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OPTIMIZING *AEGLE MARMELOS* TISSUE CULTURE: STERILIZATION, HORMONAL COMBINATIONS, AND PERMEABILIZATION STRATEGIES FOR ENHANCED SECONDARY METABOLITE PRODUCTION

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Keywords:

Callus induction, Hormonal combinations, Permeabilizing agents, Triton X-100, Tween-80, Secondary metabolites

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ABSTRACT: This study focuses on optimizing the tissue culture of *Aegle marmelos*, a medicinal plant with diverse pharmacological properties. The research fine-tuned sterilization methods, identifying a 2% sodium hypochlorite solution as the most effective. Callus induction experiments revealed the optimal hormonal combination for *Aegle marmelos* as MS medium + Auxin-2,4-D (0.5 mg/l) + Cytokinin-BAP (1.5 mg/l). Suspension cell cultures were then developed as a foundation for further experiments. The study also explored permeabilizing agents (Triton X-100 and Tween-80) in *Aegle marmelos* cell cultures, finding that Tween-80 enhances the extraction of secondary metabolites more effectively than Triton X-100. High-Performance Liquid Chromatography (HPLC) analysis validated these outcomes. In conclusion, this research significantly contributes to optimizing plant tissue culture protocols for *Aegle marmelos*, advancing scientific understanding, and offering practical insights for improving the reproducibility and efficiency of *Aegle marmelos* cell cultures. These findings hold promise for potential publication, marking notable advancements in plant tissue culture and permeabilization strategies.

INTRODUCTION: *Aegle marmelos*, commonly known as Bael or Bengal quince, possesses significant medicinal value attributed to various secondary metabolites [1–7]. Tissue culture techniques stand out as a promising avenue for the controlled and sustainable production of these bioactive compounds 8–10. Sterilization is a crucial step in establishing contamination-free cultures, providing the foundation for successful tissue culture initiation [11–13].

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Beyond microbial elimination, a proper sterilization protocol sets the stage for healthy explants that can respond optimally to subsequent hormonal treatments [11, 14]. Various methods, including surface sterilization with chemical agents or heat treatment, are evaluated for their efficacy in eliminating contaminants while preserving explant viability [11, 15].

With its broad-spectrum antimicrobial properties, sodium hypochlorite is a potential chemical sterilizer for maintaining aseptic conditions and preventing unwanted microbial interference [16, 17]. In plant tissue culture (PTC), hormonal regulation is paramount, with auxins and cytokinins playing crucial roles in growth and differentiation [18–20]. This study examines various hormonal

combinations to optimize the growth, multiplication, and expression of secondary metabolites in *Aegle marmelos* cultures. This strategic manipulation of hormonal cues is expected to be pivotal in achieving optimal tissue culture conditions.

Simultaneously, the study investigates the role of permeabilizing agents like Triton X-100 and Tween-80 in *Aegle marmelos* cell cultures. Permeabilization is a critical process that enhances the cell membrane's permeability, facilitating the extraction of intracellular compounds [21–23]. Triton X-100 and Tween-80 act by disrupting the lipid bilayer of cell membranes, allowing access to the intracellular content [22, 24–27]. Understanding how these agents affect *Aegle marmelos* cell cultures is critical for optimizing secondary metabolite extraction, which adds to the already complex field of plant tissue culture.

Among the myriad of secondary metabolites produced by *Aegle marmelos*, umbelliferone, and its derivatives garner considerable attention due to their pharmacological activities [3, 7]. Umbelliferone exhibits antioxidant, anti-inflammatory, and hepatoprotective properties, making it a potential candidate for pharmaceutical applications [28–33]. This research aims to enhance umbelliferone production through tissue culture optimization, contributing to the development of novel pharmaceutical formulations.

High-Performance Liquid Chromatography (HPLC) analysis has emerged as an important tool for investigating enhanced secondary metabolite production in *Aegle marmelos* cultures [31, 32]. HPLC, which provides precise and quantitative assessments of bioactive compounds like umbelliferone, enables researchers to track dynamic changes in secondary metabolite profiles across different culture conditions. This analysis ensures the reproducibility and consistency of secondary metabolite production, providing valuable insights into the effectiveness of the tissue culture optimization process [36, 37].

This study delves into optimizing *Aegle marmelos* tissue culture, emphasizing sterilization techniques, hormonal combinations and the use of permeabilizers to enhance secondary metabolite production. The primary objective is to establish a refined protocol for *Aegle marmelos* tissue culture that maximizes the yield of secondary metabolites with significant pharmaceutical and nutraceutical potential. The results of this research are expected to contribute to the sustainable production of bioactive compounds from *Aegle marmelos*, offering valuable insights for the pharmaceutical and biotechnological sectors.

METHODOLOGY:

Plant Material: The plant specimen of *Aegle marmelos* L. Correa, belonging to the family Rutaceae, was identified and authenticated by the Blatter Herbarium, St. Xavier's College, Mumbai, where it was confirmed to match the Blatter Herbarium specimen number Bole-21, as documented by P.V. Bole. The same authenticated specimen was used for experimentation, with leaves meticulously collected from the botanical garden of the Institute of Science, ensuring genetic uniformity for this study.

