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Isolation and Characterization of Coumarin Isolated from Endophyte, *Alternaria* Species -1 of *Crotalaria pallida* and Its Apoptotic Action on HeLa Cancer Cell Line

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Abstract

Endophyte, *Alternaria* species-1 isolated from leaf part of *Crotalaria pallida* was selected in isolation, identification and purification of coumarin. HPLC, UV-spectrophotometry, FTIR, NMR and XRD studies were carried out on characterization of endophytic fungal coumarin. *In vitro* apoptotic activity was carried out against HeLa cervical cancer cell lines. The results of MTT studies, acridine staining activity revealed the coumarin as an effective in inducing apoptotic activity in HeLa cells. The coumarin significantly inhibited the proliferation of HeLa cells and it's a concentration and time dependent manner. Significant elevation of caspase 3 and 9 and activity was observed in coumarin treated HeLa cells but no elevation or activity of caspase 7, 8, 10 was not observed. Coumarin has shown 0.156 µg/ml of significant IC₅₀ value against viable of HeLa cell lines. Thus, coumarin exerts apoptotic activity by caspase dependent apoptosis by enhancing the caspase 3 and 9 and it degraded the DNA (avoided the further replication). This is the first report in internationally that coumarin isolated from endophyte, *Alternaria* species-1, purified and characterized and its role was identified in apoptotic activity.

Keywords: Endophyte; *Alternaria* sp.; Coumarin; HPLC; XRD; FTIR; Apoptosis

Introduction

Cancer is a generic term refers to the large group of diseases characterized by abnormal growth of cells by rapidly beyond their usual boundaries, invading to other parts of the body organs leading to the formation of malignant tumors and neoplasmas. Cancer causes 1 in 8 deaths and rapidly becoming pandemic all over the globe (WHO, 2015). There were around 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million are living with cancer (IARC, 2012). This scenario of cancer has made focused on searching of new effective and economical both in usage and production of therapeutic agents. The cancer treatment is prolonged one, it is necessary to reinvestigate the therapeutics of short duration, less side effects, and economical. In this context natural products are considered to be the choice as apoptotic agents.

Coumarins are the benzopyrone compounds belong to flavonoid groups of secondary metabolites of plants. Presently there are more than 1300 coumarins have been identified in plants, bacteria, and fungi [1-3]. Coumarins have their specific fingerprints as antiviral [4], antimicrobial [5], antioxidant, anti-inflammatory [6,7], antiadipogenic [8], cytotoxic [9], apoptosis [10], antiproliferative [11], antitubercular [12] and cytotoxicity [13] agent(s). Due to these wide range of pharmacological values, coumarins and its derivatives has got more importance in synthesis and production.

Crotalaria is one of the largest genera in tropical Africa. The genus includes 690 species that are mainly situated in Africa and Madagascar [14]. Species have also been found in India, United States of America (USA) and China. *Crotalaria pallida* Aiton (Fabaceae) is an erect shrub, annual or short-lived perennial herb of 1.5 m or more tall. The plant has been used as traditional medicine; its roots have been used to treat swelling of the joints and its leaves as vermifuge [15]. Coumarins were having been reported from different species of *Crotalaria* [16,17]. In this urge, the effective isolation of coumarins by the endophytes of *Crotalaria pallida* was felt worthy. Endophytes are any ubiquitous

organisms, bacteria or fungi, occurring within plant tissues, distinct from the epiphytes that live on plant surfaces [18]. They inherit the characteristics of host plants in secreting the secondary metabolites [19-21]. These inheritance properties of endophytes are beneficial industrially in the production of important secondary metabolites.

The present study was aimed on, isolation and identification of endophytes of *Crotalaria pallida*, purification and characterization of endophytic coumarin by HPLC, UV spectra, XRD, FTIR and NMR, *in vitro* apoptotic activities on HeLa cell lines by MTT assay, acridine orange staining, analysis of caspase-3, 7, 8, 9 and 10 activity.

Materials and Method

Collection and mass production of leaf endophyte, *Alternaria* species-1

Collected the leaf endophyte, *Alternaria* species-1 from stock culture unit of Department of Biotechnology, Shridevi Institute of Engineering & Technology, Tumkur, India were mass cultured on PDB broth for large scale cultivation which was then incubated at room temperature 26 ± 2°C for 8 days.

