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Original Article

Isolation, Purification of Quercetin from *in vitro* Cell Suspension Culture of *Caesalpinia pulcherrima* and its Analysis by HPLC-DAD and NMR

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ABSTRACT

Background: *Caesalpinia pulcherrima,* belongs to Caesapiniaceae, is a known medicinal plant widely distributed in India and is used in traditional medicine for the treatment of various ailments. Many phytochemicals are reported from the plant as potential source of crude drug. **Materials and Methods:** An efficient and simple reproducible protocol was developed for callus production using leaf explants of *C. pulcherrima.* The combination of 2, 4-D, kin and BA, was used for the callus induction. Subsequently, cell suspension culture and quercetin synthesis from *in vitro* callus was attempted. Role of effect of elicitors (Sucrose, ABA and salicylic acid) in cell suspension culture was carried in MS medium containing 2,4-D + BA + kinetin. Flavonoids was purified, fractionated by HPLC-DAD and NMR. **Results:** 2, 4-D (2.5 mg/L), BA (2.5 mg/L) + kin (1 mg/mL) was effective for maximum callus induction from leaf explants. Significant cell suspension culture was noticed with liquid MS medium containing 2,4-D (2 mg/L)+ BA (1mg/L)+ kinetin (1.5 mg/L). Sucrose, ABA and salicylic acid (SA) at different concentrations influenced cell biomass and quercetin accumulation. The addition of ABA/SA along with sucrose was found to have no remarkable effect on cell biomass and also quercetin synthesis. However, cells cultured in the medium fortified with 45 g/L sucrose without ABA/SA showed the highest quercetin content (16.5 mg/g). Flavonoids was purified, fractionated by HPLC-DAD and NMR revealed the presence of 9 components such as quercetin, isoquercetin, quercetrin, rutin, quercetin 3-O-β-D-xyloside, quercetin 3-Oarabinopyranoside, quercetin 3-O-α-arabinopyranosyl (1→2) β-galactopyranoside, isorhamnetin 3-O-rutinoside and an unknown compound. **Conclusion:** *C. pulcherima* reveals significant synthesis of quercetin. Quercetin content recorded in cell suspension culture was significantly higher compared with *in vivo* plants grown in fields and the compounds were identified by NMR.

Key words: *Caesalpinia pulcherrima*, Callus, Cell suspension culture, Elicitors; growth hormones, Quercetin.

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INTRODUCTION

Flavonoids are polyphenolic pigments induce flower coloration, but also possess taste and toxicity. They function as messenger cascades and contribute defense against herbivory and pathogens. Flavonoids possess a general structure of 15 C atoms, with 2 phenyl rings and a heterocyclic ring, that are connected by carbon bridge.[1] The substituents in the heterocyclic ring C, and the varying forms of the ring are the lead characters used for their classification. Six subclasses are recognized among flavonoids such as flavones, flavonols, isoflavones, flavanones, anthocyanidins, and flavan-3-ols, which are commonly known as catechins. Multiple substituents can be present in the structural skeleton, with hydroxyl groups normally in 4', 5' and 7' positions.

Flavonols are universal group of the flavonoids and are reported in all plants except algae and fungi. Most flavonols can be found as O-glycosides and the common among then are quercetin, myricetin, and kaempferol. The biosynthesis of polyphenolic compounds is via shikimate phenylpropanoid pathways.

Caesalpinia pulcherrima Swartz. belonging to Caesalpiniaceae possess wide folklore medicinal uses. Leaves, flower, bark, and seeds are used in Indian medicine and distributed widely in India. The plant has been considered as a tonic and stimulant. The bark and leaf infusions are used as an abortifacient and cathartic. The plant is rich in active ingredients like caesalpin-type diterpenoids, sitosterol, pulcherrimin, lupeol, lupeol acetate, myricetin, quercetin, rutin, flavonoids, carotenoids, glycosides, peltogynoids, phenols, and steroids.[2]

In recent decades, there are many well established herbal and plant based medicinal practices like Ayurvedic medicine which are popular in many parts of the world. The World Health Organization reported that 80% of people in the devel-

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oping countries use herbals for their primary health needs.[3] About 40% of compounds used in pharmaceutical industry are directly or indirectly derived from plants because the chemical synthesis of such compounds is either not feasible and/or economically unviable. Therefore, a large number medicinal species are under threat of extinction because of their over exploitation. Tissue culture offers an effective and potential alternative of secondary metabolite production because the amount of secondary metabolites produced in *in vitro* cultures can be even higher than in parental plants.[4] Advances in plant tissue culture have enabled commercial scale production of plant metabolites. Many problems need to be tackled in tissue culture to increase the production of secondary metabolites in plant species. One of the major issues is heterogeneity of plant cells and difference in morphological characteristics of each individual cell from the others, this has a huge effect. Different types of cell cultures (suspension and hairy root) are used to transform the natural molecules like steroid, alkaloid, coumarin, terpenoid, lignan and many others flavouring compounds through biotransformation.[5] In this juncture present study is undertaken to analyze flavonoids in *Caesalpinia pulcherrima*, via cell suspension culture, elicitation of flavonoids, its purification, fractionation and its analyses by HPLC-DAD and NMR.

