ISOLATION, PURIFICATION AND IN VITRO CYTOTOXICITY ACTIVITIES OF COUMARIN ISOLATED FROM ENDOPHYTIC FUNGI, ALTERNARIA SPECIES OF CROTALARIA PALLIDA

Umashankar T¹, Govindappa M¹*, YL Ramachandra² Chandrappa CP¹, Padmalatha Rai S³ and Channabasava R¹

¹Natural Product Laboratory, Department of Biotechnology, Shridevi Institute of Engineering & Technology, Sira Road, Tumakuru- 572 106, Karnataka, INDIA.
²Department of P.G. Studies and Research in Biotechnology & Bioinformatics, Kuvempu University, Jnana Sahyadri, Shankaraghatta Shimoga, Karnataka -577 45, INDIA.
³Department of Biotechnology, School of Life Sciences, Manipal University, Manipal-576 104, Karnataka, INDIA.

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ABSTRACT

In the present investigation was aimed to isolate and identification of bioactive compound from Crotalaria pallida endophytic fungal Alternaria species ethanol extract and evaluated for in vitro cytotoxicity assays using onion roots, yeast and greengram germination method. MAE method was used for isolation of potent compound and subjected to purification from HPLC with standard compounds. In vitro cytotoxicity methods were used to know the toxicity level of the isolated compound. MAE method yielded major bioactive compounds along with three coumarin(s) and it was confirmed with all four tests and the 5th test confirmed as a phenolic agent. Endophytic fungal coumarin inhibited the actively growing onion root meristematic cells by possessing various abnormalities by producing highest percentage of cytotoxicity (18.05%), the death of yeast was observed in extract treated yeasts and the percentage of toxicity was 88.03% and at 400 µL of endophytic coumarin inhibited the greengram germination and there is no germination was observed. The fungal coumarin also cleaved the DNA in yeast and it is a strong evidence of toxicity in DNA level and shown apoptosis activity. For all experiments, standard coumarin was used. The endophytic fungal coumarin results were near to equal of standard. From the obtained results, the endophytic fungi Alternaria species have the ability to produce coumarin and it exhibited as strong in vitro cytotoxicity activity. It can be used as a cancer drug after test against cancer cell lines.

Keywords
Alternaria Species, Coumarin, HPLC, Cytotoxicity, Antiproliferative.

Corresponding author
Dr M Govindappa
Natural Product Laboratory,
Department of Biotechnology,
Shridevi Institute of Engineering & Technology,
Sira Road, Tumakuru-572 106, Karnataka, INDIA.
+91-7204238327,
+91-816-2212628,
endophytessiet@gmail.com

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INTRODUCTION

Fungal endophytes are micro-organisms that colonize living, internal tissues of plants without causing any immediate and overt negative effects [1]. The fungal endophytes have proven to be promising sources of many biologically active natural products [2].

_Crotalaria pallida_ is a terrestrial, annual, erect herb, up to 150 cm tall. Taproot white or brown and stem grooved, solid, glabrous. A novel antimicrobial peptide has been exhibited as a strong antimicrobial agent against human pathogenic micro-organisms [3] and lectin [4]. In the folk and Ayurvedic medicines, _C. juncea_ is used as blood purifier, abortifacent, astringent, demulcent, emetic, purgative and in the treatment of anaemia, impetigo, menorrhagia and psoriasis [5]. _Crotalaria_ species possess anticancer properties [6]. Presence of coumarin has reported from _Crotalaria madhurensis_ [7] and _Crotalaria ramosissima_ [8].

The antimicrobial, antioxidant, anti-inflammatory, lipooxygenase, xanthine oxidase (XO) and acetylcholinesterase activities and phenolic contents of different solvent extracts (ethanol, ethyl acetate, chloroform, petroleum ether and water) of _Crotalaria pallida_ were evaluated using standard _in vitro_ methods and extracts exhibited anti-HIV activity [9]. Umashankar _et al._ [10] have reported four fungal endophytes from two different parts and they have shown the presence of coumarin(s) presence. These partially purified coumarins have been shown as strong antioxidants and antimicrobial activity. The natural coumarin isolated from various plants is a ubiquitous plant metabolite with strong antioxidant, antimicrobial, antiviral, anti-inflammatory, antihypertensive, antitubercular, anticonvulsant, multiple sclerosis, antidiabetic and anticancer properties [11].

