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ENHANCED HISPIDULIN PRODUCTION *IN VITRO* FROM CALLUS CULTURE OF *MILLINGTONIA HORTENSIS* L.F.

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ABSTRACT

Stress is one of the factors that enhance secondary metabolites production in plants. This study reports the effect of altered culture conditions on production of Hispidulin in callus cultures of Millingtonia hortensis. M. hortensis L.f (Bignoniaceae) is an important tree with traditional medicinal value that grows widely in India, Burma, Southern China and Thailand. The tree is a source of Hispidulin, a bioactive flavonoid with several therapeutic properties. Hispidulin was extracted and quantified from leaves of the tree in nature and compared with the amount of the same, produced using in vitro callus. Murashige and Skoog media with combinations of auxins (2,4-Dichlorophenoxy acetic acid and Indole-3-acetic acid) and cytokinins (6-Benzylamino purine and Kinetin) was used for callus production. Combination of Indole-3-acetic acid: Kinetin (5:5 mg/L) resulted in highest amount of callus in a culture period of 6 weeks. Supplementation of 100 g/L of Polyethylene Glycol (PEG) in the culture medium resulted in an increase of Hispidulin production as compared to control in the 5th week. Extension of the culture period by 3 weeks with incubation temperature of 18^{o}C resulted in increased production of Hispidulin. Quantification was done using HPLC technique on weekly basis. A two fold increase in the concentration of Hispidulin was obtained from in vitro calli as compared to natural leaf extract. The present study offers a renewable and sustainable method for production of Hispidulin using callus culture.

KEY WORDS

Millingtonia hortensis, Hispidulin, HPLC, Callus culture.

INTRODUCTION

Millingtonia hortensis L.f is a member of family *Bignoniaceae.* A native of southern Asia and is commonly known as Indian cork tree, tree jasmine. The name *Millingtonia* honours Thomas Millington (1628-1704), an English botanist of the 18th century; *hortensis* means "grown in gardens" the tree is indigenous to Burma and Malay Archipelago, but now grows wild in most parts of India as well as being extensively cultivated as avenue tree. It is one of the handsomest tropical flowering trees (Matthew, 1991). Lifespan of flower is short and leaves shed between January and March and renewed in April – May, but the tree never remains naked (Blatter and Millard, 1954; Gamble, 1957; Hooker 1884). *M. hortensis* is the plant having various therapeutic applications. Hispidulin is the active compound which is proven to be antimycobaterial (Elkington et al., 2009), antiasthma (Anulakanapakorn et al, 1987), antimicrobial (Jetty and Iyengar, 2000), antifungal (Sharma et al, 2007), antiproliferative (Tansuwanwong et al, 2006), effects on smooth muscles (Abdalla et al, 1988) and insect larvicidal (Niveditha et al., 2013). This natural flavone is reported to be 100-fold more potent than theophylline in its property of inhibiting platelet aggregation triggered by adenosine-5'-monophosphate, arachidonic acid, paf-acether and

collagen (Bourdillat et al., 1988). Plant cell culture systems represent a potential renewable source of valuable medicinals, flavours, essences and colourants that cannot be produced by microbial cells or chemical syntheses. However, only a few cultures produce these compounds in commercially useful amounts (DiCosmo and Misawa, 1995). Different strategies, using *in vitro* systems, have been extensively studied with the objective of improving the production of secondary plant compounds (Bourgaud et al., 2001). Callus culture of the tree tissue was undertaken in the present study for the production of hispidulin.

MATERIALS AND METHODS

Plant material: *M. hortensis* was collected from St. Aloysius College, Mangalore and authenticated by Dr. Gopalakrishna Bhat (Taxonomist), Poornaprajna College, Udupi, Karnataka. A herbarium (voucher specimen no PP.574) was made and deposited to the department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

Process of extraction: Leaves were collected and kept for shade drying for 6 hrs and later dried at 45°C in hot air oven for 24 hrs. Dried leaf material (100g) was packed in a thimble and defatted by using petroleum ether and $CHCl_3$ for 3 hrs soxhlet apparatus. The defatted tissue was subjected to methanolic extraction for 6 hrs and concentrated *in vacuum.*

