Optimization of total phenolic extraction of *Chromolaena odorata* leaf for antifungal activity against plant pathogens

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ABSTRACT

Phenolic compounds are secondary metabolites mainly responsible for different pharmacological activities of the plant extracts. In this study, microwave-assisted extraction (MAE) variables were optimized for the extraction of the phenolic compounds from *Chromolaena odorata* (*C. odorata*) by using a central composite design (CCD) of response surface methodology (RSM). The optimized conditions were at 62°C for 3 min with solvent to feed ratio of 14:1 (mL/g) and ethanol concentration of 50% (v/v). The highest yield of total phenolic compounds was 75.76 mgGAE/g. Ethanol extract clearly showed antifungal activity through antifungal index. The antifungal effect *Phytophthora capsici*, *Fusarium oxysporum* and *Colletotrichum siamese* were 79.07%, 78.66% and 78.42%, in 0.5% of ethanol extract, respectively.

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1. Introduction

Chromolaena odorata (*C. odorata*) belongs to the Asteraceae family, its chemical composition contains many flavonoid compounds including flavones, flavonols, flavanons and chalcones. Flavonoids are the decisive ingredients for the biological activities which were proved to have strong antioxidant activity, combined with anti-inflammatory effects have increased effectiveness in wounds. *C. odorata* leaves also contain a high amount of essential oils. The main ingredients of *C. odorata* essential oil include: α-pinene, β-pinene, D-limonene, β-ocimene, Caryophyllene, Pregeijerene, Germacrene-D, δ-cadinene, α-copaene, Caryophyllene oxide, δ-humulene... (Moses et al., 2010). In addition, *C. odorata* also contains long-chain or cyclic polyunsaturated fatty acids. Phenolic compounds were metabolic products of arachidonic acid that were considered defense weapons against plant pathogens. Adeyemo (2018) reported that ethanolic extract of *C. odorata* showed the highest antifungal effects against

Phytophthora megakarya, followed by cold-water extracts. Zones of clearance range were from 15.00 mm to 32.00 mm for ethanolic extraction while cold water extract was from 5.00 mm to 30.00 mm at concentration from 1% to 5%.

Microwave-assisted extraction has been employed to recover phenolic compounds from *C. odorata* leaves. The MAE is a novel, unconventional extraction method that is more efficient in recovering quality yields with reduced extraction time and less solvent (Moses et al., 2010). The MAE is affected by different factors including microwave power, temperature, irradiation time, solvent concentration, and solvent to feed ratio. To optimize these factors, response surface methodology (RSM) was used as a statistical method to generate the predictive mathematical model that takes into account possible interactions between the factors under consideration (Khuri & Mukhopadhyay, 2010). A central composite design (CCD) had been employed herein because it is one of the most efficient types of RSM and is useful in avoiding experimental processes under extreme conditions, which can result in unsatisfactory results.

Although *C. odorata* has been used as a medicine in Vietnam, there are not many studies on optimizing the process of obtaining phenolic-rich extract from *C. odorata*, especially biodynamic agriculture in general, biopesticides for plant pathogenic fungi in particular. Dwijendra (2014) mentioned that the use of chemical pesticides have negative impacts on animals, humans, even the whole ecosystem. Therefore, one of the proposed solutions is to use compounds from plants, especially phenolic. These compounds are not only effective in controlling diseases in plants, but also sustainable with the ecosystem. According to Kumlachew et al. (2014), using *Ruta chalepensis* extract to inhibit the growth of anthracnose *Colletotrichum gloeosporioides* on mango. The extract reduced the development of the disease to less than 36%, while for the control the level of damage was 93.4%. Therefore, this study investigated the optimization of phenolic compounds from *C. odorata* leaves and analyzed inhibition of three plant pathogenic fungi strains. MAE is used to extract phenolics compounds from *C. odorata*. Extraction conditions including temperatures, times, ethanol concentrations and solvent to feed ratio were optimized using RSM at CCD. Then, the antifungal activity of plant extract against *P. capsici*, *F. oxysporum* and *C. siamense* was examined.

2. Materials and Methods

2.1. Plant materials, chemicals, and reagents

Fresh *C. odorata* was collected at the Research Institute for Biotechnology and Environment (RIBE), Nong Lam University Ho Chi Minh City. The leaves were washed with tap water, dried at 40°C for 3 days, pulverized to powder form, and screened to a particle size of 0.1 mm. The powdered sample was stored in a dark container at (25°C ± 2). The moisture content of the plant sample before extraction was lower than 12% water per dry sample.

