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Research Article

Cambial meristematic cells from bitter melon (*Momordica charantia* L. AMP-06): Propagation, characterization, and kuguacin J content

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Abstract: The use of plant cell culture remains an attractive means in the production of valuable phytochemicals. However, the traditionally used callus, which is dedifferentiated cells (DDCs), possesses several limitations, including inconsistent secondary metabolite production, slow growth rate, and genetic instability, which all can be circumvented by the use of innately undifferentiated cambial meristematic cells (CMCs). This paper demonstrates the isolation of CMCs from *Momordica charantia*. AMP-06. Characterization showed homogeneity of cells and the presence of small, multiple vacuoles. Untargeted Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and chemometric analyses suggest at least 25 chemical markers. Cell suspension culture growing optimizations in Murashige and Skoog (MS) medium were determined to be pH 5.8, 30 °C incubation temperature, 110 rpm agitation speed, and 30 g/L sucrose concentration. Cell growth kinetics showed a faster growth rate than previously reported growth of DDC. Analysis of a selected secondary metabolite, Kuguacin J, revealed its content in CMC to reach up to 4,924 ug/g dry weight, which is a 2,026-fold higher than the previously reported isolation yield from dried *M. charantia* leaves and vines. This shows that cambial meristematic cell culture can be utilized as a platform to produce bioactive secondary metabolites with higher content than those found in intact plants.

INTRODUCTION

The ability of plants to synthesize complex compounds with biological activities has been exploited to produce commercial drugs like the analgesic morphine, antimalarial artemether, acetylcholinesterase-inhibitor galantamine, and anti-cancer vinblastine and vincristine (Christensen, 2021; Nasim *et al*., 2022). More than a third of all US-FDA-approved therapeutic drugs are naturally produced or derived from natural products, and more than 50% of small molecule drugs developed from 1981 to 2019 are either originally derived from natural products or semi-synthetic (Newman & Cragg, 2020).

In the Philippines, *Momordica charantia* L., locally known as “ampalaya”, is one of the most commonly consumed vegetables. Folkloric knowledge on its use to treat various diseases has placed *M. charantia* at the forefront of medicinal plants in the country (DA, 2017). It is, in fact,

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one of the ten medicinal plants recognized and approved by the Department of Health (PITAHC, n.d.). Studies have shown that "ampalaya" contains a wide array of metabolites, such as alkaloids, glucosides, phenolics, flavonoids, and cucurbitane-type triterpenoids, to name a few, that account for its antioxidant, anticancer, antimicrobial, anti-inflammatory, antiviral, and other therapeutic and health-protective properties (Gayathry & John, 2022).

One of the phytochemicals from *M. charantia* with medicinal potential is kuguacin J. It was reported to demonstrate activity against chemotherapeutic multidrug resistance characterized by overexpressed P-glycoprotein. In multidrug-resistant human cervical cancer cells, KB-V1, kuguacin J showed dose-dependent increased sensitivity to vinblastine and paclitaxel resulting in increased accumulation and reduced P-gp-mediated efflux (Pitchakarn *et al*., 2012a). It has also been reported to induce G1 arrest and apoptosis in androgen-dependent and independent prostate cancer cell lines, LNCaP and PC3, but has less toxicity to the normal prostate cell line, PNT1A (Pitchakarn *et al*., 2011; Pitchakarn *et al*., 2012b). Despite this potential, in vivo studies are limited due to the low purification yields of kuguacin J (Limtrakul *et al*., 2013).

At the top of the biotechnological techniques employed today for enhanced production of valuable phytochemicals is plant cell culture technology (Ramirez-Estrada *et al*., 2016). This tool has been proven effective in the commercial production of paclitaxel from the cell culture of the Pacific yew tree (Tomilova *et al*., 2023). Other secondary metabolites produced currently at commercial scale using the technique include rosmarinic acid, ginseng, berberine, and shikonin (Eibl *et al*., 2018). Despite its utility, however, several issues continue to exist with traditionally used callus or dedifferentiated cell (DDC) culture, particularly poor growth rates and inconsistent cell biomass and metabolite yields due to genetic and epigenetic modifications that occur during the dedifferentiation process (Partap *et al*., 2023). Cambial meristematic cell (CMC) culture offers an alternative that addresses the limitations of DDC cultures. It is a lateral meristem culture isolated from cambial tissue predominantly found in plant stems, allowing for a richer and more accessible stem cell source (Ochoa-Villarreal *et al*., 2015). CMCs exhibit greater genetic stability, and their thin cell walls and rich spherical vacuoles elicit higher growth and regeneration rates as well as metabolite yield (Partap *et al*., 2023).