Callus culture: The tissue culture was compiled according to Neumann et al, (2009); Stewart, (2008); Veeresham, (2006). Murashige and Skoog, (1962) media (HiMedia) supplemented with sucrose (2%) and varying concentration of growth regulators (2,4-D, BAP, IAA and Kinetin) was prepared in double distilled water, pH adjusted to 5.8, 0.16% of phytagel was added as the gelling agent. The media was dispensed in test tubes and autoclaved at121°C, 15 lb/in^2 for 20 min. Internodes of the apical parts of the plant were selected as explants. Prior to surface sterilization the explants were subjected to Bavistin (50 % w/w carbendazim), a fungicide for 30 minutes as pretreatment. The explants were sterilized with solution of 0.1% mercuric chloride and 0.1% Sodium Lauryl Sulphate (SLS) for 7 minutes. The explants were rinsed with sterile distilled water and aseptically transferred to test tubes containing solidified media in a laminar airflow unit. The average fresh weight of the explants was maintained between 40-60 mg. 30 replicates were maintained for every test and control. The inoculated test tubes were incubated in culture room with controlled temperature of 25°C, humidity 50-60% and light:dark cycle 16:8 hours for 6 weeks. Callus produced was screened for hispulidin production. After six weeks, callus culture was kept in room temperature of 18°C for 3 weeks and quantification of hispidulin was carried out on weekly basis.

Quantification of hispidulin by HPLC:

Stock solution of standard: Standard Hispidulin was prepared by dissolving 1 mg in 0.2 ml of MeOH (HPLC Grade) and volume was made up to 1 ml by using ACN (HPLC Grade) and dissolved by sonication. Various diluted concentrations were filtered and subjected to HPLC analysis.

Stock solution of methanolic extract of leaf: Dried methanolic leaf extract (10 g) was dissolved in 2 ml of MeOH (HPLC Grade) and volume made up to 10 ml with ACN (HPLC Grade) in 10 ml volumetric flask and was dissolved by sonication followed by filtration.

Stock solution of Callus extract: Callus (1 g) was extracted with MeOH (HPLC Grade) in warm condition. MeOH was evaporated up to 5 ml residue remain in china dish and was transferred to 10 ml volumetric flask. The volume of 0.2 ml was made up to 1 ml with Acetonitrile (ACN - HPLC Grade). This final solution after filtration was subjected to HPLC analysis.

Stationary Phase (Column)	:	Hypersil C_{18} (250 mm × 4.1mm, 5µm)
Mobile Phase	:	ACN : Phosphoric Acid (pH 4.05) (40 : 60)
Flow rate	:	1 ml/min
Injection volume	:	20 µl
Detector	:	D_2 (UV detector)
Detection	:	335 nm

Linearity graph of standard was plotted (area against concentration) and area obtained for methanolic extract of leaf and callus extract were calculated by using equation of line (y = mx + c).

Statistical analysis:

Data represents the Mean ± SEM of 30 replicates of every experiment. Statistical analysis of the callus growth in tubes was carried out by one way ANOVA (Graph pad InStat software).

RESULT AND DISCUSSION

In recent years, it has become difficult to maintain an ample supply of medicinal plants due to several factors, such as ruthless exploitation from natural habitat, lack of conservation of the environment, increasing labor costs and economical or technical problems associated with the cultivation of medicinal plants. The use of tissue culture technique for biosynthesis of secondary metabolites, particularly in plants of pharmaceutical significance, holds promise for the controlled production of plant constituents (Kokate et al., 2008). In spite of recent development in synthetic chemistry, higher plants are still an important source of medicinal compounds (Debnath *et al*., 2010).

Formation of callus is the outcome of cell division and cell expansion under the influence of exogenously supplied plant growth regulator (Pierik, 1987). Calli like organs and cells are seats of metabolism and metabolic gradients. Despite changes in the physical form, they are capable of continued production of secondary metabolites e.g. Anthraquinone from Ophiorrhiza pumila tissue and cell cultures (Kitajima et al., 1998). Physical, chemical or biological stress leads to alteration in the concentration of secondary metabolites (Yeoman and Yeoman, 1996). Tissue culture media and controlled conditions reported to enhance callus growth along with secondary metabolite production of isoflavonoid in soyabean (Tuominen and Musgrave, 2006); Anthraquinones from *Ophiorrhiza pumila* tissue and cell cultures (Kitajima et al., 1998). Compact cell aggregates (Fu et al., 2005) and Methyl Jasmonate is reported to produce hispidulin in Saussurea medusa (Fu et al., 2006). Optimization of the hormonal concentration and combination are often effective. High auxin levels, although good for cell growth, are often deleterious to secondary metabolite production. Alteration in the environmental factors such as nutrient levels, light and temperature may also affect in increasing the productivity (Zenk et al, 1975). Plant growth regulators were tested in combinations of auxins, namely 2,4-D and IAA; along with cytokinins Kinetin and BAP. Amongst the combination of growth regulators tested, IAA: Kin (5:5) produced upto 1600 mg of callus from explants of 40-60 mg initial (**Fig.1A**) fresh weight in a period of 6 weeks (**Fig.1E**), The callus induced gradually covered the explants (**Fig 1B, Fig 1C**). Browning of callus was initiated at the contact region with the medium at the end of 4 weeks (**Fig. 1D**). Proliferation of callus continued till the end of 9 weeks (**Fig. 1F**) (**Table 1**). When BAP was used singly the amount of callus produced was less than 600mg/explants which is comparable to the control treatment devoid of plant growth regulators.

