Journal of Academia and Industrial Research (JAIR)
Volume 5, Issue 9 February 2017

ISSN: 2278-5213

RESEARCH ARTICLE

Phytochemical, HPTLC Finger Print Analysis and Antimicrobial Activity of Ethyl Acetate Extract of *Decalepis hamiltonii* (Wight & Arn.)

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Abstract

The present study is aimed at exploring the phytochemical analysis, HPTLC fingerprint, TLC photo documentation and antimicrobial activity of the ethyl acetate root extract of *Decalepis hamiltonii* (Wight & Arn.). The preliminary phytochemical screening was performed with ethanol, methanol, acetone, petroleum ether and ethyl acetate extract. Presence of carbohydrates, amino acids, proteins, tannins, phenols, flavonoids, alkaloids, saponins, steroids and glycosides were observed in the preliminary screening. HPTLC fingerprinting showed 9 peaks at 254 nm, the TLC photo documentation showed 9 visible spots under 254 nm, 9 spots under 366 nm and 9 spots after derivatization with vanillin-sulphuric acid reagent. The antimicrobial activity of ethanol, methanol, acetone, petroleum ether, ethyl acetate root extract of *D. hamiltonii* was carried out on gram positive and gram negative bacterial strains and two fungal strains. Among the tested organisms, *Aspergillus niger* was found to be the most sensitive organism followed by *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida albicans* and *Staphylococcus aureus* with maximum zone of inhibition ranging from 8-3 mm.

Keywords: *Decalepis hamiltonii*, phytochemical screening, HPTLC fingerprint, ethyl acetate, antimicrobial activity.

Introduction

Plants are used as medicine since time immemorial. World Health Organization (WHO) estimated that about 20,000 plant species were being used as medicine from all parts of the world out of 3,50,000 existing plant species on earth (Shivaa and Neelakantan, 2001). The secondary metabolites such as, alkaloids, flavonoids, terpenoids, anthocyanins, phenolic acids, saponins, lignins, etc., are the reason for various pharmacological properties (Ahmad *et al*., 2007). Among the modern analytical tools, HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. HPTLC is playing an important role in the present analytical world (Andola and Purohit, 2010). Medicinal plants often contain additional active principles other than the major active principles and physiologically inert substances like cellulose and starch. As the constituents derived from the medicinal plants proved to cure the human disorders, they are isolated and used for their pharmacological actions. The constituents having particular therapeutic effects are identified and isolated (Ali *et al*., 1990). Hence, the modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardization of herbals and its formulations (Anonymous, 1978).

The WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled techniques and applying suitable standards (Ahmad *et al*., 2007). Chromatographic fingerprinting techniques are most significant methods which can be used for the routine herbal drug analysis and for quality assurance. HPTLC offers better resolution and estimation of active constituents can be done with reasonable accuracy in a shorter time (Tambe *et al*., 2013). High-performance thin layer chromatography (HPTLC) based methods could be considered as a good alternative, as they are being explored as the significant tool in routine drug analysis. Major advantage of HPTLC is, its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with same or different parameters (Sethi, 1996). Since ancient period, people used various plants sources for comparing infection diseases through Antimicrobial studies (Jane, 2014).

The world is now looking towards India for new drugs to manage various challenging newer diseases because of its rich biodiversity of medicinal plants and abundance of traditional cure systems such as Siddha, Ayurveda, Unani and Naturopathy to cure diverse diseases (Salahudhin *et al*., 1998). Various infectious diseases caused by microbes can be treated with plants extracts or decoctions from various plants origins. The use of plant preparations as foodstuff, insecticides, antitumour and antimicrobial agents is some examples of immense chemical diversity in plants (Balick *et al*., 2000).

