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Elicitation an efficient tool for improving the profile of therapeutically important flavonoids from shoot cultures of *Alpinia purpurata*

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Abstract:

Polyphenolic chemicals represent a significant category of plant secondary metabolites that are prominent for their extensive variety of therapeutic activities. These chemicals are typically found in plants in trace amounts and notably, exhibit a diverse range of therapeutic properties for human health. Medicinal plants are utilized in the field of pharmaceuticals owing to their cost-effectiveness, limited occurrence of unwanted effects, and significant contribution to traditional therapeutic practices. Owing to this, there is a widespread global exploitation of these plants, resulting in the inclusion of numerous medical plant species on the endangered list. Plant tissue culture manifested itself as a promising alternative for obtaining these chemicals, and their production can be enhanced through the process of elicitation. *Alpinia purpurata* belonging to family Zingiberaceae is a medicinal plant with myriad therapeutic effects viz. antibacterial, antioxidant, vascular activity, acute hypotensive effects, cytotoxic, apoptotic properties and anti-cancer activity.

The current study assessed the impact of signalling compounds, methyl Jasmonate (MJ) and salicylic acid (SA) as elicitors, on *in vitro* shoot cultures of *A. purpurata*. Maximum growth of the *in vitro* shoot cultures and flavonoid accumulation was observed on the 25th day, as determined by growth studies conducted by harvesting shoot cultures at regular intervals of 5 days during a 40-day period. The shoot cultures were treated with elicitors for 24-96 h, on 24th day followed by post-elicitation analysis for methanol extraction with dried plant material. Each of the characteristics evaluated was significantly influenced by the type of elicitor and the duration of exposure. In comparison to the control, all treatments significantly increased flavonoid synthesis. The findings obtained from the reverse phase high performance liquid chromatography (RP-HPLC) analysis revealed that shoot cultures exposed to MJ at a concentration of 200µM for 48h displayed the highest level of all the three flavonoids Myricetin (743.16±5.99 µg/g DW), Quercetin (646.15±2.15 µg/g DW), and Kaempferol (180.25±2.12 µg/g DW). When compared to the controls a threefold increase was observed for all the three flavonoids. SA mediated elicitation of shoots after 48 h at a concentration of 100 µM, exhibited a two-fold increase for all the three-flavonoid's Myricetin (494.26±3.18 µg/g DW), Quercetin (431.12±3.25 µg/g DW), and Kaempferol (120.83±1.21 µg/g DW) compared to the control. Our findings indicated that MJ proved to be superior elicitor than SA for enhancing the synthesis of flavonoids in shoot cultures of *A. purpurata*.

Key Words: *Alpinia purpurata*, Shoot cultures, Flavanoids, Elicitation, Methyl jasmonate, Salicylic acid

Introduction:

Medicinal plants prove to be inevitable source of bioactive compounds of therapeutic value for the global population. Humans have long relied on medicinal plants as a seemingly endless source of pharmacological chemicals to treat illness and prolong life. Throughout their lives, plants are subjected to a wide variety of environmental stresses, thus they have evolved a sophisticated biochemical system to form connections and adapt to these stresses. (Hussain et al. 2022). Among the diverse array of plants, those belonging to the Zingiberaceae family, have garnered significant attention for their numerous medicinal benefits (Basu 2002; Kumar et al. 2006; Prajapati et al. 2005). *Alpinia purpurata* originally hailing from the Pacific Islands, has recently gained prominence in regions like Brazil and India (Santos et al. 2012; Brito et al. 2017). The physiological qualities of leaf extracts include antibacterial effects (Oliver et al. 2010), antioxidant properties (Jovitta et al. 2012), vascular activity, and acute hypotensive effects (Silva et al. 2014). The study conducted by Oirere et al. (2016) demonstrated cytotoxic and apoptotic properties of *A. purpurata* n-hexane leaf extract Furthermore, Palanirajan et al. (2022) found evidence supporting its potential as an anti-cancer agent in the context of MNU Testosterone Induced Prostate cancer. Additionally, Smrethe et al. (2018; Utami et al.2020) have reported its antitumor effect on struvite crystal and its inhibitory effect on struvite crystals. The extracts derived from rhizomes have demonstrated the ability to scavenge free radicals and inhibit lipid peroxidation (Subramanian and suja 2014). Immunomodulatory properties of a lectin found in its inflorescences, has been reported by Brito et al. (2017) Santos et al. (2012) discovered 42 components in *A. purpurata* essential oil, with -caryophyllene, -pinene, and -pinene being the main constituents. In another investigation, 30 monoterpenes and sesquiterpenes were discovered. Trans-caryophyllene, -pinene, -pinene, 7-epi-elinene, and camphene being the major oil components (De lira et al. 2015). *A. purpurata* dried leaf ethyl acetate and butanol extracts revealed the presence of rutin and kaempferol-3-O-glucuronide (Victorio et al. 2009). Shimoda et al. (2007) identified 6-gingerol, 8-gingerol, 10-gingerol, 4-shogaol, 6-shogaol, 10-shogaol, -pinene, 1,8-cineole, -pinene, and (E)-methylcinnamate as major phytochemical components in *A. purpurata*. The presence of alkaloids, terpenoids, flavonoids, and steroids was reported by Oirere et al. (2015) in leaf extracts of *A. purpurata*.

A. purpurata reproduces largely by clonal and sexual means, underground rhizomes produce new shoots and plants for clonal production (Hoyos, 1998). Seed germination may take three months to three years which limits sexual reproduction (De la Pea & Illsley, 2001). Seedlings are less uniform and strong than cloned shoots (Hoyos, 1998; Neal, 1965). Red ginger plant's deep red color and extended lifespan attract global flower buyers (Builes, 2003; Suárez et al. 2008). International flower markets charge US\$5.6 for red ginger stems (Wholeblossom, 2018). Lack of a reliable technology for mass-producing high-quality plant material hurts commercial manufacturing (Suárez et al. 2008). Micropropagation may enable large-scale controlled plant production. This approach reduces microbiological and insect infestations and saves space (Cardoso et al. 2018; Kane et al. 1996). Explants with pre-existing meristems have been used to micro propagate *A. purpurata* (Kochutressia et al. 2010; Vijaykumar and Namdeo, 2015).

