Materials & Methods
MATERIALS AND METHODS

Immunemodulating potential of triterpene saponins of *Centella asiatica*

**Plant material**

The fresh mature leaves of *Centella asiatica* were collected from Tirumala hills (Chittoor district, Andhra Pradesh, India). The plant material was authenticated by Taxonomist Dr. K.Madhava Chetty (Member IAAT-No.357) at Sri Venkateswara University, Tirupati, India and a voucher specimen (Ref.No.SVUBH-7001) was deposited University Herbarium Centre for future reference.

**Experimental animals and housing conditions**

Male Wistar strain albino rats (150–175 gm; obtained from Sri Venkateswara Animal Agency, Bangalore, India) were housed and maintained in clean polypropylene cages in the Departmental animal house at 26±2°C and relative humidity 44–56%, light and dark cycles of 10 and 14 h, respectively, for one week before and during the experiments.

Animals were provided with standard rodent pellet diet (Sri Sai Durga Animal Feeds, Bangalore, India) and water *ad libitum*. The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the institutional animal ethical committee (IAEC) of Department of Zoology, Sri Venkateswara University, Tirupati, India (No.50/2012-2013/(i)/a/CPCSEA/IAEC/SVU/TVJ-DPG).

**Drugs and chemicals**

**Cyclophosphamide (CYP)**

Cyclophosphamide was purchased from HiMedia Laboratories, Mumbai, India. It is an alkylating agent, the most commonly used chemotherapeutic and anticancer drug. Its cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, by producing cross links (Dollery, 1999).
For the present study the 10 mg/kg body weight dose was fixed based on the existing literature (Hou et al., 2007).

**Structure**

![Chemical structure of Cyclophosphamide](image)

**Formula**

\[ C_7H_{15}Cl_2N_2O_2P \]

**Molecular weight**

261.086 g.mol

**Systematic (IUPAC) name**

(RS)-N, N-bis (2-chloroethyl)-1, 3, 2-oxazaphosphinan-2-amine 2-oxide.

**Mechanism of action**

Cyclophosphamide is a cytotoxic chemotherapeutic drug (Colvin 2001) that acts as an alkylating agent producing reactive carbonium ions, which reacts with DNA. Initial activation reaction of CYP is carried out by microsomal oxidation system in liver producing 4-hydroxy CYP, a cytotoxic metabolite, which diffuses from hepatocytes into plasma and distributed throughout the body. Then, 4-hydroxy CYP is further converted to some other cytotoxic metabolites and phosphoramide mustard and acrolein are among them (Growchow, 1996). Phosphoramidate mustard is known to cause immune suppression (Berger, 1993). Cyclophosphamide is a unique immunosuppressant as it suppresses B-lymphocyte proliferation but can
enhance T-cell responses. These drugs are most destructive to rapidly proliferating tissues and appear to cause cell death when they tend to divide (Patil et al., 2012).

**Levamisole (LEV)**

Levamisole, marketed as the hydrochloride salt under the trade name Levamol, is an anthelmintic and immunomodulator belonging to a class of synthetic imidazothiazole derivatives. Levamol was purchased from Cipla Limited, India. For the present study the 50 mg/kg body weight dose was fixed based on the existing literature (Meera and Nagarjuna, 2009).

**Structure**

![Structure of Levamisole](image)

**Formula**

\[ \text{C}_{11}\text{H}_{12}\text{N}_{2}\text{S} \]

**Molecular weight**

204.292 g/mol

**Systematic (IUPAC) name**

(S)-6-Phenyl-2, 3, 5, 6-tetrahydroimidazo [2, 1-b] [1, 3] thiazole.

**Mechanism of action**

Levamisole is a synthetic imidazothiazole derivative that has been widely used in treatment of worm infestations in both humans and animals. As an anthelmintic, it probably works by targeting the nematode nicotinergic acetylcholine receptor. As an immunomodulator, it appears that Levamisole is an immune stimulant which has been shown to increase natural killer cells and activated T-cells. The mechanism of action of Levamisole as an anticancer drug on the immune
system is complex. The drug appears to restore depressed immune function rather than to stimulate response to above normal levels. Levamisole can stimulate formation of antibodies to various antigens, enhance T-cell responses by stimulating T-cell activation and proliferation, potentiate monocyte and macrophage functions including phagocytosis and chemotaxis, and increase neutrophil mobility, adherence, and chemotaxis. Levamisole restore depressed immune function of B lymphocytes, T lymphocytes, monocytes and macrophages (Shah et al., 2011).

