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ANTI-MICROBIAL EFFECTS OF ANDROGRAPHOLIDE USING GIBBERLIC ACID, COLCHICINE, ABSCISIC ACID INDIVIDUALLY AND IN CONSORTIA AND ITS EXPRESSION ON THE ENZYME AMYLASE

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ABSTRACT

Andrographis paniculata, traditionally known as Kalmegh or "Neelavembu and commonly as "king of Bitters" belonging to the family. Three different concentrations of Stimulants (Gibberellic acid, Colchicine, Abscisic acid and consortia) were used for treatment after 40 days of transplantation. Sampling for expression analysis was done at 2hrs, 6hrs and 24hrs after foliar application. Cytological effect of treated *A. paniculata* plants and Cytotoxicity effect using onion bulbs was also studied. The result shows the highest levels of protein in Gb3 treated plants as recorded in **10mM** concentration of **6hr** treatment **(185.13 and 175.3)**. Highest levels of carbohydrates was noticed in 10mM of 2hrs, 6hrs and 24hrs harvest periods as **117.98, 221.25 and 109.31.** The highest levels of fatty acid in Gibberlic acid treated plants was recorded at harvest treatment 24hr in 10mM **(0.130)**. It was lower when compared to untreated plants **(0.178).** In 15mM concentration, stimulation of bacteria and fungus was noticed at 6hr and 24hr. Similarly in 0.05% concentration, stimulation of fungi was recorded at 24hr. HPLC analysis of standard andrographolide showed three peaks with Retention time(Rt) of 2.5min. The A. paniculata extract of 2hr concentration of **consortia** showed two peaks at Retention time of 3min. The **Gibberlic acid** treated plant extract showed two peaks at Retention time of 3min in 24hr harvest periods of 15mM concentration. The **Abscisic acid** treated plant showed two peaks at Retention time of 3min in 24hr harvest period of 5mM concentration. In 24hr harvest period of 10mM concentration

of ABA was also showed two peaks at Retention time of 3min and 5min. Whereas a single peak were observed at Retention time of 7min.

KEYWORDS: *Andrographis paniculata*, Colchicine Gibberlic acid, Abscisic acid.

INTRODUCTION

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulphur and more rarely other elements such as chlorine, bromine, and phosphorus. Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (also called secondary metabolites). Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste.[1] Andrographolide, one of the most important diterpene lactones, abundantly found in *A. paniculata* possess great pharmaceutical value both in siddha and ayurveda systems of medicines in India and China.

Andrographis paniculata

Alkaloid *Andrographolide* from *Andrographis paniculata* plant, traditionally known as Kalmegh or "Neelavembu and commonly as "king of Bitters" belonging to the family Acanthaceae is a wonder drug in both traditional Siddha and Ayurvedic systems of medicine.

Fig 1: Andrographis paniculata.

A number **of** active principles are reported from the plant, which mainly include are diterpene lactones, flavonoids and polyphenols.[2] However, the prime constituent andrographolide has been mainly attributed for its therapeutic properties.[3] Similarly, several major plant hormones are produced through this pathway from direct biosynthesis (gibberellins) or as cleavage derivatives, such as abscisic acid and the recently discovered hormone

strigolactone.[4][5] Due to the central role this is important pathway plays in the growth and development of plants. Hence, a complete understanding of the molecular mechanisms that regulate this pathway is of tremendous importance.

STIMULANTS

Gibberelic acid

Gibberaellins are tetracyclic diterpene acids involved in the natural process of breaking dormancy and initiating germination. Usually in germination, the breakdown of starch to glucose in the endosperm begins shortly after the seed is exposed to water. Gibberellins in the seed embryo are believed to signal starch hydrolysis through inducing the synthesis of the enzyme α-amylase in the aleurone cells. In the model for gibberellin-induced protection of α-amylase, it is demonstrated that gibberellins produced in the scutellum diffuces to the aleurone cells, were they stimulate the secretion α-amylase.α-amylase then hydrolyses starch, which abundant in many seeds. Gibberellins cause higher levels of transcription of the gene coding for the α-amylase enzyme, to stimulate the synthesis of α-amylase.

Colchicine

Colchicine is a toxic natural product and secondary metabolite, originally extracted from plants of the genus *Colchicum* (autumn crocus, *Colchicum autumnale*, also known as "meadow saffron"). It was used originally to treat rheumatic complaints, especially gout, and still finds use for these purposes today despite dosing issues concerning its toxicity. Colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Since chromosome segregation is driven by microtubules, colchicine is also used for inducing polyploidy in plant cells during cellular division by inhibiting chromosome segregation during meiosis; half the resulting gametes therefore contain no chromosomes, while the other half contain double the usual number of chromosomes (*i.e.*, diploid instead of haploid as gametes usually are), and lead to embryos with double the usual number of chromosomes (*i.e.* tetraploid instead of diploid). For this reason, this type of genetic manipulation is frequently used in breeding plants commercially.

Abscisic Acid

ABA is derived from C_{40} epoxycarotenoid precursors through an oxidative cleavage reaction in plastids. The C_{15} intermediate xanthoxin is converted to ABA by a two-step reaction via ABA- aldehyde in cytosol. Abiotic stresses such as drought and salt activate the biosynthetic genes (italicized), probably through a Ca^{2+}-dependent phosphorelay cascade as shown on the

left. ABA feedback stimulates the expression of the biosynthetic genes, which is also likely through a Ca^{2+}-dependent phosphoprotein cascade.

Consortia

The combination of concentration of Gibberllic acid, Colchicine and Abscisic acid make up consortia.

Extraction Methods

The active principles are present in a proteinaceous and polysaccharide matrix, if the matrix is broken down by some method, it may facilitate the extraction of active principles, thus saving time and energy. Conventional methods of extraction like usage of heat is less capable of breaking the cell matrix; while in chemical treatment, the polysaccharide matrix can be broken by acid hydrolysis, but there are chances of degradation of active constituents. Enzymatic pretreatment to facilitate cell matrix destruction can be a better method of choice due to non requirement of heat, less energy consumption, specificity of enzymes and milder conditions.[6] Amylase enzyme was used to facilitate the enzymatic pretreatment.

Amylases

Amylase (alpha→1-4-glucan 4-glucanhydrolase, EC 3.2.1.1) is a key enzyme hydrolysing reserve starch into maltose and dextrin in the endosperm of germinating cereals. Plant extracts are increasingly used in nutraceutical products. Nutraceuticals are chemicals found as a natural component of foods beneficial to the human body in preventing or treating one or more diseases or improving physiological performance.[7]

Analysis of Protein, Carbohydrates, Fatty acids and Drug release

Proteins are large biological molecules, or macromolecules, consisting of one or more chains of amino acid residues. Proteins perform a vast array of functions within living organisms, including catalyzing metabolic reactions, replicating DNA, responding to stimuli, and transporting molecules from one location to another. A **carbohydrate** is a large macromolecule, perform numerous roles in living organisms. A **fatty acid** is a carboxylic acid with a long aliphatic tail (chain), which is either saturated or unsaturated. Fatty acids are usually derived from triglycerides or phospholipids. Fatty acids are important sources of fuel because, when metabolized, they yield large quantities of ATP.

Drug Release

Drugs are alkaloids synthesized through the MEP/DXP pathway. They have several therapeutic uses. They can be released by heat or enzymic methods. The drug released from pretreated dried crude residue is analyzed by UV method at fixed times.

Cytological study (Mitosis) A. paniculata 2n=50

The cytological study is mainly carried out in the somatic cells of the plants. It is usually bring out the information about the cells using root tip squash method.

