Research article

A non-destructive harvesting method to produce biologically active phenolics and flavonoids of *Gnidia glauca* (Fresen.) Gilg

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ABSTRACT

Introduction and Aim: *Gnidia glauca (*Fresen.) Gilg (*G. glauca*) is an abundant source of phenolic and flavonoid compounds with therapeutic potential, despite this very few investigations conducted for enhanced phenolic and flavonoid production by suspension culture. In the present study a non-destructive harvesting method was developed for phenolics and flavonoids production in cell suspension of *G. glauca* using methyl jasmonate (MeJA) as a potent elicitor for product enhancement.

Materials and Methods: cell suspension culture was initiated to evaluate the elicitor impact on cell growth, Phytochemical extraction and screening, total phenolic and flavonoid content, antioxidant assay by 1,1 diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity, was done to evaluate, its antioxidant capacity in the methyl jasmonate elicited suspension cultured sample of *G. glauca.*

Results: Methyl jasmonate elicitation in response to 50 µM dosage showed an increase in biomass production of phenolic compounds and flavonoids after 6 days of culture incubation. Qualitative screening of phytoconstituents revealed the abundance of flavonoids and phenolics tannins, confirmed by UV-Visible spectrophotometry, TLC and HPLC analyses. *G. glauca* cell suspension culture sample showed dose dependent DPPH free radical scavenging activity compared to the respective control samples.

Conclusion: The outcome of the present study shows that *G.glauca* MeJA (methyl jasmonate) elicitation at 50µM dosage elicited the highest presence of phenolics and flavonoid with potential antioxidant capability. Considering the vulnerability of *G.glauca* as enlisted in endangered plants list, the present protocol is a non-destructive harvesting method to produce biologically active phenolics and flavonoids of the plant.

Keywords*:* *Gnidia glauca* (Fresen.) Gilg; Methyl Jasmonate (MeJA); Phenolics; Flavonoids; HPLC.

INTRODUCTION

Plants respond to stress by producing secondary metabolites, which are beneficial molecules with many biological functions. Additionally, they oversee helping plants adjust to environmental changes or may act as a defense against herbivore predation or microbial pathogen infection (1). A diversity of plant secondary metabolites is a valuable source for agrochemicals pharmaceuticals, fragrances, aromas, colors, food additives, and biopesticides. Since ancient times, nature has been a source of medicinal compounds. Intervention is still most significantly higher from herbal medicine. About 65–80% of the world population relies on health care, most often in poor nations. Herbal remedies can be made from stems, roots, seeds, fruit, leaves flowers, and bark. The therapeutic properties of these plants are found in the phytoconstituents that have various physiological impacts on the human body (2).

Gnidia glauca (Fresen.) Gilg is a member genus of Thymelaeceae, also known as Mukkude, Mukkudaka, and Datpadi by vernacular names. *G. glauca* (Fresen.) Gilg an endangered medicinal plant, known to have diverse biological properties such as antimetastatic, antidiabetic, hypoglycemic, antioxidant, hepato-protective, antiinflammation, antifungal antibacterial, and antiviral properties (3, 4).

The large class of secondary metabolites known as flavonoids is broadly distributed in plants. More than 6,500 known flavonoids are abundant and protect plants against various plant stresses (abiotic and biotic), exhibit a broad range of biological activities, and are essential in the contact of plants and their environment (5). In plants, the richness of flavonoids demonstrates their fundamental significance and their function in protecting seedlings prophylactically against infections or ultraviolet radiation (6).

In vitro micropropagation provides enormous potential to develop plant derived drugs. Consequently, cell culture methods were developed as a potential approach for investigating the biosynthesis of secondary metabolites in plants (7). The callus cell suspension cultures of plants have been playing an important role in the synthesis of active secondary metabolites, also employed to produce pharmaceuticals such as antioxidant and therapeutic

compounds (8).

MATERIALS AND METHODS

Chemicals

Elicitor methyl jasmonate (MeJA) and DPPH (Diphenyl picrylhydrazyl) were procured from (Sigma-Aldrich, St. Louis), HPLC grade solvents (HiMedia, India), glassware was procured from borosil, India NaOH, Ferric chloride ($FeCl_3$), and Lead acetate (Himedia, SRL, India) Aluminium sheets TLC (Merck, silica gel 60 F_{254}) respectively, analytical grade chemicals were used in this study (HiMedia, India) all the chemical reagents are prepared freshly when required.

