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INVESTIGATION ON BIOSYNTHESIS OF SOME BIOSIMILAR DRUGS USING VARIOUS CULTURE TECHNIQUES – RESEARCH ARTICLE

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***Abstract-** Plant tissue culture technique play very significant role in the investigation on biosynthesis of biopharmaceuticals, plant metabolites etc. The present investigation based on the production of biosimilar drugs by the help of plant tissue culture technique. In this work, D.carota cell line developed in nutrient media. MS media was used as nutrient medium. Several types of modification also done in MS media with a view to enhance initiation of callus, like modified MS media, MS media with coconut water and MS media with sugar solution. MS media with coconut water (74%) show maximum initiation of callus. The biomass production in D.carota was recorded 126.48 g/L, 129.80 g/L and 121.50 g/L with MS media, MS media with coconut water and MS media with sugar solution respectively. Callus obtained from static MS media used for establishment of suspension culture. The cells were grown in culture vessels on a rotary shaker incubator. The growth of cells was studied by packed cell volume and viability of cell. The packed cell volume was recorded 13.5%, 14.5% and 10.2% in callus of MS media, MS media with coconut water and MS media with sugar solution with viability 89%, 91% and 85% respectively. The growth of D.carota cells was also studied with fix concentration of inoculum i.e. 10% packed cell volume. After 16th day of inoculation 26.17%, 29.20% and 23.20% packed cell volume was recorded in MS media, MS media with coconut water and MS media with sugar solution respectively. After the establishment of well-developed cell line, substrate solutions (Benzoic acid and chloroquine) were fed into media in different concentration i.e. 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml. The viability of the cells of culture was recorded with different concentration of substrates during the experiment. The percentage viability of D.carota cell in suspension culture was recorded 90%, 91%, 87% and 89% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml of benzoic acid fed respectively. In chloroquine fed, the percentage viability of D.carota cell in suspension culture was recorded 92%, 90%, 90% and 89% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml respectively. The samples were analysed periodically and metabolites were identified by matching with known standards using thin layer chromatography and high-performance liquid chromatography. Thin layer chromatographic studies revealed that D. carota cell culture gave one*

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metabolite each with benzoic acid and chloroquine. The metabolites formed were identified with authentic samples by Co-TLC which illustrates that D. carota transformed benzoic acid into p-hydroxybenzoic acid and chloroquine into hydroxychloroquine. The identity of metabolites was further confirmed by HPLC. The ethyl acetate fraction and chloroform fraction of D.carota were subjected to HPLC. The identification and quantitative estimation of peaks was carried out with known standards of p-hydroxybenzoic acid and hydroxychloroquine. The peaks of metabolites of ethyl acetate was found identical with known standards of p-hydroxybenzoic acid and the peaks of metabolites of chloroform fraction was found very close with known standards of hydroxychloroquine, i.e. it was assumed that chloroquine converted into hydroxychloroquine. On 20th day of incubation, accumulation of P-hydroxybenzoic acid in suspension culture was recorded 70%, 76.66%, 53.5% and 47.6% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml respectively. The yield of hydroxychloroquine in suspension culture was recorded 61%, 57.33%, 44.45% and 42.8% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml respectively after 20th day of incubation. The studies performed with media of suspension culture and control sample were showed no accumulation of p-hydroxybenzoic acid and hydroxychloroquine. Results clearly indicate that the experiment successfully optimized the ideal culture condition to induce callus and produce biomass of D.carota. D.carota cells were efficiently utilized for bioconversion of benzoic acid into p-hydroxybenzoic acid and chloroquine into hydroxychloroquine. The D.carota cells possess hydroxylation potential, which can be useful for the preparation of many products of pharmaceutical importance and this hydroxylation process only done in the presence of substrate.

Keywords - Biosynthesis, Biosimilar, D.carota cell line, Callus culture, Suspension culture, hydroxychloroquine, p-hydroxybenzoic acid.

1. Introduction –

1.1 Biosimilar

A biosimilar (also known as follow-on biologic or subsequent entry biologic) is a biologic medical product that is almost an identical copy of an original product that is manufactured by a different company.[1] Biosimilars are officially approved versions of original "innovator" products and can be manufactured when the original product's patent expires.[2]

FDA definition of a biosimilars - The FDA describes biosimilars in the following way: "The biologic product is highly similar to the reference product not withstanding minor differences in clinically inactive components" and that "there are no clinically meaningful differences between the biologic product and the reference product in terms of safety, purity, and potency of the product".[3]

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EMA definition of a biosimilar - "A biosimilar is a biological medicinal product that contains a version of the active substance of an already authorised original biological medicinal product (reference medicinal product) in the European Economic Area (EEA). Similarity to the reference medicinal product in terms of quality characteristics, biological activity, safety and efficacy based on a comprehensive comparability exercise needs to be established."[4]

1.2 Plant tissue culture

Plant tissue culture is a technique used for *in vitro* regeneration of plants. It relies on maintaining plant cells in aseptic conditions on a suitable nutrient medium. The culture can be sustained as a mass of undifferentiated cells for an extended period of time or regenerated into whole plants. Callus culture and suspension culture are the basic technique used to produce the desired metabolites of plants.[5]

Callus culture - The growth and maintenance of unorganised cell masses initiated from disorganised growth of pieces of plant tissues or the explant are called callus. Callus cultures are initiated from a small part of an organ or tissue segments called explant on a growth supporting solidified nutrient medium under sterile conditions. Any part of the plant organ or tissue may be used as the explant. Typical explants are leaf, root, stem-nodal and internodal parts, axillary buds, shoot tip, shoot apical meristem, seeds etc.

Suspension culture - Suspension culture is the culturing of isolated cells in a liquid media. The callus is removed from the original explant and transferred to a medium and placing it on a mechanical orbital platform shaker at 100-150 rpm. Agitation is required for suspension cultures for three purposes: it serves to break up the cell aggregates; it maintains a uniform distribution of the cell of various sizes and shapes, and cell clump in medium ; and it provides gas exchange for the cell to sustain cell respiration in the liquid medium.[6]

2. Material & Method

2.1 Cell Culture of *D.carota*

Plant material - *D.carota* were purchased from local market of Indore, (M.P.). The plant material was washed, dried and stored in store room.

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2.2 Culture Media

2.2.1. Selection of culture media – Miras‑ Moreno B. et al (2018) reported good growth of *D.carota* culture in MS media and they were successfully done their work on effect of terbinafine on the biosynthetic pathway of isoprenoid compounds in carrot suspension cultured cells. MS media was selected as basal medium for *D.carota* culture and growth of cells was maintained by selecting the optimum concentration of nutrients.

***2.2.2. Nutrients of MS-Media* -** Murashige & Skoog media was invented by two scientists Toshio Murashige and Folke K. Skoog in 1962 as a plant growth media. It is also known as MS Media.

It consists of several components. They contain macronutrient such as Potassium nitrate (KNO_3), Ammonium nitrate (NH_4NO_3), Magnesium sulphate ($MgSO_4.7H_2O$), Calcium chloride ($CaCl_2.2H_2O$), Potassium phosphate (KH_2PO_4). Micro-nutrient such as Ferrous sulphate ($FeSO_4.7H_2O$), EDTA disodium salt (Na_2EDTA), Manganese sulphate ($MnSo_4$. $4H_2O$), Boric acid (H_3BO_3), Zinc sulphate ($ZnsO_4.5H_2O$), Sodium molybdate dihydrate ($NaMoO_4.2H_2O$), Copper sulphate ($CuSO_4.5H_2O$), Potassium iodide (KI), Cobalt chloride ($CoCl_2$.6H2O). Inorganic element & vitamins such as Myo-inositol, Nicotinic acid, Glycine, Pyridoxine hydrochloride, Thiamine hydrochloride. All above mentioned components were collected from central store of Swami Vivekanand college of pharmacy, Indore.

2.3 Experimental

2.3.1 Preparation of Media

2.3.1.1 Stock Solutions

S.No.	Stock solution	Composition (mg/L)	Stock solution (w/v)	Stock solution (w/v)	Volume of the stock / 1000 ml of media
			10 ml	100ml	
			1X	10X	
1.	Ammonium nitrate (NH_4NO_3)	1650 mg	1650 mg	16500 mg	10 ml
2.	Calcium chloride ($CaCl_2.2H_2O$)	440 mg	440 mg	4400 mg	10 ml
3.	Magnesium sulphate (MgSO4. $7H_2O$)	370 mg	370 mg	3700 mg	10 ml
4.	Potassium nitrate (KNO_3)	1900 mg	1900 mg	19000mg	10 ml
5.	Potassium phosphate (KH_2PO_4)	170 mg	170 mg	1700 mg	10 ml

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6.	Boric acid (H_3BO_3)	6.20 mg	6.20 mg	62 mg	10 ml
7.	Cobalt chloride (CoCl2.6H2O)	0.025 mg	0.025 mg	0.25 mg	10 ml
8.	Copper sulphate ($CuSO_4.5H_2O$)	0.025 mg	0.025 mg	0.25 mg	10 ml
9.	EDTA disodium salt (Na_2EDTA)	37.30 mg	37.30 mg	373 mg	10 ml
10.	Ferrous sulphate ($FeSO_4.7H_2O$)	27.8 mg	27.8 mg	278 mg	10 ml
11.	Manganese sulphate ($MnSo_4$. $4H_2O$)	22.30 mg	22.30 mg	223 mg	10 ml
12.	Potassium iodide (KI)	0.83 mg	0.83 mg	8.3 mg	10 ml
13.	Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	0.25 mg	0.25 mg	2.5 mg	10 ml
14.	Zinc sulphate ($ZnsO_4.5H_2O$)	8.60 mg	8.60 mg	86 mg	10 ml
15.	Myo – Inositol	100 mg	100 mg	1000 mg	10 ml
16.	Glycine	2 mg	2 mg	20 mg	10 ml
17.	Nicotinic acid	0.5 mg	0.5 mg	5 mg	10 ml
18.	Pyridoxine hydrochloride	0.5 mg	0.5 mg	5 mg	10 ml
19.	Thiamine hydrochloride	0.1 mg	0.1 mg	1 mg	10 ml
20.	Sucrose ($C_{12}H_{22}O_{11}$)	2000 mg	1000 mg (100% w/v)	10000 mg (100% w/v)	20 ml

For preparation of accurate stock solution, accurate quantities of ingredients were weighed and transferred to volumetric flask of 100 ml. The chemical was dissolved in distilled water and volume makeup up to 100 ml with distilled water. The solutions were labelled and stored in refrigerator.

***Auxin Solution* -** 100 mg of 3-indole acetic acid was weighed accurately and dissolved in 5ml of ethanol in 100 ml volumetric flask. Finally, volume was makeup upto 100 ml by distilled water and kept in refrigerator.

Cytokinin Solutions - 50 mg each of benzyl adenin (BAP) and kinetin were dissolved in small volume of 0.5N HCI by slight warming and diluted separately to 100 ml using distilled water and stored in refrigerator.

