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In vitro Callus Induction and Antimicrobial activity of *Leucas vestita* Benth – an endangered species of Tamilnadu, India.

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ABSTRACT

Medicinal and aromatic plants have been the basis for traditional medicine systems. Plant derived drugs are preferred over their synthetic counter parts for various reasons such as availability, efficacy, affordability and less or no side effects. These plant drugs (phytochemicals/secondary metabolites) are produced at varying amounts in different cells, tissue types and organelles within the plant. *L. vestita* is widely used in countryside as food and also for nutritional requirement. The plant has been reported to have high content (21.3%) of protein and significant amounts of total carotenoid and β-carotene. *L. vestita* is used as substitute in jaundice, anorexia, cough, dyspepsia, asthma, conjunctivitis, diabetes, otalgia, skin diseases, scabies, toothache, wound healing, for analgesic, as anti-inflammatory, anitipyretic, stimulant, expectorant, aperients, diaphoretic, anti-rheumatic and leaves possess insecticidal activity.In spite of the medicinal importance and nutritional value of *L.vestita*, and voluminous literature available on bioprospecting of this plant, no published reports are available till-date in the pertinent literature describing micropropagation or in vitro plant regeneration of this plant. In view of the medicinal importance of *L.vestita* and the abundant occurrence of secondary metabolites, the main goal of the present study was to develop a simple and reliable protocol for the rapid in vitro propagation of *L.vestita*, that would be used as not only an effective strategy for its germplasm conservation and multiplication, but also an alternative means for the year-round production of its saponins which have medicinal and pharmacological value. The present study focused to develop callus induction from leaf and stem explants of *Leucas vestita* Benth and to evaluate its anti-microbial activity. This paper reveals the

conservation method of endangered species like *Leucas vestita* through plant tissue culture which has not been reported.

Keywords- *Leucas vestita*, callus induction; endangered species.

1.Introduction

Medicinal and aromatic plants have been the basis for Indian traditional medicine systems. Plant derived drugs are preferred over their synthetic counter parts for various reasons because of ease of use, effectiveness and less or no side effects. These plant drugs (phytochemicals/secondary metabolites) are produced at varying amounts in different cells, tissue types and organelles within the plant. The valuable medicinal properties of plant materials usually the secondary metabolites like alkaloids, flavonoids, tannins resins, fatty acids, steroids, etc (Chitra Jain *et al* 2018)

Leucas vestita Wall. ex Benth. (Lamiaceae) is endemic to India and is mostly distributed in the hilly regions of Kerala and Tamil Nadu above 1000M altitude from sea level. The plant is phylogenetically related to *L. aspera*. A perusal of published literature shows that *L. vestita* is less explored concerning its phytochemical and pharmacological characters. *Leucas vestita* ex Benth has become an endangered species, which has to be conserved and preserved for future hence been taken for the study. *L. vestita* is widely used in countryside as food and also for nutritional requirement. This plant as high substance (21.3%) of protein (Prakash et al., 1988) and major amounts of total carotenoid and β-carotene (Rajyalakshmi et al., 2001). *L. vestita* is used as substitute (Kurup et al., 1979; Garg, 1992; Sharma, 1996; Anonymous, 2003) in jaundice, anorexia, cough, dyspepsia, asthma (Singh et al., 2002; Tiwari and Yadav, 2003; Mahishi et al., 2005; Revathi and Parimelazhagan, 2010), conjunctivitis, diabetes, otalgia, skin diseases, scabies, toothache, wound healing (Nadkarni, 1954; Chopra et al., 1958; 1962; Khory and Katrak, 1996), for analgesic (Reddy et al., 1993), as anti-inflammatory (Ghani, 1998; Saundane et al., 2000), anitipyretic, stimulant, expectorant, aperients, diaphoretic, anti-rheumatic and leaves possess insecticidal activity (Ganesan et al., 2007).

Despite the medicinal properties and nutritional benefits of *L.vestita*, and enormous literature available on bioprospecting of this plant, no published reports are available till-date in the relatable literature describing micropropagation or invitro study of this plant. In the view of the medicinal importance of *L.vestita* and the

abundant amount of secondary metabolites, the main goal of the present study was to develop a simple and reliable protocol for the rapid invitro callus induction of *L.vestita*, that would be used as not only an efficient strategy for its germplasm conservation and multiplication, but also an another means for the perennial production of its flavonoids which have medicinal and pharmacological value.