Sterilization Standardization: A rigorous sterilization protocol was established to assess the efficacy of sodium hypochlorite on various *A. marmelos* explants. Different concentrations of sodium hypochlorite (4%, 3%, 2%, and 1%) were tested, and exposure durations ranged from 1 to 5 minutes. The explants underwent a sequential process, including washing with detergent (teepol), rinsing with tap water, treatment with varying NaClO concentrations, followed by ethanol (70%, 30 seconds), and thorough washing with sterile distilled water.

Preparation of Murashige and Skoog (MS) Medium: MS medium formulations were meticulously designed to investigate the influence of hormonal variations on *A. marmelos*. The following hormonal combinations were tested:

- MS medium + 2,4-D (2mg/L)
- MS medium + Kinetin (0.5 mg/L)
- MS medium + Kinetin (1.0 mg/L)
- MS medium +2,4 D (0.5mg/l) + BAP (1.5mg/l)
- MS medium + IAA (1 mg/l) + NAA (0.5 mg/l) + BAP (0.3 mg / l)

Inoculation and Incubation: Sterilized explants were meticulously inoculated in sterile MS media devoid of hormones to evaluate the survival rate, death rate, browning rate, and contamination rate. Incubation was conducted under controlled conditions, maintaining a 16/8 h light/dark cycle at 25±2°C for 7 days.

Callus Induction and Preparation of Suspension Cell Culture: Explants were systematically inoculated in sterile MS media, with different hormonal combinations, to induce callus formation. The hormonal combinations included those mentioned earlier. Incubation was carried out for an extended period (6–8 weeks) at 25±2°C, maintaining a 16–8 hour light/dark cycle. To initiate a cell suspension culture, fresh friable calluses were transferred to MS liquid sterile medium supplemented with growth hormones and maintained on a rotary shaker initially in the dark, then incubated at 25±2°C, maintaining a 16-8-hour light/dark cycle. Callus and suspension cell cultures were subcultured on a three-week cycle.

Permeabilization and Treatment: To enhance cell membrane permeability and facilitate intracellular compound extraction, permeabilization strategies were employed in21-day-old suspension cell cultures. Triton X-100 and Tween-80 solutions were utilized, with varying concentrations and treatment durations. Triton X-100 concentrations included 100, 125, 150, and 200 ppm for 30 minutes, while Tween-80 solutions were maintained at 5% for 1, 2, 3, and 4 hours (modified method of Patade *et. al.,* 2017) [26]. Treated cells underwent washing with sterilized double-distilled water and MS medium to remove the traces of adhering permeabilizing agents, followed by an incubation period in darkness for 7 days at a temperature of 25±2°C on a gyratory shaker. Control samples were non-treated.

Preparation of Standard Stock Solutions: Standard stock solutions for umbelliferone, crucial for HPLC analysis, were prepared by dissolving 5mg of powder in 5 mL of methanol. These stock solutions were used to prepare standard graphs at concentrations of 10 ppm, 20 ppm, 40 ppm, 80 ppm, and 100 ppm.

HPLC Analysis: HPLC conditions were achieved using an Agilent system equipped with a 4-solvent delivery system, quaternary pump, UV detector, and a 4-chamber in-line degasser. The analysis was performed on an Agilent ZORBAX RRHD Eclipse plus C18 column with a Methanol: Water: GAA mobile phase (54.99:45:0.1 v/v) at 300 °C. Detection was set at 325 nm, focusing exclusively on umbelliferone. Calibration curves facilitated accurate quantitative estimation.

Statistical Analysis: Three sets of 5 repeats were performed for each experiment to ensure statistical robustness. One-way ANOVA followed by post-hoc Tukey's test was conducted to assess significant differences between treatments, ensuring the reliability of the experimental findings.

These detailed and meticulously executed methods were designed to thoroughly investigate the effects of sterilization and culture conditions on *Aegle marmelos*, with a particular emphasis on optimizing tissue culture for increased bioactive compound production, particularly through HPLC analysis.

RESULT AND DISCUSSION:

Inoculation and Evaluation of Survival, Death, Browning, and Contamination Rates after sterilization with Sodium Hypochlorite:

TABLE 1: EFFECT OF SODIUM HYPOCHLORITE STERILIZATION AT VARIOUS CONCENTRATIONS FOLLOWED BY 70% ETHANOL (30 SECONDS) ON EXPLANTS OF *A. MARMELOS* IN TERMS OF SURVIVAL, DEATH, BROWNING, AND CONTAMINATION RATES