Cytotoxic Effect (Onion plants) 2n=16

The chromosomal aberrations are normally studied for cytotoxic effects. Onion plants are gives best information for this studies.

Antimicrobial study

It is the analysis of Anti-microbial components and development of broad spectrum of antimicrobial effect against micro organisms.

Quantification of Andrographolide using HPLC

The technique used for the analysis of andrographolide is the high-performance liquid chromatography (HPLC). It is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

MATERIALS AND METHODS

***Andrographis paniculata* plant growth conditions and treatments**

A well characterized genotype of *Andrographis paniculata* (Kalmegh) of Quaid-E-Millath Government College, Chennai, India was grown at a temperature 26±2°C. Relative humidity was maintained at 65-75% in whole growing periods. Thirty day old plantlet of *A.paniculata* was transplanted in the plastic pots containing soil: sand: FYM with the ration of 1:1:1. There were no chemical fertilizers and plant protection measures were adopted during the investigation. Three different concentrations of Gibberellic acid (Gb3) (5mM,10mM,15mM), Colichicine (0.02%,0.05%,0.08%), ABA (5mM,10mM,15mM) and consortia were used for treatment after 40 days of transplantation. Sampling for expression analysis was done at 2hrs,6hrs and 24 hrs after foliar application. Andrographolide estimation was done just before the blooming of plants. Control plants were grown under similar conditions without chemical treatments.

Standardization and dose optimization of enzymes

Amylase

The amount of amylase required to act on standard substrate was determined by the following method, which depends on the ability of the enzyme to hydrolyze the starch.

100mg of amylase was accurately weighed and dissolved in sufficient acetate buffer (pH=5.0) and the volume was made to 100 ml. Cornstarch (200 mg) used as standard substrate was accurately weighed and added into sufficient distilled water (warm). It was then further warmed gently with stirring and volume was made to 100 ml. The final concentration obtained was 2 mg/ml.

Method

5 ml of standard solution of starch (10 mg) was added into each of 15 test tubes, which were initially numbered serially. The tubes were placed in water bath, which was maintained initially at 40°C. When temperature of the solution in the test tubes was reached 40°C; blank, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0,8.0, 9.0 and 10 ml amylase solution was added into the test tubes from test tube number 1 to 15 respectively. The content in the tubes were mixed thoroughly and placed in water bath (40°C) to maintain the temperature. After 60 minutes, the tubes were removed and in each test tube 0.05 ml of 0.02 M iodine solution was added. The content of the tubes were mixed thoroughly. The tubes were observed for the presence of blue colour. The results were tabulated.

Table 1: Standardisation of Enzyme.

S.No	Amount of starch solution (ml)	Amount of Amylase enzyme added (ml)	O.D value
1	5	0	0.000
2	5	0.2	0.636
3	5	0.4	-0.037
4	5	0.6	-0.015
5	5	0.8	0.003
6	5	1.0	-0.404
7	5	2.0	-0.464
8	5	3.0	-0.378
9	5	4.0	0.008
10	5	5.0	-0.304
11	5	6.0	-0.030
12	5	7.0	-0.188
13	5	8.0	-0.472
14	5	9.0	-0.284
15	5	10.0	0.407

Screening of enzyme: Screening amylase enzyme at 37 °C was processed in the following steps.

Pretreatment of crude drug with enzyme

Quantity of crude drug

The quantity of crude drug taken was 2.5 g which was dispersed in 50 ml of buffer media (acetic acid buffer pH 5). Throughout the study this quantity was kept constant. The effect of amylase enzyme to facilitate drug release with respect to time was studied at 37°C, in a shaker kept at 135 rpm. Time interval of 2, 4, and 24 h were fixed for the studies.

Experimental set up for the enzymatic pretreatment

The solution of amylase enzyme was prepared in acetic acid buffer I.P (pH =5.0). 0.201 g of amylase was dissolved in sufficient acetate buffer and volume was made to 50 ml. 2.5 g of crude drug powder was suspended in 50 ml of buffer solution containing required quantity of amylase enzyme. Three conical flasks were prepared and kept for shaking on orbital shaker incubator at 37°C for time intervals of 2, and 24 h respectively. After stipulated time intervals, suspension in each conical flask was filtered using vacuum. The filtrate was used for the analysis of sugar, protein and drug release, while the residue was dried in incubator and was used for the analysis of drug release. Control were grown under identical conditions replacing gibberelic acid. Similar tests were performed and compared.

PROTEIN DETERMINATION: LOWRY et al., Method

CARBOHYDRATE DETERMINATION: Anthrone method

DETERMINATION OF DRUG RELEASE: UV Method

DETERMINATION OF FATTY ACID: Titrimetric method

Fig: 3 Andrographis paniculata control with Gb3s treated plants.

Fig: 4 *Andrographis paniculata* control with colchicine treated plants.

CYTOLOGICAL STUDY

Mitotic Effects

Healthy *Andrographis paniculata* plants grown in mud pots were chosen for the treatment. Few wounds made on the plants. The plants were sprayed with different concentrations of chemicals at one day interval for three days. Plants without treatment was considered to be control. The terminal tip of the shoot of the plant was taken as the mitotic material. Toluidine Blue stain was used. Material was taken in a watchglass along with few ml of the stain and heated using a spirit lamp. The material was squashed and observed under a microscope.

CYTOTOXICITY EFFECT

Mitotic effects

0.01 g of treated Crude powder of *Andrographis paniculata* was taken separately and mixed with 10ml of distilled water. The onion bulbs were taken and partially suspended in to the solution after required periods of 2hrs and 24hrs, the bulbs were taken out, root tips were excised. The cut root tips were placed in a watch glass with few ml of Acetocarmine stain and heated using a sprit lamp. The onion root tip were then transferred to a clear slide, squashed and observed under a microscope. The total no of cells, no of cells showing normal meiotic stages and cells showing Stimulants effect were observed and compared between treated and untreated plants. Photographs were taken.

ANTI-MICROBIAL STUDY

Preparation of Extracts

The chosen plant parts were carefully dried under shade at room and temperature for few days. The sample was ground using mortar and ethanol was added. Extraction was prepared by percolation.

Preliminary Anti-microbial study and Agar well diffusion method

Muller Hinton Agar (MHA) media was prepared and poured in sterile petri plates and allow to solidify. Sterile cork borer of 6mm diameter was used to make wells on the medium.3hrs cultures of each test organism(*Staphylococcus areus ATTC, Aspergillus flavus*) were compared with standard Mc-farland scale and the cultures was uniformly inoculated on the medium by using a sterile cotton swab. 150µl of each extract were dropped into each appropriately labeled wells. The plate were then incubated at 37c for 24 hrs. Zones of inhibition was measured after incubation.

HPLC ANALYSIS

Sample preparation: Air-dried and powdered plant material (1 g) was extracted with methanol (5 ml, 12 hr), filtered, completely dried under vacuum and 5 ml of acetonitrile was added. Sample was filtered through a millipore filter (0.45 µm). The successive extracts used for HPLC analysis.

Chromatographic condition: High Performance Liquid Chromatographic (HPLC) determination of andrographolide was done following procedure of.[8]

RESULTS AND DISCUSSION

A well characterized genotype of *Andrographis paniculata* (Kalmegh) of Quaid - e –Millath Government College, Chennai, India was grown at a temperature 26±2°C. Relative humidity was maintained at 65-75% in whole growing periods. Thirty day old plantlet of *A.paniculata* was transplanted in the plastic pots containing soil: sand: FYM with the ration of 1:1:1. There were no chemical fertilizers and plant protection measures were adopted during the investigation. Three different concentrations of Stimulants (Gibberellic acid, Colchicine, Abscisic acid and consortia) were used for treatment after 40 days of transplantation. Sampling for expression analysis was done at 2hrs, 6hrs and 24 hrs after foliar application. Andrographolide estimation was done just before the blooming of plants. Control plants were grown under similar conditions without chemical treatments.