The present study focused to develop callus induction from leaf explants of *Leucas vestita* Benth and to evaluate the anti-microbial activity of *Leucas vestita* Benth.

2. Materials and Methods

2.0 Explant Sterilization

Prior to the collection of explants the cuttings were sprayed with 0.5% bavistin and 0.04% streptomycin (two days before explant collection). Juvenile shoots were collected for callus induction. The explants that were collected include the leaf, stem and also the internodal regions. They were cut using a sterile blade and placed in distilled water to avoid drying of the explants. The size of the explants were about 3-4 cm during collection. All the experiments were carried out in the Laminar Air Flow Chamber under sterile conditions. The leaf explants were thoroughly washed under running tap water for 30 min to remove the external surface dust particles. The explants were washed with Bavistin for 1 hr followed by thorough washing with 10% (w/v) Tween 20 for 20 min. Subsequently the explants were washed with 1% (w/v) Sodium hypochlorite for 2 min and then they were disinfected with 0.3 % (w/v) aqueous mercuric chloride solution for 10 min, and rinsed 5 times with sterile distilled water, and blotted dry on sterile Whatmann No. 1 filter paper for *in vitro* culture experiment. The explants were placed on to MS medium for callus induction with auxins in the dark condition. (Murashige et al, 1962). The explants were cultured on MS medium prepared in culture tubes (25x150mm) and plugged tightly with non-absorbent cotton.

2.1. Culture Conditions

In the present study, MS salts containing 3% (w/v) sucrose as a carbon source were used in MS medium with different plant growth regulators. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl prior to adding 7.0 g/l agar and autoclaved at 121ºC for 20 min. All the cultures were maintained under

16/8h in (light/dark) photoperiod with a light intensity of 60µ E mol m^{-2} s^{-1} (cool white fluorescent light) and maintained at controlled temperature (25 ± 2ºC) (Dharishini *et al* 2014)

2.2 Induction of callus

In the present study, leaf explants were cultured on MS medium supplemented with various concentrations of 2,4-D (0.5-4.0 mg L^{-1}) in combination with BAP (1.0 mg L^{-1}) for callus induction. The induction of the callus generally starts with the bulging in the leaf and also in the internodal regions. The bulging was generally visible from the seventh day after the inoculation. And the induction of the callus was generally visible from the 14th day onwards.

2.3 Antibacterial activity:

Leucas vestita Benth. leaf extract is active against a range of microorganisms. This activity is confirmed by testing its antibacterial activity against four bacterial species namely *Escherichia coli, Staphylococcus aureus*, *Staphylococcus epidermidis,* and *K. pneumoniae.* Now the cultures of the bacterium were taken into the laminar flow hood and then they were spread over the surface of the LB media in the plates. Discs of Whattman filter paper were cut and sterilized previously. These discs were taken and dipped into the plant extract and callus extract and was kept on the media after the cultures of microorganisms were spread over it. Then the petriplates were wrapped and then it was kept overnight at room temperature for the growth of the bacterium and the formation of the inhibition zones.

Statistical Analysis

Experiments were setup in a randomized complete block design and data were collected from 2 experiments with 20 replicates. The analysis of variance (ANOVA) was carried out using SAS program by recorded the percent of response, number of shoots per explants, shoot length and number of roots per shoot. The differences in mean were analyzed by Student-Newman-Keuls Test at the P≤0.05 significance level.

Result and Discussion

Source for plant material

The explants collected from the cuttings kept in the green house of the Padmavani Arts and Science College for Women, Salem-11, Tamilnadu, India. The explants seem to respond with good callus induction.

The time replication protocol gave good results with large green fragile callus after 14 days of incubation. The callus bulging was observed after 7 days. The callus percentage is more in leaf explant segments, than in stem or internodal segments.

Surface Sterilization

The explants were carried out in various stages of sterilization process. The explants were washed using surfactant Tween 20 with two drops for 5 minutes. They were rinsed several times to remove the debris on the plant surface. The type of explants taken for the culture initiation was leaf segment. The leaves responded depending upon the nature of the texture. The young leaves responded quickly with good cell enlargement and bulging, whereas matured leaves showed very less cell bulging. The leaf explants observed to respond with 70% of callus induction, with green colored fragile callus. The indication for callus was observed from the 7^{th} day. Good callus formation was noted on the 14^{th} day from inoculation.

