https://doi.org/10.48047/AFJBS.6.14.2024.3885-3902

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

EVALUATION OF EXTRACT OF *PASSIFLORA FOETIDA* LINN. AND TO STUDY THE EFFECT OF ELICITOR ON TOTAL FLAVANOID AND TOTAL PHENOLIC CONTENT

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Volume 6, Issue 14, Aug 2024

Received: 09 June 2024

Accepted: 19 July 2024

Published: 08 Aug 2024

doi: 10.48047/AFJBS.6.14.2024.3885-3902

ABSTRACT:
Traditional systems of medicine like Ayurveda, Homeopathy and Unani system of medicines have used it as a remedial plant for different diseases. Passiflora contains a lot of phyto-constituents such as flavonoids, glycosides, alkaloids, phenolic and volatile constituents which have therapeutic potentialand clinical applications. In present investigation, it is observed that the application of elicitor (salicylic acid) on the *Passiflora foetida* Linn. leads to enhanced production level of flavonoids and phenolic content under controlled environmental conditions. The present study was performed by the application of SA on the plants grown in green house. The concentration variations in the chosen abiotic plant elicitor i.e salicylic acid in 50 µM, 150 µM and 250 µM concentrations was done in controlled green house. The percentage yield of untreated plants and 50 µM, 150 µM and 250 µM salicylic acid treated plants was found to be 10.52, 9.25, 9.28 and 10.53 respectively. The phytochemical screening of the extract of SA (150 µM) shows the presence of tannins, glycosides, Phenolic compounds, flavonoids, phyosterols and terpenoids. The total flavonoid content was calculated as Quercetin equivalent (mg of Quercetin /g of extract) (QE) at different concentration of SA as 50 µM, 150 µM and 250 µM and was found to be 30.22, 47.94 and 42.36. The maximum concentration was found at 150 µM as 47.94±0.411mg of Quercetin /g of extract and percentage change in total flavonoid content as compared to control was 70.23%. The total flavonoid content in the untreated plants was found to be 14.27±0.286. The total phenolic content was calculated as Gallic acid equivalent (mg of GA/g of extract) (GAE) at different concentration of SA as 31.41, 40.21, 37.14 and the maximum concentration was found at 150 µM as 40.21±0.503mg of GA/g of extract) (GAE) and percentage change in total phenolic content as compared to control was 56.77%. The total phenolic content in the untreated plants was found to be 17.38±0.417. TLC has been done to separate different components present in different selected solvents using solvent system Ethyl acetate: Methanol: Distilled water: Formic acid (50:2:3:6 v/v) as mobile phase and detection at 366nm in absorbance mode was found most appropriate for separation which is based on maximum number of spots observed in applied samples. Calculation of Rf values (0.63) of developed spots of applied extracts was done by observing color intensity.
KEYWORDS: Elicitors, salicylic acid (SA), *Passiflora foetida* Linn.

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 nutraceutical, cosmaceutical 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 (Gairola S. et al., 2010). *Passiflora foetida* Linnaeus is an annual climibing herb. It is also known as passion flower (Holm L.G. *et al*., 1991). It is also known as in english (love in a mist) and hindi (Rakhi flower, Krishna kamal and Jhumkalata). It is belonging to Passifloraceae family. It is also found in India, Central America, South East Asia, Arica and Brazil South America (Schoch G. *et al*., 2001). It has a lot of active phytoconstituents such alkaloids, phenols, glycoside, flavonoids, cyanogen compounds, (Dassanayake E.M. 1994, Hoffmann L. et al., 2003) C-glycosyl flavonoids (apigenin and luteolin), isovitexin, vitexin, kaempferol, isoschaftoside and kaempferol (Patil S. *et al.,* 2012, Patil A. S. *et al*., 2013). 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 (Dassanayake E.M. et al., 1994).

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. 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 (Patel H. et al., 2013). 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 (Namdeo A.G. et al., 2013).

