Elicitation of Dactylorhin–E and studying anti-cancerous potential of *Dactylorhiza hatagirea* (D.Don).

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled "**Elicitation of Dactylorhin–E and studying anti-cancerous potential of *Dactylorhiza hatagirea* (D.Don).**pursued by **DEVANSHI POPLI** (123819) in partial fulfillment for the award of degree Master of Technology in Biotechnology from Jaypee University of Information And Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institute for the award of any degree or appreciation.

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DEVANSHI POPLI

DATE:

SUMMARY

The orchid *Dactylorhiza hatagirea*, a critically endangered species, is a medicinal orchid used to cure various diseases including dysentery; diarrhea; chronic fever; cough; stomach ache; wounds; fractures; cuts; burns and general weakness. It is indigenous to the Himalayas and exclusively found in the Ladakh (altitude -3000 meters) region of Jammu and Kashmir, India. They are of great value to floriculture industry as cut flowers and potted plants. The field grown micro-shoots were taken and cultured on both liquid and solid MS media, and maximum biomass (6.29 ± 0.20) was obtained in plantlets cultured in liquid media containing BAP (3mg/l) and IBA (4mg/l). Mass multiplication of shoots and roots was observed (Devanshi et al, 2016). The in-vitro grown plantlets were hardened on potting mixture containing coco peat, vermiculite and perlite (1:1:1), for acclimatization to field conditions and further multiplication. The in-vitro grown shoots were quantified by HPLC for dactylorhin-E. As the amount synthesized is not of significant level so elicitation was carried out by using different elicitors. The maximum amount of dactylorhin-E, estimated is 0.06µg/mg. The hydro alcoholic extraction was used for quantification from root and shoots samples and was tested on human cell lines for toxicity studies. In present study we have carried out elicitation of dactylorhin-E where using yeast extract, salicylic acid and methyl jasmonate as elicitors and we found out non- significant improvement in the content of dactylorhin-E. Also, we have optimized the protocol for hydro-alcoholic extraction of the plant and tested this extract on normal cell line (HEK-293) and breast cancer cell lines (MDA-MB-231 and MCF-7) where we have discovered the anti- cancerous activity of the plant which has not been reported till now. These findings will help in the generation of herbal formulations for treating cancer and like ailments by exploring with extended experimentation further onwards.

DEVANSHI POPLI

DATE:

DR. HEMANT SOOD

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LIST OF ABBREVIATIONS

Abbreviations	Full forms
KN	Kinetin
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
BAP	6- Benzylaminopurine
NAA	Napthalene acetic acid
2,4-D	2,4 dichlorphenoxyacetic acid
IAA	3- indoleacetic acid
MS	Murashige and skoog

CHAPTER -1

INTRODUCTION

CHAPTER-1

1.1 INTRODUCTION

Dactylorhiza hategirea (D. Don) ***Soo*** is a monocotyledon plant of family Orchidaceae. It is also known as *Panch aunle, Hatajadi* (Nepali), *Aralu, Salap* (Sanskrit), *Ongu lakpa* (Sherpa) and *Lob.*

1.1.1 Geographical Distribution:

It is a medicinal orchid which is found abundantly in Hindu Kush Himalaya range. Its occurrence is alpine and sub-alpine zones with an altitude of 2800-4200 m above from sea level. Other than Nepal Himalayas, it occurs in the same parts of India, Pakistan, Bhutan and China also. In India, it is commonly found in Jammu and Kashmir, Arunachal Pradesh, Sikkim, Himachal Pradesh and Uttarakhand.

1.1.2 Morphology:

It is an erect, terrestrial herb and reaches up to the height of 60 cm, with palmately divided tuberoids.

LEAVES: Leaves are generally oblong-lingulate or lanceolate or elliptic.

ROOTS: The plant store a huge amount of water in their tuberous roots to stay alive under arid conditions.

FLOWERS: Flowers are 1.7 to 1.9 cm in length with purplish-lilac, curved spur, rose or rarely white. In many-flowered plant, cylindrical inflorescence is observed. The inflorescence consists of a compact raceme developed from axillary buds with 25 to 50 flowers.

SEEDS: It is commonly propagated by rhizomes and seeds. Seeds are extremely small (dusty) and contain little food reserves. %. A single orchid capsule contains millions of seeds, which lack metabolic machinery and do not have any endosperm. Seed germination rate is very poor, i.e. 0.2% to 0.3. In spite of a extremely large number of seeds produced, only few seeds germinate in nature. For germination, seeds require symbiosis with mycorrhiza. The flowering Period and the fruiting Period is June-July and August-September respectively.

1.1.3 Indigenous uses:

It is a medicinal orchid which is of high value and is used in Indian system of medicine, particularly ayurveda, siddha, and unani medicine. Tubers are emollient, sweet, cooling, astringent, demulcent, nervine, and rejuvenating tonic. A decoction of tuber is given in colic pain. They are also beneficial in dysentery, phthisis diabetes, hemiplegia, chronic diarrhea, seminal weakness (aphrodisiac), neurasthenia, cerebropathy, emaciation and general debility. Powder is used to relieve fever. To check bleeding it is sprinkled over wounds. Root is also used as farinaceous food and also used in urinary disorders. The bulbous roots of *Dactylorhiza hatagirea* which are identical to the tubers of Orchis macula (Orchidaceae), serve as a source of **Salep,** which is considered as a nutritive and restorative tonic. It is used traditionally in Indian subcontinent especially in the Northern region and Nepal as sexual stimulant and aphrodisiac.

1.1.4 Chemical constitution:

Tubers contain a glucoside-loroglossin, a bitter substance, starch, mucilage (45%), albumen, phosphate (2.7%), chloride, a trace of volatile oil and ash. Chemically, dactylorhins A, dactylorhin B, dactylorhin C, dactylorhin D, dactylorhin E, dactylose A, dactylose B and lipids etc. are found as major constituents.

1.1.5 Pharmacological activity:

Rhizomatous part of *D. hatagirea* has shown resistance against all Gram positive and Gram negative bacteria, but the aerial part of the plant has shown limited resistance against some of the bacteria. The rhizomatous part is more effective than the aerial part against all tested organisms, except *E. coli*, is indicated by Zone of inhibitions (ZOIs) between two parts of *D. hatagirea*. Further, it is interesting to note that one of the very resistant bacteria to synthetic drugs *E. coli*, was found to be very susceptible to the extract of this plant. This finding is unique from the other folkloric uses of *D. hatagirea*. Hence, this plant can be a possible source for sprouting newer antimicrobial compounds for treating dysentery and other stomach problems caused by *E. coli*. *D. hatagirea* can be helpful in improving the sexual behavior and performance as it is effective in improving and preventing functionality of sexual organ. The results also agree with the hype that the plant is capable of being selected as herbal cure for sexual dysfunction. A study on adult

male rats gave evidence that plant has the capability of increasing the testosterone level in adult male rats which results in increased sexual desire and arousability in them.

The classification of *D. hatagirea* is as follows:

Kingdom: Plantae

Division: Angiosperms

Class: Monocots

Order: Asparagales

Family: Orchidaceae

Subfamily: Orchidoideae

Tribe: Orchideae

Sub tribe: Orchidinae

Genus: *Dactylorhiza*

Species: *hatagirea*

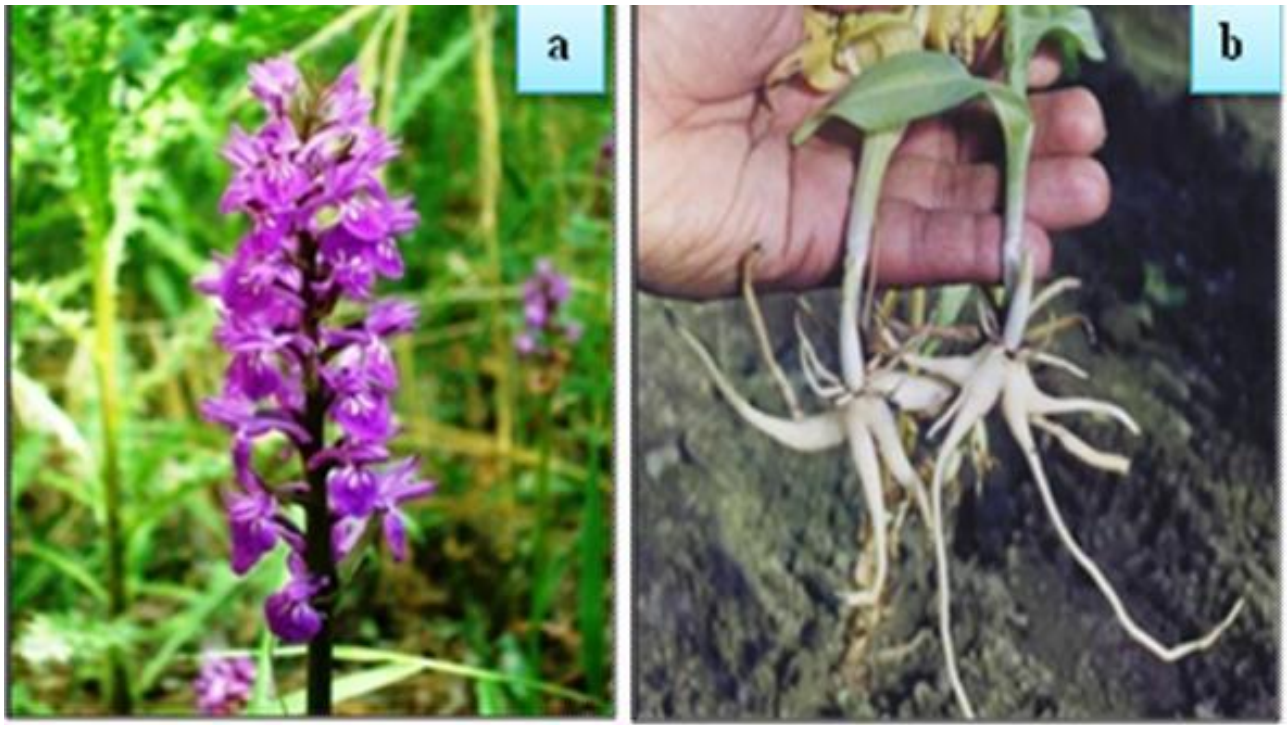

Figure 1.1 Plant morphology of *D. hatagirea* a. Inflorescence b. Tuber (Ashish warghat et al, 2014)

