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Research Article

Effect of Addition *Saccharomyces cerevisiae* Elicitor on Total Flavonoid Content and Antioxidant Activity of *Gardenia jasminoides* Cell Suspension Culture

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DPPH inhibition,
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Elicitation,
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Abstract: *G. jasminoides* is a medicinal plant with diverse properties due to the various secondary metabolites including flavonoids. Flavonoid content in gardenia leaves can be increased through *in vitro* culture methods, such as CSC combined with elicitation. This process is carried out by adding an elicitor, which provides a stress condition in the culture to produce secondary metabolites. An example of an elicitor capable of increasing secondary metabolites is *S. cerevisiae* powder. Therefore, this study aimed to examine the morphology of gardenia leaves callus, measure the growth of CSC, determine the optimum elicitation time, and evaluate the most potent concentration of *S. cerevisiae* powder. The treatments carried out include variations in elicitation duration of 0, 2, 4, and 6 days as well as *S. cerevisiae* elicitor concentrations of 0, 2.5, 5, and 7.5%. The results showed that the callus of gardenia leaves was friable and yellowish. Gardenia CSC showed two growth phases, namely exponential and stationary. The optimal elicitation period for maximizing total flavonoid content was six days, whereas the ideal period for achieving the highest antioxidant activity was two days. The highest levels of both flavonoids and antioxidant activity were observed with a 7.5% concentration of *S. cerevisiae*.

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1. Introduction

G. jasminoides is a Rubiaceae shrub widely distributed across Asia (Wiart, 2022). This medicinal plant has been proven to reduce fever, relieve pain, reduce headaches, mitigate hypertension, cure jaundice, and ease skin infections (Uddin et al., 2014). The leaves contain active compounds, including flavonoid, which has antipyretic activity (Yoga et al., 2022). Flavonoids are polyphenolic compounds distinguished by a C6-C3-C6 carbon structure. Various types of flavonoids include flavone, flavanol, flavanone, isoflavone, anthocyanidins, and chalcone, which exhibit properties such as anti-inflammatory, anti-carcinogenic, anti-microbial, and anti-mutagenic effects (Chagas et al., 2022).

Flavonoid content can be increased through in vitro culture methods by culturing leaves aseptically in an artificial medium and maintaining them in a controlled environment (Bhojwani and Dantu, 2013). Cell Suspension Culture (CSC) combined with elicitation is an in vitro method that has great potential to enhance the production of active compounds. CSC involves cultivating a crumbly

callus in a liquid medium, accompanied by shaking. Before application, the callus needs to be subcultured to increase the number available for use (Stewart, 2008).

Elicitation is a method for increasing active compounds by adding an elicitor that induces stress in the culture conditions, prompting the synthesis of secondary metabolites as cell defense mechanisms (Nikalje et al., 2021). In particular, elicitation of CSC should be conducted during the stationary phase of suspension culture, when biomass is at its highest and is expected to produce significant amounts of secondary metabolites (Krishnan and Siril, 2018).

A prominent elicitor capable of increasing the content of active compounds is *S. cerevisiae* in powder form. Some advantages include its various beneficial components, such as polysaccharides, vitamins, nucleic acids, minerals, and enzymes, which can trigger cell defense mechanisms (Alcalde et al., 2022). Additionally, *S. cerevisiae* is non-pathogenic, widely available (Dena et al., 2021), and exhibits fast growth, allowing for timely harvests (Bhatia et al., 2015).

The enhancement of secondary metabolite compounds through elicitation in CSC is affected by two key factors: the duration of elicitation and the concentration of the elicitor. Extended elicitation periods can lead to a decrease in metabolite content, while insufficient elicitation may fail to generate high levels of secondary metabolites (Karalija et al., 2020). Additionally, overly high concentrations of elicitors can induce excessive stress that may harm or kill the cells, whereas very low concentrations might not activate adequate defense mechanisms (Gonçalves et al., 2019).

When a 5% *S. cerevisiae* extract is applied to a *Morinda citrifolia* callus culture for two days, quinone levels increase by 2.18 times compared to cultures without elicitation (Purwianingsih and Hamdiyanti, 2009). In another study, *Gymnema sylvestre* CSC treated with 1.5% *S. cerevisiae* extract for three days showed a 9.3-fold increase in gymnemic acid compared to non-elicited cultures (Chodisetti et al., 2013). Bavi et al. (2022) found that adding a 1200-ppm yeast extract elicitor for three days doubled the flavonoid content in *Zataria multiflora* CSC. Research by Farjaminezhad & Garoosi (2021) demonstrated that treating *Azadirachta indica* CSC with 200 ppm yeast extract for four days resulted in a 2.5-fold increase in squalene content compared to control treatments. Additionally, Jin et al. (2023) reported that flavonoid levels in *Oplopanax elatus* CSC increased by 1.5 times when a 100ppm yeast extract elicitor was applied for four days.