The enzyme Amylase was used to extract the andrographolide by the enzymatic cell disruption method. This facilitates the release of andrographolide from the polymeric matrix. The screening of the enzyme was done by analysing sugar, protein, fat and drug release. Cytological effect of treated *A. paniculata* plants and Cytotoxicity effect using onion bulbs was also studied.

Gibberlic acid treatment and it's analysis of protein, carbohydrate, fat and drug release

The *Andrographis paniculata* plants were treated with three different concentrations of Gibberlic acid (Gb3) 5mM, 10mM, 15mM after 40 days of transplantation. Sampling for expression analysis was harvested after at 2hrs, 6hrs and 24hrs after foliar application. Each harvested sample was treated with amylase and recovery periods of 2hrs, 6hrs and 24hrs was given and tested for sugar, protein, fat and drug release.

The **protein** content in all the treatments and it's recovery periods were definitely much higher when compared to the control. The highest levels of protein in Gb3 treated plants was recorded in **10mM** concentration of **6 hr** treatment **(185.13 and 175.3)** at **2hrs**

TABLE 2: Gibberlic Acid Treatment And It's Analysis of Protein, Carbohydrate, Fat And Drug Release.

S.no	Control	Concentration of gb3	Harvest after gb3 treatment	Protein		Carbohydrate		Drug release		Fat
				2hrs	24 hrs	2hrs	24hrs	2hrs	24hrs	
1	0.174	5mM	2 hrs	56.75	**53.5**	42.25	46.25	7.5	-7.5	0.13
2	0.172		6hrs	78.63	21.63	29	44	3.75	-3.75	0.11
3	0.172		24hrs	**108.5**	21.63	84.87	103.37	6.25	37.5	0.12
4	0.174	10mM	2 hrs	25.5	54.06	117.98	117.89	4.42	4.335	0.125
5	0.172		6hrs	**185.13**	**175.3**	221.25	173.62	6	-3.75	0.105
6	0.172		24hrs	60.75	82.35	109.31	8	3	-1.875	**0.130**
7	0.174	15mM	2 hrs	0.73	7.55	8.03	12.48	**17.16**	18.98	**0.128**
8	0.172		6hrs	-41.13	-197	-170.37	-190.13	**11.25**	7.50	**0.129**
9	0.172		24hrs	-26.02	6.46	-28.981	10.402	**18.98**	10.95	**0.127**

and **24hr** recovery period after amylase pre-treatment. Similarly, **5mM** concentration of **24hr** treatment with **108.5 at 2** hr recovery period after amylase pre-treatment was also noticed. It was higher compared to other treatments and their recovery periods and untreated plants. Lower levels of protein was seen 15mM concentration at 6hr and 24hr durations.

[9]reported that Gibberlic acid (GB3), evaluated for their effects on the protein content of date palm somatic embryos, increased protein content at 3mM Gb3 (20 μM). According (10) the elevated contents of endogenous Gb3 contents positively and significantly correlated with free amino acid contents and Glutamine synthase activity and soluble protein contents in grains, suggesting that the increased protein concentration was mainly due to the enhanced enzyme activity of ammonia assimilation and substrate concentration of protein synthesis under exogenous application of Gb3.

In agree with the above reports, our results also clearly demonstrated that Gb3 was positively related to the increased protein.

The **carbohydrate** content in the GB3 treated *Andrographis* plants was also found to be much higher than the untreated control. Highest levels of carbohydrates was noticed in 10mM of 2hrs, 6hrs and 24hrs harvest periods as **117.98, 221.25 and 109.31.** 2hr recovery period after pretreatment showed higher values of carbohydrates than 24hr recovery periods. 15mM showed less carbohydrate values in all the harvest periods. This increase was mainly correlated with the exogenous application of GB3.

[11]has reported that in *Polypodium vulgare* the application of exogenous Gb3 triggers more sugar accumulation, but decrease starch content.[6] reports that this behavior of enzyme gives support to the hypothesis that polysaccharides and polypeptides polymer are interlinked with each other to form complex network. But according to the present study, it is noted that both protein and carbohydrate content was much higher in 10mM followed by 5mM and lesser content was seen in 15mM. Similarly, as the protein content increases carbohydrate content also increases. The Gibberellic acid treated plants released more **drug** than the untreated *Andrographis* plants. The highest values was noticed in 15mM concentration in all the harvest periods. 5mM and 10mM concentrations shower lesser drug release values. The results are in agreement with the reports[12], which also showed that Gb3 treated plants showed increased contents of andrographolide when compared to control.

In our report also the drug release was found to be highest. It was noticed that with increase in drug release, the content of protein and carbohydrate values were reduced and vice versa.

The **fatty acid** content in all the treatments were definitely much lower when compared to the control. The highest levels of fatty acid in Gibberlic acid treated plants was recorded at harvest treatment 24hr in 10mM **(0.130)**. It was lower when compared to untreated plants **(0.178)**. Low levels of fatty acid treated plants was seen in 5mM harvest periods. According to[13] Gibberlic acid treatment brought about a general decrease in the concentration of the phospholipids. Hale *et al* also found a decrease in total lipids (Fatty acids) in stem of peanut treated with Gibberlic acid.

Our present findings also revealed that Gibberlic acid brought about a decrease in the content of fatty acid in treated plants over untreated plants.

CYTOLOGICAL STUDY

Mitotic effct of gibberlic acid

The *Andrographis paniculata* cells observed in control and Gibberlic acid treated plants showed normal chromosomes. The cells showed normal prophase stages. All the cells were of normal size.

Thus, the stimulant Gibberlic acid did not create any mitotic defects and found to be safe for usage at all concentrations.

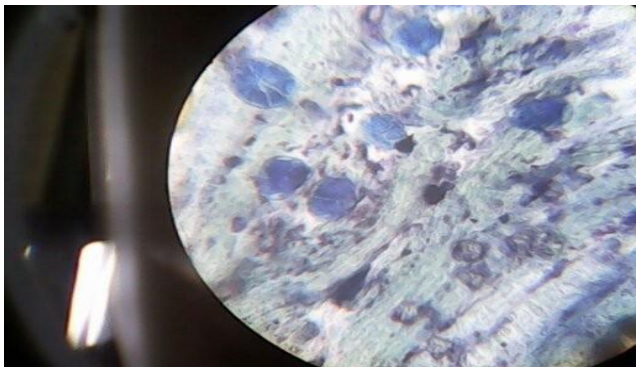


Fig: 5 Normal *Andrographis paniculata* cells.

Cytotoxicity Effect

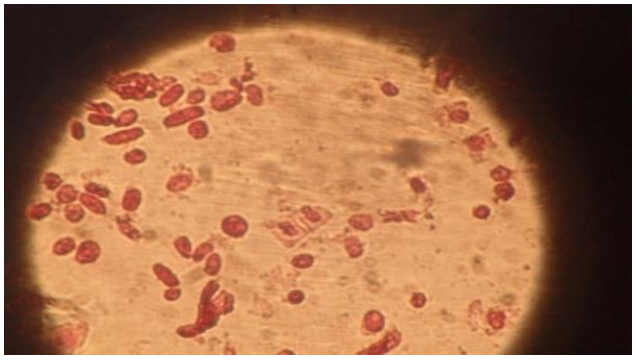


Fig 6: Normal Onion cells.

The onion cells observed in control and Gibberlic acid treated plants showed normal chromosomes. The cells showed normal Anaphase, Metaphase and Telophase stages. All the cells were of normal size.