Meanwhile, LC-MS/MS metabolomics is one of the most commonly employed methods to provide profiling and identification features, thereby identifying chemical markers. Frequently paired with chemometric tools such as principal component analysis (PCA) or orthogonal partial least squares discriminant analysis (OPLS-DA), it generates markers in the form of masses that are unique or overly expressed in a specific sample. It has been used in plant species identification, disease diagnosis and prognosis, and metabolic pathways analysis (Plumb *et al*., 2023; Rakusanova *et al*., 2024).

Previous studies have already reported on the callus culture of *M. charantia,* such as the work of Chung *et al.* (2018). However, as far as literature is concerned, this is the first time to study the isolation and culture of cambial meristematic cells from the aforementioned plant. Characterization of its cambial nature was conducted while chemical markers, generated by LC-MS/MS metabolomics, were proposed. Furthermore, kuguacin J content was analyzed to demonstrate the utility of this biotechnological system for perpetual production of bioactive phytochemicals from *M. charantia*.

METHOD

Isolation of Cambial Meristematic Cells (CMCs)

Seeds of *Momordica charantia* AMP-06 were decoated and surface-sterilized with 70% ethanol and 2% NaOCl for 3 minutes each, with sterilized distilled water rinsing after each step. They were grown in a phytohormone-free, solid Murashige and Skoog (MS) media at 26±2 °C with a 16/8-hour light/dark regimen of ~3000 lux from LED lamps.

Cambial meristematic cells were isolated according to the method previously described by Lee *et al*. (2010) with some modifications. Instead of cutting longitudinally to remove the xylem

guided by staining with lignin-specific phloroglucinol dye, the epicotyl stem of the two-week-old seedling was cut slantly at approximately 0.5 cm long, laid directly on a solid medium, and positioned such that the cambium tissue at the cut portion is exposed. MS basal medium with 8g/L agar and 0.5g/L 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.8) was used instead of B5 medium and 4g/L gelrite. The carbon source was 30g/L sucrose. Instead of picloram, the medium was supplemented with naphthalene acetic acid and kinetin at 1.0 mg/L (5.4 μM) and 0.5 mg/L (2.32 μM), respectively, after showing optimal cell proliferation based on preliminary comparison of different auxins, cytokinins, and concentration ratios (data not shown), where twenty explants were used per replicate with three replicates each. It was grown in total darkness. Seven days after inoculation, white cells proliferating at the cut area of the stem explants were pooled and triturated with forceps to transfer small pieces to fresh solid media.

Vacuole Staining and Microscopic Analysis

Neutral red was used to stain the vacuole following the method of Schwab & Hülskamp (2010). Briefly, cells were incubated with neutral red solution 0.5% (w/v) for 5 minutes, centrifuged at 10, 000 rpm for 5 minutes, and washed with pH 7.2 phosphate buffer. Cells, stained and unstained, were viewed in an inverted microscope (Evos) with 10X magnification. The experiment was conducted thrice.

LC-MS/MS-Based Chemical Marker Identification

Dried two-week-old solid medium-grown CMCs were compared with mature *M. charantia* fruit by untargeted metabolic analysis using Liquid Chromatography-Quadrupole-Time-of-Flight (LC-QToF) for chemical marker identification. Dried, pulverized biomass (0.5 g) was extracted with 2.5 mL ethyl acetate, sonicated for 30 minutes, and centrifuged for 5 minutes at 5,000 rpm. Extraction was repeated three times, then the supernatants were pooled and dried using SpeedVac. Dried extracts were reconstituted with 0.5 mL LCMS-grade methanol, centrifuged at 10,000 rpm, and transferred to LCMS vials with inserts.