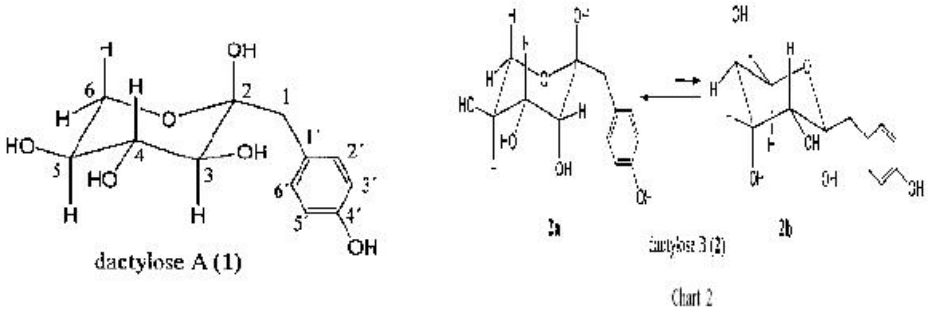

Figure 1.2 Structure of dactylose(Ashish warghat et al, 2014)

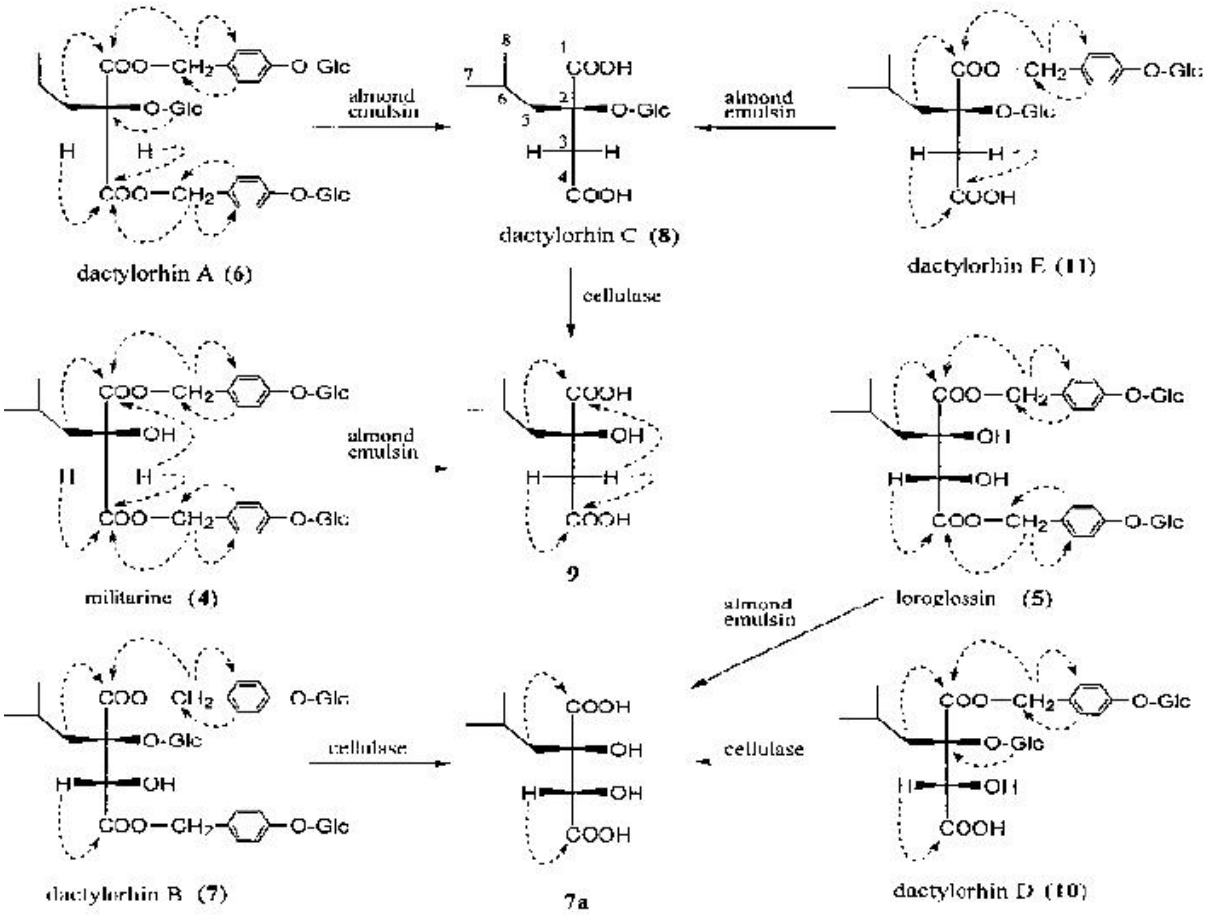

Figure 1.3 Structure of Dactylorhin (A to E) (Ashish warghat et al, 2014)

1.1.6 Conservation Status:

The plant has been listed under national priority species of medicinal herbs for cultivation and conservation by MFSC, Department of Plant Resources, Kathmandu. According to the Forest Act 1993, and Forest Regulation 1995, the rhizomatous part of *D. hatagirea* is illegal to collect, trade and process. If the collection is done with the government authority, the government royalty is Rs. 500 per piece according to Forest Regulation 1995 and its amendment 2005. Also, the plant has been listed under endangered and vulnerable species by Convention on International Trade in Endangered Species (CITIES) and Conservation Assessment and Management Plan (CMAP) respectively.

1.1.6.1 Ex-situ Conservation:

Seed and rhizomes are the sources of propagation of this plant. A gentle slope, humus soil, open moist areas, well drained sandy soil with higher content of organic matters is suitable. The light humid, moist climate of alpine and subalpine is appropriate for its growth. Collected seed are shown in nursery bed during April- May at the spacing of 40-60 cm for each seedling. 250-300 kg from a hectare is the dried yield in natural condition. For one hectare of land for cultivation, 5 kg of seed is required. 15-20 tons/ha compost fertilizer is required for manure. 3 to 4 hoeing and weeding is necessary during cultivation. The germination percentage is 85-90%.

1.1.6.2 In-situ Conservation:

Gathering of rhizome is done only after flowering of plants, for sustainable harvesting. Collection of mother plant takes place by leaving l immature tubers by filling with layer of soil with the help of sharp *kuto* (a small spade like hand tool). September to November is the harvesting period after seed ripening and fall. Proper care of the surrounding vegetation should be taken while rooting out the tubers of the *D. hatagirea*. Collection of plant should be done by applying rotating system. For harvesting, the rotation of the plant is 4-5 years. Sustainable harvestable amount is 80%. For the protection of rare and endangered orchid species, both ex-situ and in-situ approaches are important. Due to some anthropogenic activities, low rate of propagation, poor seed germination and as well as due to habitat destruction, there is a great threat to the species. For ex-situ conservation of terrestrial orchid, tissue culture is one of the most important measures. Knowledge of the morphological and physiochemical aspects of the

germination and development of a specific orchid species is of significant importance for the establishment of tissue culture techniques. Liquid media for micro propagation of this plant has also been optimized. This holds a robust potential for large scale propagation and secondary metabolite production which is required to meet the demand of national and international market (Devanshi et al, 2016).

According to a report, the annual demand of this species is approximately 5000 tons due to presence of high value secondary metabolites such as dactylorhin and dactyloses which are responsible for its pharmacological activity. This leads to over-exploitation of the species from wild habitat. Local inhabitants use this high value medicinal plant for illegal trading. The local inhabitants could gather dried roots of *D. hatagirea* at the cost of Rs. 100 to 200 per kg. 90 to 100 mature plants are exploited for 1 kg of dried roots of this plant. As a result, so many areas are there where *D. hatagirea* is present in great quantity, but now, a few individuals of this species are seen. This indicates that if the careless factors continue to function, this species might become extinct within a few years or so.

An 'elicitor' is defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds (Naik and Khayri, 2016). Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors. Though, elicitation enhances secondary metabolism in plants or plant cells in vitro but the exact mechanism of elicitation is not exactly understood. The current study is carried out with following objectives

1) Optimization of liquid MS medium for enriching biomass of *Dactylorhiza hatagirea.(MS media,IBA* 4 mg/L *and BAP* 3 mg/L*).*
2) Elicitation of dactylorhin – E in *Dactylorhiza hatagirea* using various elicitors and its quantification using HPLC.
3) Effect of hydro alcoholic plant extract on cancer cell lines.

CHAPTER-2

REVIEW OF LITRATURE

2.1 **Population status of *Dactylorhiza***

Rinchen et al [2012] carried out a study on population census of *D. hatagirea* in Suru valley of Jammu and Kashmir, India. He discovered that 11 associated species across the study sites and out of forty eight sites, hardly sixteen sites showed its occurrence. They discovered least density of *D. hatagirea* (8.00 and 6.1 ind/m2) among other associates. The maximum density was discovered in *Equisetum* sp. (3.45 to 17.2 ind/m2) followed by *Polygonum* sp. (2.6 to 4.35 ind/m2), *S. chrysanthemoides* (2.05 to 5.75 ind/m2), *M. lupalina* (1.75 to 5.6 ind/m2), *R. hirtellus* (1.5 to 4.55 ind/m2) and *P. hexandrum* (1.95 to 3.65 ind/m2)

Giri et al [2008] studied the analysis of closely associated vegetation and current status of *D. hatagirea* in Garhwal Himalaya, India. He discovered 24 herb species across the study sites and out of six study sites, only two sites showed its presence. The highest density was found in *P. alpinum* L. (141.52-201.28 ind/m2) followed by *G. trichophylla* Royle, (14.2-75.0 ind/m2), *D. cachyemyriana* Jaub. and Spach, (8.32-40.32 ind/m2), *P. depressa* Willd.(15.0-58.4 ind/m2) and *A. aptera* DC (2.80- 32.2 ind/m2) and dominant herbs in all study sites. *D. hatagirea* showed least density (0.70- 1.8 ind/m2) in all study sites.

