

## Enhancement And Evaluation Of Phenolic And Flavonoid Content In Plant Cell Suspension Cultures Of *Passiflora Foetida* Linn Using Abiotic Elicitor

**Running Title-** Enhancement and Evaluation of Phenolic and Flavonoid Content

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### ABSTRACT

By using plant tissue culture methods, many active phytoconstituents of medicinal plants are studied. Plant tissue culture proves as an efficient platform for such studies. Undifferentiated masses of cells through in-vitro callus culture development under aseptic conditions and in specific media are generated by using medicinal plants. The undifferentiated cells produce and secrete vital active pharmaceuticals when they are cultured in a suspension medium. *Passiflora foetida* Linn. (a medicinally active plant) contains different active phytoconstituents which have high therapeutic value. In this investigation, callus and cell suspension cultures from leaf explants of *Passiflora foetida* Linn. are optimized and the application of the abiotic elicitor (i.e. Salicylic acid at concentrations of 50  $\mu$ M, 150  $\mu$ M, and 250  $\mu$ M) is evaluated. At 24 and 96 hours, cell suspension cultures are harvested (intracellular and extracellular) respectively. After that phenolic and flavonoid components are analysed quantitatively using standard spectrophotometric methods using standards (i.e. gallic acid and quercetin). The results indicate the enhancement in the phenolic and flavonoid contents after 24 hours of application is seen when Salicylic acid elicitation is done at 150  $\mu$ M concentration. This finding proves that salicylic acid is very effective for enhancing the phytochemical production in *Passiflora foetida* Linn. under aseptic tissue culture conditions.

**Keywords:** *Passiflora foetida* Linn, phenolic content, flavonoid content, cell suspension culture, elicitors, salicylic acid.

### 1.0 INTRODUCTION

Herbs are used in alternative traditional system of medicines and they are the most common source of medicines in different countries. Herbs are also used as nutraceuticals, cosmeceutical and are also used for enhancing good health for facing chronic stress and it is also used to treat weakness or illness. Various plant and animal species are adversely affected by the various factors mainly the climatic ones. So, these factors affect the herbs quality and yields of active phyto-constituents present in the herbs. Some endangered plants are medicinally important because they contain a lot of active phytoconstituents. So, some biotechnological methods are used to enhance their productivity and also preserve beneficial effects of medicinally important herbs<sup>1</sup>.

Biotechnological techniques like Plant tissue culture technology are majorly used for growing the medicinally active herbs which contain some active phytoconstituents which have therapeutic value. This can be done by using elicitors, Precursors, biotransformation or by changing the medium and environmental conditions.

Elicitors play an important role for enhancing the active phytoconstituent's synthesis by different biosynthetic pathways in the herbs or plants. So, they also enhance the synthesis of commercially beneficial compounds. When any stress is produced in a plant by using elicitors then active phytoconstituents are released due to triggering of the defense response<sup>2</sup>.

