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RESEARCH ARTICLE

Estimation of Cell Suspension Culture with Enhanced Aporphine of *Annona squamosa* L.

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ABSTRACT

Annona squamosa L. is an economically important species. *Annona squamosa* L. leafs are significant as herbal substitute for diverse diseases. Present study's main objective to evaluate the enhancement of Aprophine- Alkaloid in Cell suspension culture of *Annona squamosa* L. Suspension culture with treatment of Salicylic Acid (SA) was experienced on modified Murashige and Skoog (MS) strains with specific combination of Growth Regulators (3.0 mg L-1 BAP and 4.0 mg L-1 NAA). Cell cultures were treated with different concentration of Salicylic acid (SA) (25, 50, 75 and 100 mg/L) separately. Thin Layer Chromatography (TLC) and High pressure liquid chromatography (HPLC) was performed for the quantitative analysis of isolated secondary metabolites-Aprophine in isolated control and Salicylic acid treated Cells. Retention time for the recognition of Aprophine, was reported at 5.75 min and 6.5 min respectively at 254 nm. Highest accumulation was observed in 100 µM Salicylic acid treated cell culture in comparison to control. The results of this study revealed enhanced accumulation in Cell suspension culture of *Annona squamosa* L. by using Salicylic acid.

Keywords: Aprophine, *Annona squamosa* L., Growth Regulators, Suspension culture, Salicylic acid.

INTRODUCTION

The relation of herbal medicine inaugurates with human progression. The term medicinal plants cover different kinds of plants and a number of these plants have medicinal accomplishment. The name of the genus, "Annona", came from the Latin word "anon", which means "annual product", that submits to the yielding of fruits of the varied species of this genus of *Annona squamosa* L. (Morton J. 1987) (Sandeep and Abhilasha, 2017). *Annona squamosa* L. plant is accredited through the medicinal potentials which comprise anti-fertility and antitumour potential. Leaves of

Annona squamosa has been reported that its is having an anti-diabetic activity, anti-lipidimic, insecticidal, anti-tumor, antioxidant and anti-inflammatory activity (Gajalakshmi *et al*., 2011). This is a very noticeable plant in the Ayurveda medicine for curing diverse diseases. This plant is predictably used to treat dysentery, heart problems, worm infestation, constipation, antibacterial infections, dysuria, fever and ulcers. It is originate rising extrovertly and extensively in the hilly territory, waste domain and has turned into entirely naturalized in numerous districts of Rajasthan, Punjab, and Andhra Pradesh (Pathak and Zaman 2013). Aprophine is an alkaloid which is prominently present in *Annona squamosa* L. it is highly present in leaves and tender stem (Morita et al. 2000). Aprophine has various medical applications which can apply in disease curing such from Antineoplastic Agents to Motor Dysfunction Diseases (Nabavi et al. 2017). The aim of the present study to elevate the concentration of aprophine in Cell suspension culture of *Annona squamosa* L. by using chemical elicitors to popularize its commercial use for medical purpose.

MATERIAL AND METHODS

Preparation of Explant and Callus Induction of *Annona squamosa* L.

Young fresh leaves of *Annona squamosa* L. were collected from the Herbal Garden of Jayoti Vidyapeeth Women's Uniersity, Jaipur, Rajasthan. Explants were prepared according to the method described. Young leaves were incubated in the dark at 25 ± 2ºC for three weeks for sprouting buds. Then leaf tips (about 0.5 cm) from the used as an explant for callus induction. *Annona squamosa* L. callus was induced and proliferated according to the method described by (Ali et al. 2016). Sterilized leaf explants were planted in MS (Murashige and Skoog) medium supplemented with auxin and cytokinen hormones combinations 3.0mg/l BAP (6-benzylaminopurine)+4.0 mg/l NAA (Naphthalene acetic acid) as plant growth regulator and were incubated at 25 ± 2º C and photo-period of 16/8h light/dark. Fresh Callus samples were further taken out for the preparation of Cell suspension culture.

