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Isolation of antagonistic fungi and evaluation of antifungal activity of the separated metabolite against the red rot of sugarcane pathogen

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ABSTRACT

Antagonistic potential of six rhizosphere soil fungi viz., Trichoderma harzianum, T. viride, Aspergillus flavus, A. fumigatus, A. niger, and A. sulphureus were tested against the red rot of sugarcane pathogen Colletotrichum falcatum. Out of the seven soil fungi tested, Trichoderma harzianum was found to be most effective in controlling the growth of Colletotrichum falcatum based on the colony interactions and effects of volatile and non-volatile metabolites. When HPLC fractionation of the mycelia extract of T. harzianum, T. viride, Aspergillus flavus, A. fumigatus, A. niger, and A. sulphureus were tested against the red rot of sugarcane pathogen C. falcatum. The T. harzianum peak at showed RT 4.893 maximum antifungal activity with the inhibition zone of 0.9 mm followed by Trichoderma viride(0.7mm), A. fumigatus (0.5 mm), A. niger (0.5mm), A. flavus (0.4 mm), and A. sulphureus (0.3mm). This peak may be responsible for the antifungal activity of this fraction. The fungus isolated in the present study may be exploited commercially to biocontrol this disease. Further studies on the determination of the actual biochemical compound responsible for antagonistic effect through FT-IR and GC-MS analysis is under way.

Key words: Antagonist, *Trichoderma*, *Colletotrichum*, HPLC.

INTRODUCTION

Red rot disease caused by the fungal pathogen *Colletotrichum falcatum* Went (Perfect state: *Glomerella tucumanensis* (Speg) is a threatening disease of sugarcane, causing severe yield loss in most of the sugarcane-growing states in India [1]. The sugar industry in India suffers losses of more than 500 million dollars (US) every year due to red rot disease [2, 3] and this loss is due to the reduction in the sucrose contents and weight of the cane due to red rot disease.

Plant diseases cause major production and economic losses in agriculture and forestry. The bacterial, fungal and viral infections, along with infestations by insects result in plant diseases and damage. Synthetic fungicides are widely used by the farmers to eradicate pathogens but it results in environmental hazards and have harmful side effects on human beings and animals. The chemical fungicides not only develop fungicide resistant strains but also accumulate in food and ground water as residues. In order to over come such hazardous control strategies, scientist, researchers from all over the world paid more attention towards the development of alternative methods which are, by definition, safe in to the environment, non-toxic to humans and animals and are rapidly biodegradable, One such strategy is use of Biocontrol (BCAs) agents to control fungal plant diseases. Among the BCAs, species of genus *Trichoderma* are the most promising and effective biocontrol agents. *Trichoderma* as antagonist controlling wide range of microbes was well documented and demonstrated for more then seven decades ago, but their use under field condition came much later [4], and their mechanisms of mycoparasitism is much more complex, involving nutrient competition, hypoparasitism antibiosis, and cell wall degrading enzymes. In the present study the

antagonistic potential of selected fungal isolates was tested against *Colletotrichum falcatum*. Both volatile and non-volatile compounds from antagonist fungi were evaluated against the test pathogen.

MATERIALS AND METHODS

Collection of diseased samples and isolation of *Colletotrichum falcatum*

Colletotrichum falcatum was isolated from sugarcane (*Saccharum officinarum*) collected from sugarcane cultivated field. Strains were isolated from lesions of infected stem pieces. Three 5 × 5 mm pieces of tissue were taken from the margin of infected tissues, surface sterilized by dipping in 1% sodium hypochlorite for 1 minute, immersed in 70% ethanol for 1 minute and rinsed three times with sterilized water and finally dried in sterilized tissue paper. Samples were placed on water agar and incubated at room temperature (28-30˚C). The growing edges of fungal hyphae developing from the tissues were then transferred aseptically to potato dextrose agar (PDA) [5]. Single spore subcultures were obtained for each *Colletotrichum* isolate using the procedure described by Goh [6]. When the fungus showed sporulation, spore masses were picked off with a sterilized wire loop and streaked on the surface of water agar. After incubation overnight (28-30˚C), single germinated spores were picked up with a sterilized needle and transferred to PDA and identified pure cultures were stored in agar slants.

