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Research Article

Metabolites of the rust mycoparasite, *Sphaerellopsis paraphysata* for management of rust in pearl millet

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ABSTRACT: The mycoparasite, *Sphaerellopsis paraphysata* was isolated from the uredospores of pearl millet rust pathogen *Puccinia substriata* with promising natural parasitic association. The culture had definite margin, sometimes submerged in media and sporulation occurred on the third week of incubation. The mycelium was hyaline and septate. The secondary metabolites were explored to contain the uredospore production thereby reduce the rust incidence. The solvent extraction method was used to extract the secondary metabolites produced from crude extracts of *S. paraphysata*. The fractions of the secondary metabolites were analyzed using GC-MS. The major components present were Phenol, Decane, 3-Butoxy-1,1,1,5,5,5, hexamethyl-3-(trimethylsiloxy) trisiloxane, Butanoic acid, 3-methyl-3-methyl butyl ester, which were reported to have anti-microbial, anti-fungal and anti-oxidant properties. The crude metabolite of *S. paraphysata* in the dilution of 1:5 recorded the maximum lysis of the uredospores of *P. substriata* (92.67%). Further, SEM study showed severely lysed broken cell wall which led to leakage of cellular contents. The spraying of secondary metabolites on rust infected pearl millet crop reduced the incidence of rust. The present study paved the way for the development of new biologically originated molecule for the management of pearl millet rust disease.

KEY WORDS: Pearl millet, rust, secondary metabolites, *Sphaerellopsis paraphysata*

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INTRODUCTION

The pearl millet crop is affected by several biotic and abiotic factors that cause significant reduction in the yield of the crop. The pearl millet leaf rust caused by *Puccinia substriata* is the most destructive and results in yield loss upto 76% (Wilson *et al.*, 1996). The uredial, telial and basidial stages of the macrocyclic rust pathogen are found on pearl millet. The spermagonial and aecial stages occur in alternate host viz. *Solanum melongena* (eggplant) (de Carvalho *et al.*, 2006). Since the fungicidal control of rust disease has hazardous impact on environment, there exists a huge need for alternatives like natural biological originated antagonistic organisms. The new isocyanide antimicrobial metabolites such as darlucin A (1) and B (2), were isolated from *Sphaerellopsis filum* by fermentation process, which exhibited antibacterial and antifungal activity (Zapf *et al.*,1995). Hence, the present study was focused on the isolation, separation and assay of the secondary metabolites of mycoparasite, *Sphaerellopsis paraphysata* to contain the rust uredospore production.

MATERIALS AND METHODS

Collection and isolation of mycoparasitic fungi

Mycoparasitic fungus viz., *Sphaerellopsis paraphysata* was isolated from pearl millet rust samples collected from different location of Tamil Nadu. The single pycnidial method explained by Driessen *et al.* (2004) was used in this study for the isolation of *S. paraphysata*. In this method, the pycnidia of *S. paraphysata* in the rust infected leaves were scraped and pycnidia were dipped in sodium hypochlorite for 30 sec and then mixed with water agar medium, after the solidification of the media the pycnidia dispersed randomly in the water agar were marked with a marker under stereozoom microscope and incubated at 25º C for 48 hours and then the germinated spores were located and marked which are transferred to another petridish containing V8 juice agar. Streptomycin was added to prevent the bacterial contamination. Inoculated petriplates were incubated for 20°C for 25 days. Pure culture of the biocontrol agent was obtained by single hyphal tip method (Leyronas *et al.,* 2012). The stock cultures were sub cultured at an interval of 30 days to maintain the viability of

the culture and further studies.

Extraction of *Sphaerellopsis paraphysata* crude metabolites *in vitro*

Potato Dextrose Agar (PDA) broth was prepared and uniformly transferred to 250 ml Erelynmeyer flasks as each containing 100 ml and sterilized in an autoclave at 15 lbs pressure for 20 min and cooled. The potential isolate TNAU-Sp3 was inoculated in each flask and incubated at 25ºC for 20 days. The culture filtrate of the isolate was taken and centrifuged at 10000 rpm for 15 minutes and filtered with Whatman no 1 filter paper. Solvent extraction method was used to extract the secondary metabolites present in the culture filtrate. Equal volume of ethyl acetate was added to the culture filtrate and secondary metabolites were separated using a separating funnel. The ethyl acetate extract was washed three times with 10 ml of 5% sodium bicarbonate separately and again washed three times with 10 ml of 5% sodium carbonate and finally with 10 ml of sterile water, until it became neutral. Finally sodium sulphate was added to remove the moisture each solvent was evaporated at 55ºC on a rotary evaporator separately and the crude extract (oily residues) obtained from each isolate was dissolved individually in one ml of methanol and ethyl acetate and stored in Eppendorf vials in refrigerated condition at 4ºC for further use (Almeida *et al.*, 2010).