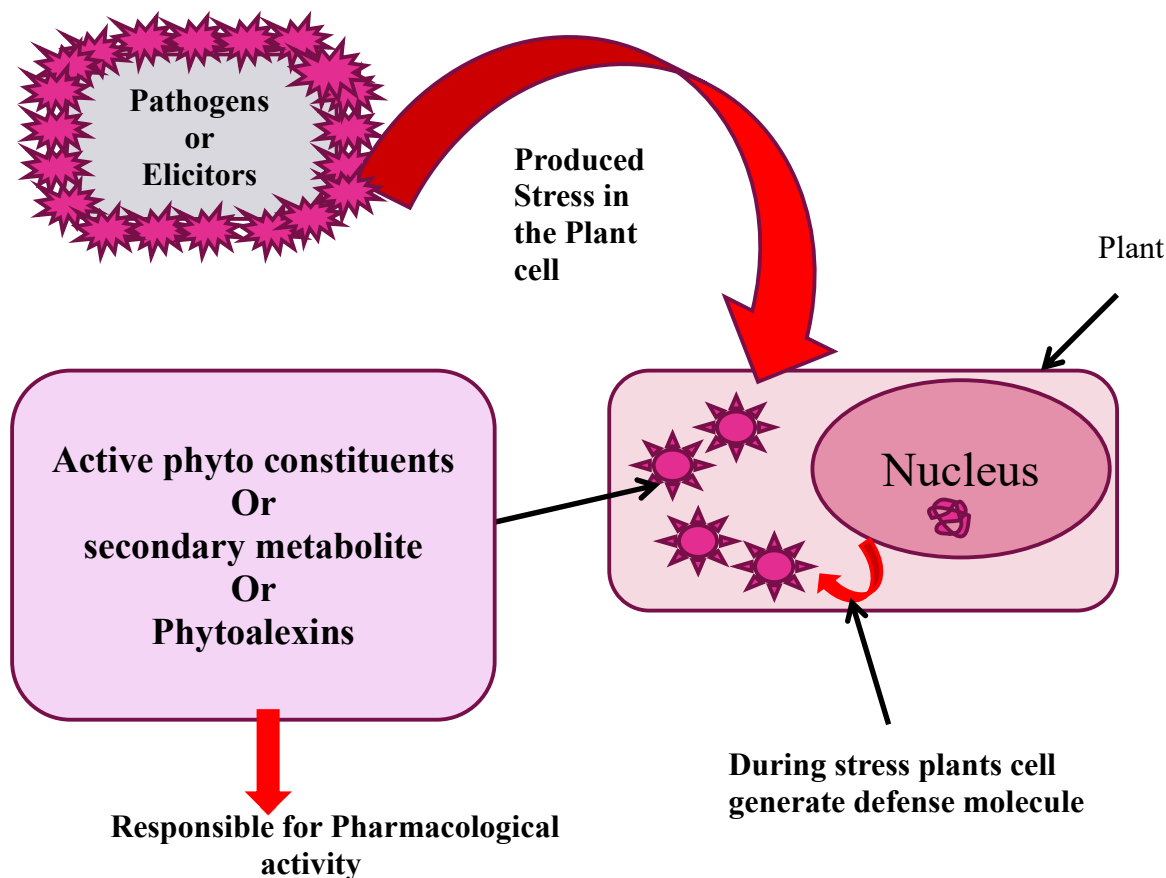


Figure:1 Mechanism action of Elicitors

Abiotic and abiotic elicitors play an important role for enhancing the active phytoconstituent's synthesis by different biosynthetic pathways in the herbs or plants. So, they also enhance the synthesis of commercially beneficial compounds. When any stress is produced in a plant by using elicitors then active phytoconstituents are released due to triggering of the defence response<sup>2</sup>. Elicitors are used for the elicitation of secondary metabolites such as alkaloids, flavonoids, tannins, volatile oils, glycosides etc. These secondary metabolites activities are conformed by their competitiveness, persistence and survival<sup>14</sup>. In medicinal plants, salicylic acid can also increase the production of secondary metabolites. The increment of gene expression of phenylalanine ammonia-lyase enzyme results in the increased level of secondary metabolites (Figure:1). When the leaves of *Crocus sativus* plant are pre-treated with salicylic acid, the level of PAL enzyme gets enhanced which is responsible for the phenolic and flavonoids accumulation<sup>15</sup>. Higher levels of many phenolic and flavonoids

content like rosmarinic acid (RA)<sup>23</sup>, salvianolic acid<sup>24</sup>, capsiate and capaicinoids<sup>16</sup> etc. are shown to be possessed by the up-regulated expression of the gene. Cell suspension culture of *Corylusavellana* was treated with abiotic elicitors (salicylic acid in concentration range of 25 and 50mg L<sup>-1</sup>) which showed increase in the concentration of phenolic component<sup>17</sup>.

*Passiflorafoetida* Linnaeus is medicinally potent vine climber which is grows in 1-6-meter-tall with has special type of eye catcher beautiful flower. Before the 15<sup>th</sup> century well-known for the Passion of Christ, and so it is also known as passion flower<sup>3</sup>. It is belonging to Passifloraceae family. It is also found in India, Central America, South East Asia, Arica and Brazil South America<sup>4</sup>. It has a lot of active phytoconstituents such alkaloids, phenols, glycoside, flavonoids, cyanogen compounds<sup>5,6</sup>, C-glycosyl flavonoids (apigenin and luteolin), isovitexin, vitexin, kaempferol, isoschaftoside and kaempferol<sup>8,7</sup>. In recent research vitexin and isovitexin both are used in management of the diabetes because it is inhibited the formation of advanced glycation end products<sup>5</sup>. *Passiflorafoetida* Linnaeus is a medicinally important medicinal plant which a various therapeutic potency. It has various pharmacological activity such as anti-oxidant, anti-inflammatory, anti-neoplastic, protective effect against neurological and psychiatric disease, protective effects against alzheimer disease, protective effects on learning cognition and depression, anti-nociceptive activity, protective activity in cardiovascular system, protective effects against diabetes, protective effects against obesity etc.



Figure: 2 *Passiflorafoetida* Linn. plant

**Taxonomic classification of *Passiflorafoetida* Linn.:**

**Domain:**Eukaryota

**Kingdom:** Plantae

**Phylum:**Spermatophyta

**Subphylum:**Angiospermae

**Class:**Dicotyledone

**Order:**Violales

**Family:**Passifloraceae

**Genus:**Passiflora

**Species:**Passiflorafoetida.

## 2.0 MATERIALS AND METHODS

### 2.1 Collection of leaf ex-plants and authentication of the plant

The leaves ex-plants of *Passiflorafoetida* Linn. were collected from the poly-green house which is constructed in the Herbarium Garden of the Smriti College of Pharmaceutical Education, Indore. The plant was authenticated by Dr. S.N. Dwivedi, Department of Botany, Janata PG College, A.P.S. University, Rewa, M.P., and Herbarium noJ/Bot./2020-091PFL was allotted on14/08/2020. The leaves ex-plants were cut into small portions (approximately 0.25cm<sup>2</sup>).



**Figure:3** Collection of leaf ex-plants

### 2.2 Callus Culture

#### 2.2.1 Leaf Ex-plants Surface sterilization

Three-month-old healthy ex-plants were used to initiate the induction of callus culture. The microorganisms were removed from the surface of the leaves of the ex-plants through the surface sterilization methods. This process started from washing the surface of the explants through running tap water for removing of all the unwanted particles such as soil and dust particles. Further the same leaf ex-plants were dipped in alcohol (70% (v/v)). This method was continued with mercuric chloride (0.1% (w/v)) for 3-5 minutes. At the completion of the surface sterilization step, the leaf ex-plants were washed with sterile distilled water (3-4 times) for the complete elimination of surface sterilizing agent. The whole process was performed under controlled environment condition (aseptic condition) which is provided through laminar air flow equipment.

#### 2.2.2 Preparation and sterilization of callus culture media

Murashige and skoog (MS) medium was augmented with agar (0.8% (w/v)) medium. The media was assimilated with different concentration of plant growth regulators such as auxin (as 3-indoleacetic acid (IAA)) in combination with various concentration of cytokinins as 6-Benzylaminopurine (BAP) was performed in order to induce the callus (Table: 1). The pH of the medium was retained between 5.5-5.7 limit and it is adjusted via the addition of 0.1NHCl / 0.1MNaOH. The digital pH meter was used for the final measurement of the pH of the medium. Prepared media (approximately 15ml) was poured into each culture tube (Jam bottle). The autoclaving (at 121°C temperature and 15 psi pressure for 15minutes) is used for the sterilization for the culture media and further it is allowed for solidification.<sup>13,22</sup>

Table:1 Leaf ex-plants of *Passiflorafoetida* Linn. is used for the induction of callus on Murashige&Skoog

medium which is supplemented with various combination and concentration of plant growth regulators such as IAA and BAP 0.5,2.0 mg/L.