Thus, the stimulant Gibberlic acid did not create any mitotic defects and found to be safe for usage at all concentration.

Colichicine Treatment And It's Analysis Of Protein, Carbohydrate, Fat And Drug Release

The *Andrographis paniculata* plants were treated with three different concentrations of Colchicine(0.02%,0.05%,0.08%) after 40 days of transplantation. Sampling for expression analysis was harvested after at 2hrs, 6hrs and 24hrs after foliar application. Each harvested sample was treated with amylase and recovery periods of 2hrs, 6hrs and 24hrs was given and tested for sugar, protein, fat and drug release.

The **protein** content in all the treatments and it's recovery periods were definitely much higher when compared to the control (Table:5). The highest levels of protein in Colchicine treated plants was recorded at **(492.00 and 122.75)** 0.02% in 24hrs harvest period of **2hrs** and **24hrs** recovery period after amylase pre-treatment. Low levels of protein was seen in 0.08% concentration of 6hr and 24hr harvest periods. This increase in protein content over control plants was definitely due to colchicines treatment.

The **carbohydrate** content in all the treatments and it's recovery periods were definitely much higher when compared to the control (Table:5). The highest levels of protein in Colchicine treated plants was recorded at **(446.25 and 47.75)** 0.02% in 24hrs harvest period of **2hrs** and **24hrs** recovery period after amylase pre-treatment. Low levels of protein was seen in 0.08% concentration of 6hr and 24hr harvest periods. This increase in carbohydrate content over control plants was definitely due to colchicine treatment.

Table3: Colchicine Treatment And It's Analysis Of Protein, Carbohydrate, Fat And Drug Release.

S.No	Control	Concentration of Colchicine	Harvest After Colchicine Treatment	Protein		Carbohydrate		Drug release		Fat
				2hrs	24 hrs	2hrs	24hrs	2hrs	24hrs	**0.158**
1	0.174	0.02%	2 hrs	17.98	17.07	17.52	18.09	7.6	7.6	0.120
2	0.172		6hrs	8.415	53.72	29.32	29.92	5.1	5.1	0.133
3	0.172		24hrs	**492**	**122.75**	**446.25**	**478.75**	6.0	6.0	**0.118**
4	0.174	0.05%	2 hrs	18.56	9.75	38.00	12.00	1.87	3.75	0.121
5	0.172		6hrs	25.51	34.34	69.20	41.46	**0.87**	**0.87**	0.129
6	0.172		24hrs	34.30	2.57	44.52	3.37	1.2	0.75	0.138
7	0.174	0.08%	2 hrs	33.37	10.96	80.85	17.51	1.23	1.23	0.126
8	0.172		6hrs	**3.69**	**21.50**	5.92	82.11	**11.34**	**12.60**	0.121
9	0.172		24hrs	4.78	26.2	**5.92**	**66.70**	-1.09	1.09	0.121

According to[6] *Andrographis paniculata* contains 20.18% of carbohydrate (starch). The enzyme, which acts on carbohydrate, can decrease the amount of the starch; and hence intern

decreases the preload of the starch for extraction of andrographolide. The action of carbohydrate is not restricted only to starch but it acts on protein also.Hence it proves that increase in protein also indirectly increases carbohydrate content.

The **drug release** content in all the treatments and it's recovery periods were definitely much higher when compared to the control (Table:5). The highest levels of protein in Colchicine treated plants was recorded at **(11.34 and 12.60)** 0.08% in 6hrs harvest period of **2hrs** and **24hrs** recovery period after amylase pre-treatment. Low levels of protein was seen in 0.05% concentration of 6hr and 24hr harvest periods. This increase in drug release content over control plants was definitely due to colchicine treatment.

Laddha *et al*., (2010) observed that enzyme which releases more amount of sugar also releases more amount of drug because of the action of the starch. But the following study revealed that the enzyme which released more amount of sugar, protein and fat release in 0.02% and 0.05% could not more release drug. But 0.08% with less sugar, protein and fat release, extracted more drug release. This difference in results might be due to the effects of colchicine. Further investigation on temperature, pH, speed of shaker, time duration, exposure to light has also to be studied.

The **fatty acid** content in all the treatments was much lower when compared to the control. The highest levels of fatty acid in Colchicine treated plants was recorded at harvest treatment 2hr in 0.02% **(0.158)**. It was much lower compared to untreated plants **(0.178)**. Low levels of fatty acid treated plants was seen in 0.05% harvest periods. Richard J. Schimmel (1974) reported that when free fatty acids (FFA) was pretreatment with colchicine did not alter basal unstimulated FFA release.The possibility that colchicine might limit production of fatty acids by accelerating the entry and metabolism of glucose Pretreatment with colchicine did not affect uptake of glucose nor its oxidation to CO2, although colchicine-treated tissues did have slightly more glucose and incorporated into the glyceride moiety of triglyceride. In the present study also free fatty acids (FFA) was reduced with colchicine treatment but did not affect the release of glucose and protein.

CYTOLOGICAL STUDY

Mitotic effects of colchicine in treated and untreated plants

The treated and untreated cells of colchicine in mitosis behaved differently. The cells observed in control plants were found to show normal chromosomes. Colchicine 0.08% 24Hr

cells showed increase in size, chromosome size reduced and thickened, yellow coloured oil like filled cells with elongated shape, shows division, few cells round up to show micronuclei condition.

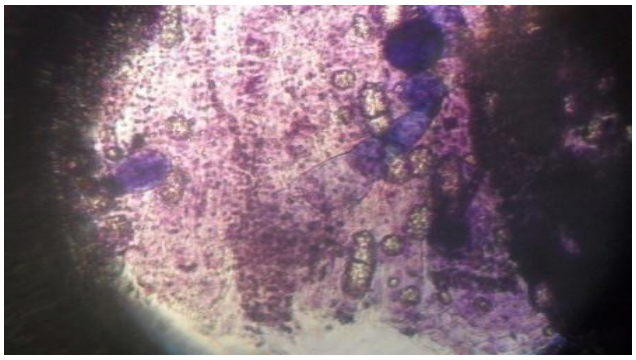


Fig 7: 0**.08% (24 hrs) concentration of colchicine.**

Colchicine 0.02% 24 Hrs., Cell walls have disintegrated, chromosomes scattered, rounded cells showing multinucleated condition increased in number, outer zone observed in some cells, yellow oil filled cells present single or grouped into four cells.

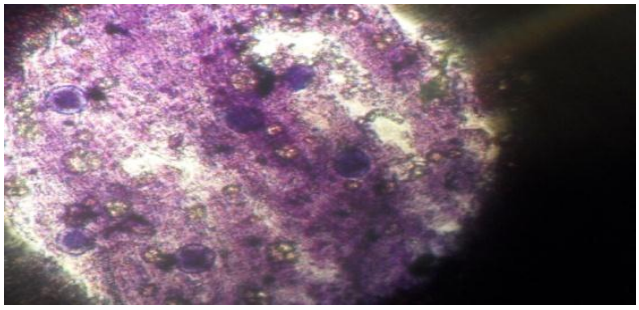


Fig 8: 0.02% (24 hrs) concentration of colchicine.

Colchicine 0.05% 24 Hrs., Cell walls with thick and short chromosomes, some cells with chromosomes show disappearance of colour giving the appearance of oil filled cells, micronucleate condition cells seen.

