APPENDIX I

Reagents preparation

MS Media (Murashige and Skoog, 1962) Stock preparation

Stock solution of organic and inorganic nutrients (Major, Minor, Iron and Vitamins) were prepared in double distilled water and stored in refrigerator. All stock solutions were prepared at twenty times (20 X) of their final concentration. The prepared stocks were stored at 4°C and the desired amount of concentrated stocks were mixed to prepare 1 litre of medium.

Plant Growth Regulators

In addition to the nutrients, necessary to add one or more growth substances (such as auxins, cytokinins, polyamines and gibberellins) to support better growth of tissues and organs. All plant growth regulators were added in medium before autoclaved.

Auxins

IAA, 2, 4-D and NAA dissolved in few drops of dilute NaOH and final volume made by sterile double distilled water (50 mL).

Cytokinins

Kinetin, ADS BAP and TDZ were dissolved in few drops of dilute NaOH or EtoH solution and final volume made by sterile double distilled water (50 mL).

Polyamines

Spermidine – a polyamine group of hormones prepared by dissolving in diluted NaOH (10 mL).

Gibberellins

Gibberellic acid (GA₃) was prepared in 95% ethanol.
## Composition of MS medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Chemical formula</th>
<th>Molecular weight</th>
<th>Amount (mg/L)</th>
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<tr>
<td><strong>Macro nutrients (Stock solution I)</strong></td>
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<tr>
<td>Ammonium nitrate</td>
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<td>Amount used (mg/mL)</td>
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<td>Gibberelic acid GA\textsubscript{3}</td>
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<td>145.2</td>
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</table>

Preparation of full strength MS medium

- Macro nutrient (Stock 1) – 50 mL
- Micro nutrient (Stock 2) – 5 mL
- Iron (Stock 3) – 5 mL
- Vitamins (Stock 4) – 5 mL
- Potassium iodide (KI) – 5 mL
- Inositol – 100mg
- Sucrose – 30g
- Agar – 7 g
All the ingredients of each stock aliquots were added and make up to 1000 mL with distilled water. Then, the pH values were calibrated using 0.1 N HCL and 0.1 N NaOH and (Agar may be dissolved in microwave oven) sterilized using autoclave at 15 lbs pressure at 121°C for 15 minutes. Plant growth hormones were added to each medium prior to autoclaving.

Preparation of $\frac{1}{2}$ strength MS medium

Macro nutrient (Stock 1) – 50.0 mL
Micro nutrient (Stock 2) – 5.0 mL
Inositol – 100.0 mg
Sucrose – 30.0 g

The above ingredients of MS stocks were added and make up to 1000mL with distilled water and prepared as above (Agar was eliminated for the liquid medium).

Sabouraud Dextrose Agar (SDA)

Peptone – 10.0 g
Dextrose – 20.0 g
Agar – 15.0 g
pH – 5.6 ± 0.2

Sabouraud Dextrose Broth (SDB)

Peptone – 10.0 g
Dextrose – 20.0 g
pH – 5.6 ± 0.2

Mueller Hinton Agar (MHA)

Beef-infusion – 30.0 g
Casein acid hydro lysate – 17.5 g
Starch – 1.5 g
Agar – 17.5 g
pH (at 25°C) – 7.3 ± 0.2
Mueller Hinton Broth (MHB)

- Beef infusions: 30.0 g
- Casein acid hydrolysis: 17.5 g
- Starch: 1.5 g
- pH (at 25°C): 7.4 ± 0.2

All the ingredients of each medium was separately dissolved in 1000 mL of distilled water. Adjust the pH values (7.4) and sterilized using autoclave at 15 lbs pressure at 121°C for 15 minutes.

Mayer’s Reagent

1.358 g of HgCl₂ was dissolved in 60 mL of d.H₂O and 5 g of KI was dissolved in 10 mL of d.H₂O. Then, the two solutions were mixed and made upto 100 mL with d.H₂O.

Fehling’s solution A

CuSO₄ (34.66 g) was dissolved in d.H₂O and made upto 500 mL using d.H₂O.

Fehling’s solution B

Potassium sodium tartarate (173 g) and sodium hydroxide (50 g) was dissolved in water and made upto 500 mL.

Ninhydrin solution

10 mg of ninhydrin was dissolved in 100 mL of distilled water.

5 % Ferric chloride solution

5 g of ferric chloride was dissolved in 100 mL of distilled water.

0.1 % Ferric chloride solution

0.1 g of ferric chloride was dissolved in 100 mL of distilled water.

Preparation of DPPH solution

0.004 mg of 1, 1-Diphenyl- 2 – picryl hydrazyl (DPPH) was dissolved in methanol. It was protected from light by covered with aluminium foil.
Preparation of 0.6 mM solution of ferrous chloride

0.009 mg of ferrous chloride (FeCl₂·4H₂O) was dissolved in 100 mL of d.H₂O

Preparation of 5mM solution of ferrozone

0.255 mg of ferrozone was dissolved in 100 mL of distilled water.

Preparation of folin ciocalteau

Dilute commercial reagent by adding equal volume of d.H₂O

Preparation of 20% sodium carbonate

20 g of sodium carbonate was dissolved in 100 mL of d.H₂O

Preparation of standard solution

10 mg of Ethylene diamine tetra acetic acid disodium salt (Na₂EDTA) was dissolved in 10 mL of methanol.

Preparation of sodium nitrite

5 g of NaNO₃ was dissolved in 100 mL of d.H₂O

Preparation of aluminium chloride

10 g of Al₂Cl₃ was dissolved in 100 mL of d.H₂O

Preparation of sodium hydroxide

4 g of NaOH was dissolved in 100 mL of d.H₂O

Preparation of CTAB buffer

2 g of CTAB was added with the mixture of 1.4mM NaCl, 20mM EDTA, 100mM Tris HCl, and adjusted the pH to 9.5
PUBLICATIONS LIST

Research papers

Book chapter
Regular Article

A Rapid Micropropagation of nodal explants of *Eclipta alba* (L.); A Multipurpose Medicinal Herb

C. Ragavendran, D. Kamalanathan and D. Natarajan*

Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem 636011, Tamil Nadu, India

*Corresponding author E-mail: mdnataraj@rediffmail.com, natarajpu@gmail.com

An efficient *in vitro* regeneration protocol was developed for medicinally important plant *Eclipta alba*. Successful regeneration and multiplication of nodal explants of *E. alba* were obtained in cytokinin enriched B5 medium. Several cytokinins [6-benzylaminopurine (BAP), kinetin (KIN), thidiazuron (TDZ), gibberellic acid (GA3) and spermidine] were supplemented alone and its combinations for obtaining better results. The best growth frequency response was achieved in the combinations of 1.0 BAP + 0.3 KIN + 1.5 GA3 (mg/L) concentration (7.4 ± 0.9 cm shoot length & 100 % regeneration). Better roots were developed in half-strength B5 medium along with IBA (1.0 mg/L) hormone and exhibits maximum root length (7.0 ± 0.8 cm) along with multiple roots (8.8 ± 0.8) at 92 %. The well-developed Plantlets were successfully acclimatized to plastic-cups containing autoclaved sand and garden soil (1:1) and kept undisturbed with plastic cover for maintaining the humidity. The plantlets were watered regularly and maintained at green house.

Keywords: *in vitro* propagation, *Eclipta alba*, Growth regulators

Abbreviations: BAP- 6-benzylaminopurine; KN - Kinetin; GA3 - Gibberellic acid; B5-Gamborg (1968); PGRs - Plant growth regulators; IBA -Indole-3- butyric acid; TDZ - Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-yl urea)

*Eclipta alba* (L.) Hassk. (Asteraceae) is a small, branched, annual herb with white flower heads, native to tropical and subtropical regions of the world. The plant is traditionally used in the treatment of liver diseases, skin disorders, premature graying of hair, and enhance the memory (Dhaka and Kothari, 2005; Banji *et al.*, 2007; Ray and Bhattacharya, 2010). The plant juice is used as a tonic and diuretic in hepatic and spleen enlargement (Baskaran and Jayabalain, 2005; Jadhav *et al.*, 2009; Singh *et al.*, 2010). The plant extracts are reported to contain several phyto-constituents like ecalbatin, alpha-amyrin, ursolic acid, oleanolic acid, ecliptasaponin, daucosterol, stigmasterol-3-Oglucoside etc. (Thakur and Mengi, 2005). The plant possesses many biological properties like anti-inflammatory and bronchodilator (Leal *et al.*, 2000) hepatoprotective and anti-hyperglycemic agent (Ananthi *et al.*, 2003), antiviral, antibacterial, spasmogenic, hypotensive, analgesic, antioxidant(Sharma *et al.*, 2001; Karthikumar *et al.*, 2007; Veeru *et al.*, 2009), cardioprotective (Baliga *et al.*, 2004) and antianaphylactic (Patel *et al.*, 2010) properties. There is an urgent need for development of
rapid and large scale production of this highly valuable medicinal herb using tissue culture technique.

*In vitro* culture techniques offer a viable tool for bulk multiplication of genetically identical plant material, large scale production and germplasm conservation of valuable plants (Tomar and Gupta, 1998). The *in vitro* technology could be a cost effective means of high-volume production of the elite planting material throughout the year, without any seasonal constrains (Ajithkumar and Seeni, 1998; Prakash *et al.*, 1999). Hence, the study was aimed to establish a rapid and reproducible *in vitro* regeneration system for *E. alba* using nodal explants on modified B5 medium.

**Materials and methods**

**Source of plant material**
The fresh and healthy young twigs (15–20 cm) of *E. alba* was collected (during November and December months) from Botanical Garden, Department of Biotechnology, Periyar University, Salem. Nomenclature of this plant was identified using standard floras. A voucher specimen was deposited in Natural Drug Research Laboratory (NDRRL), Department of Biotechnology, Periyar University, Salem.

**Surface sterilization and preparation of explants**
The nodal explants were excised aseptically from field grown plants (0.5 cm) and the surface sterilization of explants were done by standard procedures: Washed thoroughly (15 minutes) under running tap water to remove the dust particles and treated with 2 % (w/v) fungicide, (for 5–10 min) to reduce the chance of fungal contamination. About 2–3 drops of Tween–20 (Hi–Media, India) was used as surface sterilizing agents of explants followed by washing under running tap water for removal of detergents and disinfectants.

Subsequently, surface sterilization of the explants was carried out under aseptic conditions in laminar air hood. Freshly prepared Mercuric chloride aqueous solution [0.05% (w/v)] was used for 3–5 min. Then, rinsed thoroughly (3–4 times) with sterile distilled water to remove any traces of the disinfectant and edges were trimmed with sterile surgical blade. The explants were kept in sterile filter paper for drying before inoculation.

**Media used**

Modified B5 medium was used and prepared by adding aliquots of stock solutions along with 3 % sucrose as carbon source and 0.8 % agar for solidification. The pH of hormonal medium was calibrated between 5.5–5.8 using 0.1N NaOH and 0.1N HCl prior to autoclaving at 121 °C or 15 lbs for 15 minutes. Different concentrations of cytokinins and auxins were added to the modified B5 medium in combinations and alone for development of shoots and roots. The combined effect of growth hormones (BAP, Kin and gibberellic acid), were used for culturing the explants B5 medium supplemented with optimized concentration of BAP (0.5–1.0 mg/L) and different concentrations of Kin (0.3, 0.4 & 0.5 mg/L) or Gibberellic acid (1.0–1.5 mg/L) (Table1) respectively.

**Inoculation and incubation**

Nodal explants (1.0 cm) were cultured on different hormonal medium aseptically and incubated at 24 ± 2 °C under 16-h photoperiod, with light intensity of 40 mg/L μmol m⁻² s⁻¹ provided by cool white, fluorescent tubes. The regenerated shoot cultures were maintained (for 4 weeks period) and it was subcultured for multiple shoots formation. The elongation of multiple shoots was observed in same or improved medium (kept for incubation).
In vitro rooting and acclimatization
Well developed and matured shoots were excised and transferred to growth regulator (full and half strength B5 medium) for root induction and monitored regularly for root proliferation. The well-developed plantlets (5–10 cm) were carefully taken out from the medium and washed thoroughly in running tap water for removal of all medium traces. Plants were transferred to acclimatization tray or cups containing soil. Plantlets were covered with polyethylene bags to maintain high humidity and irrigated with tap water regularly and placed in greenhouse (for 7–8 weeks). Then, it was shifted to the nursery and finally transferred to the field and observe the survival rate of individual.

Statistical analysis
All the experiments were performed in triplicates (Number of Explants = 14) for mean and standard deviation (± SD) Values are calculated. The analysis of variance (ANOVA) and Duncan’s multiple range test (DMRT) were performed using INSTAT or SPSS 20 (IBM SPSS 20 statistics).

Results
Shoot regeneration and multiple shoots proliferation
The micropropagation results of nodal explants of E. alba, cultured on modified B5 medium were presented in Table 1 and figure 1. Nodal segments were cultured on B5 medium supplemented with cytokinins, BA, KIN and thidiazuron (0.5, 0.5 BAP + 0.5 KIN, 0.5BAP + 0.5 TDZ, 1.0 BAP + 0.5 S + 0.5 GA₃ and 1.0 BAP + 0.3 KIN + 1.5 GA₃ mg/L), reflect varied response for shoot induction. As per the observation, BA was found more effective than Kin and spermidine, (the number and frequencies of shoot formation (after 8 weeks) within two subcultured periods. The multiple hormones (1.0 BAP + 0.3 KIN + 1.5 GA₃ mg/L) were produced better shoot proliferation and elongation rates (7.4 ± 0.9 cm shoot length, 7.7 ± 1.1 multiple shoots) with 100 % regeneration, followed by other hormonal combinations.

<table>
<thead>
<tr>
<th>Hormone (mg/L)</th>
<th>% regeneration</th>
<th>Mean no of shoot length (cm)</th>
<th>Mean no of shoots</th>
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<tr>
<td>BAP KIN Spermidine TDZ GA₃</td>
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<tr>
<td>0.5 - - - -</td>
<td>42.85</td>
<td>3.1 ± 0.1d</td>
<td>2.6 ± 0.5f</td>
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<tr>
<td>0.5 0.5 - - -</td>
<td>71.42</td>
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<td>2.6 ± 0.0f</td>
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<tr>
<td>0.5 0.7 - - -</td>
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<td>2.0 ± 0.0g</td>
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<td>4.3 ± 0.4c</td>
<td>3.5 ± 0.5e</td>
</tr>
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<td>50.00</td>
<td>2.8 ± 0.9e</td>
<td>2.2 ± 1.2g</td>
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<tr>
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<td>64.28</td>
<td>2.8 ± 0.1e</td>
<td>2.6 ± 1.5f</td>
</tr>
<tr>
<td>1.0 - 0.5 0.5 0.5</td>
<td>92.85</td>
<td>4.0 ± 1.0c</td>
<td>4.6 ± 1.5d</td>
</tr>
<tr>
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<td>42.85</td>
<td>2.1 ± 0.0f</td>
<td>2.1 ± 0.8g</td>
</tr>
<tr>
<td>0.5 0.5 0.5 0.5</td>
<td>57.14</td>
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</tr>
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<td>2.4 ± 1.0e</td>
<td>2.0 ± 1.0g</td>
</tr>
<tr>
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<td>92.85</td>
<td>6.7 ± 1.8b</td>
<td>6.5 ± 2.0b</td>
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<td>6.1 ± 1.2b</td>
<td>5.8 ± 1.8c</td>
</tr>
<tr>
<td>0.5 0.5 0.5 0.5</td>
<td>71.42</td>
<td>3.7 ± 1.0d</td>
<td>4.4 ± 0.8d</td>
</tr>
</tbody>
</table>

- = No growth regulators added. The values are mean within a column followed by the same letter are not significantly different by Duncan’s multiple range test (p > 0.05). * Values corresponds to means [± standard error (SE)] of three independent experiments. For each experiment 14 cultures were used.
Rooting and acclimatization

*In vitro* elongated shoots of *E. alba* (2.5–3.0cm) were transferred to half-strength B5 medium supplemented with different concentrations of auxins (IBA) and combine with IAA. Different concentrations of IBA and IAA yielded different degree of roots. The B5 medium containing half strength 1.0 mg/L IBA produced better roots (92 %) (7.0 ± 0.8cm) and multiple and branching roots (8.8 ± 0.8). Plantlets having well-developed roots (5-6cm) were taken out from the culture vials and washed with running tap water. Plantlets were transferred to plastic cups containing autoclaved sand and garden soil (1:1) and kept undisturbed with plastic cover to maintain the moisture. The plantlets were watered regularly and it was periodically acclimatized to green house (Table 2, Fig 1d).

Fig. 1 Micropropagation of *E. alba* using nodal explants. (A) Shoot initiation on B5 medium supplemented with 0.5 BAP +0.5KIN mg/L (B); Shoot elongation on B5 medium fortified with (1.0 BAP + 0.3 KIN + 1.4 GA₃ mg/L) (C); Induction of multiple shoots on supplemented with 1.0 BAP + 0.3 KIN + 1.5 GA₃ mg/L (D and E); Root formation on half strength B5 medium (F); *In vitro* roots developed on B5 medium supplemented with (1.0 IBA mg/L) (G); Acclimatized plant in garden soil.
Table 2. Rooting response of *E. alba* in \( \frac{1}{2} \) strength B5 medium

<table>
<thead>
<tr>
<th>PGR concentrations (mg/L)</th>
<th>% response</th>
<th>Mean no. of roots(^a)</th>
<th>Mean root length (cm)(^b)</th>
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<tr>
<td>IBA</td>
<td>IAA</td>
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<tr>
<td>0.5</td>
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<td>71</td>
<td>3.0 ± 0.8b</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>85</td>
<td>3.6 ± 1.2b</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>50</td>
<td>2.3 ± 0.4c</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>92</td>
<td>8.8 ± 0.8a</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>42</td>
<td>3.0 ± 0.1b</td>
</tr>
</tbody>
</table>

\( - = \) No growth regulators added. The values are mean within a column followed by the same letter are not significantly different by Duncan’s multiple range test \((p > 0.05)\). Values are corresponds to mean (± SE). For each experiment 14 cultures were used.

**Discussion**

The *in vitro* propagation of *E. alba* was achieved in B5 medium supplemented with different combinations or alone of BA (0-2.0 mg/L), combined with kinetin, spermidine, thidiazuron and gibberellic acid (1.0 BAP + 0.5 S + 0.5 GA\(_3\) mg/L; 1.0 BAP + 0.5 KIN + 1.5 GA\(_3\) mg/L; 0.5S + 0.5 KIN + 0.5 GA\(_3\) mg/L). The better results of multiple shoots were observed in medium containing 1.0 BAP + 0.3 KIN + 1.5 GA\(_3\) mg/L and produced maximum number of multiple shoots (7.0/per explants) (100 %). The other combinations and concentration of BAP, spermidine, thidiazuron found to be moderate rate of shoots. The earlier studies highlights better micropropagation response of *E. alba* was reported by Shashi *et al.*, (2012) observed the maximum number of shoots in MS medium containing 13.2 mg/L BAP and 4.6 mg/L Kin (100 % with an average of 32.6 shoot buds/explant). Similarly, Baskaran *et al.*, (2004) who also reported BAP, KIN and 2iP stimulate the shoot elongation in same plant. Our results were also comparable with earlier studies of shoot regeneration of *E. alba* using various explants (Franca *et al.*, 1995; Borthakur *et al.*, 2000; Gawde and Paratkar, 2004; Dhaka and Kothari, 2005). None of them use multiple combinations of hormones in the development of an efficient propagation of *E. alba*.