Treatment groups	NaClO treatment	Duration of Treatment (minutes)	No. of explants (Leaves)	Survival rate (Mean ± SE)	Death rate (Mean ± SE)	Browning (Mean ± SE)	Contamination rate (Mean ± SE)
A1	4%	1	30	71.11± 0.93	28.89 ± 0.93	41.11 ± 0.35	1.11 ± 0.35
A2	4%	2	30	65.56 ± 0.93	34.44 ± 0.93	46.66 ± 0.60	2.22 ± 0.35
A3	4%	3	30	57.77 ± 1.26	42.22± 1.26	45.56 ± 0.35	0 ± 0
A4	4%	4	30	51.11 ± 1.53	48.89 ± 1.53	52.22 ± 0.35	0 ± 0

A5	4%	5	30	46.67 ± 1.22	53.33± 1.22	50 ± 0.60	0 ± 0
B1	3%	1	30	83.33± 0.61	22.22± 1.27	32.22 ± 2.46	1.11 ± 0.35
B2	3%	2	30	74.45± 0.70	25.55 ± 0.70	38.89 ± 0.70	2.22± 0.70
B3	3%	3	30	67.78 ± 0.93	32.22 ± 0.93	41.11 ± 1.27	1.11 ± 0.35
B4	3%	4	30	60 ± 0.61	40 ± 0.61	47.78 ± 0.70	2.22± 0.70
B5	3%	5	30	72.22 ± 4.40	27.78 ± 4.40	50 ± 1.61	1.11 ± 0.35
C1	2%	1	30	97.78± 0.70	2.22 ± 0.70	1.11 ± 0.35	3.33 ± 0
C2	2%	2	30	97.78 ± 0.35	2.22 ± 0.35	0 ± 0	0 ± 0
C3	2%	3	30	95.566 ± 1.41	4.44 ± 1.41	4.44 ± 0.35	0 ± 0
C4	2%	4	30	95.55 ± 0.70	4.45 ± 0.70	2.22 ± 0.35	0 ± 0
C5	2%	5	30	94.44 ± 0.93	5.56 ± 0.93	4.44 ± 0.35	0 ± 0
D1	1%	1	30	95.56± 0.93	4.44 ± 0.93	1.11 ± 0.35	57.78 ± 3.66
D2	1%	2	30	93.33 ± 0.61	6.66 ± 0.61	2.22 ± 0.70	44.44 ± 2.30
D3	1%	3	30	93.33 ± 0.61	6.66 ± 0.61	1.11 ± 0.35	38.89 ± 2.53
D4	1%	4	30	95.56 ± 0.35	4.44 ± 0.35	3.33 ± 0.61	32.22 ± 1.96
D5	1%	5	30	95.56 ± 0.93	4.44 ± 0.93	4.44 ± 0.35	28.89 ± 0.93

This study investigates the effects of various NaClO concentrations and treatment durations on the survival rate, death rate, browning, and contamination rate of leaf explants. The treatment groups include different NaClO concentrations (1%, 2%, 3%, and 4%) with treatment durations ranging from 1 to 5 minutes.

The choice of sterilizing agent and its concentration plays a pivotal role in ensuring both the effective elimination of microorganisms and the preservation of tissue viability [11, 15, 16]. The results clearly demonstrate that NaClO concentration and treatment duration significantly affect the survival, death, browning, and contamination rates of leaf explants **Table 1** and **Fig. 1.**

Lower NaClO concentrations (1%) had the highest survival rates but also the highest contamination rates, suggesting they are too mild for effective sterilization despite being gentle on explants. The higher contamination rates observed in lower NaClO concentrations resonate with the findings of Kuppusamy *et al.* (2019), suggesting that milder sterilization conditions may compromise effectiveness in eliminating microorganisms [11].

The 2% NaClO concentration was found to be optimal for achieving high survival rates, low death and browning rates, and minimal contamination, suggesting it is effective in balancing sterilization with the preservation of explant viability **Table 1** and **Fig. 1**. This further supports the notion that lower NaClO concentrations are associated with higher survival rates [12, 38]. Moreover, the inverse relationship between death rates and survival rates reinforces the delicate balance between effective sterilization and tissue damage [15]. Contamination rates exhibit notable variations across different treatment groups, underscoring the influence of NaClO concentration and treatment duration on microbial elimination [16]. Higher NaClO concentrations (3% and 4%) were more effective at reducing contamination but caused higher death and browning rates, indicating significant tissue damage.

Browning of explants is a crucial factor affecting tissue culture success [15]. The variation in browning rates across different NaClO concentrations and treatment durations highlights the need for precise optimization to minimize tissue damage while ensuring effective sterilization. This aligns with the findings of Xu *et al.* (2022), who emphasized the importance of cautious application of NaClO to mitigate potential hazards to tissue health during sterilization [39].

Shorter treatment durations (1-2 minutes) generally resulted in higher survival rates across all concentrations, while longer durations increased death and browning rates, indicating that exposure time to NaClO is a critical factor in maintaining explant health **Table 1** and **Fig. 1**. Thus, 2% NaClO concentration for 1-2 minutes is recommended for optimal sterilization and explant viability in tissue culture of leaf explants **Table 1.**

The results of the study were further validated through a 2-Way ANOVA with post hoc Tukey's test, which confirmed the significance of the observed outcomes. This statistical analysis reaffirmed that the different concentrations and durations of sodium hypochlorite treatment

significantly influenced the survival, death, browning, and contamination rates of *Aegle marmelos* explants **Fig. 1**. Further research could explore the fine-tuning of these parameters for different types of explants and species to improve outcomes in plant tissue culture protocols.