Isolation and Identification of Antagonist organisms

Fungal species were isolated from soil samples by using potato dextrose agar (PDA) medium. Samples were inoculated over plates by multiple tube dilution technique (MTDT) and the plates were inoculated at 26˚C for 4 days. The fungal colonies were picked up and purified by streaking and incubated at 26˚C for 7-8 days. Green conidia forming fungal bodies were selected and based on microscopic observation was identified to be *Trichoderma harzianum* Rifi., *T. viride* Pers., *Aspergillus niger* Tiegh., *A. flavus* Links, *A. fumigatus* Fresen., and *A. sulphureus* Thom. The cultures were maintained on PDA slants.

Growth inhibition assay by dual culture method

Interaction between antagonistic fungi and pathogenic fungi were determined by the method of Dennis and Webster [7]. The antagonism between the fungi isolated from soil, *Trichoderma harzianum, T. viride, Aspergillus niger, A .flavus, A. fumigas* and *A. sulphureus*, against the pathogen (*Colletorichum falcatum*) was studied by dual culture technique. In a sterile condition, mycelium was picked out using inoculation loop. *C. falcatum* was placed on right edge of petriplate containing PDA and mycelia of either *Trichoderma harzianum, T. viride, Aspergillus niger, A. flavus, A. fumigatus* or *A. sulphureus* were placed on left edge of the same petriplate and the plates were incubated at 28 ± 2ºC and observed after 7 days.

Assay for volatile metabolites of antagonist

Effect of volatile compounds from antagonist on the radial growth of *C. falcatum* was analysed. The method used to test volatile compounds from *Trichoderma harzianum, T. viride, Aspergillus niger, A. flavus, A. fumigatus* and *A. sulphureus* on *C. falcatum* was the one given by Dennis and Webster [7]. The two bottom portion of petriplates containing PDA were inoculated with mycelia of pathogen and antagonist respectively, inoculated bottom plates were placed facing each other and sealed with cellophane adhesive tape. The petriplate containing PDA without antagonist served as control. The observations on the radial growth of the test fungus were recorded after 7 days of incubation at 28 ± 1ºC. The colony diameter of test fungus in the treatment in comparison with that of check gave percent growth inhibition.

Assay for non volatile metabolites of antagonist

The effects of non volatile metabolites produced by the *Trichoderma harzianum, T. viride, Aspergillus niger, A. flavus, A. fumigatus* and *A. sulphureus* were determined by following the method of Dennis and Webster [7]. The isolates of antagonist were inoculated in 100ml sterile potato dextrose broth in 250ml conical flasks and incubated at 28 ± 2˚C for 15 days. After incubation the cultures were filtered through Millipore filter and culture filtrates were added to molten PDA medium (40˚C) to obtain a final concentration of 10% (v/v). The medium was poured into petriplates and after solidification 3mm disc of the pathogens were placed centrally and incubated at 28 ± 2˚C. Control plates were maintained without amending the culture filtrate. The percent of growth inhibition in all the above experiments were calculated by the formula

$$I = \frac{C-T}{C} \times 100$$

Where
I = Percentage of inhibition
C = Growth of mycelium in control

T = Growth of mycelium in treatment

Extraction and separation of compounds from mycelium (Watts *et al*., 1988)

Preparation of mycelial extract

The mycelial discs of *Trichoderma harzianum, T. viride*, *Aspergillus niger*, *A. flavus, A. fumigatus* and *A. sulphureus* were inoculated to in liquid potato dextrose broth and incubated in darkness for three weeks. After incubation the fungal mycelial mat was harvested by filtration, pressed between fold of filter paper and then a weighed 1 g of the fungal mycelium was extracted of 10µl in 70% acetonitrile of 10 ml. The extract was then filtered using Whatman No.1 filter paper. The filtrate was dried in vacuum. The residue was re-dissolved in HPLC solvent, i.e., acetonitrile: water: acid (65:35:1, v/v/v) and used for further analysis

Separation of components using HPLC

From the mycelial extract, 0.5 ml was injected into the rp-18 octadeclysilyl silica (DDS) column (25 x 1 cm, i.e.) with LC-UV detector (P 3000 Analytical Technologice Limited, India) and monitored at 254 nm. The flow rate was adjusted to 1.5 µl min-1. The fractionated samples were collected in vials.

