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Original article

Antifungal potential of Colchicum luteum and determination of colchicine content using HPLC for application as a fungicide

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ABSTRACT

Colchicum luteum L. is an economically important medicinal plant species of the North Western Himalaya. The corm extract of this plant is utilized in the treatment of many diseases. It is also used extensively in plant breeding program for the doubling of chromosomes. The antifungal activities of aqueous and ethanolic extracts of the corm of the medicinal plant *Colchicum luteum* were investigated in this study. These extracts were tested against the fungi, *Colletotrichum* sp. and *Fusarium oxysporum*. The ethanolic extract of corm (100 mg mL^{-1}) inhibited *Colletotrichum* sp. and *F. oxysporum* with inhibition zones of 19.2 mm and 18.3 mm, respectively, whereas the aqueous extract of corm (100 mg mL^{-1}) inhibited *Colletotrichum* sp. and *F. oxysporum* with inhibition zones of 17.1 mm and 15.2 mm, respectively. Colchicine was isolated from several plant components (corms, seeds, leaves, and flowers) and its concentration was determined using high-performance liquid chromatography (HPLC). The corm had the highest percentage of colchicine (0.191% ± 0.036) of all the plant parts examined, followed by the seeds (0.103% ± 0.021). As a result, it can be said that the corms and seeds of *C. luteum* could serve as a natural source of colchicine for the pharmaceutical industry.

1. Introduction

Medicinal herbs are generally used in the industrial production of antibiotics as well as in the preparation of traditional remedies by the local population in rural areas of Kashmir, India (Hamilton, 2004; Shinwari et al., 2014; Bano et al., 2018; Bao et al., 2021; Rather et al., 2022). The utilization of herbal plants in treatment of several diseases like burns, dermatophytes and infectious diseases has good healing results in the population. The most important weapons in fighting fungal and bacterial infections are the use of antibiotics in community (Matuska-Łyżwa et al., 2021; Wani et al., 2018b). However, the antimicrobial biopesticides have caused a dramatic change not only of the treatment of infectious diseases (Dafale et al. 2020; Lees et al., 2021: Villamizar et al., 2021). Scientists are increasingly concerned about the discovery of new antibiotic medicines against resistant infections (Spellberg et al., 2004; Uribe-Gutiérrez et al., 2021: Sala et al.,2021) The research for internal or online FDA databases has calculated the number of new antimicrobial agents authorized from 1980 till date (Wani et al., 2018a; Malik et al., 2018; Bano et al., 2021; Dullah et al., 2021). The identical analysis was carried out for newly licensed antivirals, antifungals and antibacterial from 1998 till date. Medicinal plants are increasingly reporting antimicrobial

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capabilities from many regions of the world (Senthilkumar and Reetha, 2009; Wani et al., 2018d; Wani et al., 2018c).

Colchicum luteum generally called as 'Suranjan-e-Talkh' in local language Urdu, belongs to the *Liliaceae* family (Shinwari and Gilani, 2003)". The total of 31 different alkaloids have been isolated from this genus and colchicine being the major bioactive compound. The alkaloid colchicine is used to treat the Behçet's disease and the Alzheimer's disease, treatment of rheumatism, gout, antioxidants, doubling of chromosomes (Aisen et al., 2001; Ahmad et al., 2006; Ahmad, 2010). *C. luteum* is also used widely to treat gout, rheumatism, hepatitis and spleen illnesses and blood cleansers (Chopra et al., 1986; Shinwari et al., 2003). To alleviate the world's dependency on pesticide, alternative such as biopesticide should be considered. Therefore, keeping in mind the enormous antimicrobial potential of *C. luteum,* this study was undertaken to find a natural source with antifungal potential against plant pathogenic fungi, *Fusarium oxysporum* and *Colletotrichum sp*. These pathogens cause wilt, rot, anthracnose, which affects sweet potatoes, tomatoes, legumes, melons and bananas. These fungi also cause red rot of coffee berry, sugar cane, coffee berry, crown rot of banana, and strawberry and brown blotch of cowpea (Hyde et al., 2009; Sumner, 2018; Cannon et al., 2012; Okungbowa and Shittu, 2012; Volcao et al., 2021). As far as the authors' knowledge, the antimicrobial properties of *C. luteum* extracts have not been investigated before this study on these to fungal strains, i.e, *Fusarium oxysporum and Colletotrichum* sp. using this particular plant extract.