Elicitation has not yet been performed on *G. jasminoides* callus suspension cultures (CSC). This study aims to investigate the morphology of *G. jasminoides* leaf callus as CSC material, assess the growth curve, and develop an elicitation protocol to enhance the total flavonoid content and antioxidant activity in gardenia CSC.

2. Material and Methods

2.1. *G. jasminoides* callus subculture

The friable *G. jasminoides* callus was cleaned from the remaining old medium and cut into smaller sizes. Callus was subcultured on Murashige and Skoog media with an MS composition of 4.43 g L^{-1}, sucrose 30 g L^{-1}, agar 8 g L^{-1}, and 2,4-D hormone 2 ppm. Subsequently, the sample was incubated at a temperature of 25±2 ºC and subcultured every four weeks (Gabr et al., 2017).

2.2. *G. jasminoides* CSC development

The crumbly, one-month-old *G. jasminoides* callus was cut into smaller pieces and placed in liquid MS medium containing MS composition of 4.43 g L^{-1}, sucrose 30 g L^{-1}, agar 8 g L^{-1}, and 2,4-D hormone 2 ppm. CSC was cultivated in a shaker incubator with a speed of 100 rpm and a temperature of 25±2 ºC, and suspension culture (two weeks old) was subcultured into a fresh liquid medium containing 1 mL of PCV in 20 mL of media. A shaker incubator was used to incubate the suspension culture, set at 25±2 ºC and 100 rpm (Dwivedi et al., 2016).

2.3. *S. cerevisiae* elicitor preparation

Culture of *S. cerevisiae* on PDB media aged 24 hours was inoculated in 200 mL of PDB reaching 4% of total volume. Subsequently, incubation was carried out in a shaker incubator for 24 hours followed by centrifugation for 10 minutes at a speed of 5000 rpm. The centrifuged pellets were washed with sterile distilled water and dried at 40ºC until dry. Dried *S. cerevisiae* cells were crushed into powder,

and then sterilized by autoclave at a temperature of 121 ^{o}C and a pressure of 1 atm for 20 minutes (Li et al., 2017).

2.4. Elicitation *G. jasminoides* CSC

Suspension cultures six days old were added with *S. cerevisiae* elicitor at 0, 2.5, 5, and 7.5% (w/v) then incubated in a shaker incubator for 0, 2, 4, and 6 days (Ahmed and Baig, 2014).

2.5. Secondary metabolites extraction

A filtered suspension culture was used to collect intracellular metabolites and callus cells were dried for 48 hours at 40 °C. After weighing and dissolving 200 mg of dried callus cells in 4 mL of 96% ethanol, the mixture was sonicated for 20 minutes at a temperature between 25-30 ^{o}C. Following a 10-minute centrifugation at 6500 rpm on the sonication results, the supernatant was poured into a porcelain cup and allowed to evaporate at 40 °C for a whole day. At 4 ^{o}C, the extract was kept cold (Liu et al., 2018). To collect extracellular metabolites, the leftover culture medium was dried at 40 °C for a whole day (Mendoza et al., 2018).

2.6. Determination of total flavonoid content

Total flavonoid content was determined using 0.5 mL of test solution, 1.5 mL of 96% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. A UV-Vis spectrophotometer set to 415 nm wavelength was used to measure the absorbance of the test solution after incubating for 30 minutes in the dark. Total flavonoid levels (mgQE g^{-1} extract) were determined through the quercetin standard curve and the equation y = ax+b (Sulastri et al*.,* 2018).

2.7. Determination of antioxidant activity

Antioxidant activity was determined using 0.1 mL of test solution added with 3.9 mL of 96% ethanol and 1 mL of 0.1 mM DPPH. The control solution was made with 4 mL of 96% ethanol and 1 mL of 0.1 mM DPPH. After 30 minutes of dark incubation for the test solution and control, the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Antioxidant activity was expressed as a percentage of DPPH inhibition, calculated by the formula (Hassanpour and Niknam, 2020; Nurcholis et al., 2023):

$$\%\ \text{DPPH Inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (1)$$

2.8. Statistical analysis

Data on dry weight, total flavonoid content, and percentage of DPPH inhibition were subjected to One-Way ANOVA analysis with a confidence level of 95%, then continued with the DMRT test to determine fundamental differences between treatments. All analyses were carried out with the SPSS 27.0 program (Dowom et al., 2017).