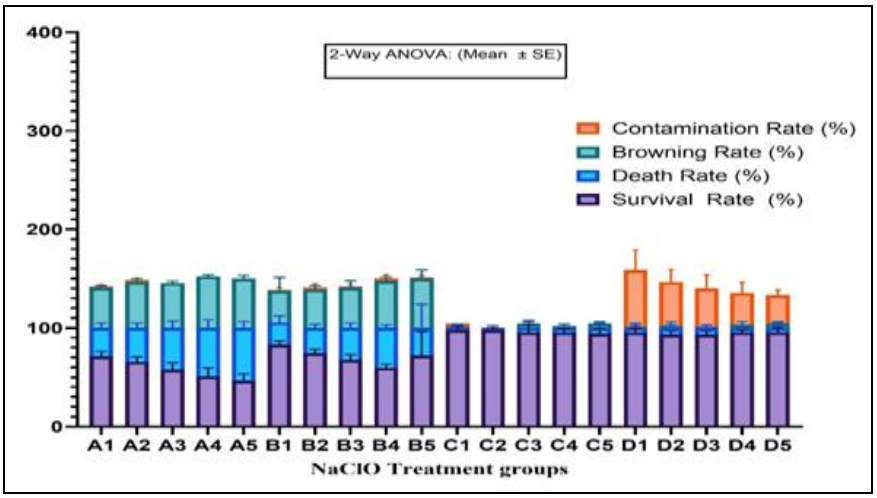

FIG. 1: EFFECT OF SODIUM HYPOCHLORITE STERILIZATION AT VARIOUS CONCENTRATIONS ON EXPLANTS OF *A. MARMELOS* IN TERMS OF SURVIVAL, DEATH, BROWNING, AND CONTAMINATION RATES

Effects of Different Growth Regulators on Callus Induction Rate (CIR) in Leaf Explants: This study evaluates the impact of various growth regulators on the callus induction rate (CIR) of leaf explants cultured on the Murashige and Skoog (MS) medium. The growth regulators tested include 2,4-D, kinetin (Kn), BAP, IAA, and NAA in different combinations and concentrations **Table 2** and **Fig. 2.**

TABLE 2: EFFECT OF GROWTH REGULATORS ON CALLUS INDUCTION RATE (CIR) OF LEAF EXPLANTS OF *A. MARMELOS*

MS+ Growth regulators (mg/L)	Number of Explants	Mean CIR	Mean ± SE
2,4-D (2mg/L)	30	70	70 ± 1.49
Kn (0.5 mg/L)	30	73.33333	73.33 ± 0.86
Kn (1.0 mg/L)	10	83.33333	83.33 ± 0.86
2,4-D (0.5 mg/L) + BAP (1.5 mg/L)	10	90	90 ± 1.49
IAA (1mg/L) + NAA (0.5 mg/L) + BAP (0.3 mg/L)	10	73.33333	73.33 ± 0.86

The CIR varied significantly among the treatments. Explants treated with 2 mg/L of 2,4-D had a CIR of 70±1.49, indicating moderate callus induction. Explants treated with 0.5 mg/L of Kn had a slightly higher CIR of 73.33±0.86, while increasing the Kn concentration to 1.0 mg/L resulted in a significantly higher CIR of 83.33±0.86, suggesting a dose-dependent effect of Kn on callus induction **Table 2.**

The highest CIR was observed with a combination of 0.5 mg/L 2,4-D and 1.5 mg/L BAP, achieving 90 ± 1.49. This combination is the most effective in promoting callus formation, likely due to a synergistic effect between auxins and cytokinins. Explants treated with a mixture of 1 mg/L IAA, 0.5 mg/L NAA, and 0.3 mg/L BAP exhibited a CIR of 73.33 ± 0.86, comparable to the CIR observed with 0.5 mg/L Kn alone **Table 2.** These results demonstrate that growth regulators' type and concentration significantly influence callus induction. While 2,4-D is effective alone, its efficiency is enhanced when combined with other growth regulators. The dose-dependent increase in CIR with Kn suggests that optimizing Kn concentrations can significantly enhance callus formation. The combination of 0.5 mg/L 2,4-D and 1.5 mg/L BAP proved to be particularly effective, highlighting the importance of auxin-cytokinin synergy in callus induction **Table 2.**

Two-way ANOVA results confirmed the significance of these findings, with the calculated F-value surpassing the critical F-value and a p-value of 0 ($p<0.05$), indicating highly significant

results. Post hoc Tukey's test further revealed that hormonal combinations significantly influenced callus induction rates, while explant type did not. Interestingly, leaf explants exhibited significantly higher callus induction rates compared to cotyledons and nodal explants. These outcomes emphasize the importance of optimizing hormonal combinations for enhancing callus induction efficiency in *A. marmelos* tissue culture.