D. hatagirea is facing extremely high risk of extinction due to overexploitation of tubers for medicinal purposes. Therefore, it is listed into the Indian Red Data Book. Near about all the species of *Dactylorhiza* unfold world-wide are listed in red data book. Therefore, there is critical need of conservation. *In situ* conservations including complete ban of its collection from its natural habitat must be implemented at an early date. The standardization of agro-technologies and cultivation in new areas will promote and boost to its conservation. The orchids are propagated through vegetative means as well as seeds. However, the rate of vegetative propagation of *D. hatagirea* is enormously slow and seed germination in nature is very insignificant, i.e. 0.2% [Vij, 2002]. Therefore, *in vitro* germination of seeds and micro propagation technology are an significant part in multiplication of orchid and conservation programmes.

2.2 Tissue culture of *Dactylorhiza*

Znaniecka and Lojkowska [2004] used mature seeds for setting up of *in vitro* collection of endangered European species, i.e. *D. majalis*. *D. majalis* seeds started to germinate after 6 weeks of culture on Fast medium.

Vaasa and Rosenberg [2004] used semi ripened seeds for preservation of *D. ruthei and D. praetermissa*. *D. ruthei* and *D. praetermissa* seeds which started to germinate after four months of culture on Norstog medium.

Rajasekaran et al [2009] studied multiplication and conservation of *Dactylorhiza hatagirea* and found satisfactory results.

Aggarwal and Zettler [2010] used green capsule for reintroduction of *D. hatagirea* symbiotically. They found 100% germination within 10 days of sowing and seedling after 3 months.

Giri and Tamta [2012] used green pod culture for conservation of *D. hatagirea*. They have tried these different medium - Knudson C (KC), Murashige and Skoog (MS), Vacin and Went (VW) and Vejsadova (VJ) media for seed germination and found much better response in MS medium supplemented with peptone (P) (1.0 g/L), morphoinoethane sulphonic acid (MES) (1.0 g/L) and activated charcoal (AC) (0.1%).

Warghat et al [2014] used seeds for in vitro protocorm development and mass multiplication of an endangered orchid, *D. hatagirea.* The surface sterilized immature seeds were cultured on germination medium. The germinated seeds further differentiated into protocorm like bodies on the same medium. The protocorm like bodies were maintained on optimal basal germination medium for further differentiation to form leaf primordia. The protocorm like bodies with leaf primordia were then cultured on regeneration medium containing MS and BM-2 media supplemented with combinations of plant growth regulators (IBA-3mg/l and KN-3mg/l. The resulting microshoots were separated from regeneration medium for mass multiplication. Maximum root number, maximum root length, maximum shoot length and maximum shoot number was observed. Plantlets with 2-3 shoots were transferred to potting mixture of cocopeat, vermiculite, and perlite (1:1:1) for acclimatization, which produced 75 shoots after 1 month of transplantation in greenhouse.

Liquid media for micro propagation of this plant has also been optimized. This holds a robust potential for large scale propagation and secondary metabolite production which is required to meet the demand of national and international market (Devanshi Popli et al, 2016).

2.3 Aphrodisiac Activity in Male Albino Rats

The results of study done by Mayank et al, 2007 suggest that aqueous extract of *D. hatagirea* causes considerable metabolic effect which is comparable to testosterone treatment. Genesis of steroids is one of the causes of increased weights of the body and sexual organ and an increase in this parameter could be regarded as a biological sign for efficiency of the herbal drugs in continuously improving the genesis of steroidal hormones.

2.4 Cell line studies:

Various cell line studies have been carried out using the breast cancer cell lines viz. MDA-MB-231 and MCF-7 such as Sharma et al, 2016 carried out a study on *Trillium Govanianum* studying for its in vitro anti-cancerous activity. Similarly, Jeevitha et al, 2010 used purified berberine against breast cancer (MCF-7), which induced apoptosis in cancer cell cells. Similarly, anti cancerous studies have been carried out in various orchids such as *Dendrobium nobile* (Jae et al, 2012)

CHAPTER- 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Plant Source:

The young micro-shoots were collected from the field grown plants of *Dactylorhiza* which were kept in glass house of Department Of Biotechnology, JUIT, Waknaghat and maintained under natural conditions.

3.2 Establishment of cultures:

In-vitro grown cultures has been established on the already defined media that is MS media+ IBA (mg/L) +BAP (mg/L) (Devanshi et al, 2016) for carrying out the mass multiplication of the plants so that, elicitation can be carried out.

3.3 Elicitation

Solid MS media with already optimized concentration of plant growth hormone were prepared for in-vitro elicitation of secondary metabolites. Plant growth hormones used were IBA(4mg/L) and BAP (3mg/L). Elicitors used were: salicylic acid, yeast extract and methyl jasmonate with different concentration as shown in Table 3.1. Yeast extract and salicylic acid was added to the media initially before autoclaving but for methyl jasmonate, which is heat sensitive, a stock solution of 10mM concentration was prepared and diluted to working solution concentration as shown in table1, which was then filter sterilized in LAF and added to autoclaved media. Sucrose and agar was added to MS media containing plant growth hormone and volume was raised tad lesser than the required volume.

pH was set between 5.6- 5.7 using 0.1N HCl and 0.1N NaOH and volume was raised to the required scale. Now, 45- 50ml of static medium (with agar) was dispersed into the culturing jars and autoclaved for 20 min at 121°C and 1.05kg /cm² pressure.

Small in- vitro grown micro-shoots were used as explants and cultured on liquid MS medium for their micro propagation under culture conditions 25±2° C and 60%-70% humidity (Devanshi et al, 2016).

Solid MS media with only plant growth hormones was used as control. The data has been collected for maximum number of shoot and shoot length.

Table 3.1: Different elicitor concentrations used for elicitation of dactylorhin-E in *Dactylorhiza hatgirea*.

MS Media + Plant Growth Regulators	Elicitor	Concentration
IBA (4mg/L) + BAP (3mg/L)	Salicylic acid	0.5mM, 1mM, 2mM
IBA (4mg/L) + BAP (3mg/L)	Yeast extract (1.5%)	0.75%, 1.5%, 2%
IBA (4mg/L) + BAP (3mg/L)	MeJa	0.075mM, 0.100mM, 0.150mM
IBA (4mg/L) + BAP (3mg/L)	Control	-

3.4 Hydro alcoholic Extraction

Shoots and roots (tissue cultured hardened) were collected from the green house of JUIT and weighed separately. Shoots and roots were grinded to form a fine dry powder using liquid nitrogen which was again weighed.

Extract of shoots and roots was prepared using Soxhlet extractor which is used when the desired compound is soluble in the solvent and impurity is insoluble in that solvent. Soxhlet Extractor has three main sections as shown in Fig 3.1 and Fig 3.2:

- **Percolator** - (Boiler and reflux) which circulates the solvent.
- **Thimble** - (usually made of thick filter paper) retains the solid.
- **Siphon tube** - periodically empties the thimble.

Extraction in Soxhlate extractor was carried out for 48 hrs at 62°C in 70% ethanol. The solvent in the solution obtained from Soxhlate extractor was then evaporated using rotary evaporator as shown in Fig 3.3. Same procedure was followed for both roots and shoots. The extract obtained

in RBF (round bottom flask) was scrapped using a scrapper which was then weighed and used for cancer cell line studies.

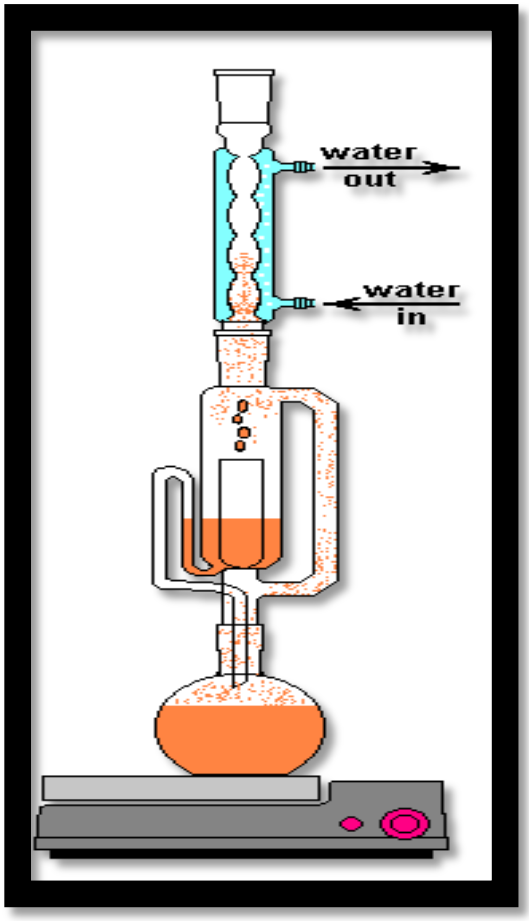

Figure: 3.1- Soxhlate Extractor

Figure: 3.2- Sample in Soxhlate extractor.

Figure: 3.3: Sample solution undergoing rotary evaporation.

3.5 HPLC quantification:

3.5.1 Sample preparation:

The cultured shoots were collected from jars after 15 days and 30 days. The samples were prepared as per the protocol given M Li et al, 2009 where in-vitro grown shoots were taken from the culturing jars and were grinded to form a fine dry powder using liquid nitrogen. 100mg of each powdered sample was left overnight in 70% methanol (10ml in 100mg of sample). Next day, the solution was centrifuged at 5000rpm for 5min. Pellet was discarded and supernatant was filtered using 0.22 micron micro filter and stored at 4°C in HPLC vials for HPLC analysis.