Cell suspension culture maintenance

Green color Callus culture with 15 days old age, were chosen to create cell suspension culture. The preliminary inoculums were prepared in an Erlenmeyer flask (150 ml) with 50 mL of modified liquid MS medium having plant growth hormones (3.0mg/l BAP+4.0 mg/l NAA). Two successive subcultures had completed by changing 40 ml of used media with the similar quantity of fresh media with 7 days intervals. Bulky clumps of callus were removed after each subculture by using a 500 micron pore nylon sieve. Following two subcultures, 10 ml of thin cell media (0.1 mL / 5 mL PCV culture) was shifted to new 40 ml media.

Salicylic acid treatment

Well developed cell cultures were transferred to the Media treated with different concentration of Salicylic acid (SA) (25, 50, 75 and 100 mg/L) separately. Elicitor treatment was maintained at 25° ± 2°C, photoperiod of 16/8h light/dark for three weeks in Incubator – Shaker. Triplicates were considered for every treatment to get accuracy of the experimentation.

Preparation of plant extracts

For TLC analysis, extraction of 1 g was done to each sample (a powdered form of cells) with 50 ml of solvent (ethanol) in a beaker during 24 hours at room temperature (Zarzycki 2014). For HPLC analysis, 5 gram powder of cells (for control and each treated sample separately) was added in 100 ml distilled water, heat it at 50 ºC for 30 minutes. After cooling process, it was mixed with 12.5 gram Magnesium oxide (Mgo) and heated again at 50 ºC for 25 minutes. Then it was cooled and 25 ml concentrated ethanol was mixed. This solution was filtered through normal filter paper followed by Whatman filter paper. Solutions were dried in petriplates. For the final step, 5 ml methanol was added in dried petriplates to make a final extract. Then extract was collected in bottles for further analysis (Stevigny et al. 2004).

Quantitative analysis of Aprophine - Alkaloid

Estimation of Aprophine through TLC detection method

Ethanolic extract of suspension culture of different concentration (Control, 25, 50, 75 and 100 mg/L Salicylic Acid) had applied as a spot on the plate 2.0-2.5 cm apart from the edge by means of a micropipette. The solvent was allowed to evaporate.

Plate development

Separation had carried out in a TLC chamber with the developing solvent i.e. mobile phase methanol: water (9: 1 v / v) to a depth of about 1.5 cm. This was a permissible to stand for at least 1 hour with a lid covering the top of the tank to make sure that the atmosphere within the tank becomes saturated with vapors of solvent (equilibration). After an equilibration, the lid was detached and the sample loaded. TLC plates were placed vertically in tank in such a manner that the sample spot should not be in contact with the solvent. The plates were taken out of the TLC chamber when the mobile phase covered two-third the length of the TLC plate and dried (Janac et al. 2008).

Analytical detection

After drying, the plates were exposed to iodine vapors by insertion in the chamber that had saturated with iodine vapors. The developed results were observed under visible light. The Rf value of the diverse spots that were recorded was calculated.

Estimation of Aprophine through High-performance liquid chromatography (HPLC)

Aporphine is an alkaloid which formulates the center of a group of quinoline alkaloids. It can subsist in any of two en-antiomeric forms such as (R)-aporphine and (S)-aporphine. Numerous diverse derivatives have been isolated and characterized from plants. Hence, it's noticeable that numerous peaks are analogous to different Aporphine derivatives would be analyzed in HPLC estimation. Consequently, by combining the peaks in connecting a particular retention time (RT) based on the reference, Aprophine molecule we predicted for the presence/induction of alkaloids in our provisions (Sarma et al. 2002).

RESULTS AND DISCUSSION

Annona squamosa L. Cell suspension culture was established by Callus culture shifting on liquid MS medium (3.0 mg L^{-1} BAP and 4.0 mg L^{-1}NAA). Cell Suspension culture was sub cultured by 2 speedy sub culturing procedures. Cell suspension culture was treated after 15th day of successive subculture in MS media having growth hormone (3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA) with various concentration of mg L^{-1} Salicylic acid separately (Figure 1). Cell suspension culture is a procedure of maintaining hereditarily similar cells in Liquid MS medium. Suspended cells are perpetually developed and used to enhance varied secondary metabolites through rising progression or by over expression of gene answerable for production of secondary metabolites (Dudareva *et al.* 2013).