Decalepis hamiltonii (Wight & Arn.) belonging to family Apocynaceae is one of the most important medicinal plants presently facing local extinction. It is endemic to Peninsular India and known by its names Makali beru or Vagani beru in Kannada and Magali kizhangu in Tamil. The roots of this plant are used in Ayurvedic medicines and for pickling as well as to make healthy cool drinks. The plant derived its name *D. hamiltonii* is from the word 'swallow-root' which means handsome. This plant has been a source of medicine since ancient period. The plant grows in a sandy-loam soil, in mixed deciduous forests in sunny areas. It occurs in thickets, forest of rock edges and boundaries up to height of 2530 m. It has been recorded in the dry and moist deciduous forests of Karnataka (Hassan, Mysore, Bellary, Tumkur and Kolar), Andhra Pradesh (Kurnool, Chittoor, Nellore, Anantpur and Cuddapah districts) and Tamil Nadu (Chengalpattu, Coimbatore, Erode, Dharmapuri, Nilgiri and Salem) at an altitude of 300-1200 m. Today it is under cultivation in fairly large areas of India, but seen less in Kathiri hills of Tamil Nadu. The roots are being used in Ayurveda, the ancient Indian system of medicine, to stimulate appetite, relieve flatulence and as a general tonic (Vadavathy, 2004). The roots are also used in folk medicine as general vitaliser and blood purifier. *Decalepis hamiltonii* roots are used as a substitute for *Hemidesmus indicus* in Ayurvedic preparations because of the similar aromatic properties (Nayar *et al*., 1978). It is also used as demulcent, diaphoretic, diuretic and tonic. It is useful in the loss of appetite, fever, skin disease, diarrhoea and in nutrition disorders (Wealth of India, 1990). It is also used in treatment of epilepsy and central nervous system disorders (Murti and Seshadri, 1942). People chew the roots against indigestion. This root extract is taken orally to rejuvenate the body (Reddy *et al*., 2006). The root powder is applied to treat wounds and bronchial asthma (Manivannan, 2010). The root extract of *D. hamiltonii* has been shown to contain significant antidiabetic and hepatoprotective properties (Naveen and Khanum, 2010). Recently it has been reported that the root of *D. hamiltonii* possess diuretic properties (Arutla *et al*., 2012). The main objective of this present study is to find out phytochemical analysis, HPTLC fingerprint, TLC photo documentation and antimicrobial activity of the ethyl acetate root extract of *Decalepis hamiltonii* (Wight & Arn.).

Fig. 1. Root of *Decalepis hamiltonii* (Wight & Arn.).

Materials and methods

Plant material: The plant root of *Decalepis hamiltonii* (Wight & Arn.), were from collected in Kathiri Hills, Erode district of Tamil Nadu. The plants were identified and authenticated by referring the standard taxonomic characteristic features according to the Flora of Madras Presidency (Gamble, 1935) and the Flora of Tamil Nadu Carnatic (Mathew, 1983). The voucher of specimen (HPRKVK 2013-199) was deposited in the Department of plant biology and plant biotechnology, Presidency College, University of Madras, Chennai, TN, India for future reference.

Preparation and extraction of plant material: The root of *Decalepis hamiltonii* was used for the preparation of extracts. The plant material was cut into small pieces, washed in water and dried at 40^0C. The dried plant pieces were then ground in mechanical grinder. The powder was sieved using a mesh sieve and stored in air tight bottles. About 50 g of the leaf powder was taken in a Soxhlet apparatus. The following series of solvents were used for the extraction: Ethanol, methanol, acetone, petroleum ether and ethyl acetate. All solvents (250 mL) used were of analytical grade (AR). The extraction carried was of hot type and was for about 48 h in each solvent. Before the successive solvent extraction, each time the powdered material was air dried below 50^0C.

Phytochemical screening

The phytochemical investigation of the different extracts of *Decalepis hamiltonii* was carried out using standard protocol (Harborne, 1973; Sofowora, 1993).

Tests for carbohydrates (Benedict's test): Crude extract when mixed with 2 mL of Benedict's reagent and boiled, a reddish brown precipitate formed which indicated the presence of the carbohydrates.