Assay of antifungal activity

The HPLC fractions (purified compounds) from mycelial extract of *Trichoderma harzianum, T. viride*, *Aspergillus niger*, *A. flavus, A. fumigatus* and *A. sulphureus* were tested for their antifungal activity against *Colletotrichum falcatum*. The mycelial extract was pipetted into different wells in PDA plates in which the test fungal pathogen *Colletotrichum falcatum* was plated previously. Inhibition zones (mm) formed around the well were measured to find out the anti fungal activity against the pathogen tested.

RESULTS AND DISCUSSION

If was found that the volatile substances emanating from the soil fungi inhibited the radial growth of the test pathogens to varied degrees ranging antagonist from 57 –78%. *Trichoderma spp,* are widely used in agriculture as biocontrol agents because of their ability to reduce the incidence of disease caused by plant pathogenic fungi, particularly many common soil borne pathogens. The result of the present study supports the findings by Papavizas [8] and Dubey [9]. It is evident from the results that out of the six soil fungi examined, the highest inhibition was found by the volatile substances produced by *Trichoderma harzianum* against *C. falcatum* (78%). The second highest inhibition (73%) was observed by the volatile substances of *T. viride*, followed by *A. niger*, (69%), *A. sulphureus* (62%) *and A. fumigatus*, (60%) and lowest inhibition (57%) was by *Aspergillus flavus* (Table-1).

Table -1 Effect of Antagonist organisms on the growth of *C. falcatum*

S. No	Antagonist Organism	% inhibition by volatile assay	% inhibition by non volatile assay	% inhibition by colony interaction
1	*T. harzianum*	78	66	69
2	*T. viride*	*73*	62	*66*
3	*A. flavus*	57	40	48
4	*A. fumigatus*	*60*	37	60
5	*A. niger*	69	52	51
6	*A. sulphureus*	62	30	56

Table – 2 HPLC fraction of mycelial extract, which showed maximum antifungal activity with their RT, Concentration, and width

S.No	Name of the Anatogonistic organism	RT (min) of the major HPLC Peak	Concentration	Width	Inhibition of mycelial growth (mm)
1	*T. harzianum*	4.893	79.54	12.855	0.9
2	*T. viride*	2.927	55.57	6.521	0.7
3	*A. flavus*	2.655	47.41	4.123	0.4
4	*A. fumigatus*	2.694	49.94	6.403	0.5
5	*A. niger*	2.803	85.25	11.057	0.5
6	*A. sulphureus*	2.694	49.94	6.403	0.3

The antagonistic effects owing to non-volatile metabolites of the soil fungi against *C. falcatum* ranged from 40 to 60%. The highest inhibition was observed by the culture filtrate of *Trichoderma harzianum* against *C. falcatum*, (66%) followed by *T. viride* (62%) and *A. niger* (52%), *Aspergillus flavus* (40%), *A. fumigatus* (37%), and *A. sulphureus* (30%) (Table - 1).

The inhibition of the radial growth of the test fungi due to non-volatile metabolites may be attributed to the production of antibiotic substances in the culture filtrates [10, 11]. It has also been reported that the antibiotic

production varies depending on the competing organisms. Results in colony interaction tests showed that radial growth inhibition of *Colletotrichum falcatum* by the soil fungi was in the range of 48 – 69 % and the highest growth inhibition was by *Trichoderma harzianum* (69%) followed by *Trichoderma viride* (66 %), *A. fumigatus* (60%), *A. niger* (60%) *A. sulphureus* (56%), and *Aspergillus flavus* (48%) (Table -1). The result of the present study supports the findings by Sivan and Chet, [12], Cruz [13].