Further, the present investigation was aimed to identify and characterize the exact coumarin present in leaf fungal endophyte, _Alternaria_ species and evaluated for _in vitro_ cytotoxicity assays to know their efficacy.

Materials and METHODS

Mass culture of fungal endophyte, _Alternaria_ species and extraction

Endophytic fungi, _Alternaria_ species were isolated from leaf part of _Crotalaria pallida_. Mass cultured the fungi using potato dextrose broth for 8 days at room temperature (26±2°C). After incubation, the fungal mycelium mat was taken for extraction using ethanol. Based on the earlier report of Umashankar _et al._ [10], 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 and analyzed the percentage of coumarin.

Identification of coumarin in _Alternaria_ species

Identification of coumarin in the extracts

Test 1

3ml 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 the reference, second was the test. To the second test tube, 0.5 ml of 10%_NH₄OH_ was added. The occurrence of intense/fluorescence under UV light is a positive test for the presence of coumarins and derivatives. The experiment was carried out for all the experiments in three replicates [12].

Test 2

5ml of the extract was evaporated to dryness and the residue was dissolved in 2ml of distilled water. The aqueous solution was divided into two equal parts in test tubes. One part was the reference. To the other test tube, 0.5ml of 10%ammonia solution was added and the test tubes were observed under UV light indicated. The occurrence of a bluish green fluorescence under UV light indicated the presence of coumarin derivatives [12].

Test 3

To the concentrated alcoholic extract of drug few drops of alcohol FeCl₃ solution was added. Formation of deep green colour, which turned yellow on addition of conc. HNO₃, indicates coumarins presence.

Test 4

The alcoholic extract was mixed with 1N NaOH solution (one ml each). Development of blue green fluorescence formation indicates presence of coumarins.

Test 5, Detection of phenols

In beakers, 5ml of each previous filtered extract were taken and 1ml of FeCl₃ (1%) and 1ml K₃(Fe(CN))₆ (1%) were added. The appearance of fresh radish blue color indicated the presence of polyphenols [13].

Isolation, identification and purification of coumarin from _Alternaria_ species

TLC

The solvent extract was centrifuged at 6000 rpm for 10 min and then filtered with Whatman No. 1 filter paper and evaporated at a constant temperature of 62°C in hot air oven until a very concentrated extract was obtained. The mobile phase was toluene: benzene: ethyl acetate (60:20:10) and visualized under UV light.
Identification of coumarin by reversed phase liquid chromatography with gradient elution

The extraction system comprised of a domestic microwave (Trio-triple) manufactured by Samsung CE108MDF-B (Bangalore, India) equipped with 230v/50Hz with a nominal maximum power of 900W, a reflux unit, 6 power levels, representation of the analysis of mean values from each level for a particular factor. Agilent technologies High Performance Liquid Chromatograph 1100 series equipped with quaternary solvent delivery pumps, diode array detector, autosampler and integration with Chemostor Software from Agilent. Stainless steel analytical columns were Inertsil® ODS-3V, C18 (250 x 4.6 mm I. D., 5 μm) (GL Science Inc, India).

Chemicals and materials

Acetonitrile (ACN, HPLC gradient grade, Rankem RFCL Limited), orthophosphoric acid (Analytical grade, Spectrochem) and methanol (MeOH, HPLC gradient grade, Rankem RFCL Limited), HPLC-grade water (Millipore). Reference standards of Coumarin >99.0% (1-2 Benzopyrone, Sigma-Aldrich Lot No.#030M1441V), p-coumaric acid >98.0% (Sigma-Aldrich Lot No.#110M1259V) and 2-Hydroxy cinnamic acid 97.0% (Sigma Aldrich Lot No.#STBB1076V) were obtained from Sigma-Aldrich, India.

Preparation of standard solution

Accurately weigh and transferred 50 mg each of mg of coumarin, p-coumaric acid and 2-hydroxy cinnamic acid standard into 100.0 mL volumetric flask, dissolve and dilute to volume with diluent. Further transfer 5.0ml of this solution into 50.0 mL volumetric flask dilute to volume with diluent.

