Optimized conditions for growth kinetics and production of pharmaceutically important Kaempferol from *Alpinia purpurata* Cell suspensions

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Abstract
Alpinia purpurata (Red ginger), a gold mine for future therapeutics belonging to the family Zingiberaceae is widely cultivated in the Asian sub-continent. Kaempferol, the major constituent of A. purpurata has been linked to many biological properties. The drug yielding potential of A. purpurata is largely due to the presence of flavonoids rutin and kaempferol with a number of therapeutic properties viz. antioxidant, anti-cancer, neuroprotective and cardio protective properties.

This study elaborates on in vitro establishment of A. purpurata and initiation of cell suspensions from the callus derived from rhizome explants. The callus obtained on MS media was supplemented with 2,4-D, BAP and Kn at 2mg/L, 0.5mg/L and 0.5mg/L respectively and was further used for initiation of suspensions. Maximum growth was obtained using optimized parameters like pH5.8, temperature of 25°C, agitation of 120rpm and 4% sucrose. Under standard culture conditions, a maximum of 13.51gFW biomass was obtained on 21st day of culture at 4% sucrose concentration. The A. purpurata suspensions when analyzed for growth along with kaempferol content were found to be maximum on the 24th day (35.285µg gFW^{-1}), indicating cell growth and flavonoid biosynthesis to be isochronous. Based on the data obtained in the present study, the suspensions of A. purpurata can be further manipulated to produce Kaempferol at higher levels.

Keywords: *Alpinia purpurata*, Callus, Cell Suspension Cultures, Kaempferol, HPLC.

Introduction

Higher plants produce approximately 1,00,000 secondary metabolites that can satisfy the pharmaceutical needs of all life forms. Based on the WHO report, 80% of the world population is dependent on traditional medicinal systems[19] in the treatment of various chronic diseases because of their safety (negligible side effects) along with failure of synthetic analogues. In order to meet the existing demand for plant based drugs, the collection of valuable flora from wild habitats is leading to reckless exhaustion thereby endangering the ecological balance. Also, due to their extreme complex nature with stereospecificity, synthetic production of these valuable phytochemicals is highly uneconomical.

Hence, tissue culture techniques like genetic transformations, cell cultures, elicitation etc. provide a reliable and continuous source for production of various phytochemicals[8]. One such medicinally significant plant is *Alpinia purpurata* (Red ginger), belonging to the family *Zingiberaceae*.

It possesses a wide range of biological properties like immunomodulatory[2], antioxidant[12], anticancer[5] etc. The parts of this plant are highly aromatic because of the secondary metabolites present in the form of essential oils. Kaempferol, a natural flavonoid present in *A. purpurata*, exhibits many pharmacological activities like anti-inflammatory, antioxidant and anticancer activity[3].

The production of secondary metabolites in intact plants is negligible and tedious, hence suspension cultures have come to the fore, as they provide a potential alternative for production of desired compounds and can be further manipulated for higher production thereby establishing a commercial process. Because of its high medicinal value, Kaempferol and its derivatives have been successfully extracted from different plants like *Crocus sativus*[5], *Justicia spicigera*[4], *Pteridium aquilinum*[6], *Acacia nilotica*[16] and *Rosa rugosa* Thunb[20]. The present study involves optimizing Kaempferol production from *A. purpurata* suspensions by standardizing the various parameters like pH, media, temperature, agitation etc.

Material and Methods

***In vitro* Establishment:** *A. purpurata* plants were procured from Rajadhani agro farms, Hyderabad. Explants were washed thoroughly under running tap water for 10mins followed by surface sterilization with 0.1% $HgCl_2$ (mercuric chloride) for 8 min and rinsed with sterile distilled water. Different plant parts like leaf, stalk, rhizome bud and root were excised and cultured on MS media supplemented with different combinations of phytohormones viz. BAP (6-Benzylaminopurine), 2-4D(2,4-Dichlorophenoxyacetic

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acid), Kn (Kinetin), NAA (1Naphthaleneacetic acid) and IAA(Indole-3-acetic acid) and incubated at standard culture conditions [25±2 °C; 16/8 h (light/dark) regime with 40–50µmol $m^{-2}s^{-1}$light].