Standardization of Antibiotic Treatment

Both antibacterial and antifungal treatments were given for the explants. Depending upon the nature of the explants, they were carried out in separate glassware for treatments. To overcome bacterial contamination on inoculation streptomycin with 0.5 mg/l and Kanamycin of 0.25 mg/l was standardized. Fungal contamination were overcome by using Benomyl 0.25 mg/l and bavistin 0.5 mg/l. the treatment were kept in rotatory shaker for 30 – 45 minutes at 125 rpms. 30 minutes was optimized as standard time interval for the explants to reduce bleaching of culture. After antibiotic treatment, they were treated with $HgCl_2$ surface sterilization process.

Standardization of $HgCl_2$

Young leaves and nodes were given 1minute treatment with 0.1% of $HgCl_2$ and large leaves were given 2 minute of $HgCl_2$ treatment, with 0.1% concentration. This treatment resulted in lesser callus induction. Hence, 0.05% $HgCl_2$, was used to avoid cell death and bleaching of explant. Good result with callus induction was seen right from the 7^{th} day onwards. The explants remained green without browning. After $HgCl_2$ treatment, the removal of mercuric traces on the plant material plays a vital role, to less down blackening of explant due to mercuric exposure. The traces of mercury were removed by rinsing several times with double distilled water in laminar airflow chamber. 5 minutes double distilled water wash was repeated 4 times i.e., around 20 minute washing in the water was carried to remove the waste debris. Later inoculated into MS media combination of plant growth regulators.

Influence of growth hormone on explants

The leaves expressed very less callus induction. Only bulging of the cell was observed in various combination with 2, 4, D and NAA concentration.

The leaf explants responded with higher callus induction, in the presence of NAA in all the three replications. High amount of callus induction was observed with NAA with BAP with green calli in large amount. The concentration of NAA that was tried in the experiments fall in the range of 0.5 -3 mg/l. BAP concentrations were tried with 0.25-2.5 mg/l. Rapid callusing of leaf explants was observed in combination of 1 mg/I NAA and 0.25 mg/l BAP. With 1 mg/l NAA, 0.5 mg/l BAP was observed to be the best for callus formation.

The callus formation from leaf segment was carried out in shoot regeneration plant growth regulators, mainly cytokinin for the production of shoots. 2.5 mg/l BAP when combined with 0.5mg/l kinetin also resulted in the formation of green callus due to embryogenesis. The callus thus obtained was transferred to media containing cytokinin for further shoot proliferation.

Callus Formation The third replication of *Leucas vestita* Benth leaf explants collected responded with high callus induction with a 60% callus initiation on 14th day from inoculation, with 1 mg/l NAA and 0.5 mg /l BAP. The cytokinin BAP with higher concentration 2.5 mg/l combined with kinetin 0.5 mg/l resulted in embryogenesis. MS medium without plant hormones was used as control. It has been shown from the previous works in various other species of *Leucas vestita* Benth that it grows better in the MS media.

Table 1: Callus response in different plant growth regulators of *Leucas vestita* Benth leaf explant

MS + Plant Growth Regulators				Observation
2,4,D mg/l	**BAP mg/l**	**NAA mg/l**	**Kinetin mg/l**	
1	-	-	-	No response
3	-	-	-	No response
-	-	1	-	Slight bulging observed
-	-	3	-	Cell enlargement seen
1	0.25	-	-	Pale green, no response
1	0.5	-	-	Pale green, no response
3	0.25	-	-	Browning
3	0.5	-	-	Browning
-	**0.25**	**1**	**-**	**Green calli**
-	**0.5**	**1**	**-**	**Green calli , cell enlargement, good response**
-	0.25	3	-	No response
-	0.5	3	-	No response
0.25	-	0.25	-	No response
0.25	0.25	0.25	-	No response
-	**2.5**	**-**	**0.5**	**Green calli with embryo formation**
0.5	1	1	-	Callus, soon browning noticed
-	-	-	-	**Control**

Table 2: Percentage of callus induction with plant growth regulators combined with MS Media in *Leucas vestita*

MS Media Combination	I Replication % Callus Induction	II Replication % Callus Induction	III Replication % Callus Induction
1.	Nil	Nil	20
2.	Nil	Nil	20
3.	10	10	20
4.	20	20	10
5.	Nil	Nil	Nil
6.	Nil	Nil	Nil
7.	10	10	Nil
8.	10	10	Nil
9.	**20**	**30**	**10**
10.	**50**	**40**	**60**
11.	Nil	Nil	10
12.	Nil	Nil	10
13.	10	10	30
14.	10	10	Nil
15.	**20**	**40**	**40**
16.	Nil	30	10
17.	**Control**	**Control**	**Control**