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Microwave-assisted extraction (MAE)

After incubation, the fungal mycelium mat was taken for extraction using ethanol. Based on the earlier report of Umashankar et al. [22], Microwave Assisted Extraction (MAE) method was used, the endophytic fungal mat mixed with ethanol was kept for extraction in microwave method at 2 cycles of 5 minutes each at 100°C.

Identification of coumarins in the extracts

Extract containing coumarins was confirmed by the confirmation tests [23]. Extracts containing phenolic compound were confirmed by the confirmation tests [24].

Test 1: 3 ml of ethanol extract was evaporated to dryness in a vessel and the residue was dissolved in hot distilled water. It was then cooled and divided into two test portions, one was reference, second was the test. To the second test tube added 0.5 ml of 10% ammonium hydroxide. The occurrence of intense fluorescence indicates the presence of coumarins and derivatives [23].

Test 2: To the concentrated alcoholic extract, added few drop of alcohol FeCl_3 solution deep green colour formed which turned to yellow on addition of conc. HNO_3 indicates the presence of coumarins.

Test 3: The alcohol extract of drug was mixed with 1 N NaOH solution (one ml each). Development of blue green fluorescence indicates presence of coumarins.

Test 4

Detection of phenols: In beakers, 5 ml of each previous filtered extract were taken and 1ml of FeCl_3 (1%) and 1 ml $\text{K}_3(\text{Fe}(\text{CN})_6)$ (1%) were added. The appearance of fresh radish blue color indicated the presence of polyphenols [24]

Isolation, purification of coumarin from *Alternaria* species-1 from high performance liquid chromatography (HPLC)

Identification, isolation and purification of coumarin were done using *Alternaria* species-1 ethanol extract based on our earlier report [13].

The obtained chromatograms were compared with standards based on retention at 18.7 min is p-coumaric acid, 30.227 min is 2- hydroxyl cinnamic acid and 31.231 min is coumarin.

UV-Visible spectrophotometry

The coumarin absorption was carried out on a UV spectrophotometer at 305 nm in quartz cell. The coumarin was well known for their absorbance in the UV region of the spectra because of the mesomeric effect of the double bonds, and their phenolic cycle. The polyphenols are detectable, then at low concentration (1–10 l M).

Fourier transform infrared spectroscopy (FTIR)

The powder material and *Alternaria* species-1 mycelia mass was ground with a specially purified potassium bromide in a mortar and pestle. The ground mixture was then pressed in a mechanical press to form a translucent pellet. The infrared spectra of the materials were recorded using Agilent (Cary-630) Fourier transform spectrophotometer in the range of 400-4000 cm^{-1} .

X-ray diffraction (XRD) studies

The synthesized sample is gently grounded in an agar mortar and pestle. The fine powder is packed into a sample holder having a

diameter of 1 cm and depth of 3 mm. The surface of the packed sample is smoothed with a flat glass and then powder X-ray diffraction spectra of the samples are recorded on X'pert Pro X-ray diffractometer. The powder XRD involves the use of the collimated beam of X-rays; with wave lengths Cu K α radiation of wavelength in the range 0.5 to 2 Å, incident on a specimen, which is diffracted by the crystalline phase in the specimen. The identification of the phase(s) of the as sample was carried out by x-ray powder diffraction (PANalytical X'pert X-ray powder diffractometer) using Cu-K α radiation at $\lambda=1.5418$ Å. A scan step of 1 s and a step size of 0.1 (in 2 θ) were applied to record the patterns in the range of 5 to 70° (2 θ).

The Powder XRD pattern has been used to determine the symmetry of the crystallographic unit cell of the coumarin.

C-13 NMR

The solid coumarin was tested by NMR. The NMR spectrum of the crystalline coumarin was done in order to be compared to other spectra. The NMR tests were recorded at 300 MHz on a Bruker spectrometer. Chemical shifts are expressed in part per million.