Callus Induction: Various parts (leaf, stalk, rhizome and root) of *A. purpurata* were cultured on MS media supplemented with different combinations of phytohormones (BAP, 2,4-D, Kn, NAA, IAA) and incubated for callus induction. Observations during callus initiation were recorded e.g. quantity, type (friable, white, brown, compact), colour and visual quality. The obtained callus was incubated at standard culture conditions.

Cell Suspension Cultures: The establishment of *A. purpurata* cell suspension cultures was carried out using four week old crystalline white friable callus. Approximately 30g of actively growing friable callus was transferred to 100 ml MS liquid media supplemented with 2,4-D, BAP and Kn. The flasks were placed on the rotary shaker under similar culture conditions with an agitation speed of 120 rpm. This uniform cell suspension culture was then used for growth kinetic studies.

Optimization of Growth conditions: Various parameters were optimized for maximizing the growth of cell suspension cultures using variables viz. sucrose concentration (3-5%), pH (5.4-6.0) and agitation speed (110-130 rpm).

Effect of Sucrose Concentration: Growth studies at various sucrose concentrations (3%, 4% and 5%) were carried out in cell suspension cultures over a period of 36 days to analyze biomass regularly at a 3-day interval and expressed as gram fresh weight (gFW). The effect of sucrose on phytochemical accumulation also was analyzed along with biomass.

Effect of pH: The effect of pH on *A. purpurata* suspensions was studied in the range of pH 5.2 – pH 6.0 to obtain maximum growth of suspension cultures.

Effect of agitation (rpm): Aeration to cultures was provided at different agitation speeds ranging from 110-130 rpm and the optimized speed was used for growth of suspensions.

Effect of Temperature: The cultures were incubated to study for optimized growth at various temperatures in the range of 22°C - 28°C.

Growth Kinetics: Growth rate and doubling time were measured by recording increase in biomass at a 3d interval for a period of 36 days. The growth pattern was analyzed by plotting a graph with biomass (gFW) against time period (days). The content of Kaempferol was analyzed along with biomass at different stages of growth and expressed as µg. gDW^{-1}.

Quantification of Kaempferol: The quantification of kaempferol was carried out using Shimadzu – LC 20AD series HPLC System. Estimations were performed on an octadecylsilane C18 column, injection volume was 20 µL with a retention time of 10 min. The kaempferol present in the *A. purpurata* suspension samples was analyzed using the gradient mobile phase: solution A: HPLC grade water with 0.1% $v.v^{-1}$ phosphoric acid and solution B is methanol. The kaempferol was detected at 360 nm with a UV-VIS detector at a retention time of 4.6 min.

The extraction protocol was carried out according to the protocol given by Victorio et al[18] in 2009 with a slight modification. The samples were analyzed by taking 200mg DW callus in HPLC grade methanol to obtain a uniform macerate. The extracts were filtered through 0.45 µm membrane filter and injected into the system while maintaining $1ml.min^{-1}$ flow rate. The presence of kaempferol in the extracts was obtained by comparing with the retention time of the standard kaempferol (sigma, ≥90%).

Statistical Analysis: The results were expressed as mean ± standard deviation calculated from the obtained triplicate data compared by least significant difference test using SAS, version 9.1.

Results and Discussion

***In vitro* plant establishment:** Various explants of *A. purpurata* after surface sterilization with 0.1% mercuric chloride were incubated under *in vitro* conditions. Only the rhizome bud explants have responded positively indicating the presence of meristematic activity in the *Zingerberaceae* rhizomatous monocot, which was similar to the earlier reports on *Alpinia* species [1]. The rhizome bud explants on MS media supplemented with 1.5 BAP ($mg.L^{-1}$), 0.5 Kn ($mg.L^{-1}$) and 0.5 NAA ($mg.L^{-1}$) have shown significant growth when compared to other explants (Fig. 1). Plants were further propagated *in vitro* on the same media combination with a subculture passage of 45 days.