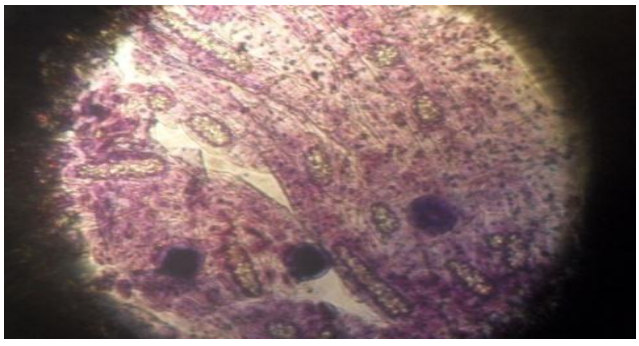


Fig 9: 0.05% (24 hrs) concentration of colchicine.

Cytotoxicity Effect

The treated and untreated cells of colchicine in mitosis behaved differently. The cells observed in untreated plants were found to show normal chromosomes. But plants treated with colchicine treatments (0.08% and 0.05%) showed cells with chromosome doubling. 0.08% colchicine showed higher levels of chromosome doubling when compared to other concentrations. The mitotic divisions in the presence of the drug showed inhibition of the spindle. The chromosomes were sensitive to the action of the drug. In 0.08% concentration of the drug, high degree of longitudinal chromosome contraction was observed associated with increase in chromosome thickness. This is because of high degree of spiralization of chromonemata. Similar conditions were also observed under other concentration of colchicine.

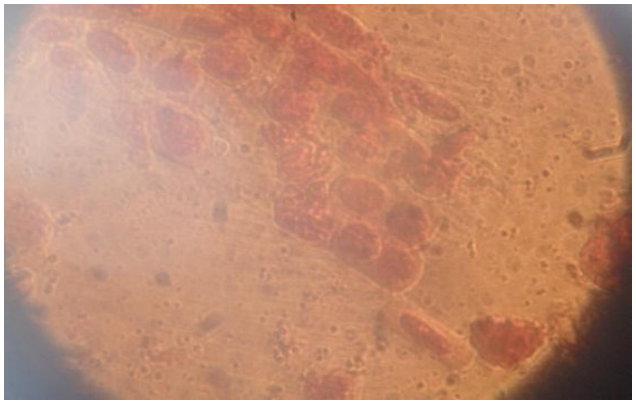


Fig 10: 0.08% concentration of colchicine.

Abscisic Acid Treatment And It's Analysis Of Protein, Carbohydrate, Drug And Fat Content

The *Andrographis paniculata* plants were treated with three different concentrations of Abscisic acid 5mM, 10mM, 15mM after 40 days of transplantation. Sampling for expression analysis was harvested after at 2hrs, 6hrs and 24hrs after foliar application. Each harvested sample was treated with amylase and recovery periods of 2hrs, 6hrs and 24hrs was given and tested for sugar, protein, fat and drug release.

The **protein** content in all the treatments and it's recovery periods were definitely much higher when compared to the control. The highest levels of protein in ABA treated plants was recorded at **15mM** at **24hr** recovery period after amylase pretreatment. It was higher compared to other treatments and their recovery periods and untreated plants.

Xie *et al.*,[15] have reported that Phyto-hormones, such as ABA are known to be involved in the regulation of protein concentration.[9] reported that Abscisic acid (ABA), increased protein content at 3 mM ABA (20 µM) in date palm somatic embryos. According to Weibing Yang *et al* (2013) the elevated contents of endogenous ABA were closely associated with the exogenous ABA. ABA contents positively and significantly correlated with free amino acid contents and Glutamine synthase activity and soluble protein contents in grains, suggesting that the increased protein concentration was mainly due to the enhanced enzyme activity of ammonia assimilation and substrate concentration of protein synthesis under exogenous application of ABA. In agree with the above reports, our results also clearly demonstrated that ABA was positively related to the increased protein content.

As enzymatic method of protein release was adopted, amylase might have triggered the release of protein.

The **carbohydrate** content in the ABA treated *Andrographis* plants was also found to be higher than the untreated control. The highest levels of carbohydrate in ABA treated plants was recorded at 15**mM** at **24h** recovery period after amylase pretreatment.. But in 5mM and 10mM, although high did not show much increase among the recovery period. The increase was mainly correlated with the exogenous application of ABA.

Table4: Abscisic Acid Treatment And It's Analysis of Protein, Carbohydrate, Fat And Drug Release.

S.no	Control	Concentration of Abscisic Acid	Harvest After Abscisic Acid Treatment	Protein		Carbohydrate		Drug Release		Fat
				2hrs	24 hrs	2hrs	24hrs	2hrs	24hrs	
1	0.174	5mM	2 hrs	15.04	23.776	28.864	28.896	0.016	0.032	0.110
2	0.172		6hrs	16.032	19.416	21.396	21.42	0.012	0.012	0.110
3	0.172		24hrs	19.425	32.65	44.05	44.325	0.05	0.025	0.110
4	0.174	10mM	2 hrs	30.08	47.552	57.728	57.792	0.032	0.064	0.110
5	0.172		6hrs	32.064	38.832	42.792	42.84	0.024	0.024	0.109
6	0.172		24hrs	38.85	65.3	88.1	88.65	**0.1**	0.05	0.110
7	0.174	15mM	2 hrs	45.12	**71.328**	**86.592**	**86.688**	0.048	0.096	**0.108**
8	0.172		6hrs	48.096	58.248	64.188	64.26	0.036	0.036	0.109
9	0.172		24hrs	58.275	**97.95**	**132.15**	**132.975**	**0.15**	0.075	0.110

[11]has reported that in *Polypodium vulgare* the application of exogenous ABA triggers more sugar accumulation, but decreases starch content.[6] reports that polysaccharides and polypeptides polymer are interlinked with each other to form complex network, thus increasing both protein and carbohydrate contents.

The results in the present study shows that the starch concentration was much higher than the protein concentration. This is due to the application of exogenous ABA. The increased results also need further investigation. The ABA untreated plants released more **drug** than the ABA treated *Andrographis* plants. The highest level of drug release in ABA treated plant was recorded at harvest treatment 24 hrs in 15mM(**0.15)**. The reports[12], showed that ABA treated plants showed increased contents of andrographolide when compared to control.

But according to the present findings, the drug andrographolide release was reduced by the ABA exogenous application.According to[6] the enzyme Amylase decreases starch and increase the andrographoloide content. But the present results disagrees with the above. According to[16][17] the higher concentration of ABA shows a decrease in 3-hydroxy-3-methylglutaryl coenzyme Thus, in agree with the above results, through the enzymatic cell disruption method by amylase the reduced levels of andrographolide was noted. But ABA did not affect the protein and carbohydrate levels. The **fatty acid** content in all the treatments were definitely much lower when compared to the control. In most of the ABA treated plants many concentration was showed same values(0.110) which was lower than the untreated plants(0.174). Steven[18] have reported that the lipid break down is repressed by Abscisic acid.

Thus, in agree with the above results, through the repression effect by ABA the reduced levels of fatty acid was noted.

Cytological Effect

The treated *Andrographis paniculata* cells of Abscisic acid in mitosis behaved differently when compared to the control plants. The cells observed in control plants were found to show normal chromosomes. Abscisic acid (15mM- 24Hr) treated Cells showed disintegration of cells. The cells of chromatin material. Chromosome slightly bigger in size.

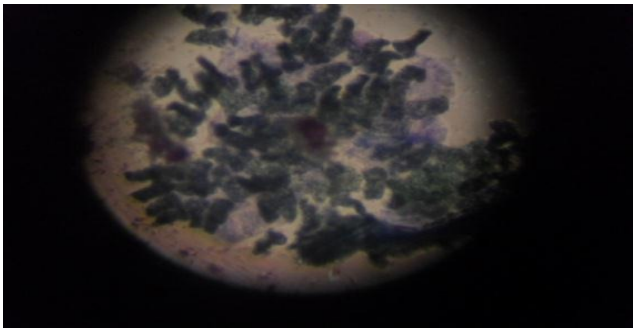


Fig 11: 15mM (24hr) concentration of Abscisic acid.

