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Original Research Article

The Influence of Aspergillus niger–Derived Dry Cell Powder on Rutin Accumulation in Aegle marmelos Cell Suspension Cultures

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Abstract: ***Background:*** Aegle marmelos (L.) Corrêa is a medicinal plant that contains bioactive compounds derived from the phenylpropanoid pathway, such as rutin, a flavonoid known for its potent antioxidant and pharmacological effects. However, slow growth and climate change limit the use of traditional extraction methods for these compounds. ***Methods:*** Callus-derived cell suspension cultures were initiated from leaf explants in Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid and kinetin. Biotic elicitation was performed with Aspergillus niger-derived dry cell powder (DCP; 0.3-1.0%). Biomass productivity was measured as dry weight. Rutin was quantified using a validated reverse-phase high-performance liquid chromatography (HPLC) method. Antioxidant activity, DPPH and ferric-reducing assay power (FRAP), and oxidative stress parameters, such as catalase (CAT), peroxidase (POD), and malondialdehyde (MDA) levels, were assessed. ***Results:*** DCP elicitation produced concentration-dependent maximal increases of 8.31-fold (rutin) at 1%. Biomass productivity was moderately reduced, suggesting a trade-off between growth and plant defense. FRAP activity, CAT, and POD activities were significantly increased, whereas the DPPH scavenging activity decreased. Lipid peroxidation decreased, as indicated by the reduced malondialdehyde (MDA) content. ***Conclusion***: A. niger DCP effectively stimulated secondary metabolism and antioxidant activity in A. marmelos cell cultures, thereby providing a sustainable platform for the production of valuable flavonoids.

Keywords: Phenylpropanoid metabolism; fungal elicitor; secondary metabolite enhancement; antioxidant enzymes; plant cell biotechnology; oxidative stress regulation

INTRODUCTION

Aegle marmelos (L.) Corr. (Rutaceae), also known as Bael, is a medicinal tree with valuable bioactive secondary metabolites, especially the flavonoid rutin (Kumar, 2023; Monika et al., 2023). These bioactive compounds are well known for their strong antidiabetic, antioxidant, and anti-inflammatory properties, making them important targets for drug development (Malabadi et al., 2024; Venkatesan et al., 2024). However, their large-scale industrial extraction is often hindered by the tree's slow growth rate and environmental fluctuations (Kawra et al., 2022; Raghavan et al., 2025). Plant cell suspension culture technology is a sustainable and scalable

biotechnological method that provides a controlled environment for continuous metabolite production (Bapat et al., 2023) Although approaches for callus induction and cell-suspension optimisation in A. marmelos exist, improving cellular product synthesis using modern technologies, such as elicitation, is an important research area (Sharma et al., 2024; Zhao et al., 2023).

Biotic elicitation using complex fungal elicitor preparations (Dry Cell Powder) has been identified as an efficient technique for activating plant metabolic pathways by mimicking natural stress responses (Humbal & Pathak, 2023; Jain et al., 2024). Elicitor Aspergillus niger have been found to induce a significant increase in metabolite production in several medicinal plants by activating defense mechanisms and increasing enzymatic activity (Elateeq et al., 2025; Nadir, 2022). Nevertheless, despite their potential, there is a lack of empirical research on the effects of A. niger DCP on the metabolic flux and defense mechanisms of A. marmelos suspension cultures.

This study aimed to establish a reliable in vitro culture system for A. marmelos and assess the effects of A. niger-derived dry cell powder (DCP) on rutin. We also examined biomass yield and antioxidant activity using DPPH and ferric-reducing antioxidant power (FRAP) assays, and linked secondary metabolite production to biological activity. Additionally, we evaluated modulation of antioxidant enzymes, including catalase (CAT) and peroxidase (POD), and lipid peroxidation via the malondialdehyde (MDA) content in the elicited cultures. The main goal of this study was to assess the effect of elicitors on the biosynthetic pathways of secondary metabolites in A. marmelos.

MATERIALS AND METHODS

Plant Material, Authentication, and In Vitro Culture Establishment

Collection, Authentication, and Voucher Deposition

A. marmelos (L.) Corrêa plant material was collected from cultivated plants at the Institute of Science, Mumbai, India. No permission was required to use the cultivation source. Taxonomic authentication was performed at the Blatter Herbarium at St. Xavier's College, Mumbai, by a taxonomist, and a voucher specimen (voucher no. BOLE-21) was deposited.

Explant Preparation and Surface Sterilisation

Fresh young leaf explants of A. marmelos were first washed under running tap water for 15 min, and then cleaned with a few drops of Teepol (wetting and cleaning agent; Sigma-Aldrich, USA) to remove debris, followed by thorough rinsing (3-4 times) with sterile distilled water. Surface sterilization was performed under aseptic conditions in a laminar airflow cabinet. The explants were first treated with 2% (v/v) sodium hypochlorite solution (NaOCl; Merck Life Science Pvt. Ltd., India) for 3 min and then thoroughly rinsed 3-4 times with sterile distilled water. The explants were then treated with 70% (v/v) analytical-grade ethanol (Merck Life Science Pvt. Ltd., India) for 20 s and rinsed 3-4 times with sterile distilled water.