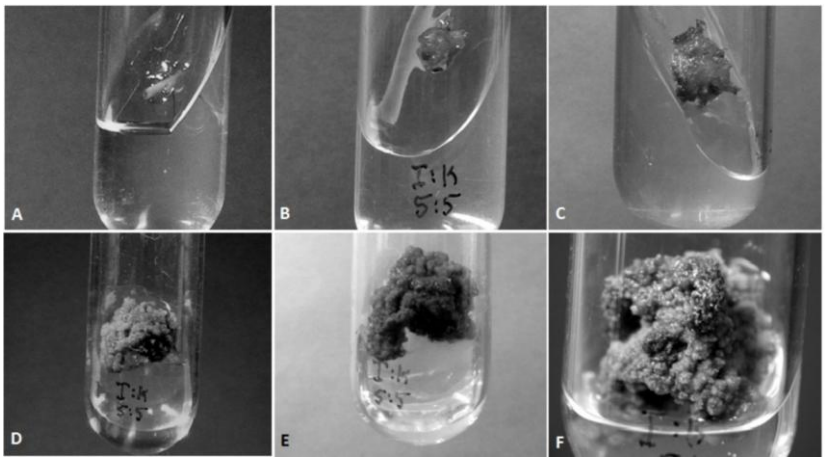

Figure 1: Growth of callus induced by IAA: Kin (5:5mg/L) over a period of 9 weeks. A. Day of inoculation; B. One week old callus; C.Two weeks old callus; D.Four weeks old callus; E.Six weeks old callus; F.Nine weeks old callus.

