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Abiotic elicitor enhanced production of phenolic and flavonoid content in cell suspension cultures of gardenia Gummifera Linn. F

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Abstract
Plant tissue culture techniques provides an excellent plate form in modern medicinal plants studies. The in-vitro callus development in a controlled environmental and media conditions generates undifferentiated mass of cells of medicinally important plant that have the capability to generate and release essential Phyto-Pharmaceuticals when undergoes cell suspension culture studies. Such methodology is of utmost importance when the plant is endangered and at the erg of extinction, Gardenia gummifera Linn. f is one such herbal plant of varied medical importance but its existence is getting declined and its over exploitation made its presence much reduced. The present study is focused on optimization of callus and cell suspension culture of leaf ex-plant of Gardenia gummifera Linn. f and the application of selected elicitors (Salicylic acid) (SA) was done in different concentrations as 100, 300, 600 µM. The intracellular and extracellular harvesting of cell suspension culture for phytochemical was done for all different concentrations at 24 and 96 hours respectively. The quantitative analysis of Phenolic and Flavonoid components was done as per the standard spectrophotometric method in which gallic acid and Quercetin used as standards. The result suggests that the elicitor (SA) had the potential to increase the accumulation of phenolic and flavonoid content at a concentration of 300 µM post 24 hours application.

Keywords: gardenia gummifera Linn. F., callus culture, cell suspension culture, elicitors, salicylic acid, phyto-chemicals

1. Introduction
Indian medicinal plant has its unique position in world in terms of number of herbs and the scientific exploration of these rich medicinal plants in the management and treatment of many dreadful diseases. These plants are serving the society as safe and effective alternative cure to many ailments and bring the quality of life based on the principles of Ayurveda. The constant utilization and exploration of herbs by many Pharma Industries and it's popularly puts an extra burden on nature for its productivity. The effect of various external factors including climate change, pollution etc. is harming its optimal growth and production limits. The results of such mentioned conditions are extinction of many valuable plant species especially those have medicinal importance. Gardenia gummifera Linn. f is amongst one such valuable herbal gem that is in the erg of its diminishment from nature and many governmental agencies have declared it as red marked plant. Since, we are living in the era of scientific and technological advancement and many approaches to protect and prevent these plants are constantly adopted by the scientific and research community. Plant tissue culture is one such methodology that can really contribute in order to preserve the medicinal plants in a controlled laboratory environment.

Plant produces many diverse categories of organic compounds in order to enhance plant's interaction with biotic environment. These secondary metabolites also help in the establishment of a defense mechanism and protect the plant from infection [1, 3].

Stress is a big factor that determines the chemical composition as well as the therapeutic activity of medicinally important plants. The stimulation of stress by the use of elicitor help to induce the production of desirable secondary metabolite by the process of elicitation and it triggers plant defense mechanism (Figure 1A and 1B) as in case of attack by pathogen. The application of elicitor improves the biosynthesis of desired and medicinally active compound in order to adapt the plant to cope up from the stress condition [4]. Elicitors are classified into two major categories biotic elicitors for example chitin, pectin, yeast extract etc, and abiotic elicitors for example heavy metals, pH, ultraviolet radiations and hormones like salicylic acid and jasmonic acids etc (Table 1).

Table 1: Classification of elicitors

Abiotic			Biotic	
Physical	**Chemical**	**Hormonal**		
UV Radiation Osmotic stress Thermal stress Salinity	Heavy metals Mineral salts Gaseous toxins		Polysaccharides	Yeast extract
			Fungal	Bacterial

Biotechnological approaches are employed for the enhancement of production of plant secondary metabolites in cells and organs culture [5, 7].

Salicylic acid an abiotic elicitor is responsible for generation of systemic acquired resistance and it also induces the production of plant secondary metabolites [8, 9]. Many researches on application of salicylic acid in in-vitro plant tissue culture condition have been explored. The production

of withanolide A, withanone, and withaferin A in hair root culture by the use of elicitor [10], similarly tropane alkaloid from Brugmansia candida [11], stilbene production in the cell suspension of V. vinifera [12] are few examples.