Waters Ultra-High Performance Liquid Chromatography (UPLC) I-Class equipped with a Xevo G2-XS Qtof mass spectrometer (Waters Corp., USA) was used for the analysis with the stationary phase, a 2.1 × 100 mm 1.8-Micron Acquity HSS T3 column, and the mobile phases composed of 0.1% formic acid in water (A) and acetonitrile (B). With a flow rate of 0.5 mL/min and an injection volume of 5 μL, the linear gradient program was set at 5% B to 95% B over a 15-minute run time with the column temperature of 40 °C. The MS analysis was carried out under the electrospray ionization (ESI) positive mode, with the capillary voltage set to 1 kV, sample cone 40 V, cone gas flow of 50 L/hr, and desolvation gas flow of 950 L/hr. Source temperature was set to 120 °C while desolvation temperature was 550 °C. Data acquisition was performed in triplicates with a 50-1,200 m/z range. Leucine enkephalin was used as the reference standard for mass correction. The analysis was carried out in MS^E mode, which simultaneously acquires the parent and fragmentation spectra in a single run time.

Chemical markers were identified following the algorithm of Waters, where data were acquired and processed through UNIFI Scientific Information System version 1.8 software (Waters Corp., USA). To create markers, tolerance for mass and retention time were set to 5.00 ppm and 0.1 min, respectively, for the full range of m/z, intensity counts, and retention time. Intensities were normalized before transferring to EZ Info software (Waters, Milford, MA, USA), where orthogonal partial least-squares discriminant analysis (OPLS-DA) was used for calculation. S-plot was generated, and unique markers were identified using Unifi's elucidation tool considering elemental composition with i-FIT confidence of 99-100%, mass error of 5 ppm, and fragmentation matches of 5 and above when matched against an in-house *M. charantia* library of reported secondary metabolites.

Optimization of Cell Suspension Culture Condition

Two-week-old CMCs grown from solid media were pooled and triturated. An inoculum size of 500 mg was transferred to a 125-mL Erlenmeyer flask with 25 mL of MS medium with the same nutrient composition as mentioned above. Varied pH (5.3, 5.8, and 6.3), temperature (20 °C±1, 25 °C±1, and 30 °C±1), continuous agitation speed (90, 110, and 130 rpm), and sucrose concentration (20, 30, 50, and 70 g/L) were evaluated to determine the optimum condition producing the highest biomass accumulation at total darkness. The two-week-old cell suspension cultures were filtered, dried at 45 °C, and weighed.

Cell growth and Kuguacin J Kinetics

A total of 30 flasks of cell suspension culture were prepared and incubated using the optimized growing parameters. Three flasks were randomly selected every three days, and the cells were filtered with Whatman 91 filter paper (pore size: 10 µm), dried at 45 °C for 48 hours until a constant mass was achieved. Dried cells from the three replicates per sampling point were pooled, pulverized, and used in the analysis of a selected secondary metabolite with reported bioactivity—Kuguacin J.

Single-point calibration system was employed for the quantification of kuguacin J. Kuguacin J standard (Chemfaces) was accurately weighed, dissolved in LCMS-grade methanol to prepare a solution of 500 ppm, and transferred to an LCMS vial. LCMS analysis was carried out according to the method previously presented.

Statistical Analysis

All the tests were conducted in triplicate, and the experimental results were expressed as mean±standard deviation. Analysis of variance (ANOVA) was determined where applicable. Comparisons among treatment means were done using Tukey's range tests at $p \leq 0.05$.

RESULTS

Vacuole Staining and Microscopic Analysis

Microscopic analysis of the cells (Figure 1-A) showed a homogenous, round cell type ranging from approximately 50 to 100 µm. Meanwhile, leaf-derived dedifferentiated cells (DDCs) were heterogeneous (Figure 1-B) as they contained a combination of round, also ranging from 50-100 µm, and elongated cells, whose length could reach up to 200 µm. When stained with neutral red, the small multiple vacuoles in CMCs were observed (Figure 1-C).

