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Growth characteristic and bioactive compound synthesis in aromatic ginger (*Kaempferia galanga* Linn) callus under ultraviolet B exposure and sucrose supplementation

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Abstract The results showed that sucrose concentration affected most growth parameters and phytochemicals formed. The fresh and dry callus weight increased with increasing sucrose concentration in the media. Growth decreased with increasing sucrose concentration above the optimum concentration (30 $g.L^{-1}$). Growth parameters, including fresh callus weight and dry callus weight of K. galanga, were affected by the auxin type and UV-B radiation. Contrary to the observed growth parameters, the increasing sucrose treatment caused a 0.05-0.39 times decreased in total phenol content, 0.1-0.75 times total flavonoid content, 0.05-0.6 times EPMC content, and antioxidant activity 0.21-0.45 times compared to that without sucrose. UV-B irradiation increased the total phenolic content (TPC) and total flavonoid content (TFC) of the callus by 1.13 and 1.7 times, respectively, compared to untreated samples. Phytochemical outcomes, such as total phenols, flavonoids, EPMC levels, antioxidant activity, and PAL enzyme activity, were influenced by auxin and sucrose concentrations. While UV-B treatment boosted total phenolics, flavonoids, and antioxidant capacity in *K. galanga* callus, it did not significantly alter EPMC formation. The combined effects of sucrose concentration and UV-B irradiation had little impact on PAL enzyme activity.

Keywords: Auxin, Sucrose, UV-B radiation, Callus growth, Phenol compounds, Callus culture

Introduction

The aromatic ginger plant, known scientifically as *Kaemferia galanga* Linn, is a medicinal plant that is widely utilized for both culinary spices and medicinal purposes. The essential oil extracted from the *K. galanga* plant is beneficial for enhancing the body's immune response and preventing and alleviating colds (Setyawan *et al*., 2013). The rhizome of *K. galanga* is known to contain essential

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oils that include α-pinene, benzene, borneol, camphene, carvone, eucalyptol, pentadecane, methylcinnamate, and ethyl p-methoxycinnamate (Tewtrakul *et al*., 2005). Additionally, it contains polyphenols, quinones, triterpenoids, saponins, tannins, and flavonoids (Nurmeilis *et al*., 2016). Among the primary components of the essential oil from *K. galanga* rhizome are ethyl cinnamate and ethyl p-methoxy cinnamate (EPMC), both of which are derivatives of cinnamic acid and exhibit various biological activities (Raina and Abraham, 2015).

Cultures of medicinal plants and species with numbers in nature reduced to a minimum were developed tweenoted. Advantages of tissue culture and promotion of biotechnology in medicinal plants. These include the clonal propagation of superior seeds, reassembly of improved cultivars by somaclonal variation, in vitro selection for biotic and abiotic stress resistance, germplasm exchange and secondary metabolite production (Kolakar *et al*., 2018). Furthermore, plant tissue culture is able to select cells with high levels of secondary metabolites and develop the suitable media formulations for interactive effects on these compounds production (Karuppusamy, 2009).

The effectiveness of plant tissue culture techniques relies heavily on factors such as carbon sources, plant growth regulators (PGRs), culture environment, light conditions, genotype, type of explant, and other variables (Mehbub *et al*., 2022). Using plant growth regulators affects the morphological characteristics of callus formed in *K. galanga*. Previous studies showed that treatment of different types of auxin (NAA and 2,4-D) showed different callus morphological characteristics. Treatment of 2,4-D auxin formed a friable textured callus with a brownish-white color. In contrast, the treatment of NAA auxin formed a compact and greenish callus due to the cell differentiation process in the callus tissue (Shofiyani *et al*., 2023).

When sugar is employed as a carbon source, it has the potential to modulate metabolism, growth, and development, as well as gene expression in adventitious roots (Erin *et al*., 2022). For in vitro culture growth, most plant explants are not autotrophic and exhibit only low photosynthetic activity; therefore, sucrose is an essential ingredient in the Murashige and Skoog (MS) medium, providing a carbon source and energy. Research by Sitorus *et al*. (2011) showed the best callus wet weight and early initiation time in *Basella rubra* by adding 40 g/L of sucrose to the MS medium. Also, it was reported that fresh weight and dry weight of calluses produced from *Zingiber officinale* Rosc were increased by increasing the sucrose concentration in the medium up to 20–50 $g.L^{-1}$ (Kherasani *et al*., 2017).

Studies have shown that the in vitro application of biotic and abiotic stress treatments can increase the production of bioactive molecules. UV-C irradiation at different doses enhanced both the production of phenolic compounds and

biomass growth of *F. indica* callus cultures. In fact, 5.4 kJ. m^{-2} of UV-C radiation was applied to cultures that deviated directly at least twice when compared to those of the non-UV-treated cultures) for 30 min were found to be rich in phytochemicals and biomass (Hashim *et al*., 2021). Similarly, Gu *et al*. (2010) reported a method to stimulate the production of bioactive secondary metabolites in mulberry (*Morus alba* L.) leaves through in vitro UV-B irradiation. HPLC fingerprinting revealed five chromatographic peaks after induction, which identified two active principles: chalcomoracin, a natural Diels-Alder adduct with antibacterial activity, and moracin N, a precursor of chalcomoracin. *Lettuce* proline level and PAL activity were also higher with enhanced UV-B (Rajabbeigi *et al*., 2013).

The objective was to examine the impact of ultraviolet B exposure and different sucrose concentrations on the growth characteristics and phenolic bioactive compound production in *K. galanga* callus. This research aimed to optimize callus culture methods to produce bioactive phenolic compounds.

Materials and methods

This research was conducted in 2022–2023 at the Basic Agrotechnology Laboratory and Plant Tissue Culture Laboratory, Faculty of Agriculture and Fisheries, Integrated Analytical Chemistry Laboratory, Universitas Muhammadiyah Purwokerto, and the Research Laboratory, Faculty of Medicine, Jenderal Soedirman University.

Planting materials

Multiplication callus multiplied on MS supported with 30 g. L^{-1} sucrose and 8 g. L^{-1} agar. For the production of a friable callus 1.5 mg. L^{-1} of 2,4-D and 0.1 mg. L^{-1} of BAP were added. To produce a compact callus, 2 mg. L^{-1} of NAA and 0.5 mg. L^{-1} of BAP were incorporated. The subculture was repeated three times for callus proliferation.

Media treatment with sucrose modification

Our experimental setup involved using MS media with modified sucrose concentrations as a callus planting medium. We used compact textured callus and friable textured callus aged four weeks as materials and treated them with sucrose concentrations at five levels: S0 (0 g.L^{-1}), S1 (15 g.L^{-1}), S2 (30 g.L^{-1}), S3 (45 g.L^{-1}), and S4 (60 g.L^{-1}). A 0.5 g portion of *K galanga* callus cells was used as an inoculum and planted into 30 ml of MS medium, modified with various

sucrose concentrations based on the treatment. The medium also includes 8 g/L agar and growth regulators, either 2,4-D or NAA, in combination with BAP.

