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The Effect Of Various Ca^{2+} Elicitor Concentrations On Catechin Content In Gambir (Uncaria Gambir (Hunter) Roxb.) Callus In Vitro

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Abstract— Gambier (Uncaria gambir (Hunter) Roxb.) is a plant that contains natural antioxidants produced from the extraction of leaves and twigs. Gambier extract primarily contains catechins, which are secondary metabolites belonging to the flavonoid group. The low content of catechin produced from traditional gambier cultivation has led to a decline in the economic value of gambier plants. Additionally, the long metabolic process of gambier in producing catechin results in low levels of catechin compounds in young plants. One effort to increase the content of catechin in young gambier plants is through elicitation of gambier callus. This study aimed to determine the optimal concentration of Ca^{2+} elicitor in increasing the catechin content of gambier callus. This research was conducted from March to July 2024 at the Tissue Culture Laboratory, Universitas Andalas, and Vahana Scientific Laboratory, Padang. This study used a Completely Randomized Design (CRD) with Ca^{2+} elicitor treatments (0, 44, 88, and 176 g/L). Quantitative data were analyzed statistically using ANOVA followed by Duncan's Multiple Range Test (DMRT) at 5% significance level. The results showed that the application of Ca^{2+} elicitor was not sufficient to induce catechin compounds in gambier callus, and there were several unidentified metabolites in the callus that did not match the catechin standards used. However, the 88 g/L treatment yielded a higher amount of metabolites compared to other treatments, as shown by the total peak area obtained from HPLC analysis, which is suspected to be catechin derivatives.

Keywords— callus, catechin, elicitation, elicitor, secondary metabolite

I. INTRODUCTION

Gambir (*Uncaria gambir* (Hunter) Roxb.) is a flowering plant in the Rubiaceae family, native to tropical regions. The economically valuable parts of the gambir plant are its leaves and branches, which contain chemical compounds such as catechins, catechu tannic acid, floursin, and quercetin. These compounds are utilized as antibacterial agents, natural pesticides, tanning agents, and raw materials in the textile industry [1].

Gambir is known for its natural antioxidant properties derived from leaf extraction. The extract contains catechins at levels of 7-33%, which are secondary metabolites in the flavonoid group. Additionally, gambir contains catechu tannic acid (20-55%), quercetin (2-4%), red catechu (3-5%), gambir floursin, ash, fats, and wax. Given the variety of compounds found in gambir, it is not surprising that it has numerous applications. Preclinical tests on gambir have shown its efficacy as an antihyperlipidemic agent [2]. In vitro

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studies indicate that catechins in gambir leaf extract can reduce cholesterol formation by inhibiting the activity of HMG-CoA reductase and lipase [3], [4].

The high catechin content in gambir leaves makes them a promising candidate for developing herbal products, cosmetics, and health beverages [2]. The Indonesian National Standard for Gambir (SNI 01-3391-1994 and its revision SNI 01-3391-2000) outlines the relationship between catechin content and gambir quality: Grade III has a catechin content of 40%, Grade II has 50%, and Grade I has 60%. This standard is based on gambir produced in West Sumatra [5].

Gambir is a key export commodity in West Sumatra, with many farmers relying on its cultivation for their livelihoods. According to the Central Bureau of Statistics of West Sumatra on 2022, the area under gambir cultivation was 29,432 ha in 2019 but decreased to 28,016 ha in 2020, with production during this period amounting to 7,582 tons. The export volume of gambir from West Sumatra in 2021 reached 16,375,611 kg, valued at approximately USD 41,404,929, reflecting an increase from the previous year when the total export volume was only 102 kg worth USD 714 [6].

Since the early 2000s, Indonesia has become a significant exporter of raw gambir; however, most exported gambir contains catechin levels below 75% [1], [7]. This issue arises from inadequate cultivation techniques; many farmers grow gambir traditionally and utilize seeds from traditional sources. Consequently, physiological quality and plant health are often neglected. As a result, plant growth may lack superior traits inherited from parent plants, leading to disease transmission and lower-quality gambir production [5], [8], [9]. This presents a challenge for researchers to find solutions to improve gambir quality amid high global demand and prices.