A wide variety of biotic, abiotic, and seasonal variables influence the production and storage of secondary metabolites (SMs) in plants. Many of these secondary metabolites are challenging to chemically manufacture due to their complicated structure and high cost. Plant cell, tissue, and organ culture is an appropriate alternative for the SM production (Rao and Ravishankar, 2002) due to the many challenges associated with producing SMs through field cultivation of plants. There has been a shift in focus toward cell culture technology as a potential source of secondary metabolism modifications for the production of bioactive compounds in response to the rising demand for such molecules (Danova and Pistelli 2022). Recent years have seen a rise in the popularity of cultivating plant cells and tissues for the production of pharmaceutically important components of economic interest.

The production of defense secondary metabolites involves a series of sequential processes, which are facilitated by various events including metabolic alterations, gene activation, accumulation of jasmonic acid (JA), activation of kinase cascades, generation of hydrogen peroxide, modulation of cytosolic calcium ion levels, as well as alterations in membrane potential and cellular signaling molecules (Sliwińska, et al. 2021). The application of tissue culture techniques in optimizing culture conditions and environment for the generation of target secondary metabolites is a fast-emerging subject within plant biotechnology. *In vitro* cultures have demonstrated their potential as a sustainable alternative for meeting market demand in a production context. Plant tissue culture techniques offer an effective alternative to address the challenges faced by phytopharmaceutical enterprises in producing bioactive substances. These approaches provide a means to obtain homogenous, sterile, and suitable plant material, thus overcoming the limitations associated with traditional methods. Cultures that lack organization, such as callus and cell suspension cultures, may encounter challenges in generating substantial

quantities of secondary metabolites. Organized cultures, specifically shoot culture and hairy root cultures, serve as significant methodologies in the generation of secondary metabolites. Several studies have demonstrated that shoot cultures have comparable characteristics to hairy root cultures in terms of their ability to produce SMs, maintain genetic stability, and establish a correlation between growth and SM production (Bhadra et al. 1998; Massot et al. 2000).

A. purpurata produces flavonols from phenylalanine via the phenylpropanoid pathway. The most common flavonols are myricetin, quercetin, and kaempferol. Leroy et al. (2018) found that all three flavonols are glycosides, conjugated chemical compounds linked to sugar molecules by glycosidic linkage. An antioxidant, myricetin prevents free radical damage. (Olszowy and wianowska 2023) It inhibits cancer cell proliferation and promotes cell death and reduces inflammation (Zhou et al. 2023). Improved cardiovascular health, endothelial function, and neuroprotection was reported by (Mahmud et al. 2023; Agraharam et al. 2022). Quercetin is well-known for its use in the treatment of cardiovascular problems, cancer, allergic reactions, inflammation, allergies, and arthritis. It is also known to have antioxidant, anti-apoptotic, anti-aging, immunomodulatory, antiviral, and anti-allergic properties (Cui et al. 2022; Shabir et al. 2022). flavonoids also play a significant role in platelet aggregation, lipid peroxidation, and the enhancement of mitochondrial biogenesis (Batiha et al. 2020; Deepika and maurya 2022), Quercetin stimulates PPAR-g to improve insulin sensitivity (Saini et al. 2010). It lessens the symptoms of asthma and fever (Sreeram et al. 2021; Sangeetha et al. 2022) and has a protective effect against testicular injury and ischemic damage to the brain (Zhang et al. 2023).Kaempferol helps neurological conditions such glioblastoma, Alzheimer's, Parkinson's disease, ischemic stroke, epilepsy, major depressive disorder, anxiety disorders, and neuropathic pain (Santos 2021) and (Zhang et al. 2023; Sohretoglu and Renda 2020) Kaempferol also displayed a positive benefits on obesity and osteoporosis. Periferakis et al. (2022) reported antifungal, antibacterial, and antiprotozoal properties of Kaempferol. Kaempferol has been identified as a potential therapeutic agent for the treatment of skin diseases, as reported by Archoo et al. (2022). Kaempferol was reported to treat hormone-regulated tumors such as ovarian, breast, cervical, hepatocellular carcinoma, and leukemia (Singh. et al. 2022)

Recent research has concentrated on elicitation-based biotechnological synthesis of valuable chemicals. Biotechnology considers methyl jasmonate (MJ) and salicylic acid (SA) crucial for plant growth and stress tolerance. Chemical elicitors enhance plant secondary metabolism. Aromatic and volatile jasmonic acid ester methyl jasmonate (MJ) signals plants to alter secondary metabolism and activate defenses (Benevenuto et al., 2019). According to (Rubio-Rodríguez et al. 2021), MJ's role shows chemical elicitor potential. SA improves resistance to abiotic and biotic stressors, but most of the studies have focused on its ability to attenuate salinity, drought, cold, and UV radiation (Dey et al. 2021). Reprogramming of the secondary metabolite production pathway occurs in response to the entry of biotic and abiotic substances into plant cells via cell membrane receptors (Ramirez-Estrada et al. 2016). This effect, as shown by Shimizu et al. (2010) and Karalija et al. (2020), increases the production of secondary metabolites. Increased formation of reactive oxygen species (ROS) occurs after an elicitor treatment is applied. ROS work as signaling molecules to control gene expression, specific protein functions, and metabolic processes. Oxidative burst is the rapid increase in reactive oxygen species (ROS) generation that occurs in response to environmental stress. When reactive oxygen species (ROS) concentrations rises, plants respond by activating their defense mechanisms. Non-enzymatic antioxidants such flavonoids, phenolic acids, and anthocyanins are also a part of this defensive system (Dong et al. 2010; Sliwińska et al. 2021).