2.3.1.2 Preparation of MS-Media

The composition of macronutrients, micronutrients and organic compounds were as follows:

Ingredients	Composition (mg/L)
Macronutrients	
Ammonium nitrate (NH_4NO_3)	1650 mg
Calcium chloride ($CaCl_2.2H_2O$)	440 mg
Magnesium sulphate (MgSO4. $7H_2O$)	370 mg
Potassium nitrate (KNO_3)	1900 mg
Potassium phosphate (KH_2PO_4)	170 mg
Micronutrients	
Boric acid (H_3BO_3)	6.20 mg
Cobalt chloride (CoCl2.6H2O)	0.025 mg
Copper sulphate ($CuSO_4.5H_2O$)	0.025 mg
EDTA disodium salt (Na_2EDTA)	37.30 mg
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8 mg

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Manganese sulphate ($MnSo_4$. $4H_2O$)	22.30 mg
Potassium iodide (KI)	0.83 mg
Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	0.25 mg
Zinc sulphate ($ZnsO_4.5H_2O$)	8.60 mg
Organic Elements	
Myo – Inositol	100 mg
Glycine	2 mg
Nicotinic acid	0.5 mg
Pyridoxine hydrochloride	0.5 mg
Thiamine hydrochloride	0.1 mg
Plant Growth Regulators	
Auxin	2 ml
Cytokinin	0.2 ml
Other	
Agar	8 gm
Distilled Water	1000 ml (q.s.)
pH	5.8

MS medium was prepared by dissolving 10 ml of each salts and organic supplement stock solutions in 800 ml of distilled water. Then PGRs were added. The pH was adjusted to 5.8 using 0.1 N NaOH or 0.1N HCl, then volume was makeup up to 1000 ml using distilled water. Added 8 gm agar & medium was heated on hot plate until the agar melt. Stirred the media occasionally until all the agar is dissolved & solution become clear. The medium dispensed in culture vessels (conical flask). The culture vessels were tightly closed with non-absorbent cotton plug and covered with brown paper. The medium was autoclaved at 15 lb (120°C) for 15 minutes for sterilization. Prolonged autoclaving at high temperature was avoided. After sterilization culture vessels were kept for solidification of the medium at 25±2°C in aseptic room. The culture vessels were kept in upright position during solidification.

2.3.2 Preparation of Natural Juice

The coconut water was obtained from fresh coconut fruit. Coconut fruit was cut by scalpel and coconut water transferred into a sterile beaker. The yield of coconut water was 250ml from coconut fruit. The coconut water was sterilized by membrane filtration (filtered through muslin cloth) prior to use.

2.3.3 Modification in Media

MS Medium was modified with a view to initiate callus in *D.carota*. The different media were prepared such as modified MS media, MS media with coconut water or sugar solution.

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2.3.3.1 Modified MS Media

The composition of macronutrients, micronutrients and organic compounds were as follows:

Ingredients	Composition (mg/L)
Macronutrients	
Ammonium nitrate (NH_4NO_3)	1650 mg
Calcium chloride ($CaCl_2.2H_2O$)	440 mg
Magnesium sulphate ($MgSO4.\ 7H_2O$)	370 mg
Potassium nitrate (KNO_3)	1900 mg
Potassium phosphate (KH_2PO_4)	170 mg
Micronutrients	
Boric acid (H_3BO_3)	6.20 mg
Cobalt chloride (CoCl2.6H2O)	0.025 mg
Copper sulphate ($CuSO_4.5H_2O$)	0.025 mg
EDTA disodium salt (Na_2EDTA)	37.30 mg
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8 mg
Manganese sulphate ($MnSo_4.\ 4H_2O$)	22.30 mg
Potassium iodide (KI)	0.83 mg
Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	0.25 mg
Zinc sulphate ($ZnsO_4.5H_2O$)	8.60 mg
Organic Elements	
Myo – Inositol	100 mg
Glycine	2 mg
Nicotinic acid	0.5 mg
Pyridoxine hydrochloride	0.5 mg
Thiamine hydrochloride	0.1 mg
Plant Growth Regulators	
Auxin	1 ml
Cytokinin	0.1ml
Other	
Agar	8 gm
Distilled Water	1000 ml (q.s.)
pH	5.8

Modified MS medium was prepared by dissolving 10 ml of each salts and organic supplement stock solutions in 800 ml of distilled water. Then PGRs were added. The pH was adjusted to 5.8 using 0.1 N NaOH or 0.1N HCl, then volume was makeup up to 1000 ml using distilled water. Added 8 gm agar & medium was heated on hot plate until the agar melt. Stirred the media occasionally until all the agar is dissolved & solution become clear.

The medium dispensed in culture vessels (conical flask). The culture vessels were tightly closed with non-absorbent cotton plug and covered with brown paper. The medium was autoclaved at 15 lb (120°C) for 15 minutes for sterilization. Prolonged autoclaving at high temperature was avoided. After sterilization culture vessels were kept for solidification of the medium at 25±2°C in aseptic room. The culture vessels were kept in upright position during solidification.

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2.3.3.2 MS Media with Coconut Water (MSC)

The composition of all components was as follows:

Ingredients	Composition (mg/500ml)
Macronutrients	
Ammonium nitrate (NH_4NO_3)	825 mg
Calcium chloride ($CaCl_2.2H_2O$)	220 mg
Magnesium sulphate (MgSO4. $7H_2O$)	185 mg
Potassium nitrate (KNO_3)	950 mg
Potassium phosphate (KH_2PO_4)	85 mg
Micronutrients	
Boric acid (H_3BO_3)	3.10 mg
Cobalt chloride (CoCl2.6H2O)	0.0125 mg
Copper sulphate ($CuSO_4.5H_2O$)	0.0125 mg
EDTA disodium salt (Na_2EDTA)	18.65 mg
Ferrous sulphate ($FeSO_4.7H_2O$)	13.90mg
Manganese sulphate ($MnSo_4$. $4H_2O$)	11.15 mg
Potassium iodide (KI)	0.415 mg
Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	0.125 mg
Zinc sulphate ($ZnsO_4.5H_2O$)	4.30 mg
Organic Elements	
Myo – Inositol	50 mg
Glycine	1 mg
Nicotinic acid	0.25 mg
Pyridoxine hydrochloride	0.25 mg
Thiamine hydrochloride	0.05 mg
Plant Growth Regulators	
Auxin	2 ml
Cytokinin	0.2 ml
Other	
Coconut Water	250ml
Agar	4 gm
Distilled Water	500 ml (q.s.)
pH	5.8

MS medium with coconut water was prepared by dissolving 5 ml of each salts and organic supplement stock solutions in up to 150 ml of distilled water. Added 250ml of coconut water. Then PGRs were added. The pH was adjusted to 5.8 using 0.1 N NaOH or 0.1N HCl, then volume was makeup up to 500 ml using distilled water. Added 4 gm agar & media was heated on hot plate until the agar melt. Stirred the medium occasionally until all the agar is dissolved & solution become clear. The medium dispensed in culture vessels (conical flask). The culture vessels were tightly closed with non-absorbent cotton plug and covered with brown paper. The medium was autoclaved at 15 lb (120°C) for 15 minutes for sterilization. Prolonged

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autoclaving at high temperature was avoided. After sterilization culture vessels were kept for solidification of the medium at 25±2°C in aseptic room. The culture vessels were kept in upright position during solidification.

2.3.3.3 MS Media with Sugar Solution (MSS)

The composition of all components was as follows:

Ingredients	Composition (mg/500ml)
Macronutrients	
Ammonium nitrate (NH_4NO_3)	825 mg
Calcium chloride ($CaCl_2.2H_2O$)	220 mg
Magnesium sulphate ($MgSO4.\ 7H_2O$)	185 mg
Potassium nitrate (KNO_3)	950 mg
Potassium phosphate (KH_2PO_4)	85 mg
Micronutrients	
Boric acid (H_3BO_3)	3.10 mg
Cobalt chloride (CoCl2.6H2O)	0.0125 mg
Copper sulphate ($CuSO_4.5H_2O$)	0.0125 mg
EDTA disodium salt (Na_2EDTA)	18.65 mg
Ferrous sulphate ($FeSO_4.7H_2O$)	13.90mg
Manganese sulphate ($MnSo_4.\ 4H_2O$)	11.15 mg
Potassium iodide (KI)	0.415 mg
Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	0.125 mg
Zinc sulphate ($ZnsO_4.5H_2O$)	4.30 mg
Organic Elements	
Myo – Inositol	50 mg
Glycine	1 mg
Nicotinic acid	0.25 mg
Pyridoxine hydrochloride	0.25 mg
Thiamine hydrochloride	0.05 mg
Plant Growth Regulators	
Auxin	2 ml
Cytokinin	0.2 ml
Other	
Sugar Solution	10 ml (100% w/v sucrose solution)
Agar	4 gm
Distilled Water	500 ml (q.s.)
pH	5.8

MS medium with sucrose solution was prepared by dissolving 5 ml of each salts and organic supplement stock solutions and 10 ml (100% w/v sucrose solution) of sucrose solution in up to 400 ml of distilled water. Then PGRs were added. The pH was adjusted to 5.8 using 0.1 N NaOH or 0.1N HCl, then volume was makeup up to 500 ml using distilled water. Added 4 gm agar & media was heated on hot plate until the agar melt. Stirred the media occasionally until all the agar is dissolved & solution become clear. The medium dispensed in culture vessels (conical flask). The culture vessels were tightly closed

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with non-absorbent cotton plug and covered with brown paper. The medium was autoclaved at 15 lb (120°C) for 15 minutes for sterilization. Prolonged autoclaving at high temperature was avoided. After sterilization culture vessels were kept for solidification of the medium at 25±2°C in aseptic room. The culture vessels were kept in upright position during solidification.

2.3.4. Callus Initiation from D.carota

2.3.4.1. Explant: Wipe down and turn on the laminar air flow 30 minute before doing work in the hood and sterilize the instrument by 90% alcohol. Cut the *D.carota* root into 3-6 cm long, discarding both ends of the root. Remove the epidermis and any blemish with scalpel.

Put the tap root section in to a sterile beaker having double distilled sterile water and shake it for few seconds. Remove the double distilled water into the waste beaker. Cut the *D.carota* section from each end and discard this end portions.

Cut 3-4 transverse section (1-5 mm thick) across the tap root and transfer each to a fresh sterile Petri dish. Cut the smaller section of explants in square shape from each of the transverse sections by cutting across the cambium. The following method is used.

a. Trim the cortex and some of the phloem from each transverse section of *D.carota*
b. Cut off *D.carota* in small strip pieces containing the cambium
c. Each strip can then be subdivided to produce small square shaped explants, each contain/ing parts of the phloem, xylem and cambium.

Put explant section in to a sterile petridish having double distilled sterile water and rinse explant section three times by double distilled sterile water. The explants were transferred to a fresh sterile petridishes and subjected to surface sterilization.

Surface sterilization: Put explants in to a sterile petridishes having 90% ethanol and rinse explants three time by 90% ethanol. After that put explant section in to a sterile container (beaker) having 0.1% solution of mercuric chloride and shake it for few minutes. They help in balancing the pH. Remove the solution into the waste beaker. After that put explants in to a sterile Petri dish having double distilled sterile water and explants were washed three-four times by double distilled sterile water.

2.3.4.2. Culture of explant: The sterilized explants were cultured on MS media. The explants were aseptically transferred to culture vessels containing 30 ml of solidified media by using sterile forceps and covered by non-absorbent cotton plugs. After inoculation culture vessels were incubated in dark at 25 ±1 °C. After initiation of callus, the culture vessels were kept in light/dark cycle upto four weeks.

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2.3.4.3. The effect of modified MS media, coconut water and sugar solution on callus initiation of D.carota: In another experiment, the surface sterilized explants were aseptically transferred to culture vessel containing 30 ml of MS medium with coconut water. The culture vessels were incubated in dark at 25 ±1 °C. After initiation of callus, the culture vessels were kept in light/dark cycle upto four weeks. All the forceps, knife, petridishes, beakers etc used were presterilized autoclaving at 15 lb/ sq. inch pressure for 15 minutes. All the operations were carried out aseptically on laminar flow bench. The extent of initiation, colour and induction was visually observed. The same protocol applied for modified MS media as well as MS medium with sugar solution.

2.3.5. Growth of Callus in D.carota: The growth of *D.carota* callus into MS media was visually observed. The callus was aseptically removed from the culture vessel into petridish and trimmed into small pieces. The callus was aseptically transferred to 100 ml conical flask containing 30 ml of media. The flasks were incubated at 25 ℃ in an artificial light with light/ dark cycle. The weekly subculturing was carried out. Five flasks were harvested every week and growth was determined on fresh weight basis.