Plant material and callus induction

Explants of *Gnidia glauca* (Fresen.) Gilg was collected from the forests of Western Ghats in Karnataka (India) and authenticated by plant taxonomy specialist, Department of Botany, Sri Jagadguru Chandrasekhar Bharathi Memorial College, Sringeri, Chikmagalur District, Karnataka, with voucher specimen (No. FSB-0982). Explants were rinsed with running tap water (3 times), and Tween-20 for 5 min, followed by rinsing with (0.1% (w/v) bavistin, and kept for 20 minutes at 150 rpm in a rotary shaker incubator followed by several rinses with sterile distilled water. Further, explants were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 3 or 5 min, followed by several rinses with sterile deionized water. Surface sterilized explants were trimmed and aseptically inoculated directly onto a culture tube containing a MS medium (Murashige and Skoog, 1962) supplemented with 0.2 mg/L 2-iP (isopentenyl adenine) and 2 mg/L 2, 4-D (2, 4-dichlorophenoxy acetic acid). Medium pH was maintained at 5.7 or 5.8, cultures were maintained at 16-hour light and 8-hour dark illumination cycle. Subculture of callus was done at every 3 weeks transferring aseptically onto fresh culture medium with the same composition and concentration of plant growth regulators.

Elicitor preparation

In this investigation, MeJA (methyl jasmonate) was used as an elicitor, initially dissolved in methanol and thereafter the final desired concentrations were made up with deionized water. Filter-sterilized elicitor solutions were used for supplementation in the medium at various concentrations (50 µM, 100 µM, 200 µM) for elicitation phenolics and flavonoids production. Among these three concentrations the optimized elicitor (MeJA) concentration (50 µM) was adopted for the further investigations**.**

Suspension cultures

Suspension cultures were established using a two-month-old friable leaf callus of *G. glauca* in liquid MS medium are illustrated in Fig.1. About 1 g. of callus was inoculated in a 250 mL conical flask containing 50 mL liquid medium with growth regulators 2 mg/L 2, 4-D and 0.2 mg/L 2 ip (9). The cultures were incubated in a rotary shaker incubator at 110 rpm in dark at 25°C and maintained for 24 days. On the day of inoculation, elicitors were added, and for every 6-day interval *i.e.*, on 6^{th}, 12^{th}, 18^{th}, and 24^{th} day, cells were harvested from suspension culture flask by filtration, then collected for measurement of cell growth and quantification of phenolics and flavonoids. Results are expressed

Fig. 1: Cell suspension culture of *Gnidia glauca* (Fresen.) Gilg

Qualitative screening for phenolic compounds and flavonoids and quantification

Cells obtained of suspension cultures were subjected for phytochemical extraction maceration (10), followed by qualitative analysis for the presence of phenolics and flavonoids using standard protocols and UV-visible spectroscopy analysis (11) and TLC (Thin layer chromatography) and RP-HPLC analyses (12). Total phenolics content was quantified and expressed as mg gallic acid equivalent (GAE)/g dry weight of suspension culture cell mass, and total flavonoids content was quantified and expressed as mg catechin equivalent (CE)/g dry weight (13,14).

In vitro free radical scavenging activity by the DPPH method

Antioxidant activity of *G. glauca* cell suspension extract was enumerated by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging method, butylated hydroxyanisole (BHA) was used as standard. Cell suspension culture extract of *G. glauca* elicited with 50 µM methyl jasmonate after 6^{th} day of incubation was taken at different concentrations (20-100 µg/mL) and evaluated for DPPH radical scavenging activity (15).

Statistical analysis

All the experiments were repeated for thrice under identical circumstances, data were subjected to analysis by Microsoft Excel tool, significance between two paired samples probability p-value and significance t-test was determined. Data were presented as Mean± SD.

RESULTS

Growth kinetics

Results of cell growth kinetics (Fig. 2) showed that in 6th day MJ-elicited sample showed a maximum biomass production of phenolic and flavonoids compare to untreated samples. Results are expressed in terms of dry cell weight (DCW). The 6th day with 50 µM was (5.53 ± 0.0057 g/L DCW), 12th day (3.0±0.04 g/L DCW), 18th day (2.5±0.016 g/L DCW), 24th day (1.8±0.01 g/L DCW) and in the control sample (without treatment) 6th day was (1.16±0.05 g/L DCW), for 12th day (1.13±0.041 g/L DCW), for 18th day (0.80±0.01 g/L DCW), and 24th day was (0.90±0.01 g/L DCW) respectively. Therefore, methyl jasmonate treated sample showed increased cell biomass 0.96, 1.56, 1.23 and 2.57 folds higher compared to untreated (control) sample and has significance p value ($p=0.02$) at level $p<0.05$.