Other chemicals & equipment

All the chemicals used in this study are of analytical grade and were obtained from Sigma (St. Louis, MO, USA), Fisher (Pitrsburg, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), and Qualigens (Mumbai, India).

For the present work Barnstead Thermoline water purification plant was used for nano pure water, Kubota KR 200000T centrifuge for centrifugation of the homogenates and Hitachi UV-2000 Spectrophotometer for measuring the optical density values were used for high quality results.

Extraction of triterpene saponins

The Centella asiatica plants collected were washed thoroughly with tap water. The leaves were separated manually, shade dried and ground into fine powder. The leaf powder was extracted with distilled water as previously reported with minor modifications (James et al., 2008).

The leaves (fresh material) were weighed, cut into strips, and placed in distilled water (1:12, w/v) to extract secondary metabolites. The mixture was placed on a magnetic stirrer for 24 h then centrifuged at 2200g for 20 min. The supernatant was decanted and concentrated tenfold by rotary evaporation at 45°C under vacuum. The yield of extract was 8.2g/100g of leaves. The triterpene saponins were spectrophotometrically (535 nm) estimated as per the method described by Shiau et al., (2009) in the extract which was found to contain the total triterpene content 2.9 gm of the extract and this was evaporated in a rotary evaporator under reduced pressure, freeze-dried and used for the study.
Materials and Methods

Acute toxicity studies

The acute toxicity of triterpene saponins extract (EXT) was determined according to guideline No.420 of the Organization for European Economic Cooperation (OECD) using male Wistar strain albino rats (150 ± 25 gm). Initial doses of 100, 500, 1000, 1500 and 2000 mg/kg bodyweight of EXT were administered to the respective five groups of six rats each and monitored for three weeks for mortality and general behavior.

Toxic symptoms or mortality were observed till the end of the study with doses of 1500 and 2000 mg/kg bodyweight. The lethal dose (LD$_{50}$) was determined as 1000 mg/kg bodyweight. Hence, the experimental dose was selected as one fourth (250 mg/kg bodyweight) of the LD$_{50}$. Moreover, previous experimental studies also reported that the doses below 250 mg/kg of EXT per day are more efficacious and without any recognized side effects (Jayathirtha and Mishra, 2004).

Induction of immune suppression to rats

The cyclophosphamide (CYP) was administered, orally (1.5 ml) at a concentration of 10 mg/kg body weight for 30 consecutive days. CYP induced immunosuppression was confirmed by the levels of serum alkaline phosphatase (ALP) and creatinine in rats.

Experimental design

In this study, a total of 30 rats were divided into five groups (n = 6 in each group); the experimental groups are summarized below:

**Group I: CON**

Normal control rats (n = 6): received standard pellet chow and water for 30 days.

**Group II: CYP**

Immunosuppressed control rats (n = 6): received cyclophosphamide (CYP) (10 mg/kg body weight, by gavage) alone for 30 days.
Materials and Methods

Group III: EXT

Extract treated rats (n = 6): received triterpene saponin extract of *C.asiatica* (EXT) (250 mg/kg body weight, by gavage) alone for 30 days.

Group IV: CYP+EXT

Immunosuppressed treated rats (n = 6): received cyclophosphamide (CYP) (10 mg/kg body weight, by gavage) and triterpene saponin extract of *C.asiatica* (EXT) (250 mg/kg bodyweight, by gavage) for 30 days.

Group V: CYP+LEV

Immunosuppressed treated rats (n = 6): received cyclophosphamide (CYP) (10 mg/kg body weight, by gavage) and levamisole (LEV) (50 mg/kg bodyweight, by gavage) for 30 days.

All rats fasted overnight but had free access to water at the last day administration of the drug.

The rats were killed under anesthesia using 85 mg/kg bodyweight ketamine and 95 mg/kg bodyweight xylazine (i.p). The selected organs such as spleen, thymus and liver were dissected out.

Methodological considerations

Hematological analysis

Blood was collected from all the animals by retro-orbital plexus puncture prior to CYP treatment, and the following parameters were determined (a) red blood cells (RBC) (b) total white blood cells (WBC) count, (c) differential count, (d) platelet count (e) haemoglobin content using an automated hemato analyzer.