MECHANISM OF ACTION OF ELICITORS

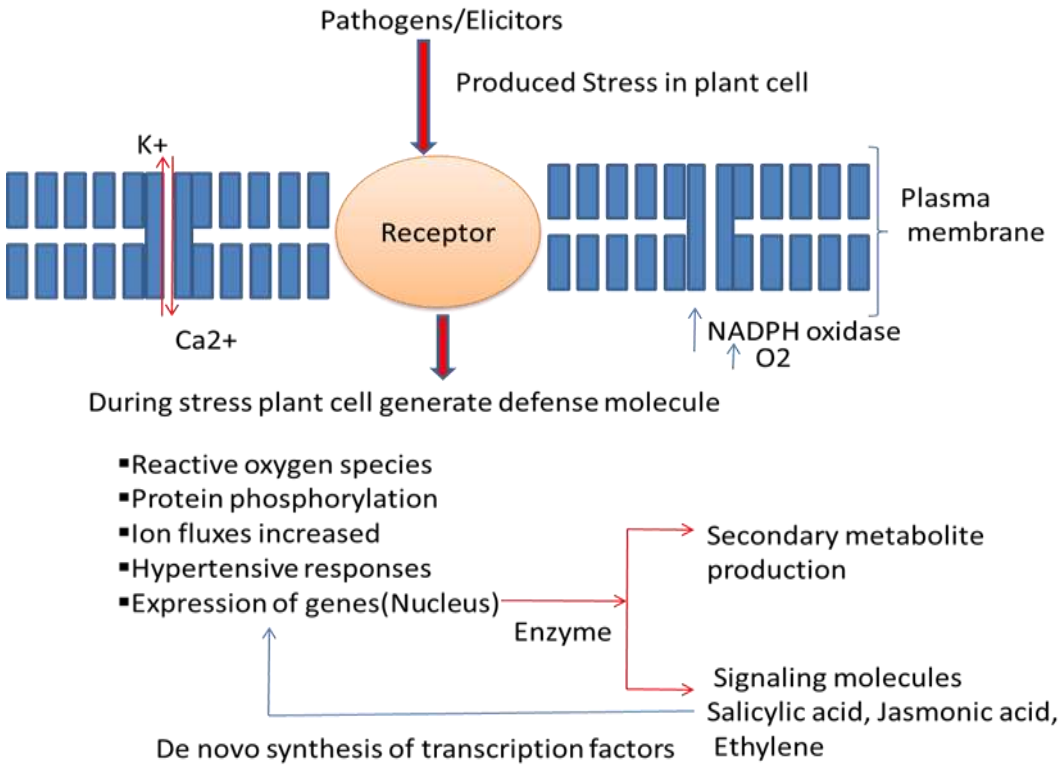

Figure: 1 General mechanism of action of Elicitors application

Each plant cell has defence response system which is capable to produce defence responses against pathogens, elicitors or environmental stresses. This defence response in plant cell is determined by their genetic characteristic and physiological state. In most common cases, plant resists to some diseases because it is genetically controlled by plant resistance genes and pathogen avirulent avirulence (Avr) genes (García-Brugger A. et al., 2006). However, defences responses in plant cell is not always produced by avirulent avirulence (Avr) genes but it can be activated by defence responses in cultivars or elicitors responses in major plant species (García-Brugger A. et al., 2006). Initially elicitors or pathogens stimulate the receptors which are present in the plasma membrane of the plant cell (Montesano M. et al., 2003). Pathogens or elicitors produce stress in the receptors of plasma membrane of the plant cell. So, during stress some defence molecules gets generated in the plant cells like reactive oxygen species (ROS), reactive nitrogen species (RNS), defence-related genes etc. Along with it, changes in plasma membrane potential and enhancement of the chlorine anionic ion and potassium cationic efflux and calcium cationic influx, rapid changes in lipid oxidation, changes in protein phosphorylation, structural defence barriers like that reinforcement and lignifications deposition in cell wall etc. and activation of De-novo biosynthesis of transcription factors also occurs which regulates the expression of genes which are involved

in the secondary metabolites production in the plant cell (Ferrari S. 2010, Smetanska I. 2005, -Zhao J.L . 2010a).

