

## PHARMACEUTICALLY ACTIVE CELL BIOMASS GROWTH PATTERN UNDER CELL SUSPENSION CULTURE OF *COMMIPHORA WIGHTII* – A CRITICALLY ENDANGERED MEDICINAL PLANT

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**Abstract:** One of the medicinal treasures of Indian Ayurveda is *Commiphora wightii* which is being used for treating many diseases due to the presence of an oleo-gum-resin (guggul gum) that is having a number of secondary metabolites which are bioactive principles for a number of medicinally important ayurvedic preparations. Over-exploitation of the plant for this resin led it to the verge of extinction and need to develop an alternative way to produce this guggul gum has become imperative. The present study was aimed to understand the behaviour and growth pattern of cell suspension culture of *C. wightii*, which can foster the way to produce secondary metabolites from *in-vitro* cultures or secondary metabolite rich cell biomass. For this, callus was initiated from immature embryos collected from seed raised mature plants and tissue culture raised mature plants on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cell suspension culture was established for both the samples in Gamborg's B5 medium with 0.5 mg/l 2,4-D and hormone free medium. Study showed a comparable growth where good growth was observed in medium containing hormone than medium without hormone. In tissue culture raised plants highest biomass was observed on 27<sup>th</sup> day which is  $17.3149 \pm 0.71$  gms in hormone supplemented medium while in hormone free medium highest biomass was obtained on 27<sup>th</sup> day which is  $14.6219 \pm 1.28$  gms. Whereas in seed derived plants highest biomass produced in medium containing hormone was on 27<sup>th</sup> day that is  $14.9060 \pm 0.73$  gms while in hormone free medium highest biomass was obtained on 27<sup>th</sup> day that is  $11.2113 \pm 0.74$  gms.

**Keywords:** cell biomass, *Commiphora wightii*, fresh weight, growth kinetics, suspension culture.

### Introduction

*Commiphora wightii* (Arn.) Bhandari (Family Burseraceae) is an important and pharmaceutically valuable medicinal plant of arid and semi-arid regions of the Indian sub – continent [URIZAR & MOORE, 2003; DENG, 2007]. It is commonly known as 'Guggul' due to the presence of an oleo-gum-resin which is composed mainly of alcohols, steroids, diterpenes and sterols [VERMA & al. 1998] and the main active constituents are guggulsterone E and Z [EL ASHRY & al. 2003]. Many biochemical studies have been done on chemical composition of this exudate showing presence of guggulsterone I, II, III, V, Z, E and ctadecan-1,2,3,4-tetrol [PATIL & al. 1972, 1973], Identified guggulsterone E & Z, guggulsterone-I, myrrhanol-A and myrrhanone-A, guggulsterone-M, dihydro guggulsterone-M, guggulsterol-Y [MESELHY, 2003], Presence of essential oils, myrecene, dimyrcerene and Polymyrcerene [JAIN & GUPTA, 2006], Presence of Quinic acid, Citric acid myo-inositol and Glycin in leaves, stem and resin through metabolite profiling [BHATIA & al. 2018].

## **PHARMACEUTICALLY ACTIVE CELL BIOMASS GROWTH PATTERN UNDER CELL ...**

Due to the presence of these diverse groups of compound *C. wightii* is used for therapeutic purpose. It is used in treating rheumatoidism and arteriosclerosis [GUJRAL & al. 1960; SATYAVATI & al. 1969]. The active constituents of this gum are used for treatment of hyper cholesterolemia, ulcers, obesity etc. [SATYAVATI, 1990], for the treatment of neurological disorders, hypertension and asthma [MAHESHWARI, 2010] and to prevent the progression of cancerous cells [SHISHODIA & al. 2007]. Antioxidant and cytotoxic activity from ethyl acetate extract of *C. wightii* under *in-vitro* condition has also been reported [ZHU & al. 2001]. It has also identified as weight loss agent [KIMURA & al. 2001] and cholesterol lowering agent [JAIN & GUPTA, 2006].