The high demand coupled with low productivity and catechin quality poses challenges for developing gambir plants [10]. Advances in technology and science can maximize active compound content in products. One approach is elicitation in culture [11]. In vitro cultured gambir leaves can produce callus as a primary material for elicitation. Callus growth can be manipulated to enhance secondary metabolite production through elicitor addition. Elicitors are active agents that trigger secondary metabolite formation by inducing plant defense responses [12]. The use of elicitors aims to stimulate secondary metabolite production by promoting phenolic compound formation [13], [14].

One effective elicitor is Ca^{2+}, which can increase epicatechin gallate concentrations during prolonged incubation [15]. The acidity level of the culture medium also influences secondary metabolism. Acidification can be achieved through various methods such as external alkalization, yeast addition, weak acid application, or by adding metal ions to the medium. Chemical compounds in the form of metal ions such as Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} can serve as abiotic elicitors by inhibiting physiological processes in plants [16]. One of the metals ion that can be used as an elicitor is Ca^{2+}. Calcium ion (Ca^{2+}) is an essential micronutrient for all living organisms, playing a structural role in cell walls and membranes, and are involved in various metabolic processes such as nutrient absorption and enzyme function. Calcium also contributes to cell formation, cell elongation, and the maintenance of membrane structure. However, excessive amounts of Ca^{2+} can induce metal stress in plants, prompting them to absorb and accumulate the ion, which in turn enhances the production of secondary metabolites [17]. Research by [15] on tea callus elicitation indicated that low concentrations of Ca^{2+} (176 g/L) could enhance epicatechin gallate levels during longer incubation compared to higher concentrations (352 g/L). Another research by [18] demonstrated that the addition of Ca^{2+} (via $CaCl^2$) to cell suspension cultures of *Cinnamomum longepaniculatum* increased the essential oil content in the cells. The application of endophytic fungal elicitors also elevated intracellular Ca^{2+} concentrations and essential oil accumulation, whereas the use of Ca^{2+} channel blockers reduced essential oil content. Additionally, other studies on rice cell cultures have shown that Ca^{2+} influx induced by elicitors plays a role in activating the biosynthesis of phytoalexins, a group of secondary metabolites important for rice plant defense [19]. Therefore, this study will utilize low concentrations of Ca^{2+} elicitors to enhance catechin production in callus culture of gambir leaf (*Uncaria gambir* (Hunter) Roxb.).

II. Materials and Methods

The source of the explants used in this study was the leaves of the gambir plant (*Uncaria gambir* (Hunter) Roxb.) from the Plant Tissue Culture Laboratory collection at Faculty of Agriculture, Andalas University. The media used were instant Murashige & Skoog (MS) with plant growth regulators 2,4-D (2,4-*Dichlorophenoxyacetic acid*), BAP (6-*Benzyl Amino Purine*) and $CaSO_4$ as an elicitor according to the treatments, sucrose at 30 g/L, pure agar at 8 g/L,. Other materials used included 1 N HCl, 1 N KOH, sterilized

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aquades, spiritus, pH paper, 96% and 70% alcohol, liquid detergent, Bayclin (NaOCl 5.25%), methanol, PTFE syringe filter (0.22 µm), C-18 column (250 x 4.6 mm, 5 µm), formic acid (0.2%), acetonitrile, catechin standard, *Epigallocatechin gallate* (EGCG) standard, *Epicatechin Gallate* (ECG) standard, glass plastic, tissue, rubber bands, label paper, HVS paper, black plastic, plastic wrap, and aluminum foil.