MATERIAL AND METHODS

Materials: Bamboo wooden stick, plastic sheets, green cotton cloths, Leaves of *Passiflorafoetida* Linn. collected from the polygreen house of Smriti college of pharmaceutical Education Indore after the treatment of different concentration of Salicylic acid. Petroleum ether, Methanol, Mayer's reagent, Dragendroff reagent, Hager's reagent, Wagners's reagent Molish reagent, Fehling solution A and B, ferric chloride, lead acetate, bromine solution, ninhydrine reagent, HCI, Zinc dust, acetic anhydride, sulphuric acid, benzene, sodium hydroxide, querecetin, gallic acid, acetic acid, folin-ciocalteu reagent, sodium carbonate, Ethanol, bath-sonicator,

DEVELOPMENT OF GREEN HOUSE

Methods: Hooped green house or poly house provided natural stress-free ventilated control environment for the growth of the *Passiflora foetida* Linn. This is developed in the medicinal garden of the Smriti College of Pharmaceutical Education, Indore in the month of November 2022. 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. It was exclusively constructed by the eco-friendly materials like bamboos and green cotton cloths due to which plant gets easily acclimatized in greenhouse. This greenhouse assures the growth of the plant and the effect of the elicitors on it. The greenhouse had a very good facility of irrigation and drainage.

Figure: 2 The greenhouse area was divided into separate blocks in which controlled and elicitor treated groups of selected medicinal plants were arranged.

Figure:3 Climber of *Passiflorafoetida* Linn.

TREATMENT OF *PASSIFLORA FOETIDA* LINN. PLANT IN POLY-GREEN HOUSE WAS DONE WITH SELECTED ELICITORS i.e. SALICYLIC ACID (SA).

The procured selected medicinal plants were cultivated in the poly greenhouse using randomized complete block design. The green house area was divided into separate blocks in which controlled and elicitor treated groups of selected medicinal plants were arranged. The selected medicinal plants were irrigated at regular interval by using the drip water system. The study started after the cultivation and acclimatization of the plants (9-10days). When the appearance of the signs of growth and development of apical leaves are visible then only the application of elicitors were started.

Varying concentration of Salicylic acid were sprayed using spray bottle at the region in the treated group plant division which is shown in Figure: 3The control group was irrigated regularly for their continuous growth. The spraying of elicitors (Salicylic acid) was continued for 45days where the growth of new apical leaves was seen.

<table>
<tr><td>SA (50µM)</td><td rowspan="3">Free Moving Space</td><td>SA (150µM)</td><td rowspan="3">Free Moving Space</td><td>SA (250µM)</td><td rowspan="3">Free Moving Space</td><td rowspan="3">Untreated plants of Passiflorafoeida Linn.</td></tr>
<tr><td>SA (150µM)</td><td>SA (250µM)</td><td>SA (50µM)</td></tr>
<tr><td>SA (250µM)</td><td>SA (50µM)</td><td>SA (150µM)</td></tr>
</table>

Figure:4 Block design of *Passiflora foetida* Linn.in poly-green house for Salicylic acid (SA) application in different concentration.

COLLECTION, DRYING AND SOLVENT EXTRACTION OF LEAVES OF *PASSIFLORA FOETIDA* LINN.

The leaves were collected from all groups after forty-five days of the exposure of the salicylic acid. Leaves of plants of all groups were collected and shade dried. The leaves were shattered and screened through 40 number mesh. After that the successive solvent extraction was performed by using soxhlet extraction. Firstly, defatting performed of plant material by using the petroleum ether for extraction for 4hr at 60°C. After that drying and levigation of the mark was done and then extraction by the use of ethanol was done. The percentage yield was calculated. The extract was allowed to dry and latter suspended in water and extracted by the use of ethyl acetateand latter used for various phytochemical analysis including

- Different identification test for the presence of various active secondary metabolites.
- Determination of total phenolic content and total flavonoids content.

- TLC Analysis

DIFFERENT IDENTIFICATION TEST FOR THE PRESENCE OF VARIOUS ACTIVE SECONDARY METABOLITES.

Phytochemical screening was performed in the extract of elicitor (SA) treated plants and control (untreated) plants as per the standard methods for the qualitative analysis of glycosides, alkaloids, saponin, tannins, flavonoids, phenol, steroids and Protein.

DETERMINATION OF TOTAL FLAVONOID CONTENT

Material: Ethyl acetate extract of *Passiflorafoetida* Linn. which is obtained from the exposed to various concentration of Salicylic acid as 50µM, 150 µM,250 µM, 5% sodium nitrate solution, 5% aluminium chloride solution, sodium hydroxide solution (1M), quercetin, UV-Visible spectrophotometer (Shimadzu 1800).