The active compounds of *A. purpurata* are concentrated predominantly in the stem and leaves. Hence, we generated shoot cultures and aimed to increase flavonoids production using an elicitation technique. The goal of the study was to see how MJ and SA affect the accumulation of flavonoids in *A. purpurata* shoot cultures. We also looked at the effect of elicitor exposure duration, which is known to affect elicitor efficiency.

Materials and Methods:

In vitro Establishment: The rhizomes of *A. purpurata* were procured from Rajadhani agro farms, located in Hyderabad, India. The rhizome, along with its buds, underwent a thorough washing process using a mild detergent and running tap water for a duration of 10 minutes. Subsequently, it was subjected to a treatment involving the application of a 0.1% w/v solution of Bavistin for a period of 5 minutes. The rhizome buds were surface sterilized by treating them with 0.1% (w/v) HgCl₂ solution for a duration of 3 minutes. Following that, the buds were subjected to an extensive rinsing procedure utilizing autoclaved double-distilled water, which was iterated ten times. The implementation of this rigorous technique was undertaken to ensure the complete eradication of any remaining HgCl₂. The rhizomes were cut to get the buds, which were then cultured on MS (Murashige and Skoog 1962) semi-solid medium containing 3% (w/v) sucrose and multiple combinations of plant growth regulators (BAP, Kn, NAA, IAA, IBA) inside a controlled laminar air flow environment.

In vitro plant propagation:

The inoculated explants were kept in a BOD incubator (Amerex, Lafayette, CA) with strict temperature and humidity regulation. A constant 25 ± 2 °C was used, along with a light intensity of 60 Mol/m²/s and a photoperiod of 16 hours of illumination followed by 8 hours of darkness (Mounika et al. 2021).

Growth Kinetics:

The aseptically established shoots were used for shoot multiplications on MS media supplemented with 1.5 BAP mg L⁻¹ + 0.5 KN mg L⁻¹ + 0.5NAA mg L⁻¹. The growth studies of shoot culture were carried out by recording the growth of the shoots for 40 days. The growth experiments were carried out using a standardized combination of 1.5 BAP mg L⁻¹ + 0.5 KN mg L⁻¹ + 0.5NAA mg L⁻¹ in MS liquid media fortified with 3% sucrose. The inoculum density was 10% w/v. The biomass and the secondary metabolite content of the samples was analyzed at an interval of 5 days for a duration of 40 day.

Production kinetics: The samples harvested from growth kinetics with a time interval of 5- days for a period of 40 days were dried and used for extraction and analysis of flavonoids viz. Myricetin, Quercetin, and Kaempferol.

Elicitor Treatment:

Methyl Jasmonate (MJ) and Salicylic Acid (SA) as signalling molecules were employed as elicitors to investigate their impact on the synthesis of flavonoids in shoot cultures of *A. purpurata*. The MJ and SA stocks were made at a concentration of 1 mM in a solution containing 50% ethanol. Prior to use, the stocks were subjected to filter sterilization using a syringe-driven filter (Millipore, Nylon, Lucknow, India) with a pore size of 0.22 µm, (Sandhya and Giri 2022). Shoot cultures that were 24 days old were subjected to treatment with MJ and SA one day prior to the attainment of maximum flavonoid production. The concentrations of MJ and SA, 50 µM, 100 µM, 150 µM, and 200 µM, were assessed to conduct elicitation tests. The shoot cultures were collected at time intervals of 24, 48, 72, and 96 h after an elicitor treatment was applied. This was done to determine the optimal duration for eliciting the highest possible response in terms of flavonoid synthesis.

Sample preparation for quantification of Myricetin, Quercetin, Kaempferol:

The shoot cultures of *A. purpurata* were utilized for the purpose of extracting and quantifying the flavonoids. Prior to extraction, the shoot samples were subjected to a drying process at room temperature for the duration of 3 days to eliminate any excess moisture. A quantity of 200mg of dried shoots was pulverized using a mortar and pestle. This was achieved by adding 20ml of methanol to the powder and subjecting it to sonication using a tip sonicator at a temperature of 60 °C for a duration of 30 minutes. The purpose of this process was to disrupt the glycosidic bonds that are present in the flavonoids. The sample was subjected to agitation for a duration of 12 hours at a temperature of 30 °C, while being shielded from light and maintained at a rotational speed of 110 revolutions per minute. Following a 12-h period, the process of centrifugation was conducted at a speed of 13,000 revolutions per minute for a duration of 10 min. Subsequently, filtration was performed using a filter with a pore size of 0.22µm. The filtrate was utilised for analysis using high-performance liquid chromatography (HPLC). The analysis of flavonoids was conducted periodically in conjunction with the development of shoot cultures and elicited shoot cultures.

HPLC analysis:

HPLC was performed to quantify the Flavonoids in the elicited and control shoot cultures according to the protocol of Idris et al. (2019). The HPLC system used in this study was Shimadzu LC 20AD series HPLC System equipped with an octadecylsilane C18 column. The HPLC model employed was Prominence model. The flow rate during analysis was set at 1 ml/min, and an injection volume of 20 µl was used. The method employed for the analysis was isocratic. The mobile phase was consisting of HPLC grade water with 0.1% orthophosphoric acid: acetonitrile in 65:35 ratio. The mobile phase was filtered to remove small particles, followed by sonication for a duration of 20 min to eliminate gas bubbles. The authentic samples of Myricetin, Quercetin and Kaempferol were procured from Sigma Aldrich, Bangalore, India. The standards were dissolved in HPLC grade methanol at a concentration of 1 mg/ml. The absorbance of the eluent was quantified using ultraviolet (UV) detector at a wavelength of 220 nm.

Statistical Analysis: The analysis of the results was conducted by utilizing triplicate data and was afterwards recorded as the mean value accompanied by the standard deviation (SD). The data comparison was conducted using SAS (Statistical Analysis System Version 9.1) by the application of the least significant difference ($p \leq 0.05$) test.