Determination of organ weights

The body weight of animals were taken before being killed, lymphoid organs such as spleen and thymus were excised, weighed, and expressed as relative organ weight.
Biochemical analysis

Liver homogenate was made in ice cold Tris buffer (0.1 M, pH 7.4) and centrifuged at 4°C and 1,200 rpm for 30 min. The supernatant was used for the estimation of Liver ALP (King, 1965), GPT (Bergmeyer and Bernt, 1980), glutathione (GSH) (Moron et al., 1979), lipid peroxidation (LPO) (Ohkawa et al., 1979).

Determination of alkaline phosphatase

The alkaline phosphates level was estimated by King, (1965) method.

Principle

Alkaline phosphatase converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-amino antipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured at 510 nm.

Reagents

- **Reagents 1**: Buffered substrate, pH 10.0
- **Reagents 2**: Chromogen reagent
- **Reagent 3**: Phenol standard, 10 mg%.

Preparation of working solution

Reconstitute one vial of reagent 1, buffered substrate with 4.5 ml of distilled water.

Procedure

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working buffered substrate</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.5</td>
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<td></td>
<td></td>
<td></td>
<td>Mix well and incubate for 3 min at 37°C</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Standard (10 mg %)</td>
<td></td>
<td>0.05</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mix well and incubate for 15 min at 37°C</td>
<td></td>
</tr>
<tr>
<td>Chromogen reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Materials and Methods

Mix well after the addition of each reagent and measure the O.D of the blank, test, standard and control at 510 nm against reagent blank.

Values were expressed as KA units.

Estimation of glutamate pyruvate transaminase (GPT)

The method of Bergmeyer and Bernt, (1980) was used to assay glutamate pyruvate transaminase.

Principle

The enzyme GPT catalyzes the reaction between 2-oxo-glutarate and L alanine forming L-glutamate and pyruvate. The pyruvate produced react with 2-4-dinitrophenylhydrazine giving a product, wit absorption maximum at 520nm.

Reagents

Buffer substrate

1.78gm of DL alanine and 30mg of α-ketoglutaric acid dissolved in 20ml buffer containing 1.25ml 0.4N NaOH. The solution was made up to 100ml with buffer (pH 7.4) and kept at 4°C.

Phosphate buffer

Dinitrophertyl hydrazine (DNPH) (pH 7.4) - 20mg% in 1N HCl, 0.4N NaOH, pyruvate standard 1 %.

Procedure

0.5ml of the substrate was incubated for 3 minutes at 37°C. After incubation, the tissue homogenate (2.5% -0.lml) prepared in cold Tris buffer (pH 7.0) was added, mixed well and was incubated for 30 minutes at 37°C. 0.5 ml of DNPH was added to this mixture and kept at room temperature for 20 minutes. The reaction was stopped by adding 5ml of 0.4N NaOH, vortexed well and kept at room temperature for 5 minutes. The absorbance was measured at 520nm.
**Materials and Methods**

The enzyme activity was expressed as a measure of pyruvate formed which was calculated from the standard curve of pyruvate. Values were expressed as U/ml.

**Estimation of protein**

The procedure described by Lowry et al., (1951) was used for the estimation.

**Principle**

The formation of a protein complex and the reduction of phosphomolybdate-phosphotungstate reagent (Folin Ciocalten Phenol Reagent) by the tyrosine and tryptophan residues of protein to from a coloured product.

**Reagents**

Solution A – 1ml CuSO$_4$ (1%) + 1ml sodium potassium tartarate (2%) and + 98ml 2% Na$_2$CO$_3$ in 0.1N NaOH.

Solution B – Folin - Ciocalten phenol reagent diluted (1:1) with dil. H$_2$O.

**Procedure**

0.1ml of tissue homogenate (2.5%) was diluted to 1.2ml with dil. H$_2$O, and mixed with 6ml of solution A. The mixture was incubated at room temperature for 10 minutes. To this solution, 0.3ml solution B was added, mixed well and kept at room temperature for 30 minutes. The absorbance was read at 680nm. The amount of protein was calculated from standard curve of BSA (bovine serum albumin).

**Estimation of lipid peroxidation**

The method described by Ohkawa et al., (1979) was used for the assay.