Ultraviolet B radiation treatment

An Exoterra TL 150 UV-B lamp (18 watts) served as the UV-B radiation source, positioned 20–30 cm from the callus, with adjustments made to achieve the desired intensity for the study. Four-week-old callus samples were treated with UV-B radiation (either exposed or unexposed). For compact callus, UV-B radiation was applied at 140 $\mu W.cm^{-2}$ for 4 hours, while friable callus was exposed at 70 $\mu W.cm^{-2}$ for 2 hours. All samples were subjected to UV-B exposure over a two-week incubation period. Those not receiving UV-B radiation were maintained under a standard 16/8-hour light/dark photoperiod. For all treatments, additional lighting was provided by a white fluorescent tube lamp (TL-D 18 W, Philips Electric®) for 16 hours daily, with a light intensity of approximately 1300±50 lux and incubation conditions set at 25±2 °C. All callus samples, both UV-B treated and untreated were harvested at the end of the six-week incubation period.

Research design of ultraviolet B radiation treatment and sucrose concentration

The study was employed using a Completely Randomized Design (CRD) in a factorial setup with two main treatment factors: ultraviolet B radiation and sucrose concentration. The first factor, UV-B radiation, included treatments with and without exposure. The second factor involved varying sucrose concentrations from 0 to 60 $g.L^{-1}$. This design combined UV-B radiation and sucrose concentration treatments on compact callus (using NAA auxin) and friable callus (using 2,4-D auxin).

Growth parameters

The growth parameters evaluated in this study: callus morphology was determined by identifying the type of callus obtained as friable or compact; callus color was determined using a scale reference for color in "Royal Horticultural Society Color Charts Edition V"; Callus dry weight: it was the dry weight of the callus and measured by oven drying of fresh samples at 60 °C, which was maintained until constant weight was reached to obtain accurate dry weight for phytochemical study. Cross-sections of the callus were also studied under the light microscope at 100x and 400x, and images were taken with an Olympus CX 23 microscope coupled with OM-20 camera.

Phytochemical analysis of callus

Sample preparation

The samples analyzed were 6-week-old *Kaempferia galanga* rhizome calluses treated with UV-B radiation per the experiment's protocol. A modified approach from Subedi *et al*., (2014) was used for sample preparation. The extract was subsequently used for phytochemical testing, which included assays for total phenol and flavonoid content, antioxidant activity using DPPH, and measured spectrophotometrically. Additionally, the EPMC content was analyzed using HPLC.

Determination of total phenol content (TPC)

The total phenol was assessed using Folin-Ciocalteu reagents, adapting the Singleton and Rossi, (1965) method with minor adjustments as per Shofiyani *et al*. (2023). Phenol content was calculated as gallic acid equivalents (GAE g^{-1}) using a standard curve of gallic acid (20–100 mg L^{-1}).

Determination of total flavonoid content (TFC)

The total flavonoid content in the ethanol extract of callus was measured using the aluminum chloride colorimetric method, following the protocol by Chandra *et al*., (2014). Quercetin served as the standard to establish a calibration curve. Flavonoid content was calculated as mg quercetin equivalent (QE.g^{-1}) of dry callus material, with all measurements taken in triplicate.

Determination of ethyl p-methoxycinnamate (EPMC) using HPLC

Test EPMC (ethyl para-methoxycinnamate) content using the Liquid Chromatography method with HPLC Shimadzu®. The instrument was optimized for wavelength 275 nm, pump pressure 4000 psi, and retention time 11.5 minutes with a mobile phase of 1% methanol acetate in water with a ratio of 70:30 with an elution rate of 1ml/minute. Expressed as mg EPMC.g^{-1} dry callus material, and all measurements were done in triplicate.

Analysis of antioxidant activity by DPPH method

The DPPH radical scavenging activity of the callus ethanol extract was determined by adding 100 µL of the sample to 200 µL of a 0.1 mM solution of DPPH. This assay was carried out in a 96-well microplate and further incubated in the dark at room temperature for 30 min. The absorbance was measured at 515 nm (Sahoo *et al*., 2014). All assays were conducted in triplicate for reliability.

Measurement of supernatant protein content

The (Lowry *et al*., 1951) method measured supernatant protein content. Bovine Serum Albumin (BSA) standards have various concentration levels from 100-1000 $\mu g.ml^{-1}$. The results were obtained with the equation $Y = 0.3465x + 0.1641$, $R^2 = 0.994$ where Y is the absorbance value. Measurements were carried out in triplicate.

Measurement of PAL enzyme activity

Measurement of PAL enzyme activity using the Zucker (1968) Method (Shofiyani *et al*., 2023). PAL enzyme activity is expressed as units (U) (U = µg of trans-cinnamic acid formed per minute per ml of extract). The specific activity of the PAL enzyme was calculated by dividing the enzyme activity value per mg of protein (unit.mg^{-1} protein). Measurements were carried out in triplicate.

Statistical analysis

Recorded data were analysed using the Costat software set, 6.400. Data that was distributed normally and homogeneous were analyzed by ANOVA. If data did not satisfy these assumptions, Kruskal-Wallis test was performed instead. Test of treatment effects was determined by the Least Significance Difference (LSD) test at 95% confidence level when F test indicated significant differences.

Results

The sucrose concentration treatment was significantly affected the callus growth parameters and the content of bioactive compounds in the callus. However, PAL enzyme activity was not affected. Meanwhile, the UV-B irradiation treatment only affected the compact callus's total phenol content (TPC). In this study, the UV-B irradiation treatment and sucrose concentration affected dry callus weight and total flavonoid content (TFC) observation parameters in both friable and compact callus.

Effect of ultraviolet B irradiation and various sucrose concentrations on K. galanga callus growth

Fresh weight callus

The types of callus showed varying responses to UV-B radiation and sucrose treatments. Sucrose treatment was significantly affected the fresh weight

of compact callus types, with the 30 g.L^{-1} sucrose treatment (S2) resulting in the highest callus weight which was not significantly different from 15 g.L^{-1} sucrose treatment (S1), which was 7.39±1.67 and 7.36±0.5 g respectively, and the lowest fresh weight callus was found in 0 g.L^{-1} sucrose treatment (S0) which was 0.87±0.02 g (Figure 1 (A)).