MATERIAL AND METHODS

Plant material

The fresh excised *Caesalpinia pulcherrima* (L.) Sw. shoot tips and young leaves were collected from their wild habitats.

Sterilization of explants

Explants were washed under running tap water for 20 min to make them free from dirt and foreign particles followed by dipping in 2% tween 20 for 10 to 15 min and then dipped in 1% Bavistin for 30 min, rinsed thrice with deionized distilled water. It was further surface sterilized using 70% ethanol for 3-5 min followed by 0.1% $HgCl_2$ for a min and then again washed with deionized distilled water thrice to remove the traces of $HgCl_2$.

In vitro culture

Sterilized explants were cultured on full, half strength MS medium fortified with 2, 4- dichloro phenoxy acetic acid (2,4-D) 0.5-5 mg/L, BA 1-5 mg/L, kinetin 0.5-2.5 mg/L and also in combination of these. Culturing conditions are maintained under 16/8 h photoperiod at 25± 2^0 C for callus induction.

Cell suspension culture

The cell suspension culture of *C. pulcherrima* was prepared by inoculating 0.5 g (fresh weight) of 9th day old cells into liquid MS basal medium containing varying concentrations of 0.5 – 2 mg/L BA, 0.5-2 mg/L 2,4-D and kin 0.5-2.5 mg/L as the cell proliferation medium.[6,7] Sterile stainless steel sieve with 850 µm pore size was used to filter the initial established cell cultures to obtain homogenous cell suspension culture. Homogenous cells or small cell aggregates that has been passed through the sieve (with diameter < 850 µm) were subsequently filtered through What man No. 1 filter paper using an air suction pump to separate the cells from the medium.

The cell cultures were placed on an shaker at 100-120 rpm in a culture room maintained at 24 ± 2°C under continuous illumination with cool white fluorescent tubes at a light intensity of 6.3-11.9 µmol photons $m^{-2}sec^{-1}$. Regularly, the cells were harvested and used as samples for subsequent studies.

Effect of sucrose

0.5 g cell biomass obtained from the cell suspension culture which was sub-cultured every 9th day in liquid MS medium fortified with 2.5 mg/L BA+ 1 mg/L 2, 4-D + 1 mg/L kin and exposed to continuous illumination was inoculated into each of the 100 ml Erlenmeyer flasks containing 20 ml liquid MS medium fortified with different concentrations of sucrose (0, 15, 30, 45, 60, 75 and 90 g/L). Six replicates were used for each sucrose concentration treatment.

Elicitation

Elicitors were employed to the suspension cells to induce the synthesis of quercetin glycosides. Physical elicitors used in this study include light (24 h), dark (24 h) and UV light treatment (45 min/day). Chemical elicitors like gibberellin (0.05- 1.5 mg/L), abscisic acid (0.05- 1.5 mg/L) and heavy metals like $CuSO_4$, and $ZnSO_4$ (25-100 µM). Organic elicitors such as coconut water (10-50%), yeast extract (0.1-0.5%), phenylalanine (100 – 500 µM) and peptone water (0.001- 0.1%). Salicylic acid (1- 100 µM) and MeJA 100- 1000 µM was also trailed. Cultures without elicitor were included as control group. Sampling was performed 6th day after addition of the elicitors up to 24th day of culture.

Cell aggregates and the liquid medium were separated as per the standard protocol. The fresh cell mass was determined after the cells were harvested. The quercetin content was determined from the harvested cells as stated below.

Effect of sucrose and elicitors combinations

0.5 g cell mass was inoculated into two diverse culture media: (a) liquid MS medium fortified with 1 mg/L BA+ 2 mg/L 2,4-D + 1.5 mg/L kin + 30 g/L sucrose (the normal sucrose content used in MS medium) and (b) liquid MS medium fortified with 1 mg/L BA+ 2 mg/L 2,4-D + 1.5 mg/L kin + 45 g/L sucrose (optimal sucrose concentration that induced the highest quercetin synthesis). SA concentration of 50 µM / 0.1 mg/L ABA was added into the medium at day zero, 3rd day and 6th day of culture. The addition of elicitors at the beginning of cell culture (0 day) was used as control. The cultures were maintained under continuous light with intensity of 6.3-11.9 µmol photons $m^{-2}sec^{-1}$ and the cells were harvested at the end of 9th day of culture. The cell mass and quercetin content and synthesis were then determined.

Quantification of flavonoids

Total flavonoids were determined by the standard protocol.[8]

Extraction, fractionation, and purification of quercetin derivatives

Cell suspension cultures were extracted with methanol at room temperature for 48 h and was filtered and concentrated under reduced pressure to yield a crude extract and was diluted in methanol water to a ratio of 9:1, and then extracted successively with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol.