2. Materials and methods

2.1Plant collection and extraction

Colchicum luteum plants were gathered from selected areas of Kashmir Himalaya located in the western Himalayas (33.27778° N, 75.3412°E), onset of March-April in the year 2018 and 2019. The identification of the collected plants was done by Dr. Haleema Bano, Assistant Professor at (Division of Environmental Sciences, SKUAST-Kashmir, Shalimar, J&K, and India). The plants were divided into different parts (corms, seeds, leaves, and flowers) and preserved at −2 °C. Corms of C. luteum were shade dried at 28 ± 3 °C. Then, they were milled by pestle and mortar until obtaining a fine powder with a particle size of 180 μm) and dissolved in ethanol and water (80:20). The extracts so obtained were filtered through four cloth layers of muslin for 20 min. The concentrated solid extracts were obtained by means of a rotary evaporator under reduced pressure. Total of five different concentrations viz., (10, 30, 50, 80 and 100 mg mL^{-1}) of both concentrated solid extracts were prepared by dissolving them in 10% dimethyl sulfoxide (DMSO) in distilled water. Finally, the produced extracts were kept at 4 °C in a refrigerator for the further examination.

2.2. Test microorganisms

The two fungal species for the antifungal test examination includes *Fusarium oxysporum* and *Colletotrichum* sp. which were obtained from the Division of Plant Pathology, SKUAST-K, Shalimar, J&K, India. The fungal species were preserved on petri plates containing potato dextrose agar (PDA) at 2 °C and sub-cultured every after 2 weeks in biocontrol plant pathology lab SKUAST-K, Shalimar. 'The fungicide Hexaconazole was used as the (positive control) for antifungal evaluation and DMSO as the negative control'.

2.3. Antifungal assessment

The antifungal assessment for ethanolic as well as aqueous extracts were done by the agar well diffusion procedure as defined by Bauer et al. (1966) and Klančnik et al. (2010). An aliquot of 100 μL of standardized inoculum of each tested fungal species was inoculated in sterile molten PDA, homogenized and poured into sterile petri dishes to provide a uniform depth of 4 mm. Inside the laminar hood, petri plates were allowed to solidify and provide uniform structure for wells. So, five wells on the periphery and one well at the center of each petri plate were made using sterile 5 mm diameter cork borers. The wells were filled with 100 μL (10, 30, 50, 80 and 100 mg mL^{-1}) of plant corm extract prepared in 10% (DMSO). The fungicide Hexaconazole (0.5 mg mL^{-1}) was loaded into the middle well (positive control) whereas 10% (DMSO) act as −ve control in an aside Petri dish (Dar et al., 2017). Thereafter, these dishes were incubated at (32 °C for 24–36 h). Finally, inhibition zones (mm) were recorded for each petri plate to determine the biopesticide activity of the of *C. luteum* plant.

2.4. Isolation and extraction of colchicine

Using a Soxhlet instrument, all dried plant parts corms, seeds, leaves and flowers were extracted with ethanol. The plant parts, before extraction, were defatted in petroleum ether before being removed with ethanol in a rotary evaporator. The plant material was extracted in a Soxhlet extractor using an ultrasonic bath for 6 h. The extract was diluted by the distilled water and afterwards partitioned using a conical 500 mL flask into petroleum ether. The chloroform extract was evaporated and the residues re-dissolved and acidified with 3% H_2SO_4 (pH 3–4) and lastly filtered through 0.45 μm filter (Hayashi et al., 1988). The resulting filtrate was the test sample ready for HPLC analysis. To avoid compound oxidation, all extracts were kept out of direct sunlight during the assay.

2.5. Quantification of colchicine by HPLC

The HPLC analysis was performed on an Agilent (1200 series) instrument with a [Sepax C18 column particle size of 5 m (4.6 m m × 250 mm)] and Empower 3 chromatography data software (Waters, Germany), which was equipped with quaternary pumps, a degasser, and a photo-diode-array detector. An Agilent (1200 series) instrument with a [Sepax C18 column particle size of 5 μm (4. 6 mm × 250 mm)]. An acetonitrile (solvent A) was used as the mobile phase and acetic acid in water (solvent B) as the stationary phase. The elution gradient was as follows: (0–7.5 min 10–60% solvent A, 7.5–10 min 60% solvent B, 10–12.5 min 60–100% and 12.5–25 min 10% solvent A). The USP (Standard of drug reference) was used to provide a colchicine reference norm. 'The flow rate was set to 1 mL/minute and the injection volume and column temperature was 40 °C μL and 40 °C, respectively'. The absorbance was monitored at 352 nm represented in Table 1. The quantitative measurements are presented in percent (%) of colchicine and are based on the relative peak positions of standards at a given retention time versus the concentration.