The natural population of this plant is declining because of relentless harvesting of resin from wild plants through tapping by local people for economic benefits. Other reasons of its declining population are slow growth and poor germination rate [YADAV & al. 1999] due to which it has been listed as critically endangered species by IUCN [VED & al. 2015, e.T31231A50131117]. Other than conventional breeding, biotechnological aspects have been explored by many researchers to conserve this valuable plant. Micropropagation has been achieved for *C. wightii* using different explants like nodal segments [BARVE & MEHTA, 1993; SONI, 2010; PARMAR & KANT, 2012], shoot tips, nodes, internodes and leaves [SINGH & al. 2010], seedling explants [YUSUF & al. 1999; KANT & al. 2010] and apical and axillary meristem [BHARDWAJ & ALIA, 2019]. Somatic embryogenesis in *C. wightii* was achieved by repetitive reciprocal transfer of callus between MS basal medium and MS supplemented with plant growth regulators [KUMAR & al. 2003]. Studies on *in vitro* guggulsterone production have been done for the production of these compounds without destructing the natural population of *C. wightii*. Production of guggulsterone in callus culture was reported that has been induced from different explants like leaves, zygotic embryos and stem [MATHUR & al. 2007a]. Guggulsterone production has also been reported in shake flask and bioreactors [MATHUR & al. 2007b]. Enhanced guggulsterone production in cultures was observed in the presence of Morphactin and 2iP [TANWAR & al. 2007], Mesquite Gum and Gum Arabic [DASS & RAMAWAT, 2009] and growth retardants with fungal elicitors [SUTHAR & RAMAWAT, 2010].

Production of secondary metabolites in suspension culture is also affected by the growth of cells in liquid medium and therefore it is necessary to understand the growth pattern of cells in culture conditions. Growth kinetics of cells in liquid medium has been studied on many plant species through Packed Cell Volume in *Phoenix dactylifera* L. suspension culture [AL-KHAYRI, 2012], Fresh weight and cell viability in *Scrophularia striata* Boiss. suspension culture [ARDESTANI & al. 2015] and Settled Cell Volume in *Sorghum bicolor* suspension culture [RAMULIFHO & al. 2019]. So, the present study was conducted to analyse the growth pattern of *C. wightii* cell suspension culture which can pave the path for obtaining healthy cell biomass and for enhancement of guggulsterone production.

## **Materials and methods**

### **Plant material**

Immature fruits were collected from healthy seed-derived plants of *C. wightii* growing at AFRI nursery and from tissue culture raised plants growing at AFRI TC field trial, Jodhpur (grown *in vitro* as in KANT & al. 2010).

### **Explant preparation**

Immature fruits were soaked in water. The floating fruits were discarded. Only the settled fruits were used as explant source. These were washed with tween 20 followed by bavestain and streptomycin (Himedia) treatment. Finally fruits were surface sterilized with 5% NaOCl (Sigma-Aldrich). Embryos were scooped out from immature fruits for inoculation.

### **Callus induction and establishment**

Embryos were inoculated on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D (Sigma-Aldrich) as reported best concentration for callus induction earlier by our team [PARMAR & KANT, 2014]. Establishment of callus was done for both the source mother plants separately by regular sub-culturing after every 4 week on same medium. Further, callus was inoculated on semi solid Gamborg's B5 medium without any hormone for induction of embryogenesis [PARMAR & KANT, 2014].

### **Establishment of cell suspension cultures**

*C. wightii* suspension cultures were initiated by inoculating non embryogenic callus (~1 g per flask) in 100-ml Erlenmeyer flasks containing 30 ml liquid medium. The medium consist of same basal salt concentrations of Gamborg's B5 medium which was used for callus induction but without agar. The cultures were initiated for both the source plants in Gamborg's B5 medium Supplemented with 0.5 mg/l 2,4-D and Gamborg's B5 medium without any hormone. The suspension cultures were incubated on a rotatory shaker (Adolf Kuhner AG LSX SMX 1200) at 120 rpm under 16-hr photoperiod in white fluorescent light and at  $25 \pm 2$  °C.

### **Suspension growth pattern analysis**

Cell suspension Culture's growth pattern analysis was done by fresh weight and dry weight methods. Cells were collected from flasks every 3<sup>rd</sup> day after inoculation on a pre-weighed Whatmann filter paper disc by filtering the suspension culture. Total weight of the filter paper and cells were determined and then weight of the filter paper was subtracted to obtain the fresh weight (FW) of cells. Filter paper containing the cells was placed in an oven at 60 °C for 24 hrs and weighed at regular intervals until the weight remains constant. Dry weight (DW) was obtained by subtracting weight of paper with dried cells from pre-weighed paper's weight.