Callus Induction and Maintenance

Callus induction was performed as described by Zhao et al. (2023). Before inoculation, the basal Murashige and Skoog (MS) medium (HiMedia Laboratories, Mumbai, India) was enriched with 3% sucrose, 0.8% agar, 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich, USA), and 1.5 mg L^{-1} Kinetin (KIN; Sigma-Aldrich, USA). The pH was adjusted to 5.8 ± 0.02 using 0.1 N NaOH or HCl, then the mixture was autoclaved at 121 °C and 15 psi for 15 minutes. Surface-sterilized leaf segments (~1 cm^2) were aseptically placed in 100 mL of sterile medium within 200 mL culture jars (Borosil Glass Works Ltd., India). Cultures were first kept in complete darkness at 25 ± 2 °C for 7–10 days to induce callus formation. Afterwards, cultures were transferred to a 16:8 h light-dark cycle (cool white fluorescent light, approximately 40–50 µmol m^{-2} s^{-1}) to facilitate the growth of green, friable callus tissue. Callus cultures were subcultured every three weeks onto fresh medium of the same formulation under identical conditions.

Establishment of Suspension Cell Cultures

Friable calli (5 g fresh weight) were transferred to 200 ml culture bottles pre-filled with 100 ml of liquid MS medium (HiMedia Laboratories Pvt. Ltd., India) supplemented with 1.5 mg L^{-1} kinetin and 1.5 mg L^{-1} 2,4-D. The culture bottles were maintained on a rotary shaker (Model RS-12R, Remi Instruments Ltd., Mumbai, India) at 130 rpm in the dark at 25 ± 1 °C. Subcultures were prepared every three weeks by transferring 25 mL of the suspension culture to 75 mL of fresh Murashige and Skoog (MS) broth.

Biotic Elicitation Using Aspergillus niger Preparation of Biotic Elicitor

The biotic elicitor was extracted from A. niger following the method described by Elateeq et al. (2025), with some modifications.(Elateeq et al., 2025) The fungus was cultivated in Potato Dextrose Broth (PDB; HiMedia Laboratories Pvt. Ltd., Mumbai, India) at 28 ± 2 °C for three weeks. The culture was autoclaved at 121 °C for 20 min to deactivate the biomass. The mycelia were then filtered through Whatman No. 1 filter paper (Cytiva, UK) and rinsed 3-4 times with 20 mL of sterile distilled water to eliminate residual nutrients. The filtered mycelia were dried in an oven at 60 °C until a constant weight was achieved. Once dried, mycelia were ground into a fine

powder using a sterile mortar and pestle. The resulting Dry Cell Powder (DCP) was stored in an airtight container at room temperature.

Elicitation Experimental Design

21 days old A. marmelos cell suspension cultures were selected based on their growth curves and were subjected to biotic stress. The treatments comprised A. niger DCP at 0.3, 0.5, 0.8, and 1.0 %, with a control at 0 %, as described by Prasad et al. (2013). All experiments were performed in triplicate for each treatment. After DCP incorporation, the suspension culture was incubated under standard conditions for 48 h before harvesting.

Biomass Productivity

Biomass productivity was determined by Settled Cell Volume (SCV) and Dry Weight (DW), as described by Kumar & Parasurama (2025). For SCV, 10 mL of cell-suspension culture was transferred to a graduated centrifuge tube and allowed to settle for 30 min without agitation, and the resulting volume was measured as SCV. The cells were harvested by vacuum filtration through Whatman No. 1 filter paper, washed with sterile water, and dried in an oven at 60 °C until a constant weight was attained. The initial dry weight (W_1) of the control culture was used as the standard for productivity. Biomass productivity (g DW L^{-1} d^{-1}) was calculated using the following formula:

$$\text{Biomass productivity} = (W2 - W1)/(t2 - t1)$$

where W_2 is the final dry weight (g), W_1 is the initial dry weight (g), and ($t_2 - t_1$) is the time interval (d).

Extraction and RP-HPLC-UV Quantification of Rutin

Preparation, Calibration, and Validation of Standard Solutions

Reference standards of Rutin (≥95% purity; Sigma-Aldrich, St. Louis, MO, USA) were weighed precisely using an analytical balance (Shimadzu Corporation, Japan). It was dissolved in HPLC-grade methanol (Merck Life Science Pvt. Ltd., Mumbai, India) to prepare stock solutions (1.0 mg mL-1). Working standards at 10, 20, 40, 60, 80, and 100 ppm were prepared by serial dilution with methanol and analyzed using high-performance liquid chromatography (HPLC). All solutions were filtered using 0.45 µm PTFE syringe filters (Whatman™, Cytiva, UK) prior to reverse-phase high-performance liquid chromatography (HPLC) analysis.

Validation: This method was validated in accordance with ICH Q2 (R1, 2005) guidelines to assess the linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) for umbelliferone and rutin under specified chromatographic conditions.(Lee et al., 2021; Patidar & Ramteke, 2024)

Linearity and Calibration Curve: Calibration curves for linearity were established using rutin working standards (10, 20, 40, 60, 80, and 100 ppm) by plotting the peak area (mAU) versus concentration (ppm), and linear regression was used to estimate the slope, intercept, and correlation coefficient (R^2).

Each concentration was injected thrice (n=3) under identical chromatographic conditions.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ were calculated from the standard deviation (σ) of the response and the slope (S) of the calibration curve using the following equations:

$$LOD = 3.3\,\sigma / S \quad LOQ = 10\,\sigma / S$$

where σ is the standard deviation of the y-intercept, and S is the slope of the calibration curve.

