MATERIALS AND METHODS
3.1. Plant collection and maintenance

Healthy plants of *Curculigo orchioides* were collected from the wild forest of Wadakanchery village in Thrissur District of Kerala, Southern part of India. These plants were planted in earthen pots containing soil, sand and manure (6:1:2) to maintain for the present investigation. Plants were frequently watered and maintained under natural shade in Botanical Garden of Sri Paramakalyani Centre for Excellence in Environmental Sciences, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu. Healthy leaves and rhizome were collected from the well established plants and used as explants during this study.

3.2. Medium and culture conditions

MS medium (Murashige and Skoog, 1962) supplemented with sucrose (3 %) become the basic media. The media was augmented with various growth regulators such as BAP, KN, 2, 4-D and NAA (Hi-media, Mumbai, India) in different concentrations ranging from 0.44 µM – 23.2 µM, depending upon the experimental design. A range of concentrations of AgNO₃ and PG (British Drug House, Chennai, India) was also used for optimizing the culture conditions for shoot proliferation. The pH of the media was adjusted to 5.8 before gelling with 0.7 % agar (Hi-media, Mumbai, India). Molten medium was dispensed into 200 ml screw-capped glass jars and 25 x 150 mm culture tubes (Borosil, Chennai, India) and capped with cotton plugs before sterilization at 121°C for 20 min. All the cultures were maintained at 25 ± 2°C and kept under a 16 hrs photoperiod provided by cool white fluorescent tubes (Philips, Mumbai, India) with a light intensity of 40 µmol m⁻². The number of explants cultured in each treatment was varied from 20 - 30 depending upon the experimental design. The duration of culture was ranging from 30 - 120 days.

3.3. Preparation of explants

Explant preparation is the most important step for successful application of micropropagation and this stage is considered to be a crucial one for overcoming the problem of microbial contamination. In plant tissue culture, microbial contamination is
a common problem caused either by exogenous or endogenous microorganisms. Effective disinfection of exogenous microbial contaminants can be performed by surface sterilization (Bonga, 1982). Hence, identification of suitable disinfectant and standardization of surface sterilization method by optimizing the duration of treatment are considered as the most significant step for the success of micropropagation. The surface sterilization protocol varies among different plant species and even among the explants of the same species (Yeoman and McLeod, 1977). Thus, the following experiments were carried out to address the problem of microbial contamination in leaf and rhizome culture of *Curculigo orchioides*.

### 3.4. Effect of disinfects on microbial contamination

#### 3.4.1. Efficacy of NaOCl and HgCl$_2$ on microbial contamination of rhizome

Efficacy of two commonly employed surface disinfectants, namely NaOCl (1 %) and HgCl$_2$ (0.1 %) were tested to eliminate microbial contamination. The rhizomes of *Curculigo orchioides* were surface sterilized with above disinfectants for various durations (3, 6, 9, 12 and 15 min) and were thoroughly rinsed with sterile distilled water for 4 to 5 times under aseptic condition. The explants were slightly trimmed, measuring about 1 cm length and implanted vertically on MS medium augmented with 4.4 µM BAP. Explants cultured without surface sterilization formed the control for this experiment. In each concentration, 90 explants were cultured as three replications each with 30 explants. The recovery of explants without microbial contamination and browning was scored after 60 days of culture and data were subjected to statistical analysis.

#### 3.4.2. Efficacy of NaOCl and HgCl$_2$ on microbial contamination of leaf explant

A similar experiment was carried out with leaf explants of *C. orchioides* as described above. After disinfection with NaOCl (1 %) and HgCl$_2$ (0.1 %), the leaf explants were trimmed, measuring about 1 cm$^2$ under aseptic condition and cultured on MS medium augmented with 4.4 µM BAP. Explants cultured without surface sterilization formed the control for this experiment. In each concentration, 90 explants
were cultured as three replications each with 30 explants. The recovery of explants without microbial contamination and browning was scored after 60 days of culture and the data were subjected to statistical analysis.

3.5. Choice of explants for regeneration of microshoots

In order to select the suitable explants for bulbils formation and shoot induction, leaf and rhizome explants of *Curculigo orchioides* were surface sterilized with 0.1 % HgCl₂ for 6 min and 15 min respectively and thoroughly rinsed thrice with sterile distilled water to remove the traces of HgCl₂. Leaf and rhizome were cut into small segments and cultured on MS medium fortified with 4.4 µM BAP. Observation on microbial contamination, browning and recovery etc were recorded after 60 days of primary culture and subjected to statistical analysis for choosing the suitable explants for further experiments.