Callus Induction: Callus induction was tried with various explants viz. rhizome, leaf and root sections from *in vitro* grown plants on MS media augmented with different phytohormone combinations 2,4-D, BAP, Kn, NAA, IAA and TDZ. The resulting white crystalline friable callus was seen protruding only from the rhizome base explants[14] while the other explants turned necrotic.

The best response for callus induction was observed on MS media fortified with 2,4-D (2.0 $mg.L^{-1}$), Kinetin(1.0 $mg.L^{-1}$) and BAP (1.0 $mg.L^{-1}$) (Table 1; Fig. 2) which has shown that the positive effect of BAP, 2,4-D and Kn combination on callus initiation was similar to the earlier reports[13,15]. The callus was maintained on the same media and sub-cultured every 28 days.

Table 1
Effect of phytohormones on callus induction from rhizome base explants

2,4-D ($mg.L^{-1}$)	BAP ($mg.L^{-1}$)	Kinetin ($mg.L^{-1}$)	IAA ($mg.L^{-1}$)	TDZ ($mg.L^{-1}$)	Response
0.5	-	0.5	0.1	0.1	++
1.0	0.3	-	0.5	0.3	++
1.5	0.5	0.5	-	0.5	+++
2.0	1.0	1.0	-	-	**++++**

*Observations were taken after 4 weeks of culture + poor ++ good +++ moderate ++++ excellent

Fig. 1: *A. purpurata* plant established under *in vitro* conditions on MS media supplemented with 1.5BAP ($mg.L^{-1}$), 0.5Kn($mg.L^{-1}$) and 0.5NAA($mg.L^{-1}$)

Fig. 2: *A. purpurata* callus from rhizome explants from *in vitro* established plants on MS media supplemented with 2 2,4-D($mg.L^{-1}$), 1BAP($mg.L^{-1}$) and 1Kn($mg.L^{-1}$)

Cell Suspensions initiation: MS liquid media containing the same combination of phytohormones for callus induction was further used for transferring healthy friable callus in order to obtain synchronous cells in actively growing stage (Fig. 3). These uniform suspensions were used for study of growth pattern in suspensions along with kaempferol production.

Optimization of conditions

1. Effect of pH, Temperature and Agitation: The growth of *A. purpurata* suspensions was optimized by studying a range of factors like pH, temperature and agitation. The optimum growth was obtained at pH 5.8, temperature of 25^0C and at 120rpm (Table 2).

pH is a very important factor affecting the biomass and flavonoid production in cell suspension cultures and slight difference in pH value can affect the overall production of biomass and bioactive compounds[22]. The agitation provided for suspensions plays a significant role by enhancing the availability of nutrients and the dispersion of unwanted gases for effective oxygenation. The present study has shown that cultures at 120rpm agitation speed have responded well which has been validated by Saurabh et al[15] wherein at higher rpm (130-160) there was reduced biomass accumulation along with kaempferol content, while lower rpm (80-100) leads to cell aggregation and finally resulting in cell death.

Temperature, an extrinsic factor greatly influences plant growth and development also acting as an abiotic elicitor in certain plant systems. The result in our present study of optimum temperature at 25°C has been confirmed by the earlier study on rice callus[10].

2. Effect of Sucrose Concentrations: The main source of carbon for secondary metabolite biosynthesis for *in vitro* plant cultures is sucrose. The different sucrose concentrations like 3%, 4% and 5% have been studied for analyzing its effect on growth and phytochemical accumulation. Higher biomass and product accumulation were observed in 4% sucrose concentration when compared to 3% and 5% in contrast with the findings of Mohammad et al[9] wherein it was reported that sucrose at a concentration of 5% had a positive effect on growth of suspensions of *Artemisia absinthium L*.