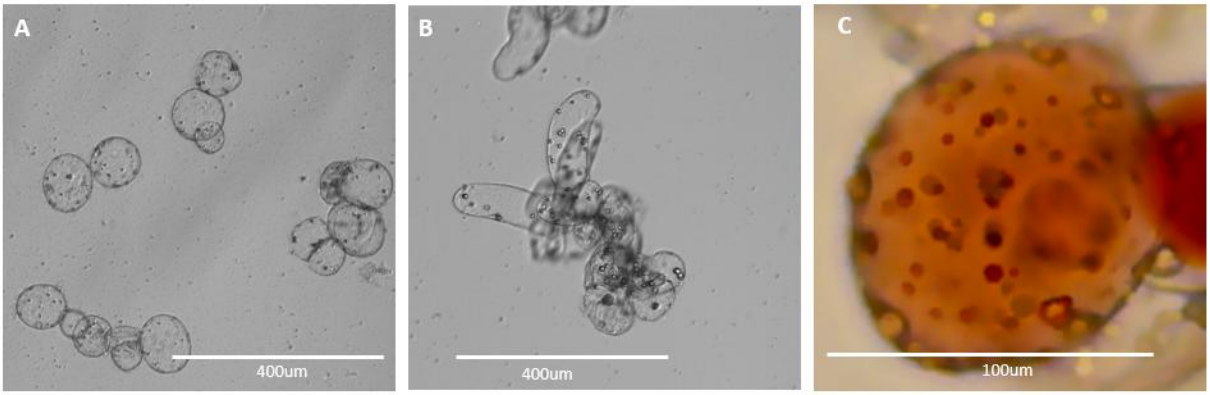


Figure 1. *Microscopic image (10X) of cambial meristematic cells (CMC) (A) showing homogeneity and leaf-derived dedifferentiated cells (DDC) (B) showing heterogeneity. Neutral red-staining of CMC shows the multiple, small vacuoles (C).*

Vacuole Staining and Microscopic Analysis

The comparison between the metabolites of *M. charantia* fruit and CMC is shown in the S-plot in Figure 2, where markers in the middle are commonly detected in the two groups, while the

more prevalent and unique to CMC are situated towards the right of the plot approaching positive 1 (highlighted in red).

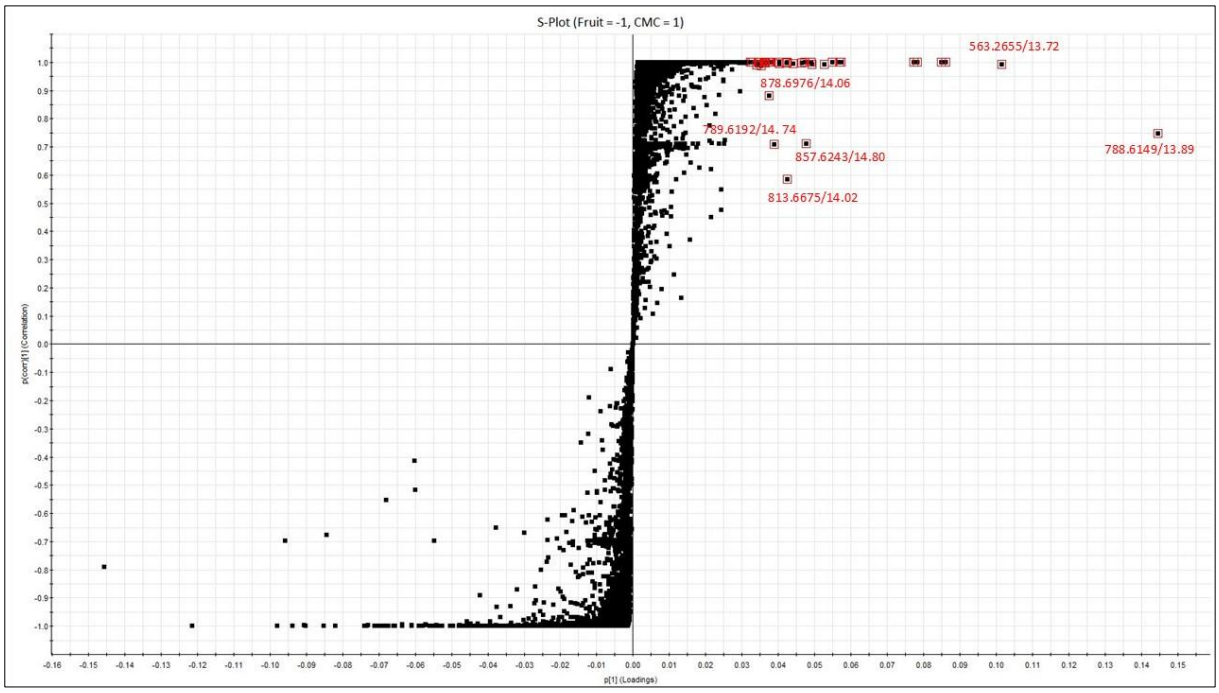
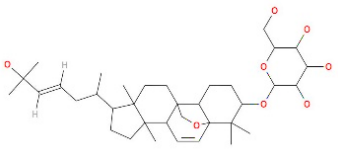
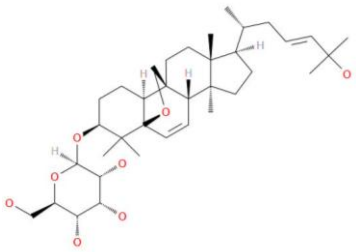
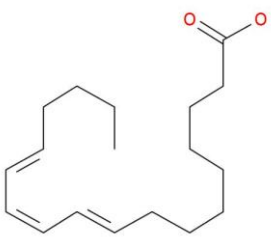