Fungal strains including *P. capsici*, *F. oxysporum*, *C. siamense* were provided by the Research Institute of Biotechnology and Environment, Nong Lam University, Ho Chi Minh City.

Ethanol, Folin-Ciocalteu reagent, gallic acid, and sodium carbonate were purchased from Sigma Aldrich.

2.2. Phytochemical screening

2.2.1. Alkaloids

The presence of alkaloids in the ethanolic extract was examined by Dragendroff's test. About 1 mL of extract was taken and added 3-5 drops of Dragendorff's. Dragendorff's reagent was prepared by 1 mL mixing solution-1 containing 0.85 g of bismuth (III) nitrate and 10 mL acid acetic in 40 mL of water, 1 mL solution-2 containing 8 g potassium iodide in 20 mL water to a solution of glacial acetic acid (4 mL) in deionization water (14 mL). If orange-red precipitate is formed after 3 min, indicating the presence of alkaloids in the crude extract.

2.2.2. Saponins

The presence of saponins in the ethanolic extract was examined by Froth test. About 0.5 mg of the extract was mixed with 5 mL of distilled water in a test tube and vigorously shaken for 2 min. Foam which persisted for 30 min and doesn't disappear upon warming indicates the presence of saponins in the extract.

2.2.3. Flavonoids

The presence of flavonoids in the ethanolic extract was examined by Shinoda Test. About 10 mg of extract was added to pinch of magnesium and 3 drops of concentrated hydrochloric acid was added. Formation of orange-pink colour indicates the presence of flavonoids.

2.2.4. Phenolic compounds

The presence of phenolic compounds in the ethanolic extract was examined by lead acetate test. About 10 mg of extract was taken and 0.5 mL of 1% lead acetate solution was added and the formation of precipitate indicates the presence of tannins and phenolic compounds.

2.2.5. Tannins

The presence of tannins the ethanolic extract was examined by ferric chloride test. About 0.5 mg of the extract, ferric chloride 5% a solution was added. Formation of dark blue precipitate indicates the presence of tannins.

2.3. MAE of *C. odorata* leaves

In all the above experiments, whether the dried *C. odorata* leaves have uniform size, were sifted through the ϕ1 mm sieve. The weight of material for several extracts was 1 g. The extraction was conducted in a microwave-assisted separator (QLABPro Closed Vessel Microwave Digestion) at different temperatures, times, ethanol concentration and solvent to feed ratio. The filtrate was obtained after centrifugation at 25°C, for 15 min (Hermle). Then used for determination of phenolic contents.

2.4. Determination total phenolic compounds

Total phenolic compounds (TPC) was determined by the method of Alara et al (2019a) with minor modifications. 30 µL of *C.odorata* extract was mixed with 100 µL of Folin-Ciocalteu reagent and 3000 µL of deionization water for 5 min, then 300 µL of 20% Na_2CO_3 was added to the mixture, filled up to 5 mL. The reaction mixture was kept in a dark room for 120 min and the absorbance was measured at 735 nm using a UV/Vis spectrophotometer (Agilent, California). Gallic acid was used as a standard in the determination of TPC from *C. odorata* leaves (gallic acid equivalents; GAE). The concentration of *C. odorata* leaf extract was calculated in relation to the gallic acid standard curve. Then, TPC was calculated by Eq. (1). The analysis was repeated three times and the results were expressed as mean ± standard deviation.

$$\text{TPC} = \frac{\text{A}}{\text{Weight of dried sample used (g)}} \quad (1)$$

with:

A: Concentration (mg/mL) × volum of solvent used (mL)

2.5. Experimental design and statistical analysis

Optimization of total phenolic content was carried out using a CCD of RSM. Experimental design includes five levels of 4 independents variables: irradiation time (2 – 4 min), extraction temperature (50 – 70°C), solvent to feed ratio (8:1 – 14:1 mL/g), ethanol concentration (40 – 60% v/v) was designed with Minitab 16.1 Statistical Software® for a total of 31 runs. In exploration studies, the marginal values of the extraction factors were determined as shown in Table 1.