Phytochemical screening for phenolic compounds

The outcomes of qualitative phytochemical screening are shown in Table 1. The abundance of flavonoids, phenolic compounds, and tannins was confirmed by qualitative analysis in *G. glauca* (Fresen.) Gilg cell suspension. Gallic acid tannins, flavonoids present are present in MeJA treated suspension sample whereas absent in untreated control sample.

Total phenolic and flavonoid content

The results of amount of phenolics and flavonoid abundant in of *G. glauca* suspension culture of elicitor treated sample reported as gallic acid equivalents and total flavonoid content was catechin equivalents respectively. Results are shown in Table 2. The highest amount of phenolic content was found to be (106.12±0.25 GAE/ mg extract.) and total flavonoids content was 153.5 ± 035 CE/mg extract) in the MeJA treated 50 µM 6th day *G. glauca* suspension culture, whereas in untreated (control) 50 µM in 6th day suspension cultured callus sample was 85.23 ± 0.18 GAE/ mg extract and 96.76 ± 0.20 CE/ mg extract respectively.

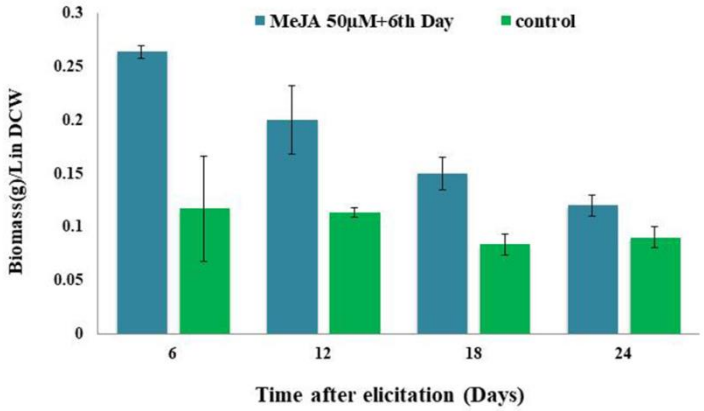

Fig. 2: Methyl jasmonate elicitation on cell growth in *G. glauca* suspension culture

Table 1: Qualitative phytochemical screening of *G. glauca* (Fresen.) Gilg cell suspension culture sample

Phytochemical test	Results	
	Control	MeJA 50µM after 6th day
I. NaOH test for flavonoids	-	+
II. Lead acetate test for flavonoids	-	++
III. $FeCl_3$ test for tannins	-	++

+ and – indicate the presence and absence of phytochemicals in the cell suspension culture sample

Table 2: Total phenol and flavonoid of *G. glauca* suspension cultured callus extract

G. glauca suspension cultured sample	Total phenol (GAE/g dry wt.)	Total flavonoid (CE/g dry wt.)
Control (untreated)	85.23±0.18	96.76 ± 0.20
MeJA +50 µM on 6th day	106.12±0.25	153.5 ± 035

Data expressed as Mean± SD; GAE: gallic acid equivalents, CE: catechin equivalents

UV-Visible spectrophotometric analysis

The results of UV-Visible spectrophotometric analysis are shown in Fig.3. The standard catechin showed absorption maxima at 281 nm, 238 nm of absorbance was 1.727, 1.565, and the control sample (without treatment) showed absorption at three different wavelengths 280 nm, 238 nm, 317 nm of absorbance was 1.727, 1.565, 0.305, while the MeJA 50µM

treated culture extract 6th day sample showed absorption at 324 nm of absorbance was (0.821) respectively. Result confirms the presence of phenolics and flavonoids in *G. glauca* suspension extract.

Thin layer chromatography (TLC) analysis

The results of thin layer chromatographic analysis (TLC) are depicted in Fig. 4, and Table 3. The characteristic bands were observed, are denoted as B-1, B-2, and B-3. The degree of retardation factor was calculated for gallic acid was 0.52, for catechin 0.58, and MeJA 50 µM 6th day culture extract sample exhibits three distinct bands and Rf value was B-10.35, B-2 0.49, and B-3 0.56 respectively, that match with standard samples in the study.