In vitro apoptosis activity on HeLa cell lines

HeLa (cervical cancer) cell lines was obtained from NCCS (National Centre for Cell Science), Pune. The HeLa cells were cultured in DMEM 10% FBS complete medium. The medium was supplemented with 10% heat inactivated fetal bovine serum and antibiotics. The cell lines were maintained at 37°C in a 5% CO_2 incubator and the media were changed frequently.

MTT assay

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. Experimental designs were done at 24 and 48h based on procedure of Umashankar et al. [13].

Detection of apoptosis by acridine orange staining

Detection of apoptosis in HeLa cell lines is characterized by the double staining with acridine orange and ethidium bromide [25]. Live cells are observed by bright dots and dead cells by orange to brown colour. 2×10^4 cells per well were seeded in 96 well plate and treated for 48 h. After incubation, the plates were centrifuged. 10 μl of 1 mg/ml Acridine orange and Ethidium bromide mixture was added to each well. Nuclei were visualized and photographed under the fluorescent microscope. The differentiation in the staining of live and dead cells was observed with these two stains. Acridine orange stains both live and dead cells. The ethidium bromide stains only the dead cells by became organization due to intercalate DNA of cells with lost or degraded DNA of cell integrity.

Determination of caspases activities

The cells were harvested and washed in PBS at 4°C after incubation. Using caspase apoptosis kit, the caspases 3, 7, 8, 9 and 10 enzymes activities were analyzed and quantified [26,27] in coumarin treated HeLa cell line. All measurements were performed 3 times, and changes

in absorbance of each caspases were measured at 405 nm using an ELISA reader.

Ten µg/ml of *Alternaria* species-1 coumarin treated the HeLa cells to induce apoptosis, cells treated with DMSO and phosphate buffered saline (pH 7.2) were used as control, standard drug 5-fluorouracil (0.26 µg) was used as positive control. After treatment, the cells were incubated for 48 h. After incubation, the cells were collected and suspended with cell lysis buffer, cells were incubated on ice for 10 min and centrifuged at 10,000 rpm for 5 min and supernatant obtained was treated with 4 ml of 4 mM pNA-conjugated substrates (DEVD-pNA) and incubated for 3.5 h at 37°C. The released amount of pNA from each treatment was measured at 405 nm using ELISA microplates reader. Based treated to untreated cells the absorbance of the relative caspase activity was calculated.

To study the inhibitory action of caspase-3, 7, 8, 9 and 10 in the extract, the sample was pre-incubated with inhibitor in cell lysate samples at room temperature ($26 \pm 2^\circ\text{C}$) for 10 min before adding caspases 3, 7, 8, 9 and 10 substrate solution separately.

Results

The endophyte, *Alternaria* species-1 have shown the presence of coumarin in all the tested methods and also confirms the presence of polyphenols, it indicates coumarin presence (Table 1). These methods are commonly using to study coumarin presence in the extract.

Identification of coumarins in the extracts by HPLC

When we compared with standard coumarins, the results confirmed the presence of two coumarins (p-coumaric acid and coumarin) at high per cent in ethanol extracts of *Alternaria* species-1 in HPLC method (Table 2). Figure 1 depicts the standard coumarins in HPLC. The ethanol extract of leaf part of *Crotalaria pallida* has shown three different types of coumarins (Figure 2). The ethanol extract of fungal endophyte, *Alternaria* species-1 yielded only two coumarins viz., p-coumaric acid and coumarin, they were identified based on their retention time and compared with standard in HPLC (Figure 3 and 4). Purified coumarin based on retention time (31.514) and obtained single peak in HPLC after collecting the bulk quantity (Figure 5). The coumarin was isolated and purified from *Alternaria* species-1 using HPLC based on retention time (31.514) and done the experiment repeatedly to collect the purified coumarin at large quantity and run the same sample in the HPLC to know its purity and to obtain pure coumarin for *in vitro* apoptosis activity. The purified coumarin was characterized by other specific analytical methods and parameters such as UV-Vis, XRD, FTIR and NMR studies. The results of these studies were very significant for purity and confirmed. These methods were used to characterize and confirm the coumarins isolated different sources [28-30].