Detection of secondary metabolites by GC-MS

The crude secondary metabolites produced by TNAU-Sp3 was analyzed through Gas Chromatography – Mass Spectrometry **(**GC-MS) (Thermo scientific Trace GC Ultra DSQ II) equipped with column (30 mm × 0.25 mm × 0.25 µm) under the following conditions: carrier gas as Helium with flow rate at 1 ml per minute and 1 µl sample injection with pre injection of solvent by AI/AS 3000 Method; split-less mode injection with 30 sec of sampling time; the column temperature maintained initially at 50ºC at the increasing rate of 10ºC min^{-1} no hold was followed by increasing upto 200ºC and kept at the same temperature for 2 minutes hold with surge pressure 3 kPa and 220ºC base temperature at right SSL method and 250ºC base temperature at right ECD method with the Aux 1 MS transfer line at 250ºC; the electron impact energy was 70 eV, Julet line temperature was set at 2,000ºC and the source temperature was set at 200°C. Electron Impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range. The total chromatogram was obtained for each sample separately. The base peak of each spectrum was compared with the base peak of the chemical components in the NIST Ver.2005 MS data library through on-line and the compounds were identified.

Assay of secondary metabolites of *Sphaerellopsis paraphysata* on uredospores germination

The assay of secondary metabolites was carried out by the methodology explained by Chaudhary *et al.* (2015). The crude extract was diluted with sterile water in the five different concentrations viz*.,* 50, 100, 250, 500 and 1000 ppm and tested its efficacy on uredospore under cavity slide method. The crude metabolites and their respective concentrations were prepared by mixing the requisite quantity of sterilized distilled water. Fresh uredospores collected from single pustule of pearl millet rust infection were dispensed uniformly in sterilized distilled water in culture tubes. Single drop of uredospores suspension was placed in the wells of series of cleaned cavity slides by using one ml pipettes. Treatment without crude metabolites served as control. Cavity slides containing spore suspension were incubated at 20 ± 10°C temperature in a BOD incubator. Four replications of each treatment maintained and four hundred uredospores were counted. The observation was made 24, 48 and 72 h of incubation using different microscopic fields for each slide. Per cent lysis of spore germination over control was calculated as per the formula described by Vincent (1947).

Efficacy of crude extracts of *Sphaerellopsis paraphysata* against pearl millet rust under glass house condition

The susceptible check of pearl millet plants cultivar CO 10 was raised in glass house. The pots consisted Farmyard manure, red earth and sand (2:1:1) and each pot was sown with five seeds. When the seedlings attained six leaf stage the leaves were sprayed with freshly collected uredospores at the concentration of 10^6 per ml as described by Kishore and Pande (2005). The plants were then covered by polythene bag for 24 hours to maintain high humidity for disease development. Seven to ten days after inoculation, the visible symptoms were noticed with small pustules of uredospores. Then the crude extract with different concentrations *viz.,* 50, 100, 250, 500 and 1000 ppm were sprayed and control was also maintained by spraying with sterile distilled water. The incidence of rust on leaves was recorded and severity was calculated as described by Ravinder Reddy (1982).

Environmental SEM analysis of *Sphaerellopsis paraphysata*

To study the parasitization of *S. paraphysata* on rust uredospores was confirmed by environmental scanning electron microscopic (ESEM) image analysis. The typical rust specimens were selected and morphologically examined. The leaf specimen was cut into a size of 5 × 5 mm and directly fixed in carbon stubs coated with gold in a high vacuum evaporator (Sputter coating) and ESEM analysis was carried out using an acceleration voltage of 8-10 kV. Image capture control was achieved using Auto-Montage v.5.0 (Synoptics) and the images were captured as a series of focal planes and montage to produce a composite focused image.