S.no.	The combination of concentration (mg/L) in Murashige&Skoog medium	
	IAA	BAP
1.	0.0	0.0
2.	0.0	0.5
3.	0.0	1.0
4.	0.0	1.5
5.	0.0	2.0
6.	0.5	0.0
7.	0.5	0.5
8.	0.5	1.0
9.	0.5	1.5
10.	0.5	2.0
11.	1.0	0.0
12.	1.0	0.5
13.	1.0	1.0
14.	1.0	1.5
15.	1.0	2.0
16.	1.5	0.0
17.	1.5	0.5
18.	1.5	1.0
19.	1.5	1.5
20.	1.5	2.0
21.	2.0	0.0
22.	2.0	0.5
23.	2.0	1.0
24.	2.0	1.5
25.	2.0	2.0

### 2.2.3 Inoculation of leaf ex-plant in callus culture medium

The adaxial face of the leaves ex-plants of *Passiflorafoetida* Linn. were inoculated at the sterile side of callus culture medium (Figure:5). It is supplemented with various plant growth regulators in the medium. The average callus formation time, fresh weight and dry weight measurement was performed.

After the inoculation of the leaf ex-plants in culture medium, it was kept for incubation for 3-6 weeks at of  $25\pm 2^{\circ}\text{C}$  and at a relative humidity upto 50-70% for 16 hrs of photo period (white fluorescent light  $60\mu\text{mol m}^{-2}\text{s}^{-1}$ ) (Figure: 4).



Figure:4 Inoculated ex-plants in callus culture media and its incubation in PTC work station

### 2.3 Cell Suspension Culture

The friable calli generated in callus culture, is used in the cell suspension culture. The maximum mass of calli was selected for the study. The fresh weight of calli (1.5gm) was inoculated in 125ml Erlenmeyer flask which previously have 25 ml Murashige and skoog liquid media. Murashige and skoog liquid media was also added with plant growth regulators in optimized concentration which is beneficial for the development of callus during experiment. The inoculated liquid Murashige and skoog media having friable calli was then kept in orbital shaker (set at 110rpm speed and  $25\pm 2^{\circ}\text{C}$ ). The condition of light was maintained manually with photoperiodic time of white fluorescent light for 16 hours, to the value of  $60\mu\text{molm}^{-2}\text{s}^{-1}$ . The suspension of cell is formed after 4 hours from the friable calli. The suspension was further continued to grow in the similar conditions for 15days.

#### 2.3.1 Sub-culturing the cell suspension culture

After 15 days,the cell suspension culture was filtered. Filtered biomass (6gms) was transferred in 500ml of Erlenmeyer flask. Murashige and Skoog liquid media was supplemented with plant growth regulator in 100ml of Erlenmeyer flask. It was kept for incubation with shaking under the same conditions. For three months the sub-culturing protocol of cell suspension culture was preceded in every 15 days.<sup>9</sup>



Figure:5 Forming cell suspension culture of *Passiflorafoetida* Linn. treated with various concentration of salicylic acid (orbital shaker).

## 2.4 Growth Pattern

The growth pattern of the plant cells in the suspension condition was evaluated through the cell suspension culture growth curve which is used to determine exponential growth phase. This analysis was started with the addition of fresh weight (1.5gms) of cells in 125ml capacity Erlenmeyer flask which contained 25ml liquid Murashige and Skoog media. Similar preparation of multiple flasks was prepared and every second day, three flasks were harvested. The harvested biomass cell was allowed for drying (at 45°C for period of 48 hours).

During the 18 days, growth curve was made by using dried weight of biomass. Calculation of the growth rate of cell was done by making the graph between natural logarithm of time and cell growth<sup>9</sup>.

## 2.5 Elicitation using Salicylic acid

Salicylic acid solution was prepared in 50% (v/v) ethanol. The sterilization of the prepared salicylic acid solution was done by passing it through micron membrane filter (0.45µ) under controlled environmental condition (aseptic) and to assure any contaminated microorganism could not enter through elicitor application. Further various concentrations of salicylic acid were prepared from this stock solution 0, 50, 150 and 250 µml/100ml of cell suspension culture<sup>10,21,20,19,18</sup>.

The various concentration of Salicylic acid was applied on the 8dayold sub-cultured cell suspension culture of *Passiflorafoetida* Linn. On the basis of the studies on the growth curve, the day for application of elicitor was selected. Those studies suggested that exponential growth phase is seen upto 10<sup>th</sup> to 12<sup>th</sup> day. On that basis, the sixth day was selected to perform elicitation by Salicylic acid. Later harvesting of cells was done on 24<sup>th</sup> hour and 96 hours after the Salicylic acid elicitor application. By using destructive sampling methods, the experiments were performed in triplicate as it shows the processing of complete samples at the end of each harvest time. Ethanol 50% (v/v) is used as a control<sup>10</sup>.

## 2.6 Analysis of Intracellular and Extracellular metabolite

### 2.6.1 Sample preparation and Extraction

The cells (harvested) collected after applying the elicitors (Salicylic acid) on 24 hours and 96 hours were used allowed to dry separately. It was grounded to fine powder biomass. Further it is extracted with 5ml of ethanol for the extraction of phytochemicals. The extracted biomass (ethanol) was kept in bath-sonicator (10minutes) for the extraction. The ethanolic extract was allowed to dry and latter suspended in water. Ethyl acetate was added in the suspended extract to collect the ethyl acetate fraction and latter used for determination of total phenolic content and total flavonoids content

In the whole process of extraction temperature was kept below 35°C (Mendoza et al, 2018). Extracellular metabolites were determined directly through the cell culture medium.