3. Results

The starting material used in CSC was a friable callus type shown in Figure 1, which had a yellowish white with a little brownish color before subculturing and a yellowish-white color after. A 1-month-old gardenia callus subcultured to MS liquid media supplemented with 2,4-D 2 ppm to initiate CSC (Figure. 2) was used to make a growth curve using a dry weight parameter every three days for 30 days incubation. Figure 3 presents two growth phases of *G. jasminoides* CSC, namely the exponential and stationary. The exponential phase showed a significant increase in dry weight which occurred on days 0 to 6, while the stationary phase experienced no significant increase or decrease in dry weight from days 6 to 30. Based on the growth curve, the elicitor could be added into *G. jasminoides* CSC on day 6, corresponding to the highest biomass.

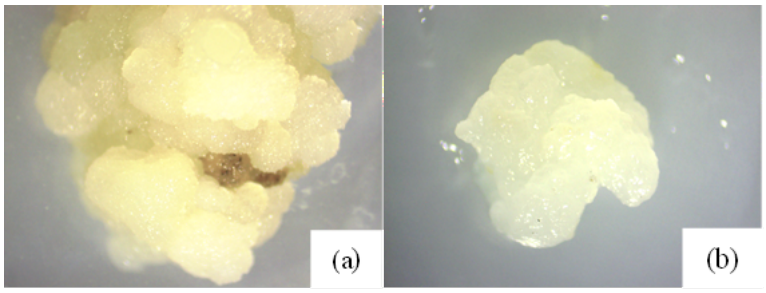

Figure 1. *G. jasminoides* callus morphology on MS media with the addition of 2,4-D 2 ppm. (a) Four weeks callus before subculture (b) One week callus after subculture.

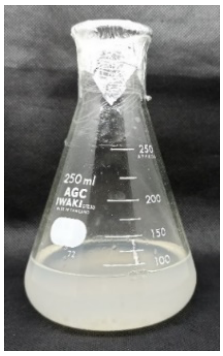

Figure 2. *G. jasminoides* CSC on MS liquid media addition with 2,4-D 2 ppm.

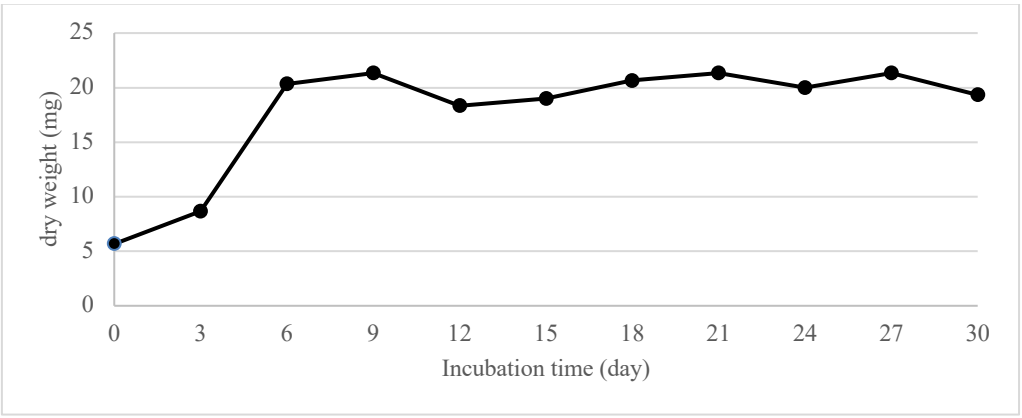

Figure 3. *G. jasminoides* CSC growth curve on MS liquid media addition with 2,4-D 2 ppm after 30 days incubation.

Elicitation on *G. jasminoides* CSC was carried out in two stages, the first was to determine the effect of elicitation time on total flavonoid content and antioxidant activity expressed as a percentage of DPPH inhibition. The dry weight of *G. jasminoides* CSC with non-elicitation and elicitation treatments is presented in Figure 4. Based on the results, there was no increase in dry weight during six days of incubation or elicitation. *G. jasminoides* CSC with elicitation had greater dry weight than the non-elicitation treatments.

As shown in Figure 5, *G. jasminoides* CSC with elicitation treatment produced higher total flavonoid content than non-elicitation on days 0 to 6. The highest total flavonoid content was obtained on the sixth day at 1.27±0.21 mgQE g^{-1} extract (7.9 times compared to non-elicitation) intracellularly, and 1.75±0.36 mgQE g^{-1} extract (2.7 times compared to non-elicitation) extracellularly. In both treatments, total flavonoid content measured in the extracellular medium was significantly higher than that in the intracellular. According to Figure 6, on days 0 to 6, elicited *G. jasminoides* CSC produced a higher percentage of DPPH inhibition than others. On the sixth day, the maximum percentage of

intracellular DPPH inhibition was recorded at 57.69±2.61%, which was 4.5 times higher than the non-elicitation treatment. On the second day, extracellular was reached 61.84±5.34%, which was 6.9 times higher than non-elicitation treatment.