A total of 16 experiments at 2 levels (upper and lower), 8 star experiments and 7 center experiments were carried out. Each experiment was conducted three times and average results were obtained. Experimental data were analyzed by MiniTab 16. The mathematical model of the influence of independent variables on the dependent variable has a quadratic polynomial function as follows:

$$Y_k = B_0 + \sum_{j=1}^{4} B_j X_j + \sum_{i,j=1}^{4} B_{ij} X_j + \sum_{j=1}^{4} B_{jj} X_j^2$$

Yk: Dependent variable (k = 1 – 4)

Xi,j: The encoding factor of the independent variable affects Yk

B_0: Regression coefficient of 0 degree

B_j: Regression coefficient of 1 degree affects the variable X_i to Y_k

B_{ij}: Coefficients of simultaneous influence of variable X_j to Y_k

B_{ij}: Regression coefficient of degree 2 describes the effect of variable X_j^2 on Y_k

Table 1. Experimental design-matrix encoding the independent variables

Variable name	Levels of evidence					
Uncoded variable	Code variable	-α	-1	0	+1	+α
A: Irradiation time [min]	U_1	1	2	3	4	5
B: Extraction temperature [°C]	U_2	40	50	60	70	80
C: Solvent:feed ratio [mL:g]	U_4	5	8	11	14	17
D: Ethanol concentration [%]	U_4	30	40	50	60	70

α = 2, U_{min}, U_{max} is the lower bound (-1) and upper bound (+1) of the independent variable, U0 = (U_{min} + U_{max})/2 is the mean value of the upper and lower bound.

2.6. Antifungal activity screening test

2.6.1. Media preparation

Potato glucose agar (PGA) medium containing 20 g D-Glucose, 20 g Agar, 200 g potato and 1000 mL distilled water were prepared. After sterilization and addition of streptomycin (0.1%), the medium was poured in a petri dish.

2.6.2. Fungal preparation

Three fungal strains including *P. capsisi*, *F. oxyporum* and *C. siamense* were inoculated on PGA medium with antibiotic supplementation at a temperature of 25°C ± 1, monitored for 5 - 7 days until fungal filaments overgrown agar plates.

2.6.3. Transplantation and Mycelial growth

Fungal plates were prepared using a disc of 9 mm diameter carrying mold spores on the surface of a petri dish containing highly enriched PGA medium of different concentration of *C. odorata* (0.1%, 0.2%, 0.3%, 0.4%, 0,5% v/v). The plates after inoculation were incubated at 25°C ± 1. Mycelium growth was observed and evaluated until the mycelium had grown to the plate of the non-enriched medium by measuring the mean of the two perpendicular diameters passing between the plates. Antifungal activity was evaluated by the formula (Chang et al., 2000):

$$I = \left(1 - \frac{d}{dc}\right) \times 100$$

where I: antifungal index, d: diameter of petri dish treated with the extracts, dc: diameter growth of the control.

2.6.4. Data analysis

Data was analysis of variance (ANOVA). These values are expressed as the mean ± standard deviation of a triplicate measurement.

3. Results and Discussion

3.1. Phytochemical of *C. odorata* leaves extract

The results obtained from phytochemical screening show that alkaloid, flavonoid, tannin, phenol and saponin were detected in all the extracts irrespective of the solvent. (Table 2). Generally, ethanol extraction has better efficiency than water extraction. Due to ethanol is considered a universal solvent, as its molecular structure allows for the dissolving of both polar and nonpolar compounds (Mark, 2018).

Table 2. Active ingredients tested in extracts of *C. odorata*

Active agents	Water extract	Ethanol extract
Alkaloids	+	++
Flavonoids	+	++
Phenolics	+	++
Tannins	+	++
Saponins	++	+

++ moderately detected; + Detected but weak.

3.2. Effect of extraction conditions on phenolic compounds

The MAE process parameters were optimized to maximize TPC from *C. odorata* leaves (Table 3). In addition, the impact of each MAE parameter and their interactions were examined. The effects of extraction factors on the TPC were presented in the following function:

Y_{TPC}= -490.265 + 43.762A + 7.284B + 20.046C + 5.099D + 0.117BC - 5.715A^2 - 0.069B^2 - 0.932C^2 - 0.049D^2

Table 4 shows the analysis of variance

Table 3. Experimental results of total phenolic compounds (TPC)