Tests for amino acids (Ninhydrin test): For the analysis of amino acid, 3 mL test solution and 3 drops 5% Ninhydrin solution were heated in water bath for 10 min. Observed for purple or bluish colour, the appearance of colour indicates the presence of amino acids.
Tests for proteins (Biuret test): About 3 mL of each test solution was added to 4% NaOH and few drops of 1% $CuSO_4$ solution into separate tubes. The tubes were observed for violet or pink colour formation.
Tests for Tannins (ferric chloride test): With 2-3 mL test solution, 5% $FeCl_3$ solution was added for bluish black colour indicates the presence of tannins.
Test for phenolic compounds (ferric chloride test): The extract was diluted to 5 mL with distilled water. To that a few drop of neutral 5% ferric chloride solution was added. A dark green color indicates the presences of phenolic compounds.
Detection of flavonoids (lead acetate test): The extracts were treated with few drops of 10% lead acetate solution. The formation of yellow precipitate confirmed the presence of flavonoids.
Tests for alkaloids (Wagner's test): About 2-3 mL extract was taken into separate tubes. To that few drops of Wagner's reagent was added and observed for reddish brown precipitate.
Test for saponins (Foam test): About 2 mL of extract was taken into test tube, shaken well, form confirms the presence of saponins.
Tests for steroids (Salkowski reaction): To 2 mL of sample, 2 mL chloroform and 2 mL Conc. H_2SO_4 were added and observed chloroform layer for red colour and acid layer for fluorescence.
Test for glycoside: To 2 mL of plant extract, 1 mL of glacial acetic acid and 5% ferric chloride was added and then a few drops of conc. sulphuric acid were added. Presence of greenish blue colour indicates glycosides.

HPTLC fingerprinting profile: High Performance Thin Layer Chromatography (HPTLC) studies were carried out following the method of Wagner and Baldt (1996) and Harborne (1998). The extracts of ethyl acetate were used in HPTLC fingerprinting profile analysis performed as per the methods described by Sethi (1996).
Sample preparation: Chloroform and ethyl acetate and 90% ethanolic extracts obtained were evaporated under reduced pressure using rotovac evaporator. Each extract residue was redissolved in 1 mL of chromatographic grade chloroform, ethyl acetate and 90% ethanol, which was used for sample application on precoated silica gel 60 F 254 aluminium sheets.
Developing solvent system: The TLC profile of ethyl acetate extract of *D. hamiltonii* was visualized under UV 254 nm, 366 nm and the photodocumentation was done using visualize (CAMACIS). The TLC profile under UV 254 nm, UV 366 nm and after derivatization with Vanillin-sulphuric acid reagent was calculated. The R_f value was calculated using the formula:

$$R_f\ Value = \frac{Distance\ travelled\ by\ the\ sample}{Distance\ travelled\ by\ the\ solvent}$$

Antimicrobial activity: Antimicrobial activities of ethanol, methanol, acetone, petroleum ether and ethyl acetate *Decalepis hamiltonii* root extract were determined by well diffusion method (Anushia *et al*., 2009). Four bacterial strains and two fungal strains were tested namely *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 15380) and two fungal strains *Aspergillus niger* (MTCC 3557) and *Candida albicans* (MTCC 227) procured from the Christian Medical College, Vellore, Tamil Nadu.

Preparation of inoculums: The media used for antibacterial test were nutrient broth. The test bacterial strains were inoculated into nutrient broth and incubated at 37^0C for 24 h. After the incubation period, the culture tubes were compared with the turbidity standard. Fungal inoculums were prepared by suspending the spores of fungus in saline water mixed thoroughly, made turbidity standard and used.
Antimicrobial susceptibility test: Bioassay was carried out by well agar diffusion method following Lalitha (2004). Fresh bacterial culture of 0.1 mL having 108 CFU was spread on nutrient agar (NA) plate using swab. The fungal strains were spread as same as the bacterial cultures, but the medium was Potato dextrose agar (PDA). Wells of 6 mm diameter were punched off on to medium with sterile cork borer and filled with 50 µL of plant extracts using micro pipette in each well in aseptic condition. Plates were then kept in a refrigerator to allow pre-diffusion of extract for 30 min. Further the plates were incubated in an incubator at 37^0C for 24 h and 28-30^0C for 3-4 d for bacterial and fungal cultures and respective solvent was used as control. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in mm. The same procedure was followed for the fungus also. The experiments were performed in triplicate.