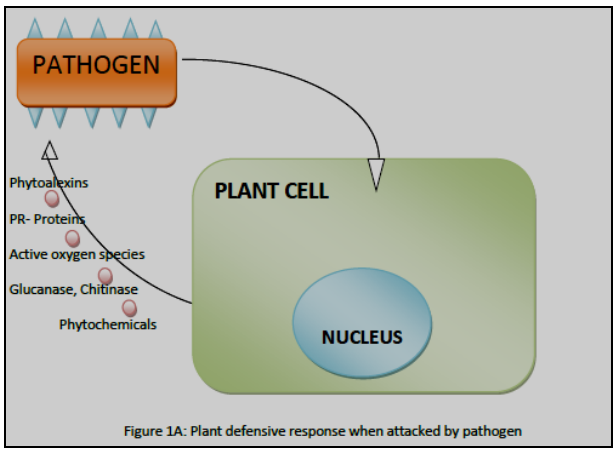

Figure 1A: Plant defensive response when attacked by pathogen

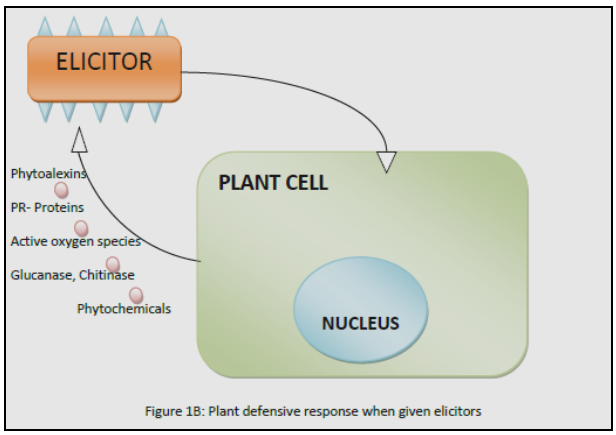

Figure 1B: Plant defensive response when given elicitors

Fig 1A and 1B: Plant cell response due to plant pathogen and elicitor

Gardenia gummifera Linn.f (Dikamali) is an essential and medicinally rich plant but is marked as endangered [13]. It belongs to family Rubiaceae. The leaves are simple, elliptical and oblong. The size of leaves are 4-8 cm long and are shinning in appearance. The flower of Gardenia gummifera Linn.f are solitary or also seen in small clusters and the color of flower is white or pale yellow. *Gardenia gummifera* Linn.f (Dikamali) is a resiniferous plant. It is also mentioned in ancient Ayurveda and found to have many health benefits. Dikamali is reported to possess gardenin-A and other essential phyto-chemicals [14]. The plant is distributed in India specially in the regions of Maharashtra, Karnataka, Kerala, Tamil Nadu.

The taxonomical classification of *Dikamali* [15]
Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Gentianales
Family: Rubiaceae
Genus: Gardenia
Species: Gardenia gummifera

2. Materials and Methods
2.1 Collection and authentication of plant
The plant was collected from the poly-house of medicinal garden of Smriti college of Pharmaceutical Education, Indore and the authentication of plant was done by Dr. S.D. Upadhyaya, Professor & Head, Department of Forestry and Medicinal Plants, J.N.K.V.V, Jabalpur, India.

2.2 Callus culture
2.2.1 Surface sterilization of ex-plant
The induction of callus was started with three-month-old healthy plantlets and the leaves were used as a source of explants. The leaves after its collection were cut in to the portions of around 0.25 cm^2. The surface sterilization was explants was done by washing the explants under running tap water followed by dipping it in 70% (v/v) alcohol and then sterilization is proceeded with 0.1 % (w/v) mercuric chloride ($HgCl_2$) for a time period of about 3-5 minutes. Finally, the explants were rinsed with sterile distilled water for around 3-4 times.