In the present study, increasing the sucrose concentration to 5% led to negative effect on biomass accumulation along with flavonoid biosynthesis in *A. purpurata* suspensions as the response is differential and species specific. The biomass increase in 4% sucrose is 1.26-fold higher in comparison to 3% and 2.34-fold higher with 5% sucrose concentrations respectively.

Fig. 3: *A. purpurata* cell suspension cultures in batch mode on MS media supplemented with 2 2,4-D (mg.L^{-1}), 1BAP(mg.L^{-1}) and 1 Kn (mg.L^{-1})

Table 2
Optimization of different parameters for in vitro cell suspensions

S.N.	pH	Temperature	Agitation	Response
1	5.1	22	100	++
2	5.2	23	110	++
3	5.6	24	115	+++
4	5.8	25	120	++++
5	6.0	26	130	++

* + poor ++ good +++ moderate ++++ excellent

Growth Kinetics: After optimizing the conditions, growth kinetic studies were carried out to analyze growth pattern for a period of 36 days with sample collection every 3days. For evaluation of this, 3g of isochronous cells were transferred to 30ml liquid media (10% w/v inoculum density). All the experiments were conducted in batch mode (Fig. 4) and the biomass accumulation was expressed as gram fresh weight (gFW). An extended lag phase was observed till 12th day and logarithmic phase started from 12th day and extended till 24th day where it has reached its peak accumulation. Later, stationary phase can be seen from 24th to 33rd day, after which there was a decline phase. A maximum of 10-fold higher biomass accumulation (31.28gFW) was found in 4% sucrose concentration.

Quantification of Kaempferol: *A. purpurata* cell suspension cultures were analyzed for the presence of kaempferol at sucrose concentrations of 3%, 4% and 5% respectively using HPLC analysis and expressed as µg.gDW^{-1}. The production of kaempferol was maximum on 24th day at 4% (35.285 µg.gDW^{-1}) followed by 3% (24.81 µg.gDW^{-1}) and 5%(17.073 µg.gDW^{-1}) sucrose concentrations (Fig. 7). Highest kaempferol production was seen in 4% sucrose concentration which is 1.42fold and 2.06-fold higher when compared to 3% and 5% sucrose concentrations respectively (Fig. 9).