### **Determination of cell viability**

Viability of cells in suspension culture was checked by Evan's blue staining method. Cells were taken from suspension culture every 3<sup>rd</sup> day after inoculation and stained with 0.4% Evan's blue dye (Himedia). Stained cells were observed under microscope (Nikon Optiphot - 2) at 20x magnification.

## **Results**

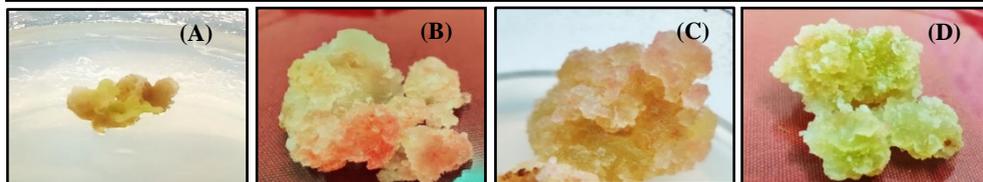
### **Establishment of callus culture**

Callus was initiated from immature embryos for both the source plants and per cent callusing was recorded (Table 1). Tissue culture raised plant's explant showed a little higher percentage than seed derived plant's explant. The non – embryogenic callus converted to embryogenic callus when cultured on Gamborg's B5 medium without any hormone (Figure 1). This showed the progressive growth of embryogenic callus on semi solid medium which can also be induced and established in suspension culture for production for guggulsterones.

**PHARMACEUTICALLY ACTIVE CELL BIOMASS GROWTH PATTERN UNDER CELL ...**

**Table 1.** Initiation of callus on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D.

S.No.	Mother plant	% Callusing	Callus colour and texture
1	Tissue culture raised plants	66.66	Pinkish white, fragile
2	Seed derived plants	58.33	Pinkish white and light brown, fragile



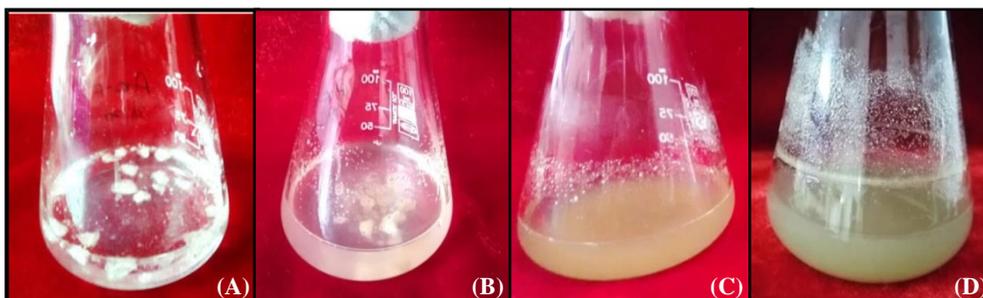
**Figure 1.** Conversion of non - embryogenic callus into embryogenic callus. (A) Induction of callus on Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D. (B) Multiplication of callus on same medium. (C) Non-embryogenic callus converting into embryogenic callus on Gamborg's B5 medium without any hormone. (D) Embryogenic callus.

**Establishment of cell suspension culture**

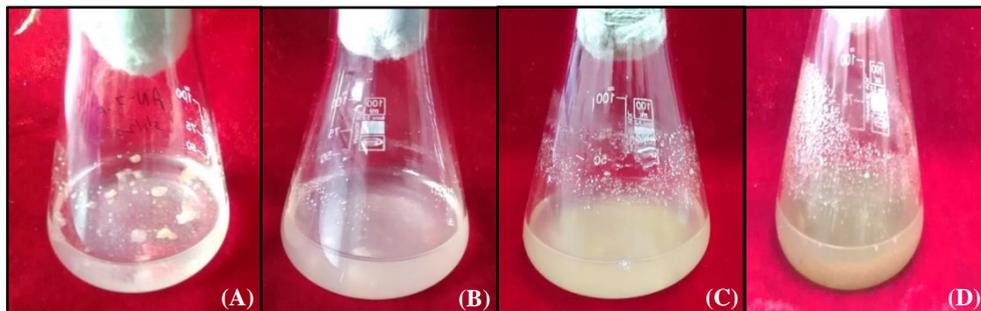
Cultures initiated from tissue culture raised plant's explant showed comparatively good growth in medium containing hormone 2,4-D (Figure 2) as compared to cultures in medium without hormone (Figure 3) because 2,4-D is responsible for higher rate of cell division. Similar pattern was observed in cultures initiated from seed derived plant's explant (Figures 4, 5). While comparing the two mother plants, tissue culture raised plants gave better response as compare to seed derived plants in terms of having dense biomass which is clearly seen in the flasks.