RESULTS AND DISCUSSIONS:

Invitro plant establishment: *In vitro* propagation is the crucial approach for burgeoning of medicinal plants in large scale. *A. purpurata* rhizome buds were surface sterilized, dissected and then transferred to MS media supplemented with various combinations of phytohormones viz. BAP (0.5-3.0 mg L⁻¹), Kn (0.5mg L⁻¹) and NAA (0.5 and 0.5mg L⁻¹).

The best response in terms of increase in shoot numbers and quantity of shoots was observed on MS media supplemented with a combination of 1.5 mg L⁻¹ BAP, 0.5mg L⁻¹ Kn and 0.5mg L⁻¹ NAA without any basal callus formation and stunted growth. A maximum number of 14.0±0.03 shoots were observed on this media combinations after 4 weeks of cultures. Lower concentrations of BAP and NAA were not effective for shoot multiplication response. However, BAP concentration of 1.5 mg L⁻¹ enhanced the shoot number in combination with Kn and NAA (Table 1). Higher concentrations of BAP (3.0 mg L⁻¹) had an adverse effect on shoot proliferation.

BAP (mg L ⁻¹)	Kinetin (mg L ⁻¹)	NAA (mg L ⁻¹)	No. Shoots
0.5	0.5	0.1	2.33±1.00
1.0	0.5	0.1	5.33±1.52
1.5	0.5	0.1	5.66±0.57
2.0	0.5	0.1	6.66±1.15
2.5	0.5	0.1	8.00±1.00
3.0	0.5	0.1	8.00±0.02
0.5	0.5	0.5	6.33±1.52
1.0	0.5	0.5	13.3±0.52
1.5	0.5	0.5	14.0±0.03
2.0	0.5	0.5	12.6±1.66
2.5	0.5	0.5	10.3±0.63
3.0	0.5	0.5	8.00±1.00

Table:01. Impact of varying concentrations and combinations of BAP, Kn, and NAA on the shoot proliferation from rhizome bud explant of *A. purpurata*.

Rhizome bud explants proved to be suitable explant source for shoot multiplication in *A. purpurata*. The prerequisite for *in vitro* micropropagation to attain its highest quality and productivity is pathogen-free explants, after surface sterilization. This research supports the finding of Hardjo and Krisnawan (2020), who also reported the regeneration of *A. purpurata* plantlets on MS medium augmented with 3.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA + 200 mg L⁻¹ PVP from rhizome bud explants. Previous reports in other members of the Zingiberaceae family, rhizome bud explants of *Curcuma longa* were employed for shoot multiplication with 2.5mg BAP L⁻¹ and 1.5 mg NAA L⁻¹ (Wu et al. 2015; Kim et al. 2004). Hore and Tanti (2018) has documented comparable findings in *Lasia spinosa* (Lour) where plantlets were regenerated using rhizome buds as explants on MS medium fortified with 3% sucrose, 3 mg L⁻¹ (BAP), and 1 mg L⁻¹ (NAA). In a study conducted by Shanthala et al. (2020), it was shown that *Curcuma* plantlets were successfully regenerated using rhizome buds as explants. The regeneration was accomplished by cultivating the explants on a MS medium with 2mg L⁻¹ BAP. Panigrahi and Parida (2023) reported the regeneration of *Kaempferia parviflora* using rhizome bud as an explant on MS medium containing Kn 3mg L⁻¹ and NAA 0.5mg L⁻¹. Shoot proliferation was seen when *Chrysanthemum rosc* was regenerated using rhizome buds as explants and cultivated on MS medium that has 1mg L⁻¹ BAP and 0.5 mg L⁻¹ NAA (Arora et al. 2012).



Figure:1. Multiple shoot formation from *in vitro* rhizome bud explant of *A. purpurata* on MS media supplemented with BAP (1.5 mg L^{-1}), Kn (0.5 mg L^{-1}), NAA (0.5 mg L^{-1}). (a)Rhizome bud explant (b) shoot proliferation after 15days (c)after 35 days



Figure : 2. *In vitro* grown *A. purpurata* plants on MS liquid media supplemented with 1.5 mg L^{-1} BAP + mg L^{-1} 0.5 Kn + mg L^{-1} 0.5 NAA after 25 day of culture.

Growth studies: The growth studies of the shoot cultures were investigated using optimal physical parameters. In this study, the growth medium used was MS liquid medium, which was enriched with 3% sucrose and a combination of 1.5 mg L^{-1} BAP, 0.5 mg L^{-1} Kn, and 0.5 mg L^{-1} NAA. In order to assess biomass, an inoculum density of 10% w/v (1.5g) was employed as the initial mass, and biomass was quantified in terms of gram fresh weight (gFW). The growth phase of cultures exerted a significant influence on both culture morphogenesis and flavonoid production. The relationship between the growth index (GI) and flavonoid production, measured in terms of fresh weight (g FW), was examined during a 40-day period, with data collected at 5-day intervals. The shoot cultures demonstrated a consistent growth pattern, as indicated by the increase in GI on gFW basis. This growth was seen to be directly proportional to the duration of the culture period. The highest biomass of 7.04 (gFW) was attained on the 25th day of culture. However, subsequent to this peak, the biomass exhibited a fall. (Fig.3)

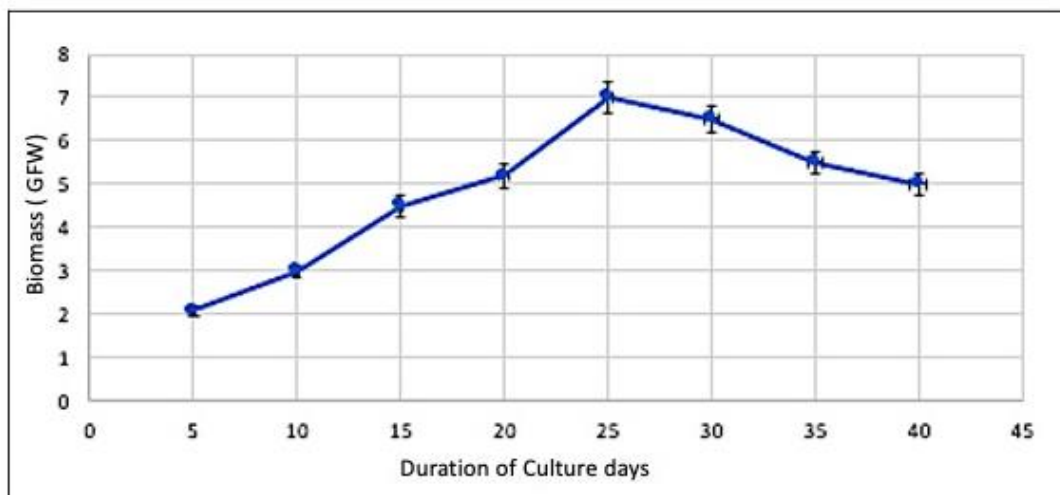


Figure: 3. Biomass increase at regular intervals in shoot cultures of *A. purpurata*.