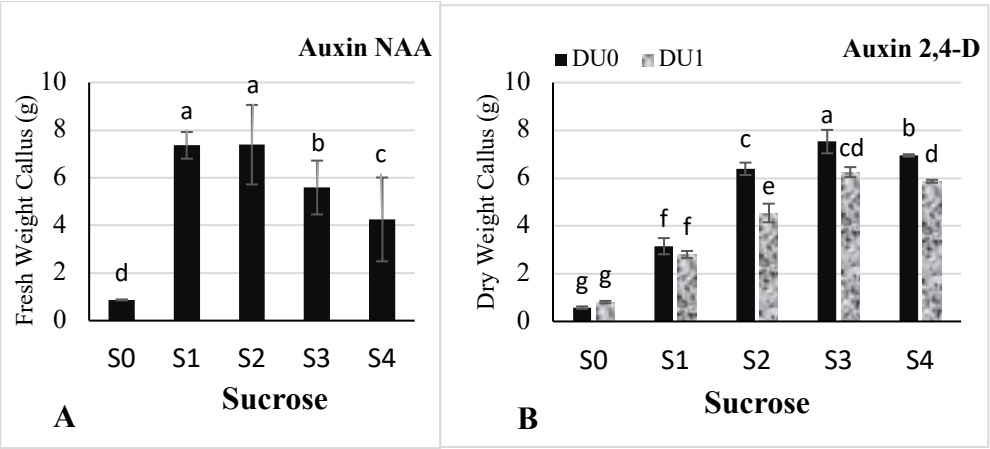

Figure 1. Average fresh weight callus (g) with sucrose treatment on compact callus (A), with sucrose and UV-B radiation treatment on friable callus (B): In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

UV-B radiation treatment and sucrose concentration interacted with the formation of fresh weight callus in friable callus, where the combination of treatment without UV-B radiation and sucrose 45 g.L^{-1} (DU0S3) was 7.53±0.49 g, which was significantly different from all treatment combinations. While the combination of treatment without UV-B and sucrose 0 g.L^{-1} (DU0S0) and the combination of UV-B and sucrose 0 g.L^{-1} (DU1S0) gave the lowest fresh callus weight, namely 0.58±0.05 and 0.81±0.05 g, respectively (Figure 1 (B)).

Dry weight callus

UV-B irradiation and sucrose concentration treatments were significantly affected the dry callus weight of fragile and compact calli. In compact textured callus, the treatment that gave the highest dry callus weight was without UV-B irradiation and 60 g.L^{-1} sucrose (NU0S4), which was 0.69±0.07 g, significantly different from all treatments. Meanwhile, the lowest dry callus weight was found in the treatment without UV-B irradiation and 0 g.L^{-1} sucrose (NU0S0) and the

treatment of UV-B irradiation and 0 g.L^{-1} sucrose (NU0S0) which were 0.05±0.02 and 0.05±0.01 g, respectively (Figure 2 (A)).

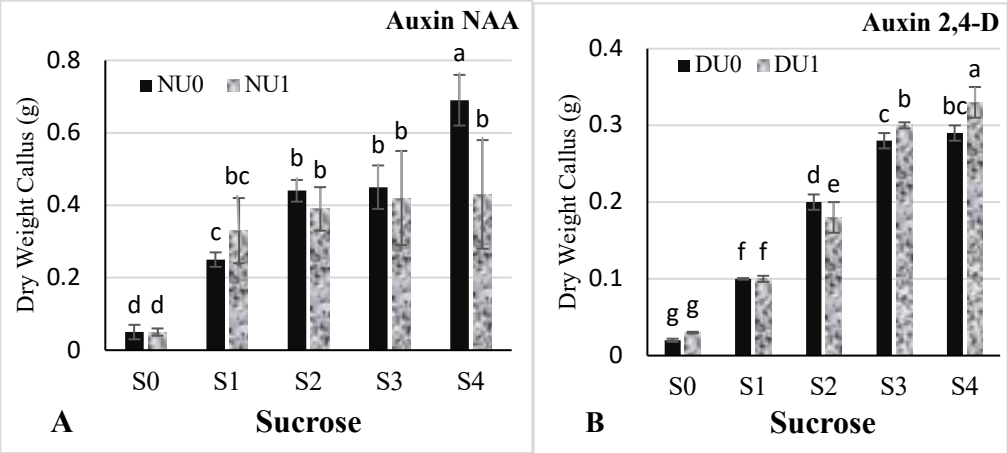

Figure 2. Average dry weight callus (g) of *K. galanga* with UV-B radiation and sucrose treatment on compact callus (A) and friable callus (B): In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

The highest average dry callus weight in friable callus was found in UV-B irradiation and 60 g.L^{-1} sucrose, which was 0.33±0.02 g, significantly different from all treatments. Meanwhile, the lowest dry weight of callus was found in the treatment without UV-B irradiation and 0 g.L^{-1} sucrose (DU0S0) and UV-B irradiation and 0 g.L^{-1} sucrose (DU0S0), which were 0.02±0.002 and 0.03±0.001 g, respectively (Figure 2 (B)).

Callus morphology

The morphology of the callus formed was more dominantly influenced by the treatments of different auxin growth regulators (NAA and 2,4-D) in this study (Figure 3). In treating the NAA auxin type, the callus formed had a compact texture with a green to brownish-green color. During the treatment of 2,4-D auxin type, the callus formed had a friable texture with a brownish-white color. Organogenesis occurred in compact callus, where shoots began forming in all sucrose and UV-B radiation treatment combinations. Specifically, roots began forming on the callus in treatment with UV-B radiation at a sucrose concentration of 45 g.L^{-1} Callus with 2,4-D treatment generally formed a brownish white to brown color with a friable texture (Figure 3).

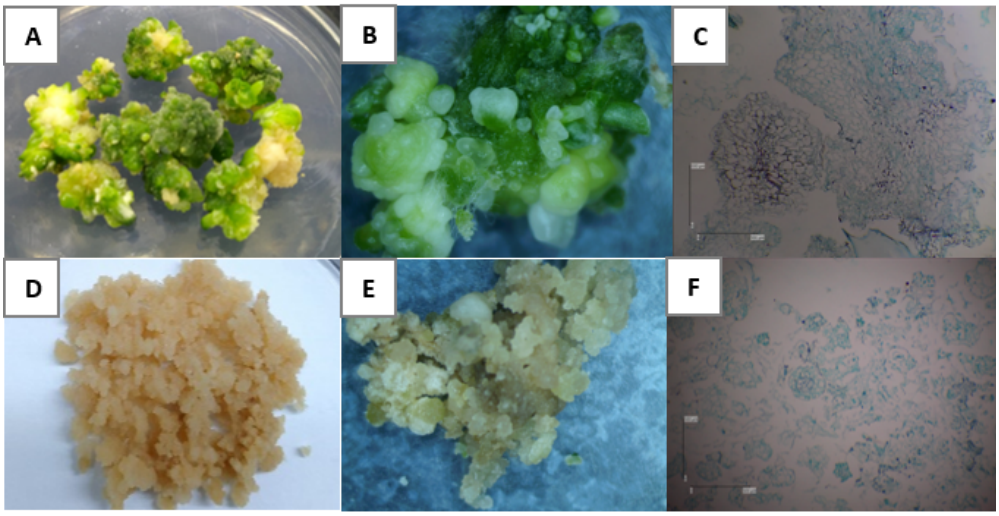

Figure 3. Compact callus texture in NAA treatments (A, B, and C) and friable callus texture in 2,4-D treatments (D, E, and F). Images C and F are cross sections of callus tissue

Effect of ultraviolet B and sucrose concentration on secondary metabolites of K. galanga callus (TPC, TFC, EPMC), antioxidant ctivity and PAL enzyme activity

Total phenol content (mg GAE.g^{-1} dry weight of callus)

The results showed that in compact callus, sucrose treatment significantly affected the total phenol content/TPC parameters of ethanol extract of galanga callus in this study. The sucrose treatment gave the highest average TPC was the 15 g.L^{-1} sucrose treatment (S1), which was 1.74 ± 0.32 mg GAE.g^{-1} DW callus, was not significantly different from the treatment without sucrose (S0), which was 1.52 ± 0.16 mg GAE.g^{-1} DW callus and different from other sucrose treatments. The sucrose concentration treatment with the lowest TPC was the 60 g.L^{-1} sucrose treatment (S4), which was1.25±0.08 mg GAE.g^{-1} callus DW (Figure 4).