A study by Kumar *et al.* (2018) demonstrated the effectiveness of Anther culture techniques in marigold (Tagetes spp.) breeding, showing that MS medium with 1.0 mg/l BAP, 1.0 mg/l 2,4-D, and 45g/l sucrose induced the highest callus formation (38.74%) in 16 days [40]. Pusa Arpita had higher embryogenic callus induction (27.70%) compared to Pusa Basanti Gainda (23.37%). For adventitious shoot buds, MS medium with 2.0 mg/l BAP, 0.5 mg/l NAA, and 30 g/l sucrose was most effective (13.32% induction) [40].

Similarly, Ahmadpoor *et al.* (2020) found that optimal sterilization and growth regulator conditions in *Melia azedarach* L. led to the highest callus yield on MS medium with 5 mg/L 2,4-D and 5 mg/L Kin, and maximum secondary metabolite production with 3 mg/L NAA and 1 g/L Kin [41].

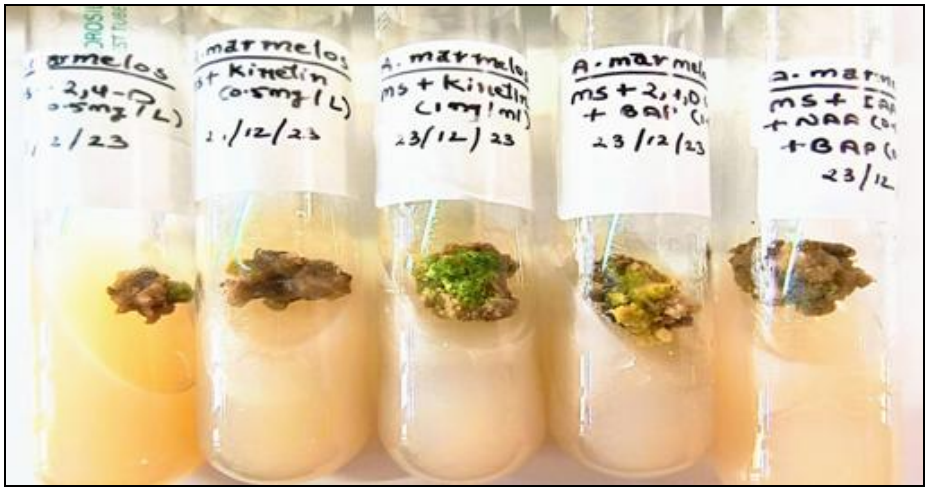

FIG. 2: CALLUS INDUCTION IN MS MEDIA+ DIFFERENT HORMONAL COMBINATIONS

Callus Growth and Characteristics in Leaf Explants over Time: During the initial week (7 days), the leaf explants exhibited callus growth ranging from 2 to 5 mm, with a fresh weight of 0.29 ± 0.026 g and a dry weight of 0.085 ± 0.062 g. At this stage, the explants were characterized by swelling. By the second week (14 days), the callus growth remained between 2 to 5 mm, but the fresh weight increased to 0.47 ± 0.011 g and the dry weight decreased to 0.026 ± 0.001 g, with notable swelling and curling of the leaf explants. This suggests an initial phase of rapid water uptake and cell expansion **Table 3.**

TABLE 3: CALLUS GROWTH AND THE DEVELOPMENT OF ITS CHARACTERISTIC OVER 49 DAYS

Explant Type	Total no. of explants	Days in Culture	Callus Growth (mm)	Callus fresh Weight (g) (Mean ± SE)	Callus Dry Weight (g) (Mean ± SE)	Callus Characteristics
Leaf	10*3	7	2-5	0.29 ± 0.026	0.085 ± 0.062	Swelled leaf explants
		14	2-5	0.47 ± 0.011	0.026 ± 0.001	Swelling and curling of leaf explants
		21	5-6	0.65 ± 0.0267	0.037± 0.0008	Friable light green callus
		28	6-8	1.26 ± 0.012	0.057 ± 0.001	Increased callus growth, Friable
		35	8-10	1.86 ± 0.046	0.076 ± 0.001	Dense and organised Callus
		41	10-12	0.25± 0.018	0.16 ± 0.009	Compact Dark green Callus
		49	12-14	0.26± 0.012	0.197 ± 0.001	Callus becoming Greenish brown

After 21 days, the callus size extended to 5-6 mm, with a fresh weight of 0.65 ± 0.0267 g and a dry weight of 0.037 ± 0.0008 g. The callus appeared friable and light green, indicating a transition towards more organized tissue growth. By 28 days, the callus size increased to 6-8 mm, with a fresh weight of 1.26 ± 0.012 g and a dry weight of 0.057 ± 0.001 g, showing significant growth and a friable texture. This phase marks robust proliferation, likely due to optimal nutrient uptake and cellular division **Table 3.**