### 2.6.2 Determination of Total Flavonoid Content

The total flavonoids content of the extracted which is obtained after the elicitation by Salicylic acid was determined by the Aluminium chloride methods through the colorimetric assay which was performed in triplicate. We have taken 2ml of distilled water and 150µl of sodium nitrate (5%) was mixed in 0.5ml solution of extract of *Passiflorafoetida* Linn. in ethylacetate was allowed to mix. It is kept for incubate for 5 minutes. After added 250µl of aluminium chloride (5%) and then slowly shaking and later added 2ml of 1M sodium hydroxide solution in this solution. Then it was kept for 15 minutes at room temperature. UV –Visible spectrophotometer methods (Shimadzu 1800) is used for the determination of absorbance of the solution at 510 nm wavelength against blank. In the same way, calculation of the total flavonoid contents of extract of cell suspension culture was done by exposing to various concentration of Salicylic acid. From a calibration curve of quercetin, the total flavonoids contents were calculated as quercetin equivalents<sup>11</sup>.

**2.6.3 Determination of Total Phenolic Content**

The phenolic content in ethyl acetate extract of *Passiflora foetida* Linn. was determined by using spectrophotometric method by using FC reagents (Folin-Ciocalteu reagent)<sup>12</sup>. 10% of Folin-Ciocalteu reagent (2.5ml) and 7.5%w/v of Na<sub>2</sub>CO<sub>3</sub> (2 ml) is added to 0.5ml of each of the three replicated ethylacetate extracts of the plant (1mg/ml). The blank was prepared by mixing 0.5ml of methanol 10% of Folin-Ciocalteu reagent (2.5ml) and 7.5%w/v of Na<sub>2</sub>CO<sub>3</sub> (2 ml). It is incubated at 45°C with constant shaking for 15 min. Spectrophotometer method at 765nm wavelength is used to measure the absorbance of the sample. The total phenolic content in sample was measured depending on the calibration curve of standard gallic acid. The content of total phenol was calculated on the basis of the calibration curve of gallic acid and the results were expressed as mg of gallic acid equivalents (GAEs) per gram of extract.

**2.7 Statistical analysis of data**

Microsoft excel is used for data analysis and reported as mean ±SEM of triplicate determination.

**RESULT AND DISCUSSION**



**Figure :6** A) Leaf explant of *Passiflora foetida* Linn is treated with different concentration of plant growth regulators. B) Callus growth seen at 21st days of the culture.

**Growth Curve analysis of Cell Suspension Culture (CSC)**

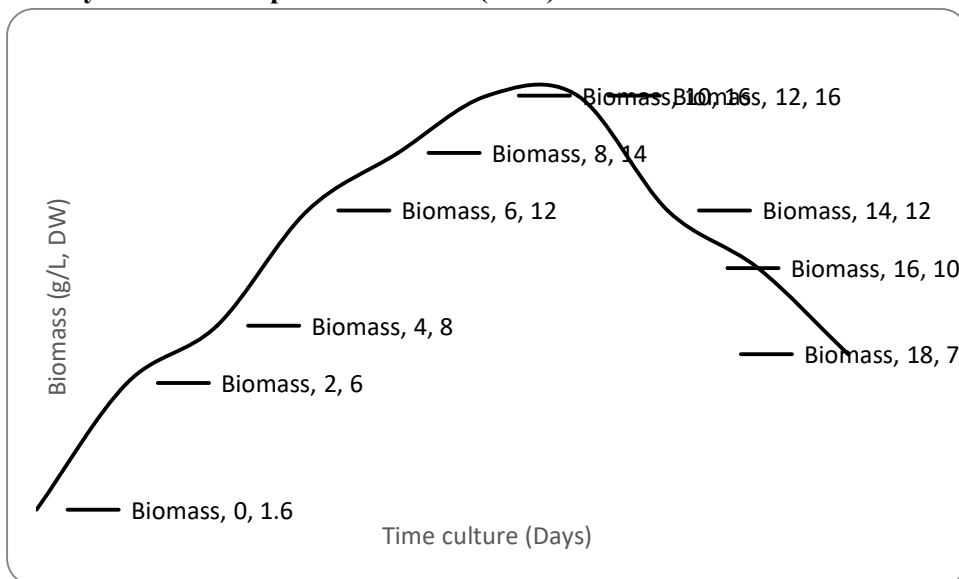


Figure:7 Growth Curve analysis of Cell Suspension Culture (CSC)of *Passiflora foetida* Linn.

### Callus culture

Table: 2 By using different combination and concentration of plant growth regulators for the measurement of average callus formation, fresh and dry weight.

S.no.	The combination of different concentration (mg/L) of plant growth regulators in MS media		Average Callus Formation in Days	The average fresh weight in grams	The average dry weight in grams
	IAA	BAP			
1.	0.0	0.0	-	-	-
2.	0.0	0.5	19 days and 12 hours	0.1144	0.0154
3.	0.0	1.0	17 days and 12 hours	0.1231	0.0040
4.	0.0	1.5	16 days and 12 hours	0.1403	0.0107
5.	0.0	2.0	18 days	0.1179	0.0142
6.	0.5	0.0	21 days	0.0450	0.0053
7.	0.5	0.5	10 days and 12 hours	0.4110	0.0474
8.	0.5	1.0	11 days and 12 hours	0.2511	0.0143
9.	0.5	1.5	10days	0.3285	0.0173
10.	0.5	2.0	8days	<b>0.5054</b>	<b>0.0628</b>
11.	1.0	0.0	18days	0.0127	0.0033
12.	1.0	0.5	10 days and 12hours	0.128	0.0303
13.	1.0	1.0	10days	0.406	0.0312
14.	1.0	1.5	13days	0.4072	0.0355
15.	1.0	2.0	12 days and 12 hours	0.3174	0.032
16.	1.5	0.0	16 days and 12 hours	0.170	0.0045
17.	1.5	0.5	10days	0.3064	0.0167
18.	1.5	1.0	12days	0.4314	<b>0.0499</b>
19.	1.5	1.5	10 days and 12 hours	0.4442	0.0159
20.	1.5	2.0	11days	0.0121	0.0044
21.	2.0	0.0	18days	0.4214	0.0418
22.	2.0	0.5	14 days and 12 hours	0.4305	0.0318