The *in vitro* raised multiple shoots were excised and transferred individually to half strength B5 medium 1.0 IBA mg/L and 0.5 IBA mg/L for better root formation. Half strength B5 medium 1.0 mg/ L IBA were found to be more effective than other concentrations. Similarly, Baskaran *et al.*, (2004) also achieved multiple roots (94.3 %) on full strength MS medium containing 9.8mg/L IBA. Likewise, Mohammad *et al.*, (2012) reported that better root formation was achieved in *Rauwolfia serpentina* using half-strength MS medium containing 0.5 mg/L (IAA).

Based on the above observation of present study, we conclude that the efficient protocol was developed for direct regeneration of *E. alba* using nodal explants. The rooted plantlets were successfully acclimatized and established in soil with survival frequency rate of 70% during hardening process (Fig 1g).

**Conclusion**

To conclude, the outcome of results will encourage large-scale micropropagation of this important medicinal herb in a short period of time to increase the biomass and yield of active principles (wedelolactone, dimethyldedelolactone) or other secondary metabolites of pharmaceutical importance occurred in the plant of *E. alba*. This protocol informs a successful and rapid technique that can be developed for the commercial propagation and conservation of this plant and for future phytomedicine production.
Acknowledgement
The authors express their sincere and heartfelt thanks to Department of Biotechnology, Periyar University, Salem for providing necessary laboratory facilities to carry out the project work successfully.

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EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF TARENNA ASIATICA (L.) O. KTZE. EX K. SCHUM.

S. KARTHIKKUMARAN1, T. SAJEESE2, T. PARIMELAZHAGAN1*, V. VINODHKUMAR2, D. KAMALANATHAN3, T. NATARAJAN1

1Bioprospecting Laboratory, Department of Botany, Bharathiar University, Coimbatore - 641 046, Tamil Nadu, India., 2Department of Microbial Gene technology, Madurai Kamaraj University, Madurai - 625 021, Tamil Nadu, India., 3Department of Biotechnology, Periyar University, Salem - 636 011, Tamil Nadu, India. Email: dpnimel@gmail.com

ABSTRACT

Objective: The study was aimed to analyze the phytochemical, antioxidant and antibacterial potentials of T. asiatica.

Methods: The leaves, barks and flowers extracts were analyzed for total phenolic and flavonoid contents. Antioxidant activities were evaluated using DPPH scavenging, ABTS* scavenging, FRAP, phosphomolybdenum reduction, metal chelating, nitric oxide radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging and lipid peroxidation assays. Antibacterial activity was examined using agar well diffusion method against pathogenic microorganisms.

Results: The total phenolic and flavonoid contents were found to be higher in acetone extract of leaves (57.21 g GAE/100 g and 619.67 mg RE/g respectively). Antioxidant assays revealed that leaves acetone extract possesses significant (p<0.05) DPPH* scavenging capacity (IC50: 20.39 µg/mL), ABTS* scavenging activity (104.35 µM TE/g), ferric reducing activity (152.13 mM Fe(II)/mg), phosphomolybdenum reduction (417.93 µg AAE/g) and metal chelating activity (20.85 µg EDTO/100 g). At a concentration of 200 µg/mL, the leaves acetone extract also showed higher nitric oxide radical (49.22%), superoxide radical (73.63%) and hydroxyl radical (69.04%) scavenging activities and inhibition for lipid peroxidation (57.38%). Leaves and flowers acetone extracts inhibited the growth of S. dysenteriae, B. subtilis and S. bovisd with an inhibition zone ≥12 mm. MIC of flowers acetone extract was found to be 20 µg/mL against both B. subtilis and S. bovisd.

Conclusion: T. asiatica contains considerable phenolic and flavonoid contents which is responsible for the evident antioxidant and antimicrobial activities. These findings validate that T. asiatica can be a natural antioxidant and antibacterial source which will address medical security.

Keywords: Antimicrobial, Antioxidant, Flavonoid, Free radical, Phenolic, Reactive oxygen species, Tarenna asiatica

INTRODUCTION

Medicinal plants have enormous therapeutic potential to heal many infectious diseases by avoiding many side effects [1]. The secondary metabolites such as phenolics, flavonoids, alkaloids, iridoids etc. present in the plant extracts, generally produced by plants for their defence mechanisms have been implicated in the therapeutic properties of most medicinal plants [2]. Over-production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in humans can result in disease development and oxidative damage to nucleic acids, proteins, and lipids. The lack of antioxidants can quench excess reactive free radicals lead to oxidative stress and thereby cancer, cardiovascular, neurodegenerative, inflammatory and Alzheimer’s diseases [3]. Recent studies revealed that synthetic antioxidants can be toxic and expensive and this generated a need to identify natural and probably safer sources of antioxidants [4].

Antibacterial activity is the ability of a substance to inhibit or kill bacterial cells. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbes has led to the screening of several medicinal plants for their potential antimicrobial activity [5]. Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects. Therefore, there is need to search new infection-fighting strategies to control microbial infections and most concern about the alarming increase in the rate of infection by antibiotic-resistance microorganisms [6]. Thus current research focuses the development of antimicrobials of plant origin which can be used as an alternative to synthetic drugs.

Tarenna asiatica which belongs to the family Rubiaceae has a significant position among medicinal plants by virtue of its several phytotherapeutic values. The ethnomedical reports reveal that T. asiatica has been used for treatment of several disorders such as boils, external ulcers and sores [7]. The leaves possess wound healing property so that the leaves infusion is given orally in plough injuries [8, 9] and it is used as antidote [10]. T. asiatica is used as one of the ingredients of preparation for paralysis by the Malayal tribe in Nalamanikadi, Eastern ghats, India; in which the young leaves of the plants were ground, made into paste and applied externally to affected portion for two to three months [11]. Sabu et al. reported the wound healing property of leaves paste of T. asiatica mixed with turmeric powder which is applied externally on the head of the children for curing wounds [12]. Moreover, the anti-inflammatory effect of aqueous suspension of leaves powder rather than their extracts was reported by Amutha and coworkers through carrageenan induced lung inflammation in rats [13].

T. asiatica leaves are also used in skin diseases whereas their fruits are smashed and applied to boils to promote suppuration [14, 15]. Besides, the classical notes on the medicinal property of its fruits points out the application of fruit juice on the eyelids to arrest infection [16, 17]. The tribal population in the hilly tracts of Eastern Ghats in Andhra Pradesh uses the stem barks of T. asiatica for vomiting [18]. Even though T. asiatica possesses enormous medicinal values, less effort was made to scientifically evaluate their potentials. Therefore, the present research work has been carried out to scientifically validate the antioxidant and antimicrobial potentials of T. asiatica.

MATERIALS AND METHODS

Collection and identification of plant material

Fresh leaves, barks and flowers of T. asiatica were collected during the month of May 2012 from Maruthamalai hills, Coimbatore district of Tamil Nadu, India. The taxonomic identity of the plant was confirmed from the Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu (No: 293). The fresh plant materials

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were washed under running tap water to remove the surface pollutants and were air dried under shade. Then they were separately homogenized into fine powder using mixer and used for further studies.

Chemicals and standard drugs
The chemicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, 2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylethromban-2-carboxylic acid (Trolox), Sodium nitroprusside, Butylated hydroxy anisole (BHA), Butylated hydroxy toluene (BHT), rutin, gallic acid, ferrous chloride, ferric chloride, ferric cyanide, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), hydrogen peroxide, ethylene diamine tetraacetic acid (EDTA) disodium salt, N-(1-naphthyl) ethylene diamine dihydrochloride, riboflavin and Muller Hinton agar medium (MHA) were obtained from HiMedia (Mumbai, India) and Sigma Aldrich (Bengaluru branch, India). All the chemicals and solvents used were of the highest purity and analytical grade.

Preparation of extracts
The freshly collected plant materials were washed thoroughly in tap water, shade dried at room temperature (25°C), powdered and used for solvent extraction. The plant samples were successively extracted with petroleum ether (for disposing lipid and pigments), chloroform, acetone and methanol using soxhlet apparatus and the air dried residue were further extracted with hot water by the method of maceration for 24 h. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. The solvents were evaporated using a rotary vacuum-evaporator (Yamoto RE300, Japan) at 50°C and the remaining solvent was removed by lyophilisation (VirTis Benchtop K, USA). The extract recovery in different solvents was expressed as percent of the plant sample dry matter. The freeze-dried extracts thus obtained were dissolved in the respective solvents at the concentration of 1 mg/mL and used for antioxidant and antimicrobial studies.

Quantification assays
Quantification of total phenolics
The total phenolics of *T. asiatica* were determined according to the method described by Makkar [19]. About 50 μL of plant extracts were taken into a series of test tubes and made up to 1 mL with distilled water. A test tube with 1 mL of distilled water served as the blank. Then, 500 μL of Folin – Ciocalteau Phenol reagent (1 N) was added to all the test tubes including the blank. After 5 minutes, 2.5 mL of sodium carbonate solution (20%) was added, vortexed well, mixed the contents, and incubated in dark for 40 minutes. The formation of blue colour in the incubated test tubes indicated the presence of phenolics which was read at 725 nm against the reagent blank. Gallic acid was used as standard to plot the graph and the results were expressed as gallic acid equivalents (GAE). The analyses were performed in triplicates.

Quantification of flavonoids
The method of Zhishen *et al.* was followed for the quantification of flavonoids in the extracts [20]. About 2 mL of distilled water was added to 500 μL of different plant extracts taken in test tubes. The blank test tube contained only 2.5 mL of distilled water. Then, 150 μL of 5% NaNO₂ was added to all the test tubes followed by incubation at room temperature for 6 minutes. After incubation, 150 μL of 10% AlCl₃ was added to all the test tubes including the blank. Again, the test tubes were incubated for 6 minutes at room temperature. Then, 2 mL of 4% NaOH was added to all the test tubes which were made up to 5 mL using distilled water. The contents in all the test tubes were vortexed well and allowed to stand for 15 minutes at room temperature. The pink colour developed due to the presence of flavonoids was read spectrophotometrically at 510 nm. The flavonoid - rutin was used as the standard for plotting the calibration curve. All the experiments were done in triplicates and the results were expressed in rutin equivalents (RE).

In vitro antioxidant assays
**DPPH** scavenging activity
The antioxidant activity of *T. asiatica* extracts were determined according to the method of Braca *et al.* [21], in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The extract aliquots were taken and made up to 100 μL with methanol. About 3 mL of a 0.004% methanolic solution of DPPH was added to all the test tubes containing samples and standards. Negative control was prepared by adding 100 μL of methanol to 3 mL of DPPH solution. The tubes were allowed to stand for 30 minutes at 27°C. The absorbance of the sample was measured at 517 nm against the blank (methanol). Radical scavenging activity of the samples was expressed as IC₅₀ which is the concentration of the sample required to inhibit 50% of DPPH* concentration.

**ABTS**⁺ scavenging activity
ABTS radical cation decolorization assay was performed for the samples according to the method of Re *et al.* [22]. ABTS*⁺ was produced by reacting ABTS (7 mM) with potassium persulfate in the dark for 12–16 hours at room temperature. Prior to assay, this solution was diluted with ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance of 0.700 ± 0.02 at 734 nm. After the addition of 1 mL of diluted ABTS solution to the samples, absorbance was measured at 734 nm against the blank (ethanol). The unit of antioxidant activity was calculated as the concentration of Trolox having equivalent antioxidant activity expressed as μM/g sample extracts.

**Ferric reducing antioxidant power (FRAP)** assay
The antioxidant capacities of different extracts of samples were estimated according to the procedure described by Puldido *et al.* [23]. FRAP reagent (900 μL), prepared freshly and incubated at 37°C, was mixed with 90 μL of distilled water and 30 μL of test sample or methanol (blank). The test tubes were incubated at 37°C for 30 minutes in a water bath. The FRAP reagent was prepared by mixing 2.5 mL of 20 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM FeCl₃, 6H₂O, and 25 mL of 0.3 M acetate buffer (pH 3.6). At the end of incubation, the absorbance readings were taken immediately at 593 nm against the reagent blank. 7 mM ferric solutions of known Fe(II) concentration, ranging from 100 to 2000 μM, (FeSO₄·7H₂O) were used for the preparation of the calibration curve. Equivalent concentration was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM concentration of Fe(II) solution.

**Phosphomolybdenum reduction assay**
The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al.* [24]. Triplicates of 150 μL of sample and different concentrations of standard (ascorbic acid in 1 mM dimethyl sulphoxide) were added with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a test tube. The test tubes were covered with aluminium foil and incubated in a water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank. A typical blank solution contained 3 mL of reagent solution and water in place of sample, and it was incubated under the same conditions as the rest of the samples. The results reported are mean values expressed as mg of ascorbic acid equivalents per gram extract.

**Metal chelating activity**
The chelating of ferrous ions by various extracts of *T. asiatica* was estimated by the method of Dinis *et al.* [25]. Briefly, 1000 μL of samples and BHT (standard) were added to 100 μL solution of 2 mM FeCl₂. The reaction was initiated by the addition of 400 μL of 5 mM FeCl₃.
ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm against the blank (deionized water). The metal chelating capacities of the extracts were expressed as mg EDTA Equivalent/100 g extract.

**Nitric oxide radical scavenging assay**

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide [26] which interacts with oxygen to produce nitrite ions which can be estimated by use of the Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide). Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with plant extract (200 µl) and incubated at 25°C for 150 min. A control solution without sample and blank (saline buffer) was conducted in an identical manner. At intervals, samples (0.5 mL) of the incubation solution were removed and diluted with 0.5 mL of Griess reagent (1% sulphanilamide, 2% H3PO4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylenediamine was read at 546 nm. The scavenging activity on nitric oxide was calculated as: scavenging activity (%) = [(A0 - A) / A0] X 100; where, A0 is the absorbance of the control and A is the absorbance of the sample extract/standard.

**Superoxide radical scavenging activity**

The assay was based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin–light–NBT system [27]. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH-7.6), 20 µg riboflavin, 12 mM EDTA, 0.1 mg NBT and 100 µl of triplicate of sample solution or BHA and BHT (standard). Reaction was started by illuminating the reaction mixture with sample extract for 90 s. Immediately after illumination, the absorbance was measured at 590 nm against the reagent blank (reaction mixture without plant sample). Identical tubes with reaction mixture kept in the dark served as blank. The scavenging activity on superoxide anion generation was measured as: scavenging activity (%) = [(A0 - A) / A0] X 100; where, A0 is the absorbance of the control and A is the absorbance of the sample extract/standard.

**Hydroxyl radical scavenging activity**

The scavenging activity of different solvent extracts of *T. asiatica* on hydroxyl radical was measured according to the method of Klein et al. [28]. A triplicate of 200 µl of different solvent extracts were added with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%, 1mL of DMSO (0.85%) V/V in 0.1 M phosphate buffer, pH 7.4. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice cold TCA (17.5% W/V), 3 mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of color formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage of hydroxyl scavenging activity was calculated by the following formula: scavenging activity (%) = [(A0 - A) / A0] X 100; where, A0 is the absorbance of the control and A is the absorbance of the sample extract/standard.

**Lipid peroxidation assay**

A modified thioarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media [29]. Malondialdehyde (MDA), a secondary product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm. Egg homogenate (500 µL of 10%, v/v in phosphate-buffered saline pH 7.4) and 200 µL of sample were added to a test tube and made up to 1.0 ml with distilled water. Then, 50 µL of FeSO₄ (0.075 M) and 20 µL of L-ascorbic acid (0.1 M) were added and incubated for 1 h at 37°C to induce lipid peroxidation. Thereafter, 0.2 mL of EDTA (0.1 M) and 1.5 mL of TBA reagent (3 g TBA, 120 g TCA and 10.4 mL 70% H2O2 in 800 mL of distilled water) were added in each sample and heated for 15 min at 100°C. After cooling, samples were centrifuged for 10 min at 3000g and absorbance of superoxide was measured at 532 nm. Inhibition (%) of lipid peroxidation was calculated using the equation: % Inhibition = [(A0 - A) / A0] X 100, where, A0 is the absorbance of the control and A1 is the absorbance of the tested sample.

**Antibacterial assay**

**Microorganisms used**

The bacterial strains namely *Klebsiella pneumoniae*, *Proteus vulgaris*, *Shigella dysenteriae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella boydi*, *Bacillus subtilis*, *Corynebacterium diphtheriae* and *Staphylococcus aureus* were collected from the Department of Biotechnology, Periyar University, Salem – 636 011, Tamil Nadu. The collected strains were stored in refrigerator at 4°C and sub-cultured in 30 days interval on Muller Hinton agar slants. Each inoculum was adjusted to McFarland standard, equivalent to 1x10⁷ CFU which was used for performing antibacterial tests.

**Agar well diffusion method**

Antibacterial screening of leaves, barks and flowers extracts of *T. asiatica* was done using agar well diffusion method against selected pathogenic microorganisms with required modifications. For this 25 mL of sterile Muller Hinton agar No. 2 containing the suspension of test organisms (50 µL) was swabbed on the molten MHA plates by using sterile cotton swab. Sample wells were made using sterile cork-borer (5 mm diameter) on each seeded plates and labeled properly. Standard antibiotic chloramphenicol (100 µg/mL) was used as positive control. Sterile DMSO (100%) was used as negative control. Then, approximately 50 µL of each extract (1 mg/mL) and chloramphenicol was separately introduced into wells and allowed to diffuse at room temperature. The plates were kept in incubator at 37°C for 24 hours. After incubation, the diameter of inhibition zone was measured by ruler and recorded in mm Eilers.

**Determination of Minimum Inhibitory Concentration (MIC)**

The minimum inhibitory concentrations were estimated by broth dilution technique [30]. The culture tubes were seeded with respective cultures and different concentration of plant extracts (leaves, barks and flowers) was added and incubated at 37°C for 24 hours. The lowest concentration of plant extracts inhibiting the growth of the organism and which did not permitting any visible growth of the inoculated test organism in broth culture, was regarded as the minimum inhibitory concentration (MIC) in each case.

**Determination of Minimum Bactericidal Concentration (MBC)**

After culturing the test organisms separately in nutrient broth containing various concentrations of the plant extracts, the broth was inoculated onto freshly prepared agar plates to assay for the bactericidal effect. The culture was incubated at 37°C for 24 hours. The lowest concentration of plant solvent extract that does not yield any colony growth on the solid medium after the incubation period was regarded as minimum bactericidal concentration (MBC) [31].

**Statistical analyses**

All the experiments were done in three replicates and the results were expressed as Mean ± Standard Deviation (SD). The statistical analyses were done by using SPSS version 17.0 by means of one way ANOVA followed by Duncan test for antioxidant studies.

**RESULTS**

The percentage yield of leaves and flowers extracts of *T. asiatica* in different solvents are presented in table 1. The better yield was obtained for methanol extraction where the yield percentage was 21.6, 15.48 and 11.78 % for flowers, leaves and barks
respectively. However, the extract recovery percent was found to be lower in the chloroform extracts of leaves (3.19 g), flowers (2.67 g) and barks (1.46 g).

**Quantification of total phenolics and flavonoids**

The results of total phenolics and flavonoid contents are shown in table 1. Acetone extract of T. asiatica leaves revealed highest phenolic content (57.21 g GAE/100 g) followed by acetone (48.26 g GAE/100 g) and methanol (43.98 g GAE/100 g) extracts of barks. In the estimation of flavonoids, the acetone extract of leaves exhibited significant amount of flavonoid content (61.967 mg RE/g) followed by acetone extract of barks (561.00 mg RE/g) and flowers (497.00 mg RE/g).