Reagents preparation

0.05% v/v of orthophosphoric acid buffer
Transfer 1.0ml of orthophosphoric acid into 2000ml water. Filter this solution through 0.45µm membrane filter and sonicate to degas.

Mobile phase-A:
0.05% v/v orthophosphoric acid buffer.

Mobile phase-B:
Mix 995 mL of Methanol and 5mL of 0.05% v/v orthophosphoric acid Buffer. Filter through 0.45µ membrane filter and degas.

Mobile phase-C:
Mix 995 mL of Acetonitrile and 5mL of 0.05% v/v orthophosphoric acid Buffer. Filter through 0.45µ membrane filter and degas.

Diluent preparation:
Mix 90:10 ratio of Methanol and water.

Chromatographic conditions

Column: Inertsil® ODS-3V, C18 (250 x 4.6 mm I. D., 5 μm)
Flow: 1.0ml/min
Column Temperature: 25°C
Wavelength: 275nm
Injection volume: 10μl
Run time: 60 minutes

<table>
<thead>
<tr>
<th>Time</th>
<th>Mobile phase-A (in %)</th>
<th>Mobile phase-B (in %)</th>
<th>Mobile phase-C (in %)</th>
</tr>
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<tr>
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<td>5.0</td>
<td>15.0</td>
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<td>65.0</td>
<td>20.0</td>
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</tr>
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<td>35.0</td>
<td>70.0</td>
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<td>40.0</td>
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<td>5.0</td>
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<tr>
<td>50.0</td>
<td>80.0</td>
<td>5.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Plant and endophytic fungal material

1.0g of powdered plant material and endophytic *Alternaria* species fungal mycelial mat (screened through mesh 44) were transferred in to 25.0ml volumetric flask. Added about 20ml of diluent and sonicate for 120minutes, cooled to room temperature diluted to volume with diluent. Filtered through Accu-Bond SPE Cartridge (Agilent Technologies, India) discard the first few ml of the filtrate.
Standard chromatogram
Retention at 18.7 is p-coumaric acid, 30.227 is 2-hydroxy cinnamic acid and 31.231 is coumarin.

Antimitotic activity using *Allium cepa* root tips
Growing *A. cepa* root tips

The fresh and healthy bulbs of onion were obtained from the local market of Tumakuru, Karnataka, India. To achieve sprouting, the bulbs were placed in contact with distilled water and extract in a 25 ml beaker at room temperature (26±2 °C) for 2 days in the dark individually. The distilled water was changed every 24 h between 9 to 10 h. Bulbs with root length of 2 cm and above (range= 2.2-3 cm) were selected for *A. cepa* test [14].

Antimitotic studies

The bulbs that developed uniform roots were used for the experiment. These roots (three roots per concentrations) were incubated with ethanol extract of *Alternaria* species and plant extract at various concentrations of 400 and 50 µg/ml. A control was set with three roots from the same bulb with distilled water and 0.2M PBS (pH 7.2) was used as a medium. Mitotic index were recorded at 12, 24 and 48 h of incubation and compared with that of control bulbs placed over distilled water and 0.2M PBS and quercetin was used as standard drug [14].

Microscopic investigations

The root tip cells were fixed with stained examined using a compound microscope. The treated roots were rinsed with distilled water and cut into segments about 1-2 cm length from the tips and fixed in ethanol: glacial acetic acid (3:1), hydrolyzed for 5 min with 1N HCl at 70 °C and stained with 2 % acetocarmine for 1 h. Stained root tips were excised and squashed on a clean glass slide with a drop of 45 % acetic acid and examined under microscope. In all the slides, 100-300 cells were counted to determine the number of cells in interphase and dividing phase. The mitotic index was calculated by using the formula:

\[
\text{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100
\]

Changes in chromosome morphology were photographed under (100 X) a light microscope with a Sony photographic camera.