2.2.2 Inoculation of ex-plant in callus culture medium
The explants were cultured in such a way that its adaxial face is at the side of MS medium [16] supplemented with 0.8% (w/v) agar medium that contains different types of auxins like 1-Naphthaleneacetic acid**,** 2,4-Dichlorophenoxyacetic acid and 3-indoleacetic acid in different concentrations in combination with various cytokinins like 6-Benzylaminopurine and Kinetin that help in the induction of callus.
The pH of the medium was 5.5-5.7 and adjusted with help of 0.1 N NaOH/0.1N HCl and measured by using pH meter. Around 15 ml. of prepared media was poured in each culture tube (Jam bottles). The sterilization was done with the help of autoclave (121ºC temperature, 15 psi pressure and 30 minutes time) and further solidified.
The cultures in which the leaf explants was inoculated were kept at 26±2°C as the temperature of incubation. The 16-hour photoperiod time with the arrangement of white fluorescent light (60 µmol m−2 s−1) was also applied during incubation period.

2.3 Cell suspension culture
The cell suspension culture protocol was performed by using friable calli that has large biomass. The portion of calli (1.5 gm fresh weight FW) was inoculated in Erlenmeyer flasks (125 ml.) that contains 25 ml. of MS liquid media supplemented with plant growth regulators as used in the callus development experiment. After the inoculation of calli in liquid MS media the flasks were kept in orbital shaker. The speed was adjusted to 110 rpm, temperature during the incubation was 25 ± 2 °C and the light conditions was photoperiodic for 16 h (white fluorescent light) (60 µmol m−2 s−1). The suspension of cell was continued to grow for 15 days. Further the cell suspension culture was filtered and around 6 gms filtered biomass were again transferred in Erlenmeyer flasks (500 ml.). The liquid MS media in flask was 100 ml. The sub culturing of cell suspension culture was continued every 15 days for around 3 months [17].

2.4 Growth curve
The growth curve of culture was determined. Erlenmeyer flasks (125 ml.) containing 25 ml. liquid MS media were inoculated with around 1.5 grams of fresh weight (FW) and the harvesting of 3 flasks were done every second day. The biomass is further dried at 45 °C for 48 hours. The growth

curve was prepared using the dried weight (DW) of biomass over the course of eighteen days. Specific cell growth rate was determined by plotting the graph between cell growth and natural logarithm of time [17].

2.5 Elicitation method

Salicylic acid solution was prepared in ethanol 50% (v/v). The solution was sterilized through passing with 0.45-micron filters. The elicitor application was carried with 6 days old freshly sub-cultured cell suspensions culture of Gardenia gummifera Linn.f since the exponential growth phase was observed upto 8^{th} -10^{th} day. Cells were harvested 24 h and 96 h post addition of the elicitor. MS liquid sterile media. Different concentration of Salicylic acid was used in this experiment as 0, 100, 300 and 600 µm/ 100 ml of cell suspension. The experiments were performed in triplicate by the use of destructive sampling method. The destructive methods indicate the processing of complete samples at the end of each harvest time. As a control ethanol 50% (v/v) was used [18].

2.6 Intracellular and extracellular metabolite analysis

2.6.1 Extraction and sample preparation

The dried cell biomass obtained from 24 h and 96 h post addition of the elicitor were grounded to give a fine powder. The extract was prepared from powdered biomass with addition of 5 ml. of methanol and kept in bath-sonicator for 10 minutes. Further the sample was centrifuges at 6000 rpm for around 20 minutes. The supernatant was collected and used in the determination of total phenols and flavonoids. The temperature during the entire extraction process was kept below 35^0C [18]. The determination of extracellular metabolites was performed directly from the cell culture medium.