Results and discussion

Phytochemical analysis: The medicinal value of the plants lies in their active substances that produce a definite physiological action on the human body. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, steroids, etc. (Britto and Sebastian, 2011). The various phytochemical tests were performed to know the secondary metabolites present in the roots of *D. hemiltonii* such as alkaloids, flavonoids, glycosides, saponins, steroids, tannins, phenolic compounds, proteins, amino acids and carbohydrates. The results obtained clearly evident the presence of good amount of carbohydrates, tannins, phenols, flavonoids, alkaloids, saponins, steroids and glycosides in all the solvent extracts, maximum phytochemicals were present in ethyl acetate extract hence it was selected for HPTLC studies (Table 1).

Table 1. Phytochemical screening of root extracts of *Decalepis hamiltonii.*

Root extracts	Ethanol	Methanol	Acetone	Petroleum ether	Ethyl acetate	Aqueous
Carbohydrates	-	+	+	+	+	-
Amino acids	-	-	-	-	-	-
Proteins	-	-	-	-	+	-
Tannins	+	+	-	+	+	-
Phenols	-	+	+	+	+	-
Flavonoids	+	+	+	+	+	-
Alkaloids	+	-	+	-	+	-
Saponins	-	-	+	+	-	-
Steroids	+	+	+	+	+	-
Glycosides	+	+	+	+	+	-

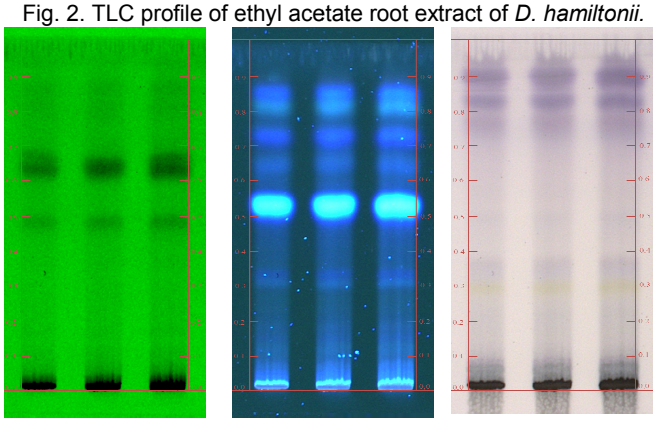
Presence (+); Absence (-).

Table 2. TLC profile of ethyl acetate root extract of *Decalepis hamiltonii.*

UV 254 nm		UV 366 nm		Vanillin-sulphuric acid	
Colour	Rf value (s)	Colour	Rf value (s)	Colour	Rf value (s)
Dark	0.02	Blue	0.02	Dark	0.02
Dark	0.04	Light green	0.04	Light violet	0.08
Dark	0.05	Light green	0.3	Light yellow	0.3
Dark	0.48	Fluorescent blue	0.5	Light violet	0.37
Dark	0.5	Dark grey	0.6	Light violet	0.5
Dark	0.6	Light green	0.63	Light violet	0.73
Dark	0.69	Dark blue	0.71	Violet	0.81
Dark	0.8	Light green	0.81	Violet	0.89
Dark	0.9	Dark blue	0.83	Grey	0.94

High Performance Thin Layer Chromatography: HPTLC photodocumentation profile of ethyl acetate extract of *Decalepia hamiltonii* under UV 254 nm, UV 366 nm after derivazation with vanilin sulphuric acid reagent are shown in Fig. 2. The R_f values and colour of the spots are shown in Table 2. TLC profile shows different types of constituents which is evident from the colour of the spots under UV 254 nm, the profile spots at R_f 0.02, 0.04, 0.05, 0.48, 0.5, 0.6, 0.69, 0.8 and 0.9 appeared as major spots and VU 366 nm, spots at R_f values 0.02, 0.04, 0.3, 0.5, 0.6, 0.63, 0.71, 0.81 and 0.83 are seen. In the derivatization with vanilin sulphuric acid reagent, the spots at R_f 0.08, 0.5, 0.73, 0.81 and 0.89 may be steroid or triterpene or their glycoside and the spot at R_f values 0.3 and 0.37 may be flavonoid or flavonoid glycoside. According to the HPTLC finger print profile of ethyl acetate extract of *D. hamiltonii* root, nine different chemical components were present (Table 3). These components with R_f values 0.03, 0.24, 0.29, 0.40, 0.51, 0.63, 0.77 and 0.84 were found to be more predominant as the intensity of peak area was with 882.8, 692.4, 2600.7, 1511.8, 1030.5, 12560.4, 10261.7 and 16338.0 AU respectively. The remaining components were found to be very less in quantity as the Rf values of AU for all other peaks were less than 882.8, 692.4, 2600.7, 1511.8, 1030.5. The ethyl extract exhibited 8 spots indicating the occurrence of at least 8 different components separated in the solvent system (Table 3 and Fig. 3). The peak at 0.28 and 0.34 may be flavonoid and their glycosides. The peaks at 0.06, 0.47, 0.57, 0.75 and 0.81 may be steroid or triterpene or glycoside.

