 1926.03 μg/g ranked distinct second and F9 with 1879.38 μg/g ranked distinct third. Thus the rank order of F8, F6 and F9 are similar for both total phenol and phenolic acid content (Fig. 3).

3.3. Physico-chemical properties of EC formulations

The solubility of mother extract (M) and its chromatographic fractions (F3-F10) in LSN with >20% a.i. (extract/fraction) was not satisfactory and with <20% a.i. bioefficacy may be poor. Therefore, the optimum a.i. content in EC formulation was 20%. The surfactant (A:B) combination (3:2) was selected as it produced the minimum creamy layer (0.4−1.3 mL) after 0.5−24 hrs (Table 3). The test results of the physico-chemical properties of different EC formulations have been summarized (Table 4). After 2 h, 0.5−0.9 mL creamy layer was estimated which falls within the standard specifications of <2 mL. The volume of foam 6−11 mL after 1 min was much lower than specified maximum value of 15 mL and thereby indicating easy spraying/spreading quality over plant surface preventing material loss and

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Table 2. Phenolic acid profile of chloroform extract fraction of *Clerodendrum* leaves.

Leaf extract/fractions	Phenolic acid content (μg/g)* in leaf extract fractions ± RSD≠								
	Benzoic acid	Caffeic acid	Chlorogenic acid	Gallic acid	P-coumaric acid	T- Cinnamic acid	T-Ferulic acid	Vanillic acid	Total (μg/g)
M	ND	ND	ND	ND	ND	ND	ND	ND	ND
F3	ND	ND	ND	ND	ND	ND	ND	ND	ND
F4	ND	ND	ND	ND	ND	ND	ND	ND	ND
F5	ND	30.21 ± 2.85	ND	ND	ND	ND	ND	ND	30.21
F6	40.7± 2.55	934.74 ± 0.13	910.31± 0.20	ND	40.23± 4.30	ND	ND	ND	1926.03
F7	ND	ND	ND	ND	128.9 ± 0.99	ND	ND	ND	128.98
F8	1036.95 ± 0.17	148.02 ± 0.61	36.33± 2.96	393.40 ± 0.56	341.± 0.30	ND	44.49 ± 1.19	165.53 ± 0.62	2166.46
F9	160.93± 0.63	ND	709.86± 0.29	ND	947.15 ± 0.05	ND	61.44± 1.64	ND	1879.38
F10	ND	70.86 ± 1.92	ND	ND	316.19 ± 0.34	ND	ND	ND	387.05

ND = Not detected.

* Data are average of three replications.

≠ values in parentheses indicate relative standard deviation (RSD)