Figure 2. *The OPLS-DA S-plot shows the highlighted potential markers of CMC with some selected masses and their retention times (m/z/RT).*

Table 1. *The putatively identified chemical markers of M. charantia AMP-06 CMC.*

$[M+H]^+$; RT	Elemental Composition	i-FIT Score (%)	Putative Identification	Monoisotopic Mass (Da)	Mass Error (ppm)	Structure
619.4193; 8.60	C36H58O8	100	Momordicoside I	618.4132	2.79	
619.4193; 8.60	C36H58O8	100	Momordicoside F2	618.4132	2.79	
279.2335; 10.82	C18H30O2	100	Punicic acid	278.2246	3.68	

Elucidation of these chemical markers by elemental composition produced 25 markers with i-FIT confidence scoring of 99-100%. Among these markers, two have putative identification based on matching with an in-house library of reported *M. charantia* secondary metabolites, as shown in Table 1. [M+H]+ ion with m/z 328.1219 and a retention time of 8.60 min has two

putative identifications (alternate assignment), namely momordicoside I and momordicoside F2. Meanwhile, [M+H]+ ion with m/z 279.2335 eluted at 10.82 min was putatively identified as punicic acid. The generated fragmentation spectra of these compounds are shown in Appendix 1, while the other 23 unidentified chemical markers are shown in Appendix 2.

Optimization of Cell Suspension Culture Condition

To produce the highest biomass quantity, pH, agitation speed, temperature, and sucrose concentration were optimized. Figure 3-A shows that pH 5.8 was the optimal pH, with its biomass being significantly (p<0.01) the highest as compared to pH 5.3 and 6.3. Continuous agitation at 110 rpm (Figure 3-B) was the optimal speed to significantly produce the highest biomass as compared to cultures agitated at 90 and 130 rpm. Figure 3-C summarizes the effects of temperature on the biomass production of CMC suspension culture, where incubation at 30°C produces the highest biomass amount. However, this is not significantly different (p>0.05) as the biomass produced with 25 °C incubation. Incubation at 20 °C was shown to be unfavorable as it produced the lowest biomass accumulation. Sucrose concentration of 30g/L produced significantly the highest biomass proliferation (Figure 3-D) compared to lower and higher concentrations.