<table>
<thead>
<tr>
<th>Parts</th>
<th>Extract yield (g/100 g dried powder)</th>
<th>Total Phenolics (g GAE/100 g extract)</th>
<th>Flavonoids (mg RE/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Petroleum 6.12</td>
<td>25.40±1.25</td>
<td>141.3±7.23</td>
</tr>
<tr>
<td></td>
<td>Ether 3.19</td>
<td>37.31±2.30</td>
<td>135.00±2.00</td>
</tr>
<tr>
<td></td>
<td>Chloroform 5.19</td>
<td>57.21±4.59</td>
<td>619.67±19.27</td>
</tr>
<tr>
<td></td>
<td>Acetone 15.94</td>
<td>20.62±3.51</td>
<td>426.00±5.29</td>
</tr>
<tr>
<td></td>
<td>Methanol 8.91</td>
<td>16.93±3.93</td>
<td>446.33±2.52</td>
</tr>
<tr>
<td>Barke</td>
<td>Petroleum 2.73</td>
<td>18.86±0.71</td>
<td>106.00±1.73</td>
</tr>
<tr>
<td></td>
<td>Ether 1.48</td>
<td>28.62±1.97</td>
<td>88.67±2.52</td>
</tr>
<tr>
<td></td>
<td>Chloroform 1.83</td>
<td>48.16±3.32</td>
<td>561.00±10.24</td>
</tr>
<tr>
<td></td>
<td>Acetone 11.78</td>
<td>43.98±1.80</td>
<td>317.00±8.72</td>
</tr>
<tr>
<td></td>
<td>Methanol 6.63</td>
<td>34.74±0.56</td>
<td>117.67±3.51</td>
</tr>
</tbody>
</table>

Values are mean of triplicate determination (n=3) ± standard deviation; GAE - Gallic Acid Equivalents; RE - Rutin Equivalents Statistically significant at p < 0.05 where a > b > c > d in each column

**Antioxidant assays**

**DPPH radical scavenging activity**

The free radical-scavenging activities of different parts of T. asiatica samples along with standards such as rutin, vitamin E and BHT were determined by the DPPH radical scavenging assay and the results are represented in figure 1. The lower IC₅₀ values indicate higher DPPH radical scavenging activities. Generally the acetone and methanol extracts of all the parts showed significant reduction of DPPH radical. However, the highest free radical scavenging activity was exerted by acetone and hot water extracts of leaves and the IC₅₀ values were 28.38 and 21.35 µg/mL respectively.

**Figure 1: DPPH radical scavenging activity of T. asiatica**

Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at p < 0.05 where a > b > c > d

**ABTS radical cation scavenging activity**

The trolox equivalent antioxidant capacity was measured using ABTS radical cation decolorisation assay; one of the most frequently employed methods for antioxidant activity. The results of ABTS radical cation scavenging activities of different extracts of leaves, barks and flowers of T. asiatica are shown in table 2. Among the different solvent extracts, the acetone extract of leaves and barks revealed appreciable level of trolox equivalent antioxidant activity (10435.44 and 7269.71 µM TE/g extract respectively). In connection to that the methanol and hot water extracts of leaves also exhibited higher activity (6540.71 and 5376.34 µM TE/g extract respectively).

**Ferric reducing antioxidant power (FRAP) assay**

The ferric reducing capacities of T. asiatica extracts are shown in table 2. The ferric reducing activity observed for the plant extracts ranged between 6.56 to 152.23 mM Fe(II)/mg extract. The significant reducing capacities were shown by the acetone of extracts of all the parts and the decreasing order of activity for the parts was leaves > barks > flowers. It was also seen that the hot water extract of leaves also showed higher activity which was estimated to be 119.05 µM Fe(II)/g extract.

**Phosphomolybdenum reduction assay**

The total antioxidant capacity of different solvent extracts of leaves, barks and flowers of T. asiatica were analyzed and are shown in table 2. Among different parts used, leaves showed higher activity in most of its solvents compared to the extracts of barks and flowers. Acetone extract of leaves and flowers (417.93 and 337.11 mg AAE/g extract) exhibited the highest phosphomolybdenum reduction compared to other solvent extracts. The methanol extracts also possessed antioxidant capacities which were 233.02, 224.01 and 221.20 mg AAE/g extract for barks, leaves and flowers respectively.

**Metal chelating activity**

The iron chelating activity of different extracts of T. asiatica are shown in table 2. The significant chelating activity was observed for the acetone extract of leaves (20.85 g EDTA/100 g extract) followed by the acetone extracts of flowers and barks 15.03 and 10.07 g EDTA/100 g extract respectively). Moreover, the methanol extract of leaves also revealed its potential in chelating iron and the activity was 14.14 g EDTA/100 g extract. On the other hand, the hot water as well as non polar extracts were not able to chelate iron and thereby failed to reduce the colour formation by iron-ferrozine complex.

**Nitric oxide radical scavenging activity**

The nitric oxide radical scavenging activity of different solvent extracts of all the samples are presented in figure 2. The higher nitric oxide radical scavenging activity was exerted by acetone extract of leaves (49.22%) followed by that of barks (44.62%) at a concentration of 200 µg/mL. However, the methanol and hot water extract of all the parts revealed comparable scavenging activities. Apart from the sample data, the standards viz. rutin and BHT exhibited significantly different activity even at a concentration of 100 µg/mL (65.00 and 58.78% respectively).

**Superoxide radical scavenging activity**

The results of superoxide anion scavenging activities of different extracts of T. asiatica are shown in figure 2. The results showed that acetone extract of leaves (73.63%) have highest superoxide radical scavenging activity compared with other solvent extracts of other parts of the plant at a concentration of 200 µg/mL. Among the different extracts of flowers, significant activity was shown by methanol extract and was 65.19%. The scavenging activity was also compared with that of natural (rutin) and synthetic (BHT) antioxidants.
Table 2: ABTS⁺ scavenging, FRAP, Phosphomolybdenum reduction and Metal chelating activities of *T. asiatica*

<table>
<thead>
<tr>
<th>Parts</th>
<th>Solvents</th>
<th>ABTS⁺ scavenging (μM TE/g extract)</th>
<th>FRAP (μM Fe(II)E/g extract)</th>
<th>Phosphomolybdenum reduction (mg AAE/g extract)</th>
<th>Metal chelating (g EDTA/100 g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Petroleum Ether</td>
<td>300.37±77.33</td>
<td>7.80±0.06</td>
<td>31.38±1.01</td>
<td>0.71±0.04</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>870.74±5.38</td>
<td>6.56±0.61</td>
<td>116.5±4.1</td>
<td>0.44±0.35</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>1043.45±23.31</td>
<td>152.1±0.74</td>
<td>471.93±6.08</td>
<td>20.5±0.52</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>6540.71±5.35</td>
<td>93.78±0.32</td>
<td>244.0±1.31</td>
<td>1.14±0.87</td>
</tr>
<tr>
<td></td>
<td>Hot Water</td>
<td>5376.34±36.51</td>
<td>119.05±8.08</td>
<td>186.9±4.61</td>
<td>1.44±0.73</td>
</tr>
<tr>
<td>Barls</td>
<td>Petroleum Ether</td>
<td>13466.2±17.54</td>
<td>8.61±2.17</td>
<td>27.40±0.93</td>
<td>0.44±0.26</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>21127.4±2.10</td>
<td>24.36±1.91</td>
<td>119.1±8.31</td>
<td>3.59±0.18</td>
</tr>
<tr>
<td>Flowers</td>
<td>Petroleum Ether</td>
<td>216.60±32.55</td>
<td>32.07±4.67</td>
<td>23.08±0.61</td>
<td>1.25±0.36</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>11069.7±4.63</td>
<td>22.34±1.84</td>
<td>96.53±1.40</td>
<td>2.93±0.24</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>397235±32.5</td>
<td>87.06±1.37</td>
<td>337.1±0.20</td>
<td>15.03±2.50</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>206211.35±5.56</td>
<td>75.98±1.12</td>
<td>221.2±4.05</td>
<td>3.17±1.30</td>
</tr>
<tr>
<td></td>
<td>Hot Water</td>
<td>349648±51.96</td>
<td>48.91±1.75</td>
<td>99.92±6.59</td>
<td>2.47±0.91</td>
</tr>
</tbody>
</table>

**Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at p < 0.05 were a> b> c in each column**

Figure 2: Nitric oxide and Superoxide radicals scavenging activity of *T. asiatica*

**PE** – Petroleum ether, **C** – Chloroform, **A** – Acetone, **M** – Methanol, **HW** – Hot water, Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at p < 0.05 where a> b> c> d in each assay

**Hydroxyl radical scavenging activity**

Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid - iron EDTA. The hydroxyl radical formed by the oxidation will react with dimethyl sulfoxide (DMSO) to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The scavenging activity of different plant parts were shown in figure 3. The OH⁺ scavenging activities of all the samples were investigated at the concentration of 200 µg/mL except for standards. The acetone extracts of leaves, barks and flowers showed significant levels of scavenging activities and were 69.04, 62.22 and 62.44% respectively. The methanol extracts of samples also exhibited moderate scavenging activities compared to other sample extracts.

Figure 3: Hydroxyl radical scavenging and Lipid peroxidation activity of *T. asiatica*

**PE** – Petroleum ether, **C** – Chloroform, **A** – Acetone, **M** – Methanol, **HW** – Hot water, Values are mean of triplicate determination (n=3) ± standard deviation, Statistically significant at p < 0.05 where a> b> c> d in each assay

**Lipid Peroxidation**

Lipid peroxidation is an oxidative deterioration process of polyunsaturated fatty acids which is induced by radical and its inhibition results by *T. asiatica* are shown in figure 3. In this assay, *T. asiatica* extracts established strong inhibition for lipid peroxidation which was higher for acetone extract of leaves (57.38%). Other extracts, like methanol extract of leaves (43.78%) and acetone extract of flowers (42.74%) also showed comparable inhibition of lipid peroxidation at 200 µg/mL. The decreasing order of inhibition for different parts of *T. asiatica* was found to be leaves > flowers > barks.

**Antibacterial activity**

The leaves, barks and flowers extracts of *T. asiatica* were tested separately for their potential inhibition against pathogenic microorganisms and the results are presented in table 3. Among the different solvent extracts high antibacterial effect was shown by the considerable zone of inhibition against *S. boidii* (14 mm), *B. subtilis* (13 mm) and *S. dysenteriae* (12 mm) which were comparable to the activity of antibiotic chloramphenicol. Leaves and flowers acetone extracts also inhibited the growth of *S. dysenteriae, B. subtilis* and *S. boidii* with an inhibition zone ≥ 12 mm. Petroleum ether, methanol and hot water extracts were not as effective as acetone extracts to retard the growth of microorganisms. The gram positive *S. aureus* was found to be sensitive to none of the extracts. On the other hand, the antibiotic chloramphenicol exhibited significant inhibition against all the microorganisms tested. The acetone extracts were found to be more active against gram negative bacteria compared to gram positive bacteria.

The acetone extracts which showed positive inhibition against the bacteria *B. subtilis* and *S. boidii* were selected for the evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for active inhibition of bacterial growth (Table 4). Initially, the extracts were prepared in different concentrations such as 10, 20, 30, 40 and 50 (µg/mL) whose activities directed to study the activities of further concentrations such as 42, 44, 46 and 48 (µg/mL). From the study, it was concluded that among the acetone extracts of different parts, flowers extract possesses higher antibacterial activity against *B. subtilis* and *S. boidii*. It was found that among the different concentration gradients 40 – 50 µg/mL of extracts showed clear broth which depicts the active inhibition on culture growth. The lowest concentration of the extract (MIC) that will inhibit the visible growth of the microorganisms was found to be 20 µg/mL for flowers acetone extract against both *B. subtilis* and *S. boidii*. It was further optimized from the clear inhibition of bacterial growth that the MBC of acetone extract was 46 µg/mL against *B. subtilis* and 44 µg/mL against *S. boidii*. The leaves and barks acetone extracts were found to have no significant effect against both *B. subtilis* and *S. boidii* as compared to that of flowers.
Table 3: Antimicrobial activity of *T. asiatica* against pathogenic microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Leaves (1 mg/ml)</th>
<th>Barks (1 mg/ml)</th>
<th>Flowers (1 mg/ml)</th>
<th>Chloramphenicol (100 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>PE C A M H</td>
<td>PE C A M H</td>
<td>PE C A M H</td>
<td>PE C A M H</td>
</tr>
<tr>
<td>C. diptheriae</td>
<td>6 - 10 - 7 - 8 - 7 - 6 - 4 - 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>8 - 12 - 7 - 7 - 6 - 13 - 8 - 6 - 12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>11 8 6 8 8 11 8 7 6 - 11 8 8 6 - 12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>- - - - - - - - - - - - - -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>- - 8 8 - 8 - 8 - 7 8 - 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. dysenteriae</em></td>
<td>10 - 13 - 10 - 12 - 8 - 12 - 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>- - 8 - 8 - 8 - 8 - 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>- - 8 - - - - 8 - 8 - 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td>11 8 12 7 6 7 10 14 7 6 8 7 12 - 6 14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PE – Petroleum ether, C – Chloroform, A – Acetone, M – Methanol, H – Hot water, ‘-’ indicates no activity

Table 4: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of acetone extracts of *T. asiatica* against *B. subtilis* and *S. boydii* cultures

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Acetone Extracts</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Barks</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. boydii</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Highly turbid; + clear; ++ moderately clear; +++ highly clear

**DISCUSSION**

Quantification of total phenolics and flavonoids

The hydroxyl groups present in the polyphenols make them good for free radical-scavenging reactions and as metal chelating agents. Moreover, the arrangement of the hydroxyl groups around the phenolic molecule is also important for antioxidant reactions [32]. The higher amount of phenolics in the acetone extracts of *T. asiatica* leaves, barks and flowers could be due to its higher solubility and it could be significantly contribute to the antioxidant capacity of that species.

Flavonoids are used as natural antioxidants in food, medicinal and non-nutritive plant materials due to their ability to inhibit and scavenge reactive oxygen species [33]. Flavonoids are potent inhibitors of molecular oxygen (O2), and also scavenge other free radicals such as OH and NO2 [34]. Flavonoids suppress the effects of active oxygen species (H2O2 and O2·-) in many other vulnerable biological systems [35]. Since *T. asiatica* possesses good flavonoid content in leaves and barks, it could be assumed that it can have a higher free radical scavenging activity which involves the transfer of electron or hydrogen atom from flavonoids to free radicals.

**In vitro Antioxidant Assays**

**DPPH radical scavenging activity**

The DPPH assay has been widely used to analyze the antioxidant activity of plant extracts and foods as free radical scavengers or hydrogen donors [36]. The antiradical scavenging activity of different extracts of *T. asiatica* would be related to the nature of phenolics, flavonoids etc. which contributes to their electron transfer or hydrogen donating ability [37]. The significant activity of acetone extract of leaves may be due to the presence of phenolic compounds in the plant parts. On the other hand, the DPPH radical scavenging efficiency of extracts from *T. asiatica* might have also been partly attributed to Millard reaction products other than phenolic constituents because they also effectively participate as radical scavengers [38]. The higher apparent antioxidant capacity of smaller molecules due to their better access to the DPPH radical site suggest that the extracts may contain more smaller compounds than larger compounds [39].

**ABTS radical cation scavenging activity**

The ABTS radical is soluble in both aqueous and organic solvents, is not affected by ionic strength and can be used to measure the antioxidant capacity of hydrophilic and lipophilic compounds in test samples [40]. Moreover, the radical is suitable for evaluating antioxidant capacity of phenolics due to their comparatively lower redox potentials (0.68 V). Many phenolic compounds can thus react with the ABTS radical because of their thermodynamic property [41]. Apart from these, Hagerman et al. have reported that the high molecular weight phenolics have more ability to quench free radicals (ABTS+) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific groups [42]. The ABTS assay indicated that the extracts of *T. asiatica* possess strong hydrogen donating ability and could serve as free radical scavengers by acting as primary antioxidants when they are ingested along with nutrients.

**Ferric reducing antioxidant power (FRAP) assay**

The ferric reducing antioxidant power assay is used to measure the antioxidant effect of any substance in the reaction medium as its reducing ability. Yen and Duh reported that the reducing power of bioactive compounds, mainly low and high molecular phenolics, was associated with antioxidant activity, specifically scavenging of free radicals [43]. It has been also proved that the potential antioxidants through *in vitro* ferric-reducing antioxidant power assay increased the total antioxidant capacity of blood plasma [44]. Thus the ferric reducing power of different extracts of *T. asiatica* reveals that there are compounds in the acetone extracts which have high affinity to the ferrous ions and thereby quench/scavenge them through redox reactions.

**Phosphomolybdenum reduction assay**

Phosphomolybdenum assay is mainly based on the ability of plant extracts to reduce Mo(VI) to Mo(V) and subsequent formation of green phosphate/Mo(V) complex at an acidic pH. Being simple and independent of other antioxidant measurements commonly employed, the application of assay was extended to plant extracts [24]. Since the antioxidant activity is expressed as the number of equivalents of ascorbic acid, the total antioxidant capacity observed for the extracts of *T. asiatica* can be correlated with its free radical
scavenging activity. The reduction of Mo (VI) to Mo (V) by the leaves, bark and flowers extracts of *T. asiatica* may be due to the electron transfer or hydrogen ion transfer by the bioactive compounds, specifically phenolics and flavonoids present in the respective plant parts.

**Metal chelating activity**

Iron can undergo Fenton reaction and produces reduced metals which may form highly harmful hydroxyl radicals and thereby contributing to oxidative stress [45]. Reports reveals that chelating agents which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [46]. Moreover, antioxidants inhibit interaction between metal and lipid through formation of insoluble metal complexes with ferrous ion. Therefore, the metal chelating assay reveals that acetone extract for all the parts have shown effective activity, suggesting that its action as antioxidant may be related to its sequestering of Fe²⁺ ions that may otherwise catalyze Fenton type reactions or participate in metal catalyzed hydroperoxide decomposition reactions.

**Nitric oxide radical scavenging assay**

Reactive nitrogen species, formed during their reaction with oxygen or with superoxides are very reactive and can be implicated for inflammation, cancer, and other pathological conditions [47]. These compounds are responsible for altering the structural and functional behavior of many cellular components. The phytochemicals possess the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health. Since, the acetone extracts of leaves and bark of *T. asiatica* showed higher scavenging activity, it is clear that it can be used for reducing the deleterious effects caused by the reactive nitrogen species in human body.

**Superoxide radical scavenging activity**

Superoxide radical acts as a precursor of more reactive oxygen species like hydrogen peroxide, hydroxyl and singlet oxygen and is known to be a very harmful species to cellular components [48]. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potent precursors of highly reactive species such as hydroxyl radical and thus the study of scavenging of this radical is important [49]. Since, the acetone extract of leaves and bark of *T. asiatica* showed appreciable percentage of scavenging activity against superoxide radical, it can be used against adverse effects caused by superoxide radical in the body. The active principles in the plant extracts eliminate the radical by its reduction to attain the octant stage or through the formation of water molecule.

**Hydroxyl radical scavenging activity**

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules like lipids, poly peptides, proteins and DNA, especially thiamine and guanosine [50]. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxyl cyclohexadienyl radical. The resulting radical can undergo further reactions, such as reactions with oxygen to give peroxy radical, or decompose to phenoxyl type radicals by water elimination [48]. Hagerman et al. have also reported that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by tannins than their specific functional groups [42]. Thus, the hydroxyl radical scavenging of *T. asiatica* acetone extracts can be directly related to the amount of phenolics and tannins present in the sample.

**Lipid peroxidation assay**

In the food processing industries, flavonoids have been shown to inhibit heat or chemical initiated lipid peroxidation as well as chelating metallic and super oxide ions [33]. The phytoconstituents in the plant extracts can reduce the highly reactive and transient peroxy radicals that are involved in lipid peroxidation [51]. There was a significant chelating capacity as the extracts reduced the concentration of the catalyzing transition metal in lipid peroxidation [52]. The active components in the acetone extracts of leaves, bark and flowers can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants and therefore inhibit lipid peroxidation [53].