The tools used in this study were culture bottles (100 ml), beakers, tweezer, dropper pipette, spatula, scalpel, scissor, bunsen burner, Erlenmeyer flasks, Petri dishes, hand sprayer, analytical scale, hot plate, magnetic stirrer, autoclave, Laminar Air Flow Cabinet (LAFC), bucket, culture rack, matches, oven, mortar, porcelain pestle, 2 ml centrifuge tube, sonicator, centrifuge, vortex mixer, micropipette, 2 ml Eppendorf tube, 0.45 µm filter, volumetric flask, 1.5 ml vial, burette, test tube, test tube rack, Agilent HPLC LC 1220 Infinity II system, camera, stationery, and STAR software Version: 2.0.1.

The experiment was designed using a Completely Randomized Design (CRD) consisting of 1 factor which is concentration of the elicitor Ca^{2+} (0 g/L; 44 g/L; 88 g/L; 176 g/L). Each treatment level consisted of 5 replications, resulting in total of 20 experimental units. Each experimental unit consisted of 5 culture bottles leading to a total of 100 culture bottles and each culture bottle contained 1 explant that were all observed. The explants were incubated in a culture room under dark conditions at a temperature of 25-27°C.

The data obtained from the study were analyzed using the F-test at a significance level of 5%, and significantly different data were further analyzed using Duncan's New Multiple Range Test (DNMRT). The variables observed were the percentage of viable explants, callus color, callus structure, and catechin content in the callus.

III. Result and Discussion

A. Percentage of Viable Explants

The percentage of viable explants reflects the ability of gambir callus to survive under various concentrations of Ca^{2+} elicitors in the callus growth medium until the end of the observation period. Viable explants were characterized by living callus, absence of browning, and the emergence of meristematic cells. Statistical analysis revealed that the percentage of viable explants showed a significant difference at the 5% level. The percentage of viable explants is presented in Table 1.

TABLE I. Percentage of Viable Gambir Callus Explants at 4 Weeks Post-Treatment with Various Concentrations of Ca^{2+} Elicitors

Consentration of Ca^{2+} Elicitors (g/L)	**Percentage of Viable Explants (%)**
0	90.00 a
44	86.40 a
88	82.80 a
176	64.80 b
KK = 9,94%	

[a] Note: Numbers in the same column followed by the same lowercase letters are not significantly different based on Duncan's Multiple Range Test (DMRT) at a 5% significance level. The percentage of viable explants was transformed using Arcsin √x %.

Viable explants were identified by fresh, healthy callus with no signs of browning or cell death. Data in Table 1 indicate that the survival rate of gambir callus treated with Ca^{2+} elicitors at concentrations of 0–88 g/L exceeded 80%, while at a concentration of 176 g/L, the survival rate dropped to 64%. This suggests that higher concentrations of Ca^{2+} elicitors negatively affect callus survival, likely due to stress caused by excessive elicitor application and prolonged exposure to stress conditions in the medium. Examples of fresh gambir callus explants up to four weeks after elicitation for each treatment are shown in Figure 1.

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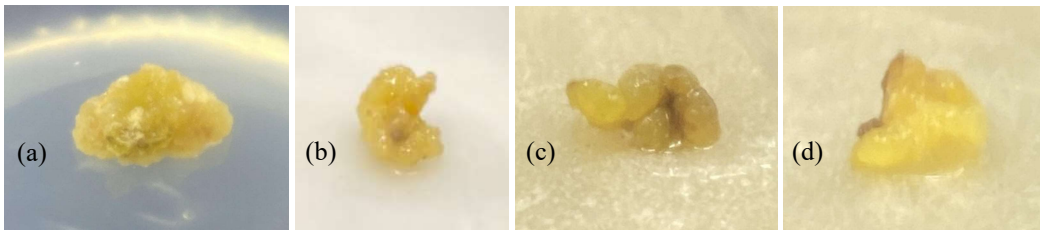

Fig. 1. Fresh gambir callus at 4 weeks post-elicitation with various concentrations of Ca^{2+} elicitors: (a) 0 g/L; (b) 44 g/L; (c) 88 g/L; (d) 176 g/L.