3.6 Cell lines and culture:

MCF-7 and MDA-MB-231 cell lines were used for studying the anti-cancerous properties of the plant and HEK-293 was used to evaluate plant extract effect on normal cell line. Cells were grown in T-25 subculture flasks containing Dulbecco's Modified Eagle Medium (DMEM) for MCF-7 and HEK-293 similarly Leibovitz (L-15) for MDA-MB-231, which were supplemented with 1% antibiotics (100-U/ml penicillin and 100µg/ml streptomycin) and 10% fetal bovine

serum (FBS). HEK-293, MCF-7 and MDA-MB-231 cells were kept at 37°C in humidified atmosphere containing 5% carbon dioxide till 70-80% confluency was observed. The cells were then trypsinized and subcultured afterwards.

3.7 Cytotoxicity studies:

The cytotoxicity studies were carried out by using 3-(4, five-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on HEK-293-293, MCF-7, and MDA-MB-231. Briefly, the cells were seeded in a 96-well plate, at a density of 1×10^4 cells/well, and kept for incubation at 37°C till 70-80% confluency was observed. After that, cells were treated with various concentrations of crude extract, viz. 250 µg/ml, 500 µg/ml, 750 µg/ml and 1000 µg/ml followed by incubation for 24 hrs. After 24hrs, supernatent was removed and 40µl of MTT (5mg/ml) solution was added to each well and again kept for incubation at 37°C for 4 hrs. Metabolically active cells have the property of reducing MTT in the presence of enzyme succinate dehydrogenase and producing purple-colored insoluble product called formazan. To solubilize the formazan crystal, 100µl DMSO was added to each well and left at room temperature for 15-20 min. To evaluate the cell cytotoxicity, absorbance (A) was taken using 570 nm as test wavelength and 630 nm as reference wavelength, using micro plate reader. Triplicate wells were assayed for each condition and standard deviations were then calculated. The cell cytotoxicity was calculated using the following formula as shown below (Equation-1):

$$\% \text{ cell cytotoxicity} = (\text{Abs.test} / \text{Abs.control}) \times 100 \qquad \text{(Eq.1)}$$

3.8 Morphological analysis through Acridine orange (AO) and Ethidium bromide (ETBR):

Further, the inhibitory concentration was further analyzed for its cell morphological changes in MCF-7, and MDA-MB-231 cell line by dual AO and ETBR staining and was compared with HEK-293 cell lines treated at same concentrations. Cells were seeded in 6-well plate at a density of 2×10^5 cells/well containing 1 ml of growth media, till 70-80% of confluency was attained, the cells were incubated at 37°C in CO_2 incubator. Cells were then treated with the inhibitory concentration and again kept for incubation, for 24 hrs. After 24hrs, the cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). Washed cells were then fixed using 4%

of paraformaldehyde (PFA) and kept for 30 min. After that, the supernatant was discarded, and cells were then treated with 1% triton × 100 for 15 min followed by again washing of cells with PBS. Cells were stained with dye mixture; comprising 5 µl of AO (1 mg/ml) and 5 µl of ETBR (1 mg/ml) for 5 min and then washed thrice with PBS. The morphological changes of the stained cell were observed using fluorescence microscope at 200X.

CHAPTER-4

RESULTS AND DISSCUSSIONS

4.1 Elicitation:

Sampling for HPLC was done after 15 days and 30 days as shown in Fig 4.1, 4.2 and 4.3. Drying, slight browning and death was observed in media supplemented with methyl jasmonate and shoots were found to be very small in size and thus not used for quantification.

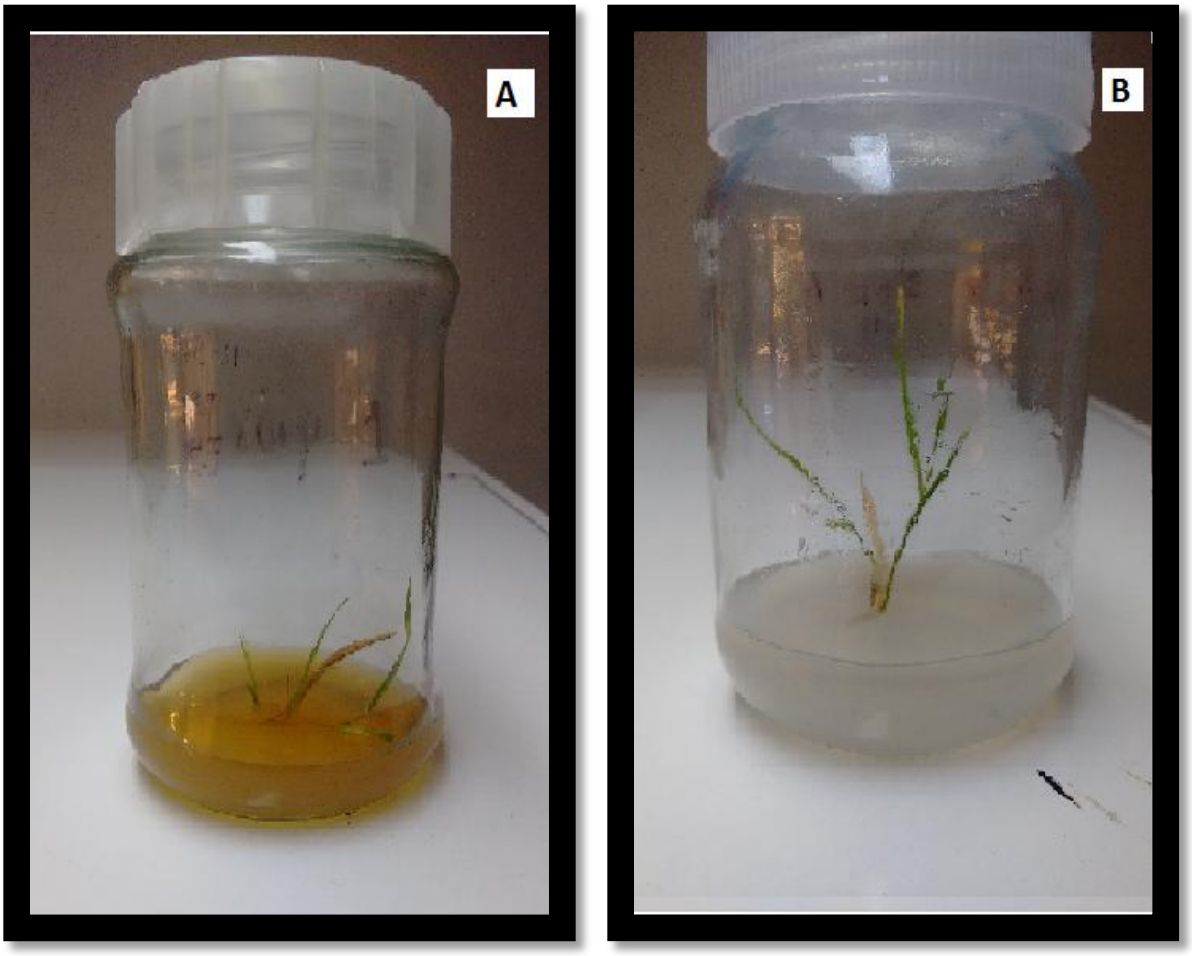

Figure 4.1 A) Cultured micro shoot on static MS media containing yeast extract on 15th day. B) Cultured micro shoot on static MS media containing salicylic acid on 15th day.

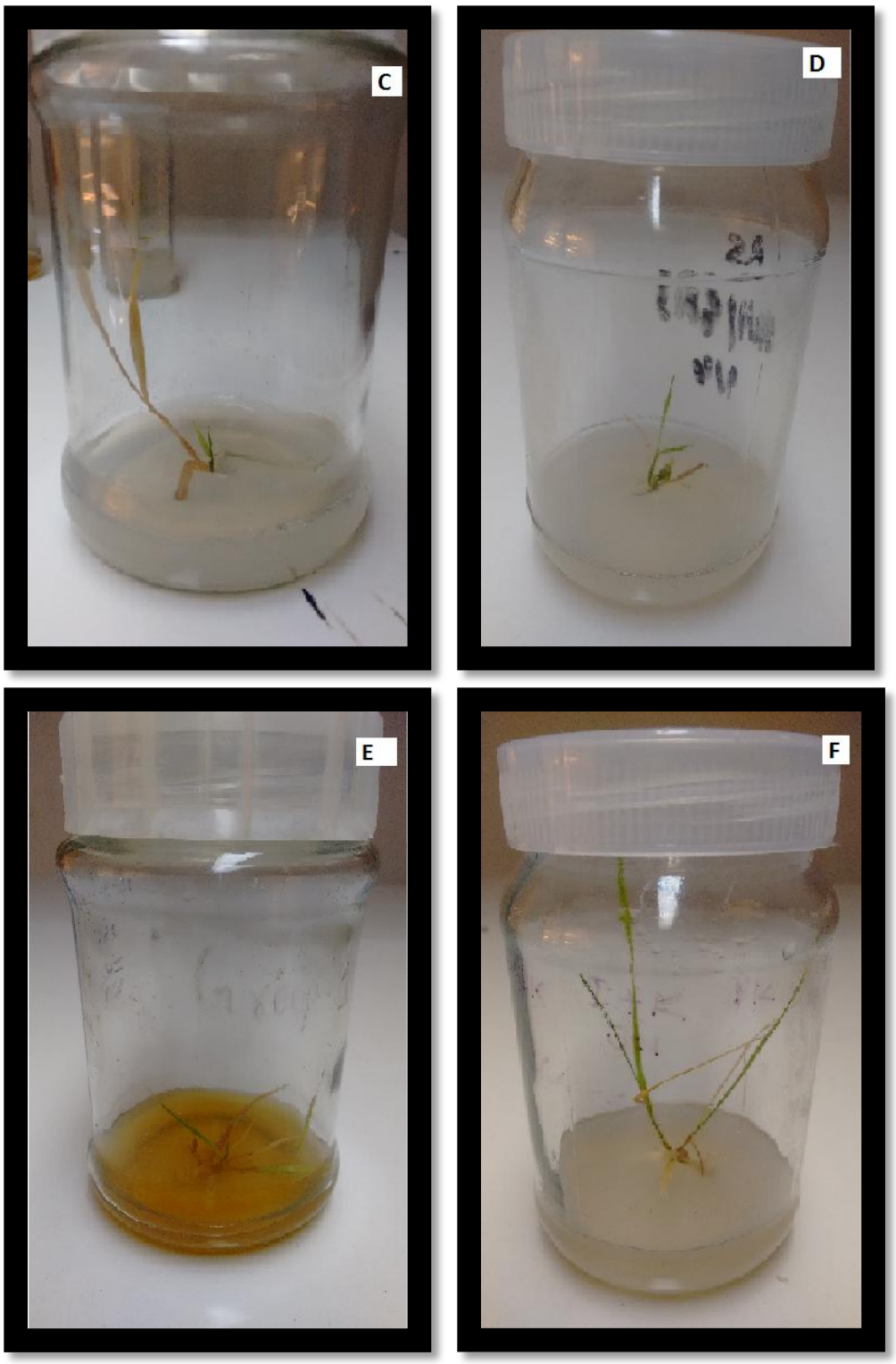

Figure 4.2 C) Cultured micro shoots on static MS media containing methyl jasmonate on 15th day. **D)** Cultured micro shoots on static MS media (control) on 15th day. **E**) Cultured micro shoot on static ms media containing yeast extract on 30th day. **F)** Cultured micro shoot on static ms media containing salicylic acid on 30th day.