Method: 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. Similarly, the total flavonoid contents of extract of cell suspension culture exposed to different Salicylic acid concentration were calculated. The total flavonoids contents were determined as quercetin equivalents from a calibration curve of quercetin (Zhishen*et al*, 1999).

DETERMINATION OF TOTAL PHENOLIC CONTENT

Material: Ethyl acetate extract of *Passiflora foetida* Linn. which is obtained from the exposed to various concentration of Salicylic acid as 50µM, 150 µM and 250 µM, 10% of Folin-Ciocalteureagent, Na2CO3, gallic acid, UV-Visible spectrophotometer (Shimadzu 1800).

Method:

The phenolic content in ethyl acetate extract of *Passiflora foetida*Linn. was determined by using spectrophotometric method by using FC reagents (Folin-Ciocalteu reagent) (Singleton VL et al 1999). 10% of Folin-Ciocalteu reagent (2.5ml) and 7.5%w/v of Na2CO3 (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 Na2CO3 (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.

Statistical analysis of data

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

THIN LAYER CHROMATOGRAPHY (TLC)

Preliminary identification of phytoconstituents was performed from the ethyl acetate extract by using thin layer chromatography.

Preparation of TLC plates:

Silica gel G (gypsum) was an adsorbent used as a stationary phase and it was taken in glass pestle mortar and added distilled water. The mixture was homogenous stirred in order to make homogenous slurry. The slurry was allowed to swell for about 15minutes. According to consistency if required distilled water then added it with constant stirring. The slurry was ready to be pouring on the base line marked TLC plate. When uniform slurry gets spread on the TLC plate.

Drying and storage of TLC plates:

The freshly prepared slurry coated TLC was allowed for air drying for the time till disappeared the transparency of the layer was achieved. Air dried TLC plate was activated in hot air oven for 30minute at 110°C. The activated TLC plates were stored in a desiccator, till required for further use.

Application of the sample:

Apply sample with help of glass Capillary in the base line of Activated TLC plate, keeping a minimum distance of about 1cm between two adjacent spots. The spots of the samples were also marked on the top of the plate in order to know their identity.

Chromatographic chamber, condition of saturation and the development of LC plates:

TLC chamber is made up of rectangular glass chamber its dimension 16.5cm X 29.5cm is used for the characterization. The filter paper, "U" shaped sheet (approximately 15cm X 40cm) is allowed to be placed in TLC Chamber for order to avoid insufficient chamber saturation. The filter paper was allowed to be absorbed (soaked) developing solvent in the developing chamber. When the filter paper got moistened, it was then allowed to be pressed against the walls of the chamber in order to adhered to the wall of glass chamber. The chamber was allowed to saturate for time period of around 24 hours before its use. The chromatographic experiment was carried out at room temperature in diffused daylight.

Developing solvent system:

In this study many developing solvent systems was tried and the satisfactory resolution system was noted and reporting of the most suitable solvent system was done (Shuayprom et al 2016).

Detector/spraying equipment:

UV chamber was used for the substance exhibiting fluorescence. The results are observed at 366nm of UV light and in day light.

RESULT AND DISCUSSION

Analysis of pharmacognostic activities of leave of *Passiflora foetida* Linn.

Table:1 Organoleptic features of dried leaves of *Passiflorafoetida* Linn.

Common name of plant	Scientific name	Parts of the plant used in study	Colour	Odour	Consistency
Rakhi flower	*Passiflora foetida* Linn**.**	Leaves	Greenish Black	Characteristic	Sticky

Table:2 Percentage yield of ethanolic extract of leaves of *Passiflora foetida* treated with Salicylic acid

Name of Solvent	**Percentage yield (Untreated plants)**	**Percentage Yield (Treated with 50µM/SA)**	**Percentage Yield (Treated with 150µM/SA)**	**Percentage Yield (Treated with 250µM /SA)**
Ethanolic extract	10.52	9.25	9.28	10.53

Table:3 Phytochemical qualitative tests of extract of leaves of *Passiflora foetida* Linn. treated with Salicylic Acid