Ethyl acetate and *n*-butanol fractions were separately chromatographed on amberlite XAD-16 columns (2 m × 8 cm). Aqueous methanol solutions (from 0% to 100%, with 10% increments) were used as the mobile phase, and 11 fractions were collected for each extract (fractions EA1–EA11 and B1–B11, for Ethyl acetate and *n*-butanol, respectively). The fractions were pooled and subjected to Sephadex LH-20 chromatography (30 cm × 45 mm) with methanol water (1:1) as the mobile phase.

Reverse-phase HPLC-DAD analyses

HPLC-DAD (High Performance Liquid Chromatography with Diode Array Detector) was used to analyze the chemical composition of ethyl aceate and *n*-butanol fractions described above. The mobile phase of HPLC-DAD analysis consisted of (A) 1% phosphoric acid in water or (B) 1% phosphoric acid in methanol. Gradients used were: 0–15 min 50–70% of B followed by 15–25 min 70–100% of B. The flow rate was

1 mL/min and the injection volume 20 µL. The UV–vis spectra were recorded from 254 to 400 nm, with detection at 254 and 365 nm.

Nuclear magnetic resonance (NMR) analysis

^{13}C-NMR spectra were run on a Bruker AV NMR instrument equipped with 5 mm ^{13}C operating at 500 MHZ with tetra methyl silane (TMS) as an internal standard.

Statistical analysis

Experiment was repeated three times and triplicates were maintained each treatments. All data are represented as mean ± SD. Data were analyzed by one way analysis of variance (ANOVA) at 5% probability level using SPSS software (version 16).

RESULTS AND DISCUSSION

Total flavonoid content in the flower and leaf extract of *C. pulcherrima* was 1.9 and 4.2 mg/g tissue respectively. Subsequently, the flavonoids were fractionated by HPLC-DAD. The analysis revealed a pool of fractions displaying varying proportions of compounds having UV absorption typical of flavonoids.

In vitro callus initiation was carried out with stem tip and leaf explants of *C. pulcherrima*. Maximum callus induction (39 - 96%) and highest callus proliferation (348±.48 – 1476 ±0.10mg FW) was observed in half strength MS medium fortified with 2,4-D (2.5 mg/L) + 2.5 mg/L BA and kin (1.5 mg/L) with 30 g/L sucrose for all the leaf explants compared to shoot tip (Figure 1a,b and c) (Table1).

Sheeba *et al.*[9] Studied about the callus induction caused by the effect of phytohormones on in *Physalis minima* and the best result in terms of % of callus induction (80.67) obtained on NAA (3 mg/L) using leaf as explants after 20 days of inoculation. Ashtari *et al.*[10] reported efficient callus induction and plant regeneration in *Ducrosia anethifolia* in stem tips on MS medium (2 mg/L NAA and 1 mg/L BA) and on MS medium containing (3 mg/L NAA and 1 mg/L BA) with stem, leaf and node. In *Lilium leucanthum*, the highest frequency of plant regeneration (96.7%) and the highest mean number of bulblets per scale (3.07) were obtained on MS medium fortified with 0.5 mg/L BA and 0.1 mg/L NAA.[11] Amali *et al.*[12] trailed MS medium supplemented with 2.0 mg/L of 2,4-D and 0.5 mg/L of kinetin developed embryogenic calli when supplemented with 1.0 mg/L of thidiazuron (TDZ). Lood *et al.*[13] initiated calli in *Lagenaria siceraria* with optimum concentration of hormones i.e., 2.0 mg/L 3.3BA + 1.0 mg/L NAA.

Bonyanpour and Khosh-Khui[14] induced calli in *Punica granatum* L. '*Nana*' from leaf explants with 1 mg/L BA and 1 mg/L NAA. Hasan *et al.*[15] induced calli and plant regeneration from shoot tips of *Cassia obtusifolia*. The highest percentage of callus induction was (96.6%) observed in the medium supplemented with 2.0 mg/L 2,4-D. The highest number of shoots was 5.0 found in the medium having 2.0 mg/L 2.4-D+0.2 mg/L Kin.

Hasan *et al.*[16] developed *in vitro* callus induction and regeneration from nodal explants of *Cassia alata*. Greenish friable callus was obtained from the excised nodal explants on MS medium supplemented with 1.5 mg/L 2,4-D within 20 days of inoculation. Tan *et al.*[17] studied the effect of plant growth regulators on callus, cell suspension and cell line selection for flavonoid synthesis from *Centella*. The highest percentage of callus induction (86.67%) was observed in MS medium fortified with 2.0 mg/L 2,4-D while highest biomass yield (0.27 g dry weight culture^{-1}) was obtained in the combination of 2.0 mg/L 2,4-D and 1 mg/L kinetin in MS medium.