Figure 4.3 G) Cultured micro shoot on static ms media containing methyl jasmonate on 30th day**. H)** Cultured micro shoots on static ms media (control) on 30th day.

Maximum shoot length was observed in MS media containing salicylic acid as shown in Table-4.1. Maximum shoot number and maximum shoot length was recorded after 30 days as shown in Table-4.1

4.2 Quantification by HPLC

Quantification of dactylorhin-E was carried out using analytical HPLC and it was observed that the amount of dactylorhin-E did not increased significantly as shown in Table- 4.1, so that, it can be utilized for commercial purposes.

Table 4.1- Effects of different elicitors on the growth of *Dactylorhiza hatagirea*

MS Media + Plant Growth Regulators	Elicitor (conc.)	Maximum no. of shoots after 30 days.	Maximum length of shoots after 30 days.	Dactylorhin-E content (µg/mg)
IBA (4mg/L) + BAP (3mg/L)	Salicylic acid (1mM)	2	15.7cm	0.070
IBA (4mg/L) + BAP (3mg/L)	Yeast extract (1.5%)	4	6.8cm	0.067
IBA (4mg/L) + BAP (3mg/L)	MeJa (0.075mM)	5	very small	Death and Drying
IBA (4mg/L) + BAP (3mg/L)	Control	6	14.2cm	0.060

4.3 Hydro alcoholic Extraction:

Brown sticky extract was obtained from both roots and shoots. Given below in Table- 4.2 are the weights of respective samples and their extract obtained after rotary evaporation as shown in Fig-4.2.

Table 4.2 : Weight of the sample and extract

SAMPLE	WEIGHT
Fresh weight of the shoot	20.20g
Dry weight of the shoot	10.33g
Weight of the shoot extract	102mg
Fresh weight of the root	19.50g
Dry weight of the root	9.580g
Weight of the root extract	200mg

Figure 4.4- Brown sticky extract after rotary evaporation.

4.4 Effect of D. hatagirea on viability of cells:

4.4.1 Cytotoxicity studies:

IC-50 value was calculated using the equation obtained from the graph plotted between concentration and absorbance.

The table- 4.3 and 4.4 shows the percentage viability of the cells at different concentrations of root and shoot extract respectively on HEK- 293 cell line and this shows that there is no significant decrease in the cell population with increasing concentration as percentage viability at 1000µg/ml (maximum concentration) is 94.44 which means for HEK-293 cell lines the plant extract is not causing any significant death of the cells at such a high concentration.

Graphs are plotted using the tables, as shown in figure 4.5 and 4.6. Using the equation obtained from the graphs IC-50 value for the root extract (9900.0) and shoot extract (6362.5) was calculated for HEK-293.

Table- 4.3 Percentage viability of HEK-293 cells treated with root extract

Conc.	Absorbance 1	Absorbance 2	Absorbance 3	Average	%age viability
0	0.658	0.661	0.752	0.690	100.00
250	0.675	0.652	0.714	0.680	98.55
500	0.625	0.698	0.642	0.655	94.88
750	0.696	0.647	0.631	0.658	95.31
1000	0.681	0.621	0.654	0.652	94.44

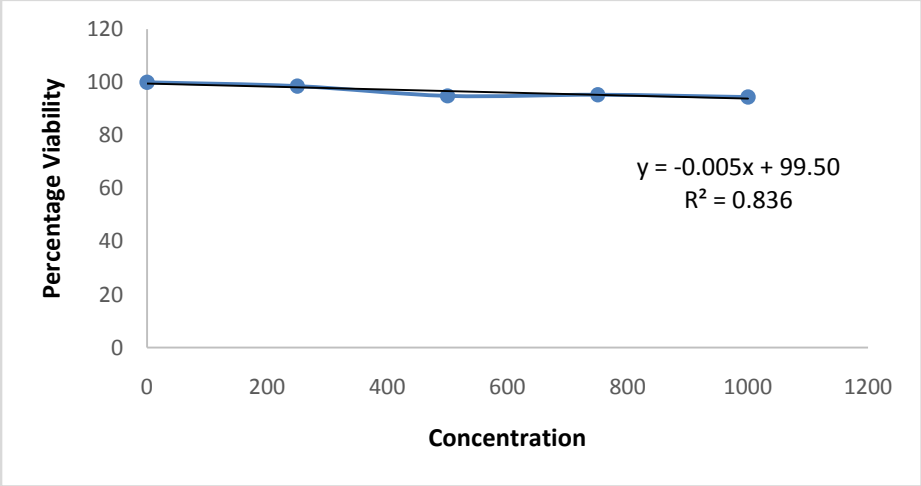

Figure 4.5 : Graph showing the effect of root extract on HEK-293 cell line.

Table- 4.4 Percentage viability of HEK-293 cells treated with shoot extract

Conc.	Absorbance 1	Absorbance 2	Absorbance 3	Average	%age viability
0	0.658	0.661	0.752	0.690	100.00
250	0.663	0.715	0.684	0.687	99.56
500	0.705	0.642	0.683	0.676	98.02
750	0.684	0.654	0.624	0.654	94.73
1000	0.614	0.653	0.647	0.638	92.41

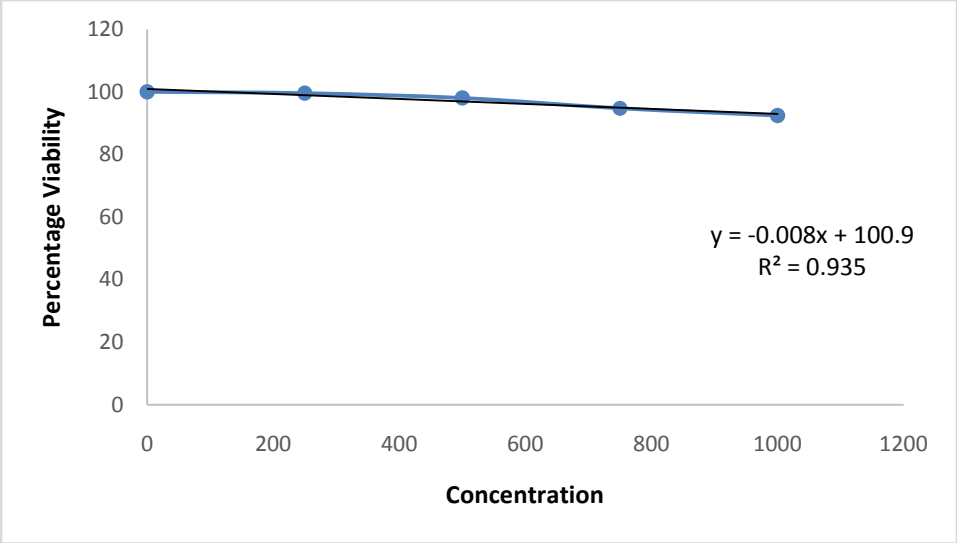

Figure 4.6 : Graph showing the effect of shoot extract on HEK-293 cell line.

Similarly, percentage viability for breast cancer cell lines viz. MCF-7 and MDA-MB-231 at different concentrations of root and shoot extract was calculated as shown in Table: 4.5, 4.6,4.7and 4.8 wherein we observed the significant decrease in cell population with increase in concentration of root and shoot extracts as depicted in the graphs shown in Figure:4.7, 4.8,4.9 and 4.10. It was observed in case of MDA-MB-231, root extract has more prominent effect in killing the cells as compared to shoot extract as percentage viability at 1000µg/ml is 82.38% in case of cells treated with root extract and 83.81 in case of shoot extract.

Table- 4.5 Percentage viability of MDA-MB-231 cells treated with root extract

Conc.	Absorbance 1	Absorbance 2	Absorbance 3	Average	%age viability
0	0.845	0.871	0.867	0.861	100.00
250	0.814	0.845	0.843	0.834	96.86
500	0.805	0.795	0.794	0.798	92.68
750	0.745	0.756	0.741	0.747	86.79
1000	0.714	0.716	0.698	0.709	82.38

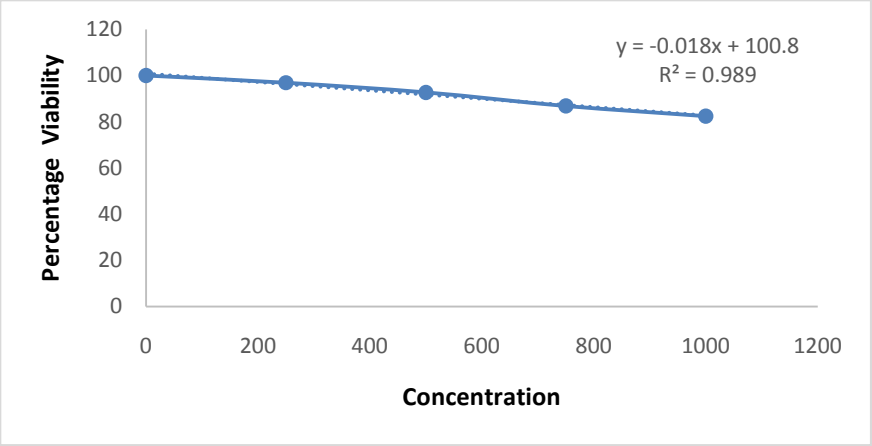

Figure 4.7 : Graph showing the effect of root extract on MDA-MB-231 cell line.

