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Research Article

CYTOTOXIC ACTIVITY OF SECONDARY METABOLITE PRODUCED BY ENDOPH TICFUNGUS *FUSARIUM SP.* OF *CROCUS SATIVUS*

Prathvi Raj[*1], Shaukat Saeed Khan[2], Madhuri Modak[1], Deepak Chauhan[3]

[1]Department of Botany, Motilal Vigyan Mahavidyalaya,Bhopal (M.P) 462001 India
[2]Department of Microbiology, Saifia Science College Bhopal (M.P) 462001 India
[3]Govt. Science and Commerce College, Benazir Bhopal (M.P) India

Correspondence should be addressed to **Prathvi Raj**

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ABSTRACT

Endophytic microbes that inhabit host plants without causing any disease and are reported to produce secondary metabolites active against many diseases. In present study a pure compound was isolated from the endophytic fungus *Fusarium sp.* which was isolated from the plant *Crocus sativus.* The compound was predicted as crocin structure was elucidated by NMR and Mass. Anticancer activity of the compound was conducted against total four human cancer cell lines, Breast MCF-7, Cervix Hela, Leukemia TH-1 and Prostate PC-3.The compound affected the leukemia TH-1 cancer cells to the tune of about 43% and PC-3 cancer cells about 23%.The result of this study suggested endophytes of *Crocus sativus* and their metabolites could be a source of bioactive natural product against human cancer cell lines.

KEY WORDS: Endophytic fungi, Fusarium *sp.*, Crocussativus, Cytotoxic activity.

INTRODUCTION

Endophytic microorganism lives inside the plants for the least a part of their life cycle without causing any visible symptoms of disease[1]. The existence of fungi inside the tissue of asymptomatic plants has been known since the end of 19th century[2]and the term of endophyte was proposed in 1886. Many endophytes have ability to synthesize various bioactive metabolites that may be used as therapeutic agents against numerous diseases [3][4][5][6].The saffron is perennial plant that belongs to the family of Iridaceaeof the genus,*Crocus* and known botanically as*Crocus sativus.* This exotic plant is a native of Southern Europe and now cultivated worldwide in many countries, particularly in Spain, Italy, France, Greece, Turkey, In Iran and Jammu and Kashmir (India). *Crocus sativus* has been used to treat several medical conditions, such as gastro-intestinal disorders,urological infections, as well as in treating malignancies[7][8]. It contains components like safranal alphacrocin of crocin Additionally *Crocus sativus* also contains amino acids, flavonoids and other chemical compounds[3]Among the total, crocin is the most important since it is the major component and has shown significant biological activities[8]The present investigation was carried out to determine the endophytic fungal biodiversity in*Crocus sativus* with aim to explore their bioactive potentialto yield

potent bioactive compounds.The aim of study work was to systematically characterize endophytic fungi obtained from the title plant andtheir bioactive potential with respect to antimicrobial activity.

METHODS

PLANT SAMPLE COLLECTION

Mature healthy plants of *Crocus sativus* were collected from different places of the saffron fields of Kishtwar district (J&K India) which is located at 33°19'N ,75°46'E,33.32°N ,75.77°E. The upland valley in the north-east corner of Jammu region and the Great Himalayan Ranges emerging in cultivation of saffron . All samples were stored in sterile polythene bags at 4°C until they were to be used.

Isolation And Identification Of Endophytic Fungi

Samples were washed thoroughly in running tap water. Surface of the plant tissues was treated by following the methodology of Murali *et al* [17]. Plant tissues were immersed in 75% ethanol for 1 minute and in an aqueous solution of sodium hypochlorite (2.5% available chlorine) for 3 minute , followed by washing with 70% ethanol for 5 seconds . The tissues were then rinsed in sterile distilled water and allowed to surface-dry under sterile conditions. The surface-sterilized samples were placed on Petri dishes containing Potato Dextrose Agar (PDA) (supplemented with streptomycin (100 µg ml−1) to inhibit bacterial growth) and incubated at temperature at around 28°C. Fungi were identified on the bases of colony (macroscopic) morphology, rate of growth, and microscopic morphology. The taxa were assigned to genera following Barnett and Hunter and Von Arx [19,20].