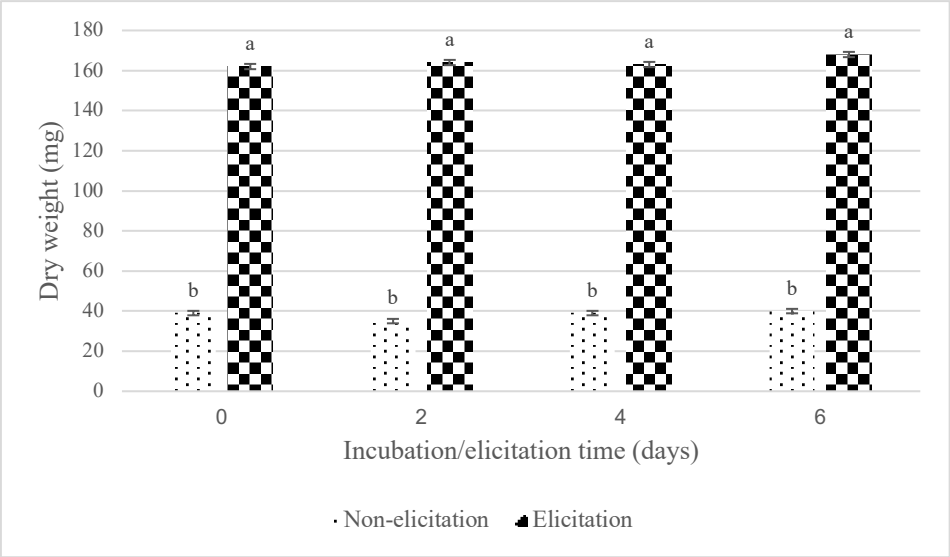

Figure 4. The dry weight of *G. jasminoides* CSC was treated with non-elicitation and elicitation. The mean± standard error (error bar) of three replicates is used to express the results. With a 95% confidence level, values followed by the same letters (a, b, c, and d) in the Duncan Test show no significant difference.

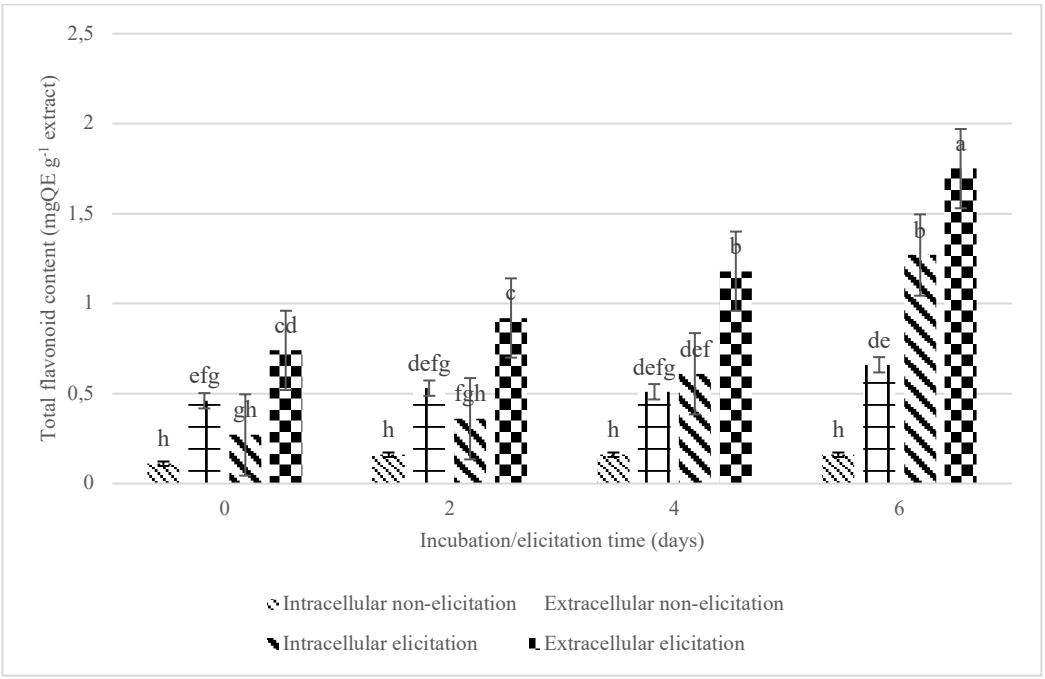

Figure 5. Total flavonoid content of *G. jasminoides* CSC treated with non-elicitation and elicitation with *S. cerevisiae* 5%. The mean ± standard error (error bar) of three replicates is used to express the results. With a 95% confidence level, values followed by the same letters (a, b, c, and d) in the Duncan Test show no significant difference.