At 35 days, the callus growth reached 8-10 mm, with a fresh weight of 1.86 ± 0.046 g and a dry weight of 0.076 ± 0.001 g, forming dense and organized structures. This peak growth indicates a high level of cellular activity and biomass accumulation. By 41 days, the callus size was slightly reduced to 10-12 mm, with a fresh weight of 0.25 ± 0.018 g and a dry weight of 0.16 ± 0.009 g, becoming compact and dark green. This reduction in size along with an increase in dry weight suggests a shift towards more mature and compact tissue with reduced water content **Table 3.**

By the end of the culture period at 49 days, the callus growth was measured at 12-14 mm, with a fresh weight of 0.26 ± 0.012 g and a dry weight of 0.197 ± 0.001 g. The callus exhibited a greenish-brown coloration, indicating aging and potential senescence. The increase in dry weight at this stage reflects a higher proportion of solid biomass relative to water content, consistent with the maturation and aging process **Table 3.**

Similar results were obtained by Fonseka & Aluthgamage, (2021) as they reported significant growth and biomass accumulation in callus cultures under optimal hormone treatments [20]. Furthermore, the characteristics of the callus observed in your study, such as the transition from a friable light green texture to a dense and organized structure, align with the findings of Ashokhan *et al.* (2020) on *Azadirachta indica* [44]. Also, Ahmadpoor *et al.* (2022) reported similar trends in their work on secondary metabolite production, where the maturation phase involved the development of denser, more organized structures with increased dry biomass [41]. Overall, these findings demonstrate a dynamic progression in callus growth and characteristics over time. Initial stages are marked by rapid cellular expansion and water uptake, leading to significant growth and biomass accumulation. The maturation phase involves the development of denser, more organized structures, while the final stage is characterized by reduced growth and increased dry biomass, indicating tissue aging. Understanding these stages is crucial for optimizing culture conditions and achieving desirable callus qualities for various biotechnological applications. Future research should aim to fine-tune culture parameters to sustain optimal callus growth and characteristics.

Two-way ANOVA with post hoc Tukey's test confirmed the significance of these differences ($p < 0.05$) among the explant types and culture durations.

HPLC Analysis: The High-Performance Liquid Chromatography (HPLC) analysis was conducted to evaluate the retention time and peak intensity (measured in milli-absorbance units, maU) of various treatments, including different concentrations of standards, Tween-80 at different time intervals (1,2,3 and 4 hours), and Triton X-100 at various concentrations (100,125,150 and 200 ppm). A control sample was also analyzed for comparison. Additionally, the fold increase in umbelliferone content in treated callus extracts by Tween 80 and Triton X-100 compared to the control was assessed.

The standards, ranging from 10 ppm to 100 ppm, exhibited consistent retention times around 3.318 to 3.399 minutes. The peak intensity increased with concentration, starting from 40.28 maU at 10 ppm and reaching up to 258.27 maU at 100 ppm **Fig. 3**. This consistent increase in peak intensity with concentration demonstrates a clear linear relationship, which is essential for the quantification of the analyte in the samples. The control sample exhibited a retention time of 3.326 minutes and a peak intensity of 48.83 maU **Fig. 4** and **5**. This serves as a baseline to compare the effects of Tween 80 and Triton X-100 treatments.

The retention times for Tween 80-treated samples were consistent around 3.318 to 3.328 minutes across different time intervals, indicating stability in the retention time. However, the peak intensities varied with time. The peak intensity initially

increased from 77.67 maU at 1 hour to 63.78 maU at 2 hours, then slightly increased to 68.9 maU at 3 hours, and significantly increased to 96.37 maU at 4 hours. This suggests that the interaction between Tween 80 and the analyte may fluctuate over time, possibly due to changes in the sample matrix or the interaction dynamics **Fig. 4.**

The fold increase in umbelliferone content in Tween 80-treated callus extracts compared to the control was significant. For instance, at 1 hour, the fold increase was approximately 1.59 times (77.67 maU compared to 48.83 maU). At 4 hours, the fold increase was about 1.97 times (96.37 maU compared to 48.83 maU) **Fig. 4.**

Triton X-100 samples exhibited a retention time ranging from 3.323 to 3.397 minutes, with peak intensities increasing with concentration. At 100 ppm treatment, the peak intensity was 54.63 maU which increased to 77.73 maU at 125 ppm then 112.84 maU at 150 ppm and gradually increased to 143.65maU at 200 ppm. The increasing peak intensity with concentration indicates a dose-dependent effect similar to the standards. The retention times remained relatively stable, although a slight variation at higher concentrations (200 ppm) was noted **Fig. 5.**

The fold increase in umbelliferone content in Triton X-100-treated callus extracts compared to the control was also notable. For example, at 100 ppm, the fold increase was approximately 1.12 times (54.63 maU compared to 48.83 maU), and at 200 ppm, the fold increase was about 2.94 times (143.65 maU compared to 48.83 maU) **Fig. 5.**

The HPLC analysis reveals that both Tween 80 and Triton X-100 treatments influence the peak intensity of the analyte. Tween 80 treatment led to up to a 1.97-fold increase, while Triton X-100 treatment resulted in up to a 2.94-fold increase as compared to control, highlighting the potential of these surfactants in enhancing secondary metabolite production in plant tissue cultures.