3.6. Control of browning of explants due to phenolic oxidation

To address the problem of blackening and browning of explants, a variety of antioxidants such as ascorbic acid, citric acid and adsorbents such as PVP and activated charcoal have been used in a number of works (Ramsay and Gratton, 2000; Liao et al., 2004; Minas, 2007). Concentration of phenolic compounds may vary in different genotypes of the same species (Glynn et al., 2004) and also plant species grown under different climatic conditions (Kjaer et al., 2001). Use of adsorbents and antioxidants during the initiation of culture has been reported as one of the promising approaches for overcoming phenolic oxidation (Weatherhead et al., 1979; Gupta et al., 1980). Other methods include choice of juvenile explants (Bon et al., 1988), incubation of cultures in the dark (Cassells and Minas, 1983) and frequent transfer of explants to fresh medium (Broome and Zimmerman, 1978). In the present investigation, the following experiments were carried out to overcome the problem of phenolic exudation.

3.6.1. Influence of charcoal on control of browning of explants

Rhizome and leaf explants collected from actively growing plants were cultured on MS medium supplemented with 4.4 µM BAP. Addition of activated charcoal (AC) to the
culture medium has been found to be useful for overcoming the problem of phenolic exudation and browning (George, 1999). Hence, different concentrations (1.0, 2.0 and 3.0 g/l) of activated charcoal was incorporated into the medium and culture medium without charcoal was served as control. Experiments were carried out with three replications, each with 20 explants. Data on the recovery of explants and loss of explants due to browning was collected after 60 days of culture and analysed statistically.

3.6.2. Effect of PVP on browning of explant

Rhizome and leaf explants were inoculated on MS medium supplemented with 4.4 μM BAP. Different concentrations of PVP (0.44 mM, 0.66 mM, 0.88 mM and 1.77 mM) were supplemented in the above medium to determine the optimum concentration of PVP for reducing the browning of explants. Culture medium without PVP was served as control. Experiments were carried out with three replications, each with 30 explants. Data on the recovery and loss of explants were collected after 60 days of culture and analysed statistically.

3.6.3. Effect of dark incubation on control of phenolic oxidation

In order to determine the influence of dark on control of browning, rhizome and leaf explants of *C. orchioides* were cultured on MS medium supplemented with 4.4 μM BAP. Freshly inoculated cultures were immediately incubated under dark for 15 days and subsequently transferred to light condition under room temperature for 45 days, bringing the total culture period to 60 days. Cultures incubated under light from the day of inoculation become the control. In each treatment, 20 explants were cultured. Observation was carried out on the incidence of browning in treatment as well as in control and data were subjected to statistical analysis.

3.6.4. Effect of antioxidants on browning of explants

Antioxidants such as ascorbic acid, citric acid and L-cysteine HCl were tested to control the browning of rhizome and leaf explants of *C. orchioides*. MS medium fortified with 4.4 μM BAP was served as basic medium. The above antioxidants were
incorporated in the medium at various concentrations (0.14 mM, 0.28 mM, 0.42 mM and 0.56 mM of ascorbic acid, 0.13 mM, 0.26 mM, 0.39 mM and 0.52 mM of citric acid and 0.16 mM, 0.32 mM, 0.48 mM and 0.64 mM of L-cysteine HCl). Explants cultured on medium without antioxidants were served as control. In each treatment, 30 explants were cultured and maintained under diffuse natural light for 60 days. Data on browning of cultures were recorded and analysed statistically.

3.7. Shoot regeneration and multiplication

Shoot proliferation is a crucial stage, which decides the successful application of micropropagation. In this stage, sufficient number of healthy shoots would be produced for *in vitro* rooting. Shoot proliferation that takes place from meristem and leaf tissues are exposed to the suitable cytokinins at an appropriate concentration. At this stage, shoot proliferation takes place either after formation of bulbils or directly from the explant. The bulbils are transferred to optimal medium for multiplication. In the present study, experiments were carried out with different cytokinins (BAP, KN) and auxin (2,4-D and NAA) to select their optimum concentration for shoot proliferation. Different adjuvants such as silver nitrate and phloroglucinol were also tested individually as well as in combination with above cytokinins to enhance shoot proliferation. Experiments were carried out to identify the best season for initiation and establishment of cultures. The details of the various experiments carried out for shoot regeneration and multiplication are detailed below.