S. no.	**Phytochemical test**	**Ethyl acetate treated extract**
1.	Glycosides	+++
2.	Alkaloids	+
3.	Saponin	-
4.	Tannins	+
5.	Flavonoids	+++
6.	steroids	-
7.	phenolic	+++
8.	Protein	+

DETERMINATION OF TOTAL FLAVONOID CONTENT WITH SALICYLIC ACID ELICITOR APPLICATION

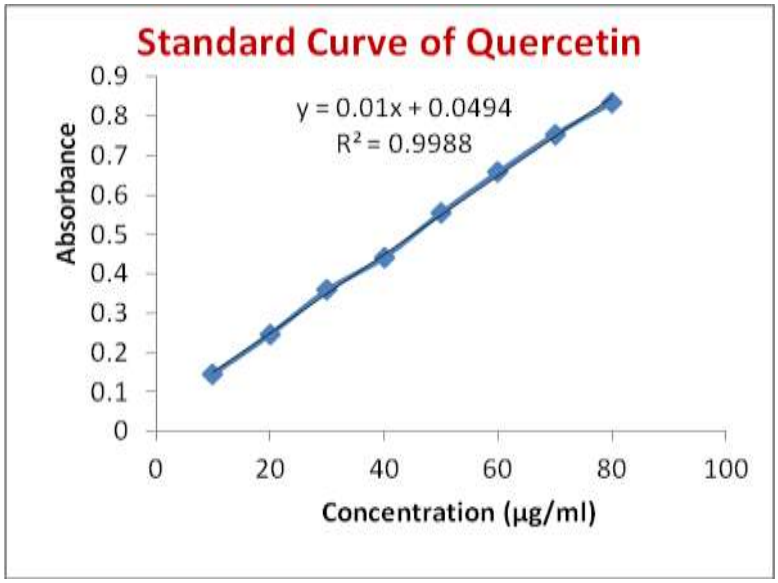

Figure:5 Standard curve of Quercetin

Quantitative Analysis of extract for its flavonoid content in SA treated *Passiflorafoetida* Linn.

Table:4 Total flavonoid content of the plant extracts (ethyl acetate extract of *Passiflora foetida* Linn.) expressed in terms of Quercetin equivalent (mg of Quercetin /g of extract) subjected to elicitor application (SA).

S.no.	Concentration of elicitor SA(µM)	Quercetin equivalent (mg of Quercetin /g of extract)	Percentage change in Total Flavonoids content content as compared to control
1.	0.00 Untreated plant (Control)	14.27±0.286	--
2.	50	30.22±0.731	52.77
3.	150	47.94±0.411	70.23
4.	250	42.36±0.407	66.31