**Principle**

Malonaldehyde (MDA) produced during peroxidation can react with thiobarbituric acid (TBA) reagent to form a pink coloured product, which has an absorption maximum at 532nm. The assay was calibrated with 1,1,3,3 tetramethoxy propane which on hydrolysis produces malonaldehyde. The results are expressed in terms of the amount of malonaldehyde produced during the reaction.
Materials and Methods

Reagents

KCl (150mM); Ascorbic acid (0.3mM)
Ferrous ammonium sulphate (0.8mM); Tris buffer (pH 7.0, 0.2M)
TBA (0.8%); SDS (8.1%); Acetic acid (20%, pH 3.5)

Procedure

0.1 ml of tissue homogenate (25%) in Tris HCl buffer (40mM, pH 7.0), KCl (30mM, 0.1 ml), ferrous iron (0.16mM - 0.1 ml), and ascorbic acid (0.06mM, 0.1 ml) in a final volume of 0.5 ml and was incubated for 1 hr at 37°C. After incubation, 0.4 ml of the reaction mixture was treated with SDS (0.2 ml, 8.1%), thiobarbituric acid (1.5 ml, 0.8%) and acetic acid (1.5 ml, 20%, pH 3.5). The total volume was then made up to 4 ml by adding distilled H₂O and kept in a H₂O bath at 95°C for 1 hr. After cooling, 1 ml of distilled H₂O and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added, and shaken vigorously. After centrifugation, the organic layer was taken and its absorbance at 532 nm was measured. The amount of MDA formed was calculated from a standard curve of malonaldehyde. Values were expressed as nmoles/mg protein/min.

Estimation of glutathione

The method described by Moron et al., (1979) was used to assay glutathione.

Principle

Glutathione (GSH) is measured by its reaction with DTNB to give a yellow coloured complex with absorption maximum at 412 nm.

Reagents

Trichloroacetic acid- 25% & 5%; Phosphate buffer (pH 8.0) - 0.2M,
Dithiobiis (2- nitrobenzoic acid) DTNB - 0.6 mM.

Procedure

To 0.5 ml of (10%) homogenate, 125 µl of 25% TCA was added to precipitate protein. The tubes were cooled on ice for 5 minutes and the mixture was further diluted with 0.6 ml of TCA, centrifuged for 10 minutes at 1500 rpm and
0.3ml of resulting supernatant was taken for GSH estimation. The volume of the aliquot was made up to 1ml with 0.2M phosphate buffer (pH 8.0). Two ml of freshly prepared 0.6mM DTNB was added to the tubes and intensity of yellow colour formed was read at 412nm.

The standard curve of GSH was prepared using concentrations varying from 5-100nm in 5% TCA for assay. Values were expressed as nmol/mg protein.

**Determination of immunemodulating markers gene expression by RT-PCR**

**Extraction of RNA**

All the reagents for RNA isolation were prepared using 0.1% overnight DEPC treated sterilized MilliQ grade water and were autoclaved at 121°C for 15 min at 15 lbs pressure. The nuclease free chemicals/reagents reserved for RNA work were handled with baked spatula. Nuclease free sterile disposable plastic ware was used for the isolation and storage of RNA. Disposable gloves were used during the preparation of buffers and reagents as well as during the protocols followed for the isolation and analysis of RNA.

**RNA isolation by TRIzol method**

TRIzol reagent (Invitrogen) was used initially to lyse the homogenized tissues as well as neutrophils and to solubilize and make nucleic acids free of proteins. To isolate total cellular RNA, the weighed (80-100 mg) tissue samples of rat liver were first grinded in a pre-chilled mortar and pestle in the presence of liquid nitrogen until tissue became a fine powder. The grinded tissue samples were further homogenized in 1ml of TRIzol reagent using tissue homogenizer. The homogenized samples were incubated at room temperature for 5 min, to permit the complete dissociation of nucleoprotein complexes. An additional isolation step was carried out to remove insoluble material from the homogenate by centrifugation at 12,000 rpm for 10 min at 4°C. The resulting pellet containing extracellular membranes, polysaccharides, and high molecular weight DNA was discarded, while the cleared homogenate supernatant containing RNA was transferred to a fresh 1.5 ml microfuge tube and was proceed further as described below.
Materials and Methods

1. To the cleared homogenate solution, 0.2 ml of chloroform was added for each ml of TRIzol reagent used.

2. The tubes were vigorously shaken by hand for 15-20 sec to mix the contents properly and incubated at room temperature for 5-10 min.