Evaluation of cytotoxic effect of coumarin on seed germination of *Vigna radiata*

Inexpensive cytotoxic assay of p-coumaric acid on sprouting seeds has been carried out using green gram (*V. radiata*) seeds by the method [15], seeds were purchased from a grocery store of good quality. For germination assays, The seeds were surface sterilized with 0.1% mercuric chloride solution for 2 minutes and washed thoroughly with tap water and then with distilled water for 30 minutes, seeds were placed in solutions of lectin from endophytic fungi, *Alternaria* sp. and plant extract at two concentrations (200 and 400 µg/ml) taken in 24-well plates and dry seeds of equal weight were added one each to microtitre wells and the plate was closed with the lid and left at room temperature for 24 h for imbibed with water. Similarly, standard anticancer agent coumarin was also prepared at various concentrations (50 and 400 µg/ml) and control plates were prepared using water. At the end of the test period (24 h), the seeds were weighed after drying them on a dry tissue paper. For morphological studies, the time of sprouting was extended to either 48 or 72 h, the length of the radicals was measured in cm at the end of 48, 72 h and growth inhibitory effect of coumarin was identified and photographs were taken.

Antiproliferative activity

Antiproliferative study was evaluated by yeast (*S. cerevisiae*) model previously reported [16].

Preparation of yeast inoculum

The yeast was inoculated with sterilized potato dextrose broth and incubated at 37 °C for 24 h, referred as seeded broth. The seeded broth diluted with sterilized distilled water, in order to get 25.4 x 10^6 cells (average).

Cell viability count

For cell viability count solution containing 2.5 ml of potato dextrose broth and 0.5 ml of yeast inoculum was prepared in separate test tubes. To each test tube 1 ml lectin extract from the endophytic fungi and plant extract was added at different concentration (400 and 50 µg/ml) respectively. To control test tube 1ml of PBS was added without extracts. All tubes were incubated at 37 °C for 12 h. Similarly for positive control standard drug quercetin was used. In the above cell suspension, 0.1% methylene blue dye was added in all tubes and cells were observed under the microscope. The number of viable cells, which does not stain and look transparent with oval shape while dead cells get stained and appeared blue in color were counted in 16 chambers of hemocytometer and percentage of cell viability was calculated using the formula [14].

\[
\% \text{ viable cells} = \frac{\text{No. of Viable Cells Counted}}{\text{Total Cells Counted}} \times 100
\]
RESULTS AND DISCUSSION

In MAE method, the *Alternaria* species was yielded highest amount (3.688 mg/g) of coumarin whereas, the plant *Crotalaria pallida* flower extract had shown 3.881 mg/g. To obtain of coumarin and related compounds from plants or endophytic fungal species from MAE is one of the best methods in present day [10, 12]. We have tested the *Alternaria* species extract to know coumarins and their derivatives and it was shown positive test for tested coumarins (coumarin, O-coumari acid and hydroxy cinnamic acid) and phenol test was also positive (Table 1). Coumarins are phenolic substances made up of fused benzene and α-pyrone rings [17]. All the coumarin(s) tests were strong evidence for presence of coumarin(s) and its derivatives.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Test 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Alternaria</em> sp.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Data based on three replicates of each experiment, +: presence

The above three coumarin(s) were visualized under UV light in TLC experiment and was compared with standard coumarin(s). The present interested compound, coumarin was compared with standard coumarin and it was confirmed (Fig 1). The results are confirmed with the earlier findings [10].

![TLC experiment](image1.png)

**Fig 1.** TLC of plant, endophytic extract and standards, A) *Alternaria* sp. B) p-Coumaric acid, C) 2-Hydroxy coumaric acid and D) Coumarin

In HPLC, the ethanol extract of *Alternaria* species was yielded three important coumarin(s) along with other phytochemicals and were identified with standard compounds based on retention time. The crude extract of *Alternaria* species was yielded low amount of all the three coumarins (Fig 2). All the coumarin(s) were identified based on retention time of the compounds compared with standard and retention time of p-coumaric acid is 18.7, 30.227 is 2-hydroxy cinnamic acid and 31.231 is coumarin (Fig 3).

![HPLC chart](image2.png)

**Fig 2.** Ethanol extract of *Alternaria* species showing different phytochemicals along with three different coumarin(s) in HPLC.
Fig 3. The standard coumarin, p-coumaric acid and hydroxy cinnamic acid peaks in HPLC.

For further, we had purified the compound, coumarin based on retention time (Fig 4) and was repeated many times to collect the same at higher concentration. Similar results were noticed by Martino et al. [18] and Govindappa et al. [9] using Melilotus officinalis and Crotalaria pallida. The purified endophytic fungal coumarin was run in the HPLC and confirmed with standard. The coumarin isolated from Alternaria species in UV spectra was similar to standard (Fig 5-6).