Table- 4.6 Percentage viability of MDA-MB-231 cells treated with shoot extract

Conc.	Absorbance 1	Absorbance 2	Absorbance 3	Average	%age viability
0	0.845	0.871	0.867	0.861	100.00
250	0.844	0.874	0.856	0.858	99.65
500	0.792	0.809	0.795	0.798	92.76
750	0.752	0.798	0.715	0.755	87.68
1000	0.698	0.713	0.754	0.721	83.81

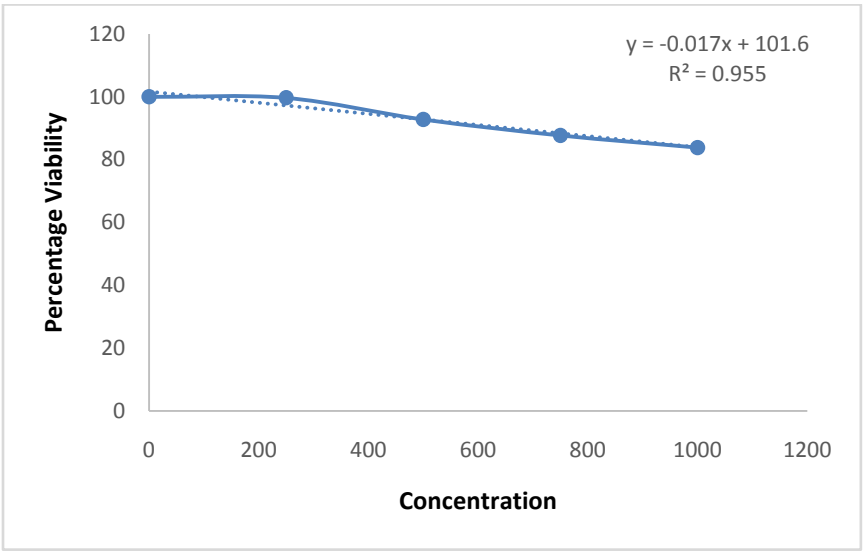

Figure 4.8: Graph showing the effect of shoot extract on MDA-MB-231 cell line.

Whereas, in case of MCF-7 the percentage viability of the cells treated with 1000µg/ml of root extract was found to be 84.24 (Table: 4.7) and similarly in case of shoot extract it was found to be 87.09 (Table: 4.8).

Table- 4.7 Percentage viability of MCF-7 cells treated with root extract

Conc.	Absorbance 1	Absorbance 2	Absorbance 3	Average	%age viability
0	0.956	0.982	0.976	0.971	100.00
250	0.948	0.958	0.984	0.963	99.17
500	0.897	0.868	0.872	0.879	90.49
750	0.904	0.847	0.849	0.866	89.22
1000	0.815	0.842	0.798	0.818	84.24

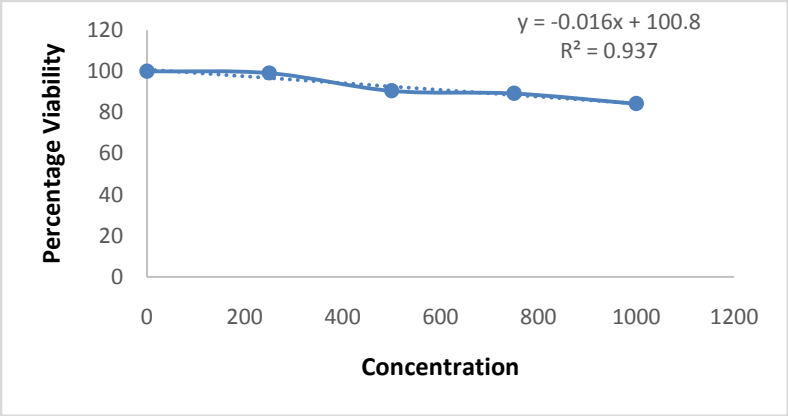

Figure 4.9: Graph showing the effect of root extract on MCF-7 cell line.

Table- 4.8 Percentage viability of MCF-7 cells treated with shoot extract

Conc.	Absorbance 1	Absorbance 2	Absorbance 3	Average	%age viability
0	0.956	0.982	0.976	0.971	100.00
250	0.914	0.945	0.954	0.937	96.53
500	0.907	0.915	0.921	0.914	94.13
750	0.897	0.906	0.914	0.905	93.23
1000	0.846	0.857	0.835	0.846	87.09

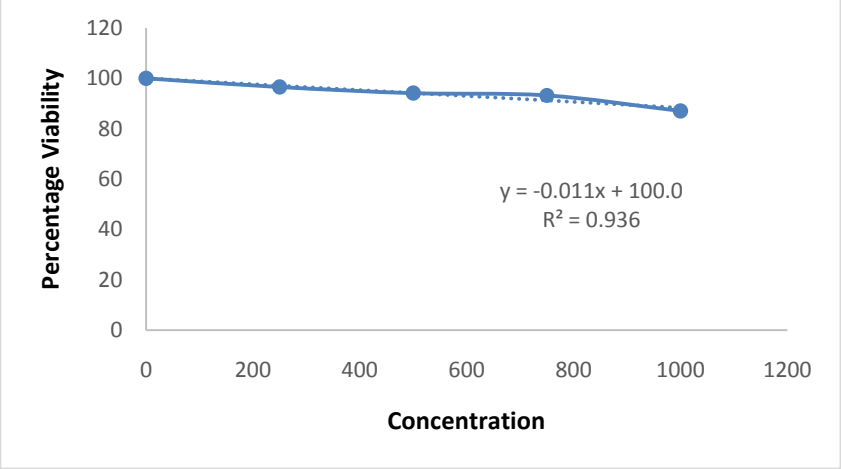

Figure 4.10: Graph showing the effect of shoot extract on MCF-7 cell line.

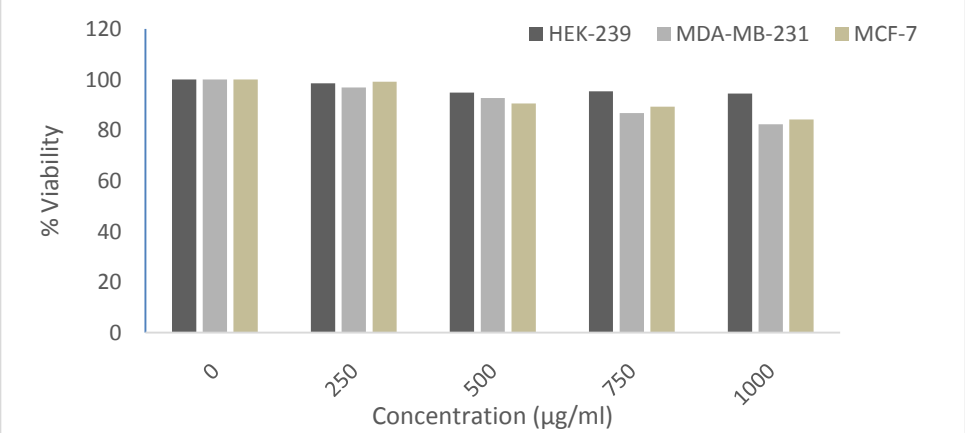

Figure 4.11: Graph showing comparative percentage viability of the different cells when treated with root extract.

The above graphs (Figure: 4.11 and 4.12) shows that the plant extract shows concentration dependent effect on the cancer cell lines viz. MDA-MB-231 and MCF-7. It has been observed that cell viability goes on decreasing with increasing concentration of root and shoot extract whereas, the effect of root and shoot extract on HEK-293 is negligible. Also, it has been observed in case of MDA-MB-231 cell line, cell number starts to decrease significantly when treated with root extract of 750µg/ml concentration as percentage viability was found to be

86.79% whereas, in case of MCF-7, 89.22 percent viability was found at 750µg/ml of concentration.

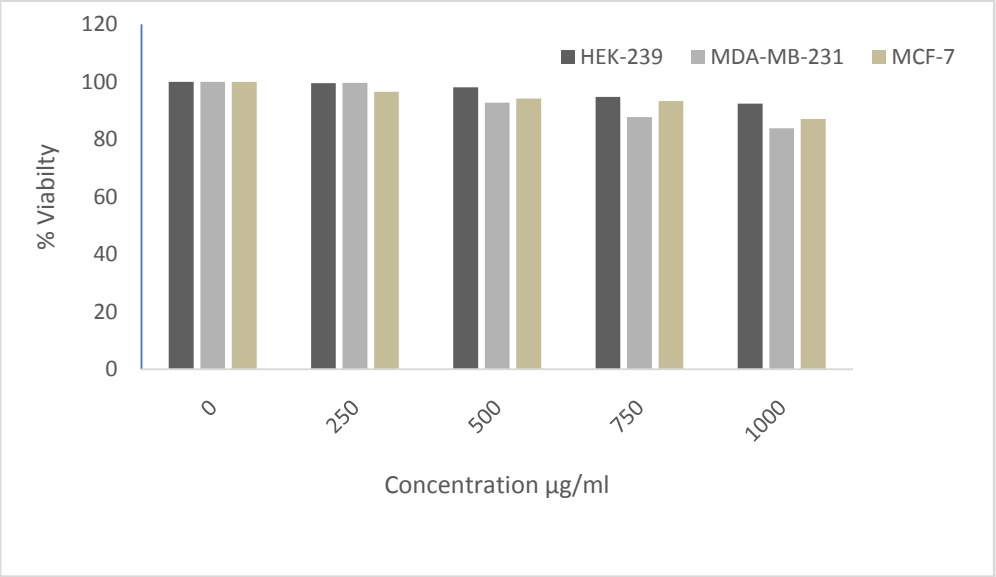

Figure 4.12: Graph showing comparative percentage viability of different cells when treated with shoot extract.