RESULTS AND DISCUSSION

Sphaerellopsis paraphysata TNAU-SP3 isolate with GenBank accession MK863554 has shown faster growth when compared to other isolates. The growth of TNAU-Sp3 (20 days to attain maximum growth) was comparatively faster than that of TNAU-Sp2 (MK 918510) (35 days to attain maximum growth). In our study, we have confirmed that the mycoparasite *S. paraphysata* found in the pearl millet rust is a hyperparasitic fungi which parasitizes the rust fungi and thus reduces the incidence of rust. Kranz and Brandenburger (1981) reported that *Sphaerellopsis filum* (anamorph of *Eudarluca caricis*) is a known mycoparasite of at least 369 species and its mode of action to degrade uredial sori, which result in stopping the propagation of uredospores and so prevents new rust infections (Gordon and Pfender, 2012).

Yuan *et al.* (1998) reported that *Sphaerellopsis filum* decreases the rust spore germination up to 98% with wide host range and used for integrated disease management in pearl millet cultivation.

Identification of compounds from secondary metabolites of *Sphaerellopsis paraphysata*

The secondary metabolites obtained from crude extracts of TNAU Sp3 was extracted with methanol and subjected to GC-MS analysis for identifying the secondary metabolites. The compound identity was verified by AMDIS software programme and NIST library 2005.

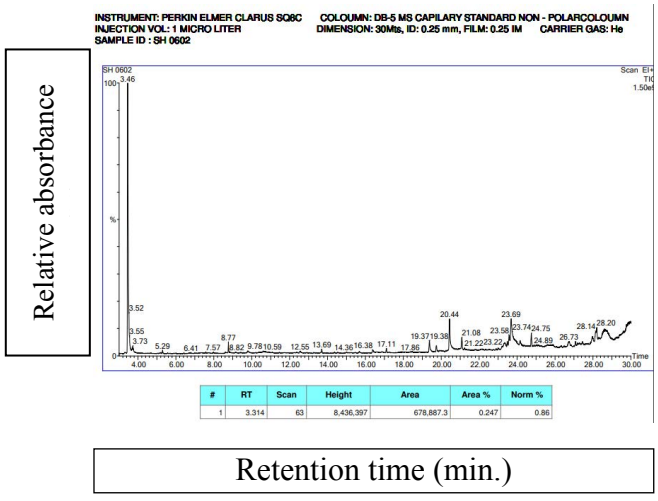

Plate 1. GC-MS chromatogram of secondary metabolites from crude extracts of *Sphaerellopsis paraphysata*

The crude extracts of TNAU Sp3 contained several secondary metabolites. The compounds with antimicrobial, antifungal and antioxidant properties were identified. They were Phenol, Decane, 3-Butoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy) trisiloxane, Butanoic acid, 3- methyl,3-methyl butyl ester, Pyridine,3-(1-methyl-2-pyrrolidinyl), 3-oxabicyclo[4.1.0] heptane-7-Carboxamide-6-methyl-N-(1-napthyl), 1-Indolizin carboxylic acid, 2-methyl-,ethyl ester, N-N-Dimethyldecanamide (Plate 1) (Table 2). The antifungal compounds were identified in our studies were reported in several studies and showing antifungal, antibacterial and antioxidant properties (Jurd *et al.*, 1971; Ansari *et al.*, 2013; Venkatesan *et al.*, 2017; Brahmi *et al.*, 2016; Gupta *et al.*, 2016; Ololade *et al.*, 2017; Srivastava, 2014 and Muhialdin *et al.*, 2016).

Effect of different concentrations of crude metabolites on uredospore germination and rust incidence

The crude metabolites extracted from *S. paraphysata* were tested with 50, 100, 250, 500 and 1000 ppm concentration against uredospores of *P. substriata.* It is clearly noticed that in all the concentration the uredospores cell wall were lysed and broken, which resulted in the leakage of cellular contents. The maximum lysis of uredospores (92.67%) was recorded in 1000 ppm followed by 500 ppm (86.67%) at 72 h after incubation. The minimum spore lysis was recorded in sterile water (2.33%) (Table 3; Plate 2). The lowest spore germination of 13.6% was recorded in 1000 ppm concentration of crude metabolites. There is no reduction in germination of uredospore in sterile water treated control (Table 3). Rust infected pearl millet cultivar CO 10 was tested to prove the toxicity of crude extract of *S. paraphysata* against *P. substriata*. The results revealed that secondary metabolites at 1000 ppm sprayed plants showed 92.6% reduction in rust symptom. Howeverm, the treatment sprayed with 50 ppm showed lowest reduction (56.3%) in rust symptom on foliage (Table 3). Uredospores treated with crude secondary metabolites at 1000 ppm showed lysis of cell and leakage of cellular contents in SEM study. The uredospores were deformed as compared to healthy uredospores not treated with crude extracts of *S. paraphysata* (Plate3). The similar results were found in spore damage of *Curvularia lunata* when it was treated by metabolites obtained from *Bryum cellulare* (Deora and Guhil, 2015). Further, this study paves the possibility for exploring the naturally occurring antifungal phytochemicals for plant disease management. The results of the SEM observations from this study showed shrunken and lysed uredospores due to the treatment of secondary metabolites. Plachecka (2005) reported that hyphal penetration of *S. filum* into the uredospore and collapsed with disintegration. Similarly the mycoparasite, *A. quisqualis* was observed in SEM by Parthasarathy (2018) who stated that the parasitized conidia of *Erysiphe pisi* with *Ampellomyces quisqualis* was shrunken and deteriorated. Yuan *et al.* (1999) reported that *S. filum* reduced the spore production of Willow rust (*Melamspora epitea*) by 98.0%.