Using diode array detector plotted the chromatographic/spectrometric data images. It was used to facilitate HPLC method to optimize and validity for detection conditions in off-line also (Figure 9). Based on linearity, sensitivity, precision, repeatability, and accuracy the method was validated. The method was used to determine seven phenolic compounds include p-coumaric acid [31,32]. To improve the quantitative analysis method established the iso-plot method and it is effective also. Using C18 column with gradient elution of phosphoric acid aqueous solution (0.05%, v/v) and acetonitrile the chromatographic separation was done, and adopted detection of isolated p-coumaric acid using wave lengths switch programme and optimized with iso-plot. Isolation and purification of coumarins were carried out by the repeated performance and collection of specific

Phytochemicals	<i>Alternaria</i> species-1 (Leaf)
Phenols	+++
Coumarin test 1	+++
Coumarin test 2	+++
Coumarin test 3	+++

*Repeated the each experiment thrice, +=Presence, ++=Medium, +++=More and -=Absence

Table 1: Identification of coumarins and phenol in leaf endophyte, *Alternaria* species-1.

Coumarins	Leaf extract	<i>Alternaria</i> species-1 (Leaf)
p-Coumaric acid	+	+
2-Hydroxy coumaric acid	-	-
Coumarin	+	+

Note: +: presence, -: absence

Table 2: Presence of two different coumarins in plant extract and *Alternaria* species-1.

Figure 1: Standard chromatogram of all the three coumarins in HPLC.

volume (v) on a third was done to coumarin using 3D plot graph. Based these studies coumarin was isolated and purified and again subjected HPLC to know the purity. Phenolic compounds absorb ultraviolet region strongly and detection and quantification the spectra was used. Detected the phenolic compounds by studying absorbance at 280 nm from the eluent after separation with selecting mobile and stationary phases. Our results confirmed by the results of Hosler et al. [24].

The coumarin absorption was carried out on a UV spectrophotometer at 305 nm in quartz cell. The coumarin was well known for their absorbance in the UV region of the spectra because of the mesomeric effect of the double bonds, and their phenolic cycle. In this range, studied absorption showed four absorption bands at 241 nm, 297 nm, 277 nm and 307 nm for the coumarin standard and coumarin from the extract of *Alternaria* species-1 (Figure 6 and 7).

FTIR

The IR spectrum of coumarin shows lactone carbonyl at 1715 cm^{-1} , $\text{vC}=\text{C}$ at 1608 cm^{-1} , 1450 cm^{-1} and $\text{vC}-\text{O}-\text{C}$ at 1254 cm^{-1} . Infrared spectroscopy involves the absorption of electromagnetic radiation in the infrared region of the spectrum which results in changes in the vibrational energy of molecules. Usually all molecules have vibrations in the form of stretching, bending, etc., the absorbed energy will be utilised in changing the energy levels associated with them [33]. The IR spectrum of coumarin shows a sharp band at 3381 cm^{-1} associated to the stretching -OH vibration. The band at 2963 cm^{-1} associated with the aromatic CH-stretching. The band present at 1715 reflecting the CO-stretching of carbonyl group of COOH (Figure 8 and 9) and it was compared with standard and the purity of the compound is similar to standard coumarin. The shows the FTIR spectrum of coumarin laser dye and compared with chemical formula of this dye. There were more than one peak obtained in region of the C-H bending vibrations out of plane ($900-600\text{ cm}^{-1}$) can support the presence of an aromatic structure. In region ($1200-1000\text{ cm}^{-1}$), there is a peak at 1028.09 cm^{-1} refers to C-H bending vibrations in of planes. The benzene rings very clear which is supportive to the peak at 1489.10 cm^{-1} that acts the $\text{C}=\text{C}$ stretching ($1400-1600\text{ cm}^{-1}$ of the benzene ring and a peak at 3061.13 cm^{-1} for C-H stretching ($3000-3100\text{ cm}^{-1}$).

XRD

The powder XRD pattern of the coumarin corresponds to

Figure 4: Separation of p-coumaric acid and coumarin from the crude extract and its area summary.

Figure 5: Purified coumarin from *Alternaria* species-1 showing 31.130 retention time.

Data Set: Coumarin from alternaria species of crotalaria pallida - RawData - G:\MGR UV\coumarin from alternaria species of crotalaria pallida.spc

Figure 6: UV-visible spectra: coumarin from the extract of *Alternaria* species-1.

