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Short Communication

QUANTIFICATION OF LEAF AND ROOT PLUMBAGIN IN *PLUMBAGO ZEYLANICA* FOLLOWED BY A COMPARATIVE STUDY WITH CALLUS AND COMMERCIAL SOURCE

Jasvinder Kaushal, Karishma Rajbhar*, Himanshu Dawda, Usha Mukundan

Department of Botany, Plant biotechnology laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (West), Mumbai, India

**Corresponding author: karishmarajbhar@rjcollege.edu.in*

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ABSTRACT

Plumbago zeylanica shows the presence of many phytochemical constituents of which plumbagin, a type of naphthoquinone, is vital and has major biological effects. Plumbagin is majorly present in the root of the plants, with a minimal concentration in other parts of the plant. However, extracting plumbagin from the roots is destructive harvesting. Thus, micropropagation could be a viable alternative to reduce the collection of plant from wild. Study from micropropagated leaf calli was considered as a steady and sustainable source of phytochemical. *P. zeyalnica* callus was subcultured in an appropriate medium with plant growth regulators to initiate root cultures. A comparative study was performed to estimate the quantities of plumbagin obtain from the wild plant root part and root created by callus culture differentiation.

Keywords: Micropropagation, *Plumbago zeylanica,* Plumbagin, TLC.

1. INTRODUCTION

Metabolites are plant products which comprise of large as well as small molecules. Fundamentally metabolites are of two types: Primary metabolite and Secondary metabolite. Metabolites which are directly involved in normal growth, development, and reproduction are the primary ones. They usually perform a typical function in the organism and are produced by all species. Some metabolites are organic in nature and not directly involved in the normal growth, development, or reproduction of an organism, are termed as secondary. They are produced in a phase of subsequent growth and may have no function in the growth (although they may have survival function). Secondary metabolite can also be produced by certain limited taxonomic group of microorganisms. These types of secondary metabolites have unusual chemical structures and are often formed as mixtures of closely associated components of a chemical family. Secondary metabolites have various uses and applications, such as, they are used as fuel, in structure integrity, signalling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own (usually as a cofactor to an enzyme), defence, and interactions with the other organisms [1].

Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather creates a long-term impairment during the organism's survivability, fecundity, or aesthetics or perhaps no significant change at all. These are often restricted to a narrow set of species within a phylogenetic group. And these also play an important role in plant defence against herbivory and other interspecies interaction. Humans have been using secondary metabolites as medicines, flavourings, and recreational drugs in the recent past. Kossel was the first to define these metabolites as opposed to primary ones. It has been clearly demonstrated that secondary products play a major role in the adaptation of plants to their environment. The compounds which have antibiotic, antifungal, and antiviral activities are prepared owing to the plant's defence mechanism against pathogens [1-3].

Plumbago zeylanica contains various phytochemicals like alkaloids, flavonoids, naphthaquinones, glycosides, steroids, saponins, triterpenoids, tannins, phenolic compounds and coumarins. The most well-known secondary metabolite from *Plumbago zeylanica* is Plumbagin (5-hydroxy-2-methyl-1, 4- naphthoquinone) which is largely present in the roots of *Plumbago* species.

The compound is yellow in colour because of the existence of naphthoquinone pigment. It is readily soluble in alcohol, acetone, chloroform, benzene, and acetic acid [2, 3].

Plumbagin is one of the simplest secondary plant naphthoquinones presents in three major families- *Plumbaginaceae, Droseraceae,* and *Ebenaceae* [4]. These plant families have highly effective biological anti-oxidant, anti-inflammatory, anticancer, antibacterial, hepatoprotective, antihyperlipidemic, antihelminthic and antifungal properties [5-7].