23.	2.0	1.0	13 days and 12 hours	<b>0.4794</b>	0.0460
24.	2.0	1.5	12days	0.4400	0.0417
25.	2.0	2.0	15days	0.2651	0.0241

**DETERMINATION OF TOTAL FLAVONOID CONTENT WITH SALICYLIC ACID ELICITOR APPLICATION**

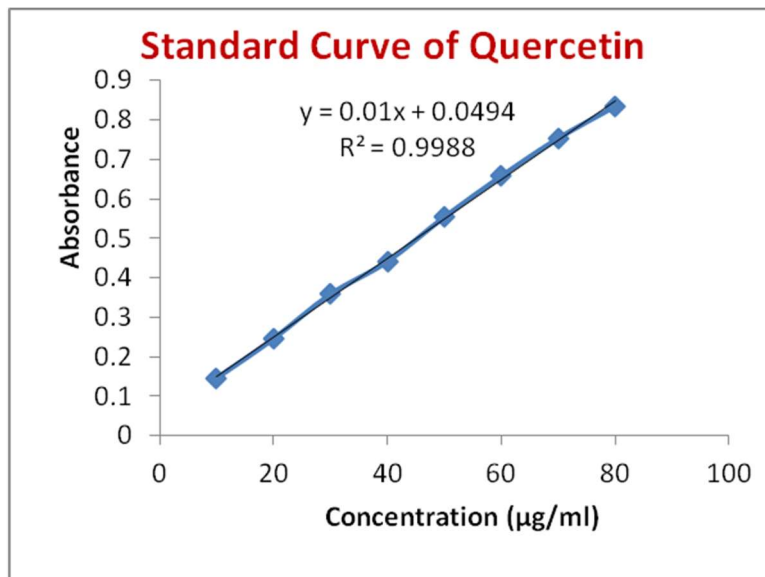


Figure:8 Standard curve of Quercetin

Table:3 Intra cellular flavonoids content of cell suspension culture of *Passiflorafoetida* Linn. with various concentration of Salicylic acid

Salicylic acid (µM)	Harvest time in hours	Intracellular flavonoid content		Harvest time in hours	Intracellular flavonoid content	
		Total flavonoid content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Flavonoid Content as compared to control		Total flavonoid content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Flavonoid Content as compared to control
0		2.104±0.173	--		2.951±0.137	--

50	24	2.711±0.107	28.84	96	3.425±0.106	16.06
150		3.141±0.141	49.28		3.771±0.120	27.78
250		2.707±0.121	28.65		3.226±0.112	9.31