Under optimum conditions, biomass increase is the result of rapid cell division and enlargement of new cells in terms of volume. This study of growth kinetics provides us with a better understanding of plant requirements and adaptation. The results of this investigation demonstrated that the growth curve of *A. purpurata* shoot cultures is sigmoidal, indicating changes in biomass growth at each time interval. A similar investigation for *Curcuma longa* shoot cultures for growth and generation of Curcuminoids was reported by (Sandhya et al. 2021). Bacoside production by *Bacopa monnieri* shoot cultures reached a peak at the 35th day as reported by Sharma et al. (2015). Similar findings were previously reported by Satdive et al. (2006), for ajmalicine production by shoot cultures of *Catharanthus roseus*.

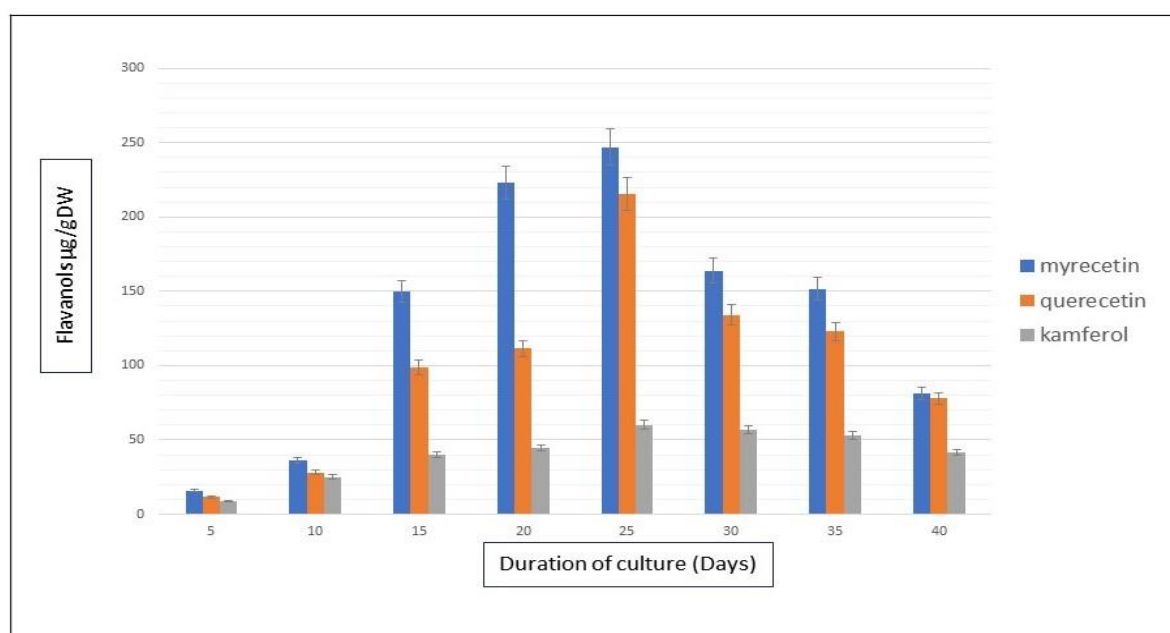


Figure:4. Production of Myricetin, Quercetin and Kaempferol from shoot cultures of *A. purpurata*.

The shoot cultures were harvested, weighed, and dried at room temperature for 3 days. The extracts were evaluated for the presence of flavonoids viz. Myricetin, Quercetin and Kaempferol. As displayed in fig.4. Shoot cultures displayed maximum content of myricetin which was $247.48 \pm 2.25 \mu\text{g. gDW}^{-1}$ followed by Quercetin which was $215.56 \pm 1.75 \mu\text{g. gDW}^{-1}$. Kaempferol was produced to lesser extent in comparison to other two flavonoids which was $60 \pm 1.16 \mu\text{g. gDW}^{-1}$. All the three flavonoids displayed similar pattern for their production which is related to the growth of the shoot cultures. The flavonoid peaks were detected in the authentic sample within the flavonoid region, with retention times of 5.2, 8.4, and 14.3.

Elicitation: Elicitation studies were carried out with two different signaling molecules MJ and SA using shoot cultures of *A. purpurata*. The effect of different concentrations of MJ and SA (50–200 μM) was evaluated on *A. purpurata* shoot cultures on the 24th day of culture in MS liquid medium a day prior to when the cultures produced maximum amount of all the three flavonoids. The controls were shoot cultures without elicitor treatment. After elicitation, biomass was recorded in terms of its Fresh weight for every 24 hours until 96 hours.