No.	Microwave-assisted extraction variables				TPC [mgGAE/g]
	A: Irradiation time [min]	B: Extraction temperature [°C]	C: Solvent:feed ratio [mL/g]	D: Ethanol concentration [%]	
1	3	40	8	40	23.97
2	5	40	8	40	36.27
3	3	60	8	40	11.16
4	5	60	8	40	25.2
5	3	40	14	40	56.79
6	5	40	14	40	62.82
7	3	60	14	40	62.35
8	5	60	14	40	70.74
9	3	40	8	60	24.72
10	5	40	8	60	38.57
11	3	60	8	60	13.08
12	5	60	8	60	25.17
13	3	40	14	60	57.97
14	5	40	14	60	63.11
15	3	60	14	60	62.13
16	5	60	14	60	71.10
17	2	50	11	50	39.12
18	6	50	11	50	59.18
19	4	30	11	50	47.09
20	4	70	11	50	42.05
21	4	50	5	50	00.87
22	4	50	17	50	78.82
23	4	50	11	30	51.44
24	4	50	11	70	53.47
25	4	50	11	50	72.09
26	4	50	11	50	71.99
27	4	50	11	50	70.24
28	4	50	11	50	72.24
29	4	50	11	50	71.60
30	4	50	11	50	70.72
31	4	50	11	50	71.61

(ANOVA) results and coefficients for TPC. The experimental data were fitted to a quadratic model. The P-value less than 0.05 indicated that the model was statistically significant. The irradiation time, extraction temperature, solvent to feed ratio and ethanol concentration all showed a significant effect ($P < 0.05$) on the yield of TPC. The P-value of the model of incompatibility test (lack to fit) is 0.087, greater than 0.05. Therefore, the regression model is compatible with the experiment.

The analytical results showed that four factors significantly affect the phenolic extraction process. This result was consistent with the general trend of the extraction of bioactive compounds from plant materials (Alara et al., 2019a). The results also showed that all four extraction factors including irradiation time, extraction temperature, solvent to feed ratio and ethanol concentration interacted with each other and affected the target function. Specifically, the effect of irradiation time, extraction temperature, ethanol concentration increased gradually to a critical value and if it continued to increase, it will decrease the overall value of the target function was decreased (Figure 1a, 1c, 1e). Higher quality phenolic compounds are mostly obtained from the plant matrix by using a binary solvent mixture (ethanol-water) because it depends on the polar-