Determination of Total Flavonoid Content: Total flavonoid content in the extracted was determined by standard aluminium chloride assay method (colorimetric assay). All the experiments were performed in triplicate. The 0.5 ml. solution of extract of Gardenia gummifera in methanol was mixed with 2 ml. distilled water and 150 µl of 5% sodium nitrate. The resultant mixture was kept for incubation (5 minutes) followed by addition of 250 µl of 5% aluminium chloride and soft shaking was done and 2mL of 1 M sodium hydroxide solution was fianlly added and the entire reaction mixture was kept at room temperature for a time period of 15 minutes. The absorbance was measured by UV-Visible spectrophotometer (Shimadzu 1800) at 510 nm against blank and the total flavonoid contents of extract of cell suspension culture exposed to different Salicylic acid concentration were calculated. In this experiment quercetin equivalents from a calibration curve of quercetin was calculated [19].

Determination of total Phenolic content: The amount of phenolics in methanolic extract of Gardenia gummifera Linn.f was determined with the help of Spectrophotometric method using Folin-Ciocalteu reagent [20]. The assay was performed in triplicate. Added 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of Na2CO3 (7.5% w/v) to 0.5 ml of each sample of methanolic extract of obtain after the extraction of cell suspension culture of Gardenia gummifera Linn.f. The blank was also prepared by admixing 0.5 ml of methanol 2.5 ml Folin-Ciocalteu's reagent (10%, Prepared in water) and 2.5 sodium carbonate (7.5%). The resulting mixture was incubated at 45°C with constant shaking for 15 min. The absorbance of the extract was calculated at 765 nm using UV-Visible spectrophotometer (Shimadzu 1800). The total phenol content in the samples was measured depending on the calibration curve of gallic acid. The results of the experiment were expressed in terms of mg of gallic acid equivalents per gram of extract.

2.7 Statistical analysis of data

The data were analyzed by the use of Microsoft Excel and reported as mean ± SEM of triplicate determination.

Results and Discussion

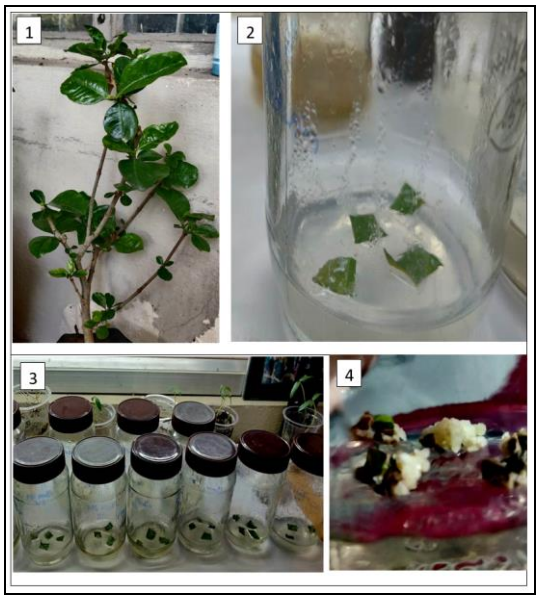

Fig 2: Morphogenetic response on leaf explants from Gardenia gummifera Linn. f (A) plant 3-month-old (B) leaf explants without PGRs (C) Leaf explants with different concentrations of PGR (D) callus grown at 21 days of culture

Growth curve of cell suspension culture

The exponential growth phase of cell suspension culture of Gardenia gummifera Linn. F was continued till day 10^{th}. Further the stationary phase is achieved that last up to day 12^{th} and afterwards the cellular death phase started. The maximum biomass content was around 14±0.23 g/L DW.

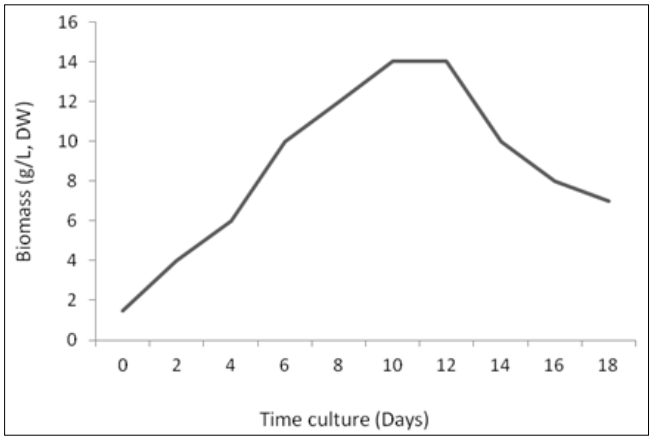

Fig 3: Growth curve of cell suspension culture of Gardenia gummifers Linn. f

Table 2: Intracellular and extracellular Phenolic content of cell suspension culture of Gardenia gummifera Linn. f with different concentration of salicylic acid (SA)