3. The samples were centrifuged at 14,000 rpm for 10 min at 4°C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an inter-phase and a colorless upper aqueous phase.

4. The clear supernatant was transferred to a fresh microfuge tube taking utmost precaution not to transfer any of the inter-phase material.

5. To precipitate the RNA from the aqueous phase, 0.5ml isopropanol and 1μl bis-acrylamide was added to the supernatant for every 1 ml of TRIzol reagent used during initial homogenization. The contents were mixed well and incubated at room temperature for 10 min.

6. It was followed by centrifugation at 14,000 rpm for 10 min at 4°C and then the supernatant was discarded. The RNA precipitated as a pellet on the side and bottom of the tube.

7. The pellet was washed once with at least 1 ml of 75% ethanol. The sample was mixed by vortexing and centrifuged at 10,000 rpm for 5 min at 4°C after adding ethanol. The RNA pellet was air dried for 10 min, after discarding the supernatant before it was dissolved in 35-40 μl of nuclease free water.

**Determination of RNA concentration and purity**

OD\textsubscript{260} and OD\textsubscript{280} of RNA extracted from liver tissue of rat using nuclease free water as a reference by Nanodrop were recorded. Purity of RNA was checked by taking the OD 260/280 ratio, which ranged between 1.9 to 2.0 as optimum for good quality DNA free RNA. Concentration of total RNA (μg/μl) was calculated using the standard formula:

\[
\text{Concentration of total RNA (μg/ml)} = (\text{OD}\textsubscript{260}) \times 40.
\]

**cDNA synthesis**

The cDNA was synthesized from the isolated RNA using SuperScript\textsuperscript{®}III First Strand cDNA Synthesis kit (Invitrogen). This kit is designed for preparation of
Materials and Methods

Full-length first strand cDNA from RNA templates that relies on a genetically engineered version of the Moloney Murine Leukemia Virus Reverse Transcriptase (SuperScript® III-MuLV RT) with low RNaseH activity. This allows the synthesis of full-length cDNA from long templates (up to 13kb).

The following reaction mixture was prepared in a tube on ice:

- Total RNA: 2μg
- Oligo (dT)₁₈ primer (0.5μg/μl): 1μl
- DEPC treated water to make: 12μl

The contents were mixed gently and centrifuged briefly in a micro centrifuge and incubated at 70°C for 5 min to linearise the RNA removing secondary structure followed by chilling. The tube was placed on ice and the following components were added in this order:

- 5X reaction buffer: 4μl
- RNAseOUT™ Ribonuclease inhibitor (20 U/μl): 1μl
- 10mM dNTP mix: 2μl
- SuperScript® III M-MuLV Reverse Transcriptase (200 U/μl): 1μl
- Nuclease free water to make final volume: 20μl

The contents were gently mixed and mixture was incubated at 42°C for 60 min for reverse transcription and the reaction was stopped by heating at 70°C for 10 min and stored at -20°C, for further use.

PCR amplification of cDNA

The cDNA of liver tissue of experimental rats was taken as template and amplification of IL-2, IFN-γ, GM-CSF, TNF-α, and β-actin (a constitutive gene as internal control) was done by using gene specific primers which were described previously (Dallman and Porter, 1993; McKnight et al., 1991; Obi et al., 2007; Salazar-Montes et al., 2000).

The following primers were obtained from Eurofins Genomics, Bangalore, India. IFN-γ: sense 5’-CTCTCTGGCTTACTGC-3’ antisense 5’-GACTCCTTT
Materials and Methods

TTCCGCTTCC-3’; **IL-2**: sense 5’-ACAAGAATCTGAAACTCC-3’ antisense 5’-GAGATGATGCTTGGACAGATGG-3’; **GM-CSF**: sense 5’-TTCTGCGGCAT TGTGGTCTA-3’ antisense 5’-AAGGGCCTTTGCCAGTTGGT-3’; **TNF-α**: sense 5’-CGAGTGACAGCCCGTGTGCTG-3’ antisense 5’-GGATGAAACAGCCAGTC GCC-3’; **β-actin**: sense 5’-ATGGATGACGATATCGCTG-3’ antisense 5’-ATGA GGATGCTGTCTGCACGT-3’.