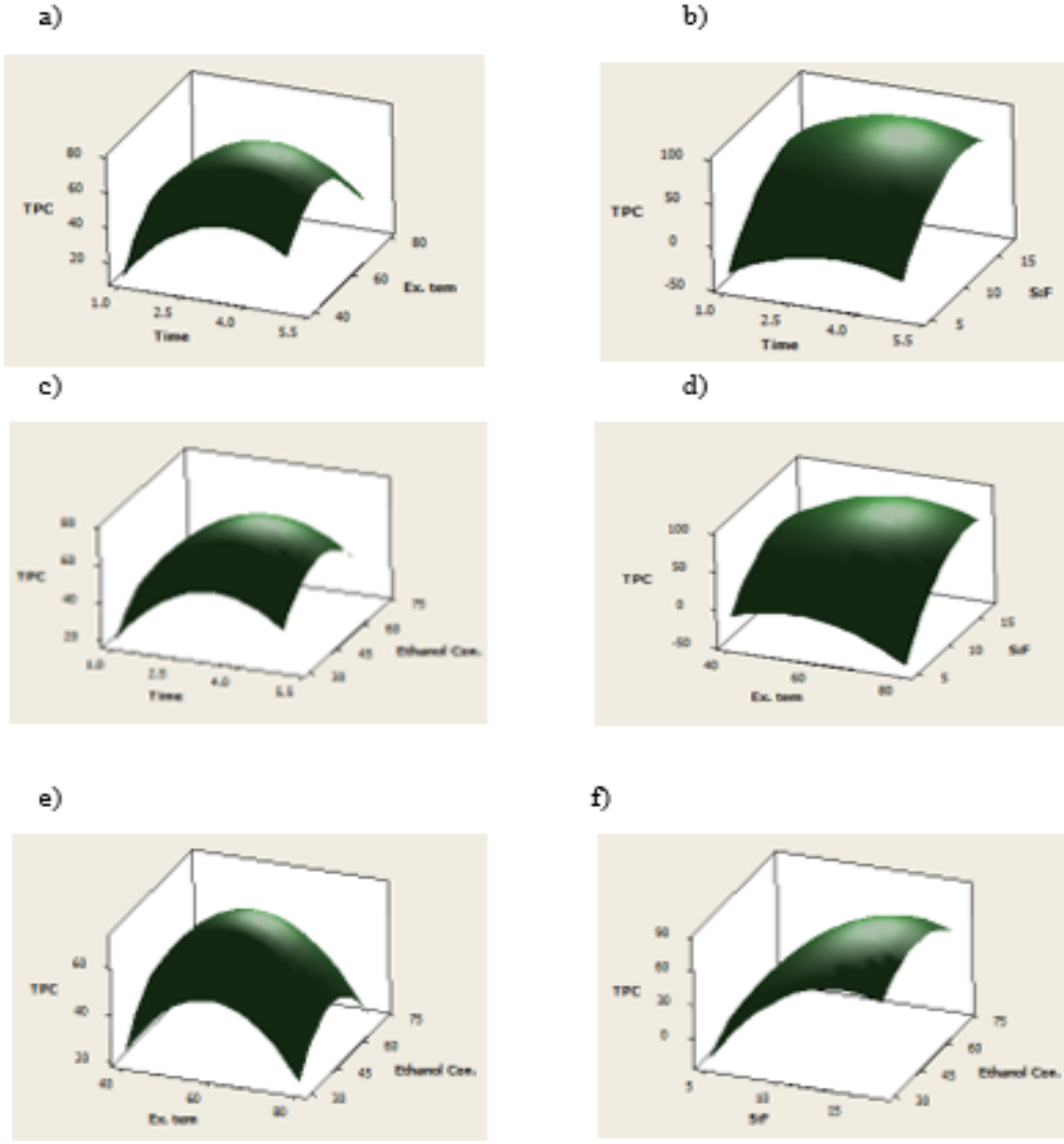

Figure 1. Response surface plots showing the influences of microwave-assisted extraction parameters on phenolic compounds. (a) irradiation time (A) versus extraction temperature (B) for total phenolic compounds (TPC); (b) A versus solvent:feed ratio (C) ; (c) A versus ethanol concentration (d) B versus C for TPC; (e) B versus D for TPC; (f) C versus D for TPC.

ity of solvents used (Alara et al., 2019a). Meanwhile, solvent to feed ratio has a positive effect on the target function (Figure 1b, 1d, 1f). Thus, from the results obtained it can be seen that within the research scope, when increasing irradiation time, extraction temperature, solvent:-feed ratio, ethanol concentration to an appropriate value will increased the value of the objective function. If an increase exceeded the optimal value, it decreased the value of the target function. For solvent feed ratio when reaching the optimum value, it did not increase the value of the target function if it continued to increase. The optimized conditions of TPC might be due to the polarity of the solvent used in extraction. A previous report showed that ethanol and water were environmentally friendly solvents that could extract a higher quantity of phenolic compounds

Table 4. Effects of the extraction factors on the target function by analysis of variance

Variable	df	TPC [mgGAEgDW^{-1}]	
		F value	P-value probability > F
Model	14	894.28	0.000
Linear			
Irradiation time (A)	1	302.01	0.000
Extraction temperature (B)	1	18.80	0.000
Solvent:feed ratio (C)	1	619.34	0.000
Ethanol concentration (D)	1	539.18	0.000
Interation			
AB	1	9.05	0.008
AC	1	1.56	0.230
AD	1	0.83	0.375
BC	1	183.76	0.000
BD	1	2.85	0.111
CD	1	0.08	0.785
Quadratic			
A^2	1	876.83	0.000
B^2	1	1263.56	0.000
C^2	1	1887.42	0.000
D^2	1	641.62	0.000
Lack to fit		3.14	0.087
R^2		99.87%	
Adjust R^2		99.76%	
Predicted R^2		99.35%	

from the plant matrix. In a similar study, Alara et al. (2019b) obtained a TPC of 88.52 mgGAE/g from *C. odorata* leaves under MAE optimized conditions of 51% (v/v) ethanol concentration, irradiation time of 3 min, temperature of 60°C and solvent-to-material ratio of 10:1 (mL/g).

In addition, the predictive models were verified by comparing the predicted and experimental data. The predicted conditions were highest at 62°C for 3 min with solvent to feed ratio of 14:1 (mL/g) and ethanol concentration of 50% (v/v). The results showed that the experimental values for TPC (75.76 mgGAE/g) did not vary significantly at the 5% level by using a paired t-test. Therefore, the regression equations derived from this study can be employed to optimize the extraction of phenolic compounds from *C. odorata* leaves.

3.3. Evaluate the antifungal ability of *C. odorata* extract

Total phenolic compounds of *C. odorata* leaves were extracted at optimum conditions then were evaporated to remove ethanol and prepared at 5 different concentrations, for evaluation of the antifungal activity against *P. capsici*, *F. oxysporum* and *C. siamense* (Table 5).