The present investigation gives the scope for the exploration of secondary metabolites and bio chemical

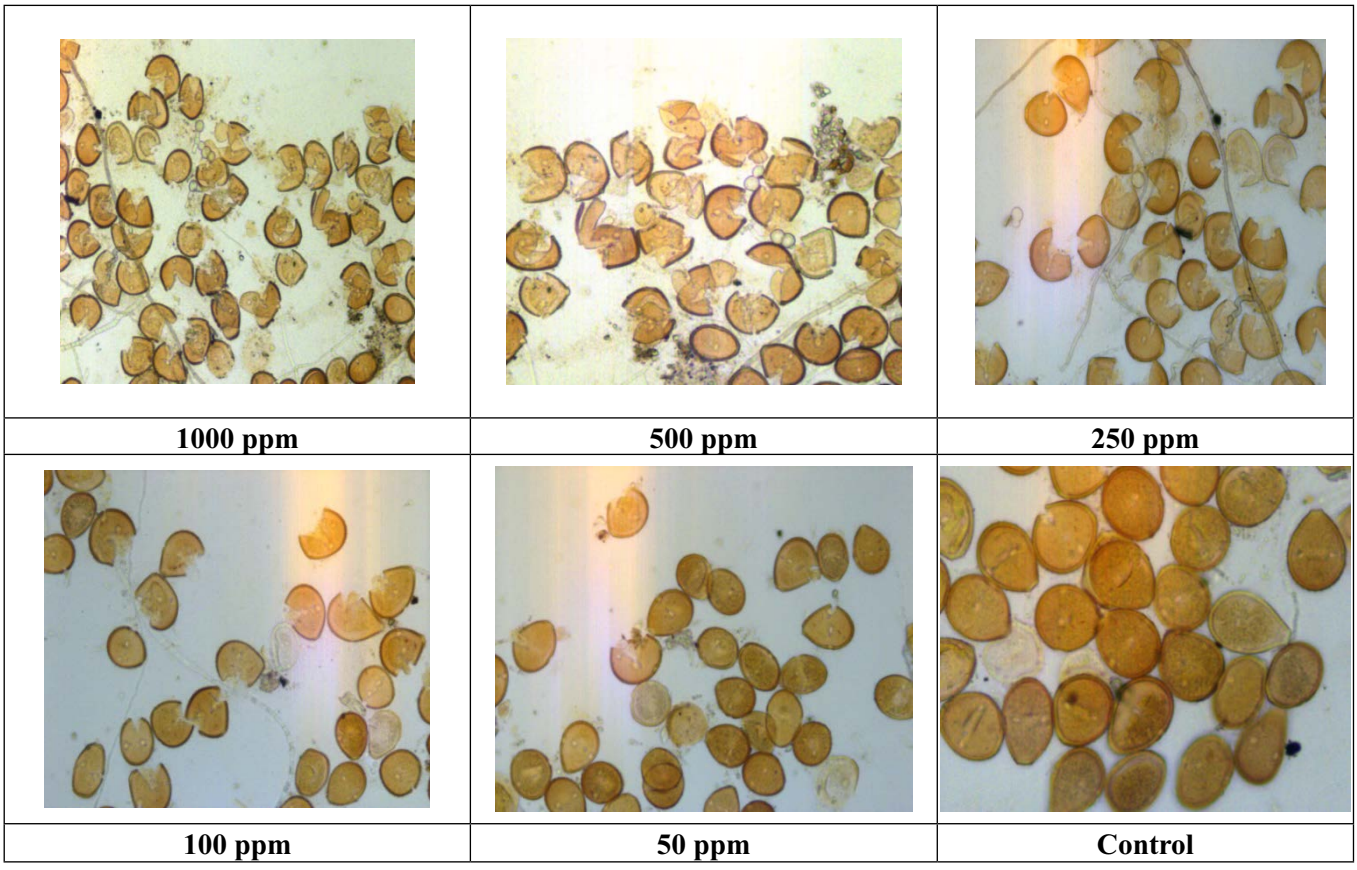

Plate 2. Bioassay of *Sphaerellopsis paraphysata* crude metabolites against uredospores of *Puccinia substriata*

Dilution
1000 ppm - Complete cleavage of spore wall