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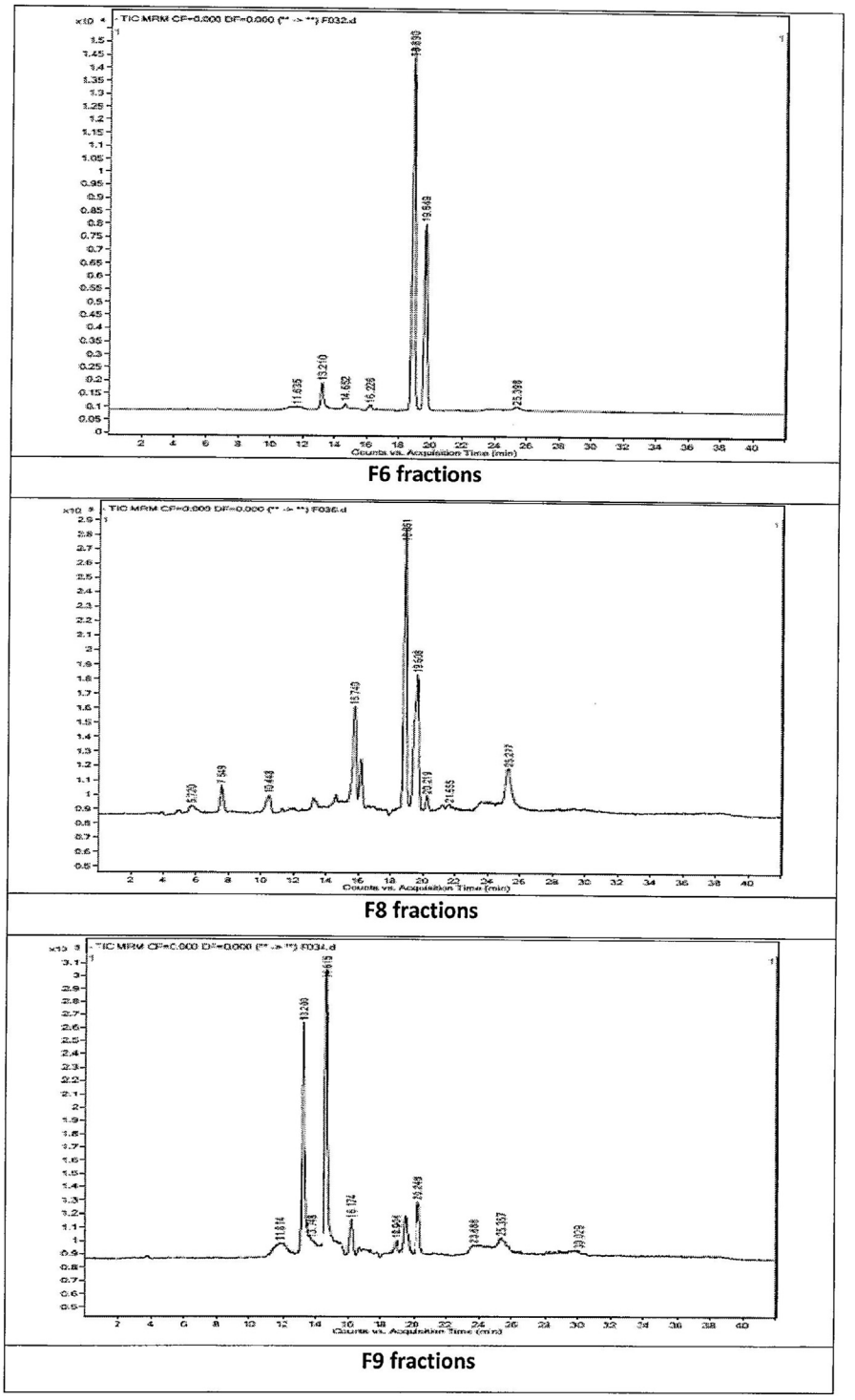

Fig. 3. LC-MS/MS chromatograms of most active fractions of *Clerodendrum* leaves.

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Table 3. Emulsion stability of *Clerodendrum* chloroform extract (20% EC).

Time (hr)	Formation of creamy layer (ml) using different surfactant ratio					
	A:B = 5:0	A:B = 4:1	A:B = 3:2	A:B = 2:3	A:B = 2.5:2.5	A:B = 0:5
0	0	0	0	0	0	0
0.5	0.8	0.7	0.4	0.6	0.7	1.1
2.0	1.4	0.9	0.7	1.2	0.9	1.8
24	4	3.4	1.3	3	3.5	4.2

A = N-alkaline sulfonate; B = K-alkaline sulfonate.

Table 4. Test results of the physico-chemical properties of EC formulation.

Parameters		Recommended Specifications (WHO and FAO, 2016)	Observed values in different EC formulation of extract (M) and fractions (F3 − F10)								
			M	F3	F4	F5	F6	F7	F8	F9	F10
Emulsion stability after	0 hrs	Initial emulsion completed	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	0.5 hrs	'Cream', maximum: <1 mL	0.4	0.3	0.4	0.5	0.5	0.4	0.3	0.5	0.6
	2 hrs	'Cream', maximum: <2 mL	0.7	0.5	0.6	0.8	0.7	0.7	0.5	0.8	0.9
	24 hrs	'Cream', maximum: <2 mL	1.3	1.1	1.5	1.7	1.3	1.5	1.2	1.6	1.8
Foaming		Maximum of 15 ml after 1 minute	6	8	7	8	7	10	6	9	11
pH value		6.0 to 8.0 (in 1% aq. dispersion)	6.53	6.53	6.32	6.23	6.89	6.88	6.91	6.53	6.51
Storage Stability in 10 ± 2 °C, 30 ± 2 °C, 54 ± 2 °C for 14 days		Average active ingredient content shall not be lower than 95%	98.2	98.5	97.4	95.1	98.3	97.6	98.3	95.7	96.1

handling problem (Mulqueen, 2003). The pH values of 1% aqueous solution ranged from 6.23-6.89 beyond that it may cause toxicity towards plant for high acidity or basicity. The a.i. (total phenol) content was higher than 95% at three different temperature conditions (at 10 ± 2 °C, 30 ± 2 °C, 54 ± 2 °C) for 14 days indicating high storage stability or thermal stability. Layer separation was <2 mL after 24 hr which indicates its good ability to form emulsion. Thus EC formulations were considered suitable for further use.

