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PRODUCTION OF STIGMASTEROL AND HECOGENIN FROM *IN VITRO* CULTURES OF *Chlorophytum borivilianum*

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Abstract- *Chlorophytum borivilianum* belonging to the family Liliaceae is commonly called safed musli. It is a perennial rhizomatous herb widely distributed in the pan tropical regions which contains pharmaceutically important saponins. Among the saponins, stigmasterol and hecogenin are considered as the major secondary metabolites and are responsible for its various biological activities viz. aphrodisiac, antioxidant, anticancer and immune booster. Present study reports production of medicinally important phytopharmaceuticals from *in vitro* cultures of *Chlorophytum borivilianum*. Leaf sheath from *in vitro* raised plants was used as explants for induction of callus on MS basal media fortified with 1mg/l of 2,4-D. Somatic embryogenesis and plant regeneration was observed from callus on MS basal media. Production of pharmaceutically important saponins stigmasterol and hecogenin was studied in callus and *in vitro* regenerated plants of *C.borivilianum*. Maximum stigmasterol production (3.265 mg/gm dry weight) was recorded from plants regenerated from somatic embryos which is 5.4 fold higher than the amount of stigmasterol in undifferentiated callus cultures (0.6 mg/gm). Maximum hecogenin production (43.55 mg/g) was recorded in plants regenerated from somatic embryos which is 27.9 fold higher than the amount of hecogenin in callus cultures (1.56 mg/gm)
Keywords- *Chlorophytum borivilianum* (Safed musli), Stigmasterol, Hecogenin, callus, Somatic embryogenesis, Quantification.

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Introduction

Nutraceutical industry in India is at a blooming stage [1,9]. The world health organization has estimated that more than 80% of the world population in developing countries depends primarily on herbal medicines for basic healthcare needs [2]. *Chlorophytum* is considered as the major herbal plant which has high commercial importance, thirteen species of *Chlorophytum* have been reported from India [2,6,10], among which *C. borivilianum* is having the highest saponin content. *C. borivilianum,* commonly known as Safed musli, belongs to family liliaceae. In India *C. borivilianum* is mainly distributed in Southern Rajasthan, North Gujarat and Western Madhya Pradesh. Major biochemical constituents of Safed musli are Saponins, among which stigmasterol and hecogenin are two major pharmacologically important saponins. *C. borivilianum* has therapeutic applications in ayurvedic system of medicine [6,9]. Fasciculate roots of *C. borivilianum* are used as tonic and constitute important ingredient of many ayurvedic and unani preparations. Roots are used for the preparation of nutritional tonic used in general sexual weakness and enhanced antioxidant property. Further, plant cell/organ cultures are restricted/altered by the environmental, ecological and climatic conditions hence cells can proliferate at higher growth rates in comparison to the conventional methods. The ability to produce morphologically and developmentally normal embryos and whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis are the potential models for studying early events in plant embryo development. Somatic embryos are induced from *in vitro* grown callus cultures by a relatively simple manipulation of the culture conditions. In this technique limited initial explants and space for multiplication was used through phenomenon based on totipotency concept. Therefore this research work was undertaken to study the secondary metabolite

production under *in vitro* condition in *C. borivilianum.*

Materials and methods

Chemicals and Reagents- MS media, Indole-3-Butyric acid, agar were from Himedia, India. Methanol of analytical/ HPLC grade, Acetic acid of HPLC grade were procured from Merck. Standard stigmasterol was supplied by Tokyo chemical industry Co. Ltd, Japan and hecogenin by MP Biochemicals, LLC, France.

Plant material- Plant material of *Chlorophytum borivilianum* was collected from Agro farms of Nandan Biomatrix Ltd., Hyderabad.

***In vitro* culture establishment-** Different explants (viz, leaf tip, leaf sheath, rhizome) were cleaned and surface sterilized with 0.1% mercuric chloride for 7 minutes and then rinsed with sterile distilled water thoroughly. The sterile explants were cultured onto Murashige and Skoog (MS) agar medium supplemented with different concentrations of 2, 4-D (0.5, 1.0, 1.5, 2.0 mg/l). The pH of this media was adjusted to 5.88 before autoclaving (121°C for 15 min at 15 lbs pressure). The cultures were incubated under controlled environmental conditions at 25±2° C with 16/8 hrs light/ dark regime. These cultures were subcultured at an interval of 28 days.

Study of different stages of regeneration from callus- Samples of callus, differentiated tissue and regenerated plant from callus were collected at different stages of development and the amount of stigmasterol and hecogenin was estimated.

Phytoconstituent extraction from *Chlorophytum borivilianum*- The dried tissue samples were ground and extracted in methanol using a soxhlet apparatus. The extract was filtered, dried and the dried powder was extracted with 1 ml of HPLC grade methanol. The samples were filtered using (0.22 µm) Millipore filters, followed by quantification. Two major saponins stigmasterol and hecogenin were quantified using HPLC.

Quantitative estimation of stigmasterol and hecogenin- HPLC analysis was carried out using the Shimadzu-LC-10AT VP series HPLC system equipped with a Supelco column (250x4.6 mm, C18, ODS with particle size of 5 µm) with a flow rate of 1ml/min. The mobile phase for stigmasterol was methanol, water and Acetic acid in the ratio of 70:30:1, stigmasterol was detected at 254 nm and hecogenin was detected at 210 nm using methanol and water in the ratio of 90:10.

Statistical analysis- The experiments were done in triplicate. Statistical analysis of data was carried out using ANOVA.