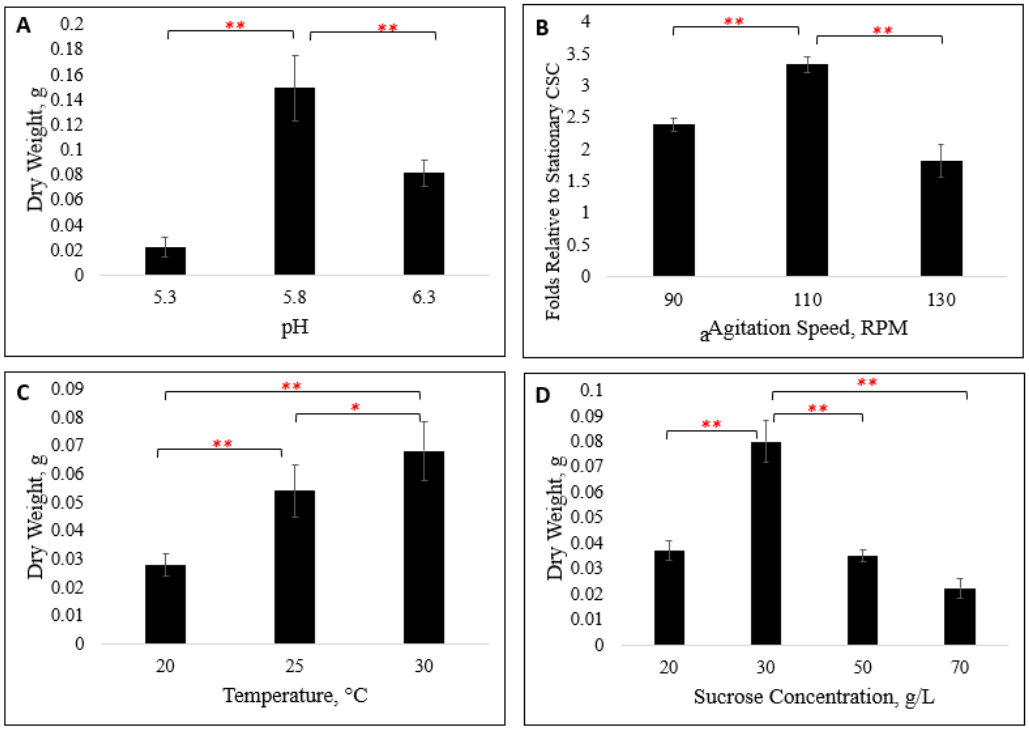


Figure 3. *Dried weight of biomass at day 14 of CSC at different pH (A), agitation speeds (B), temperatures (C), and sucrose concentrations (D). **p<0.05*

[a]Agitation speed was normalized by calculating the ratio of dry weight of agitated and stationary CSC expressed in folds since the experiment was conducted separately.

Cell Growth and Kuguacin J Kinetics

Summarized in Figure 4 (orange line) is the cell growth curve of the CMC suspension culture grown using the optimized pH of 5.8, incubating temperature of 30°C, with an agitation speed of 110 rpm, and a sucrose concentration of 30 g/L. The lag phase was observed on the first twelve days of culture, followed by the log phase on the next nine days (day 12 to 21). Stationary phase was observed for three days (day 21-24), followed by the death phase on day 24 onwards.

Kuguacin J production did not follow the same trend as the growth curve (Figure 4, black bar). Kuguacin J content was shown to be the lowest during the log phase and rapidly increased during the death phase.

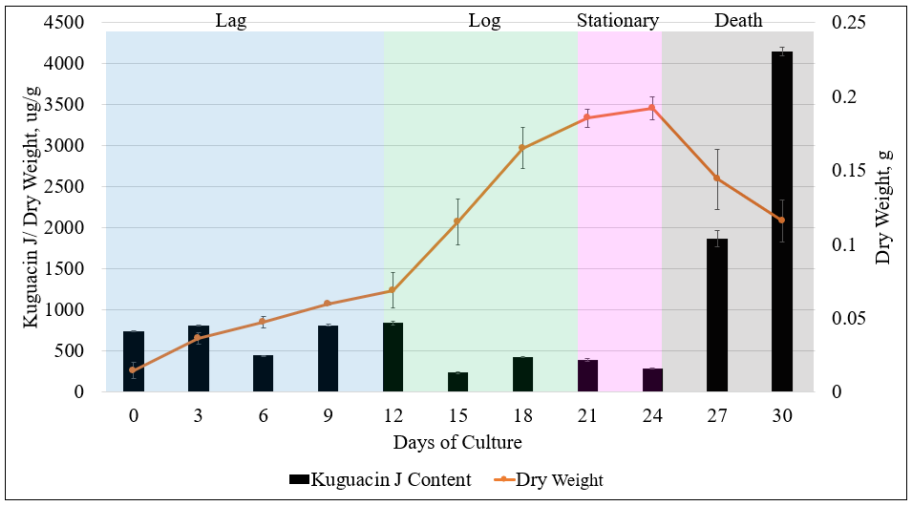


Figure 4. *The cell growth curve of CMC grown with the optimized conditions (orange line) and the Kuguacin J (ug/g dried biomass) content (black bar).*