Fig 4. Purified coumarin from Alternaria species showing 18.705 retention time.

Fig 5. Spectra of standard coumarin in UV.
By repeating HPLC method, we have collected the coumarin at high amounts for \textit{in vitro} cytotoxicity studies. The ethanol extract of \textit{Alternaria} species coumarin was found to be potent in function in antimitotic, antiproliferative and growth inhibition of green gram assays. The lower concentration of 200 µl was more significant in reducing onion root cell division and effective in reduction in index value after 48h of treatment. We have found that \textit{Alternaria} species ethanol extract was induced the antimitotic activity at various stages of cell cycle viz., chromosomal, nucleolar and cellular abnormalities, Cell shrinkage and arrest of cellular multiplication at interphase, B) Abnormal distribution of chromosomes at metaphase, C) Lagging chromosomes and chromosomal bridge at anaphase and D) Chromosomal condense and cell shrinkage at anaphase. Chromosomal fragmentation, dislocation, abnormality in movement was observed in various stages of cell division (Fig 7) and was compared with normal mitotic phases (Fig 8). The antimitotic index value decreased with treatment of \textit{Alternaria} species coumarin (18.03) was compared with standard coumarin (12.14). The results are more or similar to standard. The untreated control showed 99.01 mg/ml (Table 2). Similar results were observed with earlier reports of Sadananda \textit{et al.} [19], Channabasava and Govindappa [20] and Govindappa \textit{et al.} [14] and Umashankar \textit{et al.} [10] by using endophytic fungal extracts. The isolated coumarin had shown potential antimitogenic activity by inducing structural changes in chromosome.

![Fig 6. Spectra of isolated coumarin from \textit{Alternaria} species.](image-url)

![Fig 7. Normal mitotic phases, A) Interphase, B) Metaphase, C) Anaphase & D) Telophase.](image-url)
Fig 8. Chromosomal, Nucleolar and Cellular abnormalities due to *Alternaria* species coumarin treatment, A) Cell shrinkage and arrest of cellular multiplication at interphase, B) Abnormal distribution of chromosomes at metaphase, C) Lagging chromosomes and chromosomal bridge at anaphase and D) Chromosomal condense and cell shrinkage at anaphase.

Table 2. Antimitotic activity of coumarin isolated from *Alternaria* species extract in *Allium cepa* root tips.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Distilled water</td>
<td>99.01</td>
</tr>
<tr>
<td><em>Alternaria</em> species coumarin</td>
<td>400 mg/ml</td>
<td>18.05</td>
</tr>
<tr>
<td>Coumarin (Std)</td>
<td>1mg/ml</td>
<td>12.14</td>
</tr>
</tbody>
</table>

Data based on three replicates of each experiment.

The *Alternaria* species coumarin was evaluated against yeast in antiproliferative activity and it showed potent in inhibition of yeast cell growth. The number of dead cells was counted in coumarin treated sample and calculated using given formula. The purified coumarin of *Alternaria* species inhibited the growth of yeast cells above 76.03%, whereas standard showed 88.6% (Table 3). This indicates the purified coumarin have shown similar activity and it leads to death of yeast by inducing toxicity and death cells with debris was observed with treated cells (Fig 9). Even at low concentration of endophytic fungal coumarin induced the highest percentage of cytotoxicity compared with standard and distilled water control.

Table 3. *In vitro* antiproliferative activity of *Alternaria* species coumarin extract in yeast cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Distilled water</td>
<td>0.0</td>
</tr>
<tr>
<td>Sample A (<em>Alternaria</em> sp.)</td>
<td>10mg/ml</td>
<td>76.03</td>
</tr>
<tr>
<td>Coumarin (Std)</td>
<td>1mg/ml</td>
<td>88.6</td>
</tr>
</tbody>
</table>

Data based on three replicates of each experiment.

Fig 9. Different extracts treated yeast cells. A. control and B. *Alternaria* species coumarin extract treated yeast cells (stained cells are dead cells).