Cytotoxicity

The treated onion cells of Abscisic acid in mitosis behaved differently when compared to the control plants. The cells observed in control plants were found to show normal chromosomes. Abscisic acid (15mM- 24Hr) treated Cells show disintegration of cells. The cells devoid of chromatin material. Chromosome slightly bigger in size.chromosome became thick and short in size. Complete disintegration of the cells are seen.

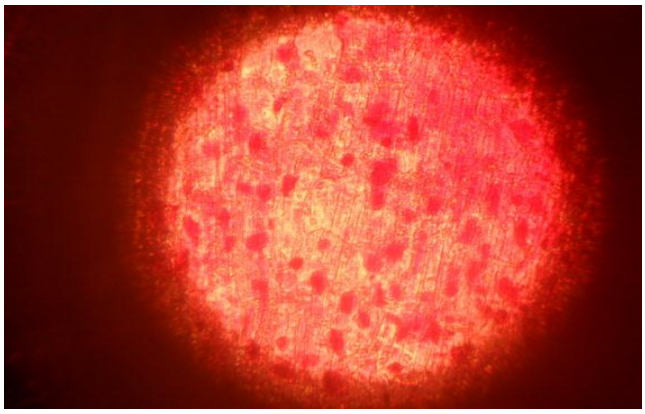


Fig: 12. 15mM(24hr) concentration of Abscisic acid.

TABLE:5 Consortia Treatment And It's Analysis of Protein, Carbohydrate, Fat And Drug Release.

S.no	Control	Harvest After Consortia Treatment	Protein		Carbohydrate		Drug Release		Fat
			2hrs	24 hrs	2hrs	24hrs	2hrs	24hrs	
1	1.268	2 hrs	**7.95**	2.46	**12.51**	2.42	0.375	0.375	0.103
2	1.304	24hrs	**12.15**	5.26	**28.11**	5.40	**1.68**	**0.84**	**0.105**

The *Andrographis paniculata* plants were treated with two different concentrations of Consortia after 40 days of transplantation. Sampling for expression analysis was harvested after at 2hrs, 6hrs and 24hrs after foliar application. Each harvested sample was treated with amylase and recovery periods of 2hrs, 6hrs and 24hrs was given and tested for sugar, protein, fat and drug release.

The **protein** content in all the treatments and it's recovery periods were definitely much higher when compared to the control (Table:7). The highest levels of protein in consortia treated plants was recorded as **12.15** in 24hrs harvest period of 2hrs recovery period after amylase pre-treatment. Low levels of protein was seen in 24hr recovery period of 2hr of harvest periods. This increase in protein content over control plants was definitely due to consortia treatment[19] have reported that both Gibberlic acid and Abscisic acid regulate

protein synthesis both positively and negatively in alurone cells largely by regulating levels of mRNA and in the case of α- amylase.

In agree with the above results, our results were confirmed that the protein content was higher when compared to the control plants.

The **carbohydrate** content in all the treatments and it's recovery periods were definitely much higher when compared to the control (Table:5). The highest levels of carbohydrate in consortia treated plants was recorded as **28.11** in 24hrs harvest period of 2hrs recovery period after amylase pre-treatment. Low levels of protein was seen in 24hr recovery periods. This increase in carbohydrate content over control plants was definitely due to consortia treatment. The highest levels of **drug** release in consortia treated plants was recorded as **1.68 and 0.84** in 24hrs harvest period of 2hrs and 24hrs recovery period after amylase pre-treatment. Low levels of protein was seen in 2hr harvest periods. This increase in drug release content over control plants was definitely due to consortia treatment.

The **fatty acid** content in all the treatments were definitely much lower when compared to the control. The highest levels of fatty acid in consortia treated plants was recorded as **0.105** in 24hrs harvest treatment. It was much lower when compared to untreated plants **(0.178)**. This was followed by 2hr treatments.

Cytological effect

The treated *A. paniculata* cells of Consortia in mitosis behaved differently. The cells observed in control plants were found to show normal chromosomes. Consortia 2hr treated Cells show normal condition. While 24 hr treated plant show fully disintegration of cells. The cells devoid of chromatin material. Chromosome slightly bigger in size.

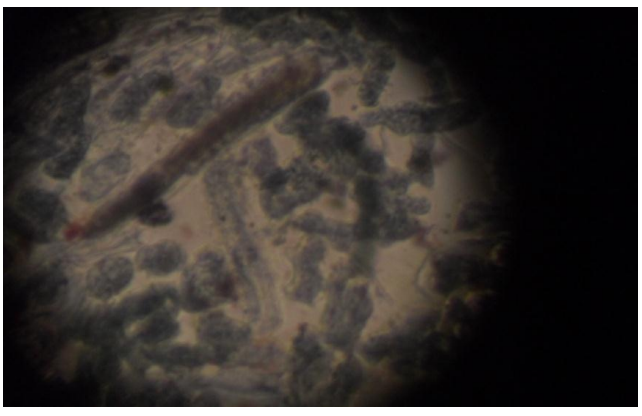


Fig: 13. 24 hr concentration of consortia.

Cytotoxicity

The cells observed in control plants were found to show normal chromosomes. Consortia 2hr treated Cells show normal condition. While 24 hr treated plant show fully disintegration of cells. The cells devoid of chromatin material. Chromosome slightly bigger in size.

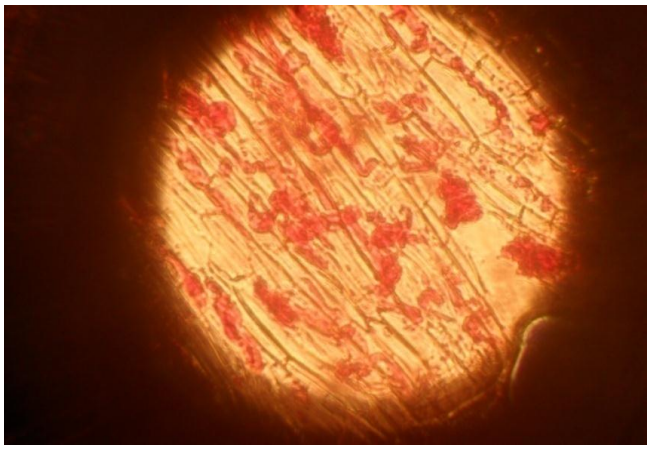


Fig 14: 24 hr concentration of consortia.

Anti-microbial activity of treated andrographis paniculata plant

The ethanolic extracts of stimulants (Gb3, Colchicine, ABA and Consortia) treated *A.paniculata* plant were assessed by using Agar well diffusion method against 2 microbial strains (Bacteria- *staphylococcus aureus*, Fungi-*Aspergillus flavus*). Gibberlic acid treated plant did not show any anti-microbial activity in both 5mM and 15mM concentration against bacteria and fungi. In 15mM concentration, stimulation of bacteria and fungus was noticed at 6hr and 24hr. Similarly, Colchicine treated plants did not show any anti-microbial activity against bacteria and fungi in 0.02% and 0.08%. In 0.05% concentration, stimulation of fungi was recorded at 24hr.

Abscisic acid treated plant extracts displayed relative antibacterial activity against *Staphylococcus aureus*, with the diameter of inhibition zone(14mm) than the others whereas Abscisic acid stimulated the growth of fungi in 5mM concentration at 24 hrs than the others. According to[20] the fungal cell growth is not inhibited by high concentration of Abscisic acid and synthesis is greatest under stress or in stationary phase cultures.