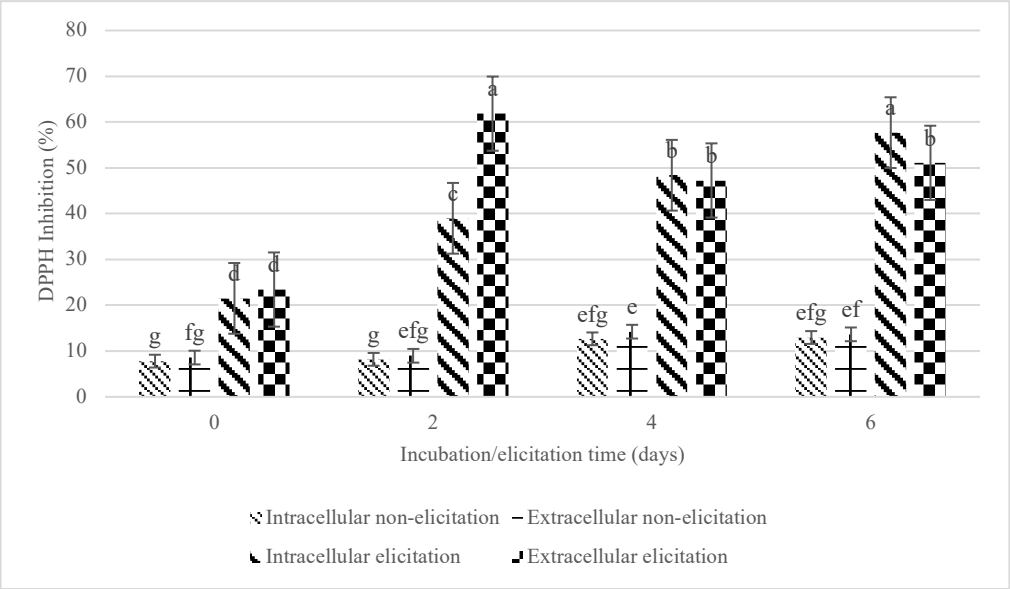

Figure 6. Percentage of DPPH inhibition of *G. jasminoides* CSC treated with non-elicitation and elicitation with *S. cerevisiae* 5%. The mean ± standard error (error bar) of three replicates is used to express the results. With a 95% confidence level, values followed by the same letters (a, b, c, and d) in the Duncan Test show no significant difference.

The effect of elicitor concentrations on total flavonoid content and antioxidant activity represented as a percentage of DPPH inhibition, was assessed during the second elicitation stage. As shown in Figure 7, the dry weight of *G. jasminoides* CSC increased with higher elicitor concentrations. *S. cerevisiae* 7.5% treatment had the largest dry weight, measuring 1574±142.30 mg, while the 0% treatment had the lowest dry weight of 126±74.54 mg.

According to Figure 8, total flavonoid content produced both intracellularly and extracellularly increased with higher *S. cerevisiae* concentrations. The 7.5% treatment yielded the highest total flavonoid concentration of 3.35±0.08 mgQE g-1 extract (8.6 times larger than *S. cerevisiae* 0%) intracellularly and 3.94±0.26 mgQE g^{-1} extract (5.4 times greater than *S. cerevisiae* 0%) extracellularly. Figure 9 showed that a higher proportion of DPPH inhibition was produced both intracellularly and extracellularly with higher *S. cerevisiae* concentrations. The greatest percentage of DPPH inhibition was obtained using 7.5% treatment, amounting to 35.33±1.09% (12.2 times compared to *S. cerevisiae* 0%) intracellularly, and 41.99±4.28% (6.1 times compared to *S. cerevisiae* 0%) extracellularly.