Precision: Precision was evaluated using intra- and inter-day analyses. Intra-day testing involved measuring three concentrations (10, 60, and 100 ppm) in triplicate on a single day. Inter-day testing involved measuring the same concentrations thrice over three days. The results were expressed as the relative standard deviation (RSD%), calculated as follows: RSD (%) = (standard deviation/mean) × 100.

Accuracy and Recovery: Recovery experiments assessed accuracy via the standard addition method by adding known amounts of umbelliferone and rutin at 80%, 100%, and 120% to pre- analyzed samples, each tested three times (n=3). The recovery percentage was calculated as follows: recovery (%) = [(Cfound − Coriginal) / Cadded] × 100, where Cfound is the concentration after standard addition, Coriginal is the initial analyte concentration in the sample, and Cadded is the amount of standard added to the sample.

Repeatability and System Suitability: Repeatability was assessed by injecting a standard solution at 50 µg/mL in triplicate (n = 3). The system suitability parameters, including retention time (tR), theoretical plates (N), tailing factor (T), and resolution (Rs) between the umbelliferone and rutin peaks, were determined.

Preparation of Cell Suspension Extract Sample Solution

Extraction of A. marmelos elicited-cell suspension biomass was performed as described by Elbouzidi et

al. (2025) Briefly, 100 mg of oven-dried, powdered biomass was extracted with 10 mL of 80% ethanol at 30 °C for 30 min using a probe sonicator (Sonics & Materials, Inc., USA) with 3-second ON/OFF pulses, followed by vortexing for 20 min. This process was repeated thrice. The mixture was then centrifuged at 13000 rpm for 15 min, and the supernatant was filtered through a 0.45 µm nylon filter before being stored at 4 °C for subsequent HPLC analysis.

HPLC Conditions Rutin Analysis

Chromatographic analysis was performed using an HPLC system (Agilent 1260 Infinity II, Agilent Technologies, USA) comprising a quaternary pump, autosampler, and UV-Vis detector. The separation was performed using a ZORBAX Eclipse Plus C18 column. The mobile phase for the analysis of rutin was a Methanol:Water (40:60, v/v) + 10 mM acetate buffer (pH 4.1), with a flow rate of 1.0 mL min^{-1}, and detection was performed at 356 nm. The injection volume was 20 µL, and the system temperature was maintained at 25 ± 1 °C. Data analysis was performed using the Agilent OpenLAB CDS ChemStation software.

Calculation of Metabolite Concentration and Content

The metabolite concentration (C, mg mL-1) in the extract was calculated from the calibration curves for rutin, using consistent chromatographic conditions. To compare the control and treated samples, metabolite concentration was scaled using the following equation: Cx = Ccontrol

× (mAUx / mAUcontrol), where Cx is the concentration in the treated sample, Ccontrol is the concentration in the control, and mAUx/mAUcontrol is the detector response. The metabolite content was expressed as mg per gram of dry biomass (mg g^{-1} DW) using the formula (C × V) / W, where C is the concentration (mg mL^{-1}), V is the extract volume, and W is the dry biomass weight of the samples. The fold increase in rutin and umbelliferone was calculated relative to the control (set at 1.00) as follows: fold increment = treatment mean/control mean.

Antioxidant Activity Assay

DPPH Radical Scavenging Assay

The DPPH radical-scavenging activity of A. marmelos extracts was determined spectrophotometrically using the method described by Baliyan et al. (2022). A constant concentration of 100 µg mL^{-1} of the extract was mixed with a 1 mM DPPH (2,2-diphenyl-1- picrylhydrazyl; Sigma-Aldrich, USA) solution in methanol (HPLC grade; Merck, India). The reaction mixture was incubated in the dark at room temperature (25 ± 2 °C) for 30 min. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer (UV-1900i, Shimadzu, Japan). The scavenging activity was calculated using the following formula: Scavenging activity (%) = [(Acontrol − Asample) / Acontrol] × 100

Where Acontrol is the absorbance of the DPPH solution without the extract, and Asample is the absorbance of the extract-treated sample.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the method described by Klimek-Szczykutowicz et al. (2020) A fresh FRAP reagent was prepared by mixing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$, and 0.3 M acetate buffer (pH 3.6) at a 1:1:10 ratio. The reagent was added to the extracts, and the mixture was incubated at 37 °C for 15 min. The absorbance was measured at 593 nm. Antioxidant activity was calculated as milligram ascorbic acid equivalents (AAE) per 100 g using a standard curve prepared with ascorbic acid (AA). The FRAP value was derived from the standard curve and reported as mg AAE per 100 g of sample using the following formula: FRAP value (mg AAE/100 g sample) = [(Asample − Ablank) / slope of standard curve] × (Vextract / Wsample) × 100

where Asample is the absorbance at 593 nm, Ablank is the blank absorbance, the standard curve slope is from ascorbic acid standards, Vextract is the final extract volume (mL), and Wsample is the dry sample weight (g).