Anti bacterial activity of *Leucas vestita* Benth

The anti bacterial activity of *Leucas vestita* was tested for various microorganisms and its efficiency was identified. The extract of the plant was taken with different solvents such as ethyl acetate, methanol and water. It was tested for its activity against a range of microorganisms such as *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis, K. pneumoniae.* (Umamaheswari *et al*, 2008). The bacterium was spread over the plate by spread plate technique and then the Whattman filter paper discs dipped in the plant extract was placed on the surface of the media. The petriplates were incubated at 37° C and it was incubated at the room temperature for the four different microbial species. It was found that the zones of inhibition formed only for *Staphylococcus aureus* and the diameter of it was found to be 2mm. Although zones of inhibition were also

found for *E.coli* and *Staphylococcus aureus* it was not found to be significant when compared to the zones obtained in the plates containing *Staphylococcus epidermidis.* Larger zone of inhibition of 14mm was recorded for *Staphylococcus aureus*. Antimicrobial activity showed increasing zone of inhibition with increasing concentration of the extract with *K. pneumonia, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* among the other microorganism.

4. Summary and Conclusion

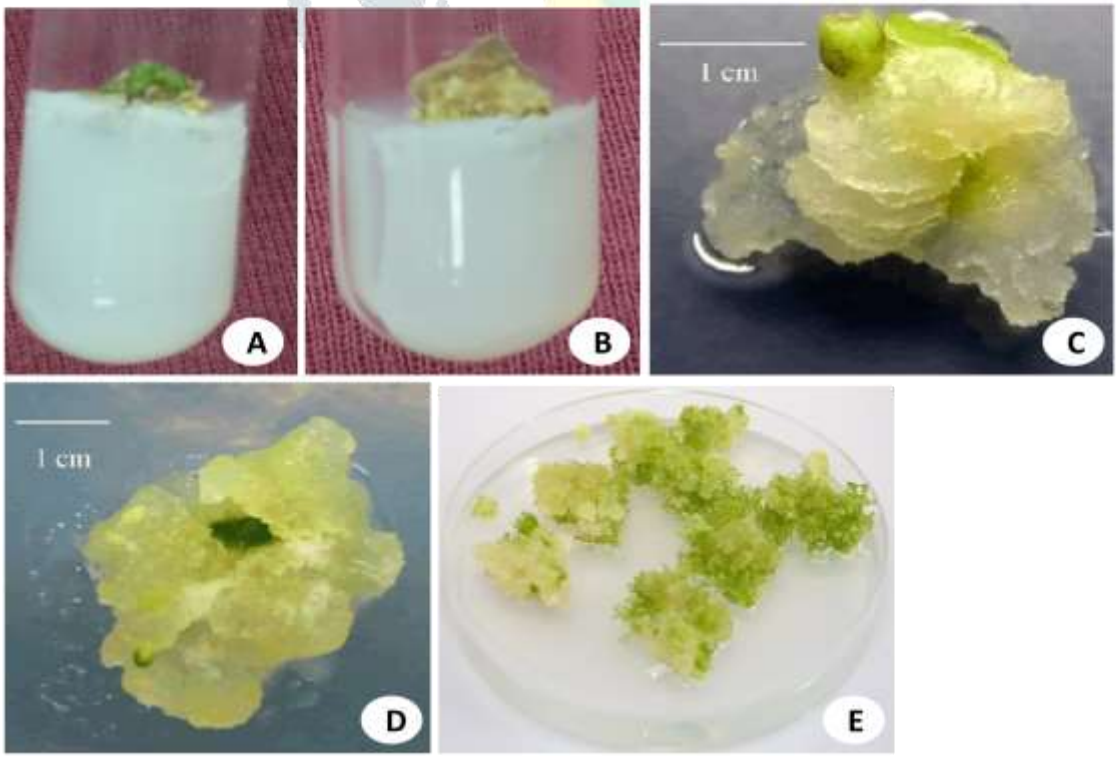
In summary, *in vitro* callus culture was achieved using leaf explants of *Leucas vestita* Benth. A reliable protocol was achieved by standardizing and by establishment of culture medium with contaminant free cultures. An efficient protocol for callus induction was achieved and the anti microbial activity of various microorganisms was studied and evaluated.