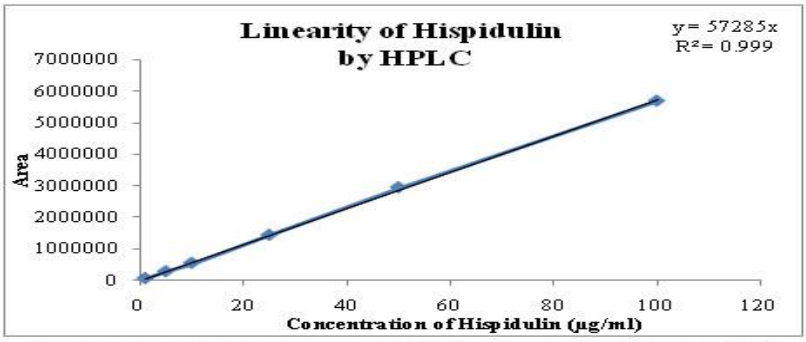

Figure 1: Linearity of various concentration of Hispidulin by using HPLC

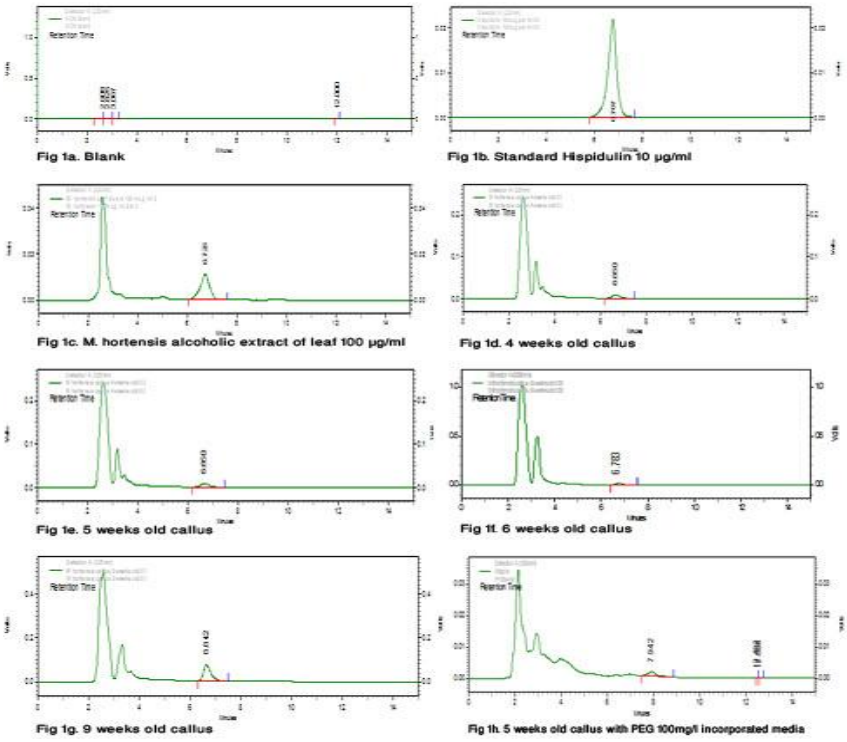

Figure 2: Linearity of various concentrations of standard Hispidulin by using HPLC

Table 1: Percentage growth of callus on MS media supplemented with combination of plant growth regulators 2,4-D:BAP:IAA:Kin, 2% sucrose and 0.16% of phytagel. Values are expressed as Mean ± SEM of 30 replicates. Values followed by uncommon alphabet, are significant different with P<0.05.

Concentration (mg/L)				Callus growth in % tubes	Weight of the callus (mg)
2,4-D	BAP	IAA	Kin		
---	---	---	---	22.00 ± 1.00[a]	< 600
5	---	---	---	41.67 ± 1.67[b]	1200-1400
5	5	---	---	55.00 ± 2.89[b]	800-1000
5	10	---	---	36.67 ± 1.67[b]	1000-1200
10	5	---	---	43.33 ± 1.67[b]	1000-1200
---	---	1	1	25.00 ± 2.89[a]	600-800
---	---	3	3	33.33 ± 1.67[a]	800-1000
---	---	5	5	83.33 ± 1.67[d]	1400-1600
---	---	5	10	41.67 ± 1.67[b]	1000-1200
---	---	10	5	50.00 ± 2.89[b]	1000-1200
---	1	---	---	25.00 ± 1.73[a]	< 600
---	2	---	---	29.67 ± 1.73[a]	< 600
---	3	---	---	38.33 ± 1.20[b]	< 600
---	4	---	---	46.00 ± 1.00[b]	< 600
---	5	---	---	35.00 ± 1.00[b]	< 600

Quantification of Hispidulin by HPLC:

Linearity was developed by using different concentrations of standard Hispidulin and plotted a graph (**Figure 2**). The amount of Hispidulin found in methanolic extract of leaf was 4.5 ± 0.45 % w/w (**Fig. 2c**) where as in callus (IAA **:** Kin 5 **:** 5), the amount of hispidulin after 4 weeks was found 1.02 ± 0.25 % w/w (**Fig. 2d**), after 5 weeks: 2.23 ± 0.32 % w/w(**Fig.2e**), after 6 weeks: 2.74 ± 0.40 % w/w (**Fig.2f**) and after 9 weeks: 8.21 ± 0.42 % w/w (**Fig.2g**).

A study on role of fungal extracts as elicitors on the Hispidulin production using suspension culture of M. hortensis reports 1.8 % increase in the 9th week upon addition of fungal extracts of Aspergillus terreus (Shreedhara, 2013). Deshpande, et al (2012) evaluated abiotic stress on callus culture of M. hortensis and reported no appreciable increase with incorporation of methyl jasmonate, but a 7% rise was observed when cultured with 100 g/L PEG in the culture medium for 9 weeks. In the current study explants were grown on MS media containing PEG 100 g/L and cultured for 9 weeks. The results obtained are as follows (Figure 3). Highest response of 3.45 ± 0.22 % w/w hispidulin was produced when cultured on medium with PEG 100 g/L at the end of 5 weeks (Fig 2h). However, the callus turned moribund when cultured for more than 6 weeks on PEG containing media due to dehydration.