Antifungal effects increased with the increasing concentration of *C. odorata* extract from 0.1 to 0.5%. Maximum antifungal effects against *P. capsici* was found to be 79.65% in 0.5% ethanol extract clearly (Figure 2). For *F. oxysporum*, the ability to inhibit fungi at concentrations of 0.1%, 0.2% and 0.3% was not statistically significant between treatments, but at concentrations of 0.4% and 0.5% differed significantly between treatments (Figure 3). Similarly, *C. siamense*'s antifungal effect of the extracts was varying such as: (Table 4) 0; 41.67; 49.67; 56.67; 63.83; 65.76 mm at 0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% respectively. Results showed that at all concentrations was a statistically significant difference (Figure 4). The interaction of polyphenols with nonspecific forces such as hydrogen bonding and hydrophobic effects, lipophilic forces, as well as by covalent bond formation, was related to microbial membranes, adhesions, enzymes, and cell envelope transport proteins (Cowan 1999; Kumar & Pandey 2013). The antibacterial activity of polyphenols may be

Table 5. Antifungal index (%) of *C. odorata extract*

Concentration (% v/v)	Antifungal index (%)		
	P. capsici (3 days)	*F. oxysporum* (5 days)	*C. siamense* (5 days)
NC	00.00^{e}	00.00^{d}	00.00^{f}
0.1%	61.63^{d}	58.23^{c}	53.33^{e}
0.2%	69.19^{c}	58.23^{c}	61.75^{d}
0.3%	75.58^{b}	58.54^{d}	69.12^{c}
0.4%	79.07^{a}	68.90^{b}	76.67^{b}
0.5%	79.65^{a}	78.66^{a}	78.42^{a}

In the same column, there is a statistically significant difference in values with different characters with $P < 0.05$. The percentage is converted by the formula $y = \sqrt{(x + 0.5)}$ before analysis ANOVA.

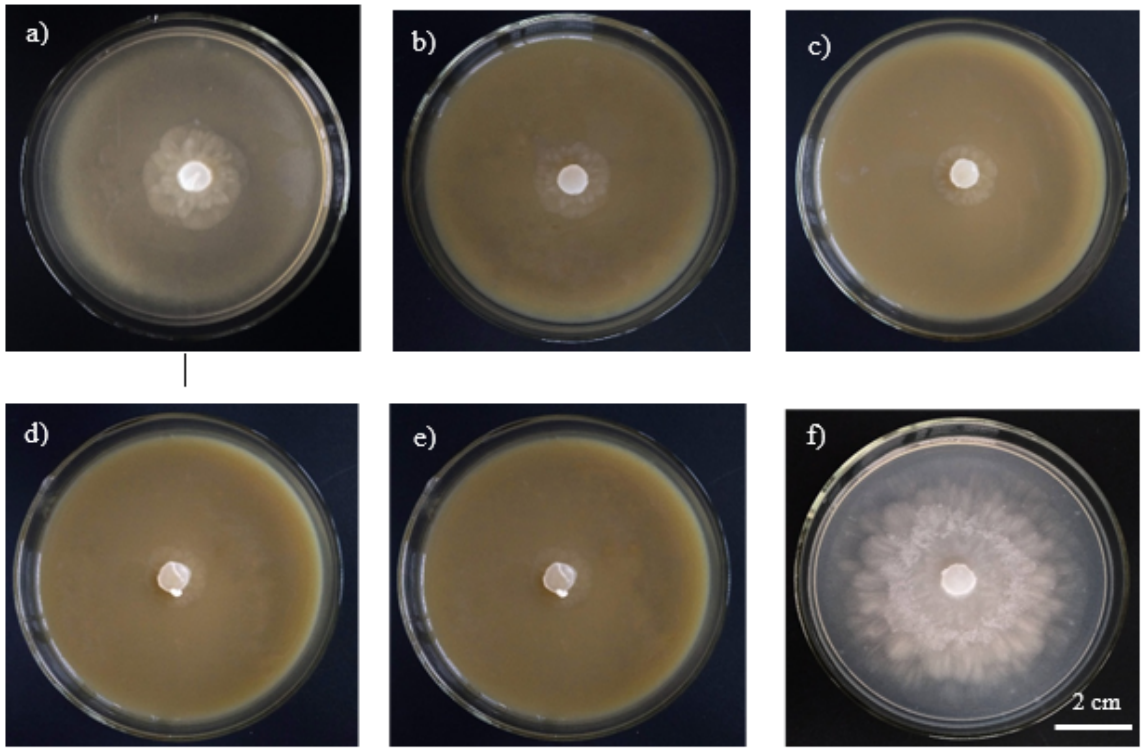

Figure 2. The antifungal ability of *C. odorata* extract against *P. capsici* at different concentration (a) 0.1% v/v; (b) 0.2% v/v; (c) 0.3%; (d) 0.4% v/v; (e) 0.5% v/v; (f) Negative Control (NC).