The antibacterial activity of the species *K. pneumonia, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* were experimental done. The results of this study reveal the fact that the organic solvent extract (methanolic extract) exhibited antimicrobial activity because of the antimicrobial principles present in it. Methanol is an effective solvent which can dissolve the active principle present in the plant. In conclusion, an efficient callus induction and proliferation protocol was established. The present study deal with plant tissue culturing and documentation of rare, endanger and threatened plants in Southern Western Ghats, Tamil Nadu, India. Finally it has been suggested that the medicinal plant *Leucas vestita* are need to be proper conservation and management plans before it is lost forever by tissue culturing. These results strongly suggest that the phytochemicals present in the plant extract leaves can act as antimicrobial agent and could be used as an alternative antimicrobial drug commercially in the near future.

Fig 1: Plant Profile

Leucas vestita

Fig 2. Invitro callus induction of *Leucas vestita* Benth from leaf explant



A. Inoculation of leaf explants in MS medium containing 1mg/l NAA.

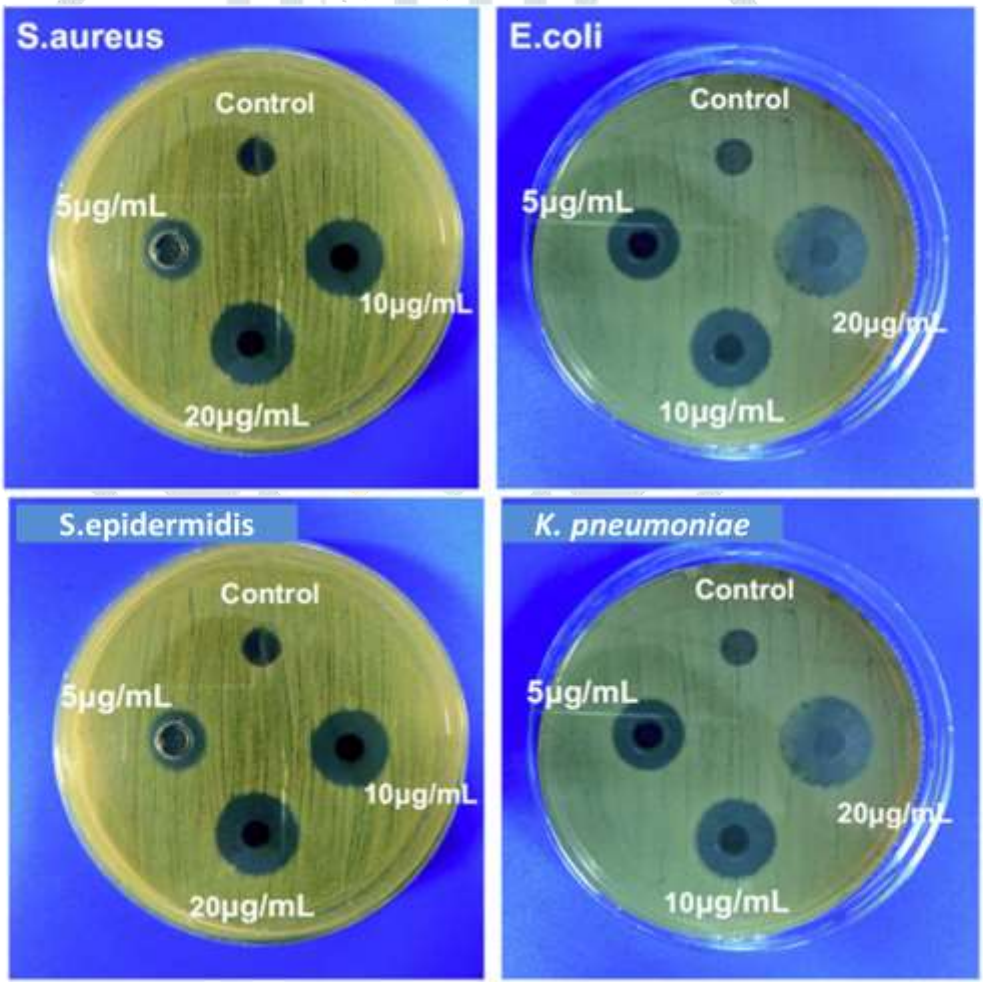
B. Callus Induction after 3 weeks of explants inoculation.

C & D. Callus proliferation after transferring to fresh medium containing MS medium with

1 mg/l NAA.

E. Multiple shoots emerging with MS medium containing BAP 0.5mg/l.

Fig 3. The anti-microbial activity of ***Leucas vestita*** Benth. from leaf callus culture.



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