DISCUSSION and CONCLUSION

Characterization of the isolated cells was conducted to validate the cambial nature of the cells. The consistent and precise secondary metabolite production in CMCs is associated with its homogeneous cells, while the presence of multiple and small vacuoles provides better resistance to shear stress, as compared to DDCs, when cells are agitated and grown in suspension culture or bioreactors (Yun *et al*., 2012). These may explain the improved biomass production in the current study versus the previously reported data for *M. charantia* DDC (Chung *et al*., 2018). The highest biomass accumulation at day 24 of CMC is 3.6-fold higher than the biomass at day 6 of its culture. On the other hand, the highest biomass accumulation of DDC at day 28 is only 0.36-fold higher than its biomass accumulation at day 7. This observation suggests faster and more pronounced growth of CMC, hence superiority over the DDC culture of *M. charantia*. Similar results were reported by Mehring *et al*. (2020) on *Ocimum basilicum* CMC reaching the log phase three days earlier than DDC and Song *et al*. (2019) on *Tripterygium wilfordii,* demonstrating higher biomass production of CMC than DDC. The faster and higher biomass production of CMC is one of its advantages over DDC.

As a preparative stage for large-scale production, optimization of growing conditions allows the use of ideal parameters for biomass accumulation. Concentration of kuguacin J, a bioactive secondary metabolite with pharmacological potential (Pitchakarn *et al*., 2011), has been monitored to be the proof-of-concept secondary metabolite produced *via M. charantia* CMC culture. Its highest concentration of 4,924 ug/g dry weight in the death phase of the culture may suggest a protective effect on senescent cells. The low concentration during the log phase is, however, an expected phenomenon since, at this stage, the cells are actively dividing and the flux goes towards primary metabolism (Malik *et al*., 2012). This defines an important parameter in improved metabolite production, where techniques, such as elicitation and precursor feeding, may be carried out on the late exponential stage. This would be a ten-day reduction of the culture time, thereby providing a cost-effective method. Nevertheless, the current study shows that kuguacin J produced in a 30-day-old CMC cell culture, 0.492%, is 2,026-fold higher than the previously reported isolation yield from dried *M. charantia* leaves and vines, 0.000243% (Chen *et al*., 2008).

In order to establish identification and authentication features of this cell line, chemical markers of *M. charantia* AMP-06 CMC are also reported herein. By LC-MS/MS with chemometric analysis, the CMC metabolic profile suggests 25 markers that may not be exclusively found in CMC but are present in relatively higher concentration compared to the fruit. This large detection disparity may be associated with the difference in the degrees of differentiation and the environmental setup where they were grown. Out of these markers, two have been

putatively identified, where only punicic acid, widely known to be present in pomegranate seed oil, has a reported therapeutic potential (Shabbir *et al*., 2017). Though it is ideal that the therapeutic component serves as the chemical marker, characteristic, and general components can also be selected for quality control purposes. These markers can then be used in the identification, quality control, and evaluation of this cell line when used in future applications.

For the first time, a cambial meristematic cell line from *M. charantia* has been established. Though further characterization must be pursued, the current study preliminarily established the cambium nature of the isolated cells by virtue of cell homogeneity and the presence of small, multiple vacuoles. Optimal growing conditions for the cell suspension culture were determined, where cell growth kinetics showed maximum biomass production at day 24. Quantitation of a selected secondary metabolite, kuguacin J, demonstrated much higher production vs previously reported isolation yield, thereby substantiating the utility of *M. charantia* CMC platform for the production of bioactive secondary metabolites.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Data Availability Statement

Research data is available and can be sent with e-mail upon request.

Authorship Contribution Statement

Zaidy T. Martin: Materials, Data Collection and Processing, Analysis and Interpretation, Literature Review, Writing. **Francisco M. Heralde III**: Conception, Design, Supervision, Materials, Critical Review.