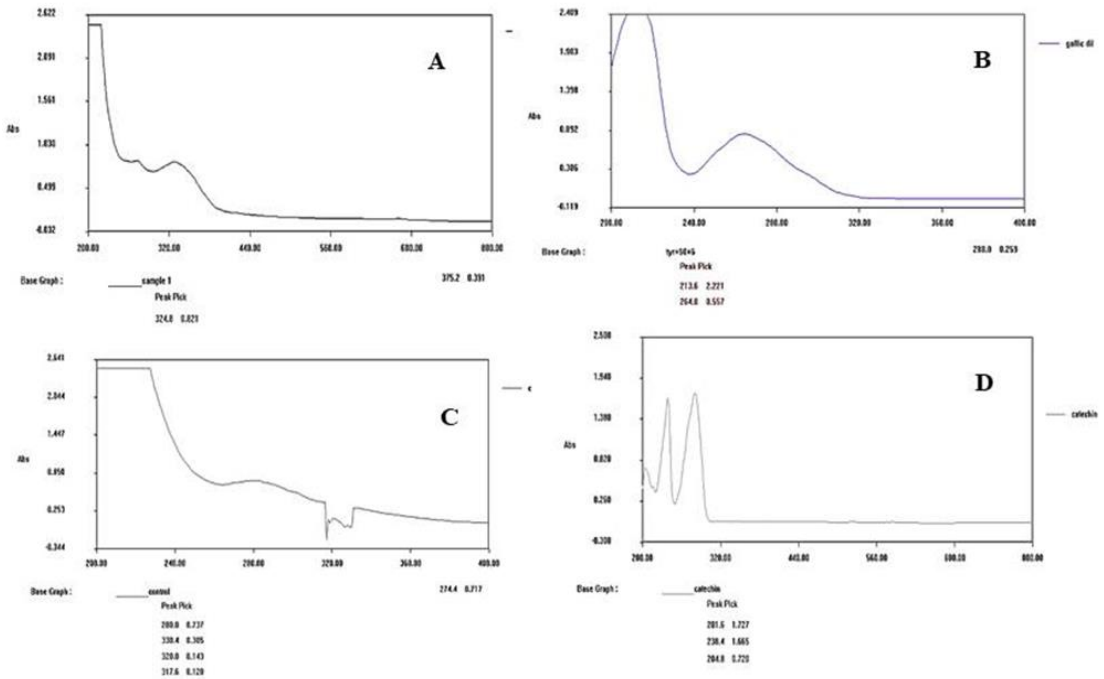

Fig. 3: UV-Visible spectrum of Cell suspension cultured callus sample of *G. glauca* (Fresen.) Gilg
A: Standard catechin; B: Standard gallic acid; C: Control; D: MeJA (methyl jasmonate) 50µM 6th day sample.

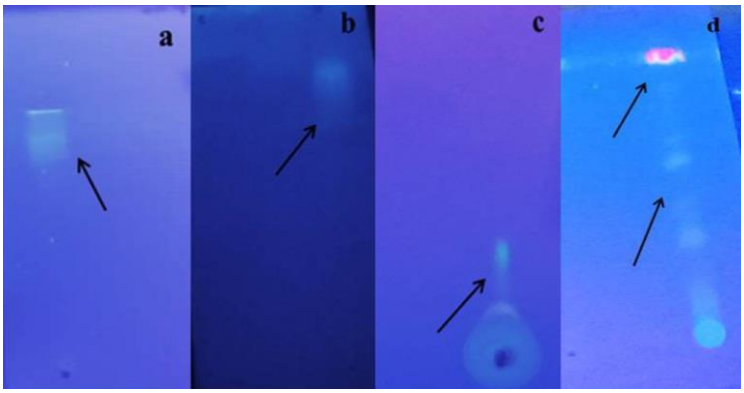

Fig. 4: TLC chromatogram of cell suspension culture extract of *G. glauca* (Fresen.) Gilg
a-standard gallic acid; b-standard catechin; c-control; d- MeJA 50µM on 6th day.