2.3.5.1. Effect of modified MS media, coconut water and sugar solution on growth of callus of D. Carota: The growth of *D.carota* callus into modified MS media, MS medium with coconut water and MS medium with sugar solution was visually observed. In separate experiment the callus of *D.carota* was aseptically inoculated to 100 ml conical flask containing 30 ml MS media with Coconut water and MS media with sugar solution. The flasks were incubated as described above, subcultured weekly and growth was determined on fresh weight basis.

2.3.5.2. Determination of fresh weight: The callus was removed with the help of long forceps into petridish, blotted dry and weight was taken.

2.3.6. Establishment of Suspension Culture of D.carota from Callus

The friable callus in active growth phase, obtained from the *D.carota* root in MS media were used initiate suspension culture in 100 ml conical flask containing 30 ml of media. The suspension culture was established in MS media. The friable callus removed aseptically from the 3-month-old culture was transferred to culture flask containing 30 ml of media. The process was repeated to other flasks and they were incubated on rotary shaker (120 rpm) at 25 ±1°C for 16 days.

2.3.6.1. Growth studies of D.carota in suspension culture with different cell inoculum: Every second day 10 ml sample was withdrawn aseptically from the flask and growth was determined by measuring packed cell volume.

2.3.6.2. Effect of coconut water and sugar solution on growth of D.carota cell in suspension culture: The effect of MS media with coconut water and MS media with sugar solution on growth was also studied. The 10 ml cell were taken from *D.carota* suspension cultured and incubated into 100 ml conical flask containing 30 ml MS of media with

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coconut water and MS of media with sugar solution. The flasks were incubated as described above. 10 ml sample was withdrawn from the flask and growth was determined by measuring packed cell volume.

2.3.6.3. Determination of packed cell volume: Ten ml of well mixed suspension was taken in graduated centrifuge tube and rotated at 180 rpm for 20 minutes. The volume of the cell settled on the bottom of the tube was recorded.

2.3.7. Determination of Viability of Cells: Accurately weighed 10 mg of fluorescein diacetate dye and dissolved into sufficient quantity of water. The volume was made-up to 100 ml. A sample of 1 ml cells was taken and stained with fluorescein diacetate (0.01%). The viable cells were stained and counted under microscope on haemocytometer.

2.3.8. Bioproduction of drug in free cell system

2.3.8.1. Plant cells: The *D.carota* cell line were taken from the stationary phase of the homogeneous suspension as developed in section **2.3.6**. The cells were inoculated into conical flask (100 ml) containing 30 ml of MS media. The MS medium was prepared as described in Section **2.3.1.2**. The cells were conditioned for one week before feeding of substrates.

***2.3.8.2. Preparation of substrates solution**:* Benzoic acid and chloroquine were taken as substrate. Accurately weighed 1000 mg (1 gm) of benzoic acid and chloroquine separately, each were individually dissolved in 200 ml of distilled water (5000µg/ml). The solutions were autoclaved & sterilized prior to use through membrane filter (0.22µ).

2.3.8.3. Feeding of substrates: The five conical flask 1, 2, 3, 4, 5 were taken which contain plant cell (10 ml PC) and MS media (50 ml) as described above. The sterilized solution 2 ml, 3 ml, 4 ml and 5 ml of chloroquine was fed to flask No. 1 to 4 respectively which led to 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml concentration of chloroquine in respective flask. Flask No. 5 was kept as control. The process was repeated to inoculate ten flasks of each concentration. The same feeding procedure undertaken with the media of suspension culture. The benzoic acid substrate was also fed in the concentration of 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml as described above. One flask containing plant cell (10 ml PC) and MS media (50 ml) was taken as control with each experiment.

2.3.8.4. Incubation of flask: The flasks were incubated in dark on a rotary shaker (120 rpm) at 25±1°C for 20 days with one control flask.

***2.3.8.5. Sampling for analysis*:** On alternate day, one flask from each concentration was collected and exposed to analysis for the presence of metabolites.

2.3.8.6. Testing of viability: On alternate day 1 ml sample (Cell + media) was withdrawn from flask of each concentration and tested for viability of cell by the method described in section 4.3.7. and using fluorescein diacetate (0.01%) dye.

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2.3.8.7. Studies of metabolites into media: In this experiment the whole process was repeated as above. During analysis, the cells were separated out from media and only media was analysed for the presence of metabolites.

2.3.9. Extraction of D.carota cell culture

The extraction was carried out with a view to extract out different substrate along with their metabolite, The *D.carota* cells fed with benzoic acid were extract out with ethylacetate. The *D.carota* cells fed with chloroquine were extract out with chloroform. The different extractions were carried out as follows.

2.3.9.1. Extraction of D.carota cell culture fed with benzoic acid: Cell (10 ml) + media (50 ml), was taken in 250 ml beaker. The pH of the media was adjusted to 5.5. Cell (10 ml) + media (50 ml) was taken into separating funnel and 20 ml ethyl acetate was added. The extraction was carried for half an hour with vigorous shaking and ethyl acetate layer was separated out from aqueous layer. The extraction was carried out three times with 20 ml of ethylacetate. The ethylacetate fractions were mixed, concentrated on evaporator and 20 ml concentrated extract was stored in vials. In other experiment the media was only extracted with ethyl acetate as described above and concentrated extract (20 ml) was stored in amber coloured vials. The flask which was kept as control was extracted by the method described above, extract was concentrated and stored in vial.

2.3.9.2. Extraction of D.carota cell culture fed with chloroquine: Cell (10 ml) + media (50 ml) was taken into separating funnel and 20 ml chloroform was added. The extraction was carried for half an hour with vigorous shaking and chloroform layer was separated out from aqueous layer. The extraction was carried out three times with 20 ml of chloroform. All the chloroform fractions were mixed, concentrated on rotary evaporator and 20 ml concentrated extract (20 ml) was stored in vials. In other experiment the media was only extracted with chloroform as described above and concentrated extract (20 ml) was stored in vials. The flask which was kept as control was extracted by the method described above, extract was concentrated (20 ml) and stored in vial.

2.3.10. Chromatographic studies

2.3.10.1. Thin layer chromatography

The ethyl acetate extract (20 ml) and chloroform extract (20 ml) obtained from the extraction of *D.carota* cell was subjected to thin layer chromatography. The screening was carried out with a view to separate and identify benzoic acid and metabolites in ethylacetate extract and chloroquine and metabolite in chloroform extract. The solvent system, detecting reagent, stationary phase reported in the literature were used as follows.

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2.3.10.1.1. Stationary phase: Silica gel G was used as stationary phase. Aqueous slurry (1 part silica gel :3 part water) was prepared and spread over the plates. The plates were dried in air and then activated for thirty minutes at 110°C in a dryer.

2.3.10.1.2. Sample solution**:** 5 ml extract withdrawn from the ethyl acetate extract (20 ml) and chloroform extract (20 ml) and was used as test solution for thin layer chromatography.

2.3.10.1.3. Solvent system: The solvent described in literature were selected with a view to separate benzoic and metabolites in ethyl acetate extract, chloroquine and metabolites in chloroform extract. The solvent system used were as follows.

Ethylacetate extract: Butanol : water : Ammonia (7:3:1.5)

Chloroform extract: Dichloromethane : n-hexene : Triethanolamine (8:2:1)

2.3.10.1.4. Detection: 10% vanillin in ethanol and concentrated sulphuric acid (2:1), used as detecting reagent for benzoic acid and its metabolites. Iodine chamber used as detecting reagent for chloroquine and its metabolites.

2.3.10.1.5. Method: The solvent systems were prepared by mixing the respective solvent and left for 15 minutes for saturation. The spots of the sample were applied on the plate, carefully, at a distance about 2.5 cm from the bottom and allowed to dry in air. The distance between the two spot was kept at least 10 mm. The plates were placed in the chromatographic chamber containing solvent system. The solvent was allowed to run until it reached at a height of about 75% from the point of spotting. The height of the solvent front was marked. The distance travelled by solvent front and distance travelled by spot was measured and R_f values were calculated.

2.3.10.2. High performance liquid chromatography

The high performance liquid chromatography was performed with a view to separate and estimate drug and metabolites.

2.3.10.2.1. For benzoic acids & its metabolites

2.3.10.2.1.1. Instrument*:* HPLC was carried out on CBM-20Alite model, SPD-20A detector A 254 nm, LC-20AD pump, Demo column, injection volume 20 µl, flow rate 1 µl/min, detector temperature 50°C.

2.3.10.2.1.2. Solvent system: For ethyl acetate fraction (containing benzoic acid and its metabolite) methanol: acetic acid: water (4:1:1) was used.

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2.3.10.2.1.3. Preparation of solution:

Test solution No. 1: 1 ml ethyl acetate fraction of *D.carota* was taken and diluted to 100 ml.

Standard solution No.1: 1-10 µg/L solution of p-hydroxy benzoic acid in ethyl acetate.

2.3.10.2.1.4. Method: The column was conditioned for thirty minutes with the mobile phase before injecting the sample. The sample of 20 µl was injected into injection port (time constant 0.5 second). The chromatograms were recorded at chart speed of 10 mm/minutes. The quantity of metabolite was estimated against authentic standards.

2.3.10.2.2. For chloroquine & its metabolites

2.3.10.2.2.1. Instrument: HPLC was carried out with CBM-20Alite model, SPD-20A detector A 254 nm, LC-20AD pump, Demo column, injection volume 20 µl, flow rate 1 µl/min, detector temperature 50°C.

2.3.10.2.2.2. Solvent system: For chloroform fraction (containing chloroquine and its metabolite) water and organic (acetonitrile : methanol : 50:50, v/v) mobile phase in 75:25 v/v ratio, with sodium 1-pentanesulfonate and phosphoric acid was used.

2.3.10.2.2.3. Preparation of solution:

Test solution No. 1: 1 ml chloroform fraction of *D.carota* was taken and diluted to 100 ml.

Standard solution No.1: 1-10 µg/L solution of hydroxychloroquine in chloroform.

2.3.10.2.2.4. Method: The column was conditioned for thirty minutes with the mobile phase before injecting the sample. The sample of 20 µl was injected into injection port (time constant 0.5 second). The chromatograms were recorded at chart speed of 10 mm/minutes. The quantity of metabolite was estimated against authentic standards.

3. Result & Discussion

The callus initiation of *D.carota* was carried out in MS media, modified MS media, MS media with coconut water and MS media with sugar solution. Modified MS media could not initiate callus. MS media, MS media with coconut water and MS media with sugar solution could initiate callus.

Growth of callus in MS media, MS media with coconut water and MS media with sugar solution was recorded in 2nd week. Fair growth was recorded with MS media and MS media with coconut water in 3rd week. Slow growth was recorded in MS media with sugar solution in 3rd week respectively.

When MS media was used, 70% of explant exhibited callus initiation with 47.6 g/L average fresh weight. MS media with coconut water was exhibited 74% callus initiation of explant with 49.2 average fresh weight. MS media with sugar solution was exhibited 65% callus initiation of plant with 45.8 g/L average fresh weight. (Table- 3.1)

The biomass yield of 126.48 g/L, 129.80 g/L and 121.50 g/L was obtained in MS media, MS media with coconut water and MS media with sugar solution respectively. (Table:3.2)

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The suspension culture was established in MS media. The packed cell volume was recorded 13.5%, 14.5% and 10.2% in callus of MS media, MS media with coconut water and MS media with sugar solution with viability 89%, 91% and 85% respectively.

The MS media suspension culture was brown in colour with cell aggregates and free cells, whereas MS media with coconut water suspension culture was creamy brown in colour with free cells and few cell aggregates. The suspension obtained from MS media with sugar solution possess minimum cell aggregates with free cells and brown in colour. (Table: 3.3)

The growth of *D.carota* cells was studied in MS media, MS media with coconut water and MS media with sugar solution with fix concentration of inoculum i.e. 10% packed cell volume. After 16th day of inoculation 26.17%, 29.20% and 23.20% packed cell volume was recorded in MS media, MS media with coconut water and MS media with sugar solution respectively. (Table: 3.4)

Thin layer chromatography of ethyl acetate fraction of *D.carota* and control was performed in isobutanol : ammonia : water (7:1.5:3) solvent system.