3.4. Bio-efficacy of 20 EC formulations and standard phenolic acids

The growth inhibitory effect of different formulations prepared from mother extract and its different chromatographic fractions on the fungus *P. vexans* was recorded and compared to the control culture plate (Table 5). Growth inhibition (GI) of the fungus was increased with increase in treatment doses (from 50 to 400 μg/mL) for the botanicals as well as for the standard fungicide, mancozeb (5−50

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Table 5. Growth of *Phomopsis vexans* on PDA media against *Clerodendrum* 20 EC and Mancozeb 75 WP.

Leaf extracts/ fractions	Growth inhibition of *Phomopsis vexans* by *Clerodendrum* extracts/fractions (20 EC) at different doses								ED_{50} (μg/mL)
	T_1 (50 μg/mL)		T_2 (100 μg/mL)		T_3 (200 μg/mL)		T_4 (400 μg/mL)		
	Radial growth* (cm)±SD	% inhibition**	Radial growth (cm)±SD	% inhibition	Radial growth (cm)±SD	% inhibition	Radial growth (cm)±SD	% inhibition	
M	3.90 ± 0.10	38.10	2.67 ± 0.05	57.54	1.43 ± 0.05	77.25	0.97 ± 0.05	84.66	74.13
F3	5.03 ± 0.05	20.11	4.90 ± 0.03	22.03	3.57 ± 0.26	43.39	2.78 ± 0.24	55.82	229.09
F4	3.93 ± 0.05	37.57	3.86 ± 0.04	38.83	2.70 ± 0.07	57.14	2.49 ± 0.22	60.42	89.13
F5	3.87 ± 0.23	38.62	2.43 ± 0.09	61.90	1.93 ± 0.05	69.31	0.77 ± 0.12	87.83	60.26
F6	3.53 ± 0.15	43.92	1.37 ± 0.05	78.17	0.90 ± 0.10	85.71	0.48 ± 0.05	92.43	54.95
F7	3.73 ± 0.05	40.74	2.83 ± 0.05	55.56	1.60 ± 0.09	74.60	1.33 ± 0.14	78.84	66.07
F8	3.40 ± 0.17	46.03	1.30 ± 0.08	79.37	0.83 ± 0.10	86.77	0.33 ± 0.02	94.71	46.77
F9	3.53 ± 0.05	43.92	1.37 ± 0.12	78.25	0.87 ± 0.05	86.24	0.43 ± 0.05	93.12	52.48
F10	3.94 ± 0.09	37.51	3.87 ± 0.05	38.62	1.97 ± 0.16	68.78	1.83 ± 0.25	70.90	87.10
Mancozeb	T_1 = 5 μg/mL		T_2 = 10 μg/mL		T_3 = 25 μg/mL		T_4 = 50 μg/mL		
	5.07 ± 0.06	19.58	3.27 ± 0.01	48.10	2.10 ± 0.07	66.67	0.87 ± 0.05	86.24	10.47
Control	6.30 ± 0.14	-	6.30 ± 0.14	-	6.30 ± 0.14	-	6.30 ± 0.14	-	
SEM	0.06		0.04	-	0.05	-	0.04	-	
CD (5%)	0.14		0.11	-	0.16	-	0.12	-	

SEM = Standard error of mean; CD = Critical difference.

* Values are average of three replications.