Plumbago zeylanica produces plumbagin in high concentrations and is readily available in India. Due to the roots producing maximum quantity of plumbagin, the compound is primarily extracted from the roots of *P. zeylanica*. However, extracting any compound from the roots of a plant results in destructive harvesting as the plant is uprooted and subsequently killed in the process. Hence, micro propagated callus, rich in plumbagin can be an alternative to this. Callus can be initiated using leaves of *Plumbago zeylanica*. Once the callus initiates, it can be allowed to grow with periodic subculturing in an appropriate medium [8, 9].

The initiated callus can be subcultured at specific intervals in an appropriate medium with plant growth regulators to initiate root cultures. Later, a comparative study was performed to estimate the quantities of plumbagin obtain from the wild plant root part and root created by callus culture differentiation. This alternative method may help in reducing the uprooting of plant for plumbagin content extraction.

2. MATERIAL AND METHOD

2.1. Preliminary screening of plumbagin

Preliminary screening of plumbagin was conducted using the extract prepared from leaf and root in a concentration of 1:10 (dilution). Standard alcoholic plumbagin was used in mg/ml concentration. Thin-layer chromatography using different types of solvent systems was performed. The solvent system used for screening was toluene: ethyl acetate: methanol with volume ratio of 8:1:1, hexane: ethyl formate with volume ratio of 9:1 and toluene: ethyl acetate with volume ratio of 9.3: 0.7. Activated aluminium silica Merck plates were used for TLC which were derivatised later with 10% methanolic KOH reagent.

2.2. Media components

Plant tissue culture media was prepared by following components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other organic supplements, solidifying agents or support systems, and growth regulators [10, 11].

Table 1: Plant culture medium (MS Modified - Murashige and Skoog)

Macronutrient Constituent (10X)	**Amount (g/l)**
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2$ $7H_2O$	4.4
$MgSO_4$ $7H_2O$	3.7
KH_2PO_4	1.7
Micronutrient Constituent (100X)	**Amount (g per 100 ml)**
$MnSO_4 7H_2O$	0.169
$ZnSO_4$	0.106
H_3BO_3	0.062
KI	0.0083
$NaMoO_4$	0.0025
$CuSO_4$	0.00025
$CoCl_2$	0.00025
Iron Source (100X)	**Amount (g per 100 ml)**
Fe-EDTA	0.43
Vitamin source (100X)	**Amount (g per 100 ml)**
Nicotinic acid	0.01
Thiamine HCl	0.1
Pyridoxine HCl	0.01
Meso-inositol	1.0
Sugar (Sucrose)	**3%**

Table 2: Plant growth regulators

Class	Name	Abbreviation	Uses and Properties
Thidiazuron	Thidiazuron	TDZ	Used for callus culture
Auxin	Indole-3-acetic acid	IAA	Used for callus induction Less concentration can stimulate organogenesis Inactivated by light Readily oxidized by plant cells
	Indole-3-butyric acid	IBA	Used for rooting Regeneration of shoots by organogenesis.
	1-Naphthaleneacetic acid	NAA	Similar like 2, 4-D, but less commonly used Synthetic analogue of IAA

2.3. Explants sterilisation protocol

Young leaves of *Plumbago zeylanica* were used as explant for micropropagation. Explants sterilization was done with a fungicide like bavistin, baciloside and surfaces were sterilised with mercuric chloride and ethanol. Selected young leaves were subjected to fungicide treatment for 1 hour in a solution containing 0.1 g bavistin and 1% baciloside. Surface sterilisation was conducted in laminar airflow. Explants were washed thoroughly with sterile distilled water to get rid of excess fungicide. Explants were treated with 0.1% mercuric chloride for 30 sec. Explants were washed thoroughly with sterile distilled water to get rid of excess mercuric chloride. Explants were treated with 70% ethanol for 30 sec. Explants were washed again with sterile distilled water for hydration.