In agree with above reports, our results also clearly demonstrated that ABA was positively related to the fungal growth. Consortia treated plant did not show any anti-microbial activity against bacteria and fungi in 2hr and 24hr. In 2hr concentration, stimulation of fungi was noticed.

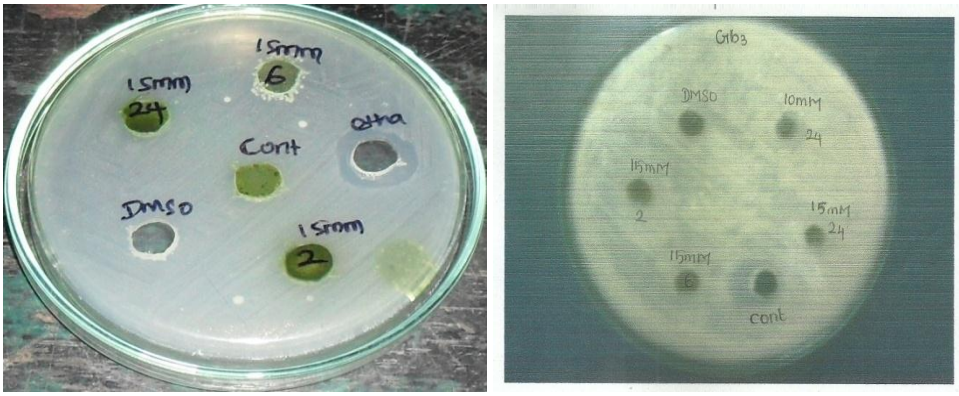
TABLE6: Anti-Microbial Activity Of Treated Andrographis Paniculata Plant.

S.NO	Stimulants	CONCENTRATION OF STIMULANTS	ANTI-MICROBIAL	
			ANTI-BACTERIAL	ANTI-FUNGAL
1	Gibberlic Acid	5mM 10mM 15Mm	X X 6hr(stimulation)	X X 24hr(stimulation)
2	Colchicine	0.02% 0.05% 0.08%	X X X	X 24hr(stimulation) X
3	Abscisic Acid	5mM 10mM 15mM	X 24hr(inhibition) X	24hr(stimulation) X X
4	Consortia	2hr 24hr	X X	2hr(stimulation) X

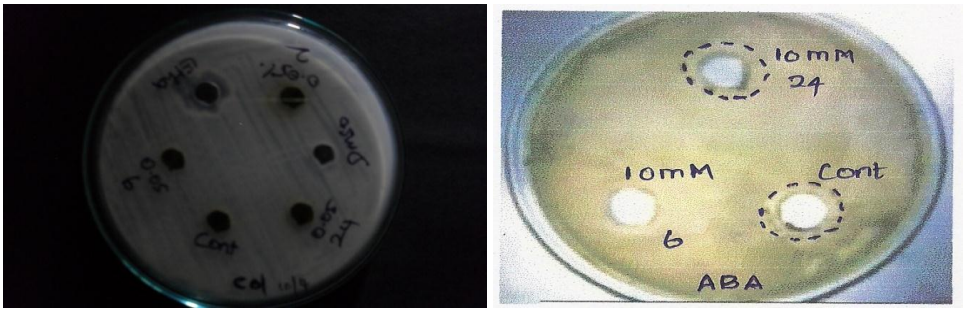
Treatments which showed anti-microbial effect were only selected for HPLC analysis

GIBBERLIC ACID	15mM	6 hr	24 hr
COLCHICINE	0.05%	- -	24 hr
ABSCISIC ACID	5mM, 10mM	- -	24 hr
CONSORTIA	- -	2hr	- -

Anti-Bacterial Study **Anti-Fungal Study**



Anti-Fungal Study Colchicine Anti-Bacterial Study: Abscisic Acid



Anti-Fungal Study Abscisic Acid And Consortia

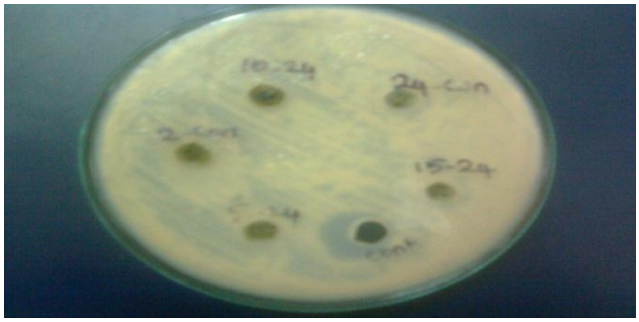
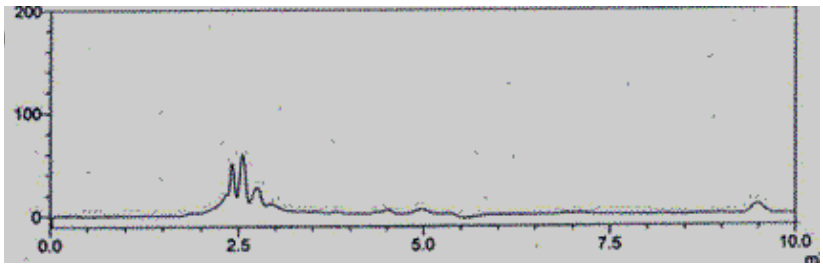


Fig 15: Gibberlic Acid.

HPLC analysis

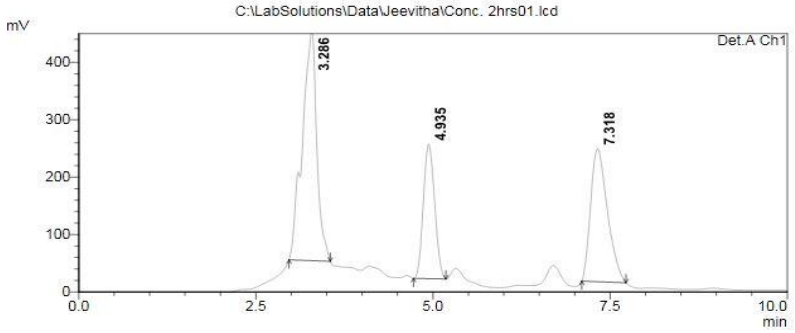
The Stimulants (Gibberlic acid, Colchicine, Abscisic acid and Consortia) treated *Andrographis paniculata* plants and plants without treatment were taken for the HPLC analysis.Chromatography performed using shimadzu HPLC and neuuslocil C18 column (5µm,250x4.6nm) with mobile phase consisting of methanol: water (65:35 v/v) at 223 nm showed the following results.

HPLC analysis of standard andrographolide showed three peaks with Retention time(Rt) of 2.5min. No peaks were observed at 5min and 7min.



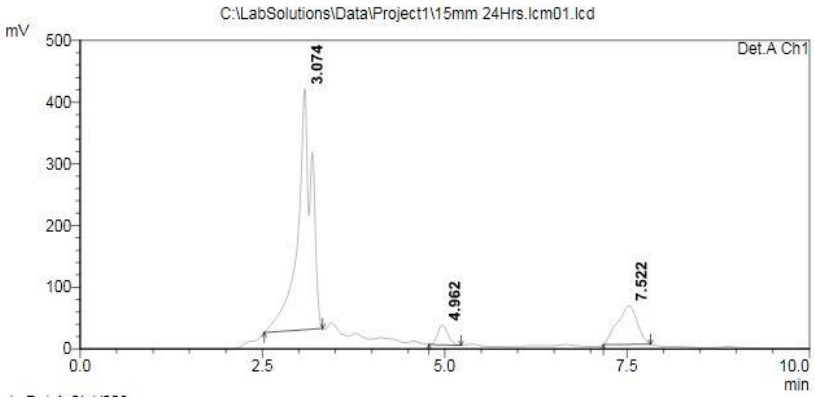
Control

The A.paniculata extract of 2hr concentration of consortia showed two peaks at Retention time of 3min.At the same time a single peak were also observed at 5min and 7min.