Aghaei *et al.*[18] compared different plant growth hormones on callus induction of stem explants in *Pistacia atlantica* subsp. Kurdica. The high efficient callus formation was observed in the medium containing different concentration of 6-BA individually. The lowest and highest percentage of callus induction were in the treatment of BA 2 mg/L with NAA 1mg/L (40%) and 6BA 1mg/L (85%), respectively. Song-Chol Mu and Gwan-Sim Mun[19] developed an efficient callus proliferation system for *Rheum coreanum*. The callus was successfully induced from rhizome explant on MS medium containing 2.4-D (0.2–0.3 mg/L). In the MS medium supplemented with a combination of BA (2 mg/L) and NAA (0.2 mg/L), single NAA (0.5 mg/L), or IBA (0.5 mg/L), a higher number of shoot, root and plantlets was achieved. All these reports confirm that the callus initiation varies among plants with respect to hormonal

Table 1: Effect of different combinations of phytohormones on callus induction (%) and callus fresh weight (mg) and callus morphology of *C. pulcherima*

Hormonal combinations	% callus induction	Callus proliferation mg FW	Nature of callus
2,4-D			
0.5	42	443±0.8	Cream friable
1	49	487±0.84	Cream friable
2	65	669±1.3	Green friable
3	54	534±0.77	Cream friable
4	40	400±0.60	Cream friable
5	39	348±.48	Cream friable
BA			
1	39	351±0.60	Cream friable
2	58	587±0.52	Cream friable
3	50	502±0.4	Cream friable
4	44	447±0.24	Cream friable
5	42	440±0.21	Cream friable
Kin			
0.5	60	601±0.73	Cream friable
1	64	639±0.8	Green friable
1.5	69	702±0.9	Green friable
2	43	448±0.6	Cream friable
2.5	40	400±0.4	
2,4-D +BA			
1 + 1	55	547±0.46	Green friable
2 + 2	63	632±0.5	Green friable
2.5 + 2.5	68	677±0.58	Green friable
3 + 3	50	490±0.3	Cream friable
2,4-D+ Kin			Cream friable
1 + 0.5	58	590±0.49	Cream friable
2 + 1	66	678±0.56	Whitish Cream friable
2.5 + 1.5	70	786±0.6	Cream friable
3 + 2	59	589±0.5	Cream friable
2,4-D+ Kin+ BA			
1 + 0.5 + 1	76	1112±0.8	Green friable
2 + 1 + 2	89.6	1234±0.9	Green friable
2.5 + 1.5 + 2.5	96.3	1476±0.10	Green friable
3 + 2 + 3	71	1095±0.98±	Green friable

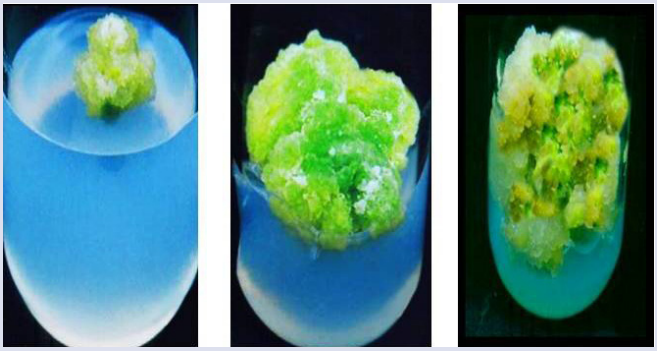

Figure 1a,b,c: *In vitro* callus induction from leaf explants of *C.pulcherima* showing green creamy friable type.

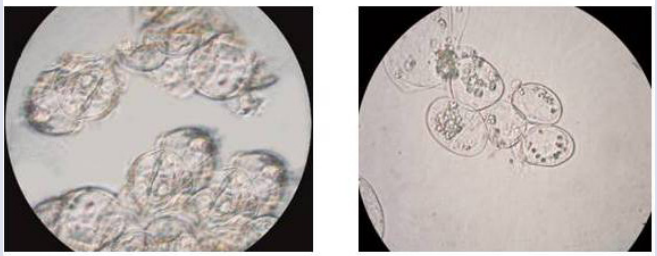

Figure 2: Viable cells from suspension culture of *C.pulcherima*

Table 2: Effect of different combinations of 2, 4-D + BA + kin on cellular biomass (g FW) of *C. pulcherima*

Hormonal combinations	Cell biomass (g FW)
2,4-D + BA + kin	
0.5 + 0.5 + 0.5	3.7±0.08
0.5 + 1 + 0.5	4±0.10
1 + 0.5 + 1	4.6±0.13
1 + 1 + 1	6±0.2
1.5 + 1.5 + 1.5	5.8±0.3
1.5 + 1 + 1.5	6.9±0.36
2 + 1 + 1.5	7.66±0.45
2 + 1.5 + 2	6.2±0.48
2 + 1.5 + 2.5	5.7±0.38

combinations and explants. Subsequently, the fresh harvested callus was proceeded to initiate cell suspension cultures.