3.8. Effect of auxin and cytokinins on shoot proliferation in leaf explant

To optimize shoot proliferation, leaf explants of *Curculigo orchioides* were inoculated on MS medium fortified with BAP and KN either alone or in combinations with 2, 4-D and NAA. Various concentrations of BAP (0.44 µM, 4.4 µM and 22.0 µM), KN (0.46 µM, 4.6 µM and 23.0 µM), 2, 4-D (4.5 µM) and NAA (5.3 µM) were used for this experiment. A total of 30 explants were used for each treatment with three replications. All the cultures were kept under diffuse natural light. Explant cultured initially for 40 days at different concentrations were observed and recovered explants showing response were transferred to fresh media of same composition and maintained.
for 90 days. Data on number of bulbils formed and sprouting percentage of bulbils were recorded and analysed.

3.9. Combined effect of growth regulators and adjuvants on shoot proliferation

3.9.1. Effect of silver nitrate (AgNO₃) on shoot proliferation

Leaf explants of *Curculigo orchioides* were cultured on MS medium supplemented with different concentrations of AgNO₃ (0.06 mM, 0.12 mM, 0.18 mM, 0.24 mM and 0.29 mM) to optimize the requirement of AgNO₃ for shoot proliferation. Presence of BAP at 4.4 µM was common to all the treatments including control. A total of 90 explants were cultured in each treatment with three replications, each with 30 explants. Explants cultured initially for 40 days at different concentrations of AgNO₃ were observed and recovered explants showing response were transferred on to fresh media of the same composition and maintained for 60 days. Data on regeneration of bulbils, number of shoots/explant, sprouting and rooting percentages were recorded and analysed.

3.9.2. Effect of phloroglucinol (PG) on shoot proliferation

Leaf explants of *Curculigo orchioides* were cultured on MS medium supplemented with different concentrations of PG (0.16 mM, 0.32 mM, 0.47 mM, 0.63 mM and 0.79 mM) to optimize the requirement of PG for efficient shoot proliferation. Presence of BAP at 4.4 µM was common to all the treatments including control. A total of 90 explants were cultured in each treatment with three replications, each with 30 explants. Explants cultured initially for 40 days at different concentrations of PG were observed and recovered explants showing response were transferred on to fresh media of the same composition and maintained for 60 days. Data on the development of bulbils, frequency of shoot regeneration and rooting percentage were recorded and analysed.

3.9.3. Combined effect of cytokinins and adjuvants on shoot proliferation

This experiment was carried out to study the synergistic effect of AgNO₃ and PG on shoot proliferation in cultured leaf explants of *Curculigo orchioides*. Leaf explants were
cultured on MS medium containing the optimized concentration of AgNO$_3$ (0.12 mM) and PG (0.79 mM) based on the results of previous experiments. In addition, two commonly used cytokinins such as BAP (4.4 µM and 22.0 µM) and KN (4.6 µM) were used along with the above adjuvants. The various combinations of media used in this experiment were as follows.

1. MS basal (control)
2. MS + 4.4 µM BAP
3. MS + 4.6 µM KN
4. MS + 0.12 mM AgNO$_3$
5. MS + 0.79 mM PG
6. MS + 4.4 µM BAP + 0.12 mM AgNO$_3$
7. MS + 4.6 µM KN + 0.12 mM AgNO$_3$
8. MS + 4.4 µM BAP + 0.79 mM PG
9. MS + 4.6 µM KN + 0.79 mM PG
10. MS + 4.4 µM BAP + 0.12 mM AgNO$_3$ + 0.79 mM PG
11. MS + 4.6 µM KN + 0.12 mM AgNO$_3$ + 0.79 mM PG
12. MS + 22.0 µM BAP + 0.12 mM AgNO$_3$
13. MS + 22.0 µM KN + 0.12 mM AgNO$_3$ + 0.79 mM PG

After 30 days of primary culture of leaf explants on the above medium, recovered explants were sub-cultured on to their respective medium and maintained for another 90 days. Data on shoot proliferation were recorded and analysed at the end of 120 days.