**Antibacterial Activity**

*T. asiatica* is a magnificent medicinal plant characterized by its significant antimicrobial activity against the tested pathogens. The different solvent extracts of leaves, bark and flowers of *T. asiatica* exhibited different range of inhibition where acetone extracts showed high degree of antibacterial activity against the selected organisms. It is well noted that chloroform and methanol extracts showed less activity than acetone extracts. This may be due to the same active substances were present in chloroform/methanol extracts in low concentrations or active substances were soluble in acetone and not present in chloroform and methanol extracts.

The earlier reports state the antibacterial activity of *T. asiatica* at the range of 8 – 14 mm of inhibition against most of the tested organisms like *P. vulgaris*, *B. subtilis* and *S. boydii* by all the sample extracts. The current observation has been deviated from other studies by the difference in the concentrations of the solvent and samples tested. It highlights the use of acetone extract for potential inhibition against most of the tested pathogens and in specific the MIC and MBC of their crude extract concentration was identified up to 46 µl of aliquot volume. Similar antibacterial activity against different bacterial pathogens was reported for few Rubiaeaceae members by Jayasinghe et al. [14] and Usha et al. [54] whereas for leaves extracts of *T. asiatica* by Choudhury et al. [55]. The earlier studies also reported the antibacterial activity of few species of Rubiaeaceae members against *Bacillus* sp., *Staphylococcus* sp and *Pseudomonas aeruginosa*. The ethanol soluble fraction of hydroalcoholic extract of *Psychotria reevesii* aerial parts exhibited an inhibition of 15.7 mm against *Staphylococcus aureus* [6]. It is evident that the extracts used in the study was highlighted in terms of concentration is considerably lower amount tested produced higher zone of inhibition by plant extracts. *B. subtilis* produces the enzyme subtilisin, which has been reported to cause dermal allergic or hypersensitivity reactions in individuals repeatedly exposed to this enzyme in industrial settings. The gram negative bacteria, *E. aerogens* are a nosocomial and pathogenic bacterium that causes opportunistic infections [56]. *K. pneumoniae* is an important cause of human infections and several diseases viz., urinary tract infections, nosomial infections, pneumonia, septicemias and soft tissue infections. The diseases caused by *K. pneumoniae* can result in death of patients who are immunodeficient. Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections and neonatal meningitis. In rare cases, virulent strains are also responsible for haemolytic uremic syndrome, periitonitis, mastitis, septicaemia and gram negative pneumonia [57]. *B. cereus* is responsible for a minority of food borne illnesses (2-5%), causing severe nausea, vomiting and diarrhea [58]. The antibacterial findings suggest that the plant extract can be effectively used to treat these infectious disease caused by these different bacteria.

Flavonoids have been referred to as nature’s biological response modifiers because of strong experimental evidences of their inherent ability to modify the body’s reaction to allergen, bacteria, virus and carcinogens. Moreover, they exhibit antihellic, anti-inflammatory, antimicrobial and anticancer activity. The high molecular weight phenolics are also known to possess general antimicrobial and antioxidant activities [59]. Therefore, it can be assumed that the phenolics, flavonoids and tannins present in the plant extracts may contribute to the activity.

**CONCLUSIONS**

The study concluded that *T. asiatica* possesses significant phenolic and flavonoid contents which contribute to the pronounced antioxidant and antimicrobial activities. The extracts have great potential as antimicrobial compounds, especially in the treatment of
infectious diseases caused by resistant microorganisms. However, it has to be considered that the results of this work can be used as a lead to continue the search of active substances in the extracts for the development of indigenous botanical resources.

REFERENCES


Indirect Propagation of *Solanum trilobatum* L using Leaf Explants

Kamalanathan Desingu and Natarajan Devarajan*

Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India

**ABSTRACT**

The present investigation was aimed to develop an efficient protocol for indirect organogenesis of *Solanum trilobatum* L. using leaf explants. Development of organs from callus and multiple shoot induction was achieved in the modified Murashige and Skoog (MS) medium enriched with NAA, 2, 4 – D alone and in combinations with various concentrations (0.5, 0.3, 1.0 + 1.0 mg L\(^{-1}\)). Morphogenic pale yellow green and greenish brown callus was obtained after 4 weeks of incubation. Multiple shoots were formed from the callus in the MS medium containing BAP combination with KIN at the concentration (1.0 + 0.3mg L\(^{-1}\)) after appropriate incubation period. The regenerated shoots were transferred to rooting medium (containing NAA + IBA with concentrations of 0.5 mg L\(^{-1}\) + 0.4 mg L\(^{-1}\) ) exhibited better roots formation. The complete plantlets were transferred to the poly cups filled with sterile sand and red soil in the ratio of 1:1 and successfully acclimatized in the field.

**Key words:** Indirect organogenesis, MS medium, Plant Growth Regulators.

**INTRODUCTION**

*Solanum trilobatum* (Solanaceae) is an erect branching herb widely distributed throughout India and has long been used in traditional system of medicines to treat various diseases [1]. Calcium, iron, phosphorus, carbohydrates, protein, fat, crude fibre and minerals are present in leaves [2]. It is used as medicine for diabetes, asthma, vomiting with blood, bilious matter phlegmatic rheumatism and leprosy [3]. The biological properties of *S. trilobatum* showed potent antibacterial, antifungal, antimitotic, antioxidant and antitumourous activities [4, 5, 6, 7]. The major phytochemicals like Sobatum, β - solamine, solaine, solasodine, glycoalkaloid and diosogenin and tomatidine were isolated [8, 9]. Several biochemical constituents are reported from *Solanum* species, which includes alkaloids, phenolics, flavonoids, steroidal saponins and their glycosides [10]. Two bioactive compounds i.e Soladunalidine and tomatidine (alkaloids) were isolated from the leaf and stem of *Solanum* species [9].

Many researchers focussed on this genus *Solanum* and documented with the direct and indirect micropropagation protocols for their medicinal properties and considerable beneficial phytoconstituents. Callus organogenesis in *Solanum tuberosum* [11], shoot tip and nodal culture of the same species *Solanum trilobatum*, [2, 12] in modified LS medium was reported. And this study was aimed to develop a rapid micropropagation protocol for the indirect organogenesis and multiplication of *S. trilobatum* using leaf explants.

**MATERIALS AND METHODS**

**Plant materials**

The fresh and healthy aerial parts of *S. trilobatum* were collected from the local garden and nomenclature was identified by Dr. D. Natarajan, Assistant Professor, Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem, Tamilnadu.
Induction of callus

*In vitro* grown leaf explants (two weeks matured) were cultured for callus induction in MS [13] media supplemented with 2% sucrose as carbon source and 0.7% agar (Hi Media) along with various concentrations of auxins (2, 4 – D (1.0 – 5.0 mg/L), NAA (1.0 – 5.0 mg/L) and combination of 2, 4 – D + NAA (0.5 + 0.5, 1.0 + 0.5, 1.0 + 1.0). The pH of the media was adjusted to 5.8 prior to autoclaving for 15 min at 121°C. The cultures were maintained under cool-white fluorescent light at 24 ± 2°C with 16 h photoperiod. The callus cultures were maintained for about 4 – 7 weeks and repeatedly subcultured in the same hormonal medium.

Organogenesis

The callus cultures were subcultured for the embryogenic development of shoot induction in the MS medium supplemented with various concentration of cytokinins and auxins such as BA alone (0.4 – 2.5 mg/L), BA + KIN (1.0 + 0.1 – 0.5 mg/L) and BA + NAA (1.0 + 0.1 – 0.5 mg/L) and incubated. The developed auxiliary buds were isolated and repeatedly subcultured on same medium for the formation of multiple shoots. For root formation, well developed shoots (3 cm) were transferred to MS media supplemented with auxins IBA alone (0.4 – 2.0 mg/L) and combination of IBA + NAA (0.5 + 0.1 – 0.5 mg/L) and incubated. Each treatment consists of 3 replications and each replication, 14 explants were used. The data was recorded after 5 weeks of culture. The well developed plantlets were transferred to polycups containing sterile sand and red soil (1:1 ratio) and watered often and transferred to green house.

RESULTS

Growth of callus cultures

The explants of *S. trifoliatum* were inoculated in MS medium supplemented with different concentrations of plant growth regulators such as NAA, 2, 4-D alone and its combination for callus regeneration. Formation of the morphogenetic varied characteristic callus cultures (like greenish brown, pale yellow green callus) was obtained within two weeks of inoculation in the medium containing hormones at the concentration of 3.0 mg L⁻¹ 2, 4 - D and 5.0 mg L⁻¹ NAA and combination of 2, 4 - D + NAA (1.0 + 1.0 mg L⁻¹). Whereas other combinations of 2, 4 - D and NAA yielded slightly friable and brownish callus. The results were observed for 4 – 8 weeks of time period without altering the culture conditions under light. The hormone 2, 4 - D + NAA combination developed better callus mass then 2, 4 – D and NAA alone at lower concentrations. The rate of growth of callus increased from the second week of culture initiation until the eighth week after the rate of callussing declined. Pale yellow green callus developed from the medium was repeatedly subcultured after every 4 weeks interval for shoot initiation (table 1; figures 1 a, b).

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Concentration (mg/L)</th>
<th>Response of callus mass</th>
<th>Callus appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA</td>
<td>1.0</td>
<td>-</td>
<td>No callus mass</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>++</td>
<td>Yellow white, smooth</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>++</td>
<td>Yellow white, hard</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>+++</td>
<td>Yellow, green hard</td>
</tr>
<tr>
<td>2, 4 – D</td>
<td>1.0</td>
<td>+</td>
<td>Pale brown hard</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>+</td>
<td>Brown hard</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>+++</td>
<td>Greenish Brown hard</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>++</td>
<td>Brown hard</td>
</tr>
<tr>
<td>2, 4 – D + NAA</td>
<td>0.5 + 0.5</td>
<td>++</td>
<td>Pale Brown hard</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.5</td>
<td>+++</td>
<td>Brown hard</td>
</tr>
<tr>
<td></td>
<td>1.0 + 1.0</td>
<td>+++</td>
<td>Pale yellow green hard</td>
</tr>
</tbody>
</table>

*, ++, +++ indicates slight, moderate and considerable callusing, - indicates no response.

Shoot multiplication

*In vitro* developed callus (4 – 7 weeks) were inoculated on MS medium fortified with different concentrations of BA (0.4, 0.8, 1.0, 1.5, 2.0 & 2.5 mg L⁻¹) alone and combination of BAP with KIN (1.0 + 0.1 – 0.5mg L⁻¹); with NAA (0.1 – 0.5 mg L⁻¹) for the development of shoots. The initiation of shoots from the callus cultures were observed after 3 – 4 weeks of incubation in the MS medium containing BA + Kin (1.0 + 0.3 mg/L). The regenerative response was noticed after 15 days of incubation. It was recorded that the growth of shoot length upto 5 - 6 cm long within 6 weeks period. The MS medium supplemented with BA (1.0 mg L⁻¹) alone and BA + NAA (1.0 + 0.5 mg L⁻¹) produced several number of multiple shoots within 3 weeks periods at the average of 85% each (Table 2) and about 80% of the shoots were continued to elongation about 3.4±0.3cm in the medium containing BA + Kin (1.0 + 0.3 mg/l). Few multiple shoots were developed at 1.0 mg L⁻¹ BA + 0.5 mgL⁻¹ KIN (80%). The average number of multiple shoots developed was higher in the concentration of 1.0 mg L⁻¹ BA + 0.5 mg L⁻¹ NAA (85%) with shoot length of about 3.02±0.2 cm. The overall response was moderate and lack of multiple shoot formation in other concentrations of medium. There was no morphological variation in the plantlets was observed after complete maturation (Figures 1 c, d).
Table 2: Effects of different concentrations of cytokinin and auxin in modified MS medium on in vitro shoot proliferation from embryogenic calli cultures of *S. trilobatum*.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentration of hormones (mg/L)</th>
<th>Duration of Regenerative response (Days)</th>
<th>Average number of multiple shoot (%)</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4</td>
<td>20</td>
<td>65</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>20</td>
<td>70</td>
<td>2.69±0.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>15</td>
<td>85</td>
<td>3.01±0.8</td>
</tr>
<tr>
<td>BA</td>
<td>1.5</td>
<td>15</td>
<td>72</td>
<td>3.26±0.8</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>20</td>
<td>65</td>
<td>2.91±0.1</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>20</td>
<td>65</td>
<td>2.83±0.1</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.1</td>
<td>20</td>
<td>65</td>
<td>2.78±0.3</td>
</tr>
<tr>
<td>BA + KIN</td>
<td>1.0 + 0.2</td>
<td>18</td>
<td>65</td>
<td>2.93±0.3</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.3</td>
<td>15</td>
<td>75</td>
<td>3.43±0.3</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.4</td>
<td>15</td>
<td>75</td>
<td>3.0±0.2</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.5</td>
<td>15</td>
<td>80</td>
<td>3.06±0.3</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.1</td>
<td>30</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.2</td>
<td>30</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.3</td>
<td>30</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.4</td>
<td>23</td>
<td>75</td>
<td>2.89±0.3</td>
</tr>
<tr>
<td></td>
<td>1.0 + 0.5</td>
<td>20</td>
<td>85</td>
<td>3.02±0.2</td>
</tr>
</tbody>
</table>

- *No response. The values are the mean ± standard deviation. Data represents the average of triplicates, each replicates consists of 14 cultures.*

Fig. 1: Indirect organogenesis of leaf explants of *Solanum trilobatum*. A. Callus initiation, B. embryogenic callus, C. organogenesis (shoot proliferation), D. multiple shoots, E. *in vitro* developed roots, F. acclimatized plant.

**Rooting and Acclimatization**

The shoots excised from the cultures (3 cm length) were transferred to MS medium containing different concentrations of IBA (0.4 – 2.0 mg L⁻¹) and combinations with NAA + IBA (0.5 + 0.1 – 0.5 mg L⁻¹) for root induction. Maximum number of roots was formed in full strength MS medium at 0.5 + 0.4 mg L⁻¹ IBA + NAA the average length of the developed roots was about 4.30±0.2 cm (Table 3). IBA + NAA showed better rooting formation than the IBA alone and more than 80% of multiple roots was observed (after 4 weeks) in higher concentration of IBA and combination IBA + NAA under controlled conditions (Figure 1e). The rooted plants were then maintained for maturation of the roots and it was further removed from the culture medium and transferred to the polycups containing sterile sand and red soil mixed in the ratio of 1:1 and watered frequently with half strength MS medium at the temperature of 24±0.2° C and gradually increase the humidity to strengthen the shoots in acclimatization. The rate of successful acclimatization was achieved as 78%.
Table 3. Effect of different concentrations of individual and combinations of IBA and NAA on root formation of S. trilobatum.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Concentration of hormones (mg/L)</th>
<th>Duration of root formation (Days)</th>
<th>Formation of multiple roots (%)</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA</td>
<td>0.4</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>15</td>
<td>80</td>
<td>3.06 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>15</td>
<td>82</td>
<td>3.05 ± 0.1</td>
</tr>
<tr>
<td>IBA + NAA</td>
<td>0.5 +0.1</td>
<td>30</td>
<td>45</td>
<td>2.86 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.5+0.2</td>
<td>20</td>
<td>70</td>
<td>2.90 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.5+0.3</td>
<td>20</td>
<td>76</td>
<td>3.18 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.5+0.4</td>
<td>15</td>
<td>85</td>
<td>4.30 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.5+0.5</td>
<td>15</td>
<td>80</td>
<td>3.55 ± 0.1</td>
</tr>
</tbody>
</table>

No response. The values are the mean ± standard deviation. Data represents the average of triplicates, each replicate consists of 14 cultures.

DISCUSSION

Reliable callus induction and regeneration of viable plants considered as a limiting step to the successful use of modern techniques in genetic improvement of the major crop [14]. Many researchers observed 2, 4 – D is the best hormone for callus induction in monocot and dicot plants [15]. The results of present study showed 2, 4 – D and combination of 2, 4 – D + NAA yielded high degree of callus mass.

The auxin 2, 4-D, by itself or in combination with cytokinins, has been widely used to enhance callus induction and maintenance [16]. Moreover, depending on 2, 4-D concentration there was a range of variations in callus initiation, percentage of explants developed from callus, callus texture, callus colour and degree of callus formation was noticed. Callus initiation on cut ends of *in vitro* cultured explants could be observed in all 2, 4-D and 2, 4 – D + NAA levels after 7- 17 days. Similar findings were reported by Yasmin *et al.*, (2003) [17].

The callus mass was subcultured in MS medium containing cytokinins for shoot induction. The BAP + KIN (0.5+1 mg/l) produced more number of multiple shoots (3.0±0.6 and shoot length of about 3.5 ± 0.2) and other concentration of cytokinins like BAP, BAP + KIN produced considerable amount of shoot formation. Similar observations about the role of cytokinins in induction of shoots from callus cultures were recorded in *Solanum* species [18, 19]. The rooting response from the shoots cultured in MS medium supplemented with auxins IBA + NAA (at the concentration of about 0.5 + 0.4 mg/l) produced maximum length of roots (4.3±0.2 cm) and 85% of multiple roots. Similarly [20, 21] were also noticed similar results in developing callogenesis and somatic embryogenesis of *Solanum tuberosum*. Kumari *et al.*, (2008) [22] reported similar kind of organogenic green callus obtained from *Ricinus communis* oil seed plant, *Eclipta alba* [23]. Similar direct regeneration of the same species of plant was reported by Jawahar *et al.*, (2004) [2] using different concentration of 6 – BA (8.88 μM/l) and KIN (9.28 μM/l) for shoot regeneration and root formation (at 9.48 μM/l IBA).

The present work concluded the indirect organogenesis protocol for the rapid  *in vitro* proliferation of *Solanum trilobatum* L, an important medicinal plant of India using leaf explants. The induction of callogenesis was strongly dependent on the auxin and cytokinin concentrations used during the subcultures. This study will support improvement of the genetic characteristics of the species and further continuation for large and commercial scale production of the plant and this study helps to avoid further loss of species from natural environment.

Acknowledgement

The authors are wishing heartfelt thanks to Department of Biotechnology, Periyar University, Salem, Tamilnadu for providing laboratory facilities to carry out this research work in a great success.

REFERENCES

ANTIOXIDANT AND HPLC PROFILE OF WILD AND MICROPROPAGATED AERVA LANATA (LINN.) JUSS. EX. SCHULT. - A COMPARATIVE STUDY

Kamalanathan D, Ragavendran C, Natarajan D*
Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem – 636 011, Tamilnadu, India

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ABSTRACT

Aerva lanata (Linn.) Juss. Ex. Schult. (Amaranthaceae) is a wonderful herb, based on their chemical constituents and pharmaceutical applications. The prime of this investigation was done with the comparison of antioxidant and phytochemical properties of in vivo and invitro propagated Aerva lanata leaves. The micropropagation results express higher rate of multiple shoots from node and shoot-tip explants in BAP alone rather combinations of BAP + KIN on modified MS medium produced comparable rates of regeneration. Phytochemical analysis revealed the presence of alkaloids, flavanoids, total phenols, tannins, and saponins. Plant extracts at lower concentration show potential antioxidant properties (DPPH, FRAP, metal chelation and phosphomolybdenum assays) of both invitro and invitro leaf extracts. The HPLC profile of plant extract shown the occurrence of four sharp peaks was confirmed the presence of phenolic compounds. The overall results indicate that micropropagated A. lanata can be used for preparation of foods and pharmaceutical formulations.