Based on the growth index, the cell culture of *C. pulcherrima* could be well maintained with an initial inoculum of 0.5 g of fresh cells inoculated into 20 mL of liquid MS medium contain 2 mg/L 2, 4-D + 1 mg/L BA + 1.5 mg/L kin in 100 mL Erlenmeyer flasks and placed on a rotary shaker at 100 - 120 rpm. Remarkable cell suspension culture was recorded with these combinations (Figure 2 and table 2). Generally, plant cell suspension cultures fail to detach completely after division, and forms multi-cellular aggregates.[20] During subculture, a sterilized stainless steel sieve with 850 µm pore size was employed to separate small cell clumps from the larger clumps. Smaller clumps were selected for the quercetin analysis

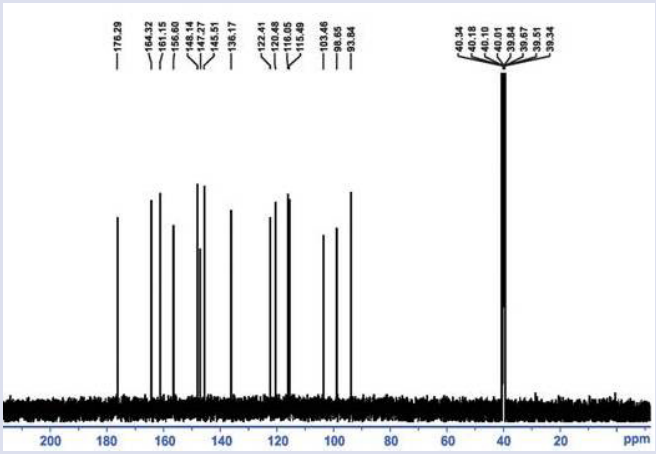

Figure 3: CNMR profile of quercetin and its derivatives of *C.pulcherima*

Table 3: Effect of different combinations of 2, 4-D + BA + kin on quercetin content (mg/g) of *C. pulcherima*

Hormonal combinations	Quercetin content (mg/g)
2,4-D + BA + kin	
1 + 1 + 1	1.24±0.04
1.5 + 1.5 + 1.5	3.1±0.08
1.5 + 1 + 1.5	5.65±0.13
2 + 1 + 1.5	6.98±0.3
2 + 1.5 + 2	3.9±0.39
2 + 1.5 + 2.5	1.5±0.06

because as cell aggregates enlarge, cells seated in the interior of a large cell clumps are not properly exposed to nutrients, light, oxygen, and other micro-environmental factors compared to younger peripheral cells. This physico-chemical gradient in turn alters the cell proliferation and phytochemical production in the culture system.

The cells cultured in MS medium fortified with 1mg/L BA along with 1 mg/L 2,4-D and 1mg/L Kin exhibited the lowest quercetin content (1.24 ±0.04 mg/g), whereas cells cultured in half MS medium fortified with 2 mg/L 2, 4-D + 1 mg/L BA + 1.5 mg/L kin produced the maximum quercetin content of 6.98 mg/g DW on 24th day (Table 3). The content of quercetin increased with the growth phase of the suspension culture. Positive correlation was noticed with biomass and quercetin content in the cell cultures.

Effect of Sucrose

Different sucrose concentration influenced the cell biomass and quercetin synthesis in cell suspension cultures of *C. pulcherrima*. However, cultures without sucrose (control) showed cell death. Medium fortified with 15 g/L sucrose triggered fresh cell mass (6.8±0.4FW) which was different from the cells cultured in medium fortified with 45 g/L sucrose i.e., optimal dried cell weight was noticed from the cells cultured in the medium fortified with 45 g/L sucrose as compared to medium fortified with 15 g/L (Table 4). Meanwhile, when the level of sucrose was further enhanced (60 g/L or more), there was a reverse trend in cell growth was noticed.

The cells cultured in medium fortified with 15 g/L sucrose yielded the lowest quercetin content (4.4±0.3). However, the cells cultured in medium fortified with high sucrose dose (90 g/L) yielded 2.5±0.04

Table 4: Effect of different concentrations of sucrose on cell biomass and quercetin content (mg/g) of *C. pulcherima*

Sucrose content (g/L)	Cell biomass (g FW)	Quercetin content (mg/g)
0	1.2 ± 0.09	0.33±0.02
15	6.8±0.4	4.4±0.3
30	8.9±0.6	11.1±0.6
45	10.4±0.8	16.5±0.8
60	5.2±0.3	5.8±0.36
75	4.2±0.38	3.9±0.24
90	3.2 ±0.2	2.5 ±0.04

mg/g quercetin content. Meanwhile, its cell biomass yield was marginal (3.2±0.02 g FW) and this resulted in to reduced quercetin. The amount of quercetin produced in the cells cultured in medium fortified with 45 to 75 g/L sucrose achieved remarkable level i.e., 16.5±0.8-3.9±0.24 mg/g, each of which was soundly different from the other experimental cell cultures sets. Among these three treatment, culture fortified with 45 g/L sucrose showed the highest growth (10.4 ±0.8 g FW) with optimal quercetin content, hence the addition of 45 g/L sucrose into the cell culture of *C. pulcherrima* could be used for increasing quercetin level (Table 4).