A study by Patade *et al.* (2017) supports our findings on the role of permeabilizing agents in enhancing metabolite production in plant cell cultures [26]. They demonstrated that using Triton X-100 and DMSO significantly increased flavonoid and campesterol release in Blumealacera. Additionally Zhang *et. al.* (2011), also highlight the significant role of Tween 80 surfactant in enhancing secondary metabolite production [25].

These findings highlight the importance of considering both the type and duration of surfactant treatments in analytical protocols. The stable retention times across treatments suggest reliable chromatographic performance, while the variations in peak intensities provide insights into the interactions between the analyte and the surfactants.

Future studies should explore the underlying mechanisms of these interactions to further optimize analytical methodologies and enhance secondary metabolite production in plant tissue cultures.

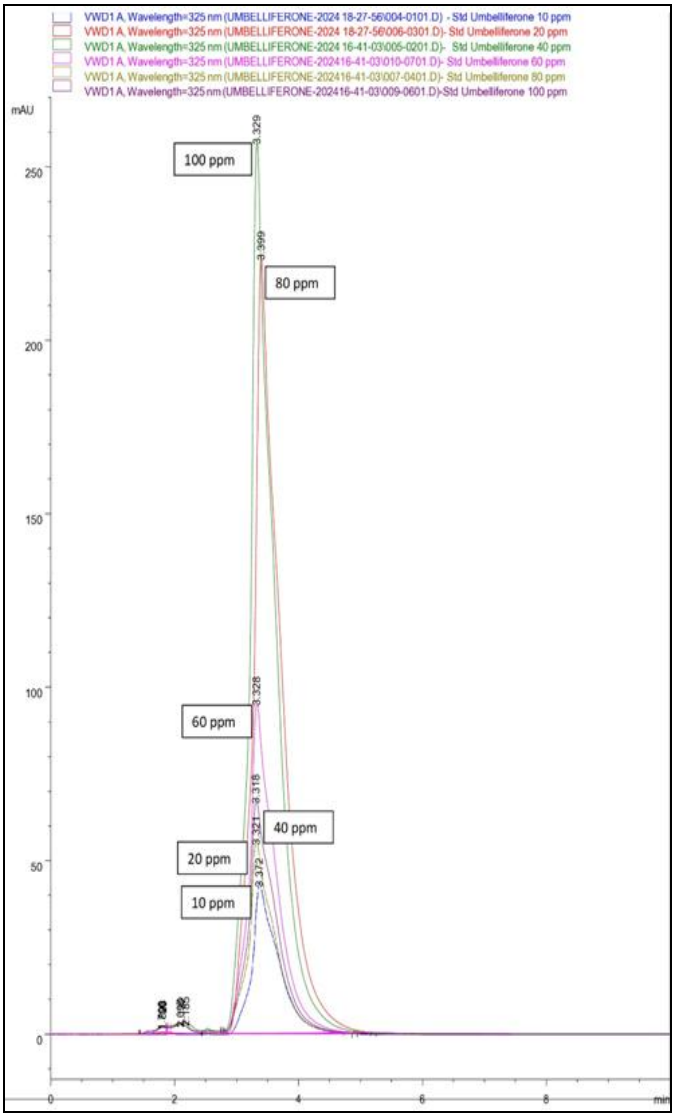

FIG. 3: OVERLAY OF HPLC ANALYSIS OF STANDARD UMBELLIFERONE

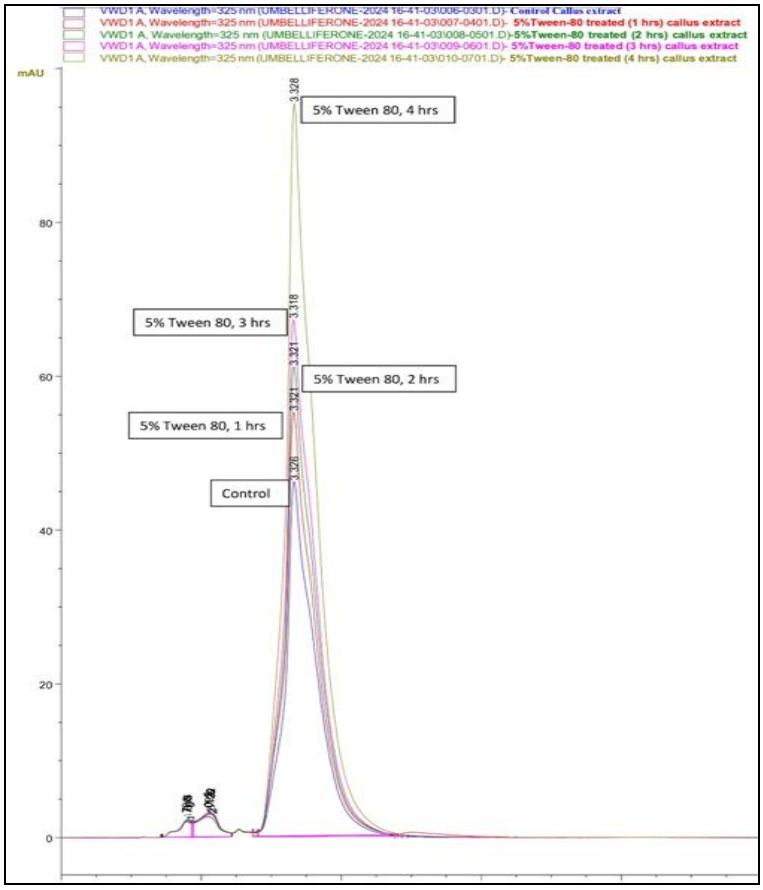

FIG. 4: OVERLAY OF HPLC ANALYSIS OF NON-TREATED AND TWEEN-80 TREATED CELL EXTRACTS

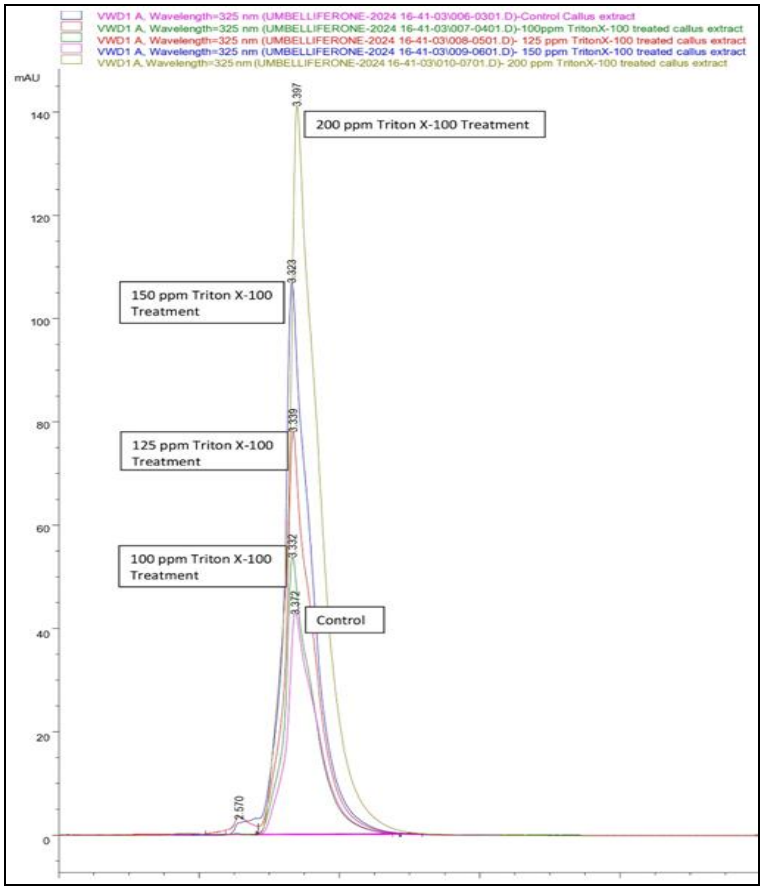

FIG. 5: OVERLAY OF HPLC ANALYSIS OF NON-TREATED AND TRITON X-100 TREATED CELL EXTRACTS

CONCLUSION: In conclusion, this study focused on investigating the effects of various treatments on plant tissue culture, specifically using *Aegle marmelos* as the plant material. The research highlighted the significance of optimizing NaClO concentrations for efficient sterilization, as lower concentrations (2%) proved most effective in promoting high survival rates with minimal browning and contamination. Furthermore, the study emphasized the importance of balancing hormonal levels for optimal callus induction protocols, with the combination of 0.5 mg/L 2,4-D and 1.5 mg/L BAP showing the highest callus induction rate.

Observations of callus growth revealed distinct developmental phases, ultimately leading to the accumulation of secondary metabolites, notably umbelliferone. Surfactant treatments, particularly Tween 80 and Triton X-100, significantly enhanced umbelliferone content in callus extracts compared to the control, indicating the potential of surfactants in augmenting secondary metabolite production in plant tissue cultures.

The findings of this research hold significant implications for advancing tissue culture methodologies and enhancing secondary metabolite yields in plant biotechnology. By understanding the optimal conditions and treatments for *Aegle marmelos* tissue culture, researchers can improve the efficiency and productivity of plant biotechnological processes. Prospects include further exploration of the underlying mechanisms behind these interactions and the optimization of conditions for various plant species and target compounds. Additionally, the insights gained from this study pave the way for potential applications in pharmaceutical, agricultural and nutraceutical industries, contributing to the sustainable production of valuable plant-derived compounds.

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