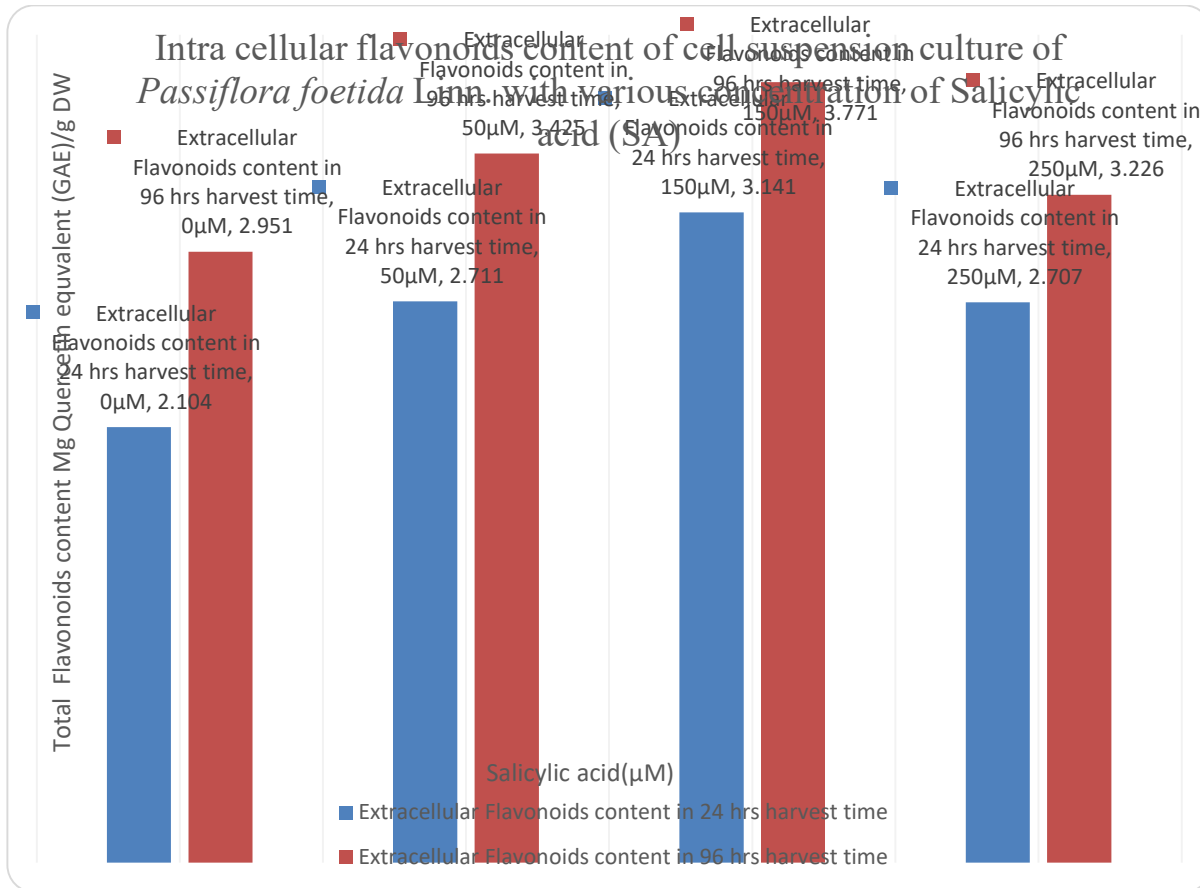


Figure:9 Intracellular Flavonoids content of cell suspension culture of *Passiflora foetida* Linn. with various concentration of Salicylic acid (SA)

Table:4 Extra cellular flavonoids content of cell suspension culture of *Passiflora foetida* Linn. with various concentration of Salicylic acid

Salicylic acid (μM)	Harvest time in hours	Extracellular flavonoid content		Harvest time in hours	Extracellular flavonoid content	
		Total flavonoid content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Flavonoid Content as compared to control		Total flavonoid content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Flavonoid Content as compared to control
0	24	2.104		24	2.104	
0	96	2.951		96	2.951	
50	24	2.711		24	2.711	
50	96	3.425		96	3.425	
150	24	3.141		24	3.141	
150	96	3.771		96	3.771	
250	24	2.707		24	2.707	
250	96	3.226		96	3.226	

0	24	2.004±0.059	--	96	2.302±0.058	--
50		2.414±0.132	20.45		2.951±0.130	28.19
150		2.52±0.127	51.6		3.312±0.102	43.87
250		2.212±0.061	10.37		2.512±0.058	9.12

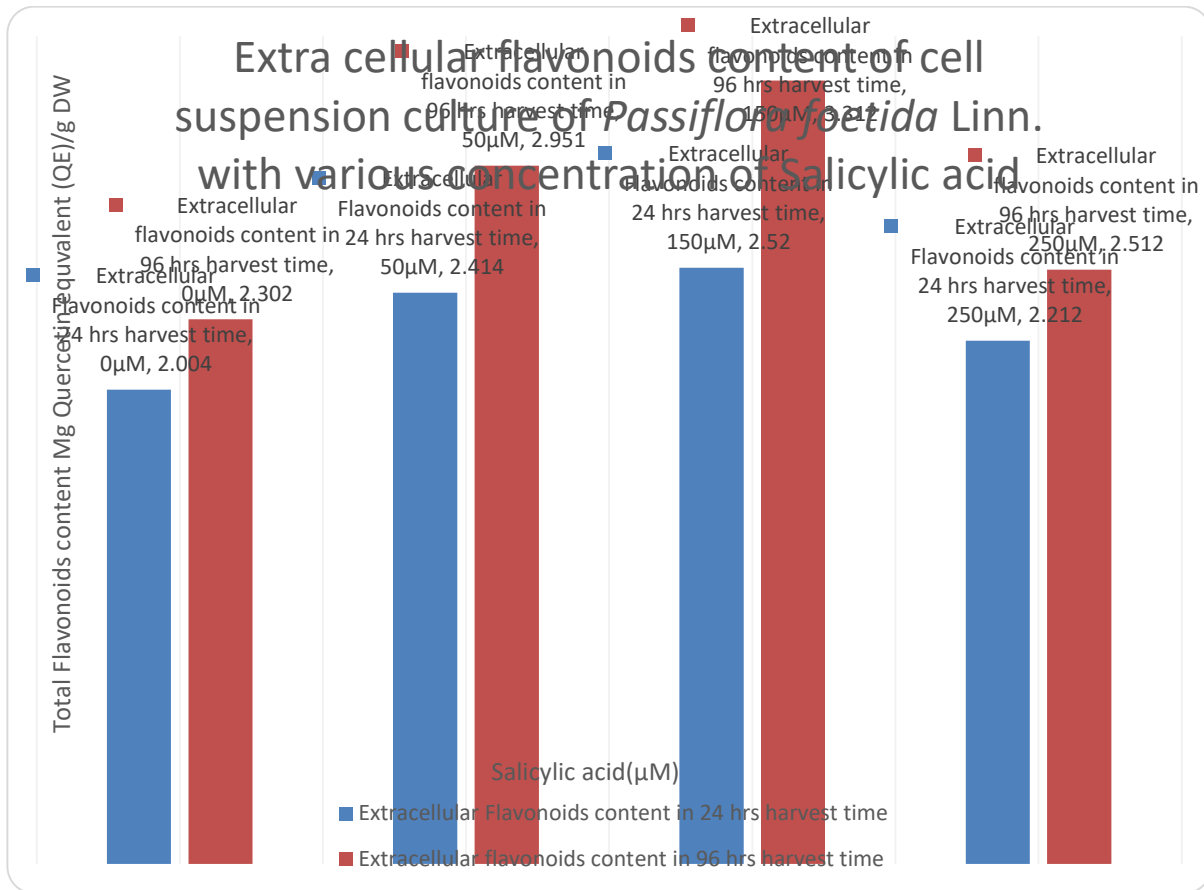


Figure:10 Extracellular Flavonoids content of cell suspension culture of *Passiflora foetida* Linn. with various concentration of Salicylic acid (SA)