Data Set: coumarin standard - RawData - G:\MGR UV\coumarin std.spc

Figure 7: UV-visible spectra: coumarin standard.

orthorhombic phase with space group Pca^z ($Z=4$) (Figure 10).

A typical analysis of a ^{13}C NMR spectrum consists of matching expected chemical shifts to the expected moieties. The chemical shifts were matched with the existed earlier reports. For coumarin, chemical shifts 103.326, 109.818 and 103.322 were characteristic (Figure 6). The

Figure 8: FTIR spectra of standard coumarin.

Figure 10: XRD patterns of the coumarin corresponds to orthorhombic phase.

Figure 9: FTIR spectra of purified coumarin from the extract of *Alternaria* species-1.

Figure 11: C-NMR pattern of the coumarin from the extract of *Alternaria* species-1.

results are compared with IR spectra of standard coumarin (Figure 11).

In vitro apoptotic activity on HeLa cell lines

In vitro apoptotic activity on HeLa cell lines shows that, the endophytic fungal coumarin from *Alternaria* species-1 has remarkable apoptotic activity on cancer cell lines. MTT assay showed that the decrease in the cell viability after 24 and 48 h. Apoptotic activity of endophytic fungal coumarin was demonstrated by its inhibition of proliferation of HeLa (cervical cancer) cells in a time and dose-dependent manner. 0.156 µg/ml of significant IC₅₀ value was noticed (Figure 11 and 12) (Table 3).

Acridine orange and ethidium bromide staining

Detection of apoptosis in HeLa cell lines stained with acridine orange and ethidium bromide treated by coumarin has given significant results. The live cells were appeared as bright green spots and dead cells are as orange to brown. Control treated cells showed most of the live cells and there are more dead cells at 48 h of treatment than the cells treated for 24 h. Staining with orange or brown is due to the ethidium bromide, which acts as intercalate agent with apoptic cell DNA (Figure 13 and 14).

Caspases are known to be involved in pivotal regulator of cellular apoptosis [33]. We have identified the effect or role of coumarin on activation of caspases 3, 7, 8, 9 and 10 by calorimetric method. The p-coumaric acid effectively induced the activity of caspases 3 and 9 in HeLa cells and no effect was observed in caspases 7, 8 and 10 activities.

From the results, conclude that the coumarin isolated from *Alternaria* species-1 treatment enhanced the cell death of HeLa cancer cell line by activating caspases enzymes in intrinsic pathways (Figure 15).

Alternaria species-1 coumarin induced apoptosis in HeLa cells was associated with typical apoptosis by nuclear DNA degradation and induction of caspase-3 and 9 activity.

Discussion

Production of secondary metabolites from endophytes is a wide area. It depends on the host plant, endophytic fungi, laboratory conditions and other optimization parameters. Based upon our earlier *in vitro* biological and HPLC screening studies [13,22], *Alternaria* sp. have been selected for isolation and purification of coumarin and its cytotoxicity on HeLa cell lines. Investigations of Taxol, the first apoptotic agent recovered by an endophytic fungi *Taxomyces andreanae* of *Taxus brevifolia* [34] and other apoptotic drugs, Paclitaxel from *Alternaria* sp. of *Taxus cuspidata*, *Ginkgo biloba* and *Taxus chinensis* [35-37], Podophyllotoxin from *Alternaria* sp. of *Sinopodophyllum hexandrum*, *Sabina vulgaris* and *Sinopodophyllum hexandrum* [38-40] strengthens this apoptotic study in relation to endophytes and the selected *Alternaria* species.