Fig. 2. TLC profile of ethyl acetate root extract of *D. hamiltonii.*



UV 254 | UV 366 nm | Visible light (Derivatization with Vanillin-Sulphuric acid).

Fig. 3. HPTLC fingerprint of EA root extract of *D. hamiltonii.*

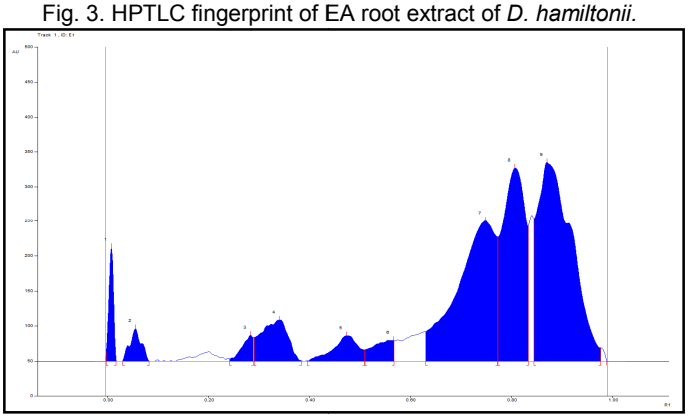


Table 3. HPTLC finger profile and Rf Values of ethyl acetate root extract of 15 µL of Et at 575 nm.

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	Area %
1	-0.00 Rf	13.9 AU	0.01 Rf	162.2 AU	14.30%	0.02 Rf	1.8 AU	1244.9 AU	2.64%
2	0.03 Rf	0.3 AU	0.06 Rf	46.4 AU	4.09%	0.08 Rf	0.7 AU	882.8 AU	1.87%
3	0.24 Rf	3.8 AU	0.28 Rf	37.2 AU	3.28%	0.29 Rf	33.8 AU	692.4 AU	1.47%
4	0.29 Rf	34.1 AU	0.34 Rf	59.0 AU	5.20%	0.38 Rf	0.7 AU	2600.7 AU	5.52%
5	0.40 Rf	0.4 AU	0.47 Rf	36.6 AU	3.23%	0.51 Rf	16.4 AU	1511.8 AU	3.21%
6	0.51 Rf	16.5 AU	0.57 Rf	29.9 AU	2.63%	0.57 Rf	29.7 AU	1030.5 AU	2.19%
7	0.63 Rf	42.2 AU	0.75 Rf	200.7 AU	17.70%	0.77 Rf	77.4 AU	12560.4 AU	26.65%
8	0.77 Rf	177.4 AU	0.81 Rf	277.2 AU	24.44%	0.83 Rf	92.5 AU	10261.7 AU	21.78%
9	0.84 Rf	204.6 AU	0.87 Rf	285.0 AU	25.13%	0.96 Rf	19.1 AU	16338.0 AU	34.67%