2.6. Statistical analyses

The data was evaluated and statistical tests were performed, by using IBM-SPSS- Statistics (Version 20) software. Three-way

Table 1
The mobile phase and its gradient mode for HPLC analysis of colchicine.

Compound	Mobile Phase	Gradient	λ max
Colchicine	(Solvent A) Acetonitrile (Solvent B) Acetic acid in water	10–60% A (0–7.5 min) 60% B (7.5–10 min)	
		60–100% A (10–12.5 min) 10% A (12.5–25 min)	352 nm

analysis of variance (ANOVA) was used to compare the variation in between the treatments).

3. Results

From all the tested concentrations of the corm aqueous extracts of *C. luetum*, the 100 mg mL^{-1} extract exhibited the highest antifungal activity towards *Colletotrichum* sp. with zone inhibition of 17 ± 0.12 mm followed by *F. oxysporum* with an inhibition zone of 15 ± 0.23 mm. Likewise, the corm ethanolic extracts also showed the highest antifungal activity against *Colletotrichum* sp. and *F. oxysporum* at 100 mg mL^{-1} with inhibition zones of 19 ± 0.29 mm and 18 ± 0.39 mm, respectively. Hexaconazole (positive control; 0.5 mg mL^{-1}) showed an inhibition zone of 32 ± 0.14 mm against *Colletotrichum* sp. and of 20 ± 0.40 mm against *F. oxysporum*. While the negative control showed zero inhibition (10% DMSO), exhibited no antifungal action towards selected tested fungal species in the separate pertiplate (Tables 2 and 3 and Figs. 1–4). At a concentration of 80 mg mL^{-1} of plant corm extract, the aqueous extracts of *C. luetum* showed the antifungal activity towards *Colletotrichum* sp. with an inhibition zone of 15 ± 0.13 mm followed by *F. oxysporum* with an inhibition zone of 12 ± 0.28 mm. Hexaconazole (positive control) on the other hand, showed an inhibition zone of 32 ± 0.2 6 mm against *Colletotrichum* sp and 20 ± 0.40 mm against *F. oxysporum*. While as ethanolic extracts of *C. luetum* showed the antifungal activity towards *Colletotrichum* sp. with an inhibition zone of 17 ± 0.1 mm followed by *F. oxysporum* with an inhibition zone of 15 ± 0.10 mm at a concentration of 80 mg mL^{-1} of plant corm extract. While, Hexaconazole (positive control) showed an inhibition zone of 31 ± 0.26 mm against *Colletotrichum* sp. and 20 ± 0.4 0 mm against *F. oxysporum*. The negative control (10% DMSO) showed no fungal activity against any of the tested fungal strains (Tables 2 and 3 and Figs. 1-4). In general, the ethanolic extract of corms (100 mg mL^{-1}) of *C. luteum* was noted to be more effective than its aqueous extract against all tested fungi (Tables 2 and 3).

Colchicine concentrations in the ethanolic extract from each part of the *C. luteum* (i.e. corm, seed, leaves and flowers) was determined by HPLC (Table 4). The concentration of colchicine concentrations varies among the different plant parts and was found the highest in corms (0.191% ± 0.036) followed by the seeds (0.103% ±0.021) (Fig. 5).

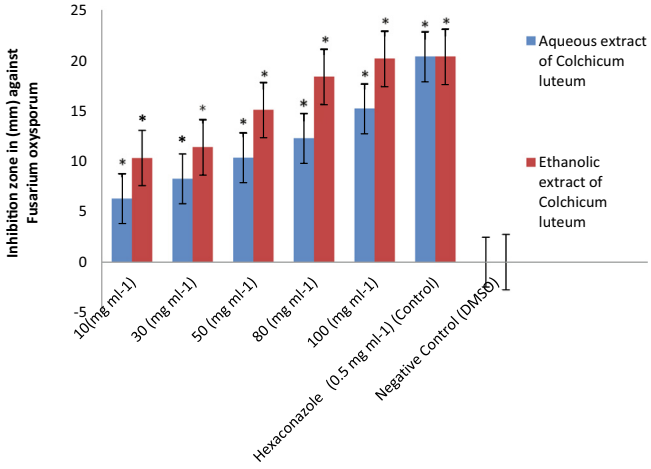

Fig. 1. Antifungal properties of Colchicum luteum against *Fusarium oxysporum*. Error bar indicates SD mean, Values with * are significantly different compared to control (P-value < 0.05).