This shows MBA-MB-231 cell line is more sensitive to root extract as compared to MCF-7. Similarly, when MDA-MB-231 cell line was treated with shoot extract percentage viability was found to be 87.68% at 750µg/ml and in case of MCF-7, it was found to be 93.23% at same concentration. The percentage viability further decreases when the respective cells were treated with 1000µg/ml of concentration of root and shoot extract. This shows root extract has is much more effective in killing the cancer cells as compared to shoot extract.

This is further confirmed by calculating IC_{50}(minimum concentration required to kill 50% of the population) using the straight line equations obtained from the graphs mentioned above, as shown in Table- 4.9 below, minimum inhibitory concentration of root extract for MDA-MB-231 was found to be 2822.2µg/ml and 3175.0µg/ml for MCF-7. Similarly, minimum inhibitory concentration of shoot extract for MDA-MB-231 was found to be 3035.2µg/ml and 4545.45µg/ml for MCF-7 whereas, for HEK-293, IC_{50} was found to be quite high as shown in Table-4.9 below.

Table: 4.9- IC_{50} value of respective cell line when treated with root and shoot extracts.

Cell Line	ROOT EXTRACT	SHOOT EXTRACT
HEK-293	9900.0	6362.5
MDA-MB-231	2822.2	3035.2
MCF-7	3175.0	4545.45

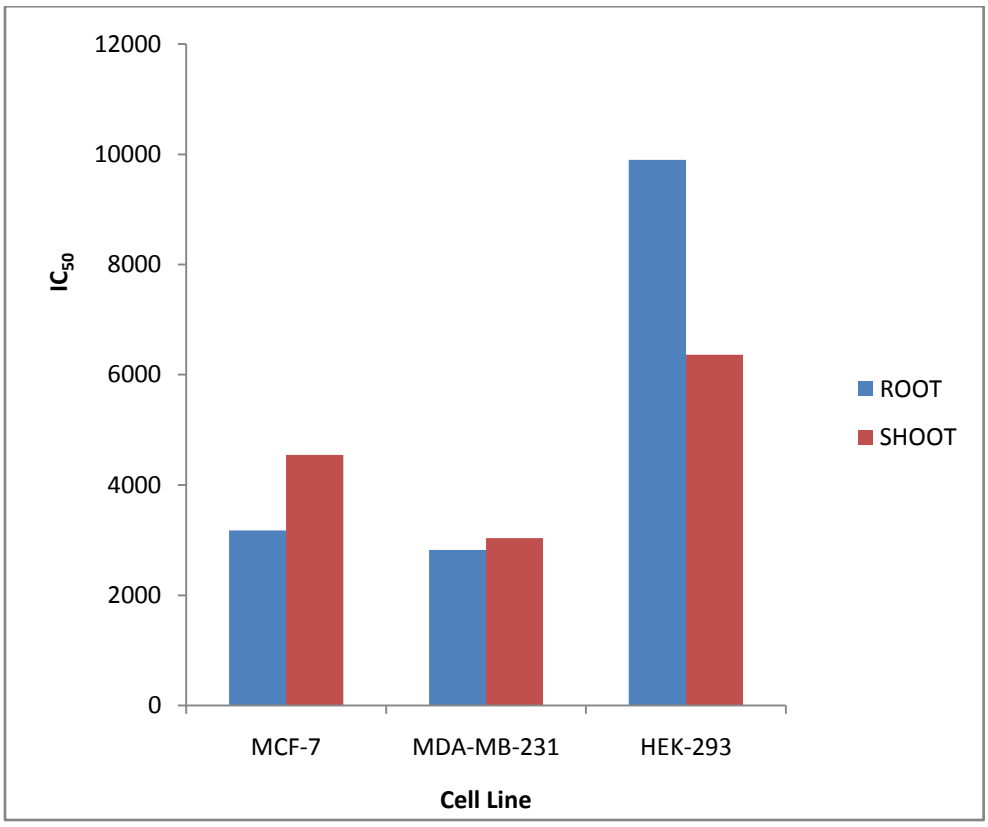

Figure 4.11: Graph showing comparative IC_{50}values of root and shoot extract on different cell-lines.

This shows that root and shoot extract do not cause any significant effect on normal HEK-293 cell line which confirms the anti- cancerous property of the plant which has not been reported by far now.

4.4.2 Morphological analysis through Acridine orange (AO) and Ethidium bromide (ETBR):

Morphological analysis through AO and ETBR shows the significant death of the MCF-7 and MDA-MB-231 cancer cells when treated with root and shoot extract with their respective IC_{50} whereas no effect was observed on HEK-293 cell lines as shown in Figure 4.12 and 4.13. AO and ETBR are the dyes used to observe morphological changes in nucleus of apoptotic cells. AO binds to both live and dead cells and ETBR stains only those cells which have lose membrane integrity.

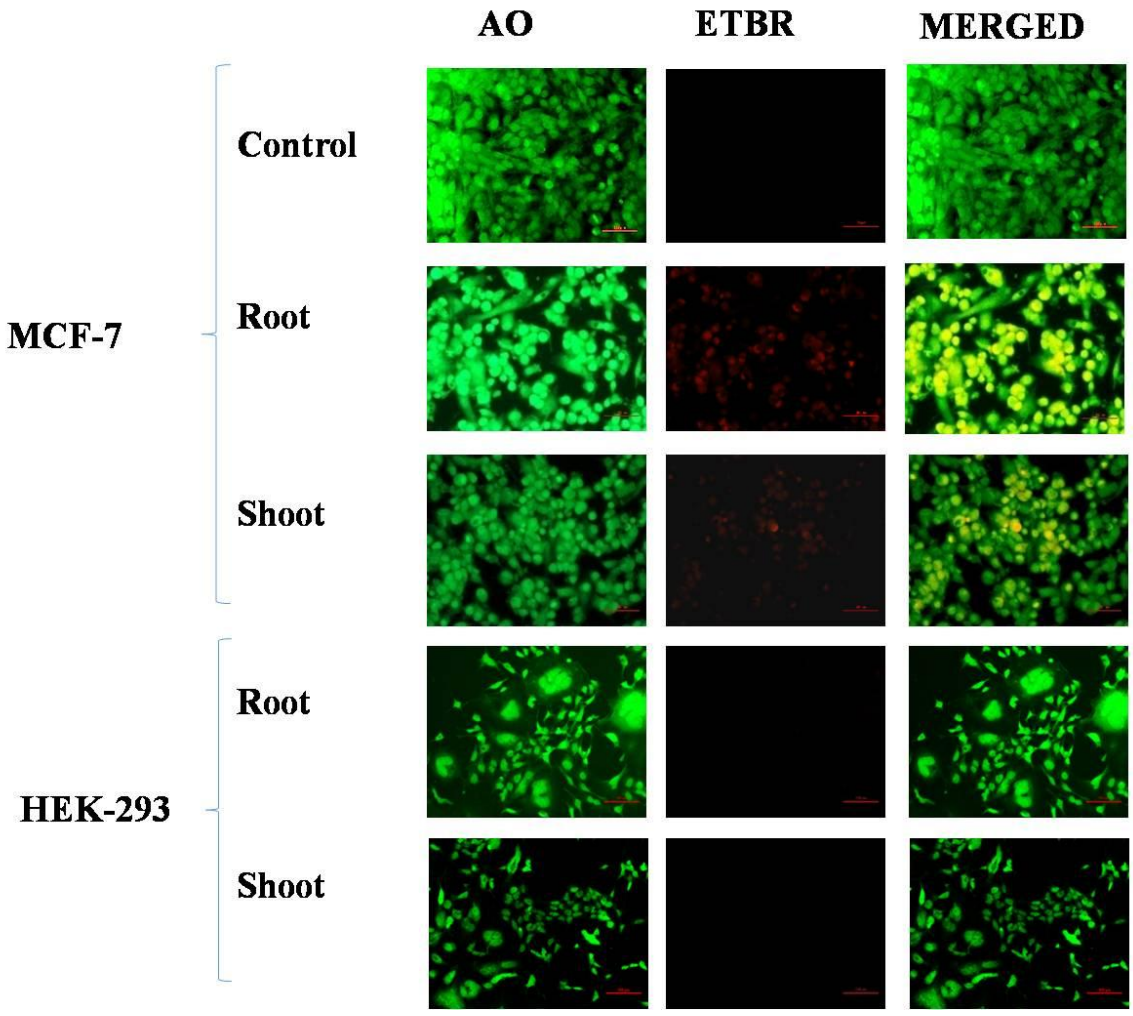

Figure: 4.12 Morphological analysis of MCF-7 by AO and ETBR staining

Cells which are stained green are viable cells and cells which are stained yellow signify early apoptotic cells and orange cells denoted late apoptotic cells. Similarly, in untreated control cells,

green fluorescence with complete uniformity was observed which depicts the normal nuclear morphology, whereas yellow and orange staining is observed in treated cells (. MCF-7 cell line treated with IC_{50} value of crude extracts was compared to HEK- 293 cell line treated with IC_{50} value of crude extracts and similarly, MDA-MB-231 cell line treated with IC_{50} value of crude extracts was also compared to HEK-293 treated with IC_{50} value of crude extracts, which shows significant decrease in cell density of the cells as shown in Figure 4.12 and 4.13.