Quantitative Estimation through TLC of Cell suspension culture of *Annona squamosa* L.

Secondary metabolites were isolated from suspension culture sample in the form of methanol extracts which were loaded to TLC plates and run in the diverse ratios of methanol and distilled water solvent system were used as mobile phase to establish the elute with finest presentation (methanol / distilled water; 9:1) . Subsequent each separation, the TLC plates were incubated at room temperature. In the current cram, the ratio of methanol and distilled water were found for suspension culture through Rf value (4.56, 0.473, 0.578, 0.7631 and 0.3157) in a appropriate solvent arrangement (methanol: distilled water; 9:1). In previous reports, equivalent conclusion of TLC studies uncovered greatest amount of constituent of the ethanol extract, as it had a highest quantity of well - determined stains. Three chief bands were realistic on UV-spray and iodine long plates. Rf value was measured as distance covered by the solute / Distance traveled by the solvent. The value of Rf for the three bands were

recorded as 0.81, 0.68 and 0.38 correspondingly. The Rf value for quinones and steroids is evident at a value of Rf ~ 0.81, geraniol at 0.68 and amino acids at 0.38 and there was no overlap of compounds. This TLC profile can provide the distinguishing impression of the *Annona squamosa* leaf (Vanitha *et al*. 2012).

Quantitative Estimation through HPLC of Cell suspension culture of *Annona squamosa* L.

High pressure liquid chromatography (HPLC) was further performed for the quantitative analysis of isolated secondary metabolites.

Effect of Salicylic acid as AN elicitor on accumulation of Aprophine Alkaloid

The responsibility of Salicylic acid treated as an elicitor for aprophine accumulation was resolute in *Annona squamosa* suspension cultures had been treated with different concentrations of Salicylic acid. According to HPLC analysis, it has been pragmatic in suspansion cultures where aprophine accumulation in suspension cultures had augmented significantly at 25 µM (677.523 ppm) 50 µM (934.71 ppm), 75 µM (582.92 ppm) and 100 µM (1000 ppm) Salicylic acid concentrations with respect to control (185.595 ppm) untreated suspension culture. Maximum aprophine accumulation in suspension cultures was obtained with 100 µM Salicylic acid concentration in comparison to control. Salicylic acid treated as an elicitor has very important role in elevating the secondary metabolite (aprophine) concentration in the cells of *Annona squamosa* (Table 1) (Figure 3-8).

Similar result were observed in various studies of other workers such as Gupta *et al*.,(2012), and Alam *et al*.,(2011). Throughout their studies, HPLC profiles of *Abelmoschus moschatus* were analyzed and two phenolic compounds gallic acid (2.77 min) and hyperzooid (7.31 min), which had different elution times, were examined. These previous studies strongly supports the analysis of the present study. Present study's results is also being supported with the reports of Kavitha and Mohideen (2017). The bioactive components of *Abelmoschus moschatus* flowers were analyzed through HPLC, UV VIS and FTIR. Phytochemical examination of *Abelmoschus moschatus* flowers explained the presence of flavonoids, terpenoids, tannins, saponins, glycosides, triterpenoids, phenol and anthroquinones. All the findings of the present study revealed that Salicylic acid (SA) showed significant effect on enhancement of Aprophine in Cell suspension culture of *Annona squamosa*. Highest accumulation was observed in 100 µM Salicylic acid treated cell culture to comparison to control. This finding support to the fact of positive regulation of salicylic acid on the enhancement of Secondary metabolite.

CONCLUSION

Annona squamosa L. is an efficiently significant species. *Annona squamosa* L. leafs are useful as herbal alternative medicine for diverse diseases. The chief purpose of this current study to assess the augmentation of Aprophine-Alkaloid in *Annona squamosa* L. Cell suspension culture. Cell Suspension culture was developed in modified Murashige and Skoog (MS) strains with growth hormones (3.0 mg L-1 BAP and 4.0 mg L-1 NAA) with treatment of different concentration of Salicylic acid (SA) (25, 50, 75 and 100 mg/L) separately. Thin Layer Chromatography (TLC) and High pressure liquid chromatography (HPLC) analysis showed that uppermost increase was in 100 µM Salicylic acid treated cell culture to comparison to control cell culture. This study revealed the accumulation of Aprophine in Cell suspension culture of study plant under Salicylic acid treatment. This study also helps to understand the future scope of Cell suspension culture for mass production of Secondary metabolites by using Elicitors.