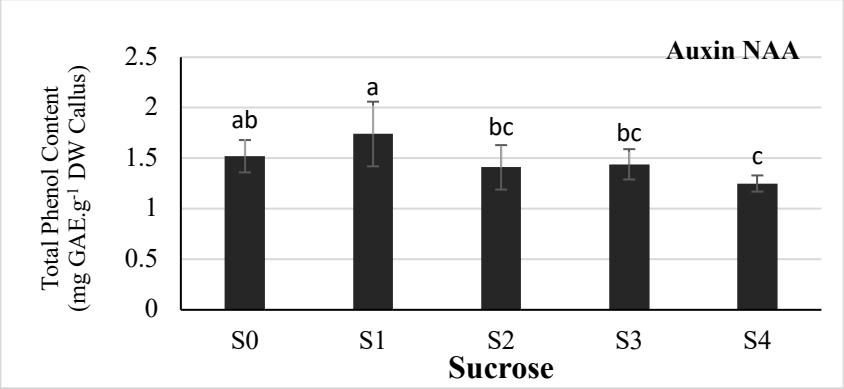

Figure 4. Average total phenol content (mg GAE.g^{-1} DW callus) of ethanol extract of *K. galanga* with sucrose treatment on compact callus: In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

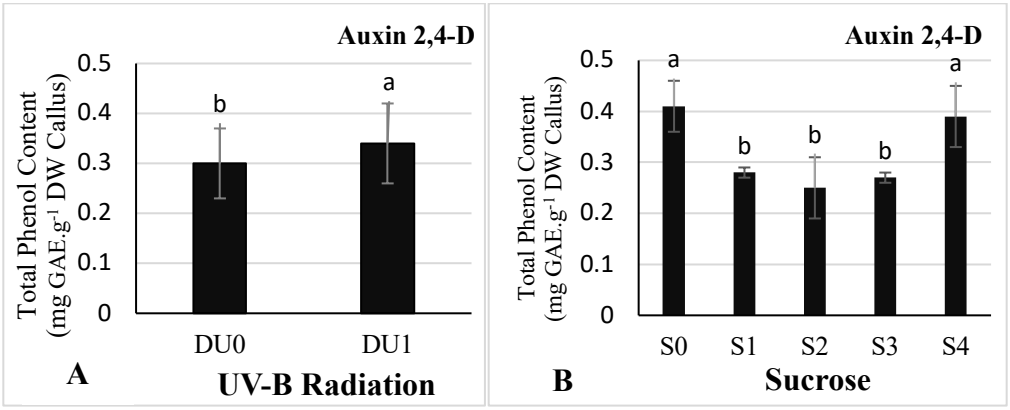

Figure 5. Average total phenol content (mg GAE.g^{-1} DW callus) of ethanol extract of *K. galanga* with UV-B radiation treatment (A) and sucrose (B) in friable callus: In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

In the friable callus, it was shown that UV-B radiation treatment and sucrose concentration individually had a significant effect on the average TPC of the ethanol extract of *K. galanga* callus (Figure 4). UV-B radiation treatment gave an average TPC of 0.34±0.08 mg GAE.g^{-1} callus DW, which was significantly different from the treatment without UV-B radiation, which was 0.3±0.07 mg GAE.g^{-1} callus DW (Figure 5 (A)). Meanwhile, the sucrose treatment that gave the highest average TPC was the treatment without sucrose (S0) and sucrose concentration of 60 g.L^{-1} (S4), which were 0.41±0.05 and 0.39±0.06 mg GAE.g^{-}

^{1} callus DW, respectively. Significantly different from the sucrose concentration treatments of 15 g.L^{-1} (S1), 30 g.L^{-1} (S2), and 45 g.L^{-1} (S3) respectively by 0.28±0.01; 0.25±0.06 and 0.27±0.01 mg GAE.g^{-1} DW callus (Figure 5 (B)).

Total flavonoid content (mgQE.g-^{1} dry weight of callus)

Regarding the total flavonoid content/TFC parameter, ultraviolet B radiation treatment, and various sucrose concentrations individually was significantly affected the TFC of ethanol extract of *K. galanga* callus both in compact callus and friable callus in this study (Figure 6 and Figure 7).

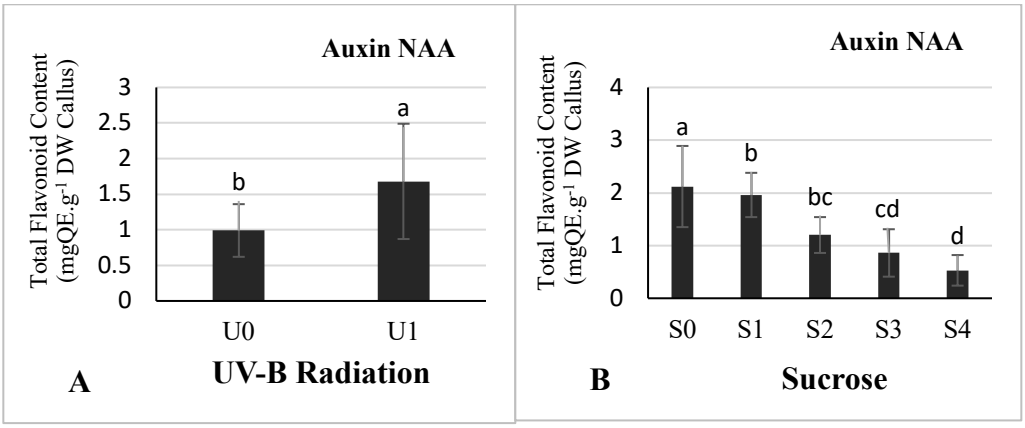

Figure 6. Average total flavonoid content (mg GAE.g^{-1} DW callus) of ethanol extract of *K. galanga* with UV-B radiation (A) and sucrose (B) treatment in compact callus: In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

UV-B radiation treatment tended to form higher flavonoids than without UV-B radiation treatment. UV-B radiation treatment on compact callus showed a TFC of 1.68±0.81 mg QE.g^{-1} dry weight of callus, which was significantly different from the treatment without UV-B radiation, which was 0.99±0.37 mg QE.g^{-1} dry weight of callus (Figure 6 (A)).