** Inhibition with respect to control.

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μg/mL). Determination of effective dose for 50% growth inhibition (ED_{50}) indicated that formulations made from fractions F5 through F9 were more potent as compared to that from mother extract (ED_{50} = 74.13 μg/mL). In contrast, the activity of formulations with fractions F3, F4 and F10 were lower than mother extract. Based on ED_{50} values, formulation with fraction F8 exhibited highest antifungal activity (ED_{50} = 46.8 μg/mL) followed by F9 (52.5 μg/mL), F6 (54.9 μg/mL), F5 (60.3 μg/mL) and F7 (66.1 μg/mL). However, none of the formulations were found to be as effective as mancozeb. Moreover, the antifungal activity in terms of ED_{50} value of the formulation was negatively correlated to total phenol (Correlation Co-efficient, r = -0.694), total phenolic acid (r = -0.692) and total flavonoid (r = -0.604) content. Thus, fraction F8 with its highest total phenol and phenolic acid content showed high antifungal activity as evidenced by its lowest $ED_{50.}$ However, F6 and F9, which ranked distinct second and third in terms of their total phenol and phenolic acid content showed reverse order in terms of their ED_{50} values. Thus it seems reasonable to assume that antifungal activity is controlled not solely by total phenol, total flavonoid and phenolic acid content. Composition of phenolic acids and flavonoids in the fractions might play a decisive role for their activity. Growth inhibition (Table 5) contributed by the individual standard phenolic acids indicated that the antifungal activity increased in a dose dependent manner for each of these phenolic acids and each of these phenolic acids was more potent as compared to mancozeb and produced diverse growth inhibition. The similar diverse anti-fungal activities of phenolic acids such as caffeic acid (Ravn et al., 1989), ferulic acid (Shi et al., 2016), cinnamic acid (Lima et al., 2018), salicylic acid (Tawata et al., 1996), Gallic acid (Nguyen et al., 2013) are reported. Additionally, antifungal activity of several flavonoids, including catechin, luteolin, and quercetin, occurring in plant extracts, is also reported by several workers (Nguyen et al., 2013; Alves et al., 2014; Roy et al., 2018; Carvalho et al., 2018). Thus, the diverse antifungal activity associated with different formulations containing varying proportion of different phenolic acids appeared to be related to their lipid solubility that allow phenolic compounds to penetrate the biological membranes while phenolic hydroxyl group with its weak acidic character may act as un-couplers of oxidative phosphorylation in respiration (Wang et al., 1989; Tomas-Barberan et al., 1990). In the present study, benzoic acid that lacks phenolic hydroxyl group and fraction F8 with highest content of benzoic acid displayed maximum antifungal activity. Again, among the standard phenolic acids tested for their potency, t-cinnamic acid without any phenolic hydroxyl group ranked distinct second in terms of activity. Benzoic and t-cinnamic acids have nearly equal lipophilic character and behave as weak acids with pKa value of 4.20 and 4.44 respectively (Kulchan et al., 2015). Thus, at physiological pH, conjugate base form is greater with benzoic than t-cinnamic acid. Thus, benzoate as compared to cinnamate allows electron transport to proceed more effectively without ATP synthesis (Niyogi et al., 2015). Therefore, carboxylic acid group in

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addition to phenolic hydroxyl group can also act as un-coupler of oxidative phosphorylation (Niyogi et al., 2015). In addition, phenolic acids with hydroxyl and carboxylic groups can interact with some functional groups on protein to form complexes with decreased solubility, rendering them less susceptible to proteolytic attack than the same protein alone (Sreerama et al., 2012). This anti-nutrient function of phenolic acids may also be related to growth inhibition of the fungus (Reddy et al., 1985).

4. Conclusion

The present study aimed to develop a plant based pesticidal formulation for production of food free from toxic chemical pesticide residues. The EC formulations made with chromatographic fractions (F8, F9 and F6) of *Clerodendrum* leaf extract exhibited significant antifungal activity against *Phomopsis vexans*, a pathogen of brinjal. Moreover, the antifungal activity associated with different fractions seems to be related with phenolic composition of the fractions concerned. The potency of botanical formulations such as F8, F9 and F6 were less than macozeb, but could be a valuable guide to improve further the potency of the formulation.

Declarations

Author contribution statement

Ramen Kole: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Soumen Saha: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ayan Mukherjee, Subrata Biswas: Analyzed and interpreted the data.

Debjani Choudhury: Performed the experiments.

Jayanta Saha, Srikumar Pal, Mitali Sarkar: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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