Isolation And Characterization Of Active Principles

Isolated fungus wasoptimized for growth in fermentation conditions on small scale i.e. preferably shake flask stage.Culture of endophytic f ungi were inoculate into 250 ml of PD broth in Erlenmeyer flasks, incubated in shaking condition for 7 days at 28°C.Total broth 4L in 16 flasks. After showing growth in flask fungus were homogenized in stirring condition in the presence of 10% methanol and extracted by partitioning with equal volume of Dichloromethane Dichloromethane (DCM) organic was evaporated to dr yness in vacuo by rotary evaporator. A neat and dried column was taken. A cotton plug was put at the base of the column and packed with silica gel (60-120).Then the extract was dissolved in minimum quantity of DCM and 1.5 g silica gel was added for slurry formation and charged into the column .The column was run with DCM up to 20th fraction. Then column was eluted with DCM by gradually increasing the percentage of Methanol. Each fraction of 25ml was collected and dried on rotavapor, the fractions were pooled on the basis of the TLC (20% and 30% methanol: DCM) pattern and the spots were visualized by spraying the chromatogram with Dragondorff reagent.Structure elucidation of the molecules was carried out by means of NMR and MASSspectrograph only.

CYTOTOXIC ACTIVITY ASSAY

The effect of secondary metabolite on cell growth was determined in a panel of human tumor cells including Breast- MCF-7, Cervix-Hela, Leukemia-THP-1 and Prostate-PC-3 using 96-well tissue culture plates [18] . The 100µl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were allowed to grow in carbon dioxide incubator (37°C, 5% CO_2, 90% RH) for 24 hours. Test materials in complete growth medium (100µl) were added after 24 hours of incubation to the wells containing cell suspension. The plates were further incubated for 48 hours in a carbon dioxide incubator. The cell growth was stopped by gently layering trichloroacetic acid (50%, 50µl) on top of the medium in all the wells. The plates were incubated at 4°C for one hour to fix the cells attached to the bottom of the wells. The liquid of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove trichloroacetic acid, growth medium low molecular weight metabolites, serum proteins etc and air-dried. The plates were stained with Sulforhodamine B dye (0.4 % in 1% acetic acid, 100µl) for 30 minutes. The plates were washed five times with 1% acetic acid and then air-dried. The adsorbed dye was dissolved in Tris-HCl Buffer (100µL, 0.01M, pH 10.4) and plates were gently stirred for 10 minutes on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.

RESULTS AND DISCUSSION

The endophytic fungi Fusarium *sp.* was isolated from the healthy corm of *crocus* sativus collected from saffron fields of Kishtwar region. Fungi were identified on the bases of colony (macroscopic) morphology, rate of growth, and microscopic morphology. The taxa were assigned to genera following Barnett and Hunter and Von Arx [19][20]

Fusarium sp. was inoculated ino16Ч1L Erlenmeyer flasks each containing 250 ml of auto-claved PD broth. These flasks incubated in a shaker with 200rpm and temperature 28°C.After seven days culture was harvested and extra cted with DCM. The crude extract of Fusarium sp. was subjected to column chromatography in a column packed with silica gel (60- 120 mesh size) to isolate pure compound. Seven fractions (F1, F2, F3, F4, F5, F6 and F7) as monitored by TLC. Fractions were screened for anticancer activity in which F6 showed activity and was selected for further separation of the compounds on a silica gel column eluted with DCM by gradually increasing the percentage of Methanol. Each fraction of 15ml was collected and dried on rotavapor. The fractions were pooled on the basis of the TLC (3% and 5% methanol: DCM) pattern and the spots were visualized by spraying the chromatogram with Dragondorff reagent.