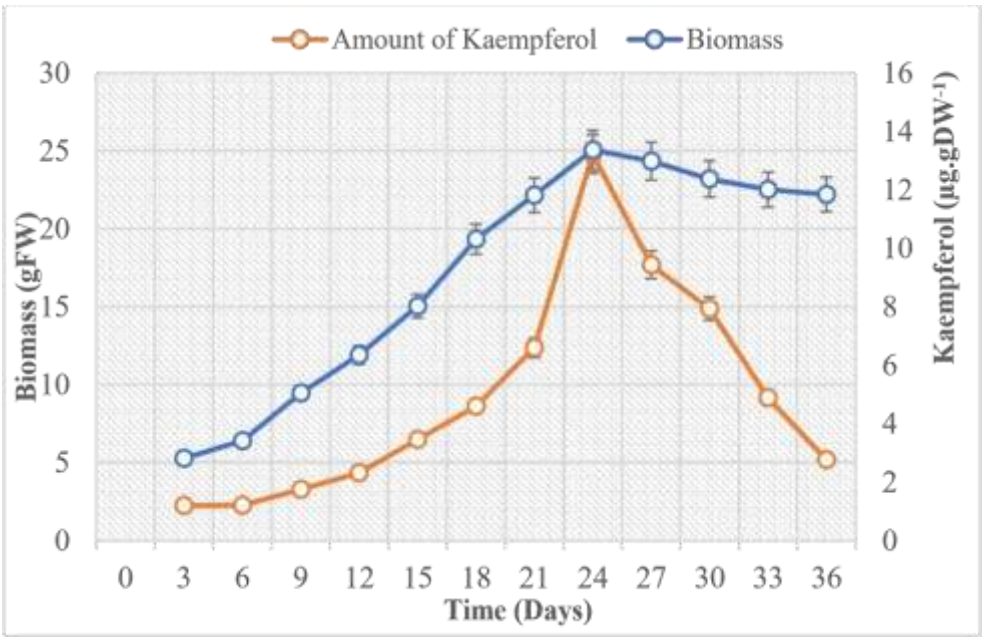

Fig. 4: Growth Vs Kaempferol production in suspension cultures of *A. purpurata* at sucrose concentration of 3%

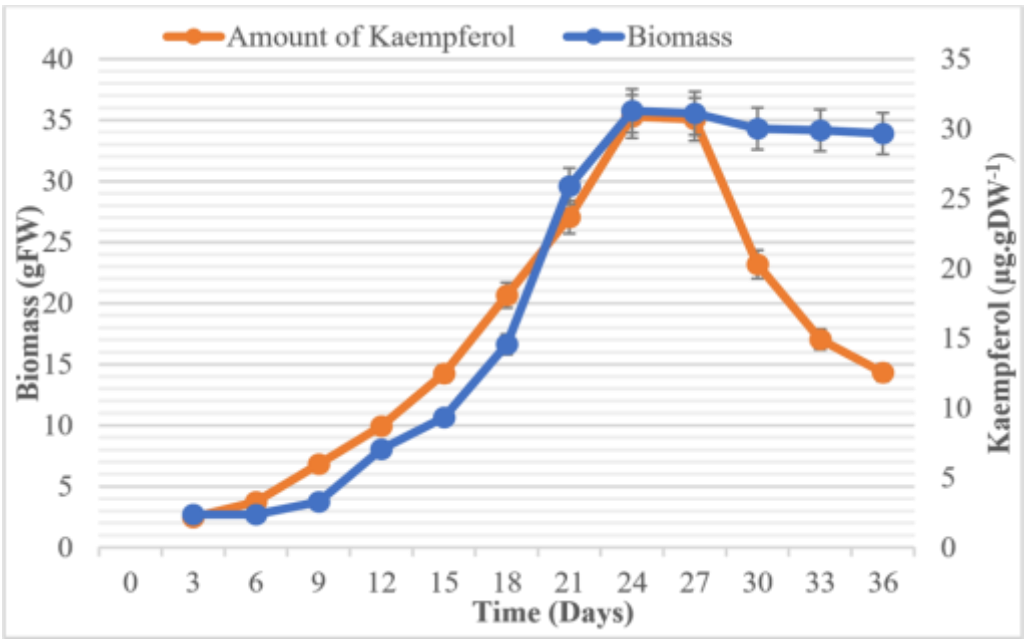

Fig. 5: Kaempferol production in relation to biomass accumulation of *A. purpurata* suspensions with 4% Sucrose