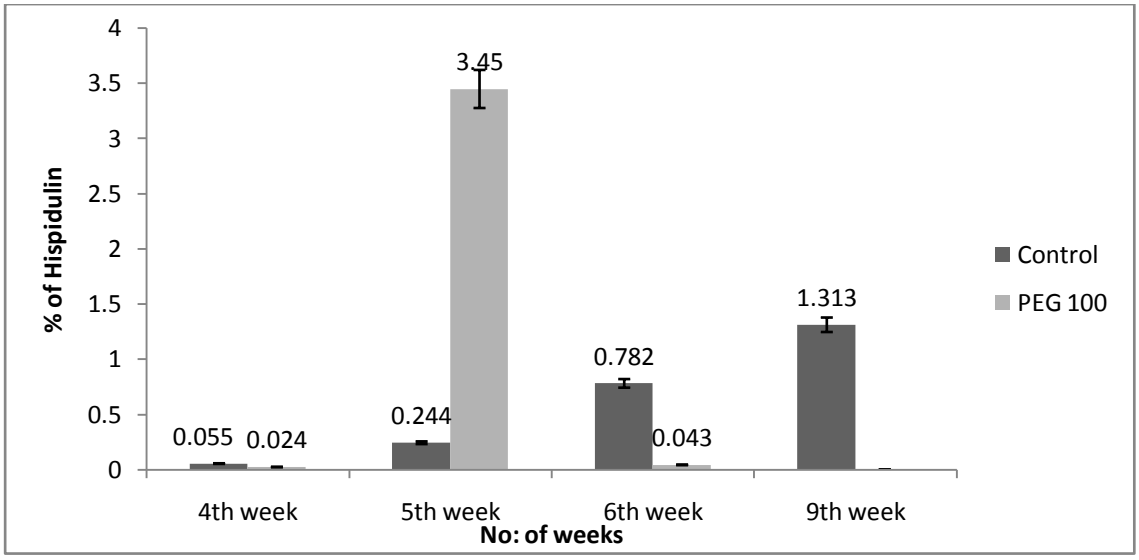

Figure 3: Comparison of Hispidulin concentration in control and PEG induced callus over a period of 9 weeks.

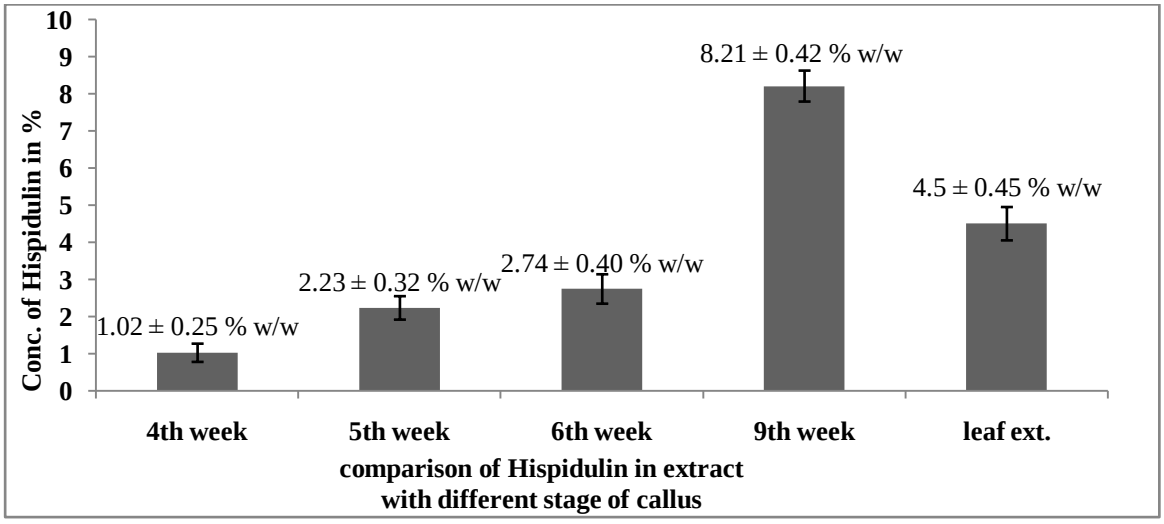

Figure 4: Comparison of Hispidulin concentration in extract from leaf and callus cultured over a period of 9 weeks.

Low temperature significantly increased antioxidant activities *in vitro* somatic embryos of *Eleutherococcus senticosus* (Shohael et al 2006). Prolonged culture has been reported to be counterproductive in case of Rhododendrol biosynthesis from *Acer nikoense* cell suspension cultures (Fujita et al., 1998). However, *Millingtonia hortensis* callus of 9 weeks on 0.16% phytagel containing medium with 18°C temperature induced increased production to 8.21 ± 0.42 % w/w (Figure 4) of the secondary metabolite Hispidulin.

CONCLUSION

Very few reports of successful production of secondary metabolite of tree tissue *in vitro* have matched levels in their natural habitat. The current study shows a two fold increase in Hispidulin content in 9 week old callus as compared to the natural leaf extract. Thus callus culture of *Millingtonia hortensis* subjected to low temperature along with prolonged culture period offers a less laborious and simple technique for enhanced production of hispidulin *in vitro*.

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