Increasing concentrations of Ca^{2+}, which is a metal ion, had a negative impact on the survival rate of gambir callus explants. The results indicate that higher concentrations of Ca^{2+} elicitors led to a decline in the percentage of viable explants. This finding aligns with [20], who stated that excessive metal ion stress—whether through high concentrations or simultaneous application with other metal ions—can inhibit callus development. Stress induced by excessive metal ions disrupts cellular metabolism and proliferation, thereby hindering callus growth.

The treatment with 176 g/L Ca^{2+} elicitors resulted in the lowest survival rate compared to other treatments. This was due to excessive application of Ca^{2+} ions beyond the tolerance capacity of the callus, leading to severe stress and subsequent stagnation or inhibition of growth, ultimately causing cell death. In general, callus responds to stress by activating defense mechanisms, such as producing phenolic compounds, which are often accompanied by browning [21], [22]. Excessive application of Ca^{2+} ions as metal elicitors can cause browning and even death in callus [22].

Observations revealed that gambir explants began showing reduced growth starting at two weeks after treatment (WAT) until the end of the observation period. This decline was marked by browning or stagnation in callus growth. Figure 2 illustrates examples of gambir callus experiencing browning.

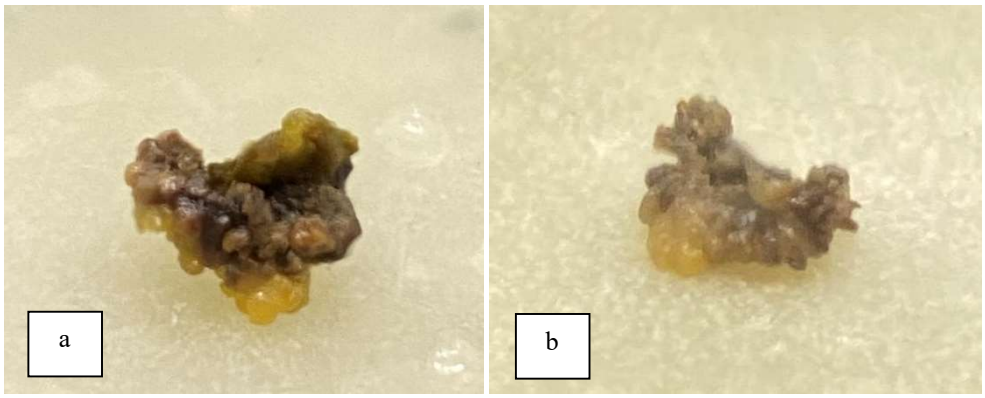

Fig. 2. Gambir callus exhibiting browning due to Ca^{2+} elicitor application: (a) gambir explant callus at 2 weeks after treatment (WST) showing initial browning at 176 g/L; (b) gambir explant callus at 4 WST under 176 g/L Ca^{2+} treatment.

Gambir callus explants exhibited signs of reduced viability through color changes from yellowish to brown and eventually black. Reference [11] noted that "browning" is a common phenomenon in woody plant explants. Under treatment with 176 g/L Ca^{2+} elicitors, gambir callus showed faster browning compared to other treatments due to stress exceeding its tolerance capacity. The defense mechanism involved producing phenolic compounds as secondary metabolites, which limited nutrient absorption by the callus. Reference [23], [24] explained that injured plants produce phenolic chemicals, and their accumulation in culture media restricts nutrient uptake by explants, leading to browning and eventual cell death. Reference [24], [25] further stated that enzymatic reactions involving oxygen result in browning due to oxidation processes within the callus.

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Reference [26] reported that browning is triggered by metabolic imbalances involving Reactive Oxygen Species (ROS), which activate peroxidase enzymes on lipid membranes, compromising membrane integrity and causing excessive accumulation of phenolic compounds. Changes in cell membrane permeability lead to enzyme and substrate release into the cytosol, initiating pigmentation or browning [27], [28].

