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Phytochemical Screening of Tissue Cultured *Holoptelea integrifolia*

Ponezhil and V. Judia Harriet Sumathy*

Postgraduate & Research Department of Biotechnology
Women's Christian College, Chennai–600006, India
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Abstract

Plant tissue culture forms the backbone of plant biotechnology, i.e. micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant Tissue Culture is an essential component of Plant Biotechnology. Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations. Today tissue culture technology is being exploited mainly for large scale production or micropropagation of elite planting material with desirable characteristics. This technology has now been commercialized globally and has contributed significantly towards the enhanced production of high quality planting material. Recently, emphasis has been on genetic transformation, especially for increased production of secondary metabolites, production of alkaloids, pharmaceutics, nematocidal compounds, and also some novel compounds not found in the whole plant, regeneration of plant resistant to herbicides, diseases, and pests, scale up of cultures in bioreactors, plants with different morphological traits, and transgenic plants for the production of vaccines etc. These developments have far-reaching implications in the improvement of medicinal plants as well. The present study was aimed to perform tissue culture and callus induction in *Holoptelea integrifolia*, observe somatic embryonic stages from callus under inverted microscope and to isolate protoplast from callus with viability test. Further investigation involves the antibacterial effect of callus extract of *Holoptelea integrifolia* on test organisms and the analysis of phytochemicals in the callus extract. Thus this study was undertaken to attempt tissue culture in the medicinal plant *Holoptelea integrifolia*, to observe the response of the plant in tissue culture techniques, to screen the phytochemicals in the callus of *Holoptelea integrifolia* and to check the antibacterial property of the callus extract on four test organisms.

Keywords: Plant Tissue Culture, Micropropagation, *Holoptelea integrifolia***,** Phytochemicals and Antibacterial property

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***Corresponding author**
V. Judia Harriet Sumathy
E-mail: sbsj@rediffmail.com
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1. Introduction

Medicinal herbs are moving from fringe to mainstream use with greater number of people seeking remedies and health benefits free from side effects. Recently considerable attention has been paid to utilize eco-friendly and bio-friendly plant based products for the prevention and cure of different human disease including microbial infection. The plant kingdom represents an extraordinary reservoir of novel molecules. The potential of higher plants, as source for new drug is thus still largely unexplored (Kumar and Kumar, 1996). Plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cells, tissues and organs under sterile and controlled environmental conditions (Murashige & Skoog, 1974).

In India, Tissue Culture research began nearly four decades ago with the first report on production of test tube fertilization (Kanta *et al*.1962). Tissue culture techniques are now being widely applied for improvement of field crop, forest, and horticulture and plantation crop for increased agricultural and forestry production. The search for biologically active compounds from natural sources has always been of great interest to the scientist looking for new sources from drugs useful in infectious diseases. It is a necessity from the scientific point of view, to establish a rational relationship between chemical composition, biological and therapeutic activities. In recent years a number of studies have been reported, dealing with antimicrobial screening of medicinal plant between chemical composition, biological and therapeutic activities. The use of plants, plant extract provides the foundation to modern therapeutic science and thus enables man to establish the empirical system of medicine. In view of the commercial importance given to the secondary metabolites in recent times, efficient production of bioactive compounds by tissue culture technology has gained popularity. The continuous and non-organized exploitation of these plants have resulted in many of them becoming rare and some even became extinct. To overcome this limitation, biotechnologists suggested the "use of cell and tissue culture technology rather than to use the whole plant" for the extraction of certain secondary metabolites.