also due to the capacity of these compounds to chelate iron, vital for the survival of almost all bacteria (Field & Lettinga, 1992). Borges et al. (2013) reported that gallic and ferulic acids induce irreversible changes in *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* membrane properties. Ting (2013) investigated the antibacterial activity and membrane interaction of 5 flavonoids (kaempferol, quercetin, chrysin, luteolin, baicalein), 2 polymethoxyflavones (tangeritin, 5,6,7,4'-tetramethoxyflavone), and 4 isoflavonoids (daidzein, genistin, ononin, puerarin) against *E. coli* and found that the antibacterial activity decreased in the following order: flavonoids > polymethoxyflavones > isoflavonoids. They also found that flavonoids rigidified the liposomal membrane, while the other compounds increased membrane fluidity. They suggested that the interaction of gallic and ferulic acids with bacterial cytoplasmic membranes causes hydrophobic changes, a decrease in negative surface charge, and local rupture and pore formation with leakage of intracellular constituents. The studies in the last decade have shown that phenolics with an OH group at the position 3 in the C-ring decrease the mem-

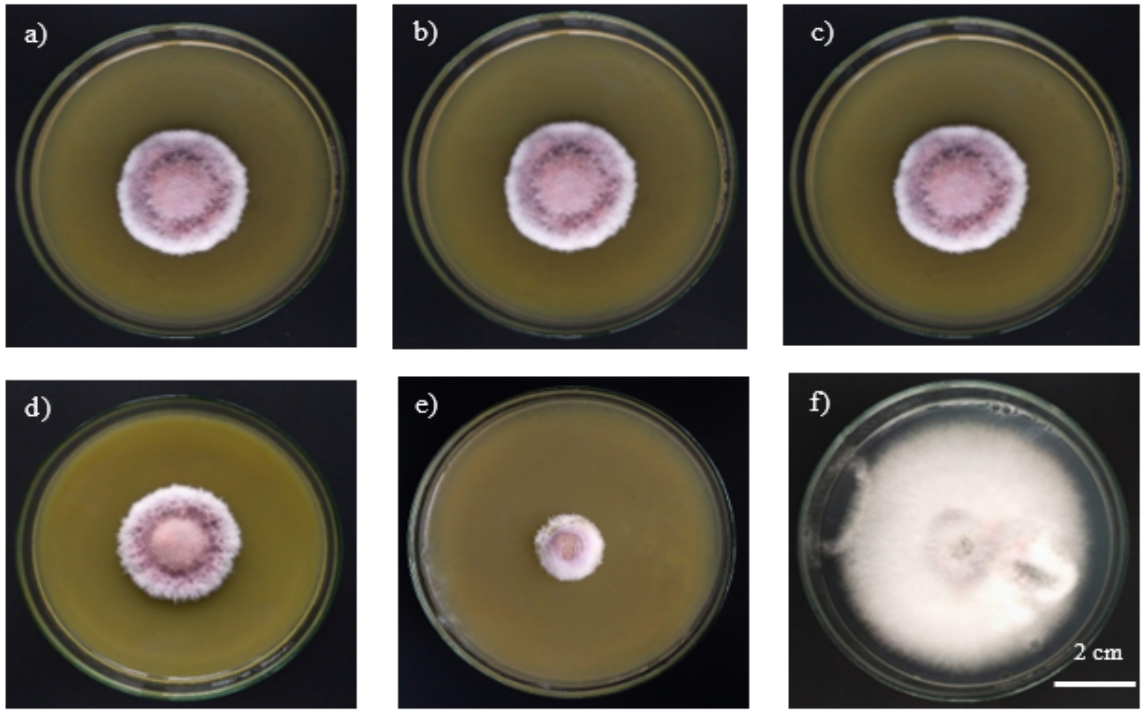

Figure 3. The antifungal ability of *C. odorata* extract against *F. oxysporum* at different concentration (a) 0.1% v/v; (b) 0.2% v/v; (c) 0.3%; (d) 0.4% v/v; (e) 0.5% v/v; (f) Negative Control.