The ethyl acetate fraction exhibited two spots (R_f value 0.51, 0.06). The authentic sample of Benzoic acid and P-hydroxy benzoic acid were run in same solvent system which exhibited 0.51,0.06 R_f value.

The chloroform fraction of *D.carota* exhibited two spots (R_f value 0.86,0.16). The authentic sample of chloroquine and hydroxychloroquine were run in same solvent system which exhibited 0.86,0.16 R_f value. (Table 3.5)

The quantification estimation was carried out with HPLC. The ethyl acetate fraction and chloroform fraction of *D.carota* were subjected to HPLC. The identification and quantitative estimation of peaks was carried out with known standards of P-hydroxybenzoic acid and hydroxychloroquine. (Fig.3.15, 3.16)

The peaks of metabolites of ethyl acetate was found identical with known standards of P-hydroxybenzoic acid. The peaks of metabolites of chloroform fraction was found very close with known standards of hydroxychloroquine, i.e. it was assumed that chloroquine converted into hydroxychloroquine.

The conversion of benzoic acid into P-hydroxybenzoic acid in suspension culture was recorded 70%, 76.66%, 53.5% and 47.6% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml respectively after 20th day of incubation. (Table- 3.6, Fig.3.17, 3.18).

The conversion of benzoic acid was not found in media of suspension culture from 2nd day to 20th day of incubation period. (Table – 3.7) The control sample also was not found any accumulation of P-hydroxybenzoic acid from 2nd day to 20th day of incubation period. (Table – 3.8)

D.carota also biosynthesis hydroxychloroquine from chloroquine. The accumulation of hydroxychloroquine in suspension culture was recorded 61%, 57.33%, 44.45% and 42.8% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml respectively after 20th day of incubation. (Table- 3.9, Fig.3.19, 3.20).

The conversion of chloroquine was not found in media of suspension culture and control sample from 2nd day to 20th day of incubation period. (Table – 3.10, 3.11)

The percentage viability of *D.carota* cell in suspension culture was recorded 90%, 91%, 87% and 89% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml of benzoic acid fed respectively. (Table – 3.12, Fig.3.21)

In chloroquine fed, the percentage viability of *D.carota* cell in suspension culture was recorded 92%, 90%, 90% and 89% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml 0respectively. (Table – 3.13, Fig.3.22)

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S.No.	Media	Colour	Weeks				Average fresh weight g/L	Extent of callus initiation (%)
			1st	2nd	3rd	4th		
1.	MS media	Brown	-	+	+++	++++	47.6	70
2.	Modified MS media	-	-	-	-	-	-	-
3.	MS media with coconut water	Creamy brown	-	+	+++	++++	49.2	74
4.	MS media with sugar solution	Brown	-	+	++	+++	45.8	65

- No initiation +Initiation of callus ++ Slow growth +++ Fair ++++ Good growth

Table 3.1 – Initiation and growth of *D.carota* in different media

Fig.3.1 – Explant of *D.carota* at 1st week in MS media.

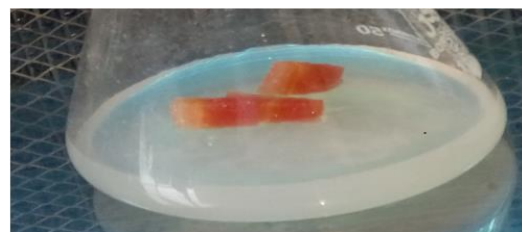

Fig.3.4 – Explant of *D.carota* at 1st week in MS media with sugar solution

Fig.3.2 – Explant of *D.carota* at 1st week in Modified MS media

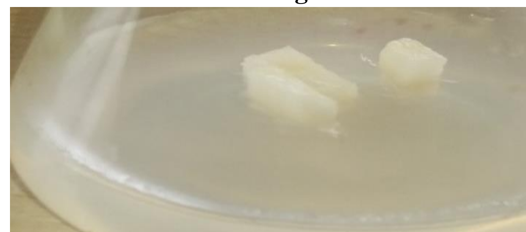

Fig.3.5 – Callus initiation in explant of *D.carota* after 2nd week in MS media

Fig.3.3 – Explant of *D.carota* at 1st week in MS media with coconut water

Fig.3.6 – Callus initiation in explant of *D.carota* after 2nd week in MS media with coconut water

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Fig.3.7 – Callus initiation in explant of *D.carota* after 2nd week in MS media with sugar solution

Fig 3.9 - Growth of callus in MS media with coconut water after 10th week at weekly subculturing

Fig.3.8 - Growth of callus in MS media after 10th week at weekly subculturing

Fig. 3.10 - Growth of callus in MS media with sugar solution after 10th week at weekly subculturing

S.No.	Callus of Media	Friability/ colour	Fresh weight g/L								
			0	1st	2nd	3rd	4th	5th	6th	7th	8th
			(Weeks) →								
1.	MS media	Brown	8.0	15.98	36.28	59.74	78.40	91.20	103.85	119.62	126.48
2.	MS media with coconut water	Creamy brown	8.0	16.92	38.78	61.80	79.30	92.90	107.680	121.52	129.80
3.	MS media with sugar solution	Brown	8.0	14.90	34.82	57.90	75.58	89.20	97.90	112.40	121.50

Table 3.2 - Yield of biomass of *D.carota* in different media.

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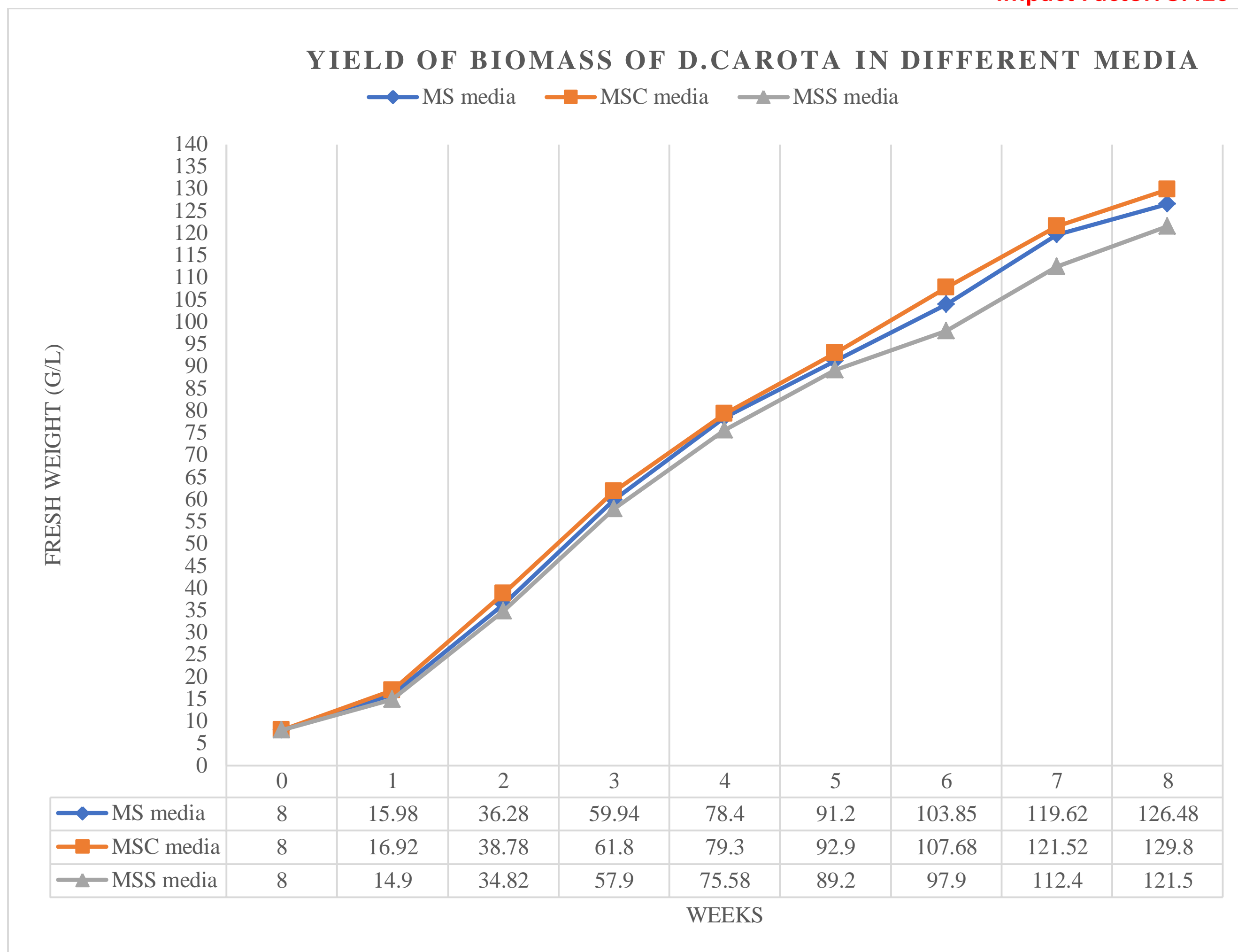

	0	1	2	3	4	5	6	7	8
MS media	8	15.98	36.28	59.94	78.4	91.2	103.85	119.62	126.48
MSC media	8	16.92	38.78	61.8	79.3	92.9	107.68	121.52	129.8
MSS media	8	14.9	34.82	57.9	75.58	89.2	97.9	112.4	121.5

Fig 3.11 – Graphical representation for yield of biomass of D.carota in different media

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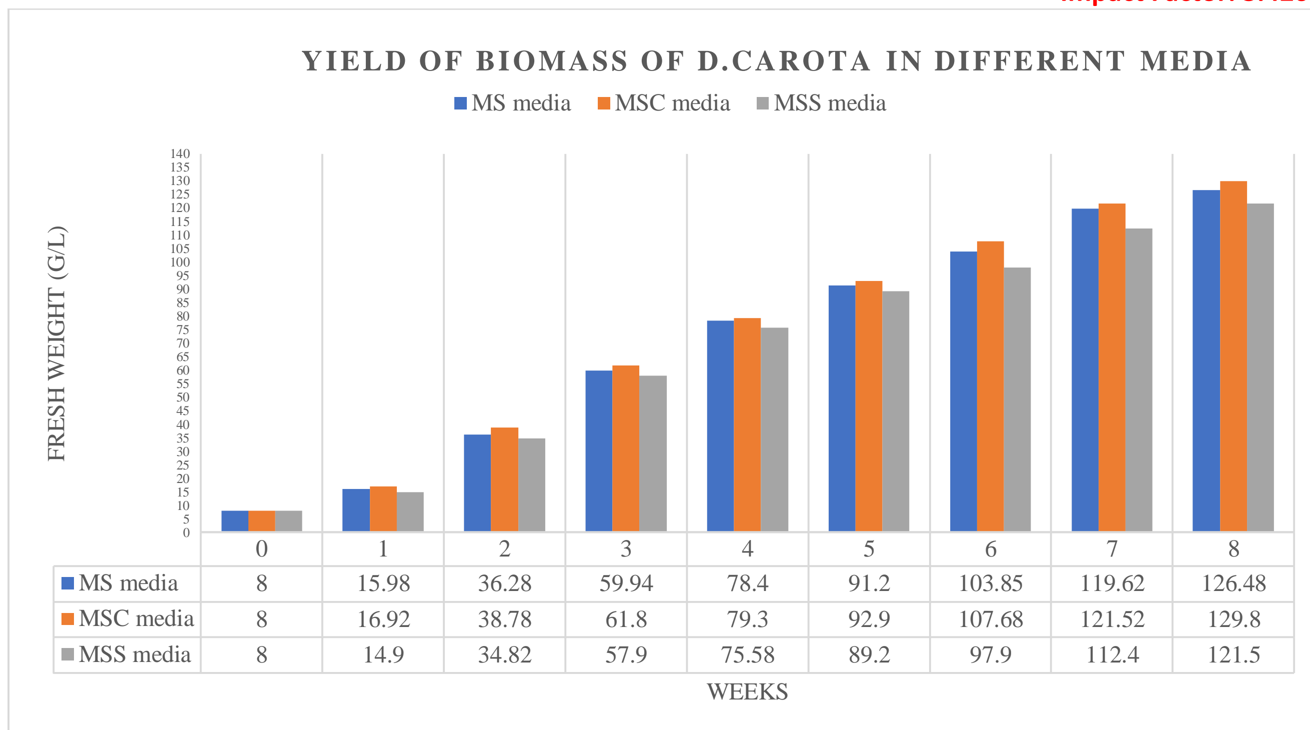