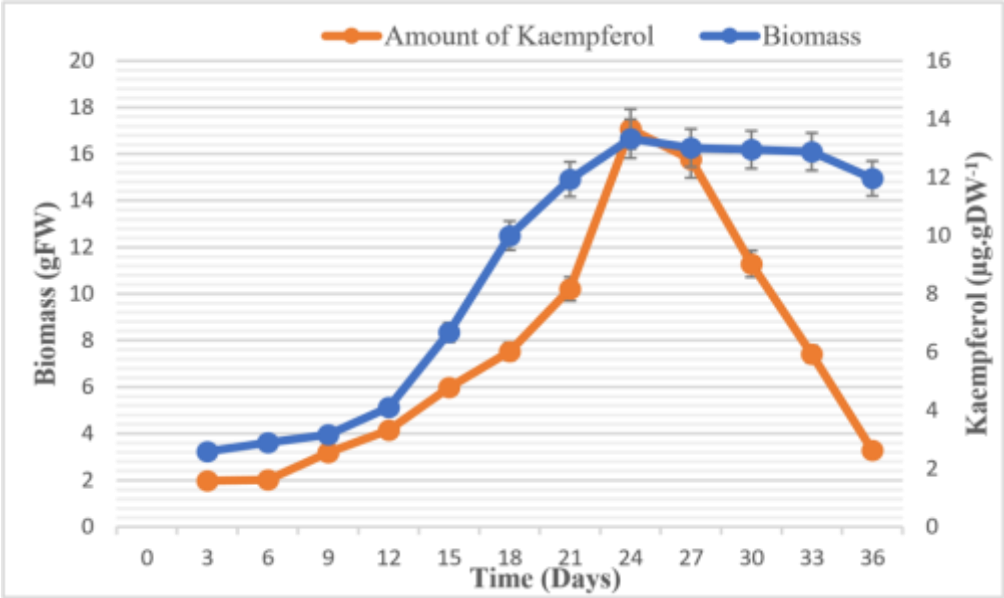

Fig. 6: Kaempferol production in relation to biomass accumulation in *A. purpurata* suspensions with 5% sucrose concentration.

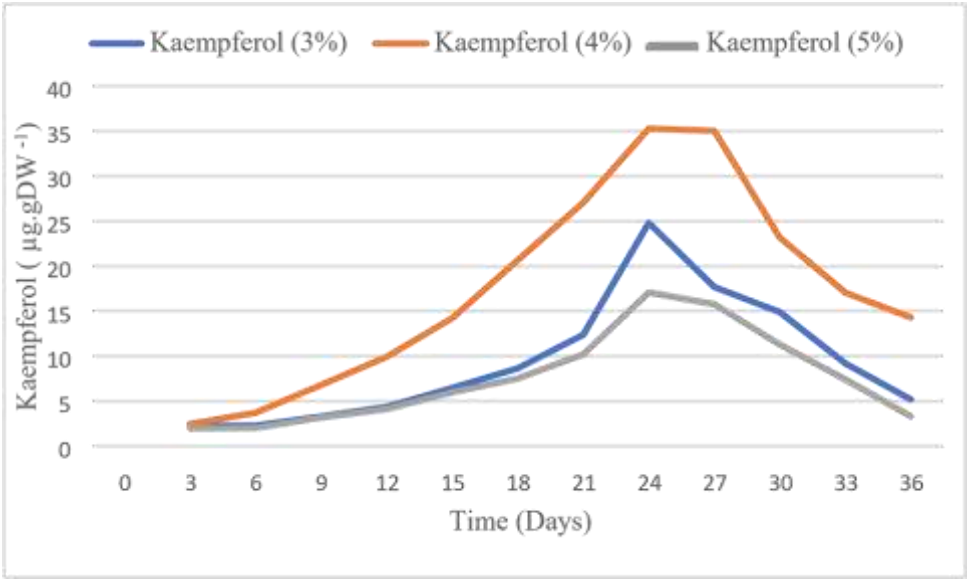

Fig. 7: Comparison of total Kaempferol content at different Sucrose Concentrations of 3%, 4% and 5% in *A. purpurata* suspensions