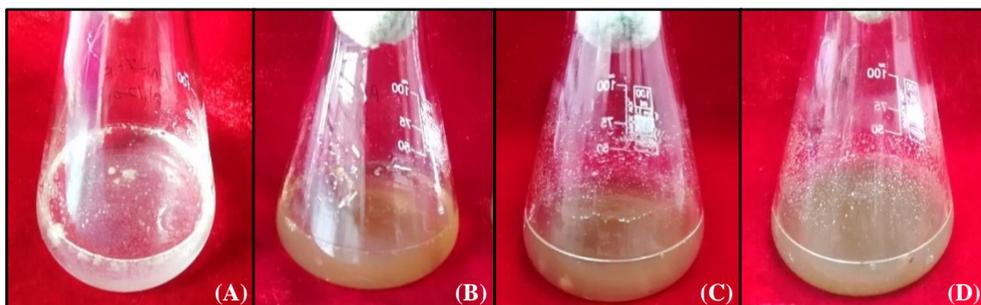
**Figure 2.** Tissue culture raised plants: Growth pattern of culture suspension in shake flask containing medium with hormone 2,4-D (A-D) A. 1<sup>st</sup> Day, B. 10<sup>th</sup> Day, C. 20<sup>th</sup> Day, D. 30<sup>th</sup> Day.



**Figure 3.** Tissue culture raised plants: Growth pattern of suspension culture in shake flask containing medium without any hormone (A-D) A. 1<sup>st</sup> day, B. 10<sup>th</sup> day, C. 20<sup>th</sup> day, D. 30<sup>th</sup> day.



**Figure 4.** Seed derived plants: Growth pattern of suspension culture in shake flask containing medium with hormone (A-D) A. 1<sup>st</sup> day, B. 10<sup>th</sup> day, C. 20<sup>th</sup> day, D. 30<sup>th</sup> day.



**Figure 5.** Seed derived plants: Growth pattern of suspension culture in shake flask containing medium without any hormone (A-D) A. 1<sup>st</sup> day, B. 10<sup>th</sup> day, C. 20<sup>th</sup> day, D. 30<sup>th</sup> day.

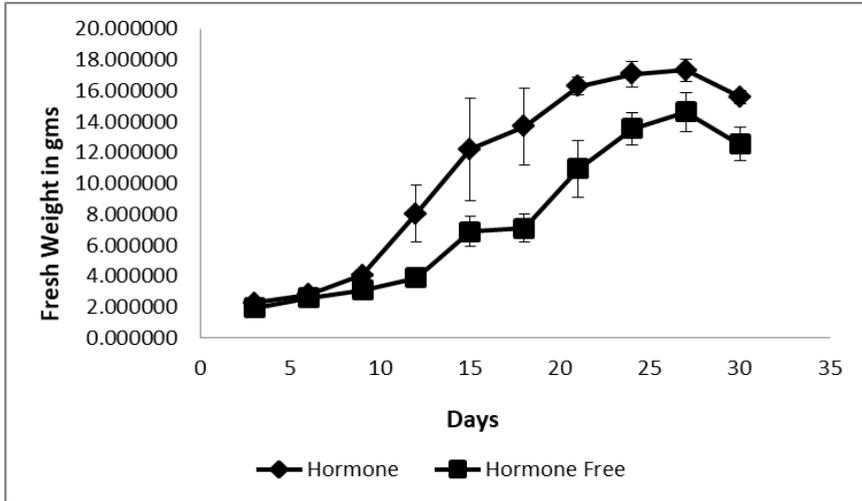
### Growth pattern analysis of suspension culture