	0	1	2	3	4	5	6	7	8
MS media	8	15.98	36.28	59.94	78.4	91.2	103.85	119.62	126.48
MSC media	8	16.92	38.78	61.8	79.3	92.9	107.68	121.52	129.8
MSS media	8	14.9	34.82	57.9	75.58	89.2	97.9	112.4	121.5

Fig 3.12 – Bar diagram for yield of biomass of D.carota in different media

S.No.	Callus of Media	Culture period (Days)	Packed cell volume (%)	Viability (%)	Physical characteristics of cells
1.	MS media	14-16	13.5	89.0	Cell aggregates, free cells, brown
2.	MS media with coconut water	14-16	14.5	91.0	Free cells, few cell aggregates, creamy brown
3.	MS media with sugar solution	14-16	10.2	85.0	Free cells, minimum cell aggregates, brown

Table 3.3 – Establishment of suspension culture from callus of *D.carota* originated in different kind of media.

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S.No.	Callus of Media	Concentration of inoculum	Packed cell volume (%)								
			0	2nd	4th	6th	8th	10th	12th	14th	16th
1.	MS media	10	10	10.59	12.56	18.59	21.82	23.58	24.56	25.90	26.17
2.	MS media with coconut water	10	10	11.20	14.90	20.10	23.80	25.10	26.90	27.90	29.20
3.	MS media with sugar solution	10	10	10.28	11.50	13.96	15.89	17.36	19.96	21.85	23.20

Table: 3.4 – Growth of *D.carota* in suspension culture media with inoculum

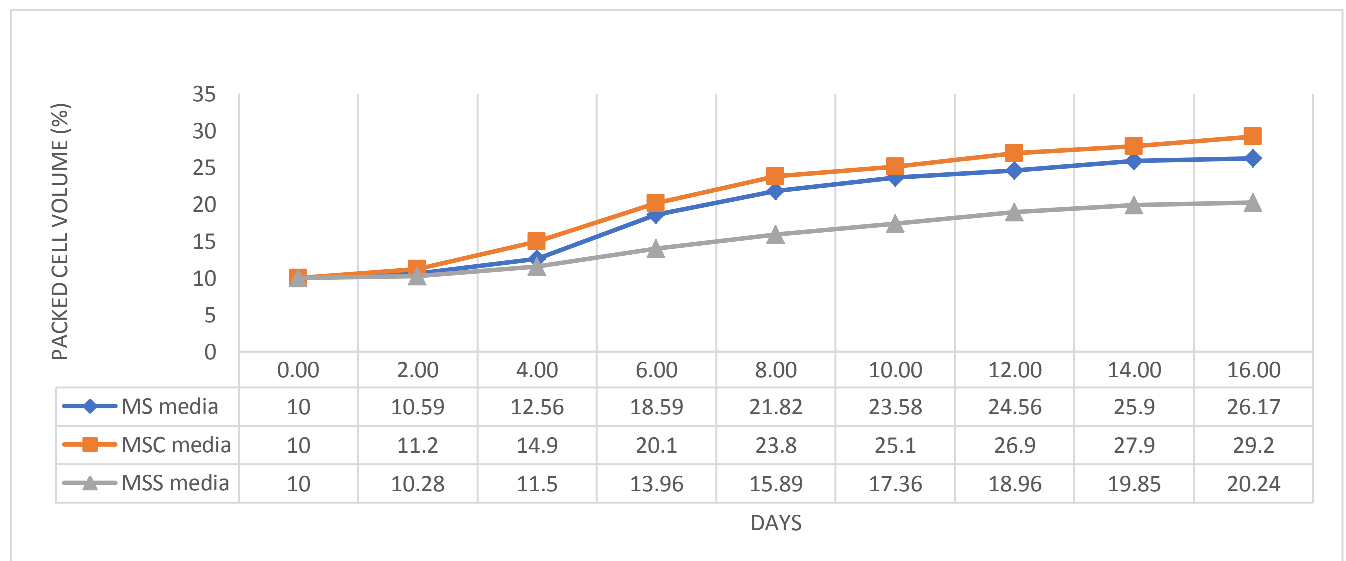

Fig: 3.13 – Graphical representation for growth of *D.carota* in suspension culture media with inoculum.

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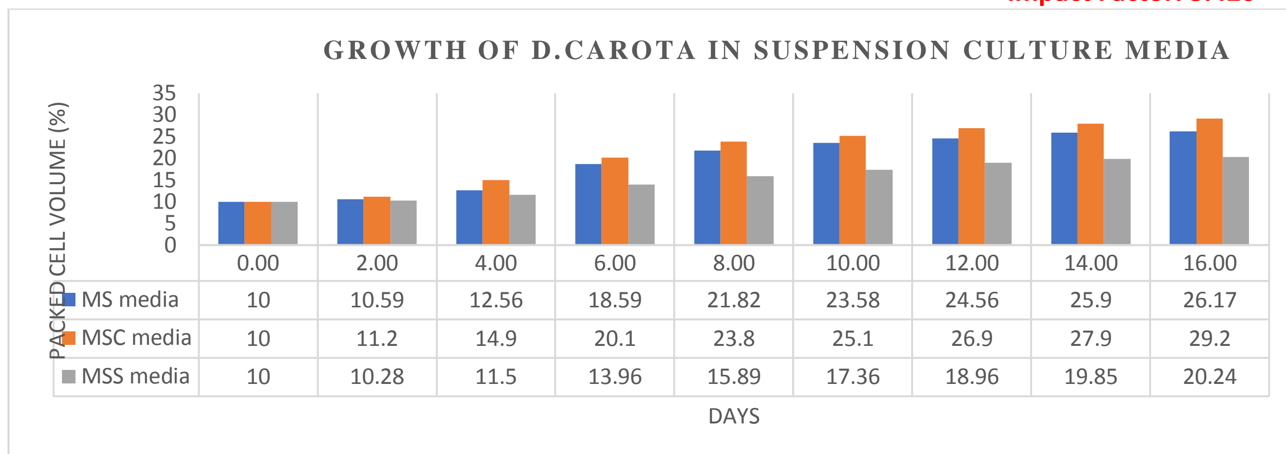

Fig 3.14- Bar diagram for growth of *D.carota* in suspension culture media with inoculum

S.No.	Fraction	No. of spot	Rf value of spot	Authentic Sample	Rf value of authentic sample	Solvent system	Detection
1	Ethyl acetate fraction	2	0.51 0.06	Benzoic acid P-hydroxy benzoic acid	0.51 0.06	Isobutanol: Ammonia: Water (7:1.5:3)	10% vaniline in alcohol + H_2SO_4 (2:1)
	Control	-	-	-	-		
2	Chloroform fraction	2	0.86 0.16	Chloroquine Hydroxychloroquine	0.86 0.16	Dichloromethane: n-hexene: Triethanolamine (8:2:1)	Iodine chamber
	Control	-	-	-	-		

Table 3.5 - TLC studies of different fraction of *D.carota*

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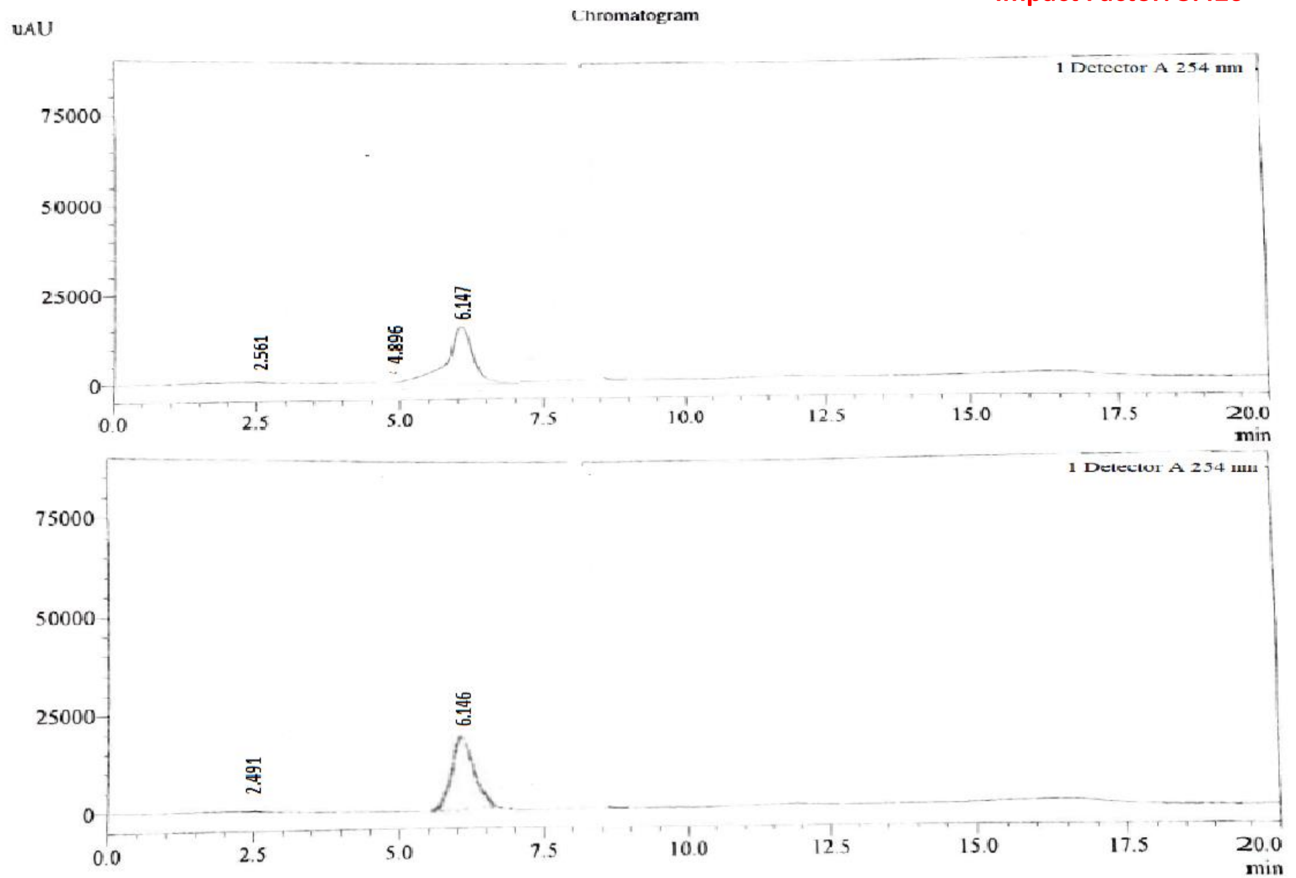