Salicylic acid (µM)	Harvest Time (Hours)	Total Phenolic Content Mg Gallic acid equivalent (GAE) /g DW	
		Intracellular	Extracellular
0	24	4.132 ± 0.020	2.513± 0.091
100		3.720± 0.093	2.726± 0.080
300		4.547± 0.128	3.112± 0.060
600		4.319± 0.144	2.361± 0.063
0	96	4.020± 0.075	3.525± 0.112
100		3.820± 0.079	3.429± 0.100
300		3.932± 0.040	2.820± 0.178
600		4.110± 0.063	2.525± 0.133

Table 3: Intracellular and extracellular Flavonoid content of cell suspension culture of Gardenia gummifera Linn. f with different concentration of salicylic acid (SA)

Salicylic acid (µM)	Harvest Time (Hours)	Total Flavonoid Content Mg Quercetin equivalent (QE) /g DW	
		Intracellular	Extracellular
0	24	3.115± 0.174	2.334± 0.065
100		2.822± 0.109	2.515±0.123
300		3.882± 0.130	3.112± 0.129
600		3.264± 0.150	2.121±0.071
0	96	3.262± 0.147	3.313± 0.068
100		3.626± 0.107	3.212± 0.120
300		3.152± 0.151	2.950± 0.103
600		3.326± 0.123	2.617± 0.068

The table 2 and 3 shows the effect of application of salicylic acid in cell suspension culture of Gardenia gummifera Linn.f on the production of phenolic and flavonoid content at extracellular and intracellular levels. The results suggest that at the intracellular levels of metabolite content accumulation there is a dose dependent effect and the maximum concentration was observed at 300 µM Salicylic acid 24 hours post elicitation similar results for extracellular level accumulation of phenolic and flavonoid content was observed with 300 µM Salicylic acid 24 hours post elicitation. But the level of intracellular accumulation is more at intracellular level.

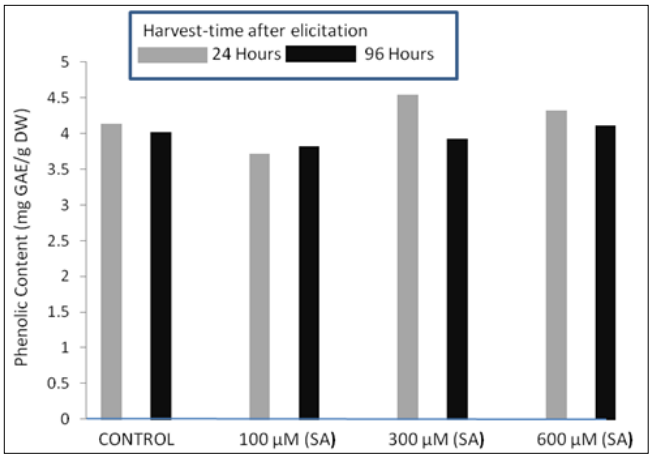

Fig 4: Effect of elicitor addition times on the intracellular phenolic compounds production in cell suspension cultures of G. gummifera Linn. f.