3.9.4. Combined effect auxin, cytokinins and adjuvants on shoot proliferation

To optimize callus induction, leaf explants of *Curculigo orchioides* were cultured on MS medium fortified with BAP (22.0 µM) and 2, 4-D (4.5 µM) either alone or in combination with AgNO$_3$ (0.12 mM) and PG (0.79 mM). A total of 30 explants were used for each treatment with three replications. All the cultures were maintained under natural diffuse light. Explant cultured initially for 40 days at different concentration
were observed for callus induction and recovered explants along with callus were transferred to fresh media of the same composition for shoot induction and maintained for 90 days. Data on percentage of callusing and number of shoots/culture were recorded and analyzed statistically.

3.9.5. Influence of explanting seasons on shoot regeneration

Leaf explants were collected during four different seasons, namely Intermediate (December – February), Summer (March – May), South West Monsoon (June – August) and North East Monsoon (September – November) during the year 2010 – 11 and 2011 – 12 and cultured on MS medium supplemented with 4.4 µM BAP. For every month, a total of 90 explants were inoculated on the above media as three replications, each with 30 explants. Explants inoculated on the above media were incubated at room temperature under natural diffuse light. Primary cultures initiated in every month were subjected to observation at the end of 60 days to determine the effect of seasons on bud break and shoot regeneration. Responding explants were maintained on the same medium for 45 days with a subculture after two weeks of inoculation. Response of leaf explants for bulbils formation and shoot induction was recorded. This experiment was repeated for two consecutive years for validation of data.

3.9.6. Effect of orientation of rhizome on shoot regeneration

In order to study the influence of orientations of rhizome on shoot regeneration, rhizomes of *C. orchioides* measuring about 1 cm were cultured in three different orientations as detailed below

A) Culture of rhizome segment by facing the distal end in contact with medium

B) Culture of rhizome segment by facing the proximal end in contact with medium

C) Culture of rhizome segment by placing on the medium horizontally
The above cultures were grown on MS medium supplemented with 4.4 µM BAP. Three replications were maintained, each with 20 explants. After 60 days of culture, data on shoot regeneration and rooting was recorded and analysed statistically.

3.9.7. Effect of auxin and cytokinins on regeneration of adventitious roots

To optimize the development and regeneration of large number of microshoots from the rhizome explant of *Curculigo orchioides*, explants were inoculated on MS medium supplemented with two different cytokinins (BAP and KN) in combination with two of the commonly used auxin (2,4-D and NAA). Various concentrations of BAP (2.2 µM and 4.4 µM), KN (2.3 µM and 4.6 µM), 2, 4-D (5.0 µM) and NAA (5.3 µM) were used for this experiment. A total of 60 explants were used with three replications, each with 20 explants. Responding explants after 30 days of primary culture were transferred on to fresh media of the same composition and maintained for 90 days, bringing the total culture period to 120 days. Data on regeneration of shoots and root development was recorded and analysed.

3.9.8. Evaluation of planting substrate on hardening

Considerable number of healthy plantlets with well developed roots obtained from the previous experiments were taken out from the culture vessels and carefully rinsed under the gentle running water to remove the medium adhering on the plantlets. These plantlets were planted on substrate containing soil, sand and vermicompost of three different ratios (6:1:2, 4:2:3, 3:3:3 and 2:2:5). The potted plantlets were grown under greenhouse by providing regular watering. After 30 days of hardening, the plants were taken out from the greenhouse and transferred to outside under natural shade for further growth and development. The performance of micropropagated plants under greenhouse as well as in open conditions was observed and recorded.
3.9.9. Statistical analysis

Most of the experiments were analysed with three replications. However, the number of explants in each treatment of various experiments was variable due to differences in final recovery of explants. The effect of different treatments on the response of explants was quantified and the level of significance was determined by the analysis of variance (ANOVA) using SPSS version 11.0 and level of differences between the treatments were assessed by Duncan’s New Multiple Range Test (DMRT) at $P \leq 0.05$.