Corresponding author
Dr. D. Natarajan M. Sc., Ph.D.,
Assistant Professor,
Natural Drug Research Laboratory,
Department of Biotechnology,
Periyar University, Salem – 636 011, Tamilnadu, India.
mdnataraj@rediffmail.com, natarajpu@gmail.com
+91 94438 57440, +91 0427-2345124 (office)


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INTRODUCTION

Antioxidant research is an important one in food and medical field. Oxidative process is the important route in the production of free radicals in food, drug, and in some living systems leads to several disorders in human body [1, 2]. Free radicals are said to cause adverse effects in immune system of the human body, excessive oxidation of cellular substrates (oxidative stress) resulting in type II diabetes, neuro-degenerative disease, cardiovascular disease and cancer etc. [3]. Currently, there is a huge demand for natural antioxidants in food industry, for replacing the synthetic preservatives used to prevent fat rancidity or colour loss [4]. Added to that, antioxidants are an important group of medicinal compounds used in food additives for inhibiting detrimental changes of easily oxidizable nutrients [5].

Amongst the antioxidants, polyphenols (such as anthocyanins, tannins, flavanones, isoflavones, resveratrol and ellagic acid) were used in the food and neuteracutical industries [6]. The antioxidants which are now being used in markets, such as Butylated Hydroxy Anisole (BHA) and Butylated Hydroxy Toluene (BHT) have been suspected for causing liver damage and other side-effects. Therefore, it is now required to development and utilization of more effective antioxidants derived or obtained from the natural origin (which will not cause adverse side effects) [7]. Natural antioxidants can protect the human body from free radicals and hold back the progress of rancidity in foods [8]. Antioxidant properties have been studied in several plants for the development of natural antioxidant formulations in the fields of food, medicine and even in cosmetic industries [9].

The large-scale production (in vitro) of bioactive compounds or extracts used as phytotherapies, pharmaceutical products, food additives and cosmetics should be encouraged because of their scientific, economical or ecological importance [10]. However, the invitro productions of bioactive metabolites or extracts are based on the interaction of environmental conditions, hormonal concentrations and the genotype of the cultured plant cells [11]. Thus, the invitro generated or cultivated plants are said to be effective source for mass extraction of many phyto-constituents of plants. Invitro propagation is used as a viable biotechnological tool for mass cultivation of plants and recommended for its ease of access to develop large scale production with a limited source and minimum period [12].

Considering the above information’s the present study was chosen a wonderful medicinal plant, Aerva lanata (L.) Juss. Ex Schultes (locally called as ‘Poolapoo’) is an erect or prostrate under-shrub with a long tap-root and many wolly-tomentose branches [13] and belongs to the family Amaranthaceae. It is used beneficial in many physiological systems such as cardiovascular and renal and influences hypertension and hyperglycemia [14]. Earlier, the plant growth is limited by its conventional germination due to its seed dormancy and growth suffer during non-seasonal periods [15] are the leading cause for the potential risks during their sustainable utilization. So far, various biological properties of A. lanata was analysed by many researchers. Antioxidant property of micropropagated A. lanata has not been documented. Hence, the current study was aimed to report the micropropagation, phytochemical composition, and its antioxidative properties of both invitro and invitro Aerva lanata leaves.

MATERIALS AND METHODS

Source of the Plant:

The explants of Aerva lanata (Linn.) Juss. Ex. Schult was collected during the month of February from Botanical garden, University campus, and it was authenticated by Botanical survey of India, Southern Circle, Govt. of India (BSI/SRC/5/23/2013-2014/Tech/2082). A voucher specimen (PU-Biotech-NDRL-03) was deposited in the department. Auxiliary buds were selected for the in vitro propagation and extraction purposes.

Micropropagation of explants:

The stem explants (node and shoot-tip) were surface sterilized aseptically and inoculated in the modified Murashige and Skoog (MS) medium [16] 3% sucrose and 0.7% agar (Hi Media) was used as carbon source and solidification purpose. Different concentrations of cytokinins and auxins were used to shoot induction. Hormones such as 6 − Benzyl amino purine (BAP) alone, combination of BAP + KIN (Kinetin) (0.5 – 1.5 mg L⁻¹ BAP, 1.0 BAP + 0.5 – 1.0 mg L⁻¹ KIN) were used for invitro propagation. Cultures were maintained under cool-white fluorescent light at 24 ± 2°C with 16h photoperiod. The shoot buds regenerated from explants were isolated and sub-cultured in appropriate time intervals (4 weeks) on same or improved medium for multiple shoots formation. The well-developed shoots (about 5 cm average) were transferred to MS medium supplemented with auxins (Indole − 3 − Butyric acid (IBA) alone and combination with 1 – Naphthalene Acetic acid (NAA) (0.5 – 2.0 IBA, 0.5 + 0.25 – 1.0 mg L⁻¹ NAA, IBA + IAA 0.5 – 1.0 mg L⁻¹ respectively) and incubated as same conditions for invitro roots. Complete invitro matured plantlets were transferred to the green house in the polycps containing Sterile soil and sand (1:1 ratio).

Extract Preparation:

Matured invitro and invitvo grown plant leaves (about 45 to 60 days) were washed and shade dried (at 50°C temperature in laboratory hot air oven for 24 hours), ground into powder using mortar and pestle. 2gms of the plant powder was extracted sequentially in 20 ml of organic solvents (like hexane, chloroform, ethyl acetate; acetone and methanol) and kept in orbital shaker for 48 hours. The extracts were filtered through Whatmann no.1 filter paper and the filtrates were reduced to 10% of its original volume.

Qualitative Phytochemical Screening:

The invitvo and invitro leaves of A. lanata were analysed for the presence of major phytochemicals (alkaloids, flavonoids, phenolic compounds, terpenoids, tannins, saponins, carbohydrates and amino acids) by standard methods [17].
Antioxidant Assay

DPPH Radical Scavenging Activity:

The antioxidant potential of plant extracts were estimated by the free radical scavenging ability or the donation of hydrogen ions with reference to the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity [18]. Different aliquots of samples (20 - 100 µl of methanol and aqueous extracts and 50 - 250 µl of solvent extracts) were dissolved in methanol and made upto 100µl. Later, it was mixed with 5ml of DPPH (0.1mM methanolic solution) and allowed to stand for 20 min (at 27°C). The change in the purple to yellow colour in all test tubes was measured spectrophotometrically (at 517 nm). A blank/ control was prepared by the same methanol solvent without adding plant extracts. The percentage of free radical scavenging activity of the samples was calculated using the formula

\[
\% \text{ DPPH Radical Scavenging Activity} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100
\]

Where, \(A_0\) = Control OD; \(A_1\) = Sample OD

More significantly, the IC50 of the extracts were also calculated.

Metal Chelating Activity:

The chelation of metal (ferrous ions) in test extracts was estimated by the modified method [19]. About 50µl of 2mM ferric chloride (FeCl3) was added with different concentration of extracts (20–100µl). And, it was allowed stand for 1 minute and made upto 1800 µl using deionized water. The reaction was initiated by the addition of 200µl of 5mM ferrozine solution. Then, reaction mixture was shaken vigorously and incubated for 10 minutes at room temperature. The absorbance of pink colour solution was measured using spectrophotometer (at 562nm). A negative control was prepared with same reaction mixture without sample. The percentage inhibition of ferrozine – Fe²⁺ complex formation was estimated. Aliquots of ethylene diamine tetra acetic acid (EDTA) (20 - 100µg/ml) were prepared as standard. And the results were calculated using the formula as mentioned below

\[
\% \text{ Metal chelating activity} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100
\]

Where, \(A_0\) = Control OD; \(A_1\) = Sample OD

Ferric Reducing Antioxidant Power Assay:

Ferric reducing antioxidant power assay (FRAP) reagent was performed in order to estimate the antioxidant capacities of phenolic extracts of samples as per the method [20]. FRAP reagent was prepared by adding 2.5 ml of 20mmol/L TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40mmol/L hydrochloric acid and 2.5 ml of 20mmol/L FeCl3.6H2O and 25 ml of 0.3 mol/L acetate buffer (pH 3.6). 90µl of plant extracts and standard was added with 270 µl of distilled water. 2.7 ml of FRAP reagent was mixed and incubated at 37°C in water bath; 30µl of methanol was used as blank. After incubation period, the absorbance was read (at 593 nm) immediately in spectrophotometer. The FRAP value is expressed as mmol Fe (II) equivalent/mg extract.

Phosphomolybdenum Assay:

Antioxidant activity was also evaluated by analysing phosphomolybdenum assay [21]. The plant extract (300 µl) was mixed with 3 ml of reagent solution (contains 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in separate vials. The vials were tightly closed and incubated in a boiling water bath (at 95°C for 90 min). A blank was also prepared in same method. Samples were allowed to cool to bring at room temperature. The development of green colour was measured at 695 nm against a blank. The results are mean values in grams of ascorbic acid equivalents per gram sample (AAE/g)

High Performance Liquid Chromatography:

The HPLC system was used for separation of the bioactive compounds from in vitro, in vivo methanolic leaf extracts of A. lanata. The chromatographic separation was performed on a C18 column (5mm, 250×4.6mm i.d.) with the column temperature set at 35°C. Linear gradient elution was used with methanol (A) and aqueous (B), with the procedure flow rate of 0.5mL/min. (gradient change from A to B as followed (v/v): 5 to 25% (0 to 5min), 25 to 55% (5 to 10 min) and 55 to 100% (10 to 15 min). The UV detector is used at 254nm. The HPLC analysis of plant sample was done by external instrumental facilities.

Statistical analysis

Each experiment was performed in triplicates and used for statistical analysis i.e mean ± standard deviation using Graph pad Prism Version. 5. Data’s were also subjected to analysis of variance and mean values at P<0.05 & 0.01, 0.0001 were considered to be significant.

RESULTS AND DISCUSSION

Micro propagation of A. lanata in modified MS medium

The regeneration of shoots and nodal explants of A. lanata was showed after two weeks of incubation. The nodal explants exhibit higher regeneration rate compared to shoot-tip explants. The BAP hormone (at 1.0mg/l) produced highest number of multiple shoots, it was recorded as 82% and the shoot length of 5.15 ± 0.2, 4.7 ± 1.1cm, and the combined effect of 1.0 + 0.5 mg/l BAP + KIN concentration show higher multiple shoot formation (94%) and the shoot length was recorded as about 4.9 ± 0.7 and 4.4 ± 0.2 cm in shoot-tip and nodal explants (Table 1; Fig.1) respectively. The other concentrations of BAP produced limited multiple shoots and shorter shoot lengths.
Table No-1: Multiple shoots formation from shoot-tip and nodal explants of A. lanata using different cytokinin combinations.

<table>
<thead>
<tr>
<th>Concentration of hormones (mg/L)</th>
<th>Mean of the shoot length (cm)</th>
<th>Mean number of multiple shoots (no’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 BAP</td>
<td>2.6 ± 0.6</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>1.0 BAP</td>
<td>5.1 ± 0.2</td>
<td>7.2 ± 1.6</td>
</tr>
<tr>
<td>1.5 BAP</td>
<td>2.1 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>1.0 BAP + 0.5 KIN</td>
<td>4.9 ± 0.7</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>1.0 BAP + 1.0 KIN</td>
<td>2.7 ± 0.9</td>
<td>5.1 ± 1.9</td>
</tr>
</tbody>
</table>

The results are mean ± S.E of triplicates, n = 14 explants/replicate. * - indicates significance with each other at P<0.05

The multiple shoots were excised and inoculated in rooting medium induces roots in the cut ends and medium containing 2.0 mg/L IBA. The highest root length and branch roots (7.2 ± 1.3 cm and 4.7 ± 1.0 mean of branch roots) was obtained in IBA fortified medium and other concentrations produced comparable rates of root proliferation (Table 2; Fig.1). The matured *in vitro* plant was acclimatized in green house successfully at a rate of 90% in polycups containing sterile sand and soil in 1:1 ratio. Hence our achievements on micropropagation with lower concentration of cytokinins were comparable with the previous report on this plant show L2 medium at 1.5mg/L BA produced higher multiple shoots (17±3 shoots & 4.7±0.2 cm length) [22]. Similarly, other reports on micropropagation of *Aerva lanata* at 2.0mg/L BAP produced 29 shootlets from 50mg callus [23]. Comparatively shoot organogenesis of about 23.6 ± 0.16 was obtained on medium containing 1.0mg/L TDZ was recorded by Varuthraju et al [15].

Table No-2: *In vitro* rooting response of A. lanata using different auxin combinations

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Concentration of hormones (mg/L)</th>
<th>Mean of root length (cm)</th>
<th>Mean number of roots (no’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA*</td>
<td>0.5</td>
<td>0.8 ± 1.1</td>
<td>0.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.7 ± 0.9</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.2 ± 1.3</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>IBA + IAA*</td>
<td>0.5 ± 0.25</td>
<td>4.0 ± 1.4</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>IBA + NAA*</td>
<td>0.5 ± 0.25</td>
<td>3.3 ± 0.7</td>
<td>3.5 ± 1.1</td>
</tr>
</tbody>
</table>

The results are mean ± S.E of triplicates, n = 14 explants/replicate. * - indicates significance with each other at P < 0.05

Figure no-1: Micro propagation of A. lanata (A) Regenerated nodal explants (B) Regenerated shoot-tip explants (C) *In vitro* flowering (D) Multiple shoots (E) *In vitro* roots (F) Acclimatized plants.

**Qualitative Phytochemical estimation of A. lanata extracts:**

The phytochemical estimation of six different solvents (hexane, chloroform, ethyl acetate, acetone, methanol and aqueous) extracts of two tested samples of A. lanata (*invivo* and *in vitro* leaf) show the presence of major phytochemicals (Table 3). Phytoconstituents (such as alkaloids, flavonoids, phenolic compounds, tannins and carbohydrates) were invariably present in all the extracts both *invivo* and *in vitro* leaf. Fixed oils were found to be absent in all the tested extracts. Terpenoids were absent in both...
methanolic and aqueous extracts of *invitro* leaf and saponins were absent in hexane chloroform and ethyl acetate extracts of both *invivo* and *invitro* leaf. This preliminary study observed the presence of diverse phytoconstituents could be reported to responsible for their potential antioxidant activity. More recently, few investigators find out methanol as well as aqueous extracts of *A. lanata* reported to contain major phytoconstituents [24, 25].

**Table No- 3: Preliminary phytochemical analysis of *A. lanata* extracts**

<table>
<thead>
<tr>
<th>Tests</th>
<th>HX FL</th>
<th>IL</th>
<th>CL FL</th>
<th>IL</th>
<th>EA FL</th>
<th>IL</th>
<th>AC FL</th>
<th>IL</th>
<th>MT FL</th>
<th>IL</th>
<th>AQ FL</th>
<th>IL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Phenolics</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aminoacids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fixed oils</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

HX – hexane; CL – chloroform; EA – ethyl acetate; AC – acetone; MT – methanol; AQ – aqueous; FL – *invivo* leaf; IL – *invitro* leaf; + = present; – = absent.

**Antioxidant potential of *A. lanata* extracts**

**DPPH radical scavenging assay:**

The free radical scavenging activity of plant extracts was performed by DPPH (1, 1, 2, 2 diphenyl picryl dihydrazine) decolorising assay for estimating IC\(_{50}\) values. As per the table 4 depicts, the IC\(_{50}\) value of methanol extract was found potent with the concentration of 53.77, 64.42µg/ml followed by aqueous extracts showing IC\(_{50}\) at 72. 22 and 75.98µg/ml of *invivo* and *invitro* leaf respectively. The radical scavenging rate was found moderate in all other extracts (acetone, hexane and ethyl acetate extracts) and the results was tabulated (Table 4). Overall output of study show lower concentration of extracts was much better radial scavenging activity than others and compared with earlier reports on methanolic and petroleum ether extracts of *A. lanata* (IC\(_{50}\) values are 70.72, 55.72% at the concentration of 500 µg/ml) [26]. Similarly our report was also correlated with other findings [18] recorded the lower concentration of extracts 0.284mg/L showed optimum IC\(_{50}\) values in ethanolic extracts of *P. scandens*.

**Table No-4: IC\(_{50}\) values of DPPH assay of field and *Invitro* leaf extracts of *A. lanata***

<table>
<thead>
<tr>
<th>Solvents</th>
<th>DPPH assay(µg/mL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>field leaf</td>
<td><em>in vitro</em> leaf</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>98.06</td>
<td>120.68</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>157.76</td>
<td>170.85</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>113.83</td>
<td>110.80</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>90.59</td>
<td>106.40</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>53.77</td>
<td>64.42</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>72.22</td>
<td>75.98</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean of triplicates. IC\(_{50}\), required concentration for inhibition of 50% of radical formed. Minimum IC\(_{50}\) values indicate maximum antioxidative property.

**Ferric reducing antioxidant power assay (FRAP):**

The capacity of a compound to reduce Fe \(^3+\)/ferric cyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity [27, 28]. The dense colour formation indicates that it having higher activity. The rate of colour formation was recorded and tabulated (Table. 5). Aqueous *invivo* leaf extract has higher (112.88 mM FeII/mg) value followed by methanol and aqueous *invitro* leaf extracts (73.48 and 67.22 mM FeII/mg). The rest of extracts show slight ferric reducing antioxidant properties. Similar results was documented [29] and show significant ferric reducing potential in ethanolic leaf extract (1,539.56 mM/mg), Overall results show *invitro* leaf extracts have notable reducing power than *invivo* leaf extracts.
Table No- 5: FRAP assay of field and *Invitro* leaf extracts of *A. lanata*

<table>
<thead>
<tr>
<th>Solvents</th>
<th>FRAP assay (mM(Fe)II/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>field leaf</td>
</tr>
<tr>
<td>Hexane</td>
<td>5.58 ± 4.04</td>
</tr>
<tr>
<td>Chloroform</td>
<td>9.69 ± 2.06</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>5.10 ± 0.22</td>
</tr>
<tr>
<td>Acetone**</td>
<td>3.53 ± 0.30</td>
</tr>
<tr>
<td>Methanol*</td>
<td>64.02 ± 2.80</td>
</tr>
<tr>
<td>Aqueous***</td>
<td>112.88 ± 5.82</td>
</tr>
</tbody>
</table>

Values are mean of triplicates ± SD, # - significance in column factors * - indicates significance with each other in row factors
* P <0.05, ***P < 0.0001.

Phosphomolybdenum assay:
Antioxidant activity of plant extracts was estimated by analysing phosphomolybdenum assay which is estimated the content of Vitamin E in the plant samples [30]. The dark green colour formation after the reaction between the reagent (ammonium molybdate and sodium phosphate with sulphuric acid) and sample extract indicates highest antioxidant activity and the results were recorded. The plant extracts were assayed for phosphomolybdenum (Table 6). Aqueous and acetone extract of field leaf show dense colour followed by *invitro* leaf aqueous extract (400.48, 356.95 and 345.90 mg AAE/g). In same way, the acetone extract 430.38 AAE/g of *P. Scandens* and [29] ethyl acetate extract (232 mg/g) of *M. vaginalis* reported higher content of Vit.E showed higher total antioxidant properties [18].

Table No-6: Phosphomolybdenum assay of field and *Invitro* leaf extracts of *A. lanata*

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Phosphomolybdenum assay (mg AAE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>field leaf</td>
</tr>
<tr>
<td>Hexane</td>
<td>40.89 ± 3.11</td>
</tr>
<tr>
<td>Chloroform</td>
<td>107.27 ± 3.40</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>80.90 ± 2.22</td>
</tr>
<tr>
<td>Acetone**</td>
<td>356.95 ± 0.78</td>
</tr>
<tr>
<td>Methanol*</td>
<td>206.29 ± 5.10</td>
</tr>
<tr>
<td>Aqueous***</td>
<td>400.48 ± 1.12</td>
</tr>
</tbody>
</table>

Values are mean of triplicates ± SD, # - significance in column factors * - indicates significance with each other in row factors
* P <0.05, ***P < 0.0001

Metal chelation assay:
Metal chelation is the process used to remove metal ions from the solution, especially to counter the poisoning by heavy metal ions. Antioxidants inhibit interaction between metal and lipid by forming insoluble metal complexes with ferrous ion [31]. Thus, the iron-chelating capacity test was measure the ability of antioxidants to compete with ferrozine in chelating ferrous ion. The *A. lanata* extracts show promising metal chelation activity in the aqueous extracts of both *invivo* and *invitro* leaves (686.68 and 685.56 EDTAE/g). The *invivo* plant extracts have reported to contain higher metal chelation property compared with *invitro* leaf extract. The lowest metal chelation was observed in hexane extracts of *invitro* leaves (Table 7).