4. Discussion

Antifungal activities of different concentrations of ethanolic and aqueous extracts of *C. luteum* corm were screened for its antifungal behavior by Ahmad et al., 2006 against different fungal strains (*Trichophyton longifusus* and *Microsporum canis*) Observations recorded by these investigators revealed that the crude extract at all the concentrations were fungistatic in nature. In present investigation it was observed that the ethanolic and aqueous corm extracts seemed to have the best antifungal activity against *F. oxysporum*

Table 2
Antifungal effect of *Colchicum luteum* (Three factorial ANOVA).

Strain solvent	Concentration	Solvent						
		C1	C2	C3	C4	C5	Mean	Factor mean of solvent
S1	Aqueous	06.36	08.39	10.29	12.40	15.44	10.58	**Aqueous = 10.91**
	Ethanol	06.52	10.38	11.55	15.45	18.55	12.48	**Ethanolic = 13.14**
	Sub mean	06.44	09.39	10.92	13.93	16.97	**12.53**	
S2	Aqueous	02.34	07.45	13.59	15.42	17.44	11.25	
	Ethanol	04.34	11.18	16.39	17.70	19.42	13.81	
	Sub mean	03.34	09.31	14.99	16.56	17.70	**11.32**	
	Mean	**04.89**	**09.35**	**12.95**	**15.24**	**17.70**		

Whereas, C.D. (≤0.05).
S1, S2: fungal strains; C1, C2, C3, C4, and C5: Different concentrations; Strain: 0.18128; Solvent: 0.18128; Concentration: 0.28662; Strain × Solvent: 0.25636; Strain × Concentration: 0.40534; Solvent × Concentration: 0.40534; Strain × Solvent × Concentration: 0.57324.

Table 3
Antifungal activity of *Colchicum luteum* extracts against different plant pathogenic fungi.

Fungi	Solvent	Plant extract Concentration (mg mL^{-1})					Positive control	Negative control
		10	30	50	80	100		
		Zone of inhibition (mm)						
Fusarium oxysporum	Aqueous	06 ± 0.29	08 ± 0.27	10 ± 0.36	12 ± 0.28	**15 ± 0.23**	20 ± 0.40	0.0
	Ethanolic	06 ± 0.21	10 ± 0.33	11 ± 0.39	15 ± 0.10	**18 ± 0.39**	20 ± 0.40	0.0
Colletotrichum Sp.	Aqueous	02 ± 0.28	07 ± 0.28	13 ± 0.31	15 ± 0.13	**17 ± 0.12**	32 ± 0.26	0.0
	Ethanolic	04 ± 0.16	11 ± 0.36	16 ± 0.82	17 ± 0.1	**19 ± 0.29**	31 ± 0.26	0.0

Negative Control: 10% DMSO; Positive Control: The fungicide Hexaconazole (0.5 mg mL^{-1}).

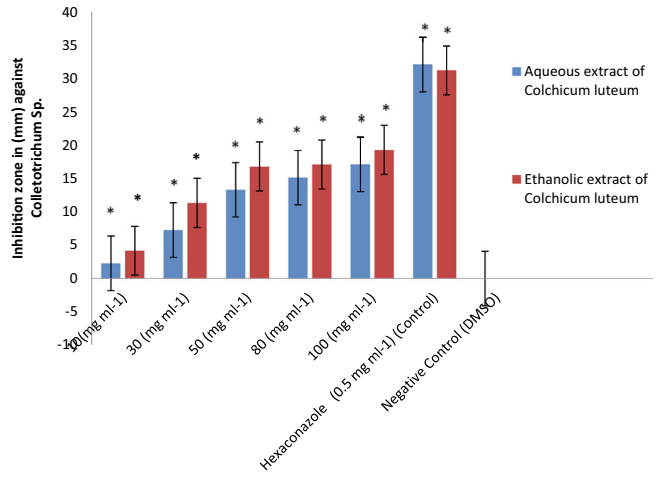

Fig. 2. Antifungal properties of *Colchicum luteum* against *Colletotrichum* sp. Error bar indicates SD mean, Values with * are significantly different compared to control (P-value < 0.05).