**DETERMINATION OF TOTAL PHENOLIC CONTENT WITH SALICYLIC ACID ELICITOR APPLICATION**

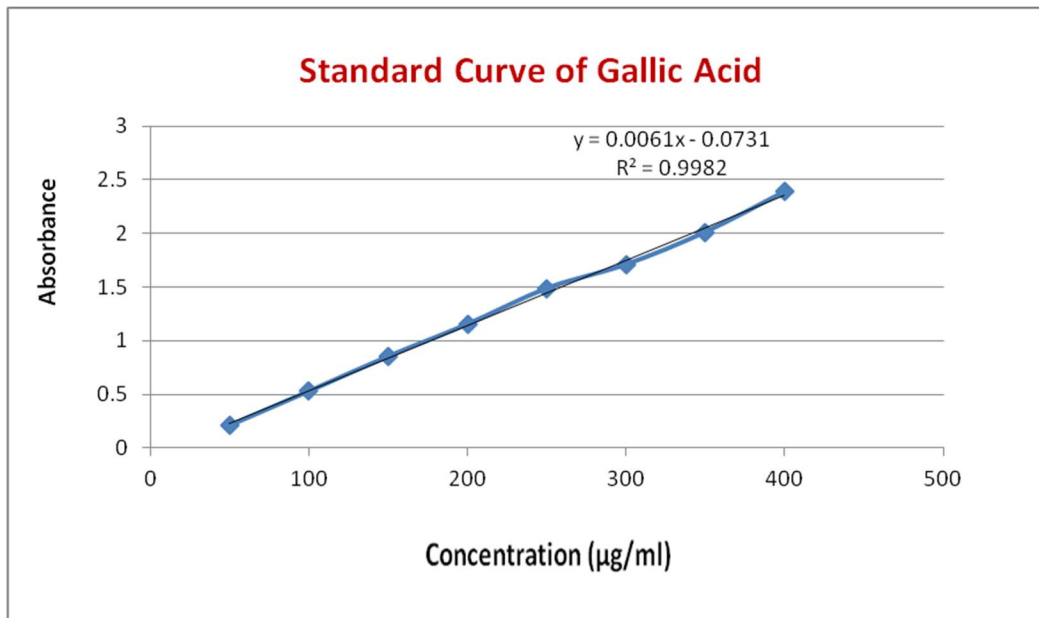


Figure:11 Standard curve of gallic acid

Table:5 Intra cellular Phenolic content of cell suspension culture of *Passiflorafoetida* Linn. with various concentration of Salicylic acid

Salicylic acid (µM)	Harvest time in hours	Intracellular Phenolic content		Harvest time in hours	Intracellular Phenolic content	
		Total Phenolic content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Phenolic Content as compared to control		Total Phenolic content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Phenolic Content as compared to control
0	24	3.123±0.019	--	96	3.252±0.217	--
50		3.610±0.091	15.59		3.710±0.068	14.08
150		3.944±0.041	26.28		4.437±0.117	36.43
250		3.317±0.143	6.211		4.107±0.61	26.29

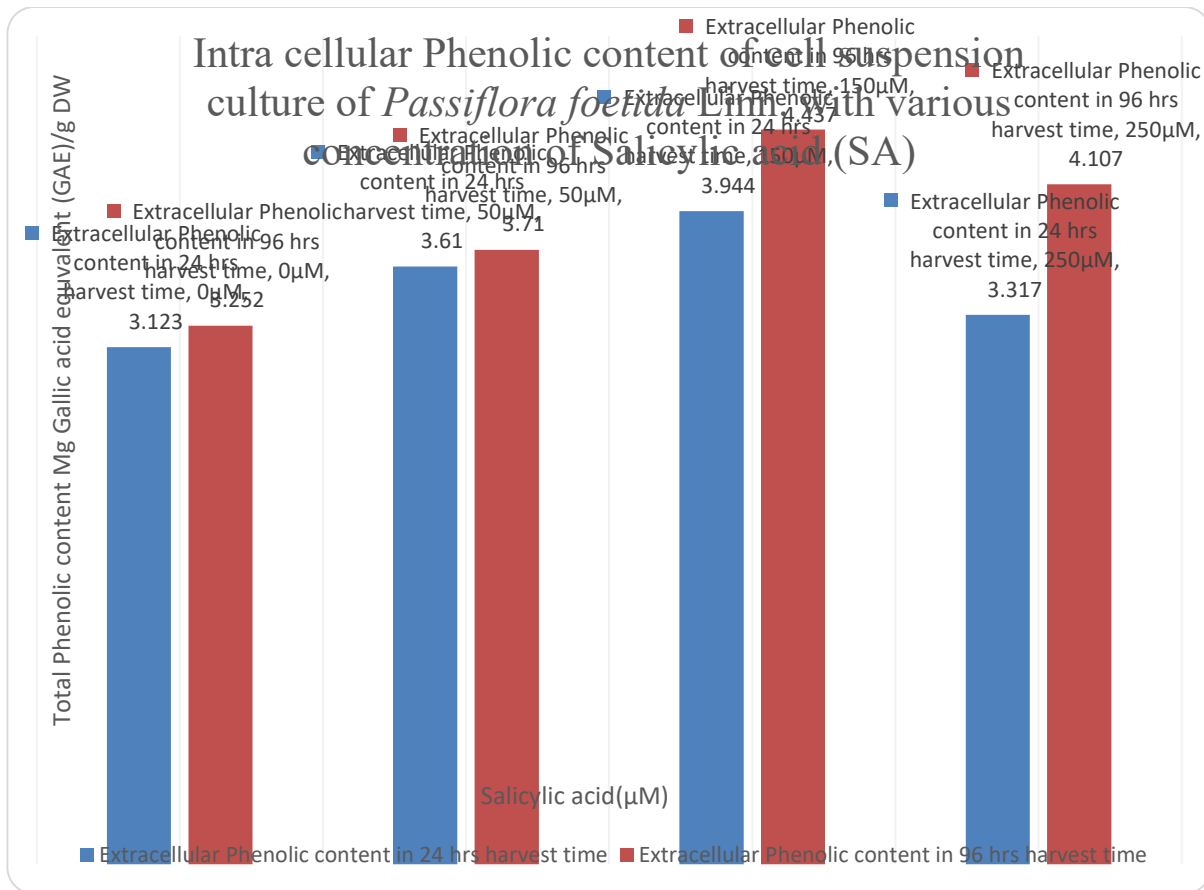


Figure:12 Intracellular Phenolic content of cell suspension culture of *Passiflora foetida* Linn. with various concentration of Salicylic acid (SA)