Meanwhile, the sucrose treatment gave the highest average TFC was the treatment without sucrose (S0), which was 2.12±0.77 mg QE.g^{-1} dry weight of callus, significantly different from other sucrose concentration treatments. Meanwhile, the lowest average TFC was found in the sucrose concentration treatment of 60 g.L^{-1} (S4), which was 0.53±0.29 mg QE.g^{-1} dry weight of callus. There was a tendency that the higher the sucrose concentration given, the lower the average TFC (Figure 6 (B)).

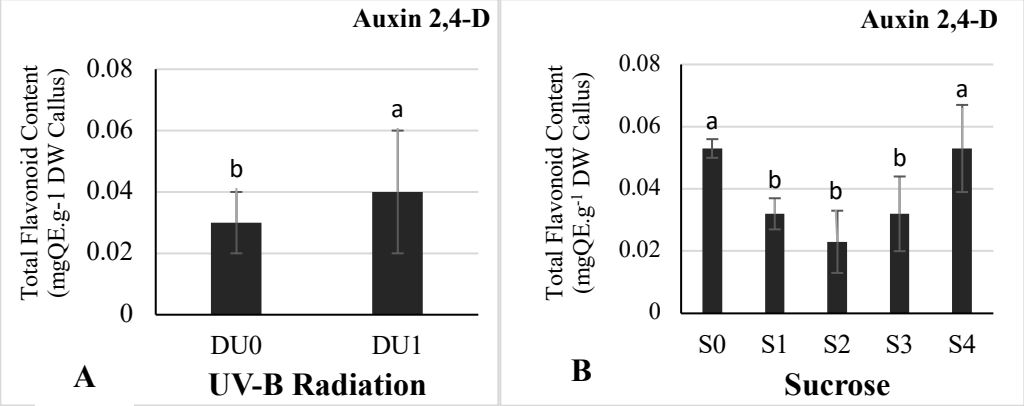

Figure 7. Average total flavonoid content (mg GAE.g-¹ DW callus) of ethanol extract of *K. galanga* with UV-B radiation treatment (A) and sucrose (B) in friable callus: In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

Formation of total flavonoids was considerably influenced by the sucrose concentration in combination with UV-B radiation treatment in ethanol extracted from *K. galanga* friable callus. Also, the amount of TFC formed in UV-B-radiated than nonradiated samples was significantly increased. The TFC of UV-B radiation treatment was 0.04±0.02 mg QE. g^{-1}.DW) of callus which was significantly different from control without UV-B (0.03±0.01 mg QE g^{-1}.DW). g^{-1} dry weight content in callus (Figure 7 (A)). On the other hand, control treatment with sucrose concentration of 60 and 0 $g.L^{-1}$ (S0) gave the highest TFC, which were 0.053±0.003 and 0.053±0.014 mg QE/g dry weight of callus, respectively. Statistical and significantly different to the treatments of sucrose concentration of 15$g.L^{-1}$ (S1), 30$g.L^{-1}$ (S2) and 45 $g.L^{-1}$ (S3) of 0.032±0.005; 0.023±0.01 and 0.032±0.012 mg QE.g^{-1} DW callus, respectively (Figure 7 (B)).

Ethyl para-methoxycinnamate content (mg.g-¹ dry weight of callus)

The sucrose concentration was significantly affected the ethyl para-methoxycinnamate (EPMC) content of ethanol extract of *K. galanga* callus with compact texture. The sucrose concentration treatment that produced the highest EPMC was the treatment without sucrose, which was not significantly different from the sucrose concentration treatment of 45 $g.L^{-1}$, namely 0.57±0.23 and 0.54±0.33 $mg.g^{-1}$ dry weight of callus, respectively. The sucrose concentration treatment that produced the lowest EPMC content was in the sucrose concentration of 60 $g.L^{-1}$, namely 0.2±0.09 $mg.g^{-1}$ dry weight of callus (Figure 8 (A)).

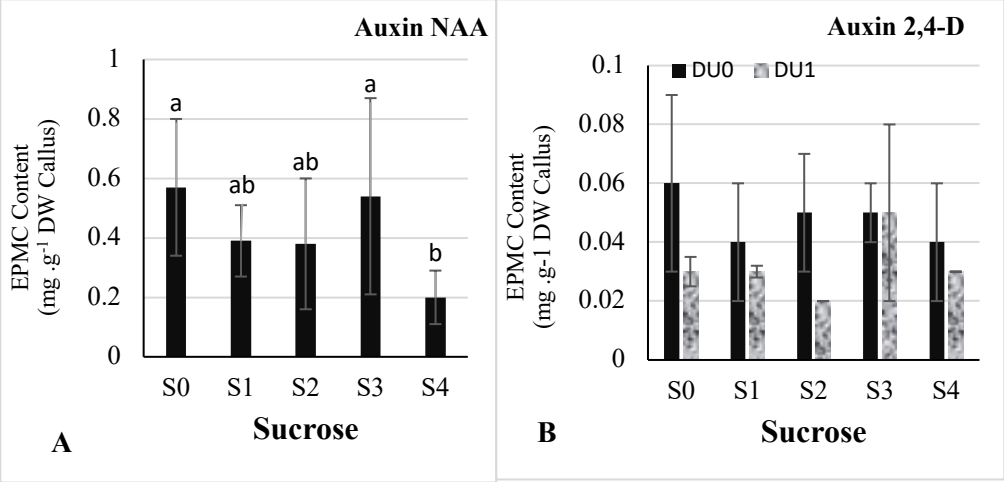

Figure 8. Mean total EPMC content (mg.g^{-1} DW callus) of ethanol extract of *K. galanga* with sucrose treatment on compact callus (A) and UV-B radiation and sucrose treatment on friable callus (B): The bar chart's mean value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

In friable calluses, UV-B radiation treatment and sucrose concentration was significantly affected and there was no interaction between the two treatments on the EPMC content of ethanol extract of *K. galanga* callus. However, there was a tendency for EPMC content in the treatment without UV-B radiation and sucrose to give a high amount, which was 0.06±0.03 mg.g^{-1} dry weight of callus, while the UV-B radiation treatment with 45 g.L^{-1} sucrose was 0.05±0.03 mg.g^{-1} dry weight of callus (Figure 8 (B)).

Antioxidant activity of callus (%)

The DPPH assay was used for determining antioxidant activity. The findings revealed that the antioxidant activity of ethanol extract from compact and friable textured *K. galanga* callus was significantly influenced by sucrose treatment. For non-compact calli, the highest mean of antioxidant activity obtained was that from no added sucrose treatment (S0) with 76.62±4.05%, statistically different from the other concentrations of sucrose tested. The lowest mean antioxidant activity was observed for the treatment with 60 g.L^{-1} sucrose concentration (S4), being 41.93±16.24%. There was a trend of the average antioxidant activity being inversely proportional to the increased concentration of sucrose treatments (Figure 9 (A)).