The relation between quercetin content and sucrose concentration may be due to the osmolytic property of sugar i.e., the increased sucrose content leads to exosmosis in the cell cultures and the supply of 15 g/L sucrose was found to be enough for cellular growth and carbon energy source for metabolism. However, enhanced sucrose level (60 g/L or higher) showed a reverse trend in cell growth of *C. pulcherrima* (ends in to plasmolysis). Similar trend was reported by Ramirez-Estrada *et al*.[21] by fortifying the culture medium of cell suspension culture with increased sugar content resulted in reduced cell growth. This may be due to the increased osmotic potential in the cell or the high viscosity of the medium which in turn inhibit the normal nutrient uptake. However, Wang *et al*.[22] stated that if a medium contain all the nutrients in excess, an increase in sugar concentration could result in a proportional increase in cell biomass. Experimenting on *Hypericum perforatum* cell cultures, they reported that growth rate increased with increase in sucrose level in the medium (15, 30, 45 and 60 g/L) for cultures with initial inocula of 15 and 50 g/wet cells/L. The present results substantiate that higher sucrose level fortified into the culture medium reduce cell biomass due to the increase of osmotic potential which subsequently reduce the nutrient uptake.

The cells culture fortified with 15 g/L sucrose exhibited the lowest quercetin content. However, the culture fortified with 90 g/L sucrose yielded decreased cellular biomass and there by the synthesis of quercetin. It is plausible that the high level of sucrose could be related to osmotic stress which results in to cell death as supported by Zhao *et al*.[23] and hence lower quercetin synthesis in *C. pulcherrima* cell cultures.

Meanwhile, the cell culture medium fortified with 45, 60 and 75 g/L sucrose showed high quercetin synthesis. Therefore, sucrose level in the culture medium should not be exceeded 75 g/L for optimal results. Wang *et al*.[22] found that for cell suspension culture of *Hypericum perforatum,* the medium that produced higher cell densities had higher sucrose levels as compared to the medium that produced higher anthocyanin accumulation. This observation was the reverse for cell suspension culture of *C. pulcherrima,* whereby the medium that enhanced quercetin synthesis had higher sucrose levels and biomass production. A relatively increased concentration of sucrose was also reported to be favorable for flavonoid production in *Fagopyrum tataricum* cell culture[23] the ajmalicine, serpentine, and quercetin in *Citrullus colocynthis* cell cultures[24] the phenol production of *Calligonum polygonoides*[25] the flavonoid accumulation in suspension cultures of *Andrographis Paniculata*[26] as well as anthocyanin production of grape cells[27] and *Camptotheca acuminate* cells.[28] Phytochemical synthesis could be induced to a certain level of osmotic stress caused by higher sucrose concentration.[23] The positive effects of osmotic potential (-0.5 to 0.9 MPa), triggered by increased sucrose concentrations in medium with anthocyanins accumulation was correlated in grapes cell cultures.[29]

Elicitation

After standardization of the cell suspension cultures to produce quercetin in optimized MS medium, application of elicitor and precursor were carried to enhance quercetin synthesis. UV-B rays (45 min treatment) increased marginally the yield of quercetin. *C. pulcherrima* in cell suspension cultures (3.4 ±0.24 mg/g DW) on 18th day which showed a significant accumulation on 24th day under physical elicitation by UV light. Meanwhile, light (24 h) or dark (24 h) treatments result no significant changes in the quercetin level in the culture medium.

Similarly, no sound quercetin synthesis was noticed with organic elicitors like peptone water and yeast extract. Meanwhile, among organic elicitors, MS media fortified with coconut water (10 - 50%) showed increased quercetin biosynthesis in *C. pulcherrima* (5.2 ±0.36 mg/g DW) cultures.

The addition of phenylalanine as precursor at lower dose enhanced quercetin content of cells in the plants, which ranged from 3.7 ±0.48 – 10.6 ±0.86 mg/g DW. Chemical elicitors, which include GA, ABA and SA at different concentrations, were successful in inducing quercetin biosynthesis in cell suspension cultures of *C. pulcherrima* (6.2±0.5- 14.6 ±1.0 mg/g DW). Impact of heavy metals was also analyzed, zinc sulphate and copper sulphate at various doses showed slight rise in quercetin accumulation (2.4±0.3 - 6±0.4 mg/g DW), but the content was lower than that of SA or ABA treatments. MeJ did not showed remarkable increase in quercetin production in cell cultures.