Tissue culture raised plants: Fresh weight analysis showed that growth of suspension culture in medium containing hormone grew with a short lag phase till day 9 that is the adaptive phase of culture to the new environment. This was followed with exponential growth till 21<sup>st</sup> day and after that stationary phase was achieved that finally culminated in the death phase marked by apoptosis due to nutrient exhaustion and cellular competition. Similarly suspension culture in medium without hormone showed quite similar growth pattern (Figure 6). Biomass produced was highest in medium containing hormone on 27<sup>th</sup> day which is  $17.3149 \pm 0.71$  gms while in hormone free medium highest biomass was obtained on 27<sup>th</sup> day which is  $14.6219 \pm 1.28$  gms. While comparing the dry weight, results was slightly different with highest dry mass on 18<sup>th</sup> day in cultures with hormone that is  $0.4462 \pm 0.00$  gms and on 24<sup>th</sup> day in cultures without any hormone that is  $0.4336 \pm 0.01$  gms. Growth pattern is shown for both the cultures in Figure 7.

Seed derived plants: Fresh weight analysis of these cultures exhibit lag phase till 12<sup>th</sup> day in medium containing hormone (2,4-D) and then grew exponentially till 27<sup>th</sup> day after that stationary phase was achieved leading to death phase. Similarly suspension culture in medium without hormone showed quite similar growth pattern with a gradual increase in growth with time (Figure 8). Biomass produced was highest in medium containing hormone on 27<sup>th</sup> day that is  $14.9060 \pm 0.73$  gms while in hormone free medium highest biomass was obtained on 27<sup>th</sup> day that is  $11.2113 \pm 0.74$  gms. Dry weight analysis

**PHARMACEUTICALLY ACTIVE CELL BIOMASS GROWTH PATTERN UNDER CELL ...**

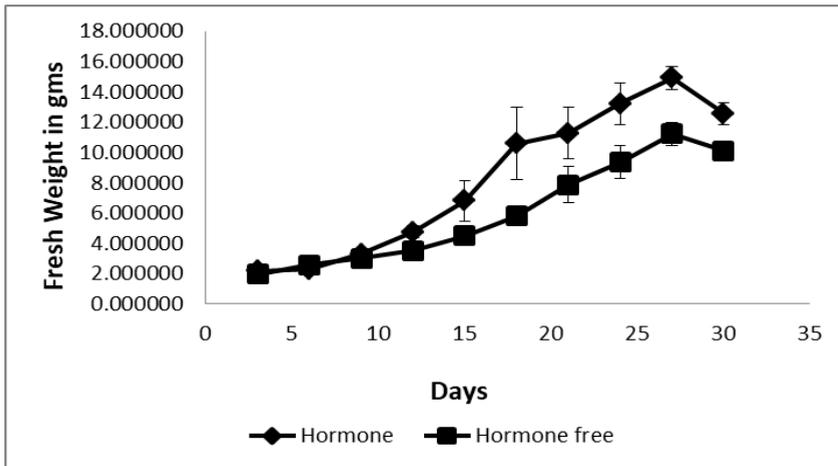
showed similar results as fresh weight method having highest dry mass on 27<sup>th</sup> day in medium with hormone that is  $0.4325 \pm 0.01$  gms and in medium without hormone highest dry mass was obtained on 27<sup>th</sup> day that is  $0.3899 \pm 0.02$  gms. Growth pattern is shown for both the cultures in Figure 9.



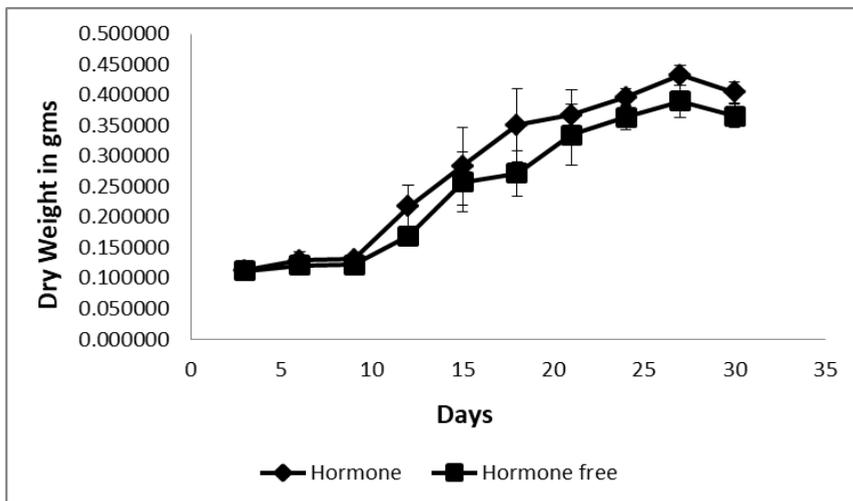
**Figure 6.** Growth pattern of culture initiated from tissue culture raised plant's explant in media containing hormone and without hormone by fresh weight method.