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Table: 1 Concentration Salicylic acid treated sample in callus and suspension culture samples present Aprophine part per million (ppm).

Concentration Salicylic acid treated sample	Suspension culture aprophine present in samples (ppm)
Control	185.595
25	677.523
50	934.71
75	582.92
100	1000

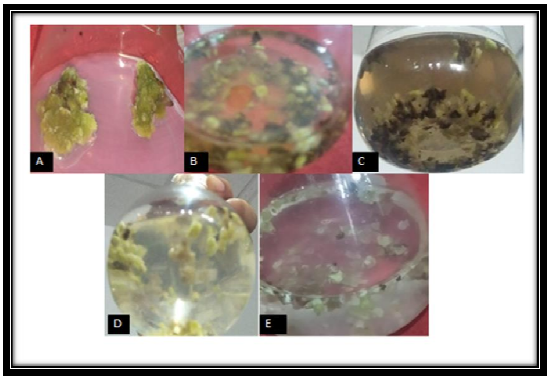

Fig. 1: Cell suspension culture of *Annona squamosa L.* A. *In vitro* developed callus of *Annona squamosa L.* B. Shifting of callus clumps on liquid MS medium (3.0 mg L^{-1} BAP and 4.0 mg L^{-1}NAA).C. Cell Suspension culture after first subculture. D. Cell Suspension culture after second subculture. E. Cell Suspension culture after 15th day of consecutive subculture in liquid MS Medium (having 3.0 mg L^{-1} BAP and 4.0 mg L^{-1} NAA) with various concentration of mg L^{-1} Salicylic acid.

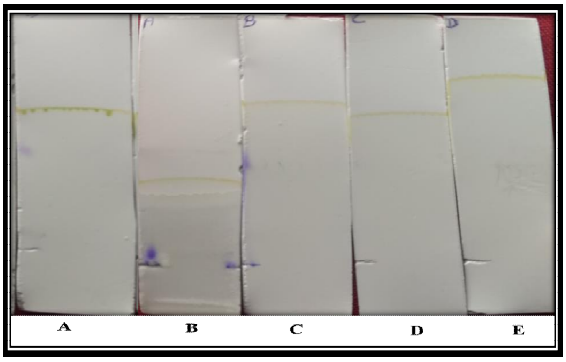

Fig. 2: Methanol extract of Cell suspension culture of *Annona squamosa* L. (A) TLC result without Salicylic Acid (SA) (B) TLC in 25 μM L^{-1} **Salicylic** Acid (SA) added medium. (C). TLC in 50 μM L^{-1} **Salicylic Acid (SA) added medium. (D). TLC in 75** μM L^{-1} **Salicylic Acid (SA) added medium. (E).** TLC in 100 μM L^{-1} **Salicylic Acid (SA) added medium**

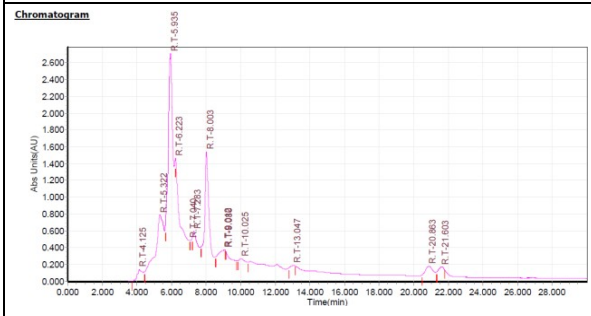

Fig. 3. Quantitative analysis through HPLC for Aprophine standard

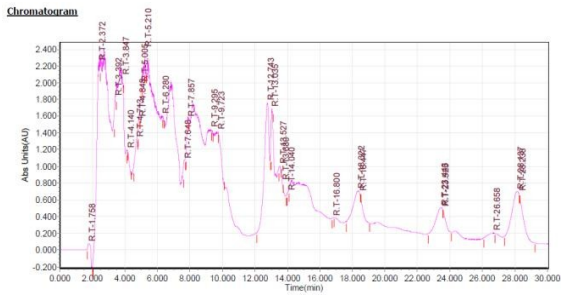

Fig. 4. Quantitative analysis through HPLC for Cell suspension culture of *Annona squamosa* L. (Control- without Salicylic Acid Treatment)