2.4. Inoculation of explants

Surface sterilized explants were kept on sterile Petri plate containing filter paper so that excess water would get drained. Sterilized explants were cut to a size of approx. 1 cm^2 by removing the edges so that dead tissues were excluded. Abaxial side of leaf sterilized explants were given few cuts and scratches, so that the veins connecting to midrib would be opened. The prepared explants were inoculated in the respective medium in such a way that the cut surfaces of the abaxial side of leaf were placed above the medium surface. Inoculated explants were kept under observation to check its growth. The micropropagation medium used for callus induction was modified MS medium supplied with Thidiazuron (TDZ) which is mostly used for callus culture. The culture was observed for the stable growth of callus.

2.5. Sub-culturing of callus in rooting medium

Selected callus was divided into approx. size of 1 cm^2 and extra agar was removed from the lower side of the callus. The prepared callus explants were inoculated in three different types of rooting medium viz. modified MS medium supplied with Indole-3-acetic acid (IAA) which is mostly used for stimulating organogenesis from callus, Indole-3-butyric acid (IBA) mostly used for rooting and regenerated shoots by organogenesis and 1-Naphthaleneacetic Acid (NAA) which mostly has similar result like 2,4-D and organogenesis from callus, but less commonly used as it is a synthetic analogue of IAA, respectively. Inoculated callus explants were kept under observation to check for their growth.

2.6. Comparative analysis of plumbagin

A comparative study of callus, root, leaf and commercially available Chitrak powder was performed to estimate the quantities of plumbagin, respectively. The detection of plumbagin was done by TLC method later followed by quantitative analysis of plumbagin by spectrophotometric method. The extract was prepared from leaf and root with the dilution of 1:10. Standard alcoholic plumbagin was used in mg/ml concentration. Extract from *Plumbago zeylanica* callus was made with 1g in 10ml alcohol and Chitrak powder (commercial) extract was prepared by refluxing 10g of powder in 100 ml alcohol for 1 hour. Above extract was also used for spectrophotometric analysis of plumbagin to estimate the amount of plumbagin present in each of the calluses from different medium.

3. RESULT

Preliminary screening of Plumbagin in root, leaf by thin layer chromatography was studied as seen in Fig. no.1 where standard plumbagin was used as a reference. The callus initiated was analysed to test the presence of Plumbagin as seen in Fig. 2 and 3. Later callus initiated where grown in different medium was analysed to quantify the Plumbagin by spectrophotometer.