The predicted product size for each primer set was: of IFN-γ (405 bp), IL-2 (400 bp), GM-CSF (298 bp), TNF-α (468 bp), and β-actin (568 bp). The concentrations of various reaction components and cycling conditions were optimized. The following reaction mixture was found to be optimum for PCR amplification in a 50 μl reaction volume:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA (50 ng/μl)</td>
<td>4.0 μl</td>
</tr>
<tr>
<td>Forward primer (200 ng/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Reverse primer (200 ng/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>PCR buffer (10X)</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>Taq DNA Polymerase (Bangalore Genei) (3units/μl)</td>
<td>0.6 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>37.4 μl</td>
</tr>
</tbody>
</table>

The above reaction mixture was subjected to PCR amplification with the following PCR cycling conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>0.5 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>57°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

PCRs were subjected to electrophoresis on 1.5% agarose gels with 1X running buffer Tris-Borate-EDTA, at 60 V for 1 hr 15 min and visualized by means of ethidium bromide staining. Bands of each target transcript were visualized by ultraviolet transillumination using GelDoc (Major Scientifics, India) and captured using a digital camera.
The ODs for each band were quantified by image J analysis software and normalized with respect to β-actin.

**Histopathological examination of intestine, liver and spleen**

A small portion of small intestine, liver and spleen were taken and fixed in 10% formaldehyde. After several treatments for dehydration in alcohol, sections having 4-µm thickness were cut and stained with haematoxylin and eosin and histopathological analysis was carried out.

**Validity of experimental procedures**

For all the enzyme studies in the present investigation, the assays were standardized by conducting preliminary test to determine the optimal pH, temperature, enzyme and substrate concentration and these optimal conditions were subsequently followed for each enzyme assay.

**Aliquots for assay**

Aliquots were selected such that initial rates were approximated as nearly as possible yet providing sufficient product to fall in a convenient range of spectrophotometric measurement.

**Enzyme units**

Enzyme activities were expressed in standard units i.e., µ moles of product formed or substrate cleaved/mg protein/min.

**Substrate requirement**

All the enzyme assays were done under the conditions following zero order kinetics unless otherwise stated.

**Beer-Lambert Law**

All the products of the reactions were measured by the spectrophotometric procedures in which the optical density (absorbance) of the resulting colored complex was proportional to the concentrations of the reaction products.
Enzyme nomenclature

The nomenclature of enzymes used in the present study is according to the report of the commission of the “International union of Biochemistry” (IUB).

Assay of dehydrogenases using INT

INT: 2 - (p-iodophenyl) - 3 (p-nitrophenyl) - 5 -phenyltetrazolium chloride): Tetrazolium salts are unique class of oxidation-reduction indicators in the study of dehydrogenases. The advantages of using tetrazolium salts as electron acceptors are:

- They are highly soluble in aqueous solutions.
- They can be reduced both aerobically and anaerobically.
- They have high redox potential which makes the reduction easier.
- They are freely permeable through membranes.

The first developed tetrazolium salt was triphenyl tertazolium chloride (TTC). Following the application of TTC, new tetrazolium salts were developed. Various tetrazolium salts receive electrons from various sites of electron transport system (Oda et al., 1958; Nachlas et al., 1960), which is due to the inherent difference in the redox potentials of various tetrazolium salts. The phenyl ring was observed to increase its redox potential.