The DNA fragmentation was observed in *Alternaria* species coumarin treated yeast cells after 48h in antiproliferative activity. Due to toxic effect of coumarin, the yeast DNA was broken. It indicates that inhibited the DNA, further DNA replication it may be due to activating topoisomerase or direct cleavage of DNA (Fig 10). The result was confirmed the findings of Govindappa et al. [2014] and Channabasava and Govindappa [20]. Most of the anticancer drugs of natural products and their synthetic compounds have activity to cleave the DNA and cause DNA damage. The natural products may suppress the DNA replication and they induced...
apoptosis. The advantage of the use of yeast as in vitro model for cytotoxic assays is that the complete genome comprises only 6250 defined genes and most importantly, many genes that are altered in human tumours have homologs and it is thought to provide a valuable resource to achieve a greater understanding about human cancer and hopefully give insights into new approaches for therapy.

The growth inhibitory effects of two different concentrations of coumarin extract exhibited remarkable and significant reduction in the length of the radical in all the treated seeds compared to control. Figure 11 depicts that the germination of green gram seeds in water, coumarin and standard coumarin treated. The development of sprouting was observed in treated with water (control) and lower concentration of endophytic fungal coumarin green gram seeds after 48 h and their dry seed weight were 56-64, 56-64, 56-64 and 56-64 in distilled water treated control, standard (2.0 mg/ml), endophytic coumarin (400 µl/ml) and endophytic coumarin (200 µl/ml) respectively. No sprouting was observed in Alternaria species coumarin and standard treated green gram had shown 2% (Fig 11). It confirms that the inhibition of root development may be due to toxicity of the compound, coumarin and it is dose dependent. After treatment, incubation periods of 24 and 72h, the average weight of dry seeds, average seed weight and average shoot length were measured. Endophytic fungal coumarin was shown less average seed weight and no growth of seeds compared to standard coumarin (Table 4). Similar results were reported by Sadananda et al. [19], they inhibited the green gram germination/sprouting by using endophytic fungal lectins and Umashankar et al. [10] have inhibited the green gram germination by using endophytic p-coumaric acid. This result indicates that the coumarin isolated from endophytic fungi, Alternaria species have similar effects when compared with standard coumarin.

![Fig 10. DNA fragmentation assay, A) Untreated lane and B) Endophytic extract treated lane.](image)

Fig 11. Green gram seed treatment with different concentrations of endophytic extracts and standard coumarin, A) Control (Distilled water), B) Coumarin 2.0 mg/ml (Std), C) Alternaria species coumarin (400 µl/ml) and D) Alternaria species coumarin (200 µl/ml).
Table 4. Observation of 24 h treated green gram seeds with different concentrations of Alternaria species coumarin extract after 72 h of further incubation (24 to 72 h).

<table>
<thead>
<tr>
<th>Observation</th>
<th>A (Control) (Distilled water)</th>
<th>B (Std) (2.0 mg/ml)</th>
<th>C (400 µl/ml)</th>
<th>D (200 µl/ml)</th>
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</thead>
<tbody>
<tr>
<td>Weight of dry seeds (mg/seed) before</td>
<td>56-64</td>
<td>56-64</td>
<td>56-64</td>
<td>56-64</td>
</tr>
<tr>
<td>Average seed weight after incubation (24+72 h) (mg/seed)</td>
<td>228-256</td>
<td>81-96</td>
<td>84-90</td>
<td>88-111</td>
</tr>
<tr>
<td>Average shoot length of seed in mm (24+72 h)</td>
<td>24-36</td>
<td>0-2</td>
<td>-</td>
<td>0-6</td>
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</table>

A) Control (Distilled water), B) Coumarin 1.0 mg/ml (standard), C) Alternaria species coumarin (400µl/ml) and D) Alternaria species coumarin (200µl/ml)

CONCLUSION

Based on the results, the leaf endophytic fungi, Alternaria species of Crotalaria pallida have yielded all the suspected three different coumarin(s) in MAE method and also other coumarin identification methods. The purified coumarin from fungal extract showed strong antimitotic activity through various mechanisms in onion actively growing root and antiproliferative activity by cause of yeast cells. The endophytic fungal coumarin had also showed inhibition of green gram germination by act as a toxic. This is the first report in nationally and internationally isolating coumarin from endophytic fungal species and its in vitro cytotoxicity assays. It can be used as a cancer drug after testing against cancer cell lines.

Conflict of interest

We declare that we have no any conflict of interest.

ACKNOWLEDGEMENTS

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