Holoptelea integrifolia

Indian Elm *Holoptelea integrifolia*, Family Ulmaceae.., is a large deciduous tree, gowing up to 18 m tall. It has grey bark, covered with blisters, peeling in corky scales on old trees. Alternately arranged leaves are elliptic-ovate, 8-13 cm long and 3.2-6.3 cm wide, smooth, with entire margins, and a pointed tip. Leaf base is rounded or heart-shaped. Stipules are lance-shaped. Crushed leaves emit an unpleasant odour. Flowers are small, greenish-yellow to brownish, pubescent, borne in short racemes or fascicles at the scars of fallen leaves. Sepals are velvety, often four. Fruit is a circular samara, 2.5 cm in diameter, with membranous, net-veined wings, and flat seed. *Holoptelea integrifolia* is used in rheumatism (stem bark) and ringworm (seed and paste of stem bark); for treating oedema, diabetes, leprosy and other skin diseases, dyspepsia, intestinal disorders, piles and sprue (bark and leaves). It is an important pollen allergen of India and sensitizes almost 10% of the atopic population in Delhi (Sharma *et al.,* 2005). Recently, an "All India Coordinated Project on Aeroallergens and Human Health" was undertaken to discover the quantitative and qualitative prevalence of aerosols at 18 different centers in the country and found *Holoptelea* to be one of the predominant allergen (Singh and Kumar, 2003). Until today, no pharmacological evaluations have been reported on this plant except one group who claimed antiviral activity (Rajbhandari *et al.,* 2001).

Somatic embryogenesis of plants originated from single cells is highly necessary and essential for gene transfer studies. A wide range of plant species are amenable to embryogenic callus initiation and regeneration through somatic embryos. The development of suitable protocols for plant regeneration is one of the main prerequisites for the genetic improvement of crop plants using biotechnological methods. (Bhanumathi, P *et.al.,* 2005). Comparatively, with respect to plant genetic transformation, plants regenerated through somatic embryogenesis are more useful than plants obtained through organogenesis (Jamine *et al*., 2007). Thus Somatic embryogenesis represents a simple and very efficient alternative means of regenerating large numbers of intact plants from tissue culture (Zimmerman, 1993).

The subject of phyto-chemistry, or plant chemistry, has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with enormous variety of organic substances that are elaborate and accumulate by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function (Vinod, *et. al.,* 2009). Plant products are classified into Primary products – aminoacids, sugar and lipids and Secondary products – alkaloids, antibiotics, volatile oils, resins, polyphenols, tannins, cardiac glycosides, sterols, saponins and terpenoid compounds. Manipulation in the culture conditions has increased the production of secondary products. Callus can be sub- cultured for years for increased production of plant products (Girish, *et.al.,* 2008).

2. Materials and Method

Composition of Murashige and Skoog media

Macro Nutrients	g/250ml
$MgSO_4.7H_2O$	3.7
$CaCl_2.2H_2O$	4.4
KNO_3	19.0
NH_4NO_3	16.5
KH_2PO_4	1.7
Micro Nutrients	
$MnSO_4.4H_2O$	1.115
$ZnSO_4.7H_2O$	0.43
$CuSO_4.5H_2O$	0.0125
$CoCl_2.6H_2O$	0.0125
H_3BO3	0.31
$NaMoO_4.2H_2$	0.0125
Iron Stock	
$FeSO_4.7H_2O$	0.278
EDTA	0.372
Vitamins	
Myoinositol	1.0
Nicotinic acid	0.010
Pyriodoxine HCL	0.010
Thiamine HCL	0.002
Glycine	0.040

1 Litre of MS Medium was prepared using standardized protocol. The glassware's were properly sterilized. The Growth Hormones were prepared and sterilized using standard protocols. Young leaves of *Holoptelea integrifolia* were taken and were washed in running tap water for 30 minutes to remove the dust and sand particles adhering to it. They were then submerged in the detergent solution (1ml of Tween – 20ml mixed with 100ml of distilled water) and were then rinsed again with tap water.The laminar air flow was wiped with 70% ethanol and UV lamp was switched on for 15 minutes before working. The explants were then submerged in 15% sodium hypochlorite solution and were then submerged in 70% ethanol for 1-3 minutes. The sterilized explants were then rinsed with 3 washes of sterile distilled water to remove completely the traces of surfactants. The water was completely removed by pressing it between the tissue paper. All the above steps were carried out in the laminar air flow. The explants was then inoculated in the MS Medium and the callus was subcultured and extracted using Soxhlet Apparatus. Antibacterial Activity Assay was carried out and the protoplast was isolated from callus using the standard Enzymatic method. Protoplast Counting and Viability Testing was calculated using the formula