Biochemical and Oxidative Stress Enzyme Analyses

Catalase (CAT) Activity

Catalase activity was determined as described by Singh (2023). The reaction mixture contained 1 mL of 0.03% hydrogen peroxide in 0.1 M HEPES buffer (pH 7.4) and 35 µL of enzyme extract. The decrease in absorbance at 240 nm was recorded for 1 min at 25 °C using UV-Vis spectrophotometry. Catalase activity (µ mL^{-1}) was calculated as (ΔA × Vtotal) / (ε × L × Vsample

× T), where ΔA is the change in absorbance per minute, ε is the molar extinction coefficient of hydrogen peroxide (43.6 $L \cdot mol^{-1} \cdot cm^{-1}$), L is the path length (1 cm), Vtotal is the total reaction volume (1.035 mL), Vsample is the enzyme extract volume (0.035 mL), and T is the reaction time (1 min). One unit of catalase activity was defined as the amount of enzyme that decomposed 1 µmol of H_2O_2 per min.

Peroxidase (POD) Activity

Peroxidase activity was assessed using the guaiacol oxidation assay, as adapted by Vodiasova et al. (2025) The reaction mixture contained 33 mM potassium phosphate buffer (pH 6.1), 16 mM guaiacol (Himedia

Laboratories, India), 2 mM H_2O_2, and 200 µL of enzyme extract. The absorbance at 470 nm was recorded after 3 min of incubation. POD activity (U mg^{-1} protein) was calculated as follows: $(\Delta A_{470} \times Vt) / (\varepsilon \times Vs \times P \times t)$. Here, "$\Delta A_{470}$ per minute" refers to the rate of change in absorbance at 470 nm, and "ε" is the molar extinction coefficient of tetraguaiacol (26.6 $mM^{-1} \cdot cm^{-1}$). The other parameters included Vt, the total reaction volume; Vs, the volume of the enzyme extract; P, the protein concentration (mg); and t, the reaction time (min).

Lipid Peroxidation (MDA Content)

Lipid peroxidation was evaluated using the TBARS method described by Abeyrathne et al. (2021) as follows: Fresh biomass was homogenized in a 0.1% solution using a Bosch homogenizer. The absorbance of the supernatant was recorded at 532 nm, and the turbidity was corrected at 600 nm. The MDA levels were quantified using a standard curve of malondialdehyde bis(dimethylacetal) (Sigma-Aldrich). The MDA concentration (nmol g^{-1} FW) was calculated as follows: $(A_{532} - A_{600}) \times V / (\varepsilon \times W)$, where A_{532} is the absorbance at 532 nm, A_{600} at 600 nm, V is the extract volume, ε is 155 mM^{-1} cm^{-1}, and W is the fresh weight of the sample.

Statistical Analysis

All experiments were performed using a completely randomized design (CRD) with three biological replicates per treatment (n = 3). Data are presented as the mean ± standard deviation (SD). The Shapiro-Wilk test was used to assess normality, and Levene's test was used to check for homogeneity of variance. Differences in elicitor concentrations were analyzed using one- way ANOVA, followed by Tukey's multiple comparison test. All analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA), with $p < 0.05$ considered significant.

RESULTS

Prior to conducting parametric statistical analysis, all datasets were tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene tests, respectively. Because the data satisfied these assumptions, a one-way ANOVA was performed, followed by Tukey's multiple-comparison test.