The detailed analytical characterization studies of coumarin confirmed its presence in endophyte *Alternaria* species-1 of *Crotalaria*

Drug concentration (µg)	% of viability		% of cell death	
	24h	48h	24h	48h
0.078	88.664 ± 0.01	87.38 ± 0.02	11.34 ± 0.02	12.62 ± 0.01
0.156	48.36 ± 0.02	39.9 ± 0.02	51.64 ± 0.02	60.1 ± 0.01
0.312	17.784 ± 0.01	14.03 ± 0.01	82.21 ± 0.01	85.7 ± 0.02
0.625	8.684 ± 0.01	9.303 ± 0.01	91.31 ± 0.02	90.97 ± 0.02
1.25	1.352 ± 0.01	1.143 ± 0.02	98.64 ± 0.01	98.87 ± 0.01
2.5	0.416 ± 0.02	0.467 ± 0.01	99.58 ± 0.03	99.53 ± 0.03
5	1.456 ± 0.03	1.61 ± 0.03	98.54 ± 0.02	98.39 ± 0.02
Vehicle control	97.8 ± 0.02	97.5 ± 0.02	2.2 ± 0.0	2.5 ± 0.0
Doxorubicin	10.13 ± 0.1	07.0 ± 0.1	89.87 ± 0.2	93 ± 0.1
IC ₅₀	0.156 µg			

Table 3: *In vitro* apoptotic activity of endophytic coumarin from *Alternaria* species on HeLa cancer cell lines.

pallida. HPLC results were found similar with the results of standard coumarin. FTIR results of endophytic coumarin were confirmed by the earlier studies reported by Murthi et al. [41]. The XRD results were also significant and confirmed by the reports of Ramaswamy [42]. Similar works were carried out in characterization of coumarin in *Justicia pectoralis* leaf [43], *Melilotus suaveolens* [44]. A cytotoxic activity of endophytic coumarin of *Alternaria* species-1 on cancerous HeLa cell lines was extensively significant having an IC₅₀ value of 0.156 µg/ml. MTT assay, acridine orange staining and caspase-3 colorimetric studies reveals that, the cytotoxicity of endophytic coumarin on viable of HeLa cells is due to DNA degradation and induction of apoptotic caspase-3 and 9 enzyme activity. Caspases trigger apoptosis through a well-orchestrated cascade [45,46]. Caspases are crucial mediators of programmed cell death (apoptosis). Among them caspase-3 is frequently associated

protease, catalyzing the target specific cleavage of many key cellular proteins [47]. Caspase -7 is executioner caspases, activates the intrinsic apoptotic pathways through cleavage of BID to induce efficient cell death, apoptotic cell development. Caspase-9 initiates apoptosis by cleaving and thereby activating executioner caspases, mitochondrial morphological changes, ROS production by cleaving and activating

BID into tBID [48]. Caspase-10 is initiate caspase in death receptor signaling [49].

The acridine orange fluorescent study clearly reveals that the cytotoxicity on HeLa cells is due to cytostasis but not through cell necrosis. Apoptotic activities of endophytic coumarin were rectified by the recent works carried out in HeLa cells with different apoptotic drugs and fungal secondary metabolites [50-52]. The natural coumarins induces apoptosis like cell death in HeLa cells mediated by the release of apoptosis inducing factor [53] but our source is different i.e. endophytic fungi. Interestingly, we also found caspase depended apoptosis from our experiment whereas, we found elevation of caspase-3 activity. The coumarin induces morphological changes, G0/G1 arrest, DNA fragmentation and increased the sub-G1 group, affected the production of reactive oxygen species and Ca^{2+} concentration, depolarization of mitochondrial membrane potential, the anti-apoptotic proteins Bcl-2 and Bcl-xL, and increased the expression of the pro-apoptotic protein Bax, decreased the mitochondrial membrane potential and promoted the release of cytochrome c and the activation of caspase-3 before leading to apoptosis in human cervical cancer cells (HeLa) [54]. Our endophytic fungal coumarin have shows some of the above results such as caspase-3 activity, toxicity and DNA fragmentation in HeLa cancer cells.

Conclusion

In conclusion, the endophytic coumarin was isolated, identified by the detailed UV-Vis, HPLC, FTIR, XRD, NMR studies. Apoptotic activity was confirmed by MTT assay, acridine orange staining and more activity of caspase 3 and 9. The mechanism of endophytic coumarin was assumed through DNA degradation and induction of caspase-3 and 9 activities without any cell necrosis. Further, optimal studies are needed to investigate *in vivo* condition, in dose fixation and other etiological studies. This is the first report on identified the coumarin produce endophyte, *Alternaria* species-1, its characterization and apoptotic activity in nationally and internationally. The endophytic fungi can be used for production of apoptotic agent, coumarin.

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