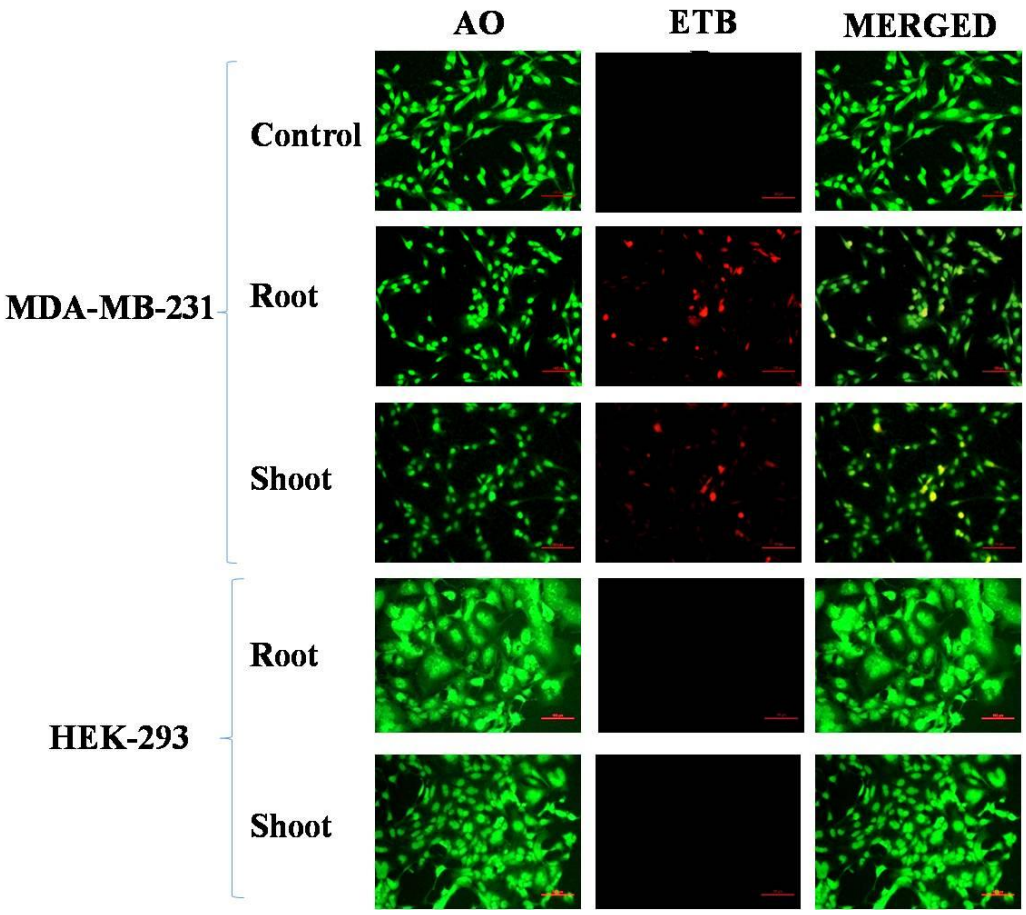

Figure: 4.13 Morphological analysis of MDA-MB-231 by AO and ETBR staining.

This confirms the anti- cancerous property of the plant with root extract having more prominent effect in killing the breast cancer cells.

DISSCUSSION:

Dactylorhiza hatgirea is a high value medicinal plant which has been used since ages for human consumption due to its exclusive effects on human body such as sexual stimulant and aphrodisiac. They are also beneficial in dysentery, phthisis diabetes, hemiplegia, chronic diarrhea, seminal weakness (aphrodisiac), neurasthenia, cerebropathy, emaciation and general debility. These unique properties of plants are due to presence of unique secondary metabolites present in them. The content of these secondary metabolites can be increased by elicitation.

Thus, we carried out elicitation of target metabolite found in this plant called dactylorhin-E (as reported by Li et al, 2009), using various elicitors such as yeast extract, methyl jasmonate and salicylic acid. These are previously used as elicitors by Neha et al (2016) in *Pichorhiza kurroa*, Harvais (1982) in orchid *Cypripedium reginae* , Deepthi et al (2015) in *Ophiorrhiza mungos* etc where they find significant increase in their respective plants.

Quantification of dactylorhin-E was carried out using analytical HPLC and it was observed that the amount of dactylorhin-E did not increased significantly so that it can be utilized for commercial purposes. Thus, we have carried out cancer cell line studies using hydro-alcoholic plant extract from tissue cultured plantets as many orchids have been reported for their anti-cancerous activity such as *Dendrobium nobile* contains denibinobin (Song et al, 2012) showed cytotoxicity against human lung carcinoma, human ovary adenocarcinoma and humanpromyelocytic leukemia cell lines. Similarly, Erianin from *Dendrobium chrysanthemum* (Ramesh et al, 2016) also shows anti cancerous activity. Anthocyanins have been identified from European Orchidaceae with the application of UV/Vis spectroscopy, various hydrolytic extraction procedures, chromatographic techniques (such as HPLC) and gel electrophoresis some of them are cyanidin glycosides 3-monoglucoside (chrysanthemin) 3,5-diglucoside (cyanin), 3,7-diglucoside (seranin) and 3-oxalylglucoside (ophrysanin), oxalyl-3,5-diglucoside (orchicyanin II), oxalyl-3,7-diglucoside (serapianin) and the intramolecular copigmented anthocyanin cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside (Strack et al, 1989). And we are very well known of the fact that anthocyanin possesses anti tumor activity against various carcinoma cells.

Hydro-alcoholic extraction has been carried out by Sharma et al (2016) for attaining highest diosgenin content from *Trillium govanianum* (nag chhatri) and studying its *in vitro* anticancerous activity. Similarly, Yadav et al (2010) carried out ethanolic extraction to study the anti cancerous activity of *Withania somnifera* on human cancer cell lines.

Anti-cancerous activity was carried out on two breast cell lines viz. MDA-MB-231 and MCF-7 by Sharma et al (2016). They have used MCF-7 and MDA-MB-231 cell lines for studying the anti-cancerous property of the plant and compared their results with normal cell line MDCK. Similarly, Jeevitha et al (2010) also studied the effect of Berberine on MCF-7 cell line and found induction of apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway.

In the present study we have also used MCF-7 and MDA-MB-231 cancer cell lines for studying the anti- cancerous property of the plant extract in order to generate a lead for further experimentation on different types of cancerous cell lines.

CHAPTER-5

CONCLUSION

CONCLUSION:

We have optimized the in- vitro conditions for the multiplication and propagation of Dactylorhiza hatagirea with its successful transplantation under field conditions. We have optimized the conditions for the elicitation of secondary metabolites and its marker compound dactylorhin-E at the lab scale whereas experimentation can be planned for the commercialization in the extension of this very project. Hydro- alcoholic plant extract was used for studying the anti- cancerous effect on breast cancer cell lines (MCF- 7 and MDA-MB- 231) along with normal cell lines (HEK- 293). This study gives scientific validation to the anti- cancerous property of *Dactylorhiza hatgirea*. It can be concluded that root extract of *Dactylorhiza hatgirea* causes more significant death of the breast cancer cells as compared to shoot extract. Also, there is no study reported by far now which shows the anti- cancerous potential of the plant *Dactylorhiza hatageria.* Infact, this study opens up the gateway that could be used for further experimentation on different cell lines or animal studies so that anti- cancerous herbal formulations can be developed.

PUBLICATIONS

1) **D Popli** and H Sood, 2016. Optimization of liquid media for increasing the biomass of *Dactylorhiza hatagirea*. 3rd prize in IEEE project presentation. 14th may 2016. JUIT, Waknaghat, Solan
2) **D Popli** and HSood . Presentation of an oral paper entitled "Optimization of liquid MS medium for enriching biomass of *Dactylorhiza hatagirea*" (2016), **IJTA 3rd international conference on Horticulture and Plant Sciences** organized by International Journal of Tropical Agriculture and Serials Publications Pvt. Ltd. New Delhi, India.
3) **DPopli**, S Sharma and H Sood . Optimization of liquid MS medium for enriching biomass of *Dactylorhiza hatagirea*. 34(5) **International Journal of Tropical Agriculture**. (2016), pp:1271-1276, ISSN0254-8755 (NAAS: 3.03).
4) S Sharma**, D Popli**, N Sharma, O. P Chaurasia and H Sood. Effect of Temperature on in Vitro Organogenesis of *Rhodiola imbricata* Edgew. – A Medicinal Herb. 5 (12) **World Journal of Pharmacy and Pharmaceutical Sciences**, 2016, pp. 1228-1243. ISSN 2278 – 4357. **SJIF 6.041.** DOI: 10.20959/wjpps201612-8266.
5) A Rajta, **D Popli**, P Kotvi, H Sood. Optimization of Culture Conditions for the Production and Germination of Artificial Seed in an Important Medicinal Plant, *Nothapodytes nimmoniana*. 3(1), **International Journal of Innovative Research in science and engineering.** 2017, pp: 70-77, ISSN (O) 2454-9665, ISSN (P) 2454-0663.

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ANNEXURE

ANNEXURE

Composition of Murashige and Skoog basal medium (MS MEDIUM)

STOCKS	CHEMICALS	ORIGINAL STRENGTH(mg/l)	STOCK(g/l)	FINAL VOLUME
A-10X	KNO_3	1900	19	
	$MgSO_4.7H_20$	370	3.7	100 ml/l
	KH_2PO_4	170	1.70	
B-20X	NH_4NO_3	1650	33	150 ml/l
C-100X	$CaCl_2.2H_2O$	440	44	10 ml/l
D-100X	Na $_2$EDTA	37.26	3.72	10 ml/l
	$FeSO_4.7H_2O$	27.85	2.28	
E-100X	KI	0.83	0.083	100 ml/l
F-100X	H_3BO_3	6.2	0.62	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.6	0.86	10 ml/l
	$CuSO_4.5H_2O$	0.025	0.0025	
	$MnSO_4.4H_2O$	22.3	2.23	
	$Na_2MbO_4.2H_2O$	0.25	0.025	
G-100X	m-INOSITOL	100	10	10 ml/l
	GLYCINE	2	0.2	
H-100X	PYRIDOXINE-HCL	0.5	0.05	
	NICOTINE ACID	0.5	0.05	10 ml/l
	THIAMINE	0.1	0.01	