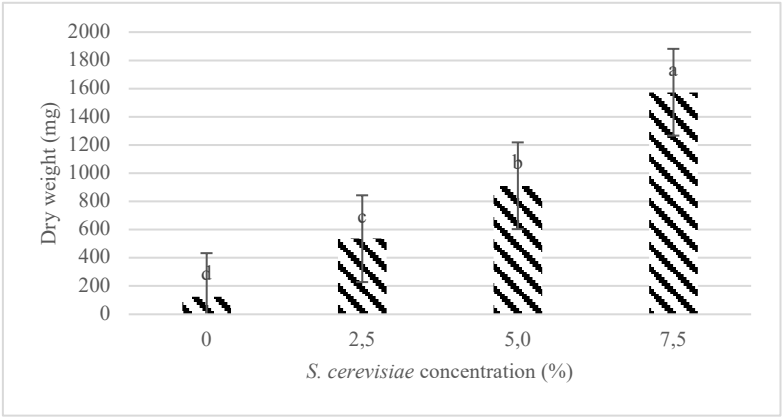

Figure 7. Effect of *S. cerevisiae* elicitor concentration on dry weight *G. jasminoides* CSC. The mean ± standard error (error bar) of three replicates is used to express the results. With a 95% confidence level, values followed by the same letters (a, b, c, and d) in the Duncan Test show no significant difference.

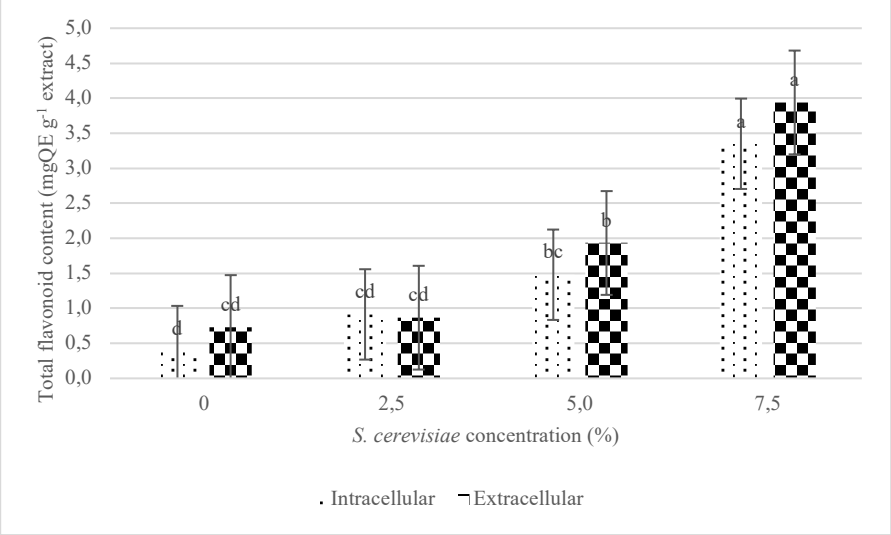

Figure 8. Effect of *S. cerevisiae* elicitor concentration on total flavonoid content *G. jasminoides* CSC. The mean ± standard error (error bar) of three replicates is used to express the results. With a 95% confidence level, values followed by the same letters (a, b, c, and d) in the Duncan Test show no significant difference.

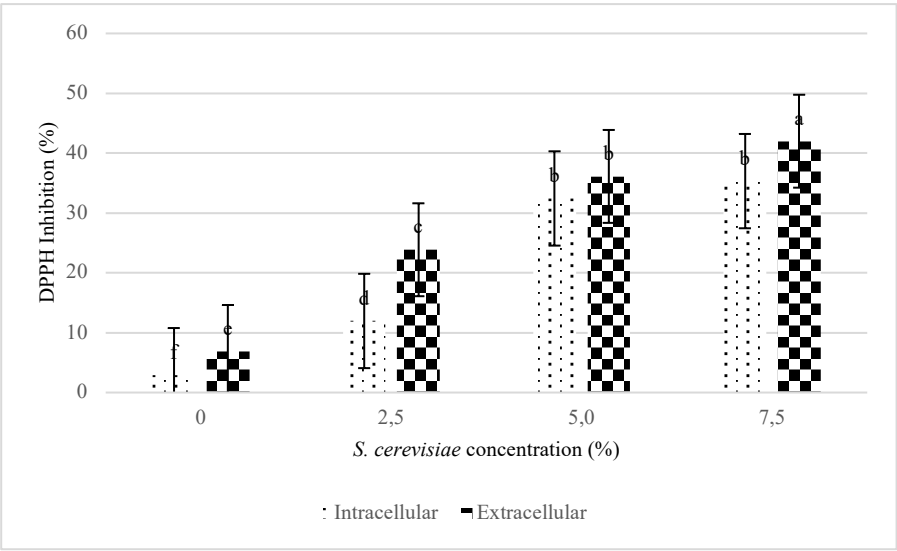

Figure 9. Effect of *S. cerevisiae* elicitor concentration on percentage inhibition DPPH *G. jasminoides* CSC. The mean ± standard error (error bar) of three replicates is used to express the results. With a 95% confidence level, values followed by the same letters (a, b, c, and d) in the Duncan Test show no significant difference.