Regardless of the parameters, the most promising effect on quercetin synthesis in cell suspension cultures was shown by chemical elicitors, SA (50 µM) or ABA (0.1 mg/L) (14.6±1.0 mg/g DW and 12.8±0.9 mg/g DW, respectively) (Table 5).

The present results were in consistent with Wang *et al*.[21] who reported a positive correlation between MeJA and flavonoid synthesis in *Hypericum perforatum*. Similarly, Thanh *et al*.[30] showed that addition of 200 µM MeJA considerably increased the ginsenoside content in *Panax ginseng* while, with the increasing concentration (above 200 µM) reduced the ginsenosides content. Bharati and Bansal[31] reviewed that the increased flavonoid caused by MeJA in cell cultures was not always due to increased biomass growth but also by the induction of the enzymes to its synthesis. But in *C. pulcherrima* cell culture, since the addition of MeJA did not increase the cell biomass and quercetin synthesis. As reported by El-Nabarawy *et al*.[32] Jasmonic acid and its derivatives play a role in the signal cascade pathway of enzymes catalyzing secondary metabolite synthetic pathways.

Effect of sucrose concentration and day of elicitation by GA/ABA

The results clearly showed that culture medium fortified with 45 g/L sucrose and 0.1 mg/L of ABA or 50 µM GA was not significantly different from the cultures grown in their absence. Cell biomass was also not influenced by the addition of these elicitors at different days into the culture media with 45 g/L sucrose. However, the amount of cell biomass produced was significantly lower than using the culture medium containing 30 g/L sucrose. Cell proliferation medium containing 30 g/L or 45 g/L sucrose with or without the addition of these elicitors did not affect quercetin biosynthesis. However, cells cultured in the medium fortified with 45 g/L sucrose without these showed the maximum quercetin content (Table 4). The reason may be the different feeding time did not influence the synthesis of quercetin. Zhang *et al*.[33] reported a controversial opinion i.e., addition of jasmonic acid at different feeding time regulates the anthocyanin accumulation in cell suspension cultures of *Vitis vinifera*. Sajjalaguddam and Paladugu[34] noticed application of phenylalanine enhances quercetin content in *in vitro cultures* of *Abutilon*

Table 5: Effect of different concentrations of elicitors on quercetin synthesis (mg/g) of *C. pulcherima*

Elicitors combinations	Quercetin synthesis (mg/g)	Elicitors combinations	Quercetin synthesis (mg/g)
UV	3.4±0.24	25	5±0.3
Light (24h)	2.2±0.2	50	6.3±0.4
Dark (24h)	1±0.07	75	6.0±0.4
Yeast extract (%)		100	5.7±0.34
0.1	1.7±0.06	GA (mg/L)	
0.2	1.8±0.07	0.05	5.2±0.36
0.3	1.8±0.07	0.1	5.9±0.4
0.4	1.7±0.06	0.5	6.2±0.5
0.5	1.5±0.05	1	7.4±0.58
Peptone (%)		1.25	10.6±0.7
0.001	1.9±0.10	1.5	10.4±0.69
0.025	2.2±0.14	ABA (mg/L)	
0.05	2.4±0.17	0.05	10.2±0.69
0.075	2.0±0.25	0.1	12.8±0.9
0.1	1.7±0.24	0.5	10±0.8
Coconut water (%)		1	10±0.8
10	1.6±0.2	1.25	8.7±0.76
20	2.0±0.3	1.5	7.4±0.66
30	2.6±0.35	SA (µM)	
40	5.2±0.5	1	10±0.8
50	4.3±0.4	10	11.7±0.9
CuSO4 (µM)		25	12±0.94
25	2.4±0.3	50	14.6±1.0
50	4.1±0.46	75	12.1±0.94
75	5.3±0.56	100	9±0.78
100	5.8±0.58	MeJA (µM)	
Phenyl alanine (µM)		100	3.3±0.15
100	3.7±0.48	200	4.0±0.18
200	4.4±0.5	300	5.1±0.2
300	6.2±0.6	500	3.7±0.1
400	10.6±0.86	750	3.1±0.08
500	9.7±0.78	1000	2.8±0.04
ZnSO4 (µM)			

indicum. Masoumian *et al.*[35] evaluated the effect of precursors on flavonoid production in *Hydrocotyle bonariensis* callus tissues. Jedinak *et al.*[36] compared flavonoid production in plant tissue cultures using biotic and abiotic factors. Shinde *et al.*[37] optimized production of isoflavones in cell cultures of *Psoralea corylifolia* using elicitation and precursor feeding.