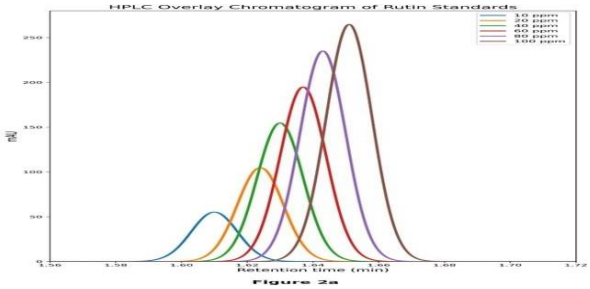


Figure 2a

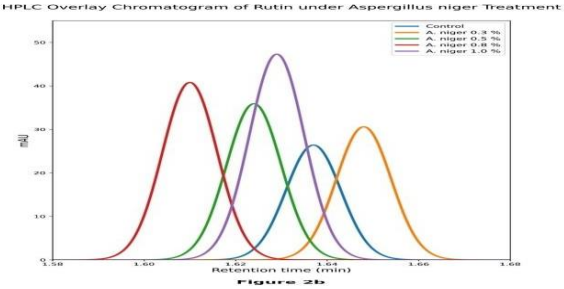


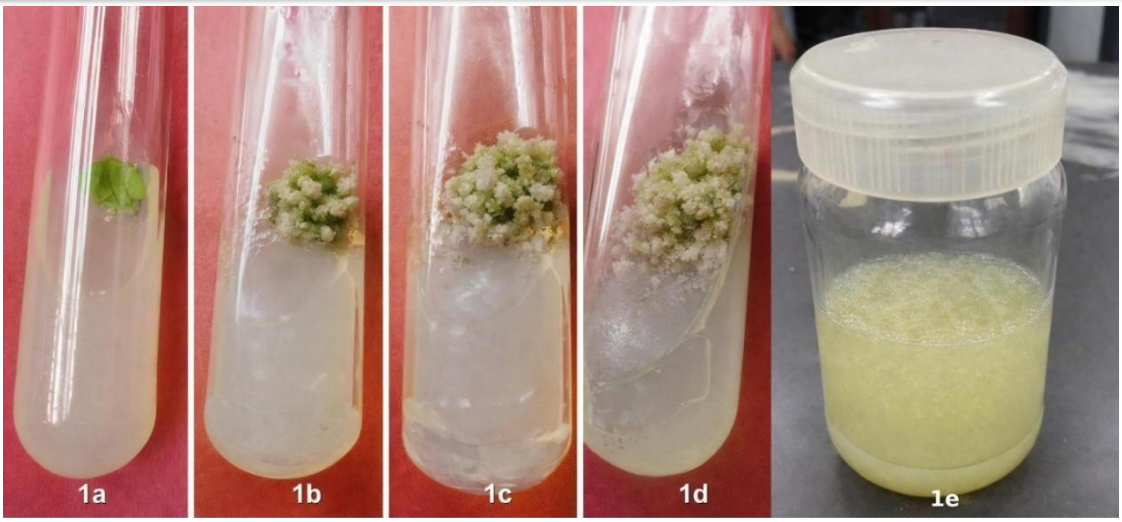
Figure 2b

Establishment of Callus and Cell Suspension Cultures

marmelos leaf explants cultured on MS medium with 0.5 mg L^{-1} 2,4-D and 1.0 mg L^{-1} kinetin showed gradual development, yielding friable callus and homogeneous suspension cultures. On day 0, the green leaf explant became a compact light-green callus by day 14 and developed into an actively growing, partially friable callus by day 21 (Fig. 1a, b, and c). Although the callus became completely friable by day 28, it exhibited a granular and over-aged morphology (Fig. 1d). A 21-day-old callus was readily used to initiate suspension cultures, and upon transfer to the liquid medium, the friable callus formed a uniform cell suspension within 21 days, demonstrating successful cell acclimatization to the liquid medium (Fig. 1e).

Fig. 1-Morphological progression of friable callus and establishment of suspension cell culture in Aegle marmelos. (1a) Initial explant on MS medium supplemented with 2,4-D (0.5 mg L^{-1}) and kinetin (1.0 mg L^{-1}) showing green leaf tissue (day 0); (1b) compact green callus at 14 days; (1c)

friable, granular callus during third subculture at 21 days; (1d) well-developed friable callus suitable for suspension initiation at 28 days; (1e) established 21-day-old suspension cell culture derived from friable callus



Chromatographic Characteristics of Rutin Standards

Fig. 2-HPLC chromatograms of rutin standards and their detection in Aegle marmelos cell suspension cultures following treatment with Aspergillus niger-derived DCP elicitor. (2a) Rutin standard; (2b) rutin under A. niger–derived DCP treatment, showing enhanced peak intensity with consistent retention.

The RP-HPLC analysis of rutin standards showed well-defined peak separation and stable retention times across the tested concentration range. Rutin consistently eluted around 1.60– 1.66 min. The detector response increased proportionally with increasing analyte concentration, and peak areas showed significant differences across concentrations ($p \leq 0.05$), confirming a concentration-dependent chromatographic response (Fig. 2a).

RP-HPLC Method Validation for Quantification of Rutin

The RP-HPLC method for quantifying rutin was validated using authentic reference standards in accordance with ICH Q2(R1) guidelines. The validation assessed parameters such as linearity, sensitivity, precision, accuracy, specificity, system suitability, and robustness. This method demonstrated good linearity across the 10–100 ppm range for the compounds, with R^2 values > 0.99. The detection and quantification limits were 9 ppm, respectively, indicating sufficient method sensitivity. All validation criteria were met, confirming the method's reliability and reproducibility. The validated method was used to quantify rutin in A. marmelos cell-suspension culture extracts (Table 1).

Table 1. HPLC method validation parameters for the quantification of rutin in Aegle marmelos cell suspension cultures elicited with Aspergillus niger–derived DCP.

Validation Parameter	Rutin	Acceptance Criteria	Conclusion
Linearity range (ppm)	10–100	—	Suitable
Regression equation	y = 3.8658x + 53.0134	—	Linear response
Correlation coefficient (R^2)	0.9948	≥ 0.99	Acceptable
Limit of Detection (LOD)	8.72 ppm	Detectable	Acceptable
Limit of Quantification (LOQ)	26.43 ppm	Quantifiable	Acceptable
Precision (%RSD, intra- day, n = 3)	0.90–1.93	≤ 2%	Acceptable
Retention time (RT)	5.7387–5.8590 min	Consistent	Stable
Specificity	No interference at rutin RT	No co-eluting peaks	Specific
System suitability (%RSD, area)	≤ 1.93	≤ 2%	Pass
Accuracy (% recovery)*	98.6–101.4	95–105%	Acceptable
Robustness**	No significant change (%RSD < 2%)	≤ 2% variation	Robust

Linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, specificity, system suitability, and robustness were evaluated in accordance with ICH Q2(R1).

Detection of Rutin in DCP-Elicited Cell Suspension Cultures

The HPLC chromatograms of extracts from A. marmelos cell-suspension cultures treated with A. niger DCP showed clear peaks for rutin, with retention times similar to those of the standards. Control cultures exhibited low peak intensities, whereas peak intensity increased with DCP concentration. The maximum peak intensity was observed at a DCP concentration of 1 % DCP, with no significant changes in retention times. Differences in peak areas among treatments were statistically significant ($p \leq 0.05$) (Fig. 2b).

Effect of A.niger- DCP on Biomass Productivity and Metabolite Accumulation

The DCP elicitor concentration had a pronounced effect on biomass productivity and metabolite accumulation in A. marmelos cell suspension cultures. As elicitor concentration increased, biomass productivity decreased somewhat, reaching its lowest point at 1% DCP, compared to the control. Conversely, rutin levels increased with higher DCP concentrations, peaking at 1% DCP. The variations observed in biomass productivity, metabolite concentrations, and content across different treatments were statistically significant ($p \leq 0.05$) (see Table 2).