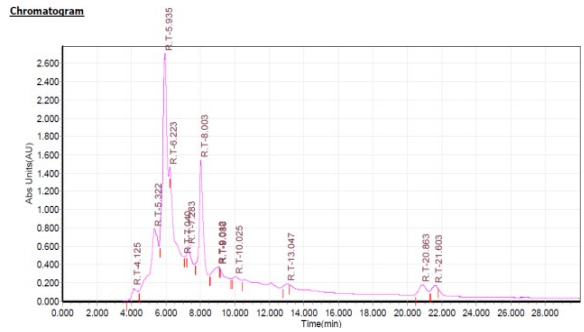

Fig. 5. Quantitative analysis through HPLC for Cell suspension culture of *Annona squamosa* L. (Treated with 25 µM Salicylic Acid)

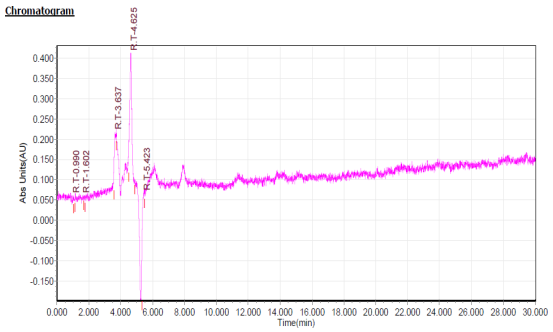

Fig. 6. Quantitative analysis through HPLC for Cell suspension culture of *Annona squamosa* L. (Treated with 50 µM Salicylic Acid)

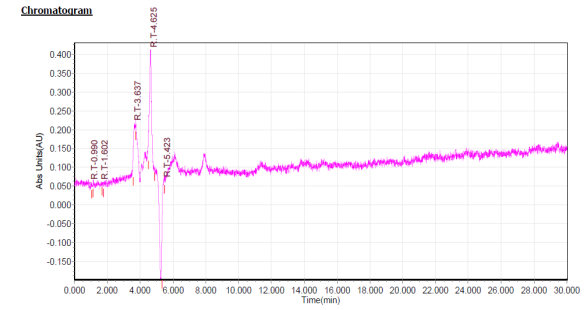

Fig. 7. Quantitative analysis through HPLC for Cell suspension culture of *Annona squamosa* L. (Treated with 75 µM Salicylic Acid)

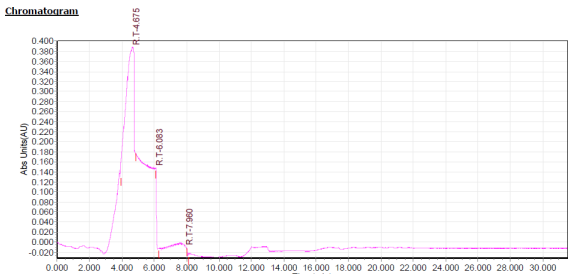

Fig. 8. Quantitative analysis through HPLC for Cell suspension culture of *Annona squamosa* L. (Treated with 100 µM Salicylic Acid)

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LITERARY REVIEW ON SHANKHA

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Abstract
Ayurveda comprise of drugs derived from herbs,minerals, metals and animals. But they can't be taken as it is, hence need to be converted into such form which will be therapeutically fit for use. *Rasashastra* is the most important and popular branch of *Ayurveda* which was developed in the medieval period. *Bhasma* are the unique dosages form of *Ras Shastra*, prepared after proper bhavana with particular herbs with particular metals and

minerals and later they are subjected to puta in a particular manner and due to its fineness and nano particle size it turn into most assimilatory, harmless and therapeutically effectual form

Key words-Rasashastra ,shankha,bhasma