Fig. -1 HPLC of Mycelial extract of *Trichoderma harzianum*

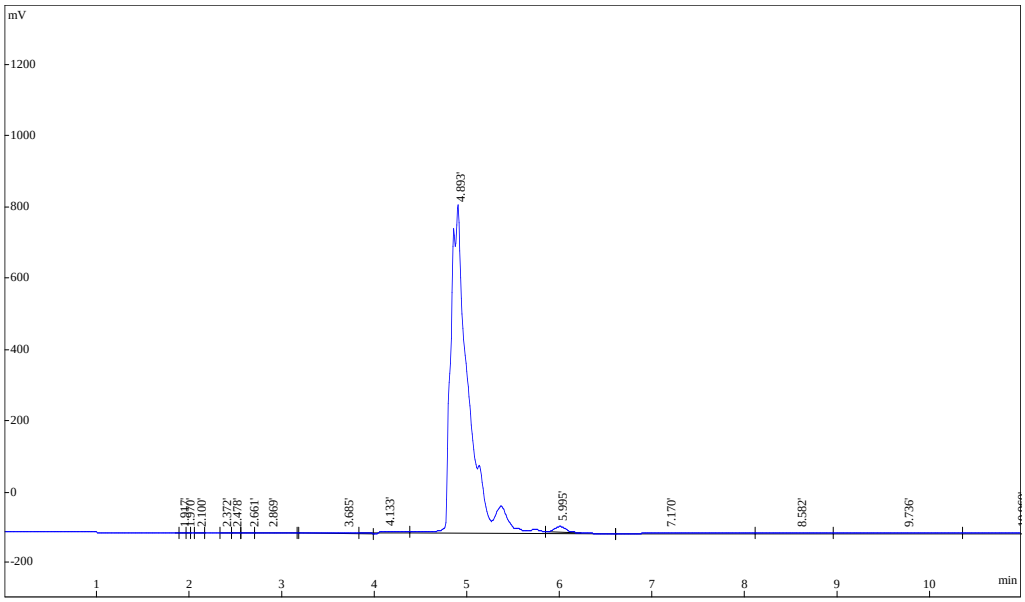
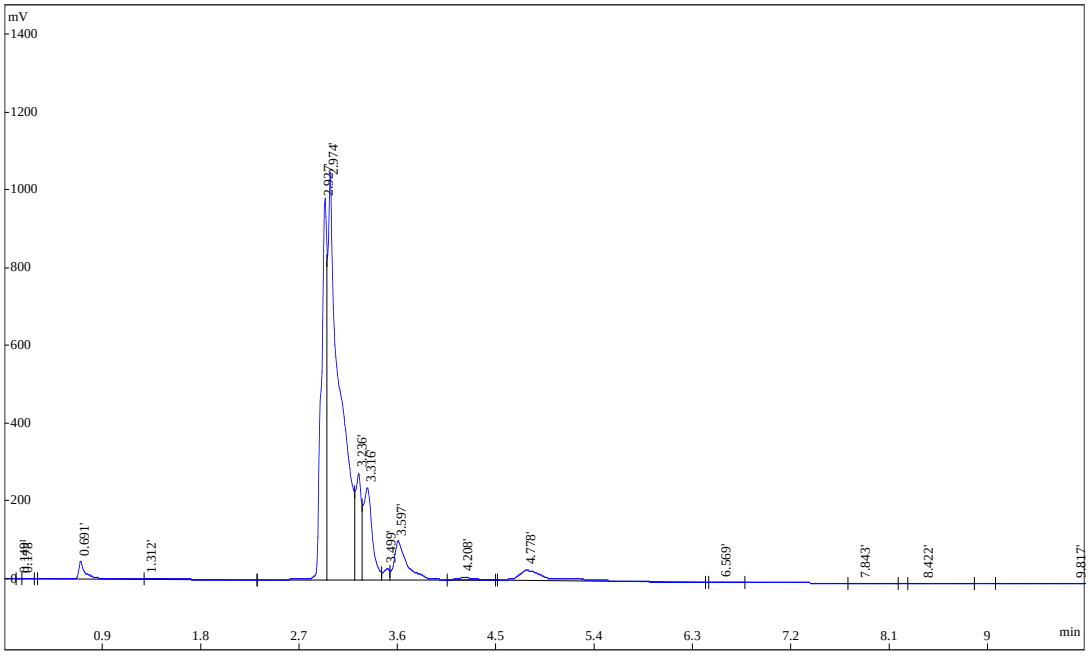