4. Discussion

The callus used as the starting material for CSC had a friable texture with a weak bond between cell walls, resulting in easy degradation and dispersion in liquid media. Subculturing was carried out every four weeks before the callus was used to activate cells and maintain the exponential phase (dos Santos et al., 2017).

G. jasminoides callus, shown in Figure 1, had a friable texture, yellowish-white with a little brownish color before subculturing (Figure 1a), and yellowish white (Figure 1b) color after subculturing. Subculture was carried out on MS media supplemented with 2,4-D 2 ppm, in line with a study by Phua

et al. (2016). 2,4-D has proven effective for inducing the formation of friable and yellowish-white callus on *Clinacanthus nutans* plants. According to Daffalla et al. (2019), friable callus has high viability, prompting the need for regular subculturing. The yellowish-white color of *G. jasminoides* callus was caused by crocin pigments (Zheng et al., 2022) and crocetin (Higashino et al., 2014), which are carotenoid compounds producing a yellow color.

The *G. jasminoides* CSC shown in Figure 2 was used to assess growth by measuring dry weight every three days over 30 days. The goal was to create a growth curve that would help identify the different growth phases of CSC and pinpoint the stationary phase for elicitation purposes (Wang et al., 2015). Dry weight was chosen as a growth parameter because it offers greater accuracy than wet weight, as it is not influenced by water content (Abbas et al., 2018). Based on Figure 3, there were two growth phases, exponential and stationary. In the exponential phase from day 0 to 6, cell division occurred continuously until a maximum number was reached. For the stationary phase which occurred from day 6 to day 30, there was no significant increase or decrease in growth but an accumulation of secondary metabolites was still observed (dos Santos et al., 2017). The elicitor should be introduced on day 6, at the beginning of the stationary phase. This phase is defined by maximum biomass and is anticipated to yield elevated levels of secondary metabolites. (Kümmritz et al., 2016).

The growth curve of *G. jasminoides* CSC depicted in Figure 3 resembled the findings of Liu et al. (2018), who used MS media supplemented with 0.5 ppm NAA and 0.3 ppm kinetin. The results indicated that the lag, exponential, stationary, and death phases occurred from days 0 to 4, 4 to 10, 10 to 12, and 12 to 16, respectively. Variations in the CSC growth curve from the same plant's callus can be attributed to the type and concentration of hormones used (Damayanti et al., 2020).

G. jasminoides CSC in both non-elicitation and elicitation treatments showed no increase in dry weight over six days of incubation, as illustrated in Figure 4. This indicates that the culture has entered the stationary phase, a point also noted by dos Santos et al. (2017). The stationary phase is characterized by a minimal difference in dry weight due to limited cell growth. Similarly, Cai et al. (2014) found that *Malus domestica* CSC elicited with yeast extract did not exhibit significant differences in dry weight after 5, 7, and 9 days of incubation. According to Figure 4, the elicited *G. jasminoides* CSC showed a higher dry weight compared to the non-elicited version. Kanthaliya et al. (2023) noted that *S. cerevisiae* has a cell wall composed of polysaccharides with high molecular weight. Consequently, this addition as an elicitor contributed to the increased dry weight of CSC. The polysaccharides did not contain any toxic substances that would negatively affect the growth of CSC.

As illustrated in Figure 5, longer elicitation times correspond to higher total flavonoid content. Al-Gendy et al. (2015) noted that extended exposure to elicitors creates greater stress conditions for CSC, stimulating the production of secondary metabolites through cell defense mechanisms. However, prolonged contact can also lead to a decrease in cell populations, resulting in reduced secondary metabolite levels. Figure 4 indicates that increased total flavonoid content is proportional to the rise in dry weight. Similarly, Kanthaliya et al. (2023) found that adding yeast extract as an elicitor to *Pueraria tuberosa* callus culture enhanced total flavonoid levels without diminishing dry weight after four weeks of incubation. The inclusion of yeast extract promoted cell division, thereby supporting both growth and the production of secondary metabolites like flavonoids.

As shown in Figure 5, total flavonoid content was significantly higher in the extracellular than in the intracellular for both non-elicitation and elicitation treatments. According to Paredes-Lopez (2018), this occurs due to cell lysis, which releases intracellular flavonoids into the culture medium. Mendoza et al. (2020) similarly reported that *Thevetia peruviana* CSC produced greater total flavonoid content extracellularly compared to intracellularly. This difference arises because glycosylated flavonoids are stored in vacuoles, while non-glycosylated ones can diffuse out through the cell membrane. Some glycosylated flavonoids stored in vacuoles include naringin, iso-naringin, hesperidin, and neohesperidin (Zhu et al., 2014). Extracellular flavonoid compounds include apigenin, luteolin, kaempferol, quercetin, isohamnetin, naringenin, and eriodictyol (Aghakhani et al., 2017).