Table:6 Extracellular Phenolic content of cell suspension culture of *Passiflora foetida* Linn. with various concentration of Salicylic acid

Salicylic acid (μM)	Harvest time in hours	Extracellular Phenolic content		Harvest time in hours	Extracellular Phenolic content	
		Total Phenolic content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Phenolic Content as compared to control		Total Phenolic content in Mg Quercetin equivalent (QE)/gDW	Percentage Change in Total Phenolic Content as compared to control
0	24	2.412±0.081	--	96	2.425±0.132	--
50		2.616±0.070	8.45		2.81±0.168	15.87
150		2.710±0.050	12.35		3.414±0.110	40.78
250		2.260±0.062	-6.301		3.129±0.090	29.03

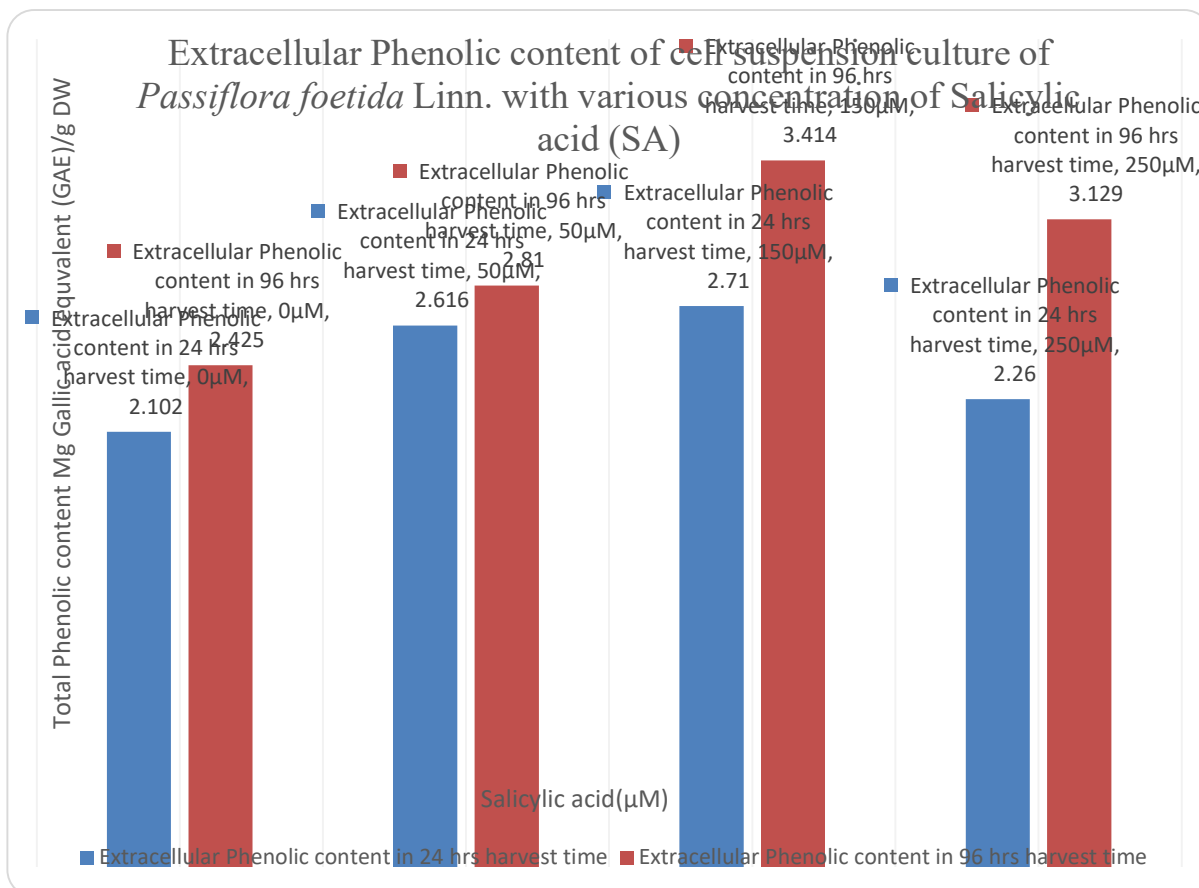


Figure:13 Extracellular Phenolic content of cell suspension culture of *Passiflora foetida* Linn. with various concentration of Salicylic acid (SA)

## CONCLUSION

For the generation of secondary metabolites, plant metabolic pathways were stimulated in the cell suspension culture of *Passiflora foetida* Linn. In this investigation, in order to enhance the production of secondary metabolites (especially phenolic and flavonoid components), different concentrations of salicylic acid (an abiotic elicitor) were used. It was concluded that salicylic acid at concentration of 150 μM was more effective compared to the control group after 24 hours of elicitor application. The activation of the shikimic acid pathway (phenylpropanoids metabolic pathway) may be involved in the mechanism behind the elicitation of salicylic acid. This investigation revealed that the production of active phytoconstituents from *Passiflora foetida* Linn (a plant having medicinal properties) is enhanced by the elicitor technology. This methodology can be used to increase industrial productivity in secondary metabolite production. Chemical characterization is necessary to specifically identify phenolic and flavonoid contents along with other phytopharmaceuticals so to completely understand the effects of abiotic elicitors on *Passiflora foetida* Linn.

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