$$\text{Viability of cells} = \frac{\text{Total number of viable cells / ml of aliquot}}{\text{Total number of cells / ml of aliquot}} * 100$$

Phytochemical Screening Test

The callus of *Holoptelea integrifolia* were air-dried and ground into uniform powder. The aqueous extract of callus sample was prepared by socking 10g of powdered samples in 20ml of distilled water for 12 hours. The extract was filtered using what man filter paper No 42. About 0.5g of the dried powdered sample was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added observed for brownish green or blue-black coloration as a confirmatory test for Tannins. Deposition of a red precipitate when an aqueous extract of callus sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of Phlobatannins. Two ml of acetic anhydride was added to 0.5g ethanol extract of callus sample with 2ml sulphuric acid. The colour changed from violet to blue or green in sample indicating presence of steroids. About 2g of the powered callus sample was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. 5ml of diluted ammonia solution were added to a portion of the aqueous solution were added to filtrate of callus extract followed by addition of concentrated sulphuric acid. A yellow coloration observed indicating the presence of flavonoids. The yellow coloration disappeared on standing. 5ml of callus extract mixed in 2ml of chloroform, and 3ml concentrated sulphuric acid was carefully added to form a layer. A reddish brown coloration was formed to show positive results for the presence of

terpenoids. 5ml of callus extract was treated with 2ml glacial acetic acid containing 1 drop of ferric chloride solution. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

3. Results and Discussion

Tissue Culture of *Holoptelea integrifolia*

The inoculation of explants on the MS media with vitamins and sugar yield the callus from leaf, after 2 weeks of incubation (Plate 1). Explant was inoculated and callus induction was initiated following that in the MS medium with 2,4 D(1.0mg/l) (Table 1). Considered increase in size was observed in the following days. Callus occupying the entire diameter of the culture tube was observed after one month.

Table 1. Concentration of Hormones used for the present study

Hormones	0.5mg/L	1.0mg/L
2,4 D	-	Callus Formation
IAA	-	-
NAA	-	-
BAP	-	-

The callus was subcultured in a medium containing MS media + 2,4 D(0.1mg/l) and an increase in size of the callus in subcultures was observed. Initially cream coloured callus appeared but later they turned dark red (Plate 2). The callus were macerated into small pieces and inoculated in MS media without Agar (Plate 3 & 4).They were allowed to incubate for 2 weeks. The cells suspension of callus was viewed under inverted microscope periodically for somatic embryogenesis. Callus growth was progressive. Fresh medium was changed periodically. The following results were obtained periodically weekly. 3rd week - early stages in the development of a proembryoid, 4th week - globular stage (Plate 5), 5th week - heart shaped stage of embryos was clearly seen under inverted microscope (Plate 6), 6th week - torpedo stage seen before plantlet formation (Plate 7) followed by rooting of the embryos (Plate 8) and shooting of the embryos induced by addition of cytokinin (BAP) with fresh MS medium without agar after 8th week (Plate 9).

Phytochemical Screening Test

The results of Phytochemical Screening Test are tabulated in Table 2 indicating the presence or absence of the Phytochemicals.