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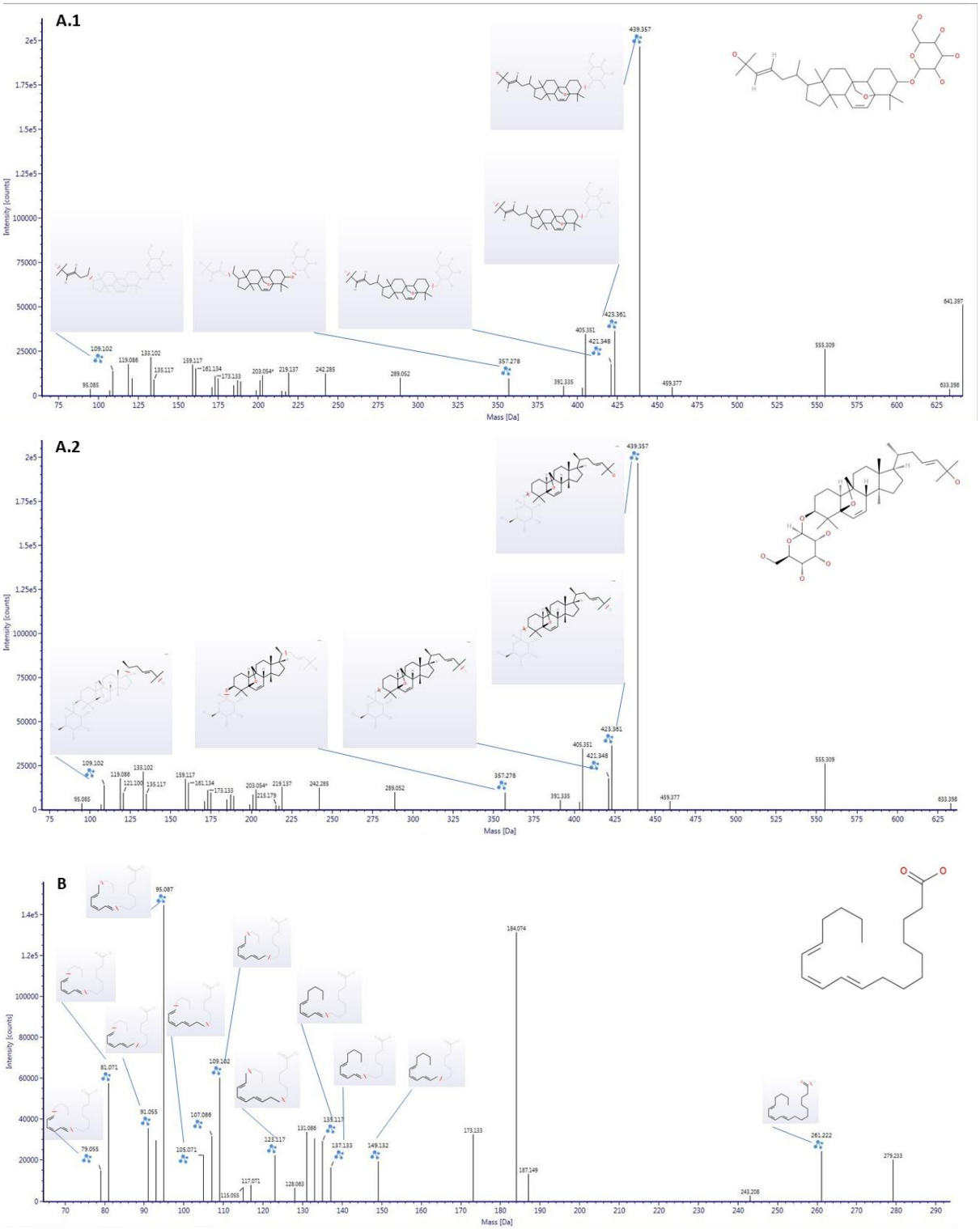
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APPENDIX

Appendix 1. *Matched fragmentation masses of the proposed chemical markers Momordicoside I (A.1) or Momordicoside F2 (A.2), and Punicic acid (B).*



Appendix 2. *The unidentified chemical markers of M. charantia AMP-06 CMC.*

M/Z	Retention Time (min)	Elemental Composition	i-FIT Score (%)
423.3628	8.03	C30H46O	100
423.3629	9.17	C30H46O	100
415.2127	9.25	C24H30O6	99.99
643.4178	9.96	C38H58O8	99.68
423.3630	10.04	C30H46O	100
405.3525	10.11	C30H44	99.24
277.2179	10.14	C18H28O2	100
573.4874	13.32	C37H64O4	100
423.3620	13.44	C30H46O	100
617.3833	13.56	C39H52O6	100
441.3373	13.68	C29H44O3	100
563.2655	13.73	C33H38O8	99.68
413.3631	13.85	C25H48O4	100
401.2912	14.07	C22H40O6	100
369.3531	14.46	C27H44	100
531.3686	14.47	C32H50O6	100
571.3611	14.48	C34H50O7	99.77
885.5510	14.54	C54H76O10	100
561.3790	14.66	C33H52O7	100
601.3715	14.66	C35H52O8	99.89
393.3527	14.76	C29H44	100
523.3611	14.83	C30H50O7	99.97
483.3687	14.85	C28H50O6	100