Table 3: Rf value (retardation factor) of MeJA- treated cell suspension culture of *G. glauca* (Fresen.) Gilg

Sample	TLC bands	RF value
I. Standard gallic acid	B-1	**0.52**
II. Standard catechin	B-1	**0.58**
III. Control, (without treatment)	B-1	**0.26**
IV. MeJA 50 µM treated 6th day culture	B-1 B-2 B-3	**0.35** **0.49** **0.56**

Note: B-1, B-2, B-3 are represented as distinct TLC bands and Rf value (Retardation factor value)

***In vitro* free radical scavenging activity by the DPPH method**

G. glauca (Fresen.) Gilg, methanolic extracts of cell suspension cultured samples were subjected to DPPH

free radical scavenging activity results are shown (Fig. 5) at various concentrations of (20, 40, 60, 80, and 100 µg/ml) (16). The percentage of scavenging activity for MeJA + 50 µM was 51.27 ± 0.27, 40.99 ± 0.75, 33.53 ± 0.61, 28.66 ± 0.76, and 18.57 ± 0.59 %, for standard BHA was 61.24 ± 0.26 %, 59.22 ± 0.25, 55.07± 0.30 50.66 ± 0.22, and 41.12 ± 0.31, for control sample was 17.92 ± 0.30, 24.44 ± 0.21, 28.32 ± 0.29, 31.24 ± 0.25, and 35.43 ± 0.60 and IC_{50} value was 2.03, 1.60, 1.03 respectively, the free radical scavenging activity was in the following order: BHA > Methyl jasmonate >control, the p-value is calculated by t-test p=0.0019 significance level at p<0.05.

Phenolic and flavonoid compounds quantification by RP-HPLC method

Results of phenolic and flavonoid quantification by RP-HPLC method are illustrated in Fig.6. The retention time (RT) of *G. glauca* (Fresen.) Gilg suspension-cultured samples (50 µM methyl Jasmonate treated, 6th day) were 2.624, 3.859 showing absorption at 240 nm. Compared with standard gallic acid and standard catechin to suspension sample of *G. glauca* 50 µM methyl Jasmonate treated, 6th day the quantified phenolic content was 64.4 µg/ml catechin content and gallic acid content was 83.3 µg/ml, respectively.

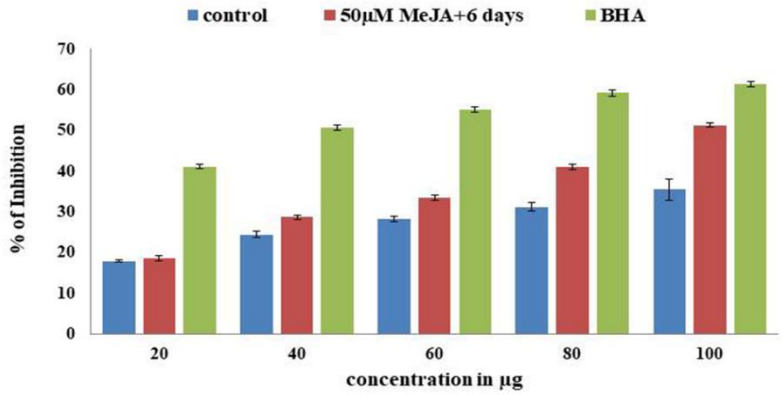

Fig. 5: *In vitro* free radical scavenging activity of MeJA elicidated suspension culture samples by DPPH method