**Figure 7.** Growth pattern of culture initiated from tissue culture raised plant's explant in media containing hormone and without hormone by dry weight method.



**Figure 8.** Growth pattern of culture initiated from seed derived plant's explant in media containing hormone and without hormone by fresh weight method.

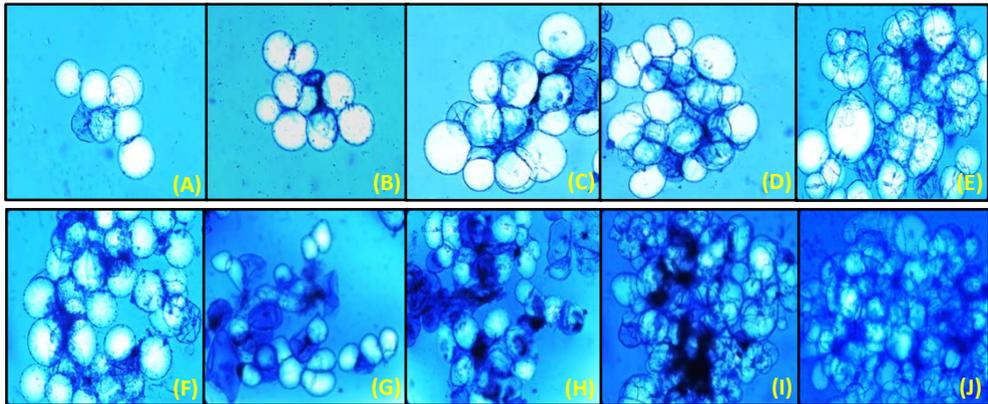


**Figure 9.** Growth pattern of culture initiated from seed derived plant's explant in media containing hormone and without hormone by dry weight method.

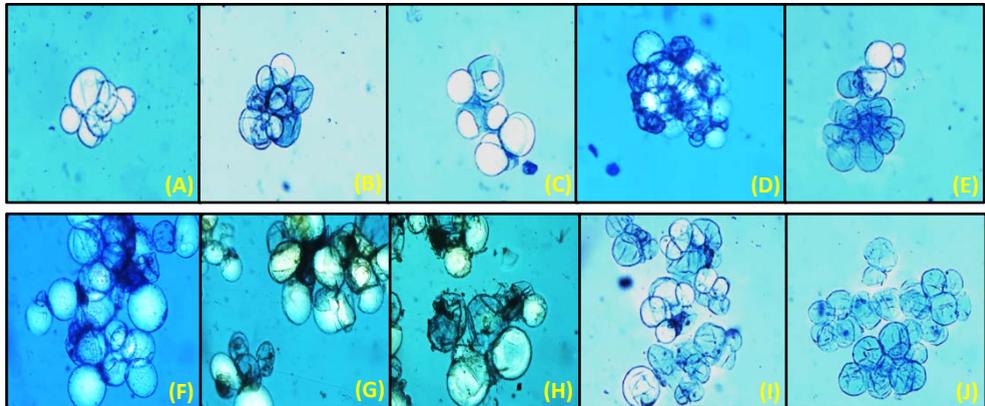
#### Determination of cell viability

Evan's blue staining revealed the viability of cells in suspension cultures of seed derived plant. In medium containing hormone a good growth was clearly seen in the culture along with more live cells in exponential phase which then decreased after the initiation of stationary phase (Figure 10). While in medium without any hormone showed slow growth along with less number of cells as compared to medium with hormone (Figure 11).