Table 2. Effect of Aspergillus niger–derived DCP elicitor treatment on biomass productivity, rutin accumulation, and fold increment in Aegle marmelos cell suspension culture.

DCP concentration (%)	Biomass productivity (g DW L^{-1})	Rutin content (mg g^{-1} DW)	Rutin fold increment	Rutin yield (mg L^{-1})
Control (0)	8.42 ± 0.31^{a}	1.26 ± 0.08^{d}	1.00	10.61 ± 0.54^{d}
0.3	7.95 ± 0.28^{b}	1.84 ± 0.11^{c}	1.46	14.63 ± 0.67^{c}
0.5	7.41 ± 0.35^{c}	2.36 ± 0.14^{b}	1.87	17.49 ± 0.72^{b}
0.8	6.88 ± 0.26^{d}	2.91 ± 0.17^{ab}	2.31	20.03 ± 0.81^{ab}
1.0	6.32 ± 0.22^{e}	3.28 ± 0.19^{a}	2.60	20.73 ± 0.85^{a}

Values are expressed as mean ± SD (n = 3). Different superscript letters within a column indicate significant differences determined by one-way ANOVA followed by Tukey's test ($p \leq 0.05$).

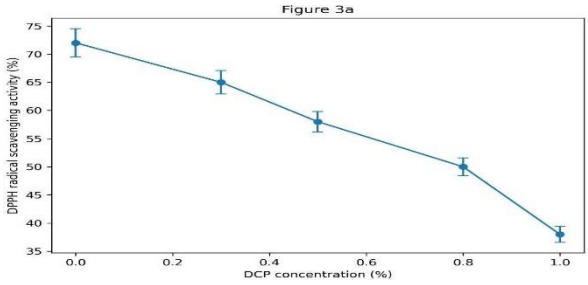
DCP-Induced Fold Increase in Rutin Content

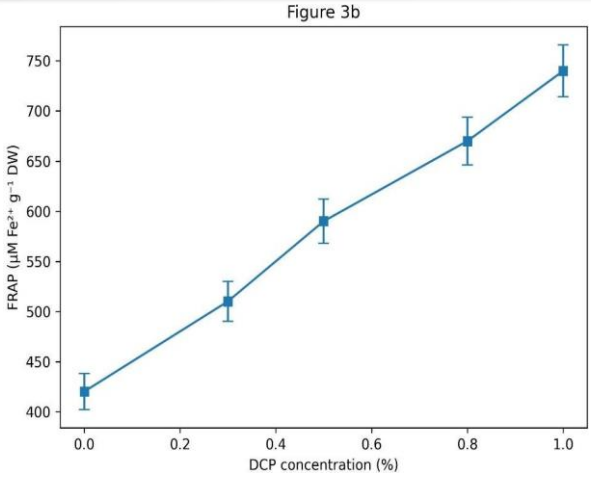
Fold-change analysis revealed a concentration-dependent increase in rutin accumulation relative to the control. Rutin content increased significantly with DCP concentration, reaching a maximum 2.60-fold increase at 1% DCP. All DCP treatments showed higher fold changes than the untreated control. The observed fold-change trend was consistent with the measured rutin concentrations and content. Differences among treatments were statistically significant ($p \leq 0.05$; Table 2).

Effect of DCP on Antioxidant Activities

The DPPH radical-scavenging ability declined steadily with increasing DCP concentration, dropping sharply at 1% DCP compared with the control. Conversely, the ferric reducing antioxidant power (FRAP) consistently increased, peaking at 1% DCP. The differing trends between the two assays were statistically significant ($p \leq 0.05$; Fig. 3).

Fig. 3-Effect of Aspergillus niger–derived DCP elicitor treatment on antioxidant activities of Aegle marmelos cell suspension culture. (3a) DPPH radical scavenging activity; (3b) ferric reducing antioxidant power (FRAP).