Fig 3.15 – HPLC profile of ethyl acetate fraction of *D.carota*

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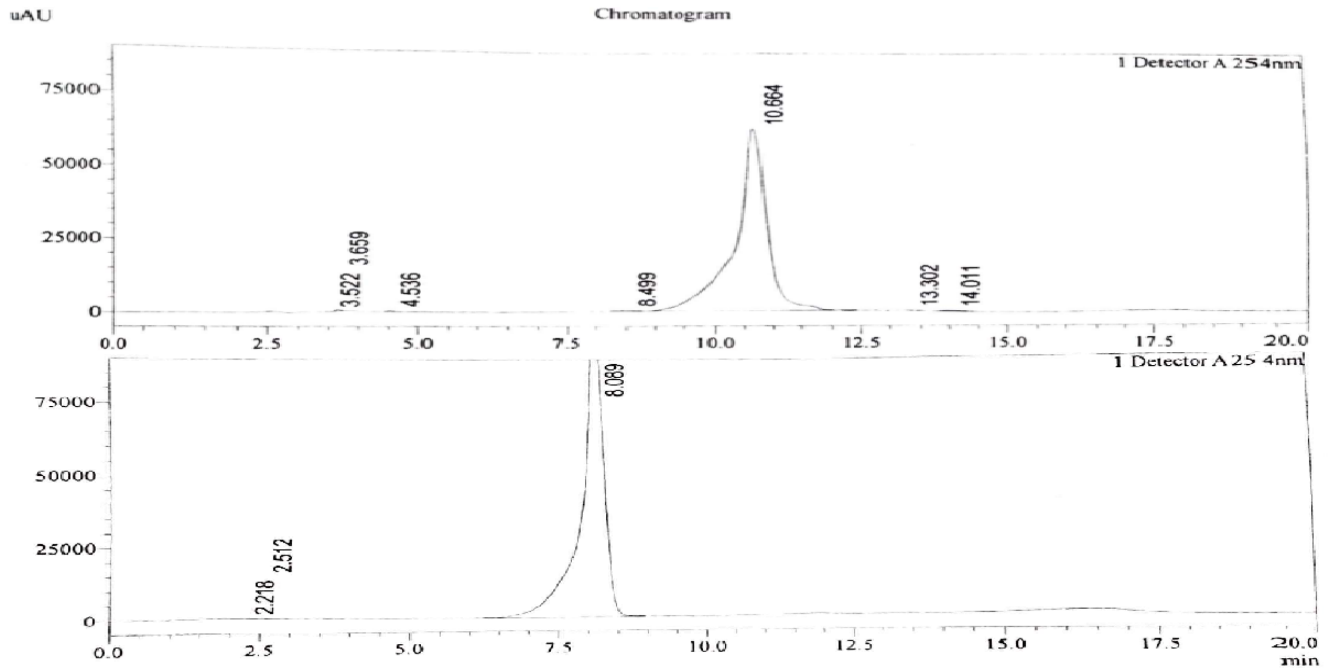

Fig 3.16 -HPLC profile of chloroform fraction of *D.carota*

S.No.	Conc. of Benzoic Acid (Per flask*)	Days →	Accumulation of p-Hydroxybenzoic acid (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	10,000 µg		0.0	1600	2100	3200	4100	5200	5700	6400	6900	7000	7100
	% yield		0.0	16%	21%	32%	41%	52%	57%	64%	69%	70%	70%
2.	15,000 µg		0.0	1800	2000	3600	4100	7100	9100	11200	11300	11500	11500
	% yield		0.0	12%	13.33%	24%	27.33%	47.33%	60.66%	74.66%	75%	76.66%	76.66%
3.	20,000 µg		0.0	2200	2500	4600	7500	8200	9600	10300	10400	10700	10700
	% yield		0.0	11%	12.5%	23%	37.5%	41%	48%	51.5%	52%	53.5%	53.5%

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4.	25,000 µg	0.0	2400	3500	4900	7900	8700	10100	10900	11400	11700	11900
	% yield	0.0	9.6%	14%	19.6%	31.6%	34.8%	40.4%	43.6%	45.6%	46.8%	47.6%

Table 3.6 - Total p-hydroxybenzoic acid accumulation in *D.carota* cell suspension culture at different concentration of benzoic acid

*Per flask contain 10 ml PC + 50 ml media

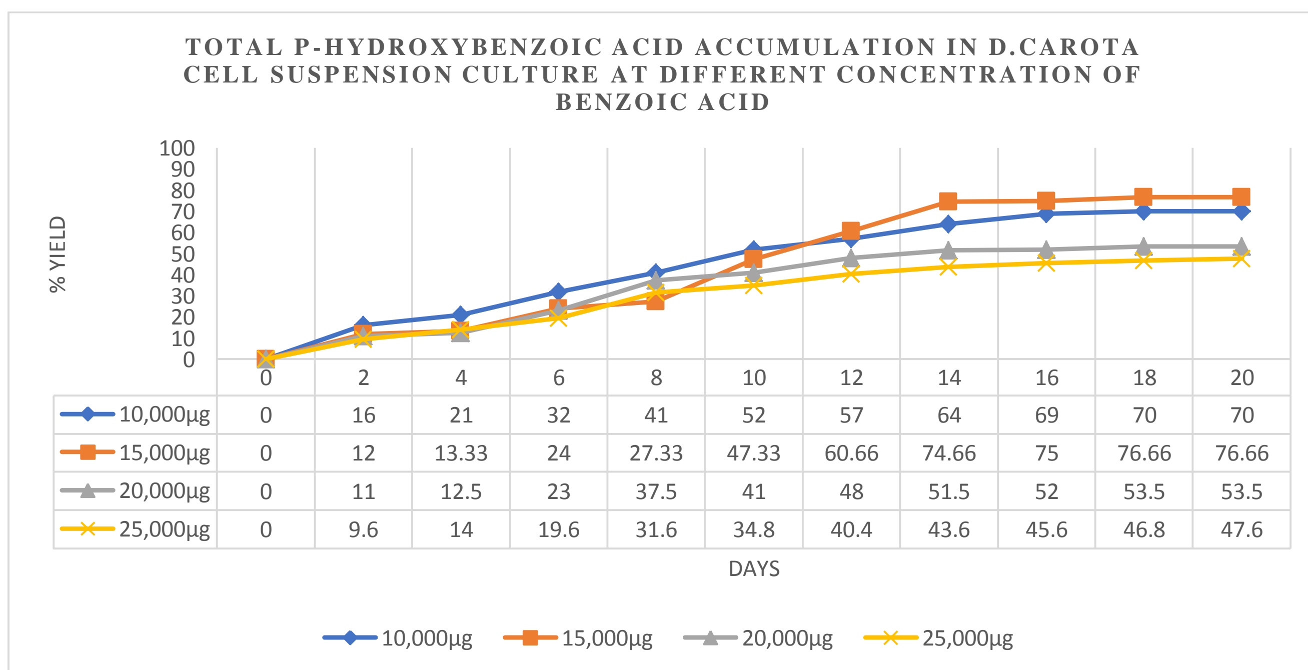

Fig 3.17 – Graphical representation for total p-hydroxybenzoic acid accumulation in *D.carota* cell suspension culture at different concentration of benzoic acid

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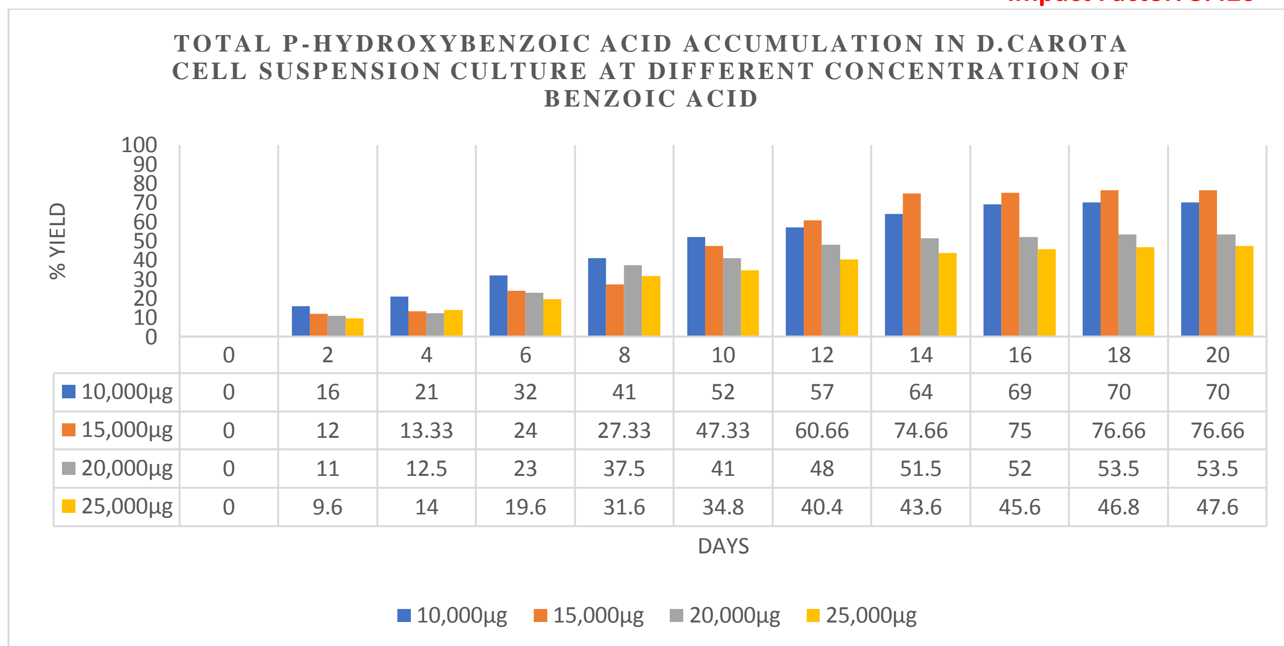

	0	2	4	6	8	10	12	14	16	18	20
10,000µg	0	16	21	32	41	52	57	64	69	70	70
15,000µg	0	12	13.33	24	27.33	47.33	60.66	74.66	75	76.66	76.66
20,000µg	0	11	12.5	23	37.5	41	48	51.5	52	53.5	53.5
25,000µg	0	9.6	14	19.6	31.6	34.8	40.4	43.6	45.6	46.8	47.6

Fig 3.18 – Bar diagram for total p-hydroxybenzoic acid accumulation in *D.carota* cell suspension culture at different concentration of benzoic acid

S.No.	Sample	Days →	Accumulation of p-Hydroxybenzoic acid (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Per flask contain 50 ml media

Table 3.7 - Accumulation of p-hydroxybenzoic acid into media of cell suspension culture of *D.carota*

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S.No.	Conc. of Chloroquine (Per flask*)	Days →	Accumulation of Hydroxychloroquine (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	10,000 µg		0.0	1200	1800	2400	2900	4200	4900	5200	5800	6100	6100
	% yield		0.0	12%	18%	24%	29%	42%	49%	52%	58%	61%	61%
2.	15,000 µg		0.0	1500	2100	3400	4200	4860	5300	6600	7200	8600	8600
	% yield		0.0	10%	14%	22.66%	28%	32.4%	35.33%	44%	48%	57.33%	57.33%
3.	20,000 µg		0.0	1600	2300	3600	4500	5100	5800	6800	7500	8800	8890
	% yield		0.0	8%	11.5%	18%	22.5%	25.5%	29%	34%	37.5%	44%	44.45%
4.	25,000 µg		0.0	1800	2500	3800	4680	5900	6900	7900	8600	9700	10700
	% yield		0.0	7.2%	10%	15.2%	18.72%	23.6%	27.6%	31.6%	34.4%	38.8%	42.8%

Table 3.8 - Accumulation of p-hydroxybenzoic acid in control samples of *D.carota*

S.No.	Conc. Of Benzoic Acid (Per flask*)	Days →	Accumulation of p-Hydroxybenzoic acid (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	10,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.	15,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3.	20,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4.	25,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3.9 - Total hydroxychloroquine accumulation in *D.carota* cell suspension culture at different concentration of chloroquine