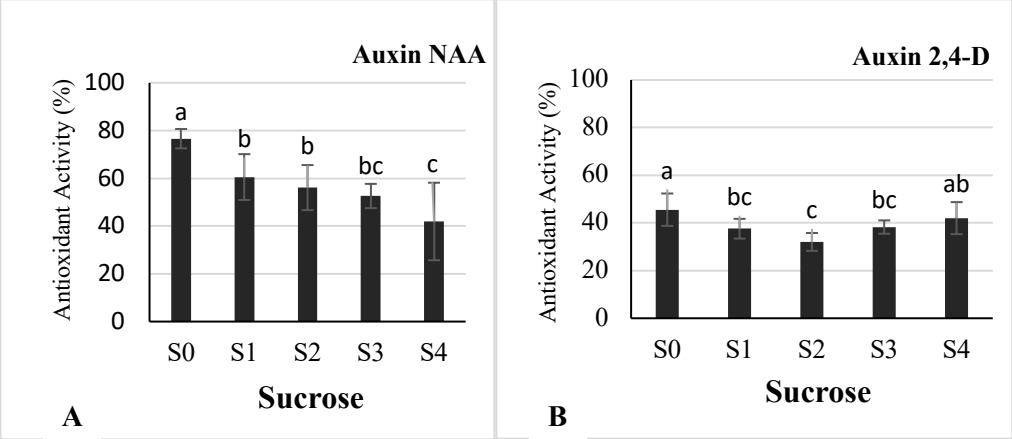

Figure 9. Average antioxidant activity (%) of ethanol extract of *K. galanga* with sucrose treatment on compact callus (A) and friable (B): In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

Meanwhile, in friable callus, the sucrose treatment that showed the highest average antioxidant activity was the treatment without sucrose (S0), which was 45.56±6.8%, which was not significantly different from the treatment with a sucrose concentration of 60 $g.L^{-1}$ (S4) which was 42.02±6.72%. The lowest average antioxidant activity was with a sucrose concentration of 30 $g.L^{-1}$ (S2), which was 32.04±3.75% (Figure 9 (B)).

PAL enzyme activity

UV-B radiation treatment and sucrose concentration was not significantly affected PAL enzyme activity in *K. galanga* callus, and there was no interaction between the two treatments. There was a tendency for compact callus treatment to show better PAL enzyme activity than friable callus. Likewise, UV-B radiation treatment provided higher PAL enzyme activity than without UV-B radiation treatment in both compact callus (Figure 10 (A)) and friable callus (Figure 10 (B)).

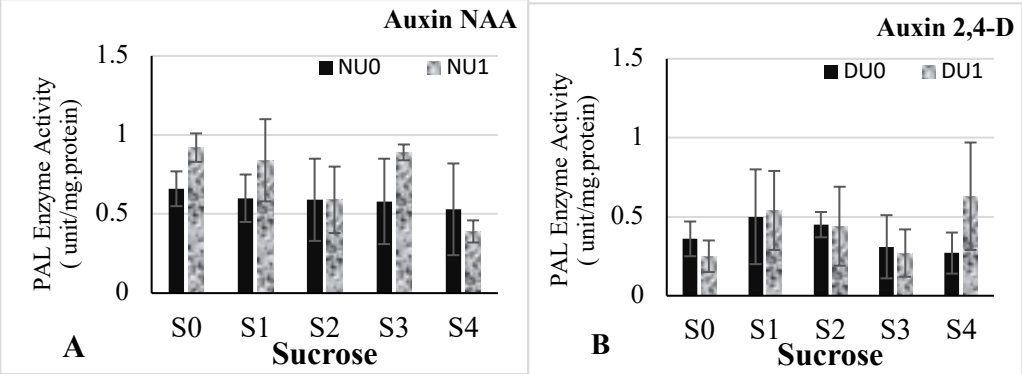

Figure 10. Average PAL enzyme activity (unit.mg-¹ protein) of ethanol extract of *K. galanga* with UV-B radiation treatment and sucrose concentration in compact callus (A) and friable (B): In the bar chart, the average value with different letters indicates significantly different treatments at P≤ 0.05 (LSD Test). Values are the average of three replications ±SD

Discussion

This study focused on the effect of combinations of UV-B and sucrose. Effects of high UV-B inflence on plant growth, morphology and physiology. However, low UV-B levels can also induce specific responses, such as enhanced production of secondary metabolites including phenolic compounds especially phenylpropanoids and flavonoids. Phenolics represent the most abundant secondary metabolites of plants, and about 20 % of the carbon (photosynthetic fixed) is used for their synthesis (Vidović *et al*., 2017). Furthermore, low UV-B levels can cause the upregulation of genes involved in stress protective processes (Roeber *et al*., 2021).

Sucrose in the medium acts as a carbon source and also induces production of secondary metabolites. Sugar acts as a signal transducer for both cells culture growth, metabolism and development. The amount of sugars, such as sucrose, maltose, fructose and glucose and the type of sugar in cells is important for growth, absorption of nutrients and secondary metabolite production. They are critical in the process of signal transduction, control gene expression and cell development (Ali *et al*., 2016).

Previous studies on the effects of different auxins, specifically 2,4-D and NAA, revealed distinct differences in callus morphology. Callus grown with 2,4-D developed a friable texture with a brownish-white color, whereas callus treated with NAA exhibited a compact, green appearance, indicating that NAA promotes differentiation (compact, green callus) depending on the photoperiod. In this study, both types of callus were maintained, with their proliferation supported by

either 2,4-D or NAA. The research further examined how each callus type responded to UV-B radiation and sucrose concentrations regarding growth and the production of phenolic bioactive compounds. The findings revealed that increasing sucrose concentration in the medium from 15 to 60 $g.L^{-1}$ led to a 4.88 to 8.49-fold rise in fresh weight for compact callus compared to conditions without sucrose. Applying UV-B radiation alongside sucrose concentrations of 15-60 $g.L^{-1}$ for friable callus increased fresh weight by 1.4 to 12.98 times compared to the setup without UV-B and sucrose. UV-B radiation and sucrose concentration also affected the average dry weight of the callus, with increases ranging from 5.00 to 13.8 times for compact callus and 5.00 to 16.5 times for friable callus. Moreover, different auxins influenced dry callus weight, with NAA treatment resulting in an average dry weight 1.91 times greater than that from 2,4-D treatment. This indicates that different auxins (2,4-D and NAA) impact dry callus weight, with NAA yielding higher dry weights and producing compact, green callus morphology.