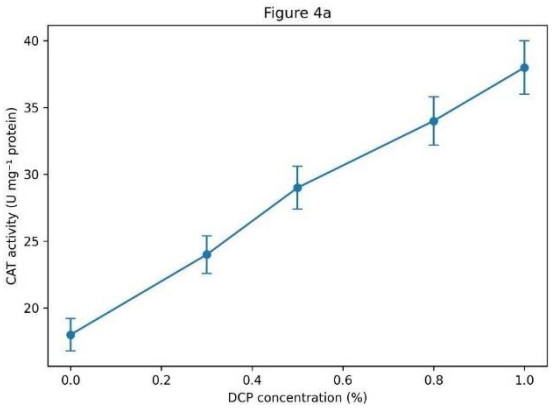


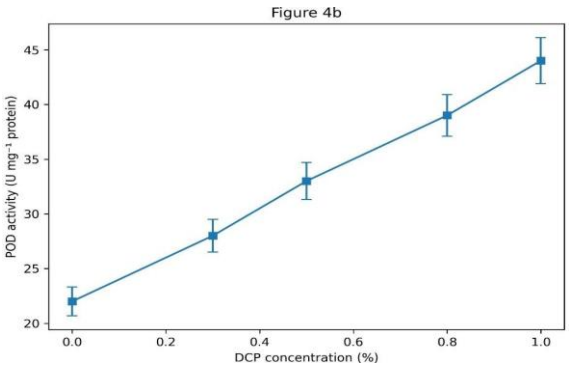


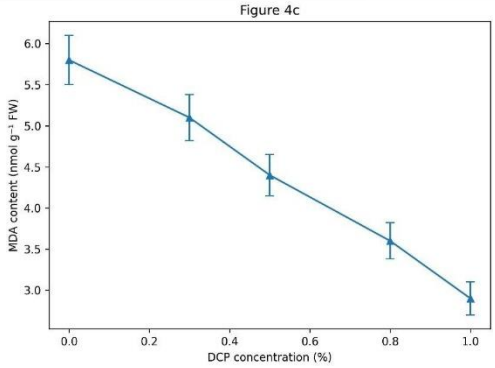
Effect of DCP on Antioxidant Enzyme Activities and Lipid Peroxidation

Catalase and peroxidase activities increased significantly with DCP concentration, peaking at 1 % DCP. In contrast, malondialdehyde content decreased significantly at higher elicitor concentrations. All enzyme and oxidative stress parameters differed significantly among treatments ($p \leq 0.05$; Fig. 4).

Fig. 4-Effect of Aspergillus niger–derived DCP elicitor treatment on antioxidant enzyme activities and lipid peroxidation in Aegle marmelos cell suspension culture. (4a) Catalase (CAT) activity; (4b) peroxidase (POD) activity; (4c) malondialdehyde (MDA) content. Data are presented as mean ± SD (n = 3).







DISCUSSION

This study shows that dry cell powder (DCP) from A. niger effectively elicits A. marmelos cells, boosting the production of bioactive compounds such as rutin. It regulates antioxidant levels, activates enzymes, and helps manage oxidative stress. DCP shifts metabolism from growth to defense, a typical plant response to biotic stress.

Suitability of the Cell Suspension System for Elicitor Responsiveness

The achievement of responsive friable callus cultures and dispersed cell suspension systems is an essential prerequisite for successful elicitor-mediated manipulation of secondary metabolism in vitro (Avila-Victor et al., 2023). Friable calli are defined as loosely packed, actively proliferating cells that readily disperse in liquid culture, thereby allowing equal access to nutrients and elicitors, which, in turn, facilitates uniform metabolic responses (Fu et al., 2025). This is especially relevant for woody medicinal plants such as A. marmelos, which often show limited response and slower adaptation in vitro (Khanal et al., 2023). The noted morphological change from compact callus to friable tissue, and finally to a stable suspension culture, indicates proper hormonal control and successful cellular dedifferentiation.(Mamdouh & Smetanska, 2022).

In the current study, day-21 callus was chosen for suspension culture because it showed optimal physiological and morphological development for cell dispersion and growth. At this stage, the callus was actively growing and partially friable. Although full friability was reached on day 28, the culture was then granular, over-aged, and showed increased cellular heterogeneity, vacuolation, partial differentiation, and decreased mitotic activity. Over-friability at this point likely led to increased cell debris and irregular aggregates, and to instability when transferred to liquid culture, further exacerbated by nutrient depletion.

Thus, day-21 callus provided the best balance of friability and cell viability, resulting in better cell growth, a more uniform suspension, and consistent growth for successful elicitation. The successful development of friable callus and suspension cultures, which facilitate increased production of secondary metabolites, has been achieved in A. marmelos (Bahnasy et al., 2025; Zhao et al., 2023). and other medicinal plants such as Ruta chapalensis (Ali, 2025), Elephantopus scaber (Kharat & Mendhulkar, 2023), and Catharanthus roseus (Linh et al., 2021), where homogeneity of suspension culture and cellular plasticity are required to elicit secondary metabolites production.

Reliability of Chromatographic Detection of Target Metabolite

RP-HPLC analysis showed high reliability, with clear, reproducible peaks for rutin in standards and cell samples. Retention times remained constant after DCP treatment, indicating the elicitor did not change its chromatographic properties, confirming the method's specificity and reliability. Rutin is a key phytochemical marker of A. marmelos, derived from the phenylpropanoid pathway, specifically the flavonoid and coumarin branches (Shetty et al., 2025). The stability of chromatographic profiles under biotic elicitor stress indicates that DCP treatment did not produce similar analogs or change downstream activity. Similar findings in other medicinal plant cell cultures show biotic elicitors increase metabolite production without altering retention times or profiles, maintaining metabolite identity under stress (Kanthaliya et al., 2023; Zhao et al., 2023). Previous studies using fungal- or polysaccharide-derived elicitors show that elicitation mainly influences flux regulation and metabolite levels, not the identity of secondary metabolites, aligning with the current findings(Elateeq et al., 2025; Linh et al., 2021; Nadir, 2022). The consistency of metabolite identity under controlled in vitro conditions indicates a highly regulated biosynthetic pathway.(Kornicka et al., 2023) These results show RP-HPLC is a sensitive, accurate method for detecting elicitor-induced changes in secondary metabolism. The unchanged flavonoid levels indicate that differences in metabolite levels are due to quantitative regulation rather than pathway modifications.

DCP-Induced Activation of Secondary Metabolism

The accumulation of rutin after DCP treatment indicates these elicitors activate defense metabolism in A. marmelos cultures. Fungal elicitors have conserved patterns recognized by plant receptors, triggering early signaling pathways such as ROS production and kinase phosphorylation (Guo & Cheng, 2022). These events then regulate the transcription of key enzymes in the phenylpropanoid pathway, leading to the accumulation of metabolites such as flavonoids (Kornicka et al., 2023; Pham et al., 2024).