## PHARMACEUTICALLY ACTIVE CELL BIOMASS GROWTH PATTERN UNDER CELL ...



**Figure 10.** Evan's blue staining of suspension culture in Gamborg's B5 medium containing hormone, that are initiated from seed derived plant's callus showing live and dead cells with the time. (A) 3<sup>rd</sup> day (B) 6<sup>th</sup> day (C) 9<sup>th</sup> day (D) 12<sup>th</sup> day (E) 15<sup>th</sup> day (F) 18<sup>th</sup> day (G) 21<sup>st</sup> day (H) 24<sup>th</sup> day (I) 27<sup>th</sup> day (J) 30<sup>th</sup> day.



**Figure 11.** Evan's blue staining of suspension culture in Gamborg's B5 medium without any hormone, that are initiated from seed derived plant's callus showing live and dead cells by the time. (A) 3<sup>rd</sup> day (B) 6<sup>th</sup> day (C) 9<sup>th</sup> day (D) 12<sup>th</sup> day (E) 15<sup>th</sup> day (F) 18<sup>th</sup> day (G) 21<sup>st</sup> day (H) 24<sup>th</sup> day (I) 27<sup>th</sup> day (J) 30<sup>th</sup> day.

### Discussion

In this study, we aimed to understand the growth pattern and behaviour of cells in suspension culture of *Commiphora wightii*. Plant cell growth in suspension culture can be determined and assessed by different methods like settled cell volume/packed cell volume, fresh weight and/or dry weight analysis, cell count/cell number [EVANS & al. 2003]. Here we have used fresh weight and dry weight method to make a growth curve to understand different phases of cell growth over a period of time. A typical growth curve is a sigmoidal curve having distinct phases like the lag phase (Adaptive phase), the exponential phase (Growth phase) and the stationary phase [GEORGE & al. 2008]. This study was done on two different mother plants and their growth was recorded in hormone (0.5 mg/l 2,4-D) and hormone free medium. In tissue culture raised plants suspension culture showed a lag phase

till 9<sup>th</sup> day followed with exponential phase till 21<sup>st</sup> day and then growth became steady with stationary phase in medium containing hormone. Whereas in medium without any hormone similar pattern has been seen but with less biomass produced. In seed raised plants suspension culture showed a different pattern with lag phase till 12<sup>th</sup> day followed by exponential phase till 27<sup>th</sup> day and then stationary phase was achieved in medium containing hormone. While in hormone free medium a gradual increase has been seen in growth of culture. After analysing the growth curve it can be suggested that sub - culturing of culture into fresh medium can be done in between 21<sup>st</sup> day to 27<sup>th</sup> day that is the end of exponential phase, which has been mentioned earlier in studies [STAFFORD & WARREN, 1991]. For maintaining a fine cell suspension culture, it is necessary to sub - culture at regular interval else the medium became exhaust or cells may produce toxic substances [BHOJWANI & RAZDAN, 1983]. In this study, it may be interesting to note that the growth of cell biomass in hormone free medium as quite comparable to that in case of hormone supplemented medium. KUMAR & al. (2004), reported the formation of resin canals which are the main source of guggulsterone synthesis in nature, at torpedo and cotyledonary stage of somatic embryos. So converting non embryogenic callus into differentiated embryogenic callus would have more amounts of guggulsterones. As we showed in figure 1, callus converted into embryogenic green callus when cultured on hormone free medium. Similarly culturing in hormone free suspension medium would also be useful in producing more amounts of guggulsterones *in vitro*. Thus going to hormone free medium is better when the targeted end product is a nutraceutical product.

### Conclusions

The cell growth under suspension state is higher in hormone supplemented medium (0.5 mg/l 2,4-D in Gamborg's B5 medium) compared to the hormone free B5 medium. Evan's blue staining also indicates higher number of living cells in hormone supplemented medium when compared to hormone free medium. This can possibly be due to a faster cell division rate in presence of hormone (2,4-D). However, the cell growth was clearly observed even without 2,4-D in hormone free medium. It was also observed that the growth rate peaks early in hormone supplemented medium compared to hormone free medium in general. Interestingly it was also observed that tissue culture raised explant material was marginally better as culture starter compared to mother plants growing in nature. The study clearly indicates that cell biomass bulked up without any hormone under suspension state is a viable way to produce cell biomass for production of guggul nutraceuticals in future. Work on augmenting secondary metabolite production in these cells holds the key on which work is under progress.

### Notes on contributors

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