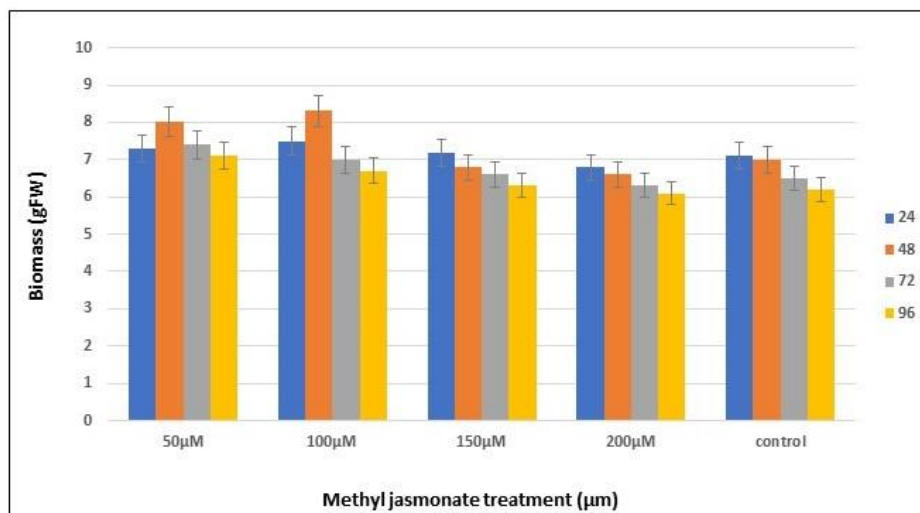


Figure: 5. Effect of MJ elicitation on biomass increase at regular intervals in shoot cultures of *A. purpurata*.

Among various concentrations of MJ, a marginal increase in biomass was noted after 24 hours. Interestingly, even a modest concentration of MJ (50 μM) exhibited a slight growth enhancement, while the most significant biomass surge was observed after 48hr of stimulation. Surprisingly, higher MJ concentrations exhibited a negative influence on biomass increase (Fig.5). A maximum biomass increase was observed $8.3 \pm 1.25 \text{ gFW}$ with 100 μM MJ concentration after 48h treatment. (Fig.5).

Kaminska et al. (2021) reported that MJ inhibits growth by reducing cell proliferation, expansion, and meristematic activity and negatively impacts biomass accumulation in *Gynura bicolor* *in vitro* cultures. In contrast, MJ in this study did not decrease growth at lower concentrations but showed a slight increase (1.1-1.3-fold) where in Shimizu et al. (2010) reported that MJ has been shown to increase *in vitro* growth, such as shoot proliferation in *Musa acuminata*, shoot length in *Ziziphora persica*, and plant height in *Artemisia annua* (Mahmood et al. 2012; Zare-Hassani et al. 2019; Alam and Albalawi 2020; Avalbaev et al. 2016).

MJ mediated elicitation when applied at different concentrations (50 μM -200 μM) for a duration of 24h-96h evoked a positive response for enhancing the production of all the three flavonoids. Substantial augmentation occurred after 48h with the 200 μM MJ concentration, with myricetin, Quercetin, Kaempferol content reaching $743.16 \pm 5.99 \mu\text{g/g DW}$, $646.15 \pm 2.15 \mu\text{g/g DW}$, $180.25 \pm 2.12 \mu\text{g/g DW}$ marking a remarkable threefold increase compared to unstimulated shoot cultures. These values were significantly higher than those of the untreated shoot cultures, which exhibited a diminishing trend in flavonoid production over time (Fig.6 a,b,c).

Lower MJ concentrations facilitated a comparatively modest rise in flavonoid production after 48 hours of elicitation. This trend was consistently observed across all three types of flavonoids. These findings underscore the effectiveness of MJ as an elicitor for fostering flavonoid production in *A. purpurata*. Remarkably, our results align with the conclusions drawn by Abdelsalam et al. (2021), who reported a staggering increase in trigonelline production with MJ (200 μM) elicitation, compared to unstimulated control shoot cultures of *Cymbopogon schoenanthus subsp.*