15-30 $g.L^{-1}$ sucrose is the best concentration for fresh weight callus and dry weight callus of *K. galanga*, whereas, the 60 $g.L^{-1}$ sucrose showed no increase in growth of fresh weight callus in this study. Consistent with (Ali et al., 2016) study, the different initial sucrose concentrations in *A. absinthium* cell suspension culture resulted in the maximum values of dry biomass at day 30 for both 3 and 5%. However, an increase in sucrose concentration caused increased inhibition of the dry weight cell. Worth to mention, that in *Zingiber officinale* Rosc callus, the growing of sucrose concentration from 20-50 $g.L^{-1}$ in MS media is a positive control for induction of wet and dry weight of the callus (Kherasani *et al*., 2017). Callus biomass was enhanced in a treatment of 30 $g.L^{-1}$ sucrose and five µM erythrose-4-phosphate, but the flavonoid content of *Gynura procumbens* Merr callus decreased after supplementing with erythrose-4-phosphate (Nurisa *et al*., 2017).

The treatment of sucrose concentrations of 45 $g.L^{-1}$ and 60 $g.L^{-1}$ showed a decrease in fresh callus weight in this study; the reduction in fresh weight was likely due to inhibition of water absorption due to increased osmotic pressure so that the callus's ability to store water was lower. Shahnewaz and Bari (2004) observed that sucrose concentration influences callus induction frequency, likely due to its role in modifying the osmotic potential of the medium rather than serving purely as a carbon source. Osmotic agents like sucrose, sorbitol, and mannitol lower the water potential in the culture medium, restricting water availability, nutrient uptake, and cellular metabolic activity (Al-Bahrany and Al-Khayri, 2002). According to Inayah (2015), excess sucrose causes cells to become saturated so that the osmotic pressure in the medium is higher than in the cells, thereby reducing cell growth and development. Kherasani *et al*. (2017)

stated that sucrose with a concentration higher than the average composition of MS medium causes the medium concentration to become more concentrated and inhibits water absorption.

According to Iraqi and Tremblay (2001), sucrose in the media is hydrolyzed into monosaccharides during the culture period. Sucrose is rapidly hydrolyzed into hexoses (glucose and fructose) by the cell wall invertase enzyme. Sucrose is vital in cells, as it produces energy during respiration, regulates membrane stabilization, regulates osmotic pressure, and helps form new plant cells (Heriansyah, 2019). Increasing sucrose concentration can increase cell growth because it guarantees the availability of carbon and energy. Conversely, low sucrose concentrations will reduce callus cell growth because energy is quickly depleted to inhibit development (Sitorus *et al*., 2011). During plant tissue culture, sucrose is an energy source for cells to maintain photomixotrophic metabolism and support optimal development (Gago *et al*., 2014). The presence of sucrose supports the maintenance of osmotic potential and water conservation in cells. The addition of sucrose helps maintain osmotic balance and water retention in cells. However, high sucrose levels can limit photosynthesis by reducing chlorophyll and epicuticular wax levels, leading to structurally and functionally abnormal stomata (Hazarika, 2006).

This study's sucrose concentration of 60 g/L yielded the highest callus dry weight, consistent with findings from Javed and Ikram (2008), who reported increased dry weight in wheat genotypes (S-24 and MH-97) as sucrose concentration rose. Similarly, Cui *et al*. (2010) found higher sucrose levels (30 and 50 $g.L^{-1}$) enhanced root biomass in *Hypericum perforatum* cultures. Conversely, Fazal *et al*. (2016) observed maximum fresh (1.22 g/100 mL) and dry callus weights (0.42 g/100 mL) at 20 $g.L^{-1}$ sucrose, with higher sucrose levels (35–50 $g.L^{-1}$) reducing biomass and cell size in *Prunella vulgaris* L. due to osmotic stress.

Good growth, as indicated by high dry weight, results from nutrient absorption and the use of growth regulators for cell development (Purnamaningsih and Ashrina, 2011). Javed and Ikram (2008), stated that increasing sucrose concentration of 4-8% significantly increased dry wheat callus weight in two wheat genotypes tested and increased free proline levels and total dissolved carbohydrates in the callus. Increasing sucrose concentration causes osmotic stress, which inhibits water absorption and increases the accumulation of proline and carbohydrates. Dry weight leads to osmotic balance, where when the concentration of the media is more concentrated, the concentration of water is more significant in the cells compared to the media, so water moves out of the cells, namely in the media (diffusion).

The average total phenol level in the media in both compact and friable calli was found to be impacted by the concentration of sucrose. In comparison to when sucrose was not added, the total phenol concentration in the compact callus increased by 1.14 times when 15 $g.L^{-1}$ (S1) of sucrose was added. A drop of 0.05-0.18 times was seen when the concentration of sucrose in the media was increased to 30-60 $g.L^{-1}$. On the other hand, in friable callus, the total phenol content decreased by 0.05-0.39 times when the concentration of sucrose was increased to 15-60 $g.L^{-1}$, and the total phenol content increased by 1.13 times when the concentration of UV-B radiation was increased.

The treatment that did not contain sucrose (S0) had the highest flavonoid concentration overall. The total flavonoid content decreased by 0.1-0.75 times when the concentration of sucrose in the compact callus increased by 15-60 $g.L^{-1}$. In comparison to no UV-B radiation treatment, the total flavonoid content increased by 1.7 times after UV-B radiation treatment. In contrast, when compared to no sucrose, a 15–45 $g.L^{-1}$ increase in sucrose concentration in friable callus resulted in a 0.4–2.6-fold reduction in total flavonoid content. When compared to when the callus was not exposed to UV-B radiation, the total flavonoid concentration increased 1.33 times.

These results indicated that the higher sucrose content added to the media causes a decrease in the total phenol content and total flavonoid content in compact and friable calluses. At the same time, UV-B radiation treatment induces an increase in phenol and flavonoid production in callus in this study. This may be because, at a sucrose concentration of 30-60 $g.L^{-1}$ most of the carbon is used to grow and accumulate organic compounds in the callus; this can be seen from this treatment's high callus growth parameters. At low sucrose concentrations, namely 0-15 $g.L^{-1}$, most of the carbon is used to form secondary metabolites rather than callus biomass.

According to these findings, compact and friable calluses have lower levels of total phenol and total flavonoid content when a higher sugar content is introduced to the media. In this work, UV-B radiation therapy simultaneously increases the formation of flavonoids and phenols in callus. This could be because the high callus development parameters of this treatment indicate that the majority of the carbon is utilized to develop and accumulate organic compounds in the callus at a sucrose concentration of 30–60 $g.L^{-1}$. The majority of the carbon is utilized to create secondary metabolites rather than callus biomass at low sucrose concentrations, namely 0–15 $g.L^{-1}$.

While higher sucrose levels decreased total phenolic content (TPC), increasing sucrose concentration from 5 to 20 $g.L^{-1}$ increased TPC from 5.4 to 12.3 $mg.g^{-1}$ DW, which is consistent with the results of Fazal et al. (2016) *Prunella vulgaris* suspension cultures. Higher sucrose concentrations (40 or 45

g.L^{-1}) increased TPC and antioxidant activity in Musa species shoot cultures, according to Ayoola-Oresanya *et al*. (2021). *Echinacea angustifolia* cultures treated with 50 g.L^{-1} sucrose showed peak phenolic and flavonoid synthesis, according to Wu *et al*., (2007). Furthermore, in *Gynura procumbens* Merr, 30 g.L^{-1} sucrose with 5 µM erythrose-4-phosphate enhanced callus biomass while decreasing flavonoid levels (Nurisa *et al*., 2017). Disaccharides increased secondary metabolite accumulation in suspension cultures of *Artemisia absinthium* L., as reported by (Ali *et al*., 2016). Cultures treated with sucrose exhibited the highest TPC (5.32 mg.g^{-1} DW), followed by those treated with maltose, fructose, and glucose.