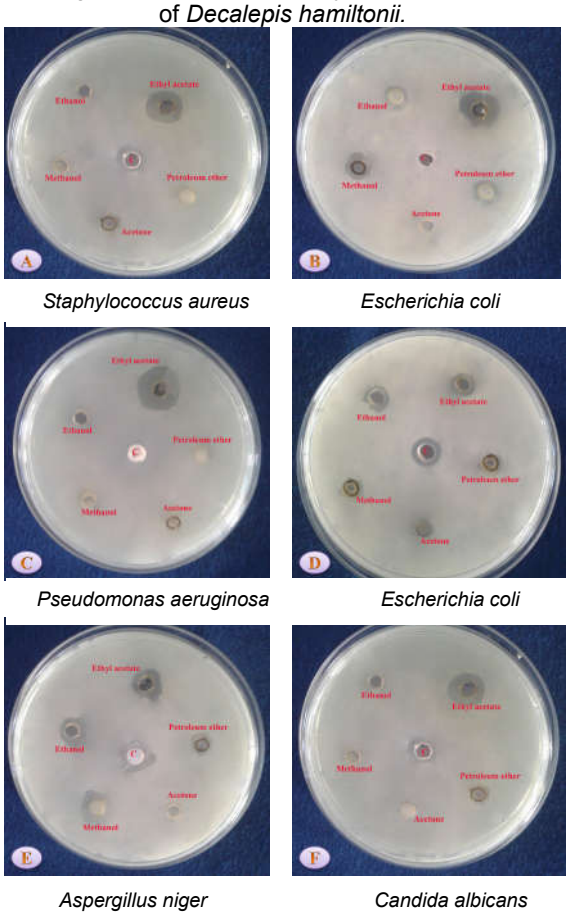
Table 4. Antimicrobial activity of root extracts of *D. hamiltonii.*

Microorganisms	Ethanol	Methanol	Acetone	Petroleum ether	Ethyl acetate
Staphylococcus aureus	-	-	-	-	5.0±0.1
Escherichia coli	4.0±0.1	6.0±0.0	-	5.0±0.1	7.0±0.0
Pseudomonas aeruginosa	-	-	-	-	8.0±0.1
Klebsiella pneumoniae	3.0±0.0	5.0±0.1	-	5.0±0.1	4.0±0.0
Aspergillus niger	6.0±0.1	6.0±0.0	-	4.0±0.0	5.0±0.1
Candida albicans	-	-	-	4.0±0.1	6.0±0.0

Values include cup border diameter (6 mm); Values are mean of three replicates (*Mean ± SD; n=3).

Antimicrobial activity: Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Iwu *et al*., 1999). Maximum zone of inhibitions was observed in *Pseudomonas aeruginosa* in ethyl acetate *Decalepis hamiltonii* root extract recording maximum zone of inhibition of 8.0±0.1 mm. Moderate inhibition was observed in ethyl acetate extracts of *E. coli*, *C. albicans*, *S. aureus*, *A. niger*, *K. pneumoniae* recording 7.0±0.0 mm, 6.0±0.0 mm, 5.0±0.1 mm, 5.0±0.1 mm and 4.0±0.0 mm respectively. Minimum zone of inhibition was recorded against *K. pneumoniae* (3.0±0.0 mm). No zone of inhibition was observed in acetone extract (Table 4 and Fig. 4).

Fig. 4. Antimicrobial activity of root extracts of *Decalepis hamiltonii.*



Staphylococcus aureus *Escherichia coli*

Pseudomonas aeruginosa *Escherichia coli*

Aspergillus niger *Candida albicans*

Conclusion

Phytochemical analysis of the root extract of *Decalepia hamiltonii* revealed the presence of carbohydrates, amino acids, proteins, tannins, phenols, flavonoids, alkaloids, saponins, steroids and glycosides. According to the HPTLC finger print profile of ethyl acetate extract of *D. hamiltonii* root, nine different chemical components were present. Maximum zone of inhibitions was observed in *Pseudomonas aeruginosa* in ethyl acetate *Decalepis hamiltonii* root extract recording maximum zone of inhibition of 8.0±0.1 mm. The isolation of pure compounds and determination of the bioactivity of individual compounds will be further performed.

Acknowledgements

Authors are extremely grateful to Dr. R. Dhamotharan, HOD and Dr. S. Ravikumar, professor, PG and Research Department of Plant Biology and Plant Biotechnology, Presidency College (Autonomous), University of Madras, Chennai, India for their immense guidance and support.

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