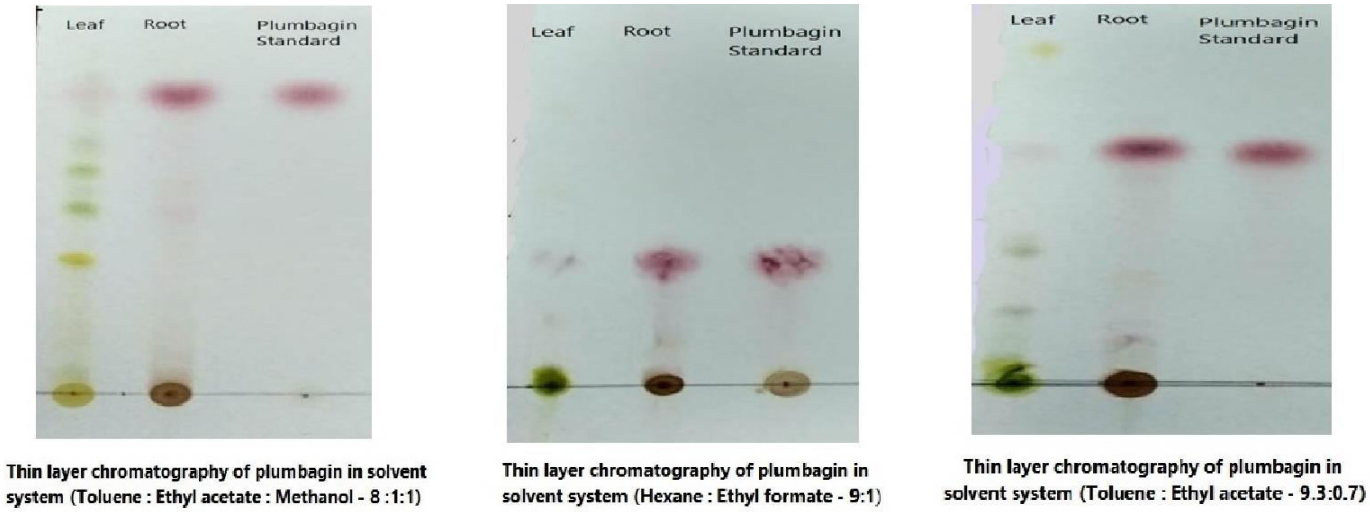

Fig. 1: Screening of plumbagin

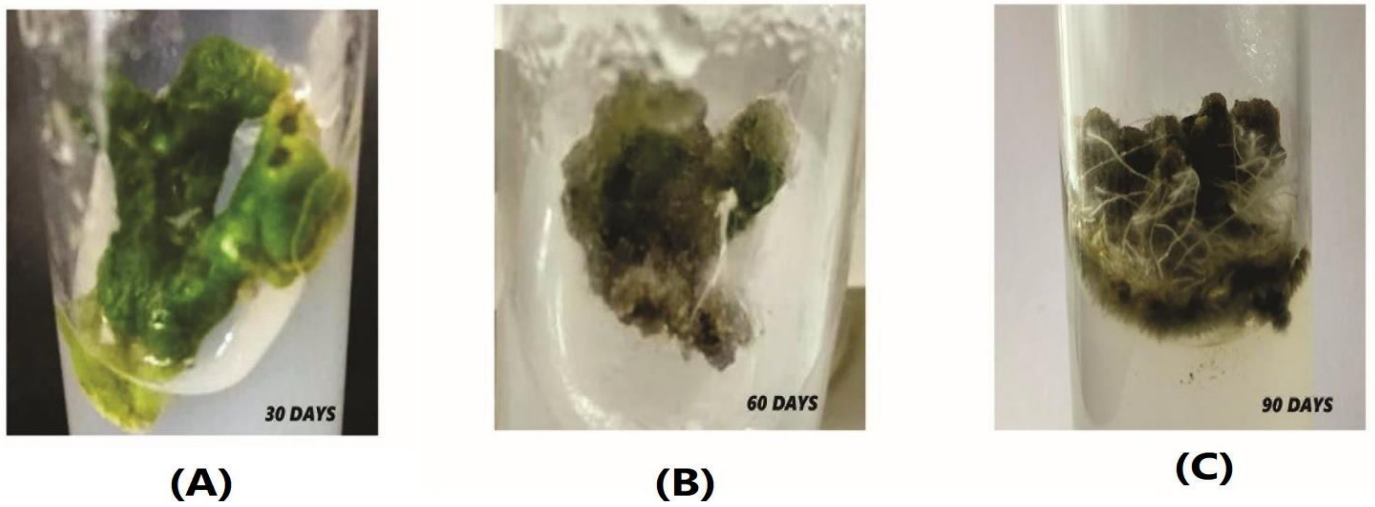

Fig. 2: *Invitro* culture of *Plumbago zeylanica* (30 days - A), Callus in TDZ and Callus in NAA 60 days (B) and 90 days (C)