B. Callus Color

Observations of the color of gambir callus explants treated with various concentrations of Ca^{2+} elicitors (0–176 g/L) revealed a range of colors, including green, yellowish-green, white, brownish-yellow, and brown. The initial callus color upon transfer to the treatment medium was yellowish-green. Over time, the callus darkened to a deep brown or even black, with young callus appearing as a compact yellowish color. Callus color can serve as an indicator of its quality; bright-colored callus indicates healthy growth, while dark-colored callus suggests poor growth or potential death [29].

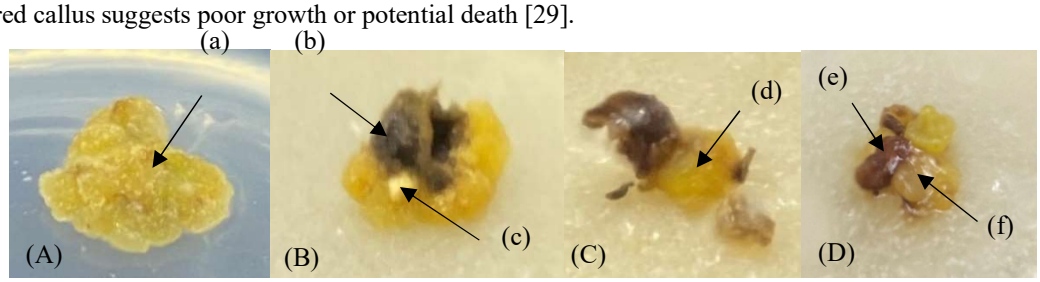

Fig. 3. Color of Gambir Callus at 4 Weeks Post-Elicitation: (a) yellowish-green; (b) dark brown; (c) white; (d) clear yellow; (e) brown; (f) brownish-yellow at various concentrations of Ca^{2+} elicitors: (A) 0 g/L; (B) 44 g/L; (C) 88 g/L; (D) 176 g/L

Initially, the callus was green and yellowish-green but later changed to brownish-yellow and eventually black. The final colors of the callus varied among treatments. The control group (0 g/L Ca^{2+}) produced green and yellowish-green callus, indicating good condition and active cell division with chlorophyll presence. In contrast, the 44 g/L treatment displayed yellowish-green and brownish-yellow colors with some browning areas. Treatments at 88 g/L and 176 g/L showed more extensive browning, resulting in brown and black (dead) areas due to phenolic compound metabolism. This color variation indicates changes in cell growth phases influenced by Ca^{2+} elicitor application, which acts as a stressor for callus growth. Reference [30] noted that using abiotic elicitors can exacerbate browning in callus.

Young cells typically yield yellowish callus; however, as they grow, they turn brown [24], [31]. Reference [32] indicated that brown explants have high phenolic content, influenced by elicitor application in the medium. Phenols react with light and oxidize into quinone compounds, resulting in browning [31].

The observed color changes in callus over time were likely due to its response to stress from Ca^{2+} elicitors. Results indicated that higher Ca^{2+} concentrations led to darker brown colors due to increased phenol accumulation from excessive ion absorption. Ca^{2+} assists polyphenol oxidase enzymes, promoting phenol conversion to quinones [33]. Darker callus colors suggest higher secondary metabolite biosynthesis activity [33], [34].

Darker callus generally indicates slower growth, aligning with [35], who stated that color can indicate growth status. This color change likely results from reduced chlorophyll (green pigment) due to Ca^{2+} elicitor application. Reference [20] explained that color changes are also influenced by growth media; stressed callus appears older than fresh callus. Thus, older callus in a given medium shows greater secondary metabolite biosynthesis activity [36].

Browning signifies the presence of phenolic compounds. Reference [37] noted that oxidized phenols form quinones, which trigger browning in cultured callus. The intensity of browning correlates positively with oxidative enzyme hyperactivity related to tissue defense against oxidative stress.

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Phenolic compound formation alters callus color [38]. Excessive phenol production followed by oxidation by oxidase enzymes and polymerization can harm the callus by inhibiting growth and triggering tissue death [39]. Callus browning is influenced by cell or tissue age and phenolic chemical production [40].