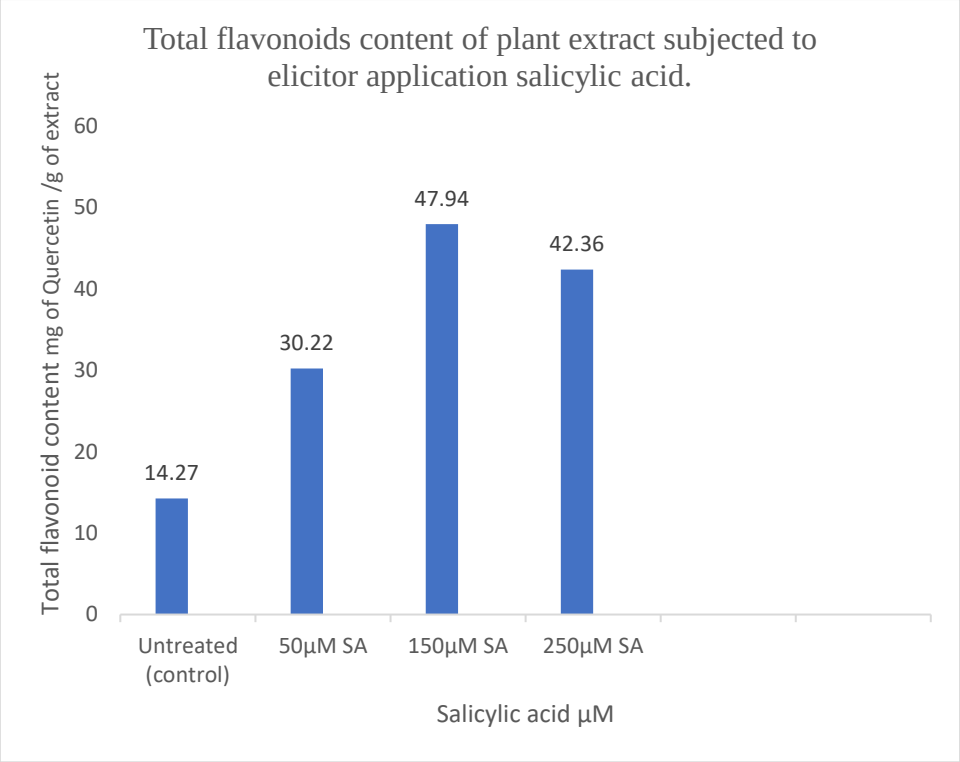

Figure :6 Total flavonoids content of plant extract at various concentration of Salicylic acid application.

DETERMINATION OF TOTAL PHENOLIC CONTENT WITH SALICYLIC ACID ELICITOR APPLICATION

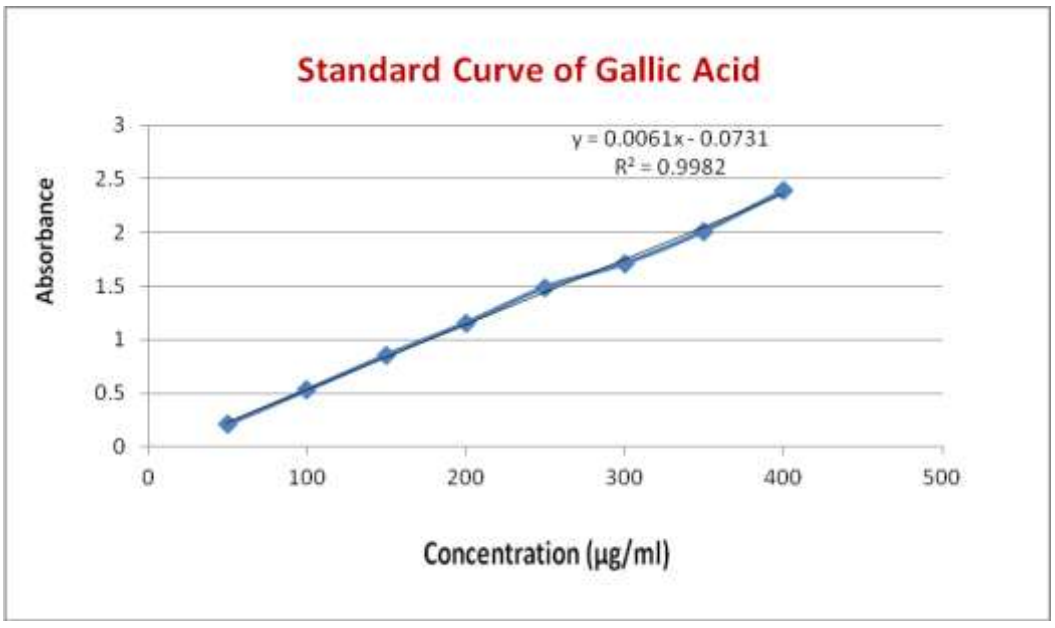

Figure:7 standard curve of gallic acid

Quantitative analysis of extract for its phenolic content in SA treated *Passiflora foetida* Linn.

Table:5 Total phenolic content of the plant extracts (Ethyl acetate extract of *Passiflora foetida* Linn) expressed in terms of Gallic acid equivalent (mg of GA/g of extract) subjected to elicitors application (Salicylic acid).