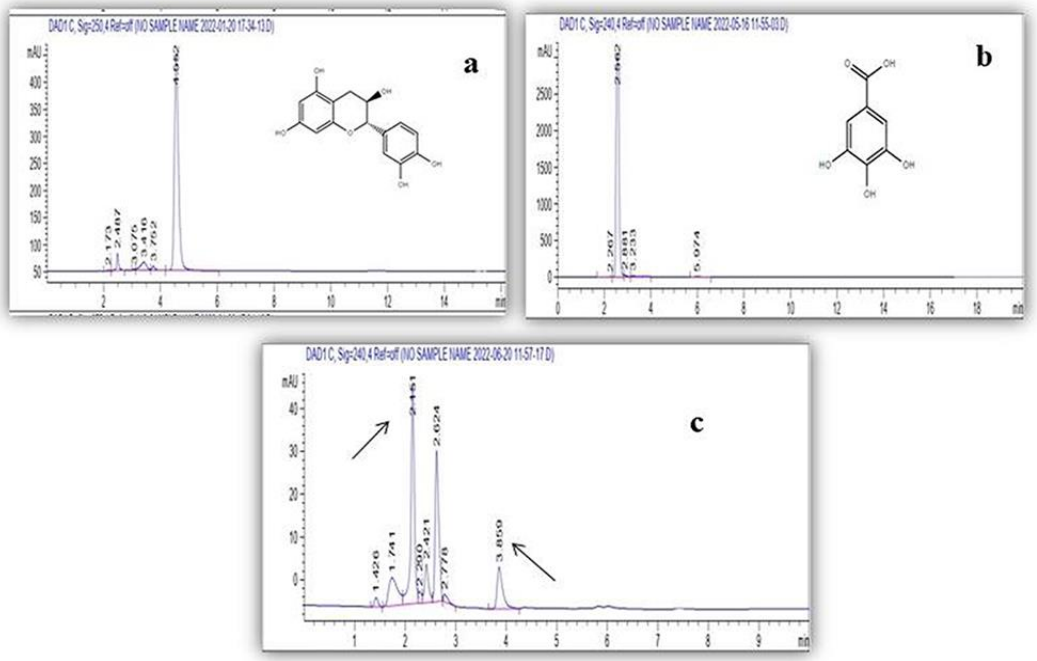

Fig. 6: RP-HPLC analysis of callus cultured flavonoid fractions of *G. glauca* (Fresen.) Gilg
a-Standard Gallic acid; b-standard catechin; c- *G. glauca* suspension elicited with 50µM MeJA for 6th day

DISCUSSION

Several investigations have proved that elicitor mediated elicitation is superior for the biosynthesis of secondary metabolites. In current investigation concentration dependent consequences of methyl jasmonate on cell propagation and flavonoid biosynthesis in the *G. glauca* cell suspension culture callus sample was examined, increasing the cell biomass implies that the elicitor effect has a positive correlation with an elicitor concentration and culture duration., the highest cell growth was observed at 50 µM concentration, which positively affects the product enhancement and revealed the dynamics of phenolic

acid and flavonoid accumulation in *G. glauca* (Fresen.) Gilg (16).

The phytochemical analysis is vital for evaluating the therapeutic potential bioactive molecules (17). In the current investigation, the phytochemical analysis of *G. glauca* (Fresen.) Gilg suspension culture results revealed the presence of phenolic and flavonoid compounds and tannins (14, 15). *G. glauca* (Fresen.) Gilg, are a rich source of phenolics and shows effective antioxidant properties due to its anion radicals, was enumerated by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging potential (18). The total amount of phenolic and flavonoid constituents was evaluated in suspension cultured callus samples of *G. glauca* (Fresen.) Gilg in which the highest phenolic and flavonoid content was observed, this may directly contribute to significant antioxidant activity and a positive interrelationship between flavonoid content and antioxidant activity.

In the current study, the quantitative analysis of phenolic compounds from *G. glauca* (Fresen) Gilg., cell suspension cultured callus samples were performed by UV-Visible, thin layer chromatography (TLC) and HPLC-DAD method, to demonstrate the presence of gallic acid and catechin content (19, 20). As a result, in methyl jasmonate-treated suspension cultured callus sample, the highest amount of phenolic and flavonoid content may positively have effect on stress tolerance in plants.

In the present study, MeJA-induced cell suspension was evaluated in *G. glauca* based on the concentration and duration exposure to the cell suspension. Cell growth was increased at a concentration of 50 µM, which induces the greatest metabolic changes to produce phenolic and flavonoid compounds. However, the current research is first report and probably become a potential or alternate strategy for understanding the effect of abiotic elicitor on *G. glauca* cell suspension, for enhancing flavonoids production.

CONCLUSION

In conclusion, the results of the current investigation confirmed that the dose-dependent of methyl jasmonate (MeJA) elicitation for determining the phenolics and flavonoids is superior based on the systematic comparative study, we determine that the MeJA elicitation 50µM on the 6th day in *Gnidia glauca* (Fresen.) Gilg, could enhance the biomass production of phenolic and flavonoid compounds. Qualitative screening for phenolic compounds and flavonoids and quantification by UV-VIS (UV-Visible), TLC and RP-HPLC are evaluated. In present study methanolic extract of cell suspension culture callus sample *G. glauca* was found to have substantially more phenolic and flavonoid constituents, the free radical scavenging activity was demonstrated, a strong positive correlation exists between flavonoid content and antioxidant activity, with this the cell suspension culture technique employed as alternative method for biomass production of phenolic acids and flavonoids. Considering the vulnerability of *G. glauca* as enlisted in endangered plants list, the present protocol is a non-destructive harvesting method to produce biologically active phenolics and flavonoids of the plant.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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