C. Callus Structure

Observations of the morphology of gambir callus, specifically its texture under various concentrations of Ca^{2+} elicitors, showed that the callus formed had a compact structure. The compact texture of gambir callus explants can be seen in Figure 4.

The results indicate that all treatments with Ca^{2+} elicitors produced callus with a compact texture. Compact callus is characterized by tightly packed and robust cells, making them difficult to separate [39]. This structure is influenced by media composition, plant hormones, and the type of plant used as the explant source [41]. Compact callus contains cells with larger vacuoles and thick polysaccharide cell walls [20].

The formation of compact callus is also affected by plant growth regulators in the initiation media. Reference [42] explained that compact callus texture is a response to cytokinin, which plays a role in nutrient transport. In this study, the induction media used contained 2,4-D (an auxin) and BAP (a cytokinin). Cytokinin facilitates the transport of water and nutrients through phloem vessels and alters osmotic potential within cells, causing cell walls to harden and resulting in compact callus texture [43].

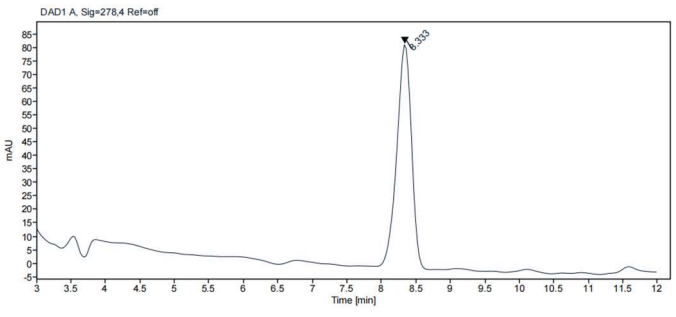
The addition of elicitors during the elicitation phase did not alter the compact structure of gambir callus. Compact callus structure also influences growth. Reference [44] noted that compact callus tends to have slower cell division compared to friable callus. [45] similarly stated that compact callus forms from actively dividing cells but later experiences reduced proliferative activity due to natural auxins present in explants.

Despite slower cell division, compact callus is preferred for secondary metabolite production. Reference [46] demonstrated that compact callus produces higher amounts of secondary metabolites than friable callus due to its dense cell structure, higher enzymatic activity, and more efficient metabolite accumulation.

D. Catechin Content in the Callus

Gambir is renowned for its catechin content, which serves as a valuable antioxidant compound utilized in industrial applications. To determine the catechin levels in gambir callus cultured with different concentrations of Ca^{2+} elicitors, catechin content was analyzed using HPLC. The results are presented in Tables 2 and 3.