500 ppm – 60% of spore wall cleaved
250 ppm – 40% of spore wall of cleavage
100 ppm – 25% of spore wall cleavage
50 ppm – Mild cleavage of spore wall
0 ppm – Healthy spores

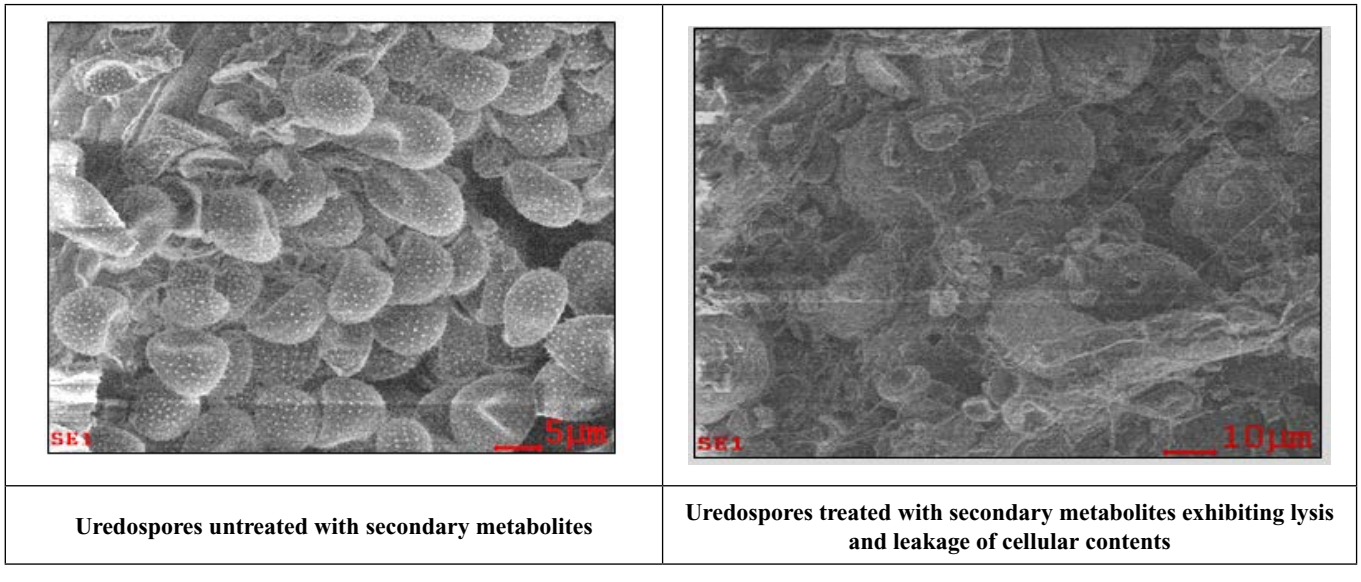

Plate 3: Scanning electron microscopy on the influence of secondary metabolites of *Sphaerellopsis paraphysata* on rust pustules