Fig.- 2 HPLC of Mycelial extract of *Trichoderma viride*



HPLC fractionation of mycelial extract of Antagonistic Organisms

HPLC analyses of mycelia extract showed few major and minor peaks. Among them, the major peak was obtained at the retention time 4.893 min by the HPLC analysis extract of *T. harzianum* (Fig.1). And for *T. viride* it was at RT 2.927 (Fig.2), for *A. niger* at RT 2.803(Fig.3), for *A. falvus* at RT 2.655 (Fig.4) for *A. fumigatus* at RT 2.694 (Fig.5), and for *A. sulphureus* at RT 2.6.94 (Fig.6). The HPLC results indicated the presence of more than one compound in all the extracts. The result of the present study supports the findings by Madhanraj, et al [14].

Fig. -3 HPLC of Mycelial extract of *Aspergillus niger*

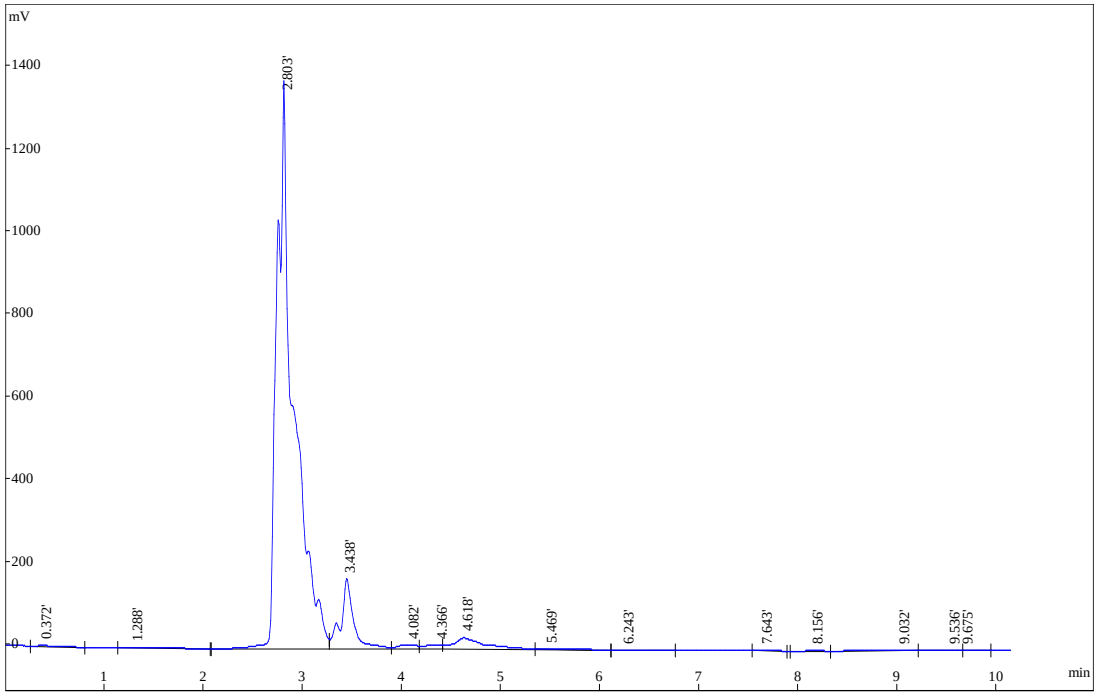
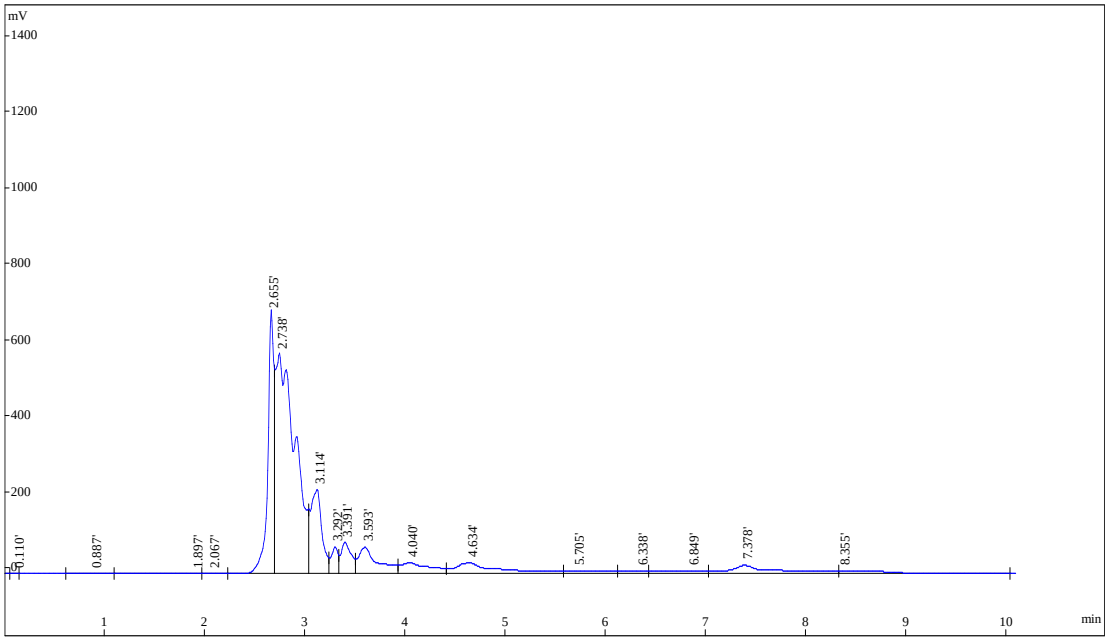