The rise in rutin levels shows a coordinated boost of the phenylpropanoid pathway, not a metabolic disruption. Elicitor-induced phenylpropanoid production, observed in plant cell cultures treated with fungal extracts such as A. niger, results from activating pathway-specific genes rather than altering metabolite identity.(Nadir, 2021, 2022) Overall, these findings show that A. marmelos suspension cultures are highly responsive to biotic stimuli, even when undifferentiated.

Growth–Defence Trade-off Under Elicitor Stress

The results of this study indicate a clear inverse relationship between biomass accumulation and secondary metabolite production, reflecting a typical growth-defense trade-off. When exposed to elicitor stress, resources shift from primary growth to the production of defensive secondary compounds (Kandoudi & Németh-Zámboriné, 2022). This adaptive response aligns with the growth-differentiation balance hypothesis, which states that investing in chemical defenses often comes at the expense of biomass growth (Watts et al., 2023). Although it usually slows cell division, it raises metabolite levels per unit biomass, improving biosynthetic efficiency and overall yield (Watts et al., 2023). Consequently, elicitor-based methods hold practical promise for the production of secondary metabolites. Recent studies on plant in vitro systems support this trend, showing that elicitors can trigger defense responses and increase secondary metabolite accumulation, while also influencing growth in medicinal plant cells and tissue cultures (Majeed et al., 2024; Trunjaruen et al., 2022)

Qualitative Shifts in Antioxidant Capacity

The differences observed in the antioxidant assay results, indicated by decreased DPPH radical scavenging and increased ferric reducing antioxidant power (FRAP), reflect qualitative rather than quantitative variations in the antioxidant activity. These differences can be attributed to distinct reaction mechanisms: hydrogen-atom transfer for DPPH scavenging and electron donation for FRAP. Elicitor-induced changes in secondary metabolism are known to influence phenolic compound levels and composition (Baliyan et al., 2022; Donoso-Bustamante et al., 2025). In elicited plant cell cultures, such variations in antioxidant content often correlate with increased accumulation of phenylpropanoid pathway-derived compounds, such as flavonoids, which have strong reducing properties (Hawrylak-Nowak et al., 2021). In this study, the higher FRAP activity likely corresponds to increased rutin levels, a well-known electron donor with a high ferric-reducing capacity. These findings support the notion that elicitation can selectively activate distinct antioxidant mechanisms by altering the phenolic composition.

Enzymatic Antioxidant Activation and Oxidative Stress Regulation

Elevated catalase (CAT) and peroxidase (POD) activities following DCP treatment suggest effective activation of antioxidant enzyme defenses in A. marmelos cell suspension cultures. During elicitor recognition, transient reactive oxygen species (ROS) act as key signalling molecules for defense responses and signal transduction; however, excessive ROS can cause damage, necessitating tight regulation by antioxidant enzymes (Dvořák et al., 2021). The reduction in malondialdehyde (MDA), a key indicator of lipid peroxidation, shows that the combined increase in enzymatic antioxidants and secondary metabolites effectively prevents oxidative damage. This finding reflects controlled oxidative priming rather than oxidative stress. Similar patterns—elevated CAT and POD activities accompanied by decreased lipid peroxidation- are widely observed in elicitor-treated plant cell cultures and are considered standard markers of elicitor-triggered defense responses in vitro (Nahar et al., 2022; Wang et al., 2024).

4.7. Integrated Interpretation and Future Perspective

Collectively, these data demonstrate that DCP produced by A. niger induces metabolic and biochemical responses in A. marmelos cell-suspension cultures. The increase in rutin, combined with improved enzymatic antioxidant systems and reduced oxidative stress, suggests the effective activation of defense-related phenylpropanoid pathways in elicitor-treated cultures. The coordinated regulation of secondary metabolite synthesis, antioxidant capacity, and redox balance confirmed the role of fungal elicitors in triggering a comprehensive defense response in undifferentiated plant cells. These findings highlight the potential of elicitor-based strategies for increasing the yield and quality of secondary metabolites in vitro. From an applied perspective, the sustained biosynthetic responsiveness of A. marmelos suspension cultures to fungal elicitation supports their use as reliable platforms to produce pharmacologically significant secondary metabolites. Further research on optimizing the pathway control and bioprocessing could improve the consistency and practical applicability of this approach.

CONCLUSION

This study demonstrated that dry cell powder (DCP) derived from Aspergillus niger acts as an effective biological elicitor, stimulating secondary metabolism in Aegle marmelos cell suspension culture. Treatment of cultures with DCP significantly increased levels of key secondary metabolites in the phenylpropanoid pathway, such as rutin, enhanced antioxidant activity, and enhanced enzymatic defense responses. Although higher elicitor concentrations reduced biomass yield, the notable increase in secondary metabolite production reflected a metabolic shift toward secondary production, consistent with the growth-defense trade-off hypothesis. The concurrent increase in antioxidant enzyme activity and decrease in lipid peroxidation suggest that the elicited cultures can maintain redox balance even under biotic stress. These findings highlight fungal-based elicitation as a potent and controllable method for the sustainable production of pharmacologically active secondary metabolites in A. marmelos.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to the conduct, writing, or publication of this manuscript.

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