3.10. Determination of genetic fidelity of micropropagated plants

3.10.1. Isolation of genomic DNA

Micropropagated plants obtained through five different modes of regeneration (Table 2) from different explants of *C. orchioides* were subjected to genetic analysis. Leaves from the above sources were collected and isolation of genomic DNA was performed using DNA isolation kit by following the protocol of manufacturer (Helini Biomolecules, Chennai, India). Fresh leaves (25 mg) were cut into smaller pieces and taken into 1.5 ml centrifuge tube and lysis buffer-1 (400 µl) and proteinase K (40 µl) was added before grinding the sample into fine paste using micropestle. The mixture was incubated in water bath at 70ºC for 10 min and allowed to attain room temperature. Then, 3 M sodium acetate (100 µl) was added. Sample was transferred into Pure – Fast column and spin at 10000 rpm for 1 min. Supernatant was discarded and 500 µl wash buffer 1 was added before centrifugation at 10000 rpm for 1 min. This step was repeated twice with wash buffer 2 (750 µl). After discarding the supernatant, column was again centrifuged at 10000 rpm for 1 min to remove ethanol. Elution Buffer (100 µl) was added and centrifuged for 2 min and DNA samples were stored at - 20º C.
Table 2: Type of explants, medium composition and mode of micropropagation of C. orchioides

<table>
<thead>
<tr>
<th>Explant type</th>
<th>Optimized media</th>
<th>Mode of regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizome</td>
<td>MS+ BAP (4.4 µM)</td>
<td>Rhizome &gt; direct plantlets</td>
</tr>
<tr>
<td>Leaf</td>
<td>MS + 2,4-D (4.5 µM) + BAP (22.0 µM)</td>
<td>Leaf &gt; callus &gt; plantlets</td>
</tr>
<tr>
<td>Leaf</td>
<td>MS + BAP (4.4 µM) + AgNO3 (0.12 mM) + PG (0.79 mM)</td>
<td>Leaf &gt; bulbils &gt; plantlets</td>
</tr>
<tr>
<td>Leaf</td>
<td>MS+ BAP (4.4 µM) + AgNO3 (0.12 mM) + PG (0.79 mM)</td>
<td>Leaf &gt; direct plantlets</td>
</tr>
<tr>
<td>Terminal Part of the leaf of in vitro plant</td>
<td>MS + BAP (4.4 µM) + AgNO3 (0.12 mM) + PG (0.79 mM)</td>
<td>Plantlets from terminal part of leaf from in vitro plants</td>
</tr>
</tbody>
</table>

3.10.2. Polymerase Chain Reaction (PCR)

PCR amplification was performed with 25 ng of genomic DNA of C. orchioides extracted from leaf tissues of micropropagated plants obtained through five different modes of regeneration (Table 2). In this experiment, about 10 ISSR primers were used for PCR amplification (Table 3). About 25 µl PCR master mix (10 x Taq buffer, 2 mM MgCl₂, 0.4 mM dNTP mix, and 2U Taq DNA polymerase) were mixed with ISSR primers (1 µl each), genomic DNA (1 µl), nuclease free water (23 µl) to make final volume to 50 µl. The PCR was programmed as 94°C for 3 min, followed by 35 cycles of 94°C for 1 min for a denaturation step, an annealing step of 1 min at 48°C and an extension step of 72°C for 2 min, ending with 5 min at 72°C.
Table 3: Primers used for amplification of Inter Simple Sequence Repeats of genomic DNA isolated from the leaf tissues of micropropagated plants regenerated through five different modes of regeneration.

<table>
<thead>
<tr>
<th>Name of the primer</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-ISSR-01</td>
<td>5’-GTGTGTGTGTGTGG-3’ (GT)₆GG</td>
</tr>
<tr>
<td>Co-ISSR-02</td>
<td>5’-GAGAGAGAGAGACCC-3’ (GA)₆CC</td>
</tr>
<tr>
<td>Co-ISSR-03</td>
<td>5’-GTGTGTGTGTGTCC-3’ (GT)₆CC</td>
</tr>
<tr>
<td>Co-ISSR-04</td>
<td>5’-CTCTCTCTCTCCTCTAC-3’ (CT)₈AC</td>
</tr>
<tr>
<td>Co-ISSR-05</td>
<td>5’-CTCTCTCTCTCCTGC-3’ (CT)₈GC</td>
</tr>
<tr>
<td>Co-ISSR-06</td>
<td>5’-CACACACACACACAC-3’ (CA)₆AC</td>
</tr>
<tr>
<td>Co-ISSR-07</td>
<td>5’-CACACACACACACAGG-3’ (CA)₆GG</td>
</tr>
<tr>
<td>Co-ISSR-08</td>
<td>5’-GAGAGAGAGAGAGG-3’ (GA)₆GG</td>
</tr>
<tr>
<td>Co-ISSR-09</td>
<td>5’-ACACACACACACACTG-3’ (AC)₅TG</td>
</tr>
<tr>
<td>Co-ISSR-10</td>
<td>5’GAGGAGGAGGAGGAGGC-3’ (GAG)₅GC</td>
</tr>
</tbody>
</table>

3.10.3. Agarose gel electrophoresis

Electrophoresis was performed in 2% agarose (Himedia, Mumbai, India) gel containing 1X TAE (Tris Acetate EDTA) buffer and ethidium bromide (Himedia, Mumbai, India) at 50 volts. The molecular weights of various PCR products were determined by comparing with 1 kb plus DNA ladder 250 bp (Helini Biomolecules, Chennai, India).