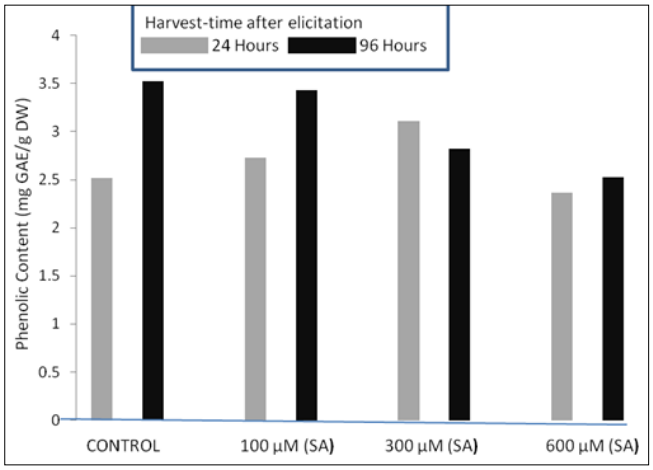

Fig 5: Effect of elicitor addition times on the extracellular phenolic compounds production in cell suspension cultures of G. gummifera Linn. f.

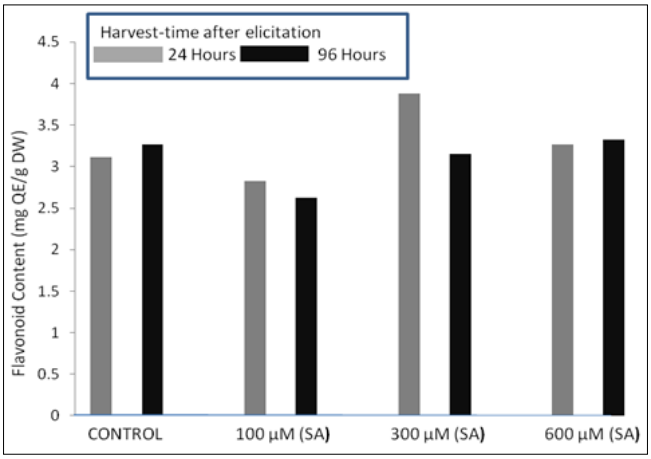

Fig 6: Effect of elicitor addition times on the intracellular flavonoid compounds production in cell suspension cultures of G. gummifera Linn. f.

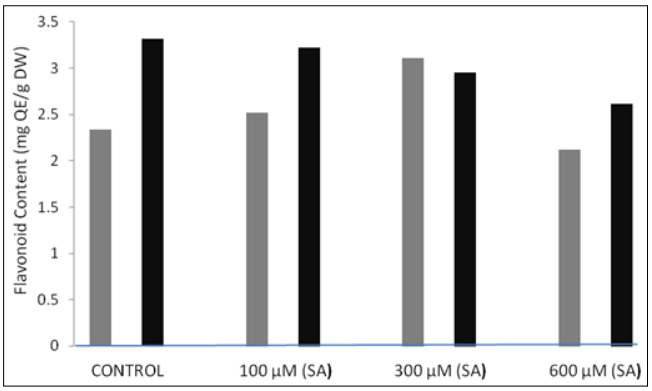

Fig 7: Effect of elicitor addition times on the extracellular flavonoid compounds production in cell suspension cultures of G. gummifera Linn. f.

Conclusion

The generation of total phenolic and flavonoid content through plant metabolic pathways was stimulated in the cell suspension culture of Gardenia gummifera Linn. f. The abiotic elicitor used in the study was salicylic acid and the

different concentrations of SA was tested for their ability to enhance the production of secondary metabolites particularly phenolic and flavonoid. It was found in the study that salicylic acid at the concentration of 300µM was effective as compare to untreated group post 24 hours of elicitor application. The possible mechanism behind the elicitation effect of SA is may be due to the activation of phenylpropanoids metabolic pathway. The findings of the study explain us that the use of elicitor technology along with scale up process can greatly affect the massive production of phyto-pharmaceuticals from Gardenia gummifera Linn. f. As discussed earlier the plant is in endangered category so the use of this methodology the Industrial productivity of secondary metabolite from plant can be enhanced. Further chemical characterization on specific phenolic and flavonoid content is necessary along with other phyopharmaceuticals in order to draw the complete sketch of outcome of abiotic elicitor on gummifera Linn. f.

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