Result

Initiation of callus: Different explants viz: leaf, leaf sheath, rhizome were tried for callus initiation on MS media supplemented with different concentrations of 2,4-D (0.5---2.0 mg/l). Only leaf sheath explant responded for callus induction rest of the explants did not show any sign of dedifferentiation. Among different concentrations of 2,4D, 1 mg/l 2,4-D proved to be the best.

Fig. 1- Formation of callus from leaf sheath, explant on MS medium supplemented with 1mg/l 2,4-D.

Table 1-Response of different explants and 2,4 –D concentrations for callus induction.

Phytohormone	Concentration (mg/l)	Explants	% response
2,4 – D	0.5	--	Nil
	1	Leaf sheath	43.6 ± 0.45
	1.5	Leaf sheath	46.9 ± 0.30
	2	Leaf sheath	45.2 ±0.35

Fig. 2a-

Fig. 2b-

Somatic embryogenesis from callus on MS basal media devoid of 2,4- D.

The callus was further maintained on the same media for few subculture (4-5) passages. When transferred to media devoid of 2,4-D the callus gave rise to somatic embryos after 21 days of inoculation. These somatic embryos on further growth gave rise to plantlets on MS basal media.

Fig.3-

Fig.4-

Plant regeneration from somatic embryos on MS basal media devoid of 2,4-D.

Production of Stigmasterol at different stages of development in *Chlorophytum borivilianum*-

Stigmasterol a pharmaceutically important saponin of *Chlorophytum borivilianum* was estimated at different stages of differentiation from callus to regenerated plant. Maximum amount of stigmasterol was observed in regenerated plants (3.265 ±0.13 mg/

gDCW) whereas (0.6±0.1 mg/gDCW) was obtained from early callus an (0.92±0.1 mg/gDCW) from late callus.

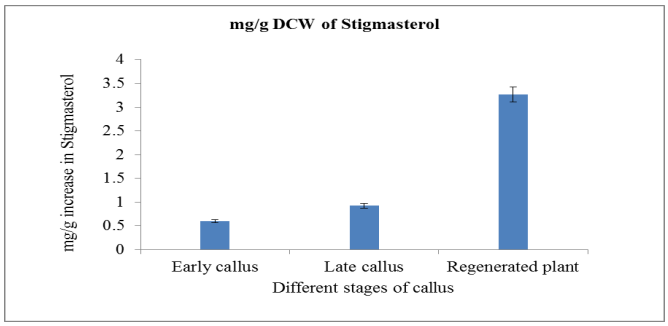

Fig. 5- Production of stigmasterol at various stages of differentiation

Production of Hecogenin a different stages of development in *Chlorophytum borivilianum*

Hecogenin a pharmaceutically important saponin of *Chlorophytum borivilianum* was estimated at different stages of differentiation from callus to regenerated plant. Among the callus extracts tested for the presence of hecogenin, a maximum of 43.55±0.52 mg/gDCW was observed in regenerated plant when compared to the early callus (1.56±0.49 mg/gDCW) and late callus (3.28±0.32 mg/gDCW).

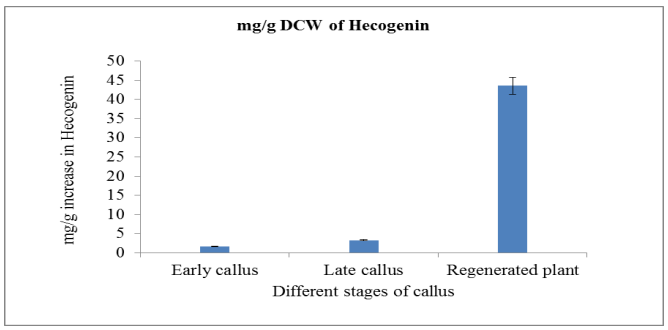

Fig. 6- Production of Hecogenin at various stages of differentiation

Discussion

This is the first report of production of stigmasterol and hecogenin at different stages of differentiation from callus cultures of *Chlorophytum borivilianum*. The amount of stigmasterol and hecogenin was found to be very low in case of young callus (0.6±0.1 mg/gDCW stigmasterol and 1.56±0.49 mg/gDCW of hecogenin), which increased with further differentiation of callus to plantlets. Callus after 48 days accumulated 0.92±0.08 mg/gDCW stigmasterol and 3.28±0.25 mg/gDCW hecogenin. However, maximum stigmasterol (3.265±0.13 mg/gDCW and hecogenin (43.55±0.52 mg/gDCW) could be observed from plants regenerated from somatic embryos. The stigmasterol content from regenerated plants was 5.4 fold higher when compared to the early callus and that of hecogenin is found to be 27.91 fold higher when compared to the early callus.

Anasori and Asghari in 2008 similar report on the production of gingerol and zingiberene along the differentiation process. This study indicates that accumulation of secondary metabolite compounds result from complex metabolic changes accompanying the differentiation of cells in plants wherein biosynthetic pathways are initiated.

The positive role of 2,4-D for callus induction has been reported in other species (Yasuda et al., 1988; Salvi et al., 2001; Shirgurkar et al., 2006). In Ceropegia candelabrum L. 2,4-D and BAP/Kn combinations were efficient in callus induction from internode and leaf explants (Beena et al., 2003). Higher concentrations of NAA and BAP stimulated callusing at basal end in case of Clitoria ternatea L. (Gyana ranjan rout., 2004). This shows that different plant species exhibit differences towards phytohormones and explants for the callus induction.

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