The fractions were pooled evaporated to dryness in va cuo by rotary evaporator.The yield of pure fraction wa s 7mg. Pure fraction was subjected for NMR and MA SS spectrograph. The molecular formula $C_{44}H_{64}O_{24}$ was assigned by HR ESI MS *m/z* 976.960 [M+H]+ (calcd . 976.964); ^{1}HNMR (400MHz,DMSO-d_6)=δ 6.89 (s, 2H), 6.77 (s, 2H), 6.53 (s, 2H) 6.48-6.45 (m, 4H), 6.34 (s, 2H), 5.40 (*d*, *J*=9.8Hz, 2H), 4.30-4.28 (m,2H), 3.70-3.60 (m, 14H), 3.49-3.35 (m, 6H), 3.20-3.18 (m,2H), 1.90 (s, 2XCH_3 , 6H) , 1.77 (s,2XCH_3, 6H). ^{13}CNMR (125MHz, DMSO-d_6) =δ 166.8, 141.9, 138.5, 138.5, 132.4, 131.0, 129.4, 128.6, 103.8, 93.8, 76.5, 75.3, 73.2, 67.8, 62.0, 16.0, 14.2. The compound was predicted as crocin not sure all the technique which was necessary for elucidation of structure were not possible because of insufficient quantity.

Cytotoxicity effect of endophytic fungal pure compounds was conducted against total four human cancer cells. Compound effect the Leukemia THP-1 cancer cell about 43% and PC-3 cancer cells about 23%. Results are shown in Table.1.

Table 1: Anticancer activity of compound isolated from fusarium sp.

Tissue Type		Breast	Cervix	Leukemia	Prostate
Cell line Type		MCF-7	Hela	THP-1	PC-3
Code	Conc.	% GROWTH INHIBITION			
Compound	100	10	12	43	23

Figure 1: *Anticaner activity of compound*

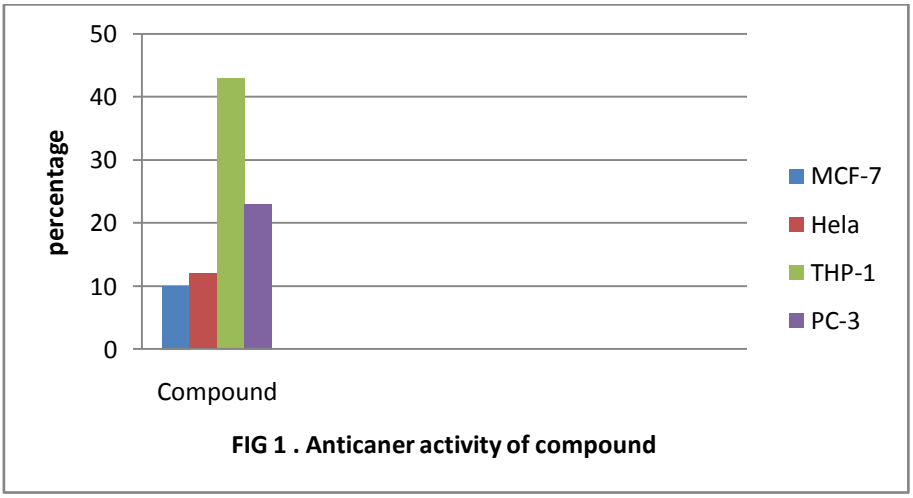

FIG 1 . Anticaner activity of compound

CONCLUSION

In this work pure compound predicted as crocin was isolated from the DCM extract of the endophytic fungus Fusarium *sp.* The compound was subjected to spectroscopic analysis NMR and Mass only due to insufficient quantity. The pure compound showed anticancer activity against leukemia THP-1 cancer cell line. The result indicates that potential of the endophytic fungus Fusarium *sp.* isolated from the plant *Crocus sativus* may be source of crocin. Some issues such as the mechanism of action of crocin, the physiological and ecological role of this fungus, isolation of other compounds from this fungus and efficient strategies for increasing the content of crocin, yield in fermentation culture of *Fusarium sp.* and isolation of endophytic fungus from *Crocus sativus* screening for active compound like crocin need to be further investigated. The worker is thankful to the Principal of M.V.M Bhopal who cordially permitted him for the Lab to work on endophytic fungi and their secondary metabolites.

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