and *Colletotrichum* sp. The extracts in the present investigation were far superior in contrast with the tested extracts against *Fusarium oxysporum* by other authors (Rongai et al., 2015). Mammadov et al., 2009; Theer et al., 2021 while investigating the antimicrobial activity of *Colchicum* species (ethanol extract) observed that the ethanol extract had a weak inhibitory effect against tested bacteria. *S. aureus* but the same extract showed higher efficacy (58%) against the bacterium *Bacillus subtilis*. The plant extracts obtained from *Ribes nigrum* showed a good antifungal activity against *F. oxysporum* with the efficacy of 78.6 % as reported by Şesan et al., (2017). Another investigation revealed the better antifungal activity of ethanol and acetone extract of leaves of few medicinal plants against *F. oxysporum* (Neela et al., 2014). Butanoic and ethanolic extracts of the tested plant *Ocimum basilicum* inhibited the growth of *F. oxysporum* to a higher extent against (Isaac and Abu-Tahon, 2014).

Table 4
Colchicine content in different plant parts of *Colchicum luteum* by HPLC.

Plant	Plant parts	% Value of colchicine (w/w)
Colchicum luetum	Corm	0.191 ± 0.036
	Seeds	0.103 ± 0.021
	Leaves	0.084 ± 0.030
	Flowers	0.056 ± 0.010

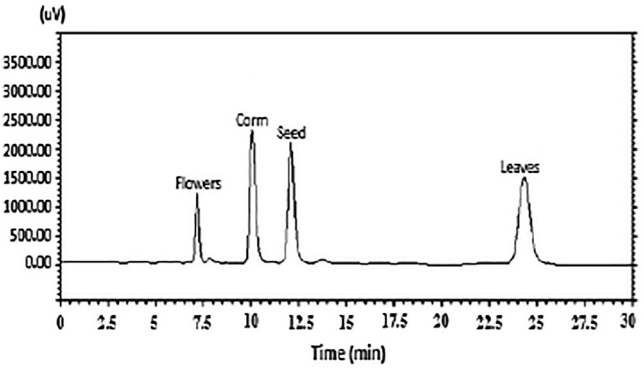

Fig. 5. HPLC chromatogram comparison of colchicine extracted from different parts of *C. luteum* at λ 352 nm.

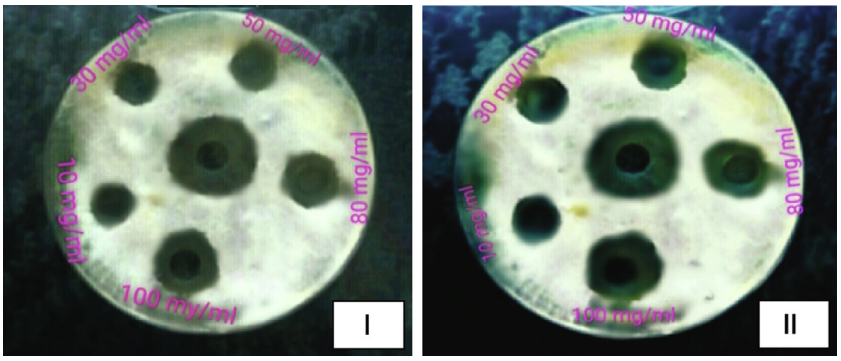

Fig. 3. Inhibition zones around the agar wells against *Fusarium oxysporum*. Aqueous extracts (I) and Ethanolic extracts (II) and Middle well indicates Positive control (Fungicide).