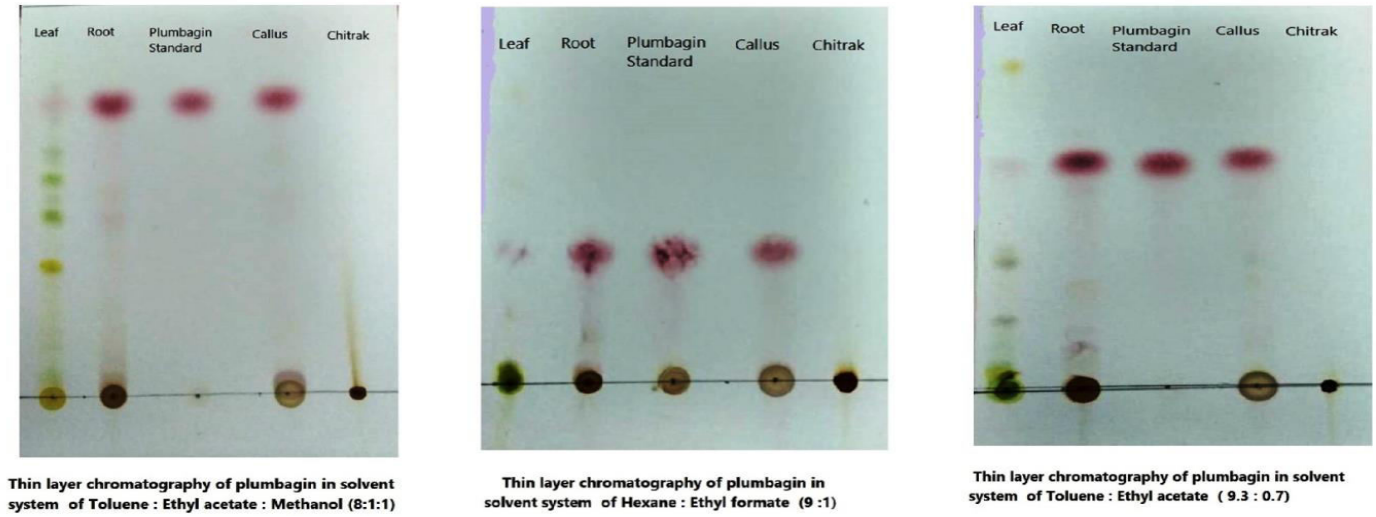

Fig. 3: Comparative TLC analysis of plumbagin

Table 3: Plumbagin Amount in callus (STD graph having equation *y=0.1734x+0.0456* with R^2 of 0.9931)

Rooting medium	Plumbagin (mcg/g)
2 IAA	52.09±2.02
IBA	99.58±2.33
NAA	200.50±2.90

4. CONCLUSION

Preliminary screening of plumbagin with thin layer chromatography of the extract prepared from leaf, root and standard plumbagin using different types of the solvent system was performed. As seen in Fig. 1, the Rf value of plumbagin was observed to be 0.725 in the solvent system toluene: ethyl acetate: methanol with the volume amount ratio of 8:1:1 for leaf, root and standard plumbagin. Rf value of plumbagin in a solvent system containing hexane: ethyl formate was observed to be 0.3005 with volume amount ratio of 9:1 for leaf, root and 0.3062 for standard plumbagin. Rf value of plumbagin in a solvent system of toluene: ethyl acetate was observed to be 0.58 with volume amount ratio of 9.3: 0.7 for leaf, root and standard plumbagin.

Micropropagation of *Plumbago zeylanica* for callus initiation in MS with TDZ resulted in the production of callus within 10-15 days and was fully grown on day 40. Later the callus was subcultured for rooting in a different medium of IAA, IBA and NAA. It was observed that NAA medium was the second-best medium after TDZ medium for callus growth with the initiation of roots as seen in Fig. 2. Comparative TLC analysis of plumbagin (Fig.3) from different parts of the plant, callus with different medium and commercial Chitrak powder showed a comprehensive picture of the presence of considerable amounts of plumbagin. The commercially collected sample of plumbagin source named as Chitrak failed to show the presence of plumbagin or it may be in an undetectable amount as seen in Fig. 3. For better support, the plumbagin amount was analysed using a preparative TLC. The quantification of plumbagin with the help of spectroscopy has supplemented the observed result. Using the slope of standard plumbagin (*y=0.1734x+0.0456)* it is observed that 2ppm of IAA has 52.09±2.02 mcg/g, 1ppm of IBA has 99.58±2.33 mcg/g and 1ppm of NAA has 200.50±2.90 mcg/g amount of plumbagin present. The data was useful enough to interpret that NAA medium is best to obtain higher amounts of plumbagin as compared to IAA and IBA.

Conflict of interest

None declared

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