Table No-7: Metal chelation assay of field and *Invitro* leaf extracts of *A. lanata*

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Metal chelation assay (mM EDTA E/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>field leaf</td>
</tr>
<tr>
<td>Hexane</td>
<td>87.10 ± 1.01</td>
</tr>
<tr>
<td>Chloroform</td>
<td>112.69 ± 6.36</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>193.27 ± 1.05</td>
</tr>
<tr>
<td>Acetone*</td>
<td>186.85 ± 4.41</td>
</tr>
<tr>
<td>Methanol*</td>
<td>182.19 ± 6.35</td>
</tr>
<tr>
<td>Aqueous*</td>
<td>686.68 ± 13.37</td>
</tr>
</tbody>
</table>

Values are mean of triplicates ± SD, # - significance in column factors * - indicates significance with each other in row factors
* P <0.05,

More recently, other investigators reported the antioxidant property of *A. lanata* leaf extracts. Fractions of ethanol extract show potent (DPPH) antioxidant property (IC$_{50}$ value at 0.542) was reported [32]. Another study [33] reported ethanolic extracts of *A. lanata* shows effective free radical scavenging activity, the IC$_{50}$ values were found to be 219.65µg in superoxide radical-scavenging
activity, 223.72μg in hydroxyl radical-scavenging activity and 168.13μg in DPPH radical-scavenging activity respectively. Likewise, the antioxidant potential and phytochemical screening of aqueous, ethanol & aqueous extracts of A. lanata was done by Ragavendran et al. [24] and their results shows high 2. 2 – diphenyl -1-picrylhydrazyl radical scavenging activity (IC<sub>50</sub> 110.74 μg/ml) and metal chelation activity (IC<sub>50</sub> 758. 17μg/ml) in ethanol and aqueous extracts. Total phenolic and DPPH scavenging of Aerva tomentosa was evaluated [34] and the results highlights methanol extract have been reported as 34.5 ± 0.726 mg gallic acid equivalent/g extract and DPPH value of methanolic extract show RC<sub>50</sub> value as 6.0μg/ml. Likewise, Mammen and Sane [35] reported higher antioxidant potential of curcumin with lower IC50 values in DPPH assay in wild plants. So far, none of the researchers report the antioxidant property of micropropagated A.lanata.

High performance Liquid Chromatography (HPLC):

The HPLC result reveals presence of good percentage of bioactive compounds. The HPLC profile of A. lanata (<i>invitro and</i> <i>in</i><i>vivo</i> leaves) show four major peaks (at a wavelength of 254 nm) due to sharpness of peaks and proper baseline and recorded its retention time (Rt min), percent area and heights (Table 8 & 9). The results show the highest peak value indicates phenolic content of samples. Similarly [36] estimated the vanillin content in the suspension cultures of A. lanata by HPLC methods. Previously, the presence of betacyanin pigments in methanol extract of A. lanata by HPLC technique was reported [37]. Further confirmation study is required to estimate the total phenolic compounds responsible in antioxidant activity of both plants (<i>invitro and</i> <i>in</i><i>vivo</i>).

Table No- 8: HPLC Analysis in methanolic extracts <i>in vivo</i> leaf of Aerva lanata

<table>
<thead>
<tr>
<th>Peak#</th>
<th>Ret. Time</th>
<th>Area</th>
<th>Height</th>
<th>Area %</th>
<th>Height %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>6779</td>
<td>271</td>
<td>24.102</td>
<td>25.819</td>
</tr>
<tr>
<td>2</td>
<td>2.586</td>
<td>9325</td>
<td>328</td>
<td>33.153</td>
<td>31.207</td>
</tr>
<tr>
<td>3</td>
<td>3.168</td>
<td>6878</td>
<td>274</td>
<td>24.455</td>
<td>26.077</td>
</tr>
<tr>
<td>4</td>
<td>4.305</td>
<td>5144</td>
<td>177</td>
<td>18.289</td>
<td>16.897</td>
</tr>
<tr>
<td>Total</td>
<td>28126</td>
<td>1050</td>
<td>100.000</td>
<td>100.000</td>
<td></td>
</tr>
</tbody>
</table>

Table No- 9: HPLC Analysis in methanolic extracts <i>in vitro</i> leaf of Aerva lanata

<table>
<thead>
<tr>
<th>Peak#</th>
<th>Ret. Time</th>
<th>Area</th>
<th>Height</th>
<th>Area %</th>
<th>Height %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.369</td>
<td>6640</td>
<td>327</td>
<td>14.385</td>
<td>24.482</td>
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<tr>
<td>2</td>
<td>2.931</td>
<td>13987</td>
<td>342</td>
<td>30.302</td>
<td>25.610</td>
</tr>
<tr>
<td>3</td>
<td>4.664</td>
<td>25531</td>
<td>667</td>
<td>55.313</td>
<td>49.907</td>
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<tr>
<td>Total</td>
<td>46158</td>
<td>1336</td>
<td>100.000</td>
<td>100.000</td>
<td></td>
</tr>
</tbody>
</table>

Figure No-2: HPLC Chromatogram of methanolic <i>in vivo</i> leaf extracts of Aerva lanata
CONCLUSION

The study attempts comparative antioxidant (DPPH, Ferric reducing antioxidant power assay, phosphomolybdenum and metal chelation) potentiality of wild and micropropagated A. lanata. The outcomes of results highlight the use of micropropagated plants with enhanced antioxidant properties. Based on the HPLC peak values, phenolic groups identified as bioactive compounds. Present study encourage positive correlation between the phytochemicals (phenolic and flavonoids) involved in the antioxidant properties are need to be analyzed and prove the evidences of higher antioxidant properties of micro-propagated plants. Further study pertaining to the antioxidant properties on individual fraction of bioactive compounds are under progress.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

ACKNOWLEDGEMENT

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REFERENCES


Regular Article

In vitro propagation of Aloe barbadensis Miller, a miracle herb

Jayakrishna, C., Karthik, C., Barathi, S., Kamalanathan, D. and Indra ArulSelvi, P*

Department of Biotechnology, Periyar University, Salem-11, Tamil Nadu, India
*Corresponding Author: iarulselvi@gmail.com

Aloe vera has valuable medicinal properties and is commercially used in pharmaceutical, cosmetic and food industries. An efficient micro propagation method has been developed in Aloe vera plants using the shoot tip explants cultured on MS medium with different phyto hormonal supplements for shoot proliferation and rooting. The shoot proliferation was found best (80%) in the MS medium containing Benzyl amino purine (BAP) 2.0 mg/L. Seventy percent of adventitious root formation was observed in half strength MS medium supplemented with Indole butyric acid (IBA). After two weeks, in vitro grown plants were transferred to the poly-cups containing 1:1 ratio of soil and sand respectively for hardening and then transferred to garden showed 75% of survival.

Key words: Micro propagation, Aloe vera, BAP, IBA, MS medium.

Aloe barbadensis Miller (=Aloe vera L.) belongs to Liliaceae, which has medicinal and cosmetic properties (Gui, 1990). It is a large succulent perennial plant growing up to 1.5 meters in height, with a strong fibrous root and at-large stem supporting a rosette of narrow lance late leaves. The leaves are whitish green on both sides and bear spiny teeth on the margins. The yellow to orange drooping flowers grow in along raceme at the top of the flower stalk. The fruit is a triangular capsule containing numerous seeds (Kay and Thida, 2005). In nature, A. vera is propagated through lateral buds which is slow, very expensive and low income practice (Meyer and Staden, 1991). It is a perennial succulent xerophyte, which is developing water storage tissue in the leaves to survive in dry areas of low or erratic rainfall. The innermost part of the leaf is a clear, soft, moist and slippery tissue that consists of large thin-walled parenchyma cells in which water is held in the form of viscous mucilage (Josias, 2008).

The plant contains the important antioxidant vitamins (A, C and F), B (thiamine, niacin, B2 (riboflavin), B12, choline and folic acid. The leaf pulp and liquid fraction of A. vera act against various microorganisms (Baby Joseph and Justin Jaj, 2010). The Chinese describe aloe’s skin and the inner lining of its leaves as a cold, bitter remedy which is downward draining and used to clear constipation due to accumulation of heat; the gel is considered cool and moist. In Ayurvedic medicine of India, aloe is used internally as a laxative, antihelmintic, hemorrhoid remedy, and uterine stimulant (menstrual regulator); in combination with licorice root, to treat eczema or psoriasis (Kathi and Victoria, 1999).

Cultivation of high value medicinal plants had created new dimension in the field of agriculture. Regeneration of A. vera in nature (in-vivo) is too slow and insufficient to meet the industrial demand (Arvind, 2010). Also they experienced a
slow increase due to limited availability of raw material with high quality (Campestrin, 2006). Therefore, there is a need to develop suitable and alternative method for traditional propagation like in- vitro propagation for rapid plant production (Natali 1990; Roy and Sarkar, 1991; Abrie and Staden, 2001). Micro propagation using stem and lateral shoot pieces of A. vera had already been proved successful (Natali, 1990; Roy and Sarkar 1991; Mayer and Staden 1991; Aggarwal and Barna 2004). However; source of explants, size, age, genotype, media composition, culture conditions and phenolic content of explants and media discoloration greatly affect shoot regeneration from different genotypes of the same species. The prime objective of this study was to carry out the alternative protocol for rapid in vitro propagation of this medicinally important A. vera.

Shoot proliferation
The explants were cultured on Murashige and Skoog (MS) medium supplemented with different hormones of concentrations from 10 - 40 μg/L of BAP alone. The pH of the medium was adjusted to 5.8 and autoclaved for 15 min at 121 lbs. Then the surface sterilized explants were inoculated in the culture medium and incubated at 24 ±0.2°C for 16 hours photo period and were sub cultured in order to increase budding frequency. After 4 weeks of incubation, the regenerated shoot was transferred to increased hormonal concentration for shoot elongation. Serial sub-culturing with certain time interval was used for multiple shoot formation.

Rooting of micro shoots
Newly formed shoots measuring 2-3cm in length were excised individually from the parent explants and transferred to rooting media with different IBA+NAA (1.5+2.0 μg/L) concentrations. The development pattern of roots was observed throughout the entire culture period.

Acclimatization
After 20 days of root formation on rooting media, the plantlets were shifted for acclimatization. Pots were kept ready filled with garden soil, compost and sand in the proportion of 1:1:1 ratio respectively. Then the plants were transplanted into the pots with special care. The plant was kept in surface chamber for initial periods and then, to the glass house having 80% humidity and 31°C temperature for 10 days. Then it was taken to shade house with less humidity. After 45 days, the plants were transferred to the soil.

Results
The explants began to show the signs of shoot proliferation after 6 days of culturing. All explants gave aseptic culture. Plants were free from fungal as well as bacterial contamination. New buds
appeared from the axils of leaves and developed into shoots, only after 4 weeks of culturing (Fig.1A). Shoot tip explants grown on medium with different concentrations of BAP and the multiplication of shoots was found best on MS medium with 2 mg/L BAP and 4.0 mg/L BAP and the emergence of shoots took place in 2 weeks. (Fig.1B) However, the higher concentration of BAP showed low response compared to low concentration of BAP. Shoot proliferation on medium with 4mg/L BAP + 1mg/L KIN also showed a better response (Table 1, Fig1B). Medium having 50 µg/L concentrations of IBA and combination of Auxin and cytokinin (NAA+ IBA) was found to be the best medium for shoot and root proliferations in A. vera (Table 2, Fig. 1C and ID). The small rooted shoots were transferred from in vitro conditions to plastic pots and placed under net to keep the environment wet and shade. Survival of plantlets was observed after one month (Fig. IE).

![Image](image_url)

**Figure 1. In vitro propagation of Aloe barbadensis** A: Inoculated explants showing multiple shoot formation after 6 days of incubation; B: Shoot Elongation; C: Root Induction; D: Branch roots of multiple shoots; E: Acclimatization

**Discussion**

The present study implies that, for shoot proliferation, generally the growth regulators like Auxin and Cytokinbin influence the process seriously. The regeneration of shoots from the explants were observed within 7 days of incubation and the roots were observed after 7 days of incubation under controlled conditions i.e. temperature at 24±2°C and 16 hours photo period with light. Similar result was also reported in A. vera by Arvind et.al. (2010). The multiple shoots were observed in the MS medium containing 2.0 mg/L and 4.0 mg/L BAP without any additional supplements of other hormones and
charcoal. MS medium with growth hormones, produced shoots within 3 days of transfer. However, medium without growth regulator produced shoots after 5 days (Coudhary and Mukundan, 2001). Maximum shoots were obtained at the concentration of BAP 2.0 - 4.0mg /L. Similar results were given by Meyer and staden report (1991). An interesting result was observed in combination of KIN and IAA, in average produced 10 shoots and roots. The highest number of roots per culture was found in MS medium containing 1.5 mg/L IAA. The IAA induced roots were able to produce many lateral roots which can survive as ex vivo plants in acclimatized conditions with soil and sand. The acclimatised plants showed highest percentage of plant survival.

Table 1. Effect of different concentration of BAP and NAA on multiple shoot formation in Aloe vera.

<table>
<thead>
<tr>
<th>Growth regulators (mg/L)</th>
<th>% of explants producing shoots</th>
<th>Average of branches/shoots</th>
<th>Mean of shoots length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>80</td>
<td>32</td>
<td>1.43 ± 0.30</td>
</tr>
<tr>
<td>4.0</td>
<td>70</td>
<td>15</td>
<td>1.13 ± 0.11</td>
</tr>
<tr>
<td>BAP+NAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 + 0.5</td>
<td>70</td>
<td>12</td>
<td>1.26 ± 0.20</td>
</tr>
<tr>
<td>3.0 + 0.5</td>
<td>75</td>
<td>16</td>
<td>1.70 ± 0.60</td>
</tr>
</tbody>
</table>

The experiment consists of 14 explants and repeated for three times and their mean values are calculated.

Table 2. Effect of various Concentrations of IBA and NAA in half strength MS on rooting of micro shoots in Aloe vera.

<table>
<thead>
<tr>
<th>Hormone con. (mg/L)</th>
<th>% of shoot rooted</th>
<th>Average of roots formed</th>
<th>Mean of root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>70</td>
<td>10</td>
<td>1.26 ± 0.20</td>
</tr>
<tr>
<td>2.5</td>
<td>70</td>
<td>08</td>
<td>1.16 ± 0.57</td>
</tr>
<tr>
<td>IBA + NAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 + 1</td>
<td>60</td>
<td>09</td>
<td>1.56 ± 0.51</td>
</tr>
<tr>
<td>2.0 + 1</td>
<td>65</td>
<td>12</td>
<td>1.66 ± 0.76</td>
</tr>
</tbody>
</table>

The experiment consists of 14 explants and repeated for three times and their mean values are calculated.

Other pervious report on the micropropagation of A. vera using more than one type of medium for initiation and multiplication are available (Aggarwal, et al., 2004). In the present study a simple two step protocol was established using MS with BAP for shoot initiation and multiplication and IAA for rooting in A. vera. This protocol could be used for the massive in vitro production of the plantlets of the A. vera.

References


In vitro propagation of nodal and shoot tip explants of Passiflora foetida L.  
An exotic medicinal plant  

C. Ragavendran, D. Kamalanathan, G. Reena and D. Natarajan*  

Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem-636011, Tamilnadu, India.

ABSTRACT  
Passiflora foetida L. (Passifloraceae) is an angiosperm plant with high medicinal value. The present study was developed an efficient micropropagation protocol by using node and shoot tip explants of Passiflora. Explants are surface sterilized and inoculated into culture medium with different concentrations of growth regulators. The MS medium supplemented with the hormone 6 – Benzyl adenine at the concentration of 1.5 mg/l was found to be ideal for optimal growth of the inoculated shoot and nodal cultures (100%) and MS medium supplemented with 1.0 mg/l IBA hormones was best suited for induction of roots in vitro (90%). The rate of successful acclimatization was about 78% in the Sand: Soil: Compost (at the ratio of 1:1:1) of the hardening process.

Keywords: Passiflora foetida L, MS medium, Growth regulators, Multiple shoots, In vitro roots.

INTRODUCTION

P. foetida L. (Passifloraceae) commonly known as Passion fruit is an exotic, fast-growing perennial creeping and spreading vine [Mossukkattan, Poonapudukku (in Tamil) and stinking passion flower (in English)], found in riverbeds, dry forest floors, way side thickets, covering the top of thorny shrubs and also growing near hamlets (Komathi et al., 2011). The specific name, foetida means “stinking” in Latin and refers to the strong aroma emitted by damaged foliage (Flavia Guzzo et al., 2004). The plant is widely distributed in worldwide, especially dominant in Southeast Asia and it consider as a weed in the Pacific Region, West Africa and Central America. The leaves are three- to five-lobed and viscid – hairy. When crushed, these leaves are emitting pungent odor that some people consider as unpleasant. The flowers are white to pale cream colored, about 5 – 6 cm diameter. The fruit is globose, 2–3 cm diameter, yellowish-orange to red when ripe, and has numerous black seeds embedded in the pulp; the fruit are eaten and the seeds are dispersed by birds (Ulmer and McDougal 2004).

P. foetida has quick and effective action in burn wounds and is recommended by Brazilian drugs centre as an anti-rheumatic (Emiim Baby et al. 2010). It is used as a bactericide, antisynergic and antilithic and used for medicinal purposes as a sedative, as well as a food source. It is used for mood disorders (depression, anxiety, and stress) (Rasool et al. 2011). Young leaves and plant tips are edible. The dried leaves are used in Vietnamese folk medicine to relieve sleeping problems. The whole plant is used in the treatment of insomnia and anxiety. In India, the plant is traditionally used for diarrhea, throat and ear infections, liver disorders, tumors, itches, fever, skin diseases and for wound dressing (Ingale et al. 2010). Aqueous extracts of leaves or whole plants have been used as remedy for colic, colds, diarrhea, asthma, and sleeping problems (Joy 2010). Considering the above views, the present study was designed to produce a reliable protocol for in vitro propagation of node and shoot tip explants of P. foetida.
MATERIALS AND METHODS

Source of plant materials
The explant of *P. foetida* was collected during the month of February, in Botanical garden, Periyar University campus, Salem, Tamil Nadu and it was authenticated by Dr. D. Natarajan Assistant Professor, and the voucher specimen was deposited in Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem.

Explant selection and sterilization
The auxiliary buds of *P. foetida* were removed (from healthy & young plant parts) and nodal and shoot tip explants were dissected using a sterile surgical blade or scissors. The explant segments were used for inoculation purpose. The explants were processed initially by washing with running tap water for about 10 – 15 minutes, to remove the dust particles. The explants were treated with a solution containing 1-3 drops of Tween 20 or commercial detergents (teepol) for 10 minutes. Then, it was washed thoroughly with sterile distilled water. The explants were treated with 5% Bavistin solution for 7 minutes and again washed with sterile distilled water. Later, the explants were surface sterilized with a solution containing 0.1%HgCl₂ for 3 – 10 minutes. Then, the explants were finally rinsed thoroughly with sterile distilled water about 3 – 5 times for removal of any chemicals present on the surface of the explants.