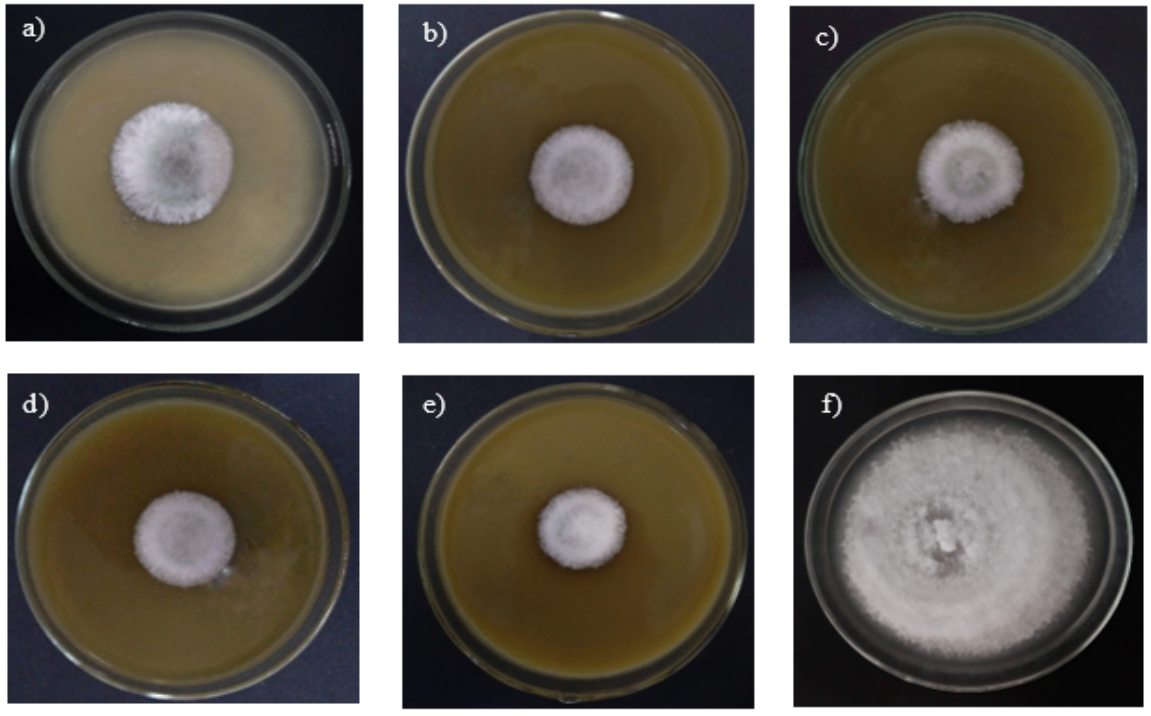

Figure 4. The antifungal ability of *C. odorata* extract against *C. sianmense* at different concentration (a) 0.1% v/v; (b) 0.2% v/v; (c) 0.3%; (d) 0.4% v/v; (e) 0.5% v/v; (f) Negative Control.

brane fluidity leading to membrane rigidification (Tsuchiya, 2010; Ting, 2013). Many studies have used membrane models to elucidate the interaction of polyphenols with the phospholipids of the bilayer lipid membrane. In addition, *F. oxysporum* YT1 and PT1 were cultured in vitro in the presence of *C. odorata* leaf extract. Mycelium growth was reduced at concentrations of 20, 30 and 40 g/L but stopped at 50 g/L. (Kra, 2009). The above was resulted contribute to affirm that *C. odorata* leaves extract has good antifungal ability as reported by Adeyemo (2018) using *C. odorata* extract by maceration to inhibit *P. megakarya* from 15.00 mm to 32.00 mm at concentrations of 1% and 5% respectively for positive control 41.00 mm.

4. Conclusions

Optimal total phenolic compounds TPC of *C. odorata* leaves was 75.76 mgGAE/g at 62°C for 3 min with solvent to feed ratio of 14:1 (mL/g) and ethanol concentration of 50% (v/v). Ethanol extract clearly showed antifungal activity through antifungal index. The highest antifungal effect on *P. capsici*, *F. oxysporum* and *C. siamense* were 79.65%, 78.66% and 78.42% in 0.5% of ethanol extract, respectively. The findings demonstrated the potential of using *C. odorata* leaf extract as a source of biopesticide production, not only the environment but also the ecosystem and biodiversity.

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Conflict of interest declaration

The authors have declared no conflict of interest.

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