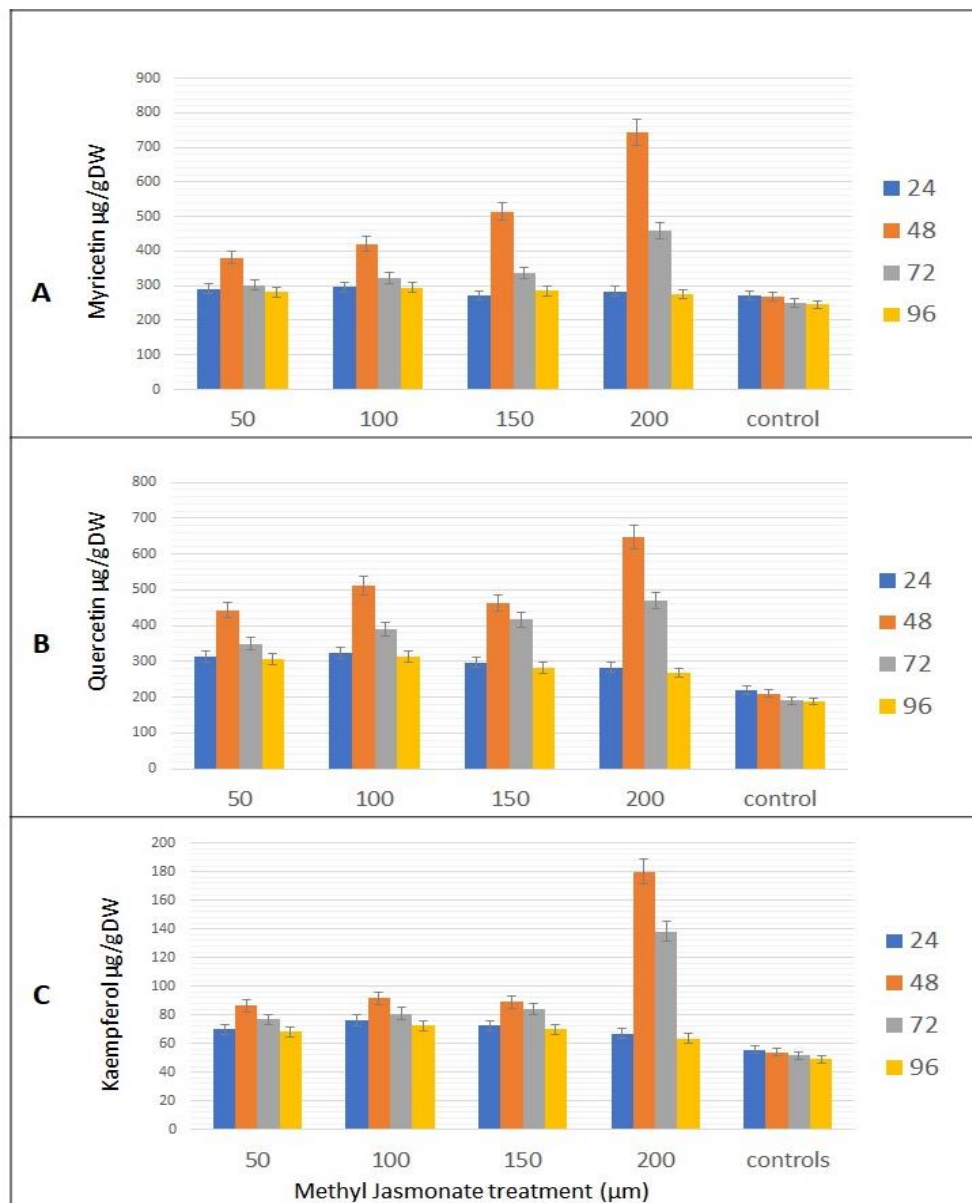


Figure:6. Effect of Methyl Jsmonate on a. Myrecetin b. Quercetin c. Kaempferol production in shoot cultures of *A. purpurata*.

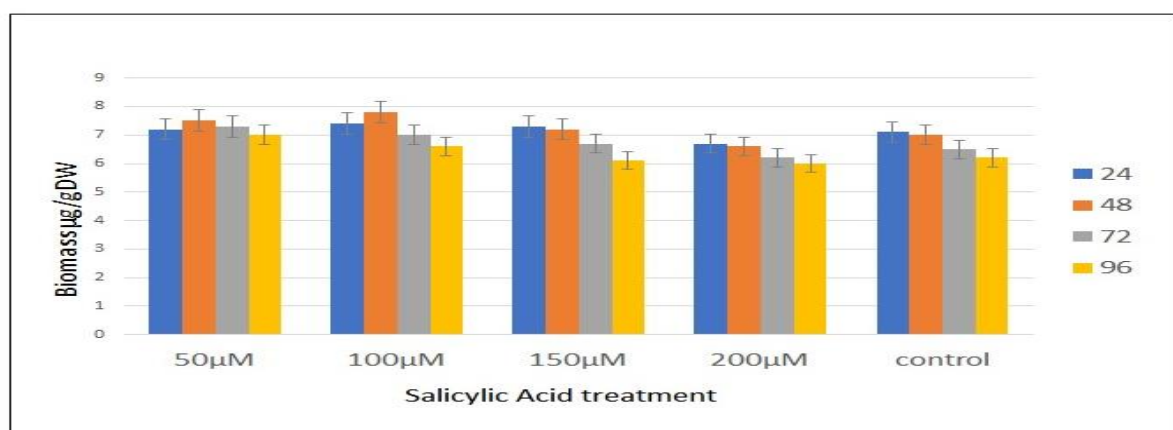
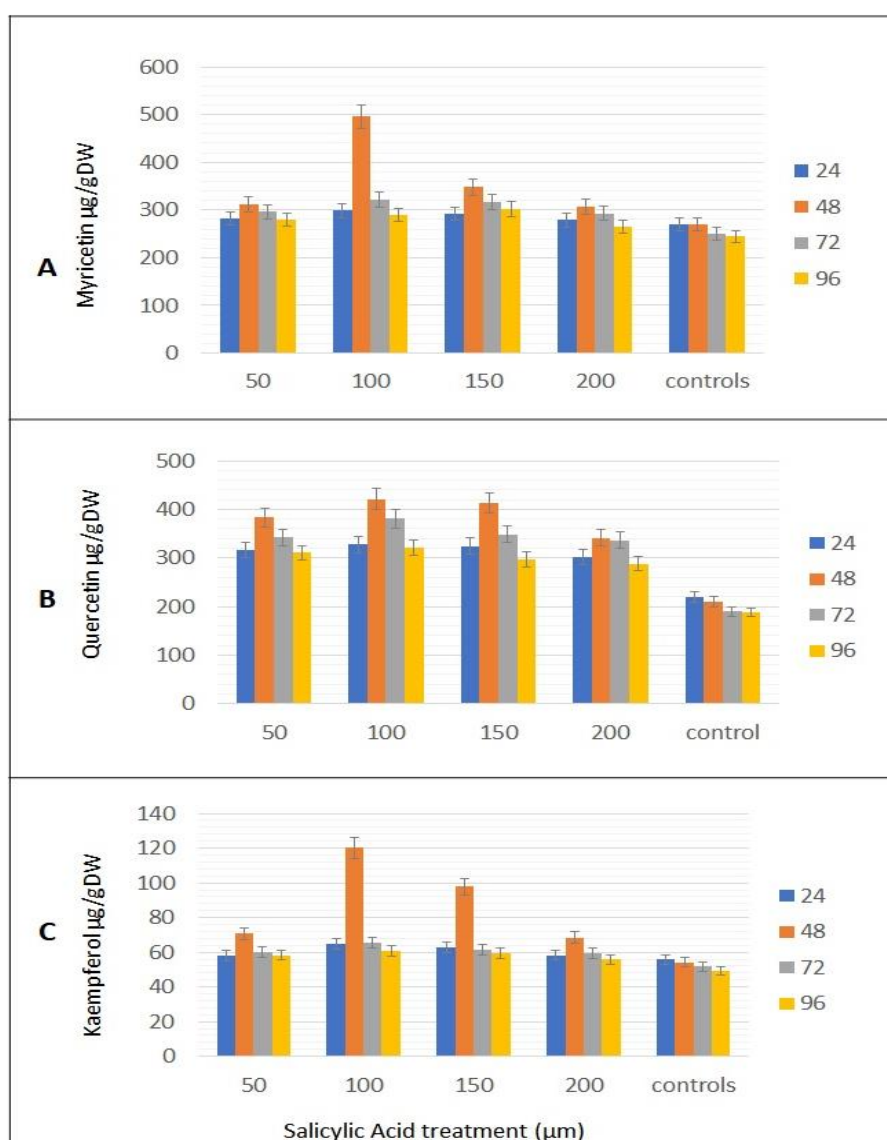


Figure: 7. Effect of Salicylic Acid elicitation on Biomass increase in shoot cultures of *A. purpurata*.