3.10.4. Documentation

Gels were visualized under UV Transilluminator and photographed using the gel doc equipment (Alphal Mager gel documentation, USA). Among ten ISSR primers, four primers produced clear and reproducible bands. The common as well as unique bands were taken into consideration for determining the genetic variability of micropropagated plants developed through different modes of regeneration.
3.11. Phytochemical analysis of micropropagated plants

3.11.1. Preparation of samples

To investigate on the medicinal properties of tissue culture plants of *C. orchioides*, rhizomes of micropropagated plants were cut into small pieces before exposing them to gradual drying at 35°C in a hot air oven for 24 hours. The dried samples were made into fine powder using sterile mortar and pestle. The powder samples were mixed with 95% ethanol 1:20 (w/v) and continuously stirred for 3 to 4 days to facilitate the extraction of bioactive compounds. Extracts were filtered through sterile Whatman filter paper (Himedia, India) and air dried. Samples were dissolved again with ethanol (95%) and used for thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC).

3.11.2. Thin Layer Chromatography (TLC)

TLC plates (20 cm length and 14 cm width) were prepared by thoroughly mixing the silica gel (Loba Chem. India) with sterile double distilled water 1:3 (w/v) and dried in a hot air oven at 100°C for 30 minutes. Ethanol extract of various samples measuring 100 µl were drawn from various samples and spotted on silica gel plate. Benzene (CDH Analytical reagent, India) was used as a solvent for chromatography analysis. Loaded samples on TLC plate was immersed at one end inside the development chamber containing the above solvent and left for 45 minutes for the separation of compounds and stained with iodine vapor in airtight chamber and TLC plate with visible spots were documented by photography using a digital camera (Sony, Japan). In addition, TLC plate was also exposed for UV observing additional compounds that fluorescent under exposure to UV light.

3.11.3. High Performance Liquid Chromatography (HPLC)

Powder forms of various samples were prepared by following the methods as described for TLC analysis. These samples were mixed with methanol (1:20) and continuously stirred for four days and finally filtered through 0.2 µm membrane. HPLC
was performed using Shimadzu (Japan) HPLC system equipped with a main column of analytical – shim-pack CLCOCTA DECYL SILANE (ODS-C18) (46 mm 10 x 25 cm) and guard column was shim-pack G-ODS (4 mm 10 x 1 cm). Stationary phase of the HPLC was silica gel (reversed phase) and mobile phase was methanol (100 % HPLC grade). The maintained column head pressure for all the samples was 125 kg + 1 cm². Hamilton Microlitre Syringe (Japan) was used to inject 20 µl samples into the column. The maintained flow rate was one ml per minute and run time was 15 minutes. The chromatogram was viewed and the peak was observed at 280 nm.

3.11.4. High Performance Thin Layer Chromatography (HPTLC)

To evaluate flavanoids from the rhizome of micropropagated plants of *C. orchioides*, HPTLC was performed. To prepare a stock solution of gallic acid, 10 g of gallic acid (Loba Chemicals Pvt Ltd, Mumbai, India) was dissolved in 100 ml of 95 % ethanol in a sterile volumetric flask. From this stock solution, seven different concentrations of gallic acid (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml and 70 µg/ml) was prepared by drawing 1 – 7 ml of aliquots from the stock solution and the final volume was adjusted to 10 ml by adding 95 % ethanol. These solutions were used as standard (Shrikumar *et al.*, 2005).

Chromatography was performed on silica gel F254 HPTLC pre-coated plates. Samples were applied on the plates as band of 7 mm width using a Camag Linomat V sample (Switzerland) applicator at the distance of 14 mm from the edge of the plates. The mobile phase was constituted with ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v). After development, plates were dried and sprayed with polyethylene glycol (PEG). The fingerprints were developed at 366 nm in fluorescence mode with Win Cats and Video Scan software.