S.no.	Concentration of elicitor SA(µM)	Gallic acid equivalent (mg of GA /g of extract) (GAE)	Percentage change in Total Phenolic content as compared to control
1.	0.00 Untreated plant (control)	17.38±0.417	--
2.	50	31.41±0.376	44.66
3.	150	40.21±0.503	56.77
4.	250	37.14±0.388	53.20

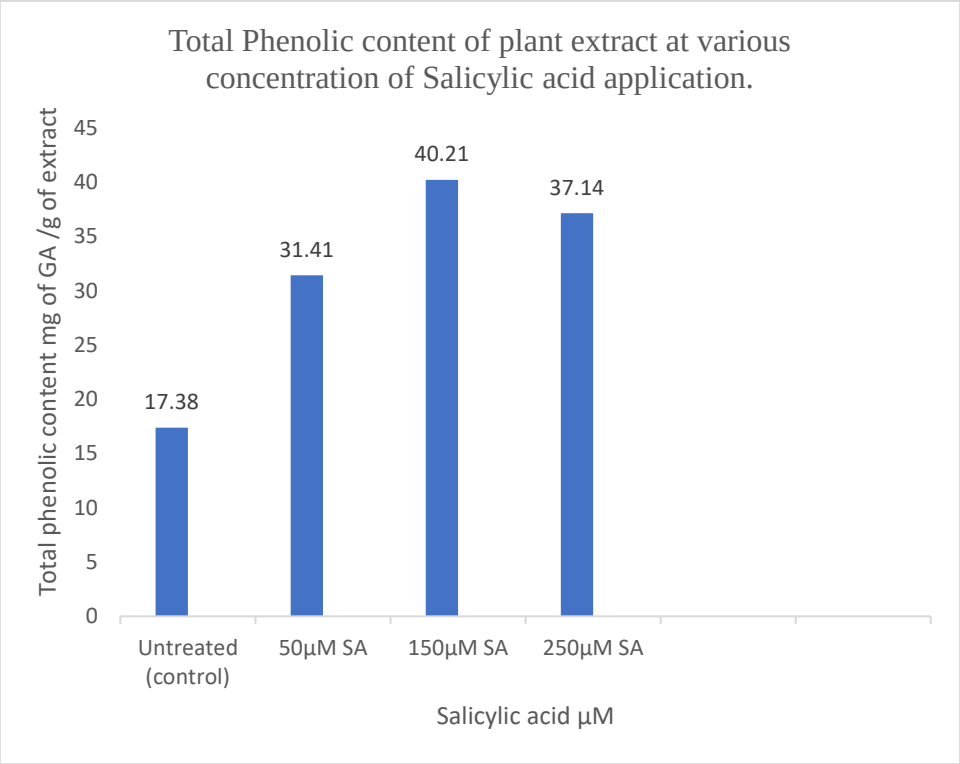

Figure:8 Total Phenolic content of plant extract at various concentration of Salicylic acid application.

Table:6 Thin Layer Chromatography of Ethyle acetate extract of *Passiflora foetida* Linn.

Extract	Mobile Phase	Rf value
Ethyl	Ethyl acetate:	0.63

acetateextract of *Passiflora foetida* Linn. (Treated with Salicylic acid)	Methanol: Distilled water: Formic acid (50:2:3:6 v/v)	

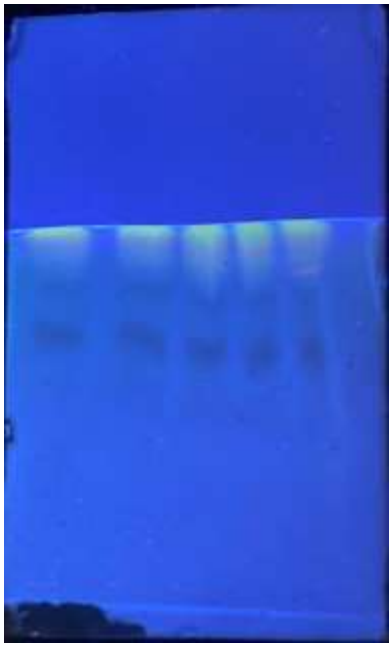

Figure:9Thin Layer Chromatography profiling of elicitor treated extracts**.**

(UV) =Ultraviolet 366 nm

The present study was performed by the application of SA on the plants grown ingreen house. The concentration variations in thechosen abiotic plant elicitor i.e. salicylicacid in 50 µM, 150 µM and 250 µM concentration was done in the controlled green house. The percentage yield of untreated plants and 50 µM, 150 µM and 250 µM salicylic acid treated plants was found to be 10.52, 9.25, 9.28 and 10.53 respectively.

The phytochemical screening of the extract of SA (150 µM) shows the presence of tannins, glycosides, Phenolic compounds, Flavonoids, phyosterols and Terpenoids (Table 24, 25).