As illustrated in Figure 6, a longer elicitation period corresponds to a higher percentage of DPPH inhibition. These findings align with (Açıkgöz, 2020), who noted that the addition of yeast extract enhanced DPPH inhibition in *Ocimum basilicum* CSC elicited for 10 days. The peak results were observed on the tenth day of elicitation. The increase in DPPH inhibition percentage is attributed to the elicitor, which creates stress conditions. As a result, cell defense mechanisms enhance the synthesis of flavonoid and phenolic compounds that function as antioxidants.

The percentage of DPPH inhibition in *G. jasminoides* CSC, as shown in Figure 6, decreased on the fourth day of elicitation in the extracellular. Mendoza et al. (2018) reported a reduction in extracellular antioxidant activity in *T. peruviana* CSC, which was accompanied by a decrease in total extracellular phenolic levels. According to Mendoza et al. (2020), secondary metabolites that are not stored in vacuoles or secreted extracellularly are vulnerable to oxidation reactions, leading to a decline in extracellular antioxidant activity. As indicated in Figure 5, there was no reduction in total flavonoid content on day 4 of elicitation. Therefore, the observed decrease in DPPH inhibition percentage can be attributed to a decline in other secondary metabolite compounds besides flavonoids. Krasteva et al. (2022) noted that various secondary metabolite compounds present in *G. jasminoides* CSC may be subject to degradation, including phenolics such as syringic, caffeic, ferulic, and chlorogenic acid.

As shown in Figure 7, the dry weight of *G. jasminoides* CSC increased with higher concentrations of elicitor, likely due to the non-toxic effects of *S. cerevisiae* and its ability to promote cell division. Isah (2016) also noted that increasing the concentration of yeast extract used as an elicitor resulted in greater fresh weight for *Nothapodytes nimmoniana* CSC. Additionally, Figures 8 and 9 demonstrate that both total flavonoid content and the percentage of DPPH inhibition rose with higher elicitor concentrations.

The addition of more elicitors led to an increase in total flavonoid content, creating greater stress on CSC. As a result, cells activate additional defense mechanisms by producing secondary metabolite compounds (Zaman et al., 2022). The higher percentage of DPPH inhibition was due to the increased accumulation of antioxidant compounds (Ahmad et al., 2019). Bavi et al. (2022) noted that introducing an elicitor to CSC induces stress and increases Reactive Oxygen Species (ROS) levels. Excessive ROS can harm cells, triggering defense mechanisms to synthesize secondary metabolites that act as antioxidants to reduce DPPH levels.

The increase in total flavonoid content was directly proportional to the higher percentage of DPPH inhibition, indicating that the flavonoid compounds accumulated from the addition of the elicitor acted as antioxidant compounds to reduce DPPH levels (Zaman et al., 2022). A previous study by Zehra et al. (2021) did not identify a specific mechanism by which biotic elicitors like *S. cerevisiae* enhance total flavonoid levels and antioxidant activity. The fundamental principle of elicitation with biotic elicitors is that plant cell receptors recognize components such as polysaccharides, chitin, or flagella as foreign, thereby activating systemic resistance. This process is initiated by the accumulation of salicylic acid, which triggers the expression of the Pathogenesis-Related gene 1 (NPR). The NPR gene interacts with transcription factors that activate Pathogenesis-Related (PR) genes, leading to the activation of plant defense mechanisms and the production of secondary metabolites.

Conclusion

In conclusion, the optimal elicitation protocol for maximizing total flavonoid content was determined to be six days, while two days was found to be ideal for enhancing extracellular and intracellular antioxidant activity. The greatest total flavonoid levels and antioxidant activity were achieved using *S. cerevisiae* at an elicitor concentration of 7.5%. This method can be utilized by the industry to develop cosmetic and health products that incorporate active plant compounds. Future research could focus on identifying specific types of flavonoid and antioxidant molecules that can be enhanced through elicitation.

Ethical Statement

Ethical approval is not required for this study because it does not deal with either human subject or animal.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Funding Statement

The authors declare that this study was self-funded

Author Contributions

Giovanny Okta Francisca contributed to data collection, original draft preparation, and visualization. Ines Septi Arsiningtyas was responsible for data analysis and methodology. Exyupransia Mursyanti contributed to conceptualization, methodology, supervision, and the review and editing of the manuscript.

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