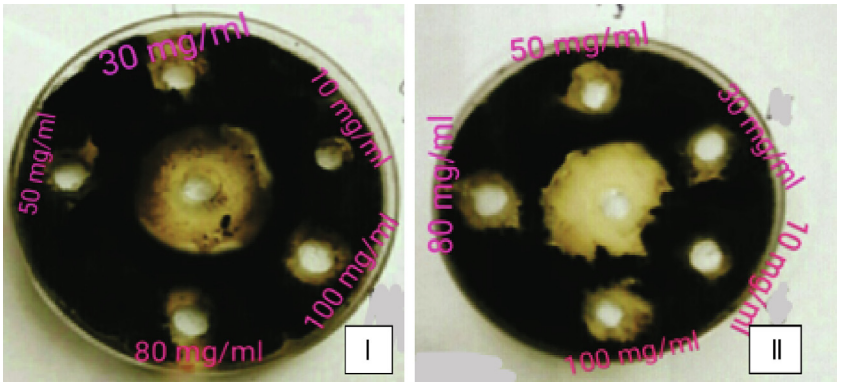

Fig. 4. Inhibition zones around the agar wells against *Colletotrichum* sp. Aqueous extracts (I) and Ethanolic extracts (II) and Middle well indicates Positive control (Fungicide).

Our findings show that *C. luetum* extracts are extremely efficient in preventing and fungal growth of *F. oxysporum* and *Colletotrichum* sp. This suggests that the extract derived from *C. luetum* corms might be used to prevent pathogen spread, infectivity, and persistence in the host. These findings are in line with the findings of Bhutia et al. (2016). The antifungal activity of plant extracts against the *Colletotrichum* sp. has been carried out by various authors (Bussaman et al., 2012; Silva et al., 2008.; Maqbool et al., 2010; Sangeetha et al., 2013; Cruz et al., 2013; Padder et al., 2021; Bhat et al., 2021) and findings from their investigations revealed a broader variation of sensitivity of the various strains of *Colletotrichum* sp. to different concentrations of the both ethanolic and crude extracts of different plant parts. Our findings showed that *C. luetum* extracts are extremely efficient in preventing the fungal growth of *F. oxysporum* and *Colletotrichum* sp. This suggests that the extract derived from *C. luetum* corms could be used to prevent pathogen spread, infectivity, and persistence in the host. Furthermore, our findings showed the best antifungal potentional of plant extract against the selected fungal species with a foundation to a larger investigation field highlighting the importance of *Colchicum* members as producers of fungicidal or fungistatic ingredients against various phytopathogenic entities like *F. oxysporum* and *Colletotrichum* sp.

The chemical-based fungicide uses for the control of phytopathogens have raised the concerns of fungicide resistance and bioaccumulation of these xenobiotic compounds in the environment (Padder et al., 2021). Therefore, the ecologically sound strategies need to be expounded for the control of phytopathogenic fungi and bacteria for a sustained agriculture, plant based organic extracts the slow but prolonged activity during their application (Rongai et al., 2015)

Colchicine levels in *Anatolian colchicum* and other species of *Colchicum*, were observed to be in the range from 0.039% to 0.3% (w/w) in previous studies (Toplan et al., 2016; Felipe et al., 2014; Ahmad, 2010. Other investigators like Alali et al. (2006) found that the underground parts of *C. tunicatum*, other species of *Colchicum* contain the colchicine content of 0.12% (w/w) as well as 0.13% (w/w) during their and vegetating and flowering stages, respectively, while that of aerial parts was only about 0.04% (w/w) and 0.02% (w/w), respectively. Our findings demonstrated the occurrence of the alkaloid colchicine at higher concentrations in various parts of *C. luteum* compared to previous authors (Siddiqui et al., 2019). The colchicine concentration in different parts of plant sections ranged from 0.051 percent to 0.695 percent which largely deferred from the findings of other investigators (Sharma et al., 2019; Saleem et al., 2020; Salehi et al., 2021). Therefore, the present investigation confirmed the presence of a potential alkaloid colchicine in the plant extracts of was *C. luteum*, furthermore this study supports the use of plant extracts in the treatment of infectious diseases and has provided important insights for the identification of new plant-based antifungal medicines.

5. Conclusion

The *C. luteum* corm extract showed significantly positive actions against the *F. oxysporum* and *Colletotrichum sp*. Ethanolic extracts demonstrated the best inhibition potential against fungal species, thus the plant extract has lot of fungicidal potential. Hence, *C. luteum* could be useful to fight against several fungal diseases in agriculturally important crops such as *Fusarium* wilt. This study recommends the antifungal potential of *C. luteum* extract as a source of effective bio -fungicide as an organic remedy to various phytopathogens. More research is needed to describe the antifungal effects of *C. luteum* against *F. oxysporum* and *Colletotrichum* sp., as well as the other potential pathogenic fungi. The method for identifying bio-fungicides in higher plants reported in this study is simple, quick, and allows for simultaneous examination of a large number of plant species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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