The total flavonoid content was calculated as Quercetin equivalent (mg of Quercetin /g ofextract) (QE) at different concentration of SA as 50 µM, 150 µM and 250 µM and was found to be 30.22, 47.94 and 42.36. The maximum concentration was found at 150 µM as

47.94±0.411mg of Quercetin /g of extract and percentage change in totalflavonoid content as compared to control was 70.23%. The total flavonoid content in theuntreated plants was found to be 14.27±0.286. The total phenolic content was calculated asGallic acid equivalent (mg of GA/g of extract) (GAE) at different concentration of SA as 31.41, 40.21, 37.14 andthe maximum concentration was found at 150 μM as 40.21±0.503mg of GA/g of extract) (GAE) and percentage change in total phenolic content as compared to control was 56.77%. The total phenolic content in the untreated plants was found to be 17.38±0.417.

The stress conditions in plants are commonly regulated by many types of defensive mechanisms. Phytoconstituents available in plants also contribute along with pathogen related proteins (PRP) etc. The triggering of defence responses in plants occurs as soon as they undergo stress. The stress may be associated with invasion of plant pathogen. Medicinally important plant like *Passiflora foetida* Linn. used in the study can be artificially stressed through the use of elicitors. This causes elevation in the level of phytochemicals. It is also observed in few cases that some new metabolites can also be generated which are commonly referred as phytoalexins. *Passiflora foetida* Linn. was used in many formulations due to its rich content of flavonoids, phenolics and terpenoids. But environmental factors affect the yield of the plant negatively and need some alternate methodology to enhance its valuable components. The present field study is anapproach to increase the level of flavonoid and phenolic content by the use of salicylic acid as an elicitor in various concentrations (50 μM, 150 μM and 250 μM). Elicitors (salicylic acid) increase the production of active phytochemical constituents (Parimi R et al 2017) in response due tobio-chemical stress. Various types of abiotic trigger responses produce the various enzymes which play active role in Salicylic acid biosynthesis and that result in helping the plant to fight against various types of environmental stresses (El-Gaied LF et al 2013). The salicylic acid treatment increases the response of some active secondary metabolites like phenolic and flavonoids compounds. The most probable mechanism responsible for this activity is the induced production of hydrogen peroxide by salicylic acid that leads to stimulation of the phenylalanine ammonium lyase enzyme which is responsible for the production of phenolic and flavonoids compounds.

CONCLUSION

Plants have played important role in ancient traditional system as well as in modern medicinal research. Many people rely on herbal medicines for the treatment of

differentdiseases and wellness of the health (Winter J.M. et al., 2012, Yuan H. et al., 2016). Plants contain a lot of miraculous medicinal active phytoconstituents which are very important in different sectors such as cosmeceutical, nutraceutical and pharmaceutical (Nasri H. et al., 2014). Demand of such products is increasing because they do not show any side effects (Atanasov A.G. et al., 2015). Elicitors play an important role in 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 (Patel H. et al., 2013). *Passiflora foetida* Linn is a medicinally active plant. It belongs to Passifloraceae family. In the present study, it is observed that the application of elicitor (salicylic acid) on *Passiflora foetida* Linn leads to enhancement in the production level of flavonoids and phenolic content. Additionally, the specific flavonoids and phenolic compounds are qualitatively identified through the chemical characterization. To separate and identify different phytochemicals present in the extract, thin layer chromatography is used. Thin layer chromatography works as a diagnostic tool to identify different phytochemicals which are present in the plant. TLC has been done to separate different components present in different selected solvents using hit and trial method. Various solvent systems have been applied. Out of these solvent systems, solvent system Ethyl acetate: Methanol: Distilled water: Formic acid (50:2:3:6 v/v) as Mobile Phase and Detection at 366nm in absorbance mode was found most appropriate for separation which is based on maximum number of spots observed in applied samples. Calculation of Rf values (0.63) of developed spots of applied extracts was done by observing color intensity. The analysis of the other phytochemicals generated here is required to get the knowledge of complete effect of salicylic acid on *Passiflora foetida* Linn.

CONFLICT OF INTEREST:

No conflict of interest is declared by the authors.

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