The ^{13}C-NMR spectrum showed carbonyl group at 176.2 ppm and aromatic carbon group from 93.8-164.3 ppm (Figure 3.). The corresponding ^{13}C NMR peak positions for isolated compound were showed resemblance with the pure quercetin which was also confirmed by previous literatures. 93.8, 98.6, 103.4, 115.4,116.0 (*Ar-C*) , 120.4, 122.4, 136.1, (Ar-C) , 145.5, (Ar-C) , 147.2, (Ar-C) , 148.1, (Ar-C) , 156.6, (Ar-C), 161.1, (*Ar-C*) , 164.3, (Ar-C) and 176.2, (*Ar-C=O*) . Thus, it can be confirmed that the isolated compound is found to be quercetin. Structural characterization of purified compounds using NMR analysis showed the presence of nine compounds which includes, quercetin, isoquercetin, quercetrin, rutin, quercetin 3-O-β-D-xyloside (Reinutrin), quercetin 3-Oarabinopyranoside (Guajaverin), quercetin 3-O- α-arabinopyranosyl (1→2) β-galactopyranoside, and Isorhamnetin 3-O-rutinoside (Narcissoside) (Figure 3).[38,39,40] Besides the expected products one unknown quercetin glycosides from *in vitro* cultures were noticed by NMR analysis in the plants, of which the biological significance remains to be explored.

CONCLUSION

Quercetin is a naturally occurring flavone found in many medicinal species in the form of quercetin glucosides. Quercetin possesses multiple therapeutic values. *In vitro* culture offer valuable tool in enhancing the synthesis of secondary metabolites. The present investigation reveals an

optimal protocol for the synthesis of quercetin in *C. pulcherima*. Quercetin content recorded in cell suspension culture was significantly higher compared with *in vivo* plants grown in fields. Further, the compound was identified by NMR analysis. Future studies are warranted to fractionate the lead molecule, its purification and evaluation of its biological potentialities.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest pertaining to this work.

ABBREVIATION USED

HPLC- DAD: High-Performance Liquid Chromatography with Diode-Array Detection; **NMR:** Nuclear Magnetic Resonance; **2,4-D-2,4:** Dichlorophenoxyacetic acid; **BA:** Benzyl Amino Purine; **Kin:** Kinetin; **NAA:** Naphtalic Acetic Acid; **TDZ:** Thidiazuron; **IBA:** Indole-3-butyric acid; **DW:** Dry weight; **FW:** Fresh weight; **MeJA:** Methyl jasmonate; **ABA:** Abscisic acid; **SA:** Salicylic acid; **$HgCl_2$:** Mercuric chloride; **$CuSO_4$:** Copper sulphate; **$ZnSO_4$:** Zinc sulphate.

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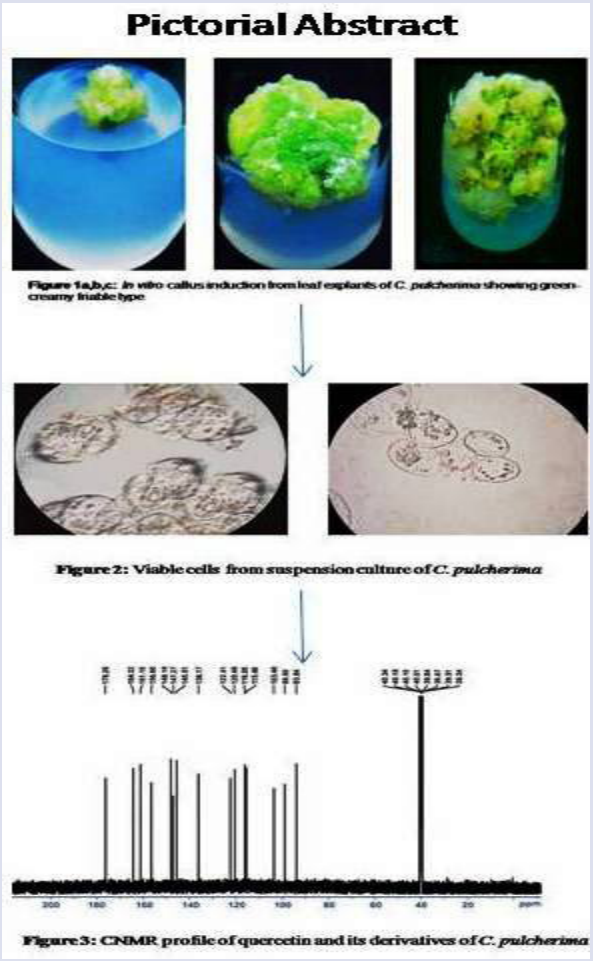
GRAPHICAL ABSTRACT



SUMMARY

- The present investigation reveals an optimal protocol for the synthesis of quercetin in *C. pulcherima.*
- Quercetin content recorded in cell suspension culture was significantly higher compared with *in vivo* plants grown in fields.
- The compound was identified by NMR analysis and showed the presence of nine compounds which includes, quercetin, isoquercetin, quercetrin, rutin, quercetin 3-O-β-D-xyloside (Reinutrin), quercetin 3-Oarabinopyranoside (Guajaverin), quercetin 3-O- α-arabinopyranosyl (1→2) β-galactopyranoside, and Isorhamnetin 3-O-rutinoside (Narcissoside)

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