In this study, UV-B radiation increased the total phenolic and flavonoid content of *K. galanga* callus. Schreiner *et al*. (2012) noted that physiological age, UV-B dose, and environmental conditions influence metabolite levels. With prolonged exposure and incubation, the callus color shifted from green to yellow, likely due to chlorophyll degradation and phenolic accumulation (Karvansara and Razavi, 2019). Similarly, (Abbasi *et al*., 2021) found that *Fagonia indica* cultures exposed to UV radiation showed elevated phenolic and flavonoid content compared to non-irradiated cultures.

UV-B radiation promotes the accumulation of total phenolics in *Lactuca undulata* callus, leading to significantly higher phenolic concentrations in treated samples compared to controls (Bojnoordi *et al*., 2023). Additionally, UV-B exposure affects gene expression, plant physiological processes, and the accumulation of secondary metabolites (Schreiner *et al*., 2012). The high energy of UV-B radiation induces cellular damage by breaking chemical bonds, generating reactive oxygen species (ROS), and impacting DNA, proteins, and chloroplasts. Plants respond by producing UV-absorbing compounds in their epidermal cells to mitigate these harmful effects (Treutter, 2005). For instance, *Ocimum basilicum* exhibits increased phenolic content and enhanced enzyme activity after UV-B exposure (Ghasemzadeh *et al*., 2016). The stimulation of secondary metabolism by UV-B is linked to enzymes such as L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), which participate in the biosynthesis of phenolic and flavonoid compounds (Abbasi *et al*., 2021; Tiecher *et al*., 2013).

UV-B radiation treatment did not affect EPMC production in compact or friable textured callus. Sucrose concentration treatment in *K. galanga* callus produced various EPMC content. Increasing sucrose concentration caused a decrease in EPMC content in callus, except at a concentration of 45 g.L^{-1}, which showed no significant difference with the treatment without sucrose. There was a 0.05-0.6-fold decrease in EPMC content in callus with an increase in sucrose

concentration in the media. While in friable textured callus, increasing sucrose concentration did not significantly affect the EPMC content of the.

Similar to findings by Bojnoordi *et al*. (2023), chlorogenic acid levels remained unchanged across different UV-B exposure times after 5 and 15 days of incubation. Conversely, Yildirim (2020) observed decreased chlorogenic acid and increased rosmarinic acid upon UV-B exposure. Keskin and Kunter (2009) demonstrated that 10 minutes of UV exposure sufficed for trans-resveratrol production in *Vitis vinifera* callus cultures. Bojnoordi *et al*. (2023) emphasized the importance of exposure duration, incubation, and callus age in UV-B-induced secondary metabolite production. Genetic diversity among plants leads to varied responses to UV-B radiation (Zlatev *et al*., 2012), with some benefiting and others adversely affected, as metabolite levels can depend on environmental and physiological factors (Schreiner *et al*., 2012).

Sucrose concentration affected EPMC production in this study. Treatment without sucrose (S0) produced the highest EPMC levels, which were not significantly different from the 45 $g.L^{-1}$ sucrose treatment. The high production of EPMS in callus without sucrose (S0) is possible because the synthesized carbon framework is used mainly to form secondary metabolite compounds, in this case, EPMC. In this study, the natural characteristics of *K. galanga* plants affect EPMC production in callus. In aromatic ginger plants, EPMC is accumulated in the rhizome, where the EPMC content increases in line with the increasing age of the rhizome and the cessation of plant growth. There was no tissue growth in the treatment without sucrose due to the limited source of carbon nutrients needed for callus growth. When carbon availability is limited, the biochemical regulatory mechanism of carbon flux control is directed at producing secondary metabolites, in this case, EPMC production in callus. According to Caretto *et al*. (2015), photosynthesis actively produces the carbon skeleton, which is then utilized for growth (primary metabolism) or defense (secondary metabolism) based on the prevailing environmental conditions. Additionally, EPMC production in new callus is triggered once callus growth reaches a stationary phase, initiating the formation of EPMC compounds within the callus.

The proportion of DPPH radicals scavenged by the ethanol extract of *K. galanga* callus was used to calculate antioxidant activity. The antioxidant activity of compact or friable textured calluses was not substantially impacted by UV-B radiation treatment. Nonetheless, the study's callus ethanol extract's antioxidant activity was impacted by the sugar concentration. When compared to callus without sucrose treatment, the antioxidant activity of callus extract decreased by 0.21–0.45 times in compact callus and by 0.1-0.29 times in friable textured callus when the quantity of sucrose increased by 15–45 $g.L^{-1}$.

The presence of phenolic and flavonoid chemicals generated within the callus affects its antioxidant activity. According to earlier research, increasing sucrose concentrations decreased the total phenolic and flavonoid content, which was associated with a decline in antioxidant activity. This finding is consistent with the results of Ali *et al*. (2016), who demonstrated that total phenolic content is essential for antioxidant activity in cultures induced by sucrose.The culture's antioxidant response to varying sucrose levels positively correlated with total phenolic and flavonoid production, suggesting that sucrose stimulates the synthesis of these compounds as potential antioxidant metabolites. Additionally, research by Rosane *et al*. (2018) on Alternanthera plants revealed that radiation exposure had minimal impact on total antioxidant capacity, as measured by DPPH, indicating that UV-B radiation did not significantly affect free radical scavenging activity.

The difference in callus response to various sucrose concentration treatments and UV-B radiation on growth and secondary metabolite production cannot be separated from the enzyme activity that works in forming bioactive compounds in *K. galanga* callus. In this study, various levels of sucrose concentration (0-60 $g.L^{-1}$) and UV-B radiation elicitation did not significantly affect the PAL enzyme activity in *K. galanga* callus. Increasing sucrose concentration decreased PAL enzyme activity in compact and friable textured callus. However, there was a tendency for UV-B radiation administration to increase PAL enzyme activity compared to without UV-B radiation, although statistically, it was not significant. The no significant difference in UV-B radiation treatment was possible because light treatment for 16/8 hours (light and dark) was given for all treatments tested in this study. As is known, PAL enzyme activity is greatly influenced by the presence of light by increasing the expression of the PAL gene (phenylalanine ammonia-lyase) (Salisbury and Ross, 1992). In addition, light in young plant tissue that is not yet well-differentiated shows that PAL enzyme activity is slow, resulting in low metabolism of phenylpropanoid compounds (Higuchi, 1990).

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Conflicts of interest

The authors declare no conflict of interest.

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