Table 2. Phytochemical Screening Test

Phytochemicals	Callus of *Holoptelea integrifolia*
Tannins	+
Saponin	+
Steroids	-
Phlobatannin	+
Terpenoid	+
Flavonoid	+
Cardiac glycosides	+

The brownish colour which appeared on the addition of 0.1 % $FeCl_2$ solution on callus extract indicates the presence of tannins (Plate 14). Red precipitate was deposited on boiling aqueous extract of callus with 1% aqueous HCl, indicating the presence of phlobatannins (Plate 15). The frothing disappeared on the addition of drops of olive oil and then the emulsion was formed indicating the presence of saponin (Plate 13). A yellow coloration was observed indicating the presence of flavonoids (Plate 12). No color change from violet to blue or green, indicates the absence of steroids. A reddish brown coloration of the interface was observed, indicating the presence of terpenoids (Plate 11). Brown ring formed indicates the presence of cardiac glycosides (Plate 10). The antimicrobial assay of ether extract of callus was performed by agar diffusion method and the zone of inhibition was measured in mm (Table 3, Graphs 1 - 4 & Plates 16 and 17).

Table 3. Zone of Inhibition

Micro-organisms	10 hrs callus extract (mm)	20 hrs callus extract (mm)	30 hrs callus extract (mm)	40 hrs callus extract (mm)
Pseudomonas aerogenosa		8	10	12
Staphylococcus aureus	-	6	9	11
Bacillus sps	-	8	10	12
Escherichia coli	-	-	7	9

Protoplast Isolation and Viability Counting

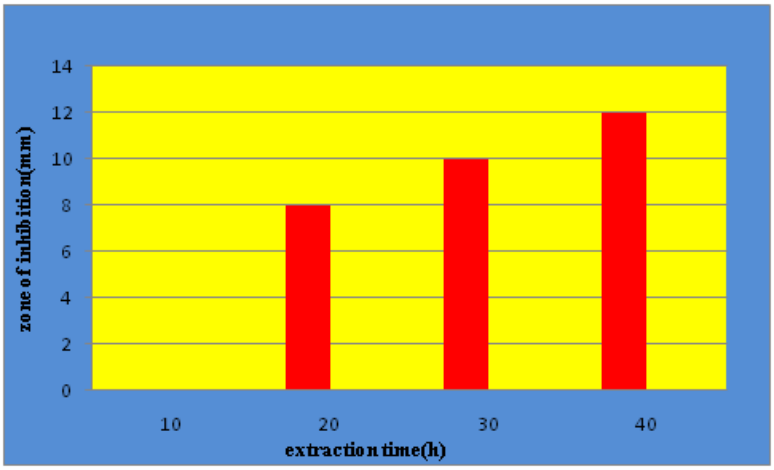
Protoplast was isolated from the four times subcultured callus. The callus which was fresh was taken and several trials were carried out. The release of protoplasts depends upon the concentration of the cellulose enzyme. 200µl of solution (enzyme suspension + Evan's blue solution) was loaded in haemocytometer.

$$\text{Viability of cells} = \frac{\text{Total number of viable cells / ml of aliquot}}{\text{Total number of cells / ml of aliquot}} * 100$$

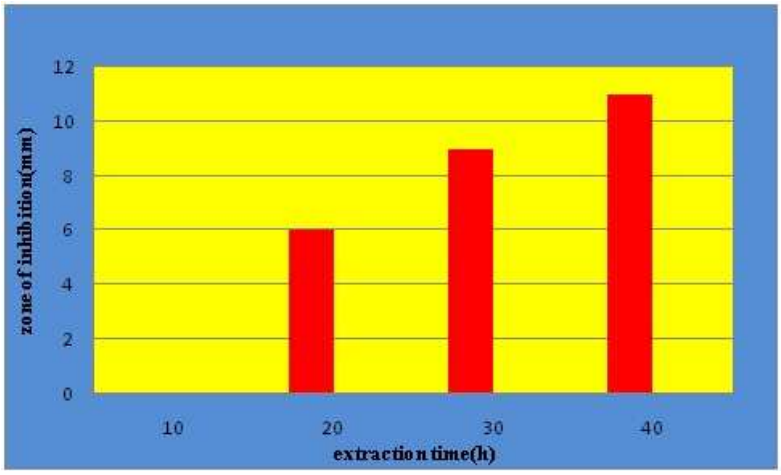
Total number of viable cells/ml of aliquot = 300
Total number of cells/ml of aliquot = 335