Media preparation
The MS (Murashige and Skoog 1962) medium was prepared by adding the aliquots of macro, micro nutrients, vitamins and amino acids. Along with inositol, carbon source (3% sucrose) and gelling agents (0.8 % agar or clarigel) was added and finally the addition of phytohormones like auxins and cytokinins (such as 2, 4 – Dichlorophenoxy acetic acid, 1- Naphthalene acetic acid, and 6 – Benzyl adenine, Adenine sulphate, Kinetin etc) to the shooting and rooting medium. The pH of the medium was adjusted to 5.6 - 5.8 using 0.1N NaOH and 0.1N HCL. Then, the liquid medium was dispersed equally in culture tube (each 10 ml) was air tight closed with cotton plug. Then, the medium was autoclaved at 15 lbs or 121 °C for 20 minutes. The mouth of culture tube was sealed tightly after autoclaved, left undistributed to cool the culture and labeling on the cotton plug. The culture test tubes were being kept under the sterile conditions under laminar air flow (Tissue Culture Chamber).

Explants Inoculation and Incubation
The explants were transferred to the cutting board and trimmed to remove the dead cells from the cutting edges in order to eliminate the contamination and inoculated healthy explants into the MS medium supplemented with specific cytokinins and auxins. The inoculated culture test tubes were incubated photoperiod of 16/8hrs light and dark conditions at 24±2°C. Cool-white fluorescent lights of 3000 lux provided light conditions; observation and recording were made every day starting from sixth day of shoot initiation. The responses of tissue/explants were carefully observed for parameters of Morphogenetic changes, i.e., new leaves and shoot induction and multiple shoot formation.

Multiple Shoots Formation
Shoot tips were established on basal salt medium (MS medium) supplemented with 30 g/l sucrose, 8g/l agar, and the medium containing 1.5 mg/L 6- Benzyl adenine (6 - BA) induced multiple shoots (1-2) with maximum percentage of responding cultures (95%). Shoot tip and inter nodal segments (1cm) were sub cultured on media similar to those used for initiation.

*In-vitro* rooting
The shoots developed in the culture tubes were maintained for 4 – 5 weeks and monitored for the continuous elongation of the shoots up to the desired level. After, the formation of multiple shoots, 3 – 5 cm length of cultures were dissected and inoculated in a medium containing ½ strength MS medium by eliminating the vitamins and amino acids and full strength MS medium supplemented with various concentration of IBA for root formation. Then, the cultures were incubated as similar to earlier steps.

Acclimatization
Rooted shoots are removed from culture and placed in the polycups containing sand: soil: compost at the ratio of 1:1:1. The humidity must be gradually reduced over time because tissue cultured plants are extremely susceptible to
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wilting. This was initially treated with ½ MS stock solution and gradually adding sterile and normal water and the survival rates were monitored.

RESULTS

Shoot Proliferation

Shoot tip and nodal explants of *P. foetida* showed regenerated *in-vitro* cultures after 6 days inoculated on MS medium fortified with different concentrations of 6 - BA (MS basal, 0.5, 1.0, 1.5 & 2.0 mg l⁻¹) alone and combination with KIN (1.5 B + 0.5mg l⁻¹K). It was recorded that the growth of shoot (length upto 5 - 6 cm long) within 3–6 weeks periods. The MS medium supplemented with BA (1.5 mg l⁻¹) alone developed more number of multiple shoots (3.16 ±0.6) developed within 2 – 3 weeks emerged directly from the explants. Out of these shoots, about 80% are continued to elongation. The response was good at 1.5 mg l⁻¹ and 2.0 mg l⁻¹ 6 - BA at the rate of 100 % and 95 % respectively. A few multiple shoots were developed at 1.0 mg l⁻¹ 6 - BA alone and combination of 6 – BA with Kin at the concentration of 1.5 + 0.5 mg l⁻¹. Overall, the response was moderate and lack of multiple shoot formation in other concentrations of medium. Auxiliary buds were isolated and repeatedly subcultured on the same hormone for continuous elongation and multiplication (Table 1; Figure 1) of shoots.

Root Proliferation

For root induction, the shoots excised from the cultures (2 – 3cm length) were transferred to the ½ and full strength MS medium containing different concentrations of IBA (0.4 – 2.0 mg l⁻¹), and maximum number of roots were formed in full strength MS medium (after 15 days of incubation). The MS medium supplemented with the concentration of 1.0 mg l⁻¹ IBA produced more roots at the rate of 90 % and the maximum roots length was about 4±0 cm (Table 2). Whereas, the rooting response of other concentrations of IBA (0.5, 1.5, 2.0 mg l⁻¹) produced 3.0 ± 0, and 2.0 ± 0 CM at the rates of 80, 70, 60 % respectively. But, the MS basal medium which produced the fewer number of roots which limits to elongate after certain length of roots. The well developed roots were observed at 80% (after 4 weeks of incubation) in higher concentration of medium under controlled conditions (Figure. 1).

Table 1: *In-vitro* shoot formation of Shoot tip and Nodal explants of *P. foetida* L.

<table>
<thead>
<tr>
<th>Hormones (mg/l)</th>
<th>Type of Explants</th>
<th>Regenerative Response (days)</th>
<th>Growth Rate (%)</th>
<th>Rate of Multiple Shoots (%)</th>
<th>Elongation (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS basal</td>
<td>Shoot tip, Nodal</td>
<td>12</td>
<td>60</td>
<td>1.8 ± 3.2</td>
<td>3.7 ± 0</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td>12</td>
<td>65</td>
<td>2.0 ± 1.8</td>
<td>3.9 ± 0</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>8</td>
<td>75</td>
<td>2.1 ± 0.8</td>
<td>4.2 ± 0</td>
</tr>
<tr>
<td>1.5</td>
<td>+</td>
<td>6</td>
<td>100</td>
<td>3.6 ± 0.6</td>
<td>7.3 ± 0</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>7</td>
<td>95</td>
<td>3.1 ± 2.1</td>
<td>5.8 ± 0</td>
</tr>
<tr>
<td>1.5B + 0.5K</td>
<td>+</td>
<td>6</td>
<td>85</td>
<td>2.1 ± 0.1</td>
<td>4.5 ± 0</td>
</tr>
</tbody>
</table>

* = Slow response, + = Well growth. Values are given as the mean ±SE. n = 14 explants

Table 2 *In-vitro* Root Development from regenerated shoots of *P. foetida* L.

<table>
<thead>
<tr>
<th>Hormones (mg/l)</th>
<th>Root initiation (days)</th>
<th>Rate of root formation (%)</th>
<th>Rate of multiple roots (%)</th>
<th>Elongation (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS basal</td>
<td>30</td>
<td>60</td>
<td>1.8 ± 0</td>
<td>1.7 ± 0</td>
</tr>
<tr>
<td>0.5 IBA</td>
<td>12</td>
<td>80</td>
<td>2.0 ± 0</td>
<td>3.0 ± 0</td>
</tr>
<tr>
<td>1.0 IBA</td>
<td>13</td>
<td>90</td>
<td>5.0 ± 0</td>
<td>4.0 ± 0</td>
</tr>
<tr>
<td>1.5 IBA</td>
<td>16</td>
<td>70</td>
<td>4.0 ± 0</td>
<td>2.0 ± 0</td>
</tr>
<tr>
<td>2.0 IBA</td>
<td>14</td>
<td>60</td>
<td>3.0 ± 0</td>
<td>2.0 ± 0</td>
</tr>
</tbody>
</table>

Values are given as the mean ±SE. n = 14 explants

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Figure 1: Direct organogenesis of nodal and shoot tip explants of *Passiflora foetida* L.


**DISCUSSION**

*Passiflora* species is almost exclusively propagated through seeds (Meletti 2002), by cuttings and grafts (Silva et al. 2005). But, there are problems related to the seed physiological quantity, unequal germination that directly damages the plant germination rates (Negreiros et al. 2004). Although many reports on the successful propagation on this species results in many reliable protocols were reported. However, the reason for plant responses in micropropagation is still poorly understood. Few species are growing through somatic embryogenesis and direct organogenesis, depending on the culture conditions (Dodsworth 2009). The present study was focused on micropropagation of *P. foetida* using shoot tip and nodal explants in modified MS medium tries to study the hormonal roles in direct regeneration of plant *in vitro*.

The *in-vitro* propagation of *P. foetida* was achieved in MS medium supplemented with different concentrations of 6-BA (1.0 – 2.0 mg/l), and multiple shoots emerged from the shoot-tip and nodal explants of *P. foetida* after 7 days of inoculation. The medium containing 1.5 mg/l of BA induced maximum number of multiple shoots with highest percentage of cultures (100%). The other concentration of BA and its combination were found to be low to moderate growth rates for shoot proliferation. Similarly Komathi et al. (2011) achieved maximum shoot tip from *P. foetida* (76%) in MS medium containing BAP and NAA at 3.0 and 0.5 mg/l and rooted in the MS medium containing the auxin, IBA and IAA alone at 1.0 mg/l. On the other hand, Isuta (2004) also reported the elongation of *Passiflora* shoots at 2.2 mg/l BAP. Yara Britto (2012) was successfully achieved indirect regeneration of the same species (*P. foetida*) from zygotic embryos. Fernando et al. (2007) developed a protocol for direct formulation of leaf primordial...
without any promeristem or cell proliferation phases. These reports were similar to our studies of successfully reproducing more shoots from single node and shoot tip explants.

Also few other reports focused on observation of the indirect organogenesis from the same genus Passiflora by Silva et al. (2009) and reported that somatic embryogenesis from zygotic embryos of P. cincinnata. Pinto et al. (2010) and Reis et al. (2007) developed a reliable protocol for somatic embryogenesis in the Passiflora. Such kind of reports was the representation of hormonal regulation of plant growth in In-vitro propagation. The present work was suggests that complete reproducible protocol using the PGR 6 - Benzyl adenine and Indole 3 butyric acid in shoot and root induction in direct regeneration of P. foetida.

Based on our results the hormone Indole 3 Butyric acid was found to successful in in-vitro rooting. 1.0 mg/l IBA was more effective than other concentration of IBA in roots from shoot cultures. The percentage of rooting response was also highest (90%) in the same concentration of auxin. The well developed plantlets were transferred to polycups containing the mixture of soil, sand and compost (in the ratio of 1:1:1) for hardening.

CONCLUSION

The present study was believed that the protocol developed for the in-vitro propagation of P. foetida in MS medium supplemented with the hormone 6-Benzyl adenine at the concentration of 1.5 mg/l was found to be ideal for the growth of inoculated shoot and nodal cultures. And, similarly the MS medium supplemented with 1.0 mg/l IBA hormone was best suited for the induction of roots in vitro with a 78 % of successful acclimatization will support the researchers and other herbalists for mass development for their beneficial uses in traditional system.

Acknowledgement

The authors are gratefully acknowledged to Department of Biotechnology, Periyar University, Salem for providing necessary lab facilities to carry out the research work successfully. The first author was acknowledged to UGC (UGC NON-SAP Research (Student) Grant) for releasing financial support to the project work.

REFERENCES

Screening of antibacterial potential of leaves and leaf derived callus extracts of Solanum trilobatum L. an important medicinal plant

D. Natarajan * and D. Kamalanathan

Natural Drug Research Lab, Department of Biotechnology, Periyar University, Salem – 636011, Tamil Nadu, India.

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ABSTRACT

The aim of present investigation was deals with the antibacterial activity of leaves and leaf derived callus extracts (ethyl acetate, ethanol, chloroform, and acetone) from the S. trilobatum L. were tested against nine human pathogenic bacteria using agar well diffusion technique. A better inhibition zone was recorded in chloroform extract than other solvents of both extracts. Based on the results, it clearly indicates that most of the extracts were effective against S. aureus, S. typhi, S. dysentriae, S. sonii, C. diphtheriae and S. boydii. The other solvent extracts (such as ethanol, acetone and ethyl acetate) showed moderate to least inhibitory effect against these organisms. The results from our findings indicated that S. trilobatum is one of the potential medicinal plant used for therapeutic purpose. Among the two extracts tested, callus extracts was found to be superior to field grown plants.

Key words: Solanum trilobatum L, Field plant, Callus, Pathogens, Antibacterial activity.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an enormous number of modern drugs have been isolated from natural sources especially from plants; many of these isolates were based on the uses of the agents in traditional medicine. This plant based, traditional medicine system continues to play an essential role in health care, with about 80% of the world’s inhabitants relying mainly on traditional medicines for their primary health care (1). The World Health Organization (WHO) has also recommended the evaluation of plants for effectiveness against human diseases and for the development of safe modern drugs (2).

According to the literature cited the callus has the potential to show secondary metabolite activity and can often be compared with the field plant (3). The success of this strategy, however, depends on the validation of micro propagated plants through pharmacological screening on their suitability for use in traditional medicine.

Solanum trilobatum Linn (Family: Solanaceae), a thorny creeper with bluish white flower, widely distributed throughout India and has been used in Siddha system of medicines to treat various diseases (4). It has been widely used as an expectorant and in the treatment of respiratory diseases including bronchial asthma (5), febrile infections, and tuberculosis (6). The methanolic extract of S. trilobatum has been shown to possess antioxidant activity (7) and hepatoprotective activity (8). Sobatam, the partially purified petroleum ether extract of S. trilobatum has been reported to be very effective in protecting UV induced damage, radiation-induced toxicity and inducing tumour reduction in mice. (9-10). Solasodine and sobatum isolated from S. trilobatum plant has been shown to possess anti inflammatory activity (11). The methanolic extract has been reported to be very effective in protecting Penaeus monodon post larvae from bacterial attack (12) and the acetone extract has been shown to possess ovicidal activity against Culex quinquefasciatus and Culex tritaeniorhynchus (13); and oviposition deterrent and skin repellent activity against Anopheles stephensi (14).

In view of this medicinal and biological importance of Solanum trilobatum, the present investigation was undertaken to screen the antibacterial properties of different solvent extracts of micro propagated (callus) and outdoor grown Solanum trilobatum were tested against selected human pathogenic microorganisms.

MATERIALS AND METHODS

Plant material and sterilization

The fresh and healthy aerial parts of S. trilobatum were collected from the garden and nomenclature was identified by Dr. D. Natarajan, Assistant Professor, Department of Biotechnology, Periyar University, Salem, Tamil Nadu. The explants (leaves) were excised aseptically with sterile scissors and washed with running tap water (for 2 – 5 min) followed by treatment with 5% (w/v) Bavistin fungicide (for 2 min) and rinsed thoroughly with sterile distilled water, then surface sterilized initially with 0.25 % (v/v) Sodium hypochlorite (for 5 min) and rinsed thoroughly with sterile distilled water (2 – 3 times). Explants were surface disinfected with 0.1% HgCl2 (w/v) solution (for 3 – 10 min) and rinsed thoroughly with sterile distilled water (3 - 5 times),and trimmed with sterile blade and wet explants are dried in No.1 whatman filter paper before inoculation (15).

Induction of callus

Explants were inoculated for callus in MS media supplemented with 2 – 3% sucrose as carbon source and 0.7% agar (Hi Media) along with various concentrations of auxins and cytokinins (2, 4 – D (0.1 – 0.3 mg L-1), NAA (0.1 – 0.5 mg L-1) respectively. The pH of the media was adjusted to 5.8 prior to autoclaving for 15 min at 121°C. Cultures were maintained under cool-white fluorescent light at 24 ± 2°C with 16 h photoperiod. The different kinds of callus mass was obtained at various concentration of NAA, 2, 4 – D, and combination of NAA + 2, 4 D. The 1.5 months old callus tissues (MS medium containing 2, 4 – D + NAA 1.0 + 1.0 mg L-1) of in vitro leaf explant (S. trilobatum) were extracted and used in this study. Simultaneously, the field grown (1.5 months old) S. trilobatum leaves were selected for in vitro screening of antimicrobial properties.

Preparation of Extracts

The known amount of field grown and callus mass samples (1.0 g) were collected, dried and subjected to cold extraction by using different solvents like ethanol, chloroform, acetone and ethyl acetate (each 10 ml) at room temperature for 24 – 48 hrs in from the various solvents. The extracts were evaporated and yielded black paste and stored at room temperature for further use.

*Corresponding author.
Dr. D. Natarajan,
Assistant professor,
Department of Biotechnology,
Periyar University,
Salem 636 011,
Tamil Nadu, India.
Micro organisms used
Nine pathogenic organisms viz. three Gram positive (Staphylococcus aureus, Bacillus subtilis, Lactobacillus acidophilus), and six Gram negative (Salmonella typhi, Shigella boydii, Shigella dysenteriae, Shigella sonnei, Corynebacterium diptheriae, and Klebsiela pneumoniae), bacterial strains were used to determine the antibacterial activity of the crude extracts.

Antimicrobial assay tests
The screening of antibacterial activity of both field leaf and callus tissue was done by using agar well diffusion method (216) with few modifications. The Muller Hinton agar media was poured into sterilized petriplate and allowed to solidify. The young bacterial cultures were spread on the top of each petriplate using sterilized L. rod. After, the well (5mm diameter) was made aseptically over the top of plates using sterile borer (5mm diameter). The each well is filled with two concentration of plant and callus extracts (25 & 50 µl/well). The antibiotic Amoxicilllin (50 µl/well) is served as positive and DMSO (50 µl/well) act as negative control. These plates were incubated for 24 - 48 hours. After incubation period, the zone of inhibition around the each well was measured.

RESULTS

In vitro Growth of callus
The leaf explants of S. trilobatum were incubated in MS medium supplemented with different concentrations of 2, 4-D, NAA alone and its combination for callus regeneration. Greenish brown hard, pale yellow green callus was obtained within two weeks of inoculation of explants on 0.3 mg L-1 2, 4-D and 0.5 mg L-1 NAA and combination of 2, 4-D + NAA (1.0 + 1.0 mg L-1) yield morphogenic pale yellow brown callus. Whereas, other combinations of 2, 4-D and NAA yielded slightly friable and brownish callus (Table 1; Figure 1).

Table 1. Effects of NAA and 2, 4-D on callus formation from leaf explants of S. trilobatum

<table>
<thead>
<tr>
<th>Hormone Name</th>
<th>Concentration (mg L-1)</th>
<th>Response of callus mass</th>
<th>Callus appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA</td>
<td>0.1</td>
<td>++</td>
<td>No callus mass</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>+++</td>
<td>Yellow white, hard</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.1</td>
<td>+</td>
<td>Pale brown hard</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td></td>
<td>Brown hard</td>
</tr>
<tr>
<td>2, 4-D+NAA</td>
<td>1.00+1.00</td>
<td>+++</td>
<td>Greenish Brown hard</td>
</tr>
</tbody>
</table>

+, ++, +++ indicates slight, moderate and considerable callusing, - indicates no response.

Antimicrobial activity
The results of antibacterial activity of different solvent extracts from the leaves of field grown and callus of S. trilobatum was tested against Gram-positive and Gram-negative bacterial pathogens were presented in table 2. All the extracts exhibited different degrees of antibacterial activity against most of the tested organisms. The results indicated that all the organisms were found to be more susceptible from lower to higher concentrations of extracts. The results clearly indicates that most of the extracts were effective against S. aureus, S. typhi, S. dysenteriae, S. soni, C. diptheriae and S. boydii. Among the two extracts tested, Callus tissue extracts were found to be more active when compared to field grown plants.

The chlorofom extracts from callus of S. trilobatum inhibited the growth of several Gram-negative bacteria i.e S. dysenteriae (18mm, 20mm), S. typhi (16mm, 20mm), C. diptheriae (13mm, 15mm) and S. aureus (12mm, 14mm) respectively. Solvent extracts of S. trilobatum leaves at two different concentrations exhibited mild to moderate inhibition over the growth of tested bacterial pathogens. The chlorofom leaf extract of S. trilobatum was found to be more effective against the all tested microorganisms with the inhibition zone ranging from 22 to 8 mm. It was found that chlorofom leaf extract exhibited highest degree of activity against S. dysenteriae (each 16mm), S.

typhi (each 16mm), S. Soni (each 22mm). The callus extracts prepared from acetone had broad spectrum activity against all the tested organisms with better inhibitory effect ranging from 13 to 8 mm. The ethanol leaf extracts showed moderate inhibitory activity against the tested bacterial pathogens except S. dysenteriae. Similarly, the extracts of acetone and ethyl acetate have showed considerable inhibitory activity against the selected pathogenic organisms. Besides, all extracts were inactive against the one gram positive organism i.e. L. acidophilus. Hence, all the leaf and leaf derived callus extracts have been expressed better antibacterial property against more than one microorganisms tested.