TABLE II. CHROMATOGRAM OF GAMBIR CALLUS USING THE HPLC TESTING METHOD

Treatments	**Figure**
Catechin standard	

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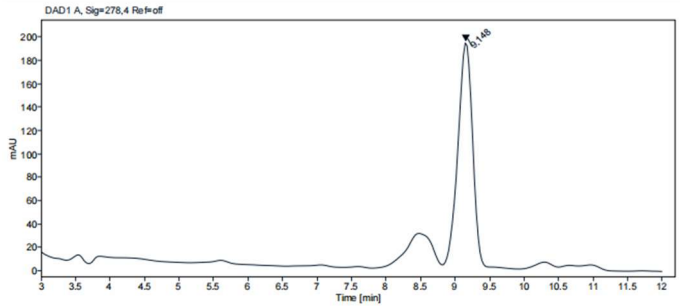
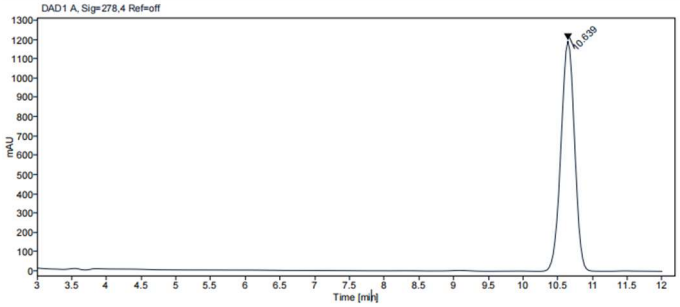
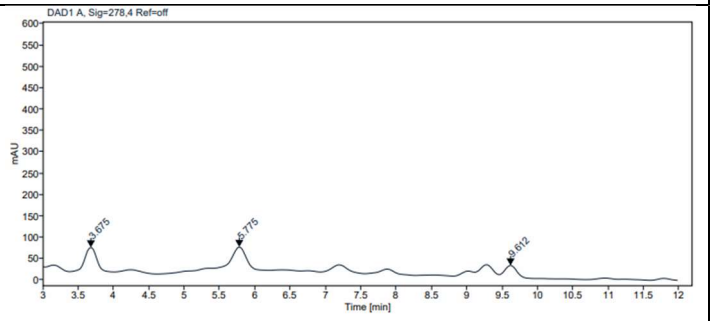
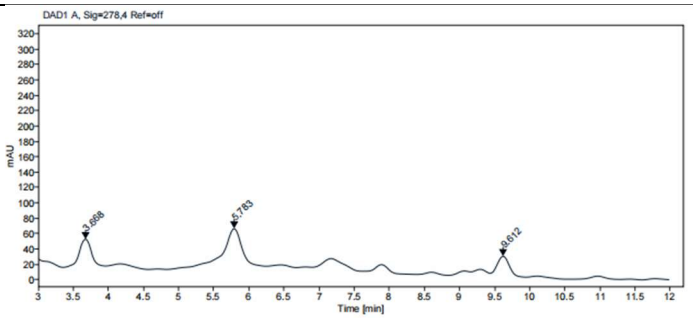
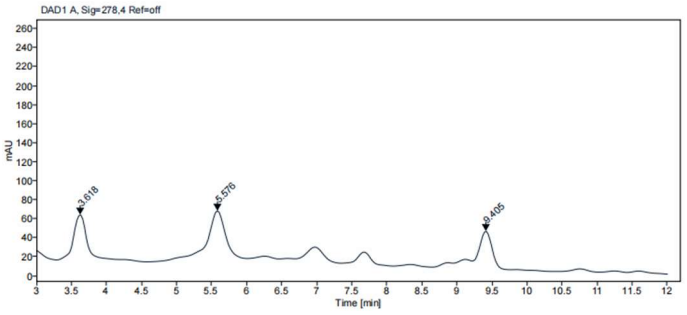
Epigallocatechin gallate (EGCG) standard	
Epicatechin Gallate (ECG) standard	
0 g/L Ca^{2+}	
44 g/L Ca^{2+}	
88 g/L Ca^{2+}	

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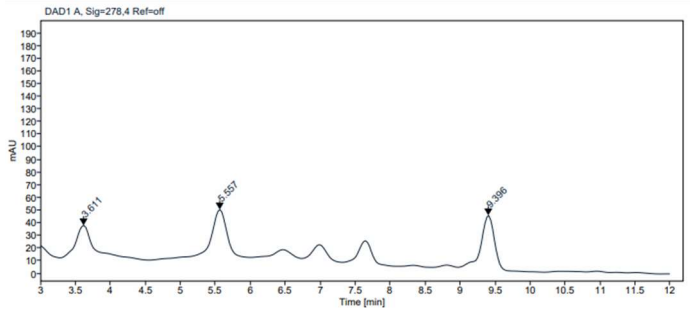
176 g/L Ca^{2+}	