$$\text{Viability of cells} = \frac{300}{335} * 100 = 89.5\%$$

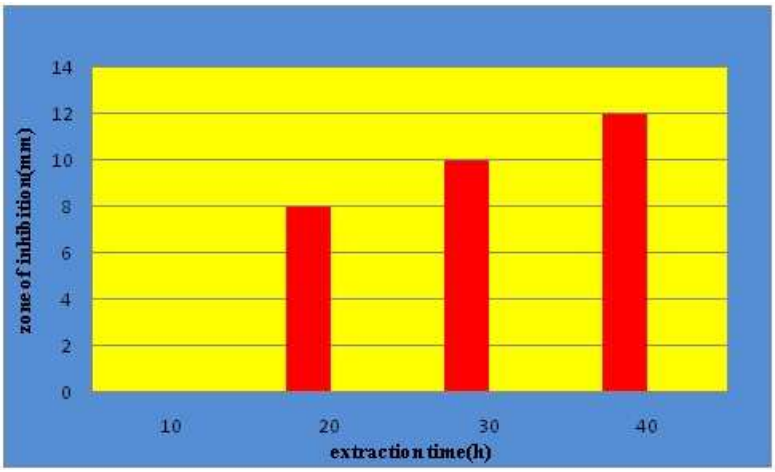
Percentage of viability was found to decrease when kept in the enzyme solution. Hence it is advised to be left in the protoplast culture solution.