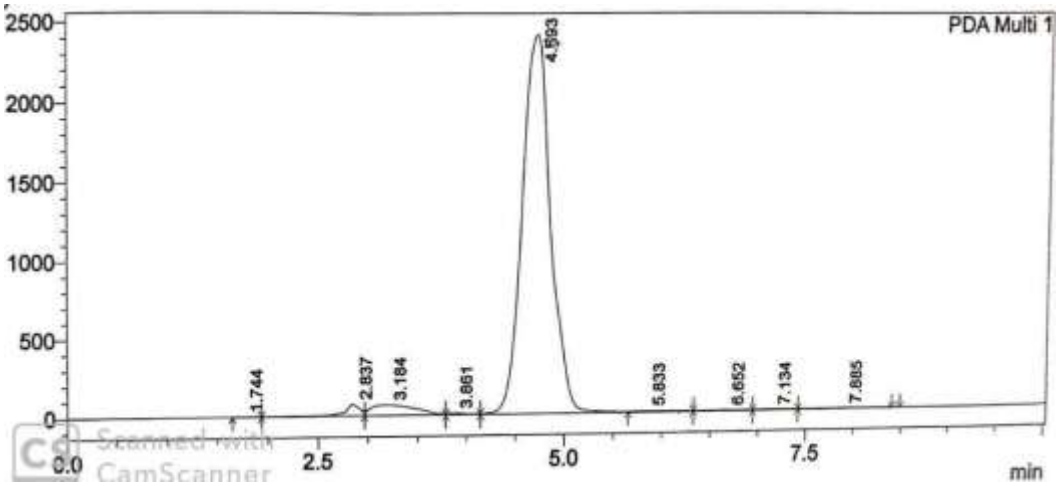

Fig. 8: Kaempferol authentic

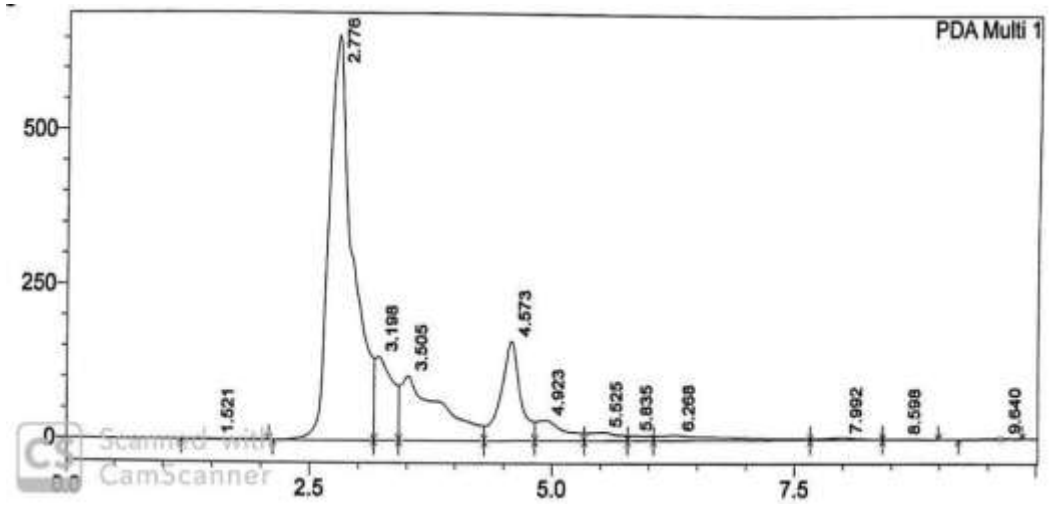

Fig. 9: Kaempferol production from *A.purpurata* suspensions grown on MS media supplemented with 4% sucrose concentration

Based on the growth pattern analysis in correlation with kaempferol quantification, the present study illustrates that kaempferol production and growth curve follow similar pattern. Extraction of Kaempferol from natural sources has garnered the attention of various researchers from around the globe due to the associated therapeutic properties. Kaempferol has earlier been extracted by enzymatic hydrolysis from tea seed[11] by supercritical fluid extraction (SFE)[7]. Hence, this study provides a great opportunity for enhanced accumulation of Kaempferol from suspensions with scope for higher production using further manipulation.

Conclusion

Kaempferol, a flavonoid antioxidant, possesses high therapeutic potential as it plays a significant role in eradicating ROS (free radicals), which are prime disease-causing agents. Suspension cultures offer major advantage for production of unique and specified phytochemicals that can be further enhanced by different elicitation techniques.

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