TABLE III. RESULTS OF CATECHIN ANALYSIS IN GAMBIR CALLUS USING THE HPLC METHOD

Treatments	RT (min)	Peaks Height (mAU)	Area
Catechin standard	8,333	82,716	1194,046
Epigallocatechin gallate (EGCG) standard	9,148	189,957	2722,220
Epicatechin Gallate (ECG) standard	10,639	1197,268	16167,328
0 g/L Ca^{2+}	3,675	56,170	387,821
	5,775	50,888	632,316
	9,612	22,117	190,851
44 g/L Ca^{2+}	3,668	34,731	626,161
	5,783	44,960	660,327
	9,612	20,007	214,348
88 g/L Ca^{2+}	3,618	46,922	575,098
	5,576	49,517	799,250
	9,405	35,248	391,357
176 g/L Ca^{2+}	3,611	24,091	337,531
	5,557	37,472	542,166
	9,396	39,511	448,083

According to these tables, all treatments revealed the presence of unidentified compounds in the chromatograms with average retention times of 3.6 minutes, 5.6 minutes, and 9.6 minutes. These retention times differ from those of the three catechin standards, which appeared at 8.3, 9.1, and 10.6 minutes, indicating that the compounds detected in the gambir callus are not catechins present in the standards used. This suggests that several secondary metabolite compounds emerged in the callus that could not be identified by the catechin standards, potentially representing other catechin derivatives. The analysis also included standards for catechin derivatives, namely Epigallocatechin gallate (EGCG) and Epicatechin gallate (ECG), which appeared at retention times of 9.1 and 10.6 minutes, respectively, differing from the compounds found in gambir callus.

The findings contrast with those of [4], who reported a catechin content of 98.75% in gambir leaves based on HPLC analysis. This discrepancy arises from the sample type used; this study utilized callus samples composed of undifferentiated young cells, resulting in simpler compounds being produced. [47] identified eight types of catechins using UHPLC in tea (*Camellia sinensis*), including Gallocatechin (GC), Epigallocatechin (EGC), Catechin (C), Epigallocatechin gallate (EGCG), Gallocatechin gallate (GCG), Epicatechin gallate (ECG), Catechin gallate (CG), and Epicatechin (EC). Based on this information, it is suspected that the compounds appearing at retention times of 3.6, 5.6, and 9.6 minutes may correspond to Gallocatechin (GC), Epigallocatechin (EGC),

and Gallocatechin gallate (GCG). However, identification of these compounds remains unconfirmed due to a lack of pure catechin standards during the study.

Catechins are a class of flavonoids commonly found in gambir. The study indicated that flavonoid activity was present in gambir callus, evidenced by color changes to brown due to secondary metabolite biosynthesis activity [48]. However, complex catechins were not detected in the gambir callus; instead, only catechin derivatives were present due to the nature of callus cells—undifferentiated and actively dividing [49]. Consequently, their ability to produce secondary metabolites remains limited.

The peak area observed in HPLC represents the vertical distance between the baseline of the chromatogram and the highest point of a specific compound's peak, reflecting its relative abundance in the sample. While peak area can estimate compound concentration, it is not always reliable due to factors such as injection volume and detector sensitivity; thus, further quantitative analysis is needed for a comprehensive representation of compound quantities.

The highest peak area observed at 88 g/L Ca^{2+} indirectly indicates relative abundance within the sample; however, identification remains pending due to standard stock limitations. The presence of this prominent peak may result from stress management within the callus leading to secondary metabolite production like flavonoids. At 88 g/L Ca^{2+}, callus stress management reached an optimal level; however, at higher concentrations like 176 g/L, callus growth declined rapidly due to excessive browning and cell death by harvest time.

Additionally, harvesting time influences catechin availability within callus; at 88 g/L with a harvesting time of four weeks post-treatment (WSP), optimal conditions for harvesting were achieved. In contrast, at 176 g/L after four WSPs, callus quality deteriorated due to accelerated browning compared to other treatments, resulting in severe browning and cell death [20].

IV. Conclusion

The application of Ca^{2+} elicitors has not been able to induce catechin compounds in gambir callus. Additionally, several metabolite compounds appeared in the callus that could not be identified by the standard catechins used. However, the treatment with 88 g/L resulted in a higher availability of metabolite compounds compared to other treatments, as indicated by the total peak area generated from HPLC testing, which is suspected to include other catechin derivative compounds.

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