DISCUSSION
The present study was carried out to evaluate the efficacy of S. trilobatum L. (Field grown plants and callus) leaf extracts against nine human pathogenic bacterial strains. Our results revealed that chloroform, acetone and ethanolic leaf extract of (callus and field grown) S. trilobatum L possess considerable growth inhibitory effect against tested microorganisms. Plants have provided a source of inspiration for novel drug compounds as plants derived medicines have made significant contribution towards human health (17).

The chloroform callus extract showed higher inhibitory activity against many pathogenic organisms and other solvents (such as ethanol, acetone and ethyl acetate) showed...

moderate to least inhibitory effect against same organisms. The zone of inhibition (mm) of the callus extracts showed comparatively higher than the field leaf extracts of same solvents against same microorganisms. The antibacterial activity of leaf and fruit extracts of S. trilobatum was tested against various pathogens like S. aureus, E. coli, P. aeruginosa and K. pneumonia using extracts of ethanol, acetone and ethyl acetate extracts and showed better inhibitory activity (18). The extracts of leaves of S. trilobatum have shown significant activity against two tested organisms (S. aureus (21mm) and P. aeruginosa (20mm)). The results of our findings showed callus chloroform extract expressed moderate to better antibacterial activity against S. aureus(12 mm, 14 mm), S. sonii(22 mm), S. typhi and S. dysentriae (16 mm). In fine, the leaf and leaf derived callus extracts were more susceptible to gram negative organisms. Various workers have already shown that Gram positive bacteria are more susceptible towards plant extracts as compared to Gram negative bacteria (19, 20). These differences may be attributed to fact that the cell wall in Gram positive bacteria is a single layer, whereas the Gram negative cell wall is multilayered structure (21).

Our results are comparable to the results of the antibacterial assay of S. trilobatum L. were tested against the Gram negative bacterial strain, Bacillus subtilis, was more susceptible to the strain of S. aureus (12 mm) was least susceptible (22). A study on the extracts of different solvents of Petunia leaf and callus against E. coli, P. aeruginosa, B. subtilis, S. aureus and reporting that callus chloroform extract possessing high antibacterial potential against the selected pathogens (23).

Other researchers contributed that antibacterial effect of callus and natural plant extracts of Perunma serratifolia L., Bacopa monnieri L. and Aloeophybus cobbe L. plants extracts against selected human pathogens like B. subtilis, E. faecalis, E. coli, K. pneumoniae and fungal cultures using various solvents (such as chloroform, acetone, methanol, ethanol and water) was showed enhanced antimicrobial activity (25, 26, 27). The antimicrobial activity of Rapolafia tetraphylla and Physalis minima leaf and callus extracts (using chloroform, petroleum ether, methanol, absolute alcohol, benzene) were tested against pathogenic fungi and bacteria reported the chloroform extracts of both plants were found more effective against bacterial and fungal cultures (28). Cytological and antimicrobial activity of embryogenic callus induced from leaf cultures of Tinospora cordofilla (Willd.) Miers (29).

Based on our findings the callus extracts of S. trilobatum showed higher inhibitory against several gram negative organisms and suggested that the use of this plant material (in vitro) are recommended for various pharmaceuticals and derivatives of drugs in future.

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REFERENCES


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SCRENNING OF ANTIBACTERIAL AND PHYTOCHEMICAL ANALYSIS OF LEAF AND LEAF DERIVED CALLUS EXTRACTS OF SEBASTIANIA CHAMAELEA (L.) MUELL. ARG

Reena Ganesan, Kamalanathan Desingu, Ragavendran Chinnasamy and Natarajan Devarajan*
Natural Drug Research Laboratory, Department of Biotechnology, Periyar University, Salem-636011, Tamilnadu, India.

**ABSTRACT**

Medicinal plants are widely used as a potent source for isolation of several drugs and formulations in treatment of many diseases. The present study was aimed to carry out preliminary phytochemical and antibacterial activity of six different solvents extracts from leaf and leaf derived callus of *S. chamaelea*. The maximum percentage of callus mass was achieved in modified MS medium supplemented with different concentration of 2, 4 – D (2.0, 3.0, 4.0 and 5.0 mg/l). *In vitro* antibacterial activity of leaf and callus extracts were tested against 12 bacterial cultures by agar well diffusion method. The acetone, methanol and ethyl acetate extracts of leaf and leaf derived callus show maximum inhibitory effect. The preliminary phytochemical analysis reflects the presence of phenolic compounds, carbohydrate, alkaloids, phytosterols, fats and oils, terpenoids. The result highlights among two extracts, leaf extract show negligible activity than callus extracts. The present study conclude that *invitro* raised plants can be utilized for isolation of antimicrobial drugs than wild plants.

**Keywords**
Sebastiania chamaelea, Callus, Antibacterial activity, Phytochemical analysis

**Corresponding author**
Dr. D. Natarajan, M. Sc., Ph.D,
Assistant Professor,
Natural Drug Research Laboratory,
Department of Biotechnology,
Periyar University, Salem – 636 011, Tamilnadu, India.
Mail: mhnataraj@rediffmail.com, natarajpu@gmail.com
Mobile: +91 94438 57440 Fax: +91 0427-2345124 (office)

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INTRODUCTION
According to World Health Organization (WHO), about 80% of the populations in many third world countries still use traditional medicine (medicinal plants) for their primary health care, due to poverty and lack of access to modern medicine [1]. 80 % of the people (6.1 billion) of the world live in less developed countries (more than 3.9 billion peoples) will likely use medicinal plants on a frequent basis. Nowadays, several antimicrobial drugs are available and particularly the antimycotic drugs present limited use by a number of factors, such as low potency, poor solubility, emergence of resistant strains and drug toxicity [2,20]. Therefore, the search for discovery of new antimicrobial agents is necessary and stimulates the research of new chemotherapeutic agents in the medicinal plants. *Sebastiania chamaelea* (Euphorbiaceae) has been selected for the present study, based on its usage in the traditional, folklore and ethnobotanical importance [3, 4]. The significant herbal uses of *S. chamaelea* are the decoction of the plant in ghee was given as tonic and applied to the head in vertigo. The juice of the plant is astringent and is used as a remedy for syphilis and diarrhea [5, 6]. *S. chamaelea* accounted for 77.5% of human necessary amino acids of which arginine stands highest with 60% of free amino acids, may promote the role for its medicament activity [7]. Now-a-days the importance and raising demands for plant based medicine, there is an urgent need to isolate biomolecules from plants by raising callus mass (*via* tissue culture technique), helped to increase the content of bioactive molecules and also conserve the plants in natural environment. Hence the present study was aimed to study the antibacterial and phytochemical nature of *in vivo* leaf and leaf derived callus extracts of *S. chamaelea* under laboratory condition.

MATERIALS AND METHODS

Collection of explants
*S. chamaelea* was collected from Botanical garden, Periyar University campus, Salem and the nomenclature was identified by Dr. D. Natarajan, Assistant Professor, Department of Biotechnology, Periyar University, Salem, Tamilnadu. The identification was done by referring standard flora’s and compared with available herbarium records. The herbarium specimen was deposited in NDRL, Department of Biotechnology, Periyar University, Salem.

Medium preparation
MS medium was prepared by adding aliquots of modified compositions [8] along with supplements of different PGR’s at various concentrations (auxins like 1-Naphthalene acetic acid, 2. 4-Dichlorophenoxyacetic acid and cytokinins like 6-Benzyladinine, Kinetin). The media were supplemented with 3% sucrose as a carbon source and 0.8% agar for solidification purpose. The pH of medium was adjusted to 5.70 ±0.5 using 0.1 HcI or 0.1 NaOH. The media was autoclaved at 121°C for 15 minutes at 15 lbs/In² pressure. Then, it was kept undisturbed for solidification and used for inoculation and subculture purposes.

Explants sterilization and inoculation
Young and healthy shoots were excised from *S. chamaelea* using sterile blade and explants were subjected to surface sterilization. The explants were cut into 1-1.5 cm length and washed under continuous flushing of running tap water for 15 min to remove the surface contaminants and soil particles. Then, the explants were submerged in 5% Bavistin solution with continuous and gentle stirring for 10 min and washed 2 times with sterile distilled water. Then, the explants were disinfected with solution containing 1-3 drops of Tween 20 or commercial detergents (teepol) for 10 minutes. Finally, the explants were sterilized with solution containing 0.1% HgCl₂ for 7 min, inside laminar air flow chamber and again washed with autoclaved distilled water for 3 times to remove all the traces of sterilant and dried in Whatmann No:1 filter paper. Explants were aseptically transferred on modified MS medium and kept for incubation.

Incubation and sub-culturing
All the cultures were maintained at a temperature of 24±2.0°C with light intensity of 3000 lux for a photoperiod of 16:8 hours of light and dark period in every 24 hour cycle. The shoots and callus developed in the culture bottles were maintained for 4 weeks period and regularly sub-cultured for multiple shoot formation and higher callus mass. The 1.5 months old callus (maximum growth index) of *S. chamaelea* was used for the screening of antibacterial and phytochemical analysis.

Extract preparation
The tissue cultured regenerative callus as well as naturally grown leaves of *S. chamaelea* were collected and shade dried. 5g powder of each sample was soaked separately in 25mL of methanol, acetone, chloroform, ethyl acetate, hexane and ethanol solvents for 24 h at room temperature. Then, the mixture was filtered through Whatman No-1 filter paper. Green colored filtrate of *in vitro* grown calli and *in vivo* grown plant leaves were evaporated to dryness.
Bacterial cultures collection
The bacterial pathogens (namely *Shigella dysenteriae*, *Vibrio vulnificus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Shigella boydii*, *S. sonnei*, *Escherichia coli*, *Coryn bacterium diphtheriae*, *Salmonella typhi*, *Bacillus subtilis* and *Klebsiella aerogenes*) collected from clinical laboratories of Salem District, Tamilnadu. The bacterial cultures were maintained on nutrient broth (NB) at 37°C and periodically sub-cultured.

Antibacterial assay
The antibacterial activity was performed by agar well diffusion method described by Natarajan *et al* [9] with few modifications. Sterile molten Mueller Hinton Agar (MHA, HiMedia) was poured into sterile petridish and allowed to solidify. Young bacterial broth culture (16 hours old) was spread on surface of MHA plates. A small deep well was made by using cork borer (5.0 mm diameter) around the plates and marked. Then, it was filled with different extracts of callus and leaf. The plates were allowed to stand for one hour for pre-diffusion of extracts and incubated for overnight at 37°C. The standard antibiotic Cefotaxime (1mg/ml) was used as positive control. At the end of incubation period, the diameter of inhibition zone was measured in millimeter [10].

Preliminary phytochemical analysis
The preliminary phytochemical tests were performed on the chloroform, acetone, methanol, hexane, ethanol and ethyl acetate extracts of *S. chamaelea* using standard methods [11].

RESULTS AND DISCUSSION
Callus development
Leaf explants were cultured in the MS medium containing higher concentrations of auxins which are responsible for initiation of callus (2, 4 – Dichlorophenoxy acetic acid alone, 1 – Naphthalene acetic acid alone and combination of both at the concentrations ranging from 1 – 5 mg/l and combinations at the concentrations of 1.0 + 0.5, 1.0 + 1.0 and 1.5 + 1.5 mg/l 2, 4 – D + NAA respectively). The various characteristic callus mass was observed during the course of investigation and the results were presented (Table 1&figure 1). The explants exhibit some degree of morphological changes and developed calluses from their cut edges after 7 days of inoculation followed by development of callus (after 2 weeks of inoculation). The culture was maintained in the same conditions and produced more number of callus mass. 2, 4 – D yield pale yellow soft, yellow green soft and hard callus mass in the medium at the concentrations of 2.0, 3.0, 4.0, 5.0 mg/l. Relatively, 2.0 mg/l 2, 4-D yield lower amount of callus compared to other concentrations. Whereas, the hormone NAA was yield yellow and yellow green soft callus in the concentrations of 3.0 – 5.0 mg/l. But 1.0 and 2.0 mg/l NAA fails to produce callus. The combinations of 2, 4 – D and NAA also yielded pale yellow and yellow green soft callus at the concentrations of 1.0 + 0.5, 1.0 + 1.0 and 1.5 + 1.5 mg/l. The callus developed from respective hormones are repeatedly sub cultured (after 4 – 6 weeks periods) for the maintenance of their mother culture and used as explants for the somatic embryogenesis from callus and also used in the sustainable manner for assay of biological properties. Duangporn and Siripong [12] developed callus from *Phyllanthus acidus* on MS medium supplemented with 2,4-D 1 mg IG1 and kinetin 1 mg IG1 to support the growth of callus cultures. Zi-Song Yang [13] induced the callus on Murashige and Skoog’s (MS) medium supplemented with 3.0 mg 1-1 2, 4-D from *Euphorbia helioscopia* (Table 1& figure 1).

<table>
<thead>
<tr>
<th>Table 1: Development of callus from <em>S. chamaelea</em> and their characteristics</th>
</tr>
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<tbody>
<tr>
<td><strong>PGR</strong></td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>2, 4 – D</td>
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<tr>
<td></td>
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<td></td>
</tr>
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<td></td>
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<tr>
<td>NAA</td>
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<tr>
<td></td>
</tr>
<tr>
<td>2, 4 – D + NAA</td>
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</table>
PGR – Plant growth regulator, – =Indicates the no callus formation, + = trace callus growth, ++ = moderate callus growth, +++ = high callus growth

![Image of callus formation](image)

**Figure 1:** Callus formation in leaf explants of *S. chamaelea*.
A – Pale yellow callus, B – Pale yellow green hard callus, C – Pale yellow green soft callus, D – green and hard embryogenic callus.

**Antibacterial activity**
The antibacterial activity of *S. chamaelea* extracts shown significant reduction in bacterial growth in various plant extracts. Ethyl acetate extracts of both *in vitro* and *in vivo* extracts shows maximum inhibitory activity against the human pathogens like *K. aerogenes*, *E. coli*, *S. sonnei*, *C. diptheriae*, *S. typhi* and moderate effect on *S. dysentriae* and *V. vulnificans*. Whereas, methanol extracts exhibit maximum activity on *L. acidophilus*, *S. typhi* and *C. diptheriae*. While, hexane extracts expressed maximum activity on *S. dysentriae*, *V. vulnificans* and minimum activity was noticed against *S. typhi*, *K. pneumoniae* and nil activity on *E. coli* and *K. aerogenes*. Acetone extracts have reported to contain high activity on *K. aerogenes*, *C. diptheriae* and minimum effect on *E. faecalis*. Chloroform extracts showed maximum activity was noticed on *K. aerogenes*, *E. coli* and minimum activity on *C. diptheriae*. The same extracts show no activity against *S. typhi*. Ethanol extracts express maximum antibacterial activity on *L. acidophilus*, *S. sonnei* and minimum activity on *E. coli*, *C. diptheriae* and *S. typhi*. Higher inhibition zone of plant extracts (leaf and callus extracts) were noticed against very few bacterial pathogens (table & figure 2). This study was correlated with other findings focused on antibacterial activities of the same plant (*S. chamaelea*) and the result highlight methanolic leaf extract showed better activity against *B. subtilis*, *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* [3]. Likewise, Natarajan and Kamalanathan [14] reported that comparison of the antibacterial activity of different extracts from leaves and leaf derived callus of *Solanum trilobatum* and most of the extracts were effective against *S. aureus*, *S. typhi*, *S. dysentriae*, *S. sonnei*, *C. diptheriae* and *S. boydii*. Antimicrobial activity of *Moringa oleifera* was tested against some bacterial and fungal pathogens was done by Tamanna tulreja [15] and the results show higher activity was observed in *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*. Madhavi et al. [16] also reported that phytochemical constituents of *Artocarpus communis* showed better antimicrobial activity against pathogenic bacteria’s.
Figure 2: Antibacterial activity of *in vitro* and *in vivo* extracts of *S. chamaelea*

A. *Klebsiella aerogenes* B. *Shigella sonnei*, *C. Salmonella typhi*, D. *Escherichia coli*

Table 2: Antibacterial activity of *in vitro* and *in vivo* extracts of *S. chamaelea*

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Extracts</th>
<th>Zone of Inhibition (mm)</th>
<th>Cefotaxime (1 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexane</td>
<td>Chloroform</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>11.6±0.5</td>
<td>11±1.0</td>
<td>07±1.0</td>
</tr>
<tr>
<td>V. vulnificus</td>
<td>9±1.0</td>
<td>13.3±1.0</td>
<td>11±1.0</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>8.3±0.5</td>
<td>8.3±1.0</td>
<td>8±1.0</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>6±1.0</td>
<td>8±1.0</td>
<td>7.6±1.0</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>7.3±1.0</td>
<td>11±1.0</td>
<td>7.6±1.0</td>
</tr>
<tr>
<td>S. boydii</td>
<td>1.1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>8±1.0</td>
<td>6.6±1.0</td>
<td>6±1.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
<td>10.3±0.4</td>
<td>-</td>
</tr>
<tr>
<td>C. diphtheriae</td>
<td>7.6±2.0</td>
<td>9.6±0.5</td>
<td>6±3.0</td>
</tr>
<tr>
<td>S. typhi</td>
<td>7±0.5</td>
<td>7±0.4</td>
<td>-</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>-</td>
<td>8±1.0</td>
<td>-</td>
</tr>
<tr>
<td>K. aerogenes</td>
<td>-</td>
<td>11.3±0.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Phytochemical screening
Phytochemical analysis was done for all six extracts (hexane, methanol, ethanol, ethyl acetate, chloroform and acetone) by standard protocols and the results shown saponin was present in all extracts except ethyl acetate extract (in vitro callus). Phenolic compounds and carbohydrate was present in most of the extracts. Alkaloids, phystosterols, fats and oils were present in both (in vitro and in vivo) extracts. Protein was absent in all the extracts. Terpenoids are present in all the extracts except hexane extract (both in vitro and in vivo samples) was reported (table 3). Similarly, Shanthi Sree et al. [3] studied the preliminary phytochemicals in S. chamaelea and reported to contain some compounds like flavonoids, phenols, tannins, steroids, some glycosides and saponins respectively.

Table 3: Phytochemical analysis of in vitro and in vivo extracts of S. chamaelea

<table>
<thead>
<tr>
<th>Extracts</th>
<th>In vitro (callus)</th>
<th>In vivo(Leaves)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HX</td>
<td>CL</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenolic</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Phenolic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fats and Oils</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

= Absent, + present, HX-Hexane, CL-chloroform, EA-ethyl acetate, AC-acetone, ET-ethanol, MT-methanol

CONCLUSION
The inhibitory activity of plant extracts were generally depends upon the concentration of extracts, type of plant parts and tested microbes [17, 18, 19]. It may be a reason for the variation in the inhibitory activity of extracts of S. chamaelea. This study conclude that the enhanced antibacterial activity was noticed by leaf and leaf derived callus extracts may be due to the presence of more amount of secondary metabolites in callus when compared to natural products. Nevertheless, our results indicate the ability to utilize plant biotechnology techniques towards development of desired bioactive metabolites in in vitro culture instead of using wild plants in pharmaceutical purposes. The outcome of study was recommends further investigation is needed for isolation and identification of antimicrobial principles (bioactive compounds) from in vitro raised plants by chromatographic and spectral analysis.

CONFLICTS OF INTEREST
We declare that we have no conflict of interest.

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