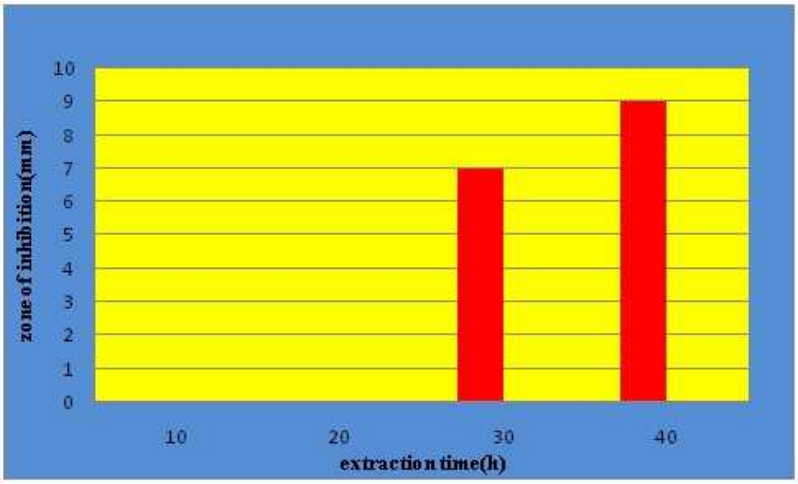
Graph 1. ***Pseudomonas aerogenosa***



Graph 2. ***Staphylococcus aureus***



Graph 3. ***Bacillus subtilis***



Graph 4. ***Escherichia coli***

Plate 1. Induction of callus from Leaf explants

Plate 2. Subculture of Callus

Plate 3. Cell Suspension Culture of Callus

Plate 4. Cell Suspension in Karl's Flask

Somatic Embryogenic Stages from Callus of *Holoptelea integrifolia*

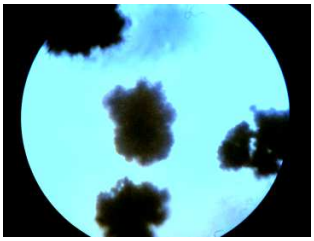


Plate 5. Globular Stage

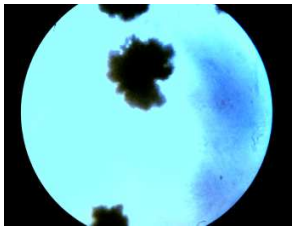


Plate 6. Heart Shaped Stage

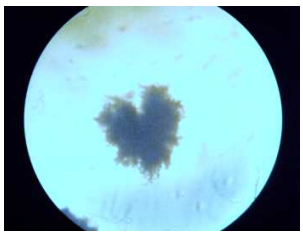


Plate 7. Torpedo Stage

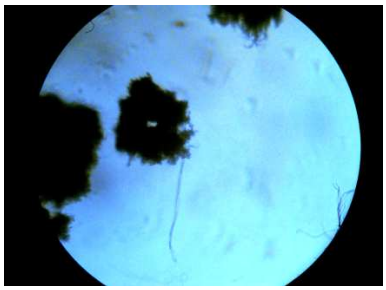


Plate 8. Initial rooting from Embryo

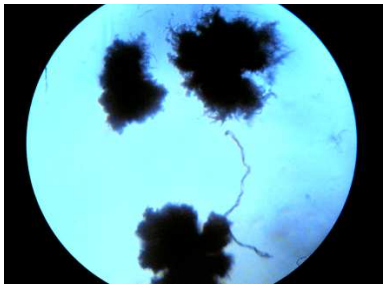


plate 9. Initial Rooting and Shooting from Embryo

Phytochemical Screening of Callus from *Holoptelea integrifolia*

Plate 10. Terpenoids

Plate 11. Cardiac Glycosides

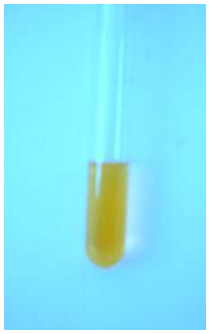


Plate 12. Flavonoids

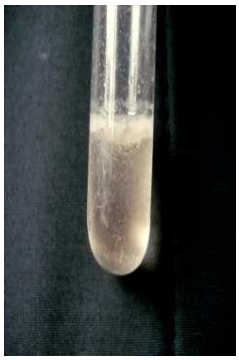


Plate 13. Saponin

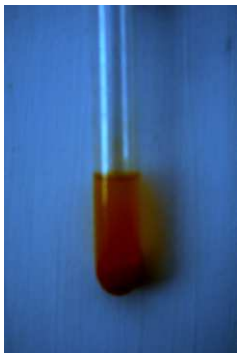


Plate 14. Tannins

Plate 15. Phlobatannins

Plate 16. Callus Extraction in Soxhlet Apparatus

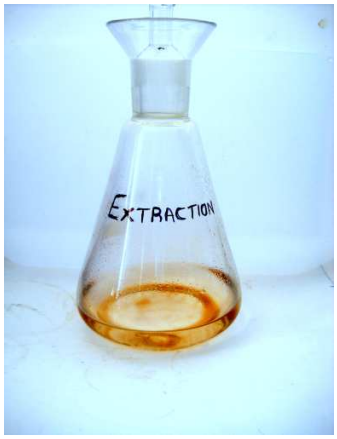


Plate 17. Callus Extraction with Diethyl Ether

4. Conclusion

The plant shows good results in callus induction, cell suspension culture and viability of protoplast. Good progression was seen in indirect somatic embryogenesis but it takes time to obtain plantlet stage in cell suspension. The indirect somatic embryogenesis of the plant can be further studied to produce synthetic seeds and plant regeneration in tissue culture. The plant studied here can be seen as potential source of useful drugs. The antimicrobial activity of callus extract for treatment of diseases has been done. The presence of tannins, phlobatannins, flavonoids, saponin, terpenoids, cardiac glycosides shows good antimicrobial activity in callus. Future studies may be necessary to standardize the plant in tissue culture and to elucidate the phytochemistry of the active principle in callus from *Holoptelea integrifolia.*

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