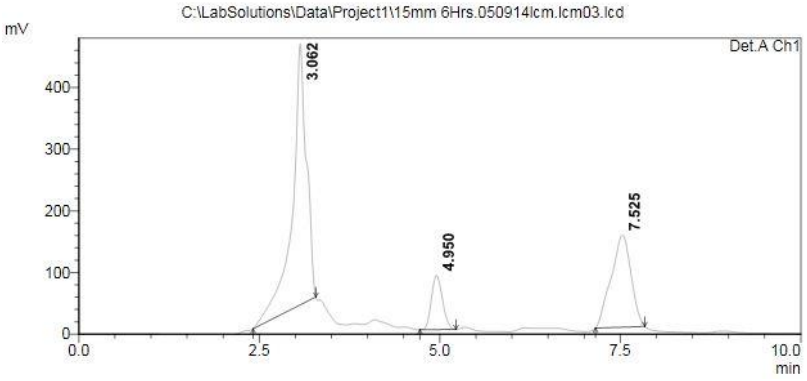


Consortia (2hrs).

The Gibberlic acid treated plant extract showed two peaks at Retention time of 3min in 24hr harvest periods of 15mM concentration. where as a single peak were observed at 5min and

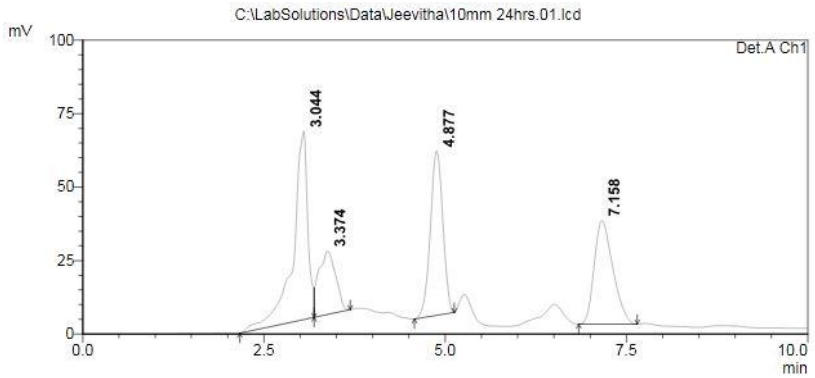


Gibberlic acid 15mM-24hr.

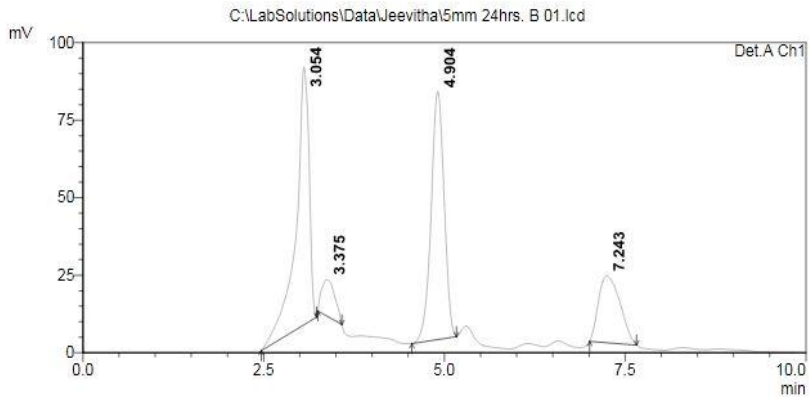


Gibberlic acid 15mM-6hr.

7min.In 6hr harvest periods of 15mM concentration of Gibberlic acid treated plant showed a single peak at Retention time of 3min, 5min and 7min.



Abscisic acid 10mM-24hr.



Abscisic acid 5mM-24hr

The Abscisic acid treated plant showed two peaks at Retention time of 3min in 24hr harvest period of 5mM concentration. A single peak were also observed at Retention time of 5min and 7min.In 24hr harvest period of 10mM concentration of ABA was also showed two peaks at Retention time of 3min and 5min.whereas a single peak were observed at Retention time of 7min **Colchicine 0.05%-24hr** Colchicine treated plant showed a single peak at Retention time of 3min, 5min and 7min in 24hr harvest period of 0.05% concentration. (This was expressed in Table.9).

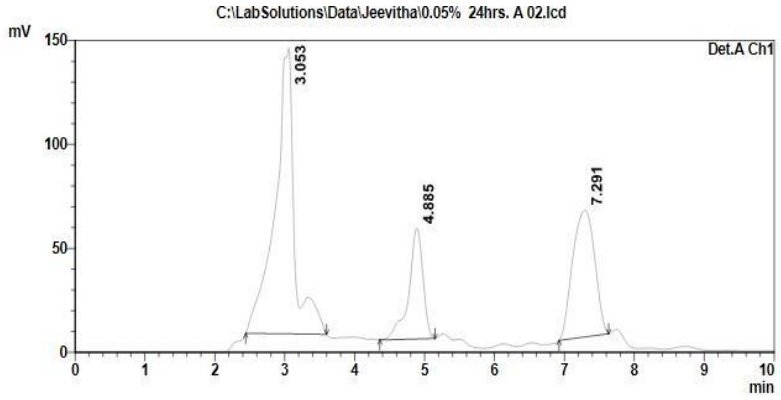


Table 7: Hplc Analysis of Treated And Untreated Andrographis Paniculata Plant.

S.no	Active compound	Retention time	Area
1	ANDROGRAPHOLIDE-STANDARD	2.50min	351676
2	*Andrographis paniculata*-Consortia(2hr)	3.286min	6089575
3(i)	*Andrographis paniculata*-Gb_3(15mM-6hrs)	3.062min	5755894
(ii)	Gb_3(15mM-24hr)	3.074min	5460660
4	*Andrographis paniculata*-Colchicine(0.05%)	3.053min	1993899
5(i)	*Andrographis paniculata*-ABA(5mM-24hr)	3.054min	1029951
(ii)	ABA(10mM-24hr)	3.044min	909493

Among these treated extracts, consortia showed more effective with the highest area was recorded as 6089575 when compared to the other concentration as well as control.

According to Alwar Vidyalakshmi and Subramanian Ananthi (2013) the plant growth hormones, which enhanced the andrographolide content in *A.paniculata* plants clearly indicates that andrographolide can be produced under *invitro* conditions by treating the callus with Napthalene-acetic-acid(NAA).According to Amudha and Subhashini., (2012) have reported that colchicine enhanced andrographolide content in *A. paniculata* plants and have confirmed by HPLC quantification.

The present results also clearly demonstrated that stimulants (Gibberlic acid, Colchicine, Abscisic acid and Consortia) positively showed increased andrographolide content.

CONCLUSION

From the present study it is concluded that protein, carbohydrate and drug release increased over the untreated plants. Cytotoxic effect showed chromosomal aberrations in *Andrographis paniculata* cells treated with colchicine and Abscisic acid. Squash preparation also showed chromosomal aberrations in *Andrographis paniculata* cells treated with colchicine, Abscisic acid and consortia. Gibberlic acid, Colchicine and Consortia treated plants stimulated the bacterial and fungal growth in microbial study. Abscisic acid showed inhibition towards bacteria. Quantification of andrographolide using HPLC also proved that the stimulants enhanced the drug content to several levels.

Hence this approach can be used to increase the drug content using stimulants and thereby decrease the exploitation of such medicinal plants.

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