Salicylic Acid (SA) was used as an elicitor at (50 μ M -200 μ M) for shoot cultures of *A. purpurata* for enhancing the biomass. A marginal increase in biomass was observed after 24h. Remarkably, even a modest concentration of SA (50 μ M) exhibited a slight growth enhancement, while the most substantial biomass surge was observed after 48 hours of stimulation with 100 μ M of SA. The higher SA concentrations exhibited a negative influence on biomass increase. A maximum biomass increase was observed to be 7.8 ± 1.35 gFW with 100 μ M concentration SA after 48h treatment.

In shoot cultures of *H. hirsutum* and *H. maculatum*, SA had a negligible impact on biomass production when compared to the control, according to Coste et al. (2011).

SA applied at different concentrations for enhancing the production of flavonoids was analysed at an interval of 24, 48, 72 and 96h. SA at a concentration of 100 μ M after 48h elicitation displayed maximum up surge in Myricetin production which was 494.26 ± 3.18 μ g/g DW. Similarly, the same concentration after 48h also evoked maximum response for production of Quercetin, which was 431.12 ± 3.25 μ g/g DW. Maximum Kaempferol production was found to be 120.83 ± 1.21 μ g/g DW which was twofold higher in comparison to unelicited shoot cultures (Fig. 8a, b, c).

Figure:8. Effect of Salicylic Acid on production of a. Myrecetin, b.Querecetin, c.Kaempferol from shoot cultures of *A. purpurata*.

SA evoked a positive response at lower concentration, but higher concentrations also induced the synthesis of flavonoids, albeit to a lesser degree. The result of this study highlights the effectiveness of SA as a stimulus for increasing the production of flavonoids in *A. purpurata*. In a study conducted by Bayraktar et al. (2016), it was observed that the introduction of 100 μ M SA into the growth medium resulted in a significant increase in the stevioside content of *Stevia rebaudiana* plants. The stevioside content reached to be 8.89 times greater than the stevioside content in the control plants.

Our findings suggest MJ as a more effective stimulant in promoting the synthesis of three specific flavonoids in *A. purpurata* shoot cultures when applied at a concentration of 200 μ M for a duration of 48 hours. Notably, it is plausible that the reported toxicity to plant cells, as documented by Wang et al. (2001), may be a contributing factor. The application of 100 μ M SA led to an increase in flavanol accumulation, but it also resulted in a decreased shoot growth index (GI) as compared to the treatment with MJ. In this experiment, a maximum dose of 200 μ M of MJ was utilized. The observed value was 2.44 times greater than that of the control group. In a study conducted by Sivanandhan et al. (2012), it was found that exposing the culture to a concentration of 100 μ M of MJ for a duration of 4 hours resulted in a significant increase in the production of withanolide A (14-fold), withanolide A (11-fold), and withanone (12-fold) during the fifth week of culture, as compared to the control group and SA 100 μ M demonstrated the highest efficacy as an elicitor in augmenting the synthesis of withanolides, specifically withanolide A (16-fold increase), withanolide A (13-fold increase), and withanone (14-fold increase). These fold increases were 1.14 to 1.18 times greater than the control group. The exposure time to SA 100 μ M was 4 hours, and the observations were made during the 5th week of culture. According to a study conducted by Diwan and Malpathak (2010), it was shown that the application of MJ at a concentration of 10 μ M resulted in increasing furanocoumarin content in *Ruta graveolens* shoot cultures. In a study conducted by Moharramnejad et al. (2019), there was an observation made that the application of 50 μ M MJ and 100 μ M SA had a significant impact on the synthesis of stevioside in *Stevia rebaudiana* plantlets. The stevioside production in the treated plantlets was found to be around 3.8 times greater when compared to the control group. According to the results of an investigation that was carried out by Jirakiattikula et al. (2021), it was determined that the most favorable concentration for the accumulation of pinostrobin, total phenolic compounds, and total flavonoids in *in vitro* shoots of *Boesenbergia rotunda* was achieved through elicitation with MJ at a concentration of 200 μ M. Similarly, the present study also revealed the same pattern, where MJ has shown the higher response to flavonoid production (Myricetin, Quercetin and Kaempferol) when compared to SA.

Conclusion:

Medicinal plants demonstrate significant potential for the production of novel bioactive chemicals with pharmacological importance, which contributes to the advancement of future medications and other commodities. Elicitation techniques have been found to be highly effective in increasing the synthesis of these therapeutically active secondary metabolites using plant cell cultures. Biotic and abiotic elicitors have emerged as promising biotechnological strategies for augmenting the synthesis of bioactive chemicals. The present work reports for the first time the utilization of MJ and SA as elicitors in shoot cultures *A. purpurata*, with the aim of improving flavonoid biosynthesis through the application of elicitation techniques, in order to fulfil the growing market requirements for pharmaceutical products. The exploration of elicitation strategy to enhance the synthesis of phytopharmaceuticals from shoot cultures of *A. purpurata* represents a novel and promising field of investigation. The primary function of elicitors and their impact on the improvement of *A. purpurata*'s harvest index of secondary metabolites (SMs) may suggest a prospective strategy for the generation of both high-quality and high-quantity pharmaceutically active flavonoids viz. Myricetin, Quercetin, Kaempferol. It can be instrumental for metabolic engineering of flavonoid biosynthesis by transcriptome analysis followed by differential gene expression analysis utilizing high flavonoid producing elicited and unelicited shoot cultures of *A. purpurata*.

Declarations:

Conflict of interest: The author declares no competing interests.

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