Fig.- 4 HPLC of Mycelial extract of *Aspergillus flavus*



Antifungal activity of HPLC fractionation of mycelial extract

Among the different fractions, tested form their antifungal activity the peak at RT 4.893 obtained from *T. harzianum* showed the highest growth inhibition (0.9 mm) followed by that of *T. viride* (0.7 mm), *A. niger* (0.5 mm), *A. fumigatus* (0.5 mm), *A. flavus* (0.4 mm), and *A. sulphureus* (0.3 mm) against *C.falcatum*.

The present study indicates the potential application of *T. harzianum*, and *Aspergillus* species against the red rot pathogen *C. falcatum.* Further studies on the molecular characterization of the specific compound that is responsible for the antifungal activity through FT-IR and GC-MS analysis is underway.

Fig. -5 HPLC of Mycelial extract of *Aspergillus fumigatus*

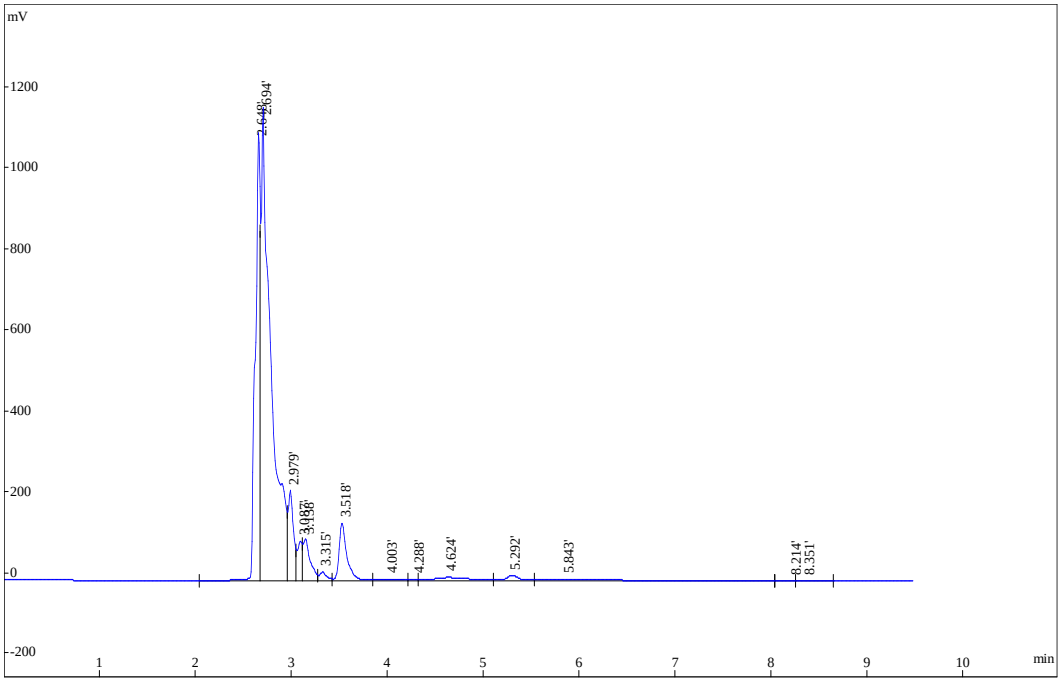
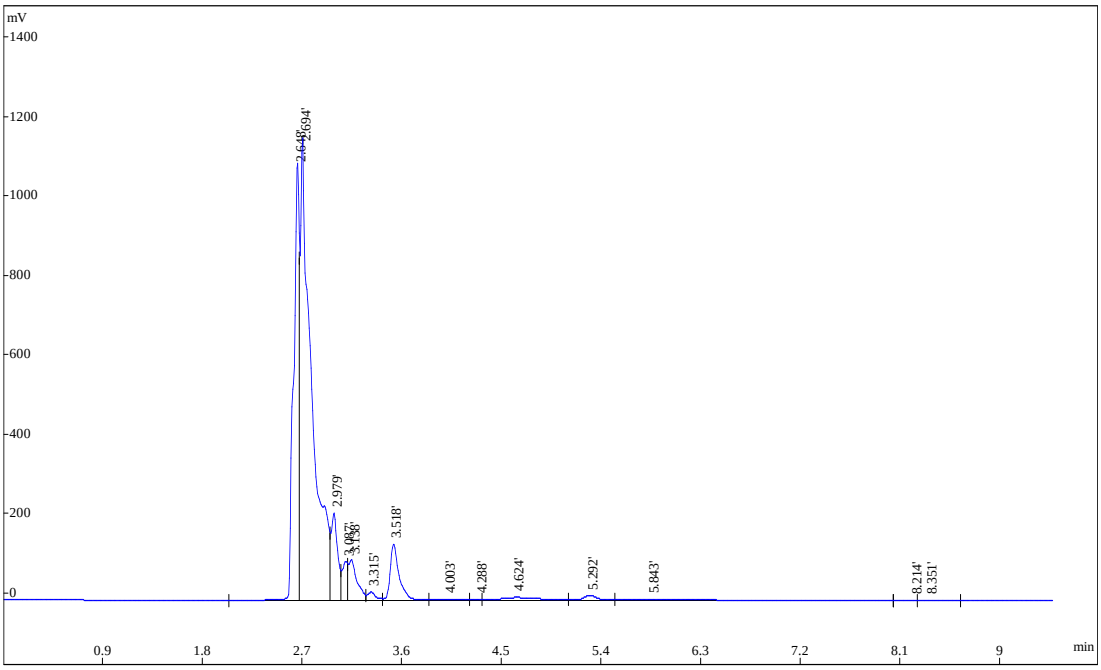


Fig. -6 HPLC of Mycelial extract of *A. sulphureus*



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