*Per flask contain 10 ml PC + 50 ml media

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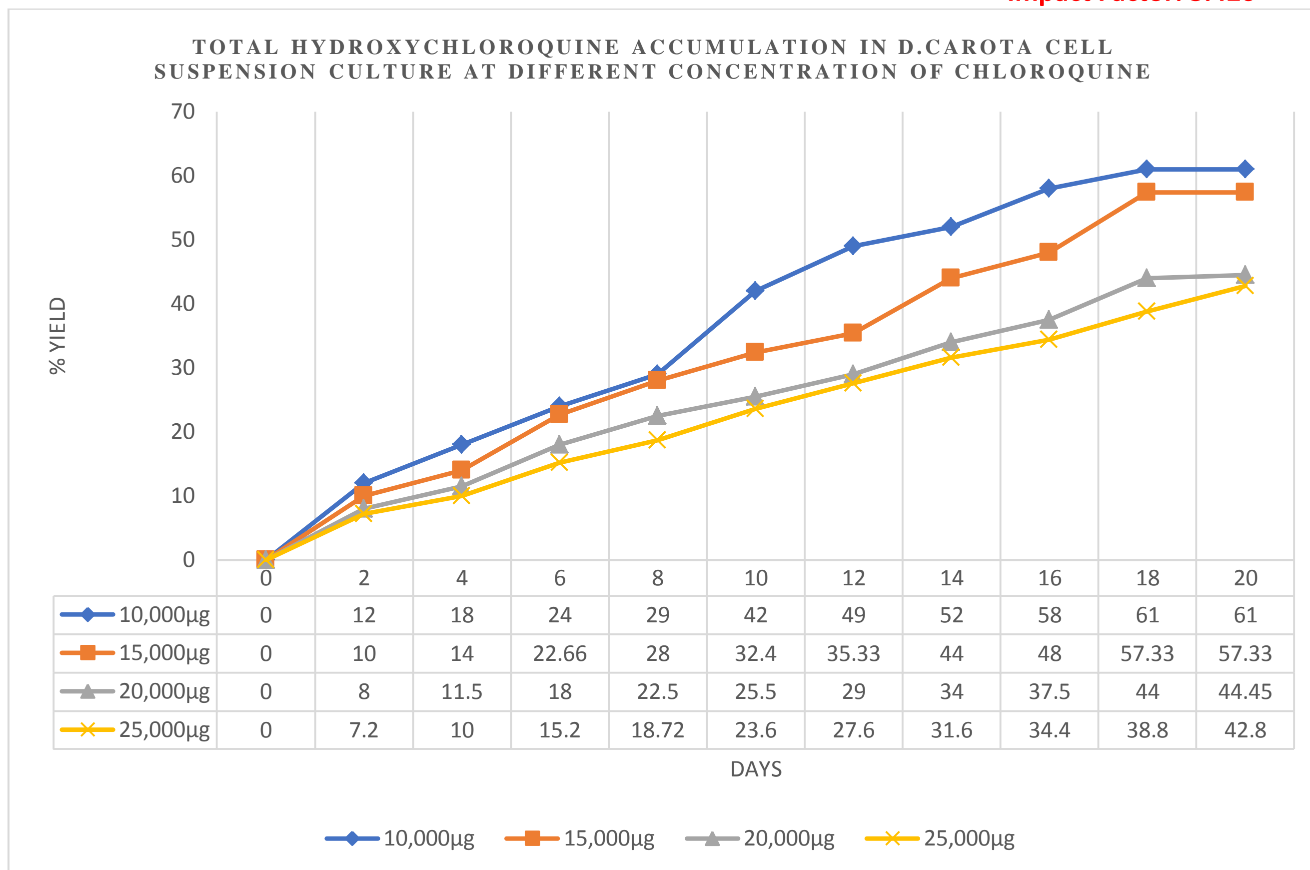

	0	2	4	6	8	10	12	14	16	18	20
10,000µg	0	12	18	24	29	42	49	52	58	61	61
15,000µg	0	10	14	22.66	28	32.4	35.33	44	48	57.33	57.33
20,000µg	0	8	11.5	18	22.5	25.5	29	34	37.5	44	44.45
25,000µg	0	7.2	10	15.2	18.72	23.6	27.6	31.6	34.4	38.8	42.8

Fig 3.19 – Graphical representration for total hydroxychloroquine accumulation in *D.carota* cell suspension culture at different concentration of chloroquine

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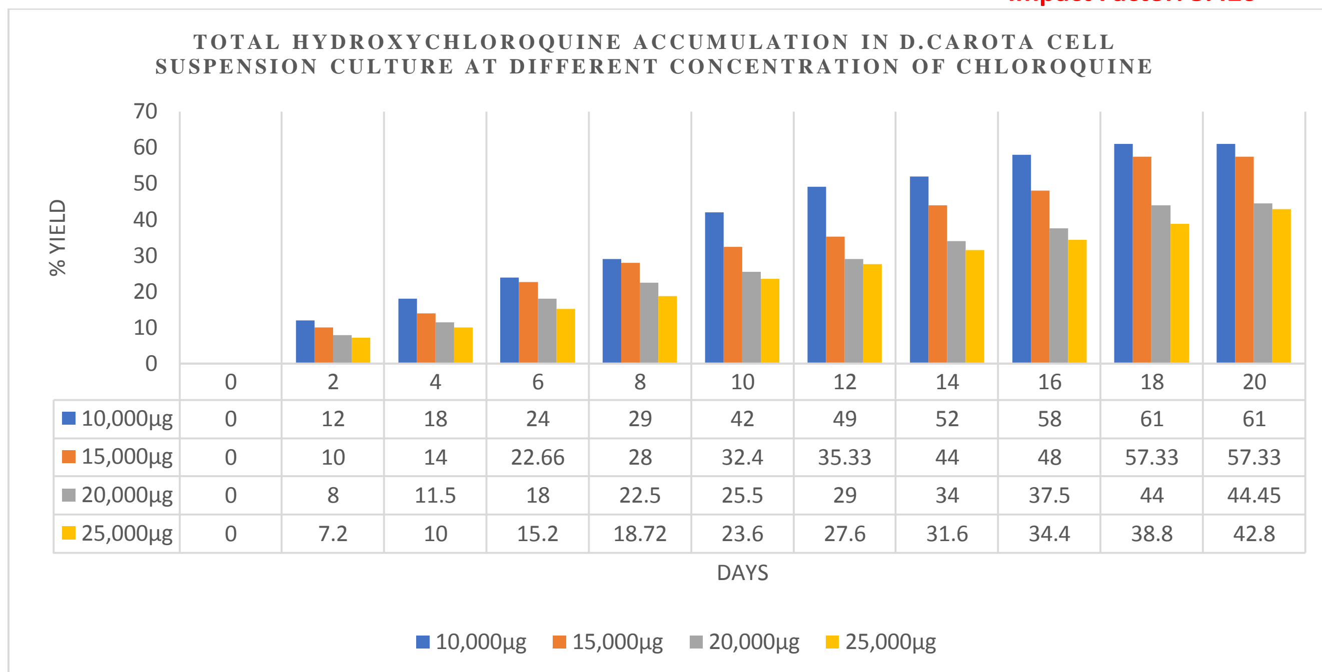

	0	2	4	6	8	10	12	14	16	18	20
10,000µg	0	12	18	24	29	42	49	52	58	61	61
15,000µg	0	10	14	22.66	28	32.4	35.33	44	48	57.33	57.33
20,000µg	0	8	11.5	18	22.5	25.5	29	34	37.5	44	44.45
25,000µg	0	7.2	10	15.2	18.72	23.6	27.6	31.6	34.4	38.8	42.8

Fig 3.20 – Bar diagram for total hydroxychloroquine accumulation in *D.carota* cell suspension culture at different concentration of chloroquine

S.No.	Conc. of Chloroquine (Per flask*)	Days →	Accumulation of Hydroxychloroquine (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	10,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.	15,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3.	20,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4.	25,000 µg		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Per flask contain 50 ml media

Table 3.10 - Accumulation of hydroxychloroquine into media of cell suspension culture of *D.carota*

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S.No.	Sample	Days	Accumulation of Hydroxychloroquine (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% yield		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3.11 - Accumulation of hydroxychloroquine in control samples of *D.carota*

S.No.	Conc. Of Benzoic Acid (Per flask*)	Days →	Percentage viability of *D.carota* cells (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	10,000 µg		90%	85%	85%	82%	88%	92%	94%	89%	91%	88%	90%
2.	15,000 µg		90%	85%	89%	84%	92%	90%	92%	87%	87%	89%	91%
3.	20,000 µg		90%	90%	85%	87%	90%	92%	91%	85%	89%	89%	87%
4.	25,000 µg		80%	80%	81%	85%	85%	81%	81%	82%	82%	90%	89%

*Per flask contain 10 ml PC + 50 ml media

Table 3.12 - Percentage viability of *D.carota* cells in suspension culture at different concentration of benzoic acid

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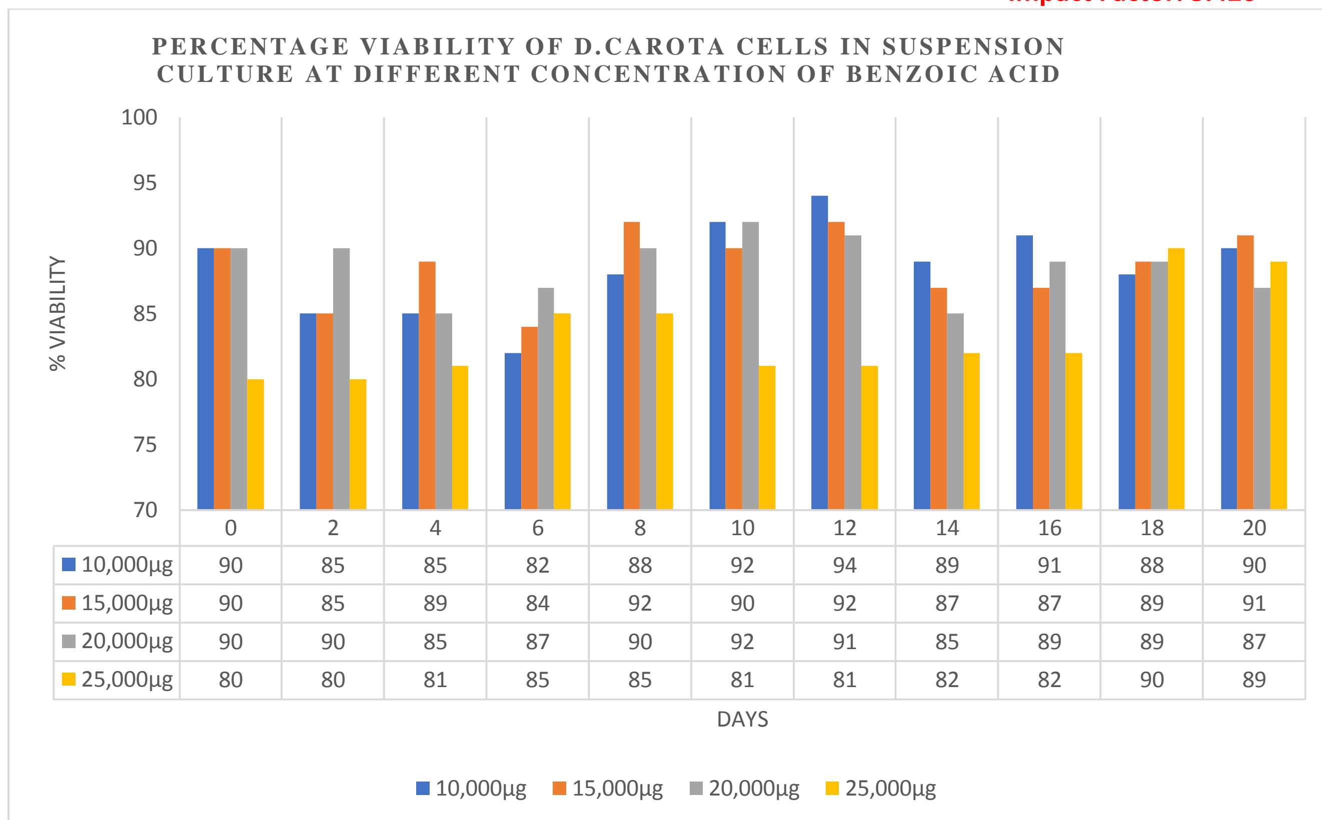