Table 1. Morphological characters of *Sphaerellopsis paraphysata*

S.No	Isolate	Cultural morphology	Mycelium	Size of the conidia (µm)	Size of the pycnidia (µm)
1.	TNAU-Sp1	Cottony, White, raised colonies, with definite margin.	Hyaline septate	11.1 x 4.65	141.21 x 130.73
2.	TNAU-Sp2	Colonies were initially cottony, white but later turned greyish on sporulation, raised with definite margin.	Hyaline septate	10.67 x 5.12	117.12 x 106.78
3	TNAU-Sp3	White but centre turns grayish on sporulation	Hyaline, septate	12.25 x 4.68	173.6 x 163.56
4	TNAU-Sp4	Whit, cottony, raised colonies with definite margin	Hyaline, septate	12.12 x 5.25	165.8 x 153.46
5	TNAU-Sp5	Greyish colonies with white centre, margin was defenite	Hyaline, septate	11.20 x 2.16	134.87 x 123.04
6	TNAU-Sp6	Greyish, raised colonies having definite margin	Hyaline, septate	12.84 x 2.05	132.1 x 110.40.6
7	TNAU-Sp7	Whit, cottony, raised colonies with definite margin,exude water droplets on sporulation.	Hyaline, septate	12.51 x 3.61	141.09 x 120.6
8	TNAU-Sp8	Cottony, White, raised colonies, with definite margin.	Hyaline, septate	9.12 x 4.61	98.76 x 87.54
9	TNAU-Sp9	Cottony, White, raised colonies, with definite margin	Hyaline, septate	10.21 x 4.32	191.1 x 187.24
10	TNAU-Sp10	Cottony, White, raised colonies, with definite margin.	Hyaline, septate	12.42 x 4.02	191.1 x 187.24
11	TNAU-Sp11	Initially white and turns grayish on sporulation, definite margin	Hyaline, septate	11.68 x 4.18	134.1 x 115.56
12	TNAU-Sp12	Colonies were initially cottony, white but later turned greyish on sporulation, raised with definite margin	Hyaline, septate	11.39 x 4.32	126.7 x 104.56

Table 2. Secondary metabolites production in the crude extract of *Sphaerellopsis paraphysata* revealed through GC-MS

Compound	Retention time	Peak Area Percentage	Molecular weight	Molecular formula	Properties	References
Silane, triethyl (2-phenylethoxy)-	3.314	0.247	236.43	$C_{14}H_{24}OSi$	Anti fungal	Wang *et al.* (2012)
Phenol	3.459	28.748	94.113	C_6H_5OH	Anti microbial, Anti fungal.	L.Jurd *et al.* (1971) Ansari *et al*. (2013)
Decane	3.729	1.314	142.286	$C_{10}H_{22}$	Anti bacterial	Hameed *et al*. (2015)
3-Butoxy-1,1,1,5,5,5-hexame-thyl-3-(trimethylsiloxy) trisiloxane	3.859	0.237	368.767	$C_{13}H_{36}$	Anti oxidant	Sujatha *et al*. (2017)
Butanoic acid, 3-methyl-, 3-methylbutyl ester	5.294	0.257	172.26	$C_{16}H_2OO_2$	Anti bacterial	Brahmi *et al*. (2014)
Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	8.771	0.750	162.231	$C_{10}H_{14}N_2$	Anti fungal and Anti bacterial	Pavia *et al.* (2000)
10,12-Tricosadiynoic acid, TMS derivative	9.781	0.433	418.727	C_2H	Anti microbial	Divya Gupta *et al.* (2016)
Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	15.678	0.221	394.674	$C_{26}H_{50}O_2$	Anti microbial	Rajani Srivastava *et al.* (2014)

N,N-Dimethyldodecanamide	13.693	0.366	227.38	$C_{14}H_{29}NO$	Anti oxidant, Anti bacterial	Ololade *et al*. (2017)
Tetra decanoic acid	16.389	0.481	229.368	$C_{14}H_{28}O_2$	Anti microbial and anti bacterial	Abubakar *et al*. (2016)

Table 3. Effect of different concentrations of crude metabolites on uredospores of *Puccinia substriata* in cavity slide and under glass house condition

S.No	Crude Extract Concentration (ppm)	% spore lysis (hours after incubation)			% uredospore germination	% reduction of rust symptoms on foliage
		24	48	72		
1.	1000	32.7[a]	63.34[a]	92.67[a]	13.6[e]	92.6[e]
2.	500	27.36[b]	47.64[b]	86.67[b]	18.6[d]	83.4[c]
3.	250	24.64[c]	41.61[c]	84.67[c]	21.6[d]	78.9[c]
4.	100	21.66[d]	36.13[d]	73.66[d]	25.3[c]	61.6[b]
5.	50	18.61[e]	31.43[e]	62.66[e]	40.0[b]	56.3[b]
6.	Control (Sterile water)	0.0[f]	0.0[f]	2.33[f]	99.0[a]	0.0[a]

*Values are means of three replications
Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$

fractions based formulation to control the rust diseases. The secondary metabolites based products are biologically originated and could serve as ideal components for ecofriendly management.

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