Fig 3.21 - Bar diagram for percentage viability of *D.carota* cells in suspension culture at different concentration of benzoic acid

S.No.	Conc. Of chloroquine (Per flask*)s	Days →	Percentage viability of *D.carota* cells (Per flask*)										
			0	2nd	4th	6th	8th	10th	12th	14th	16th	18th	20th
1.	10,000 µg		91%	85%	92%	91%	87%	89%	90%	92%	91%	93%	92%
2.	15,000 µg		90%	92%	91%	87%	89%	92%	91%	92%	90%	91%	90%
3.	20,000 µg		89%	90%	91%	92%	90%	87%	89%	88%	89%	89%	90%

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4.	25,000 µg	86%	86%	88%	90%	91%	90%	91%	90%	90%	89%	89%

Table 3.13 - Percentage viability of *D.carota* cells in suspension culture at different concentration of chloroquine

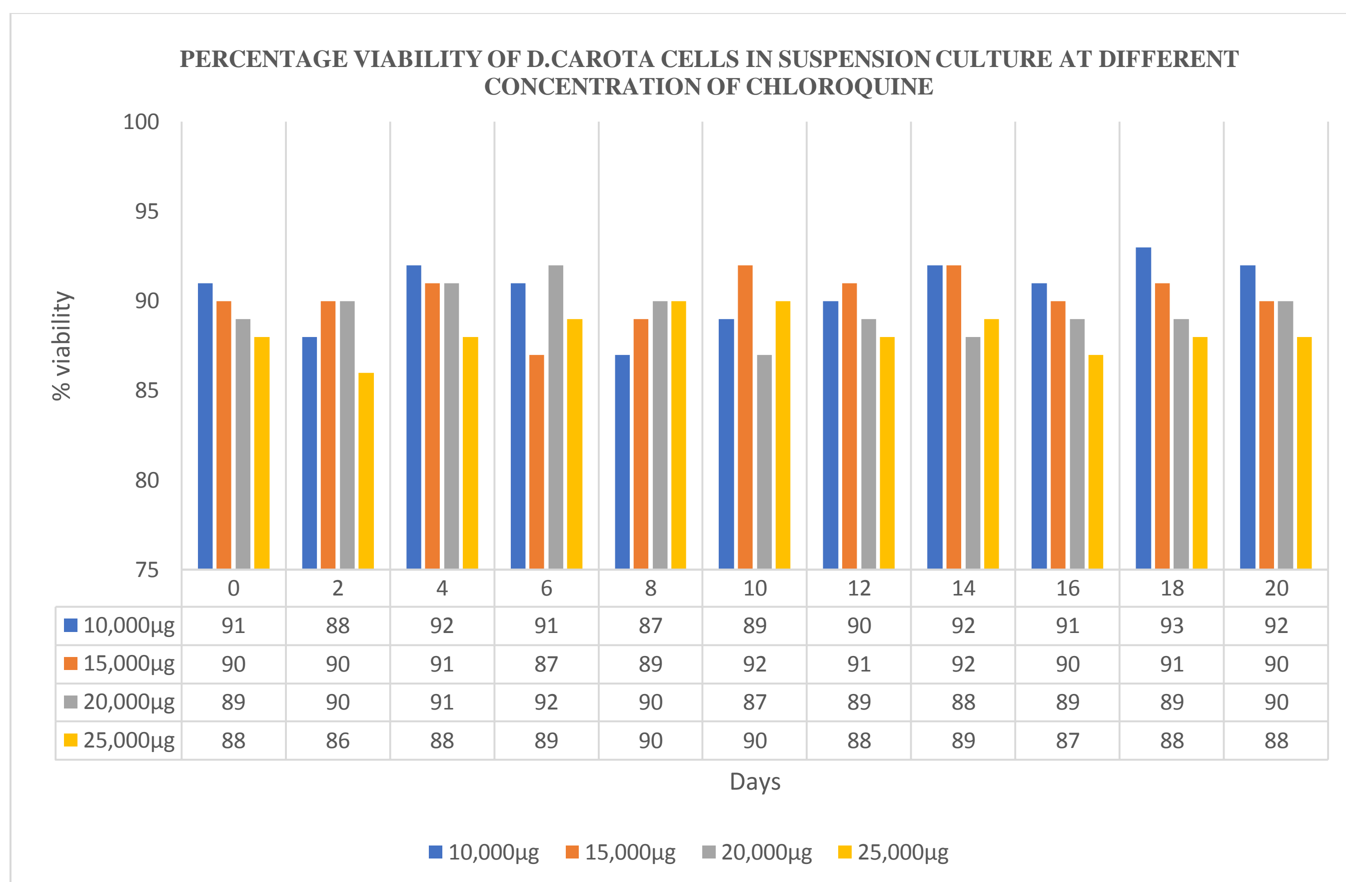

Fig 3.22 – Bar diagram for percentage viability of *D.carota* cells in suspension culture at different concentration of chloroquine.

4. Summary & Conclusion

Plant tissue culture technique play very significant role in the investigation on biosynthesis of biopharmaceuticals, plant metabolites etc. The present investigation based on the production of biosimilar drugs by the help of plant tissue culture technique. The present work was undertaken

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with a view to perform investigation on biosynthesis of some biosimilar drugs using various culture techniques.

The *D.carota* root were procure from the vegetable market of Indore region. The procured *D.carota* root was washed, dried, remove the epidermis and any blemish with scalpel. Then converted into explant and surface sterilized using 90% ethanol and 0.1% mercuric chloride solution. The sterilized explants were cultured on nutrient media. The MS media which is mostly used in plant tissue culture techniques was selected for present investigation. This media was consisting of all necessary component like macronutrient, micronutrient, vitamins and plant growth regulators necessary to prompt growth of cells and development of callus. The MS media was prepared by mixing of all components in prescribed quantity. Agar was used for static culture. The media was sterilized by autoclaving at 15 lb for 15 minutes. After sterilization culture vessels were kept for solidification of the medium at 25±2°C in aseptic room.

MS Medium was also modified with a view to rapid initiation of callus in *D.carota.* The different media were prepared such as modified MS media, MS media with coconut water or sugar solution. The sterilized explants were aseptically transferred into all above mentioned medium and culture vessels were covered by non-absorbent cotton plugs. After inoculation culture vessels were incubated in dark at 25 ±1 °C. After initiation of callus, the culture vessels were kept in light/dark cycle up to four weeks. The initiation of callus was visually observed under light. Different modification was performed on MS media for callus initiation. MS media with coconut water (74%) show maximum initiation of callus. Weekly subculturing of active callus was undertaken for growth study. Subculture vessels were incubated in light dark cycles. Growth was assessed on the basis of increase in fresh weight of callus.

The biomass production in *D.carota* was recorded 126.48 g/L, 129.80 g/L and 121.50 g/L with MS media, MS media with coconut water and MS media with sugar solution respectively. The MS media with coconut water have highest biomass yield. The effect of natural juice on biomass

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production was studied. The coconut water was obtained from coconut fruit. The juices were added to modified M-S media. The media formed by the help of coconut water was showed significant value in the initiation of callus. The suspension culture of *D.carota* was established to obtain uniform cell line. Callus obtained from static MS media used for establishment of suspension culture. The cells were grown in culture vessels (conical flasks) on a rotary shaker incubator (120 rpm at 25 ° ± 1° C). The growth of cells was studied by packed cell volume and viability of cell. For packed cell volume, ten ml of well mixed suspension was taken in graduated centrifuge tube and rotated at 180 rpm for 20 minutes. The volume of the cell settled on the bottom of the tube was recorded. For determining viability of cells, weighed 10 mg of fluorescein diacetate dye and dissolved into sufficient quantity of water. The volume was made-up to 100 ml. A sample of 1 ml cells was taken and stained with fluorescein diacetate (0.01%). The viable cells were stained and counted under microscope on haemocytometer.

The packed cell volume was recorded 13.5%, 14.5% and 10.2% in callus of MS media, MS media with coconut water and MS media with sugar solution with viability 89%, 91% and 85% respectively. The growth of *D.carota* cells was also studied with fix concentration of inoculum i.e. 10% packed cell volume. After 16th day of inoculation 26.17%, 29.20% and 23.20% packed cell volume was recorded in MS media, MS media with coconut water and MS media with sugar solution respectively. After the establishment of well-developed cell line, substrate solutions were fed into media in different concentration i.e. 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml. Benzoic acid and chloroquine were taken as substrates for investigation. The viability of the cells of culture was recorded with different concentration of substrates during the experiment.

The percentage viability of *D.carota* cell in suspension culture was recorded 90%, 91%, 87% and 89% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml of benzoic acid fed respectively. In chloroquine fed, the percentage viability of *D.carota* cell in suspension culture was recorded 92%, 90%, 90% and 89% with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and

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25,000 µg/ml respectively. The samples were analysed periodically and metabolites were identified by matching with known standards using thin layer chromatography and high-performance liquid chromatography. Thin layer chromatographic studies revealed that D. carota cell culture gave one metabolite each with benzoic acid and chloroquine. The metabolites formed were identified with authentic samples by Co-TLC which illustrates that D. carota transformed benzoic acid into p-hydroxybenzoic acid and chloroquine into hydroxychloroquine.

The identity of metabolites was further confirmed by HPLC. The ethyl acetate fraction and chloroform fraction of *D.carota* were subjected to HPLC. The identification and quantitative estimation of peaks was carried out with known standards of P-hydroxybenzoic acid and hydroxychloroquine. The peaks of metabolites of ethyl acetate was found identical with known standards of p-hydroxybenzoic acid and the peaks of metabolites of chloroform fraction was found very close with known standards of hydroxychloroquine, i.e. it was assumed that chloroquine converted into hydroxychloroquine. In free cell system, bioconversion was initiated after 2nd day of incubation. On 2nd day, 16%, 12%, 11% and 9.6% percentage yield were recorded with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml concentration of benzoic acid and 12%, 10%, 8% and 7.2% percentage yield were recorded with 10,000 µg/ml, 15,000 µg/ml, 20,000 µg/ml and 25,000 µg/ml concentration of chloroquine.

The maximum bioconversion of benzoic acid in p-hydroxybenzoic acid was 76.66% with 15,000 µg/ml concentration and chloroquine into hydroxychloroquine was 61% with 10,000 µg/ml concentration respectively. The studies performed with media of suspension culture and control sample were showed no accumulation of p-hydroxybenzoic acid and hydroxychloroquine.

Results clearly indicate that the experiment successfully optimized the ideal culture condition to induce callus and produce biomass of *D.carota*. *D.carota* cells were efficiently utilized for bioconversion of benzoic acid and chloroquine. Bioconversion of substrates were very quick as it started after 2nd day of incubation. The COOH group present in benzoic acid is metadirecting

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therefore hydroxylation at para position is highly specific which is otherwise difficult chemically. Chloroquine is derivative of 4-aminoquinoline, at tertiary amino, they bonded with two ethyl group. In bioconversion of chloroquine into hydroxychloroquine, a hydroxy moiety adds on one of the N-ethyl group of chloroquine and they converted into hydroxychloroquine.

The results clearly indicate that, the *D.carota* cells possess hydroxylation potential, which can be useful for the preparation of many products of pharmaceutical importance and this hydroxylation process only done in the presence of substrate. The high rate of bioconversion suggests the possibility of finding additional examples of culture substrate combinations with high bioconversion yield.

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