Isolation and molecular characterization of fungal endophytes

Selection of host medicinal plant and collection site

Healthy leaves and stems of *Pterocarpus santalinus* Linn.f were collected from Tirumala Hills (3,200 feet (980 m) above sea level; 13° and 14°N, 17°E) of Seshachalam Biosphere Reserve in the Eastern Ghats, India. Tirumala hill region represents one of the major centers of plant diversity with a substantial number of endemic genera. So far there are no reports on the endophyte ecology of these plants. The host plant *Pterocarpus santalinus* was selected in the present study based on following brief properties: *Pterocarpus santalinus* is endemic, endangered, globally threatened medicinal tree taxa, known as “Red Sanders” in India.
Noteworthy, *Pterocarpus santalinus* is found to be rich in saponins, triterpenoids and flavonoids (Narayan et al., 2007). The plant extract is prescribed for vomiting, treating eye diseases, mental aberrations, and ulcers. The heartwood of Red sanders is known to have antipyretic, anti-inflammatory, anthelmintic, tonic, hemorrhage, dysentery, aphrodisiac, and diaphoretic activities (Arokiyaraj et al., 2008). It has also been used as a cooling agent. Ethanol extract of stem bark was reported to possess anti-hyperglycaemic activity (Vinay Kumar et al., 2010). Bioactive compounds such as pterocarpol, santalins A and B, pterocarptriol, ispterocarpolone, pterocarpodiolones with β-eudeslo and cryptomeridol were identified in the heartwood (Kodithuwakku et al., 2011). The isoflavonoids, terpenoids, related phenolic compounds, β-sitosterol, lupeol, (-) epicatechin (Kesari et al., 2004) and auron glycosides viz., 6-OH-1-methyl-3',4',5'-trimethoxyaurone-4-O-rhamnoside and 6,4'-dihydroxyaurone-4-O-neohesperid- doside, and isoflavone glycoside 4',5'-dihydroxy 7-methyl isoflavone 3'-O-beta-D-glucoside are also present in *P. santalinus* (Krishnaveni and Sirinivasa Rao, 2000). To mention, the plants that are endemic, having an unusual longevity or that have occupied a certain land mass are also more likely to lodge endophytes than other plants. Furthermore, the plants growing in areas of great biodiversity also have the prospect of housing endophytes with great biodiversity (Yu et al., 2010). Endophytic fungi associated with *P. santalinus* that grow on organic substrates in the harsher and or extreme conditions, might include specialists that have evolved specific adaptations, some of which could also be of technical interest. For this reason, systematic investigation of endophytic fungi associated with *P. santalinus* is necessary, and will not only play a role in enhanced natural product production, but also provides new natural products and further allow us to know genetic information.

**Collection of plant material**

The symptomless and apparently healthy individual plants were randomly chosen from the collected site. The host plant was identified by Professor Madhava Chetty of the Sri Venkateswara University, and deposited (voucher specimen: No.SVU-BH-2062) in the Herbarium of the Department of Botany, Sri Venkateswara University, Tirupati, India. The plant samples were collected from
different aerial parts, viz. stem and leaf. The samples were immediately placed in plastic bags and taken to the laboratory store at 4 °C for isolation of endophytic fungi within 48 h of collection.

**Standardization of surface sterilization protocol**

The collected plant samples were washed thoroughly under running tap water until the surface adherents were removed. A total of 300 plant bits (150 bits per each tissue) of the selected medicinal plant species were separately excised and subjected to surface sterilization. Four different sterilization protocols referred by Schulz et al., (1993), Crous et al., (1995) Arnold et al., (2000) and Suryanarayanan and Vijaykrishna, (2001) were employed with minor modification like concentration of sterilizing agent and treatment time in order to standardize the surface sterilization of plant parts. In order to test the effectiveness of surface sterilization, the uncut surface sterilized and non-sterilized samples were imprinted onto potato dextrose agar with 100 μg/L streptomycin (PDAS) in Petri dishes at 28°C for 1 week. In addition, 10 ml of the both non-sterilized samples and sterilized sample rinsing water were centrifuged separately for 10 min at 5000 rpm. The supernatant was removed and added 500 μl sterilized water in the both centrifugal tubes; 100 μl of this volume from each tube was then plated onto individual PDAS (Xiong et al., 2013). The efficacy of surface sterilization protocols was calculated as the number of colonies observed on petri plate upon plating sterilized sample rinsing water as inoculum by number of colonies observed on petri plate upon plating non-sterilized sample rinsing water as inoculum ×100. In addition, the number of endophytic fungi obtained from the treated plant samples of *P.santalinus* in all treatment was also considered. This study was initially undertaken to select the most effective sterilization protocol to be followed throughout the investigation. The surface sterilization was validated upon occurrence of no mycelial growth with the surface sterilized plant samples.

**Isolation of fungal endophytes**

The fungal endophytes were isolated using a modified method described by Arnold et al. (2000). The material was thoroughly washed in sterile water, surface-
Materials and Methods

disinfecte the 70% ethanol for 1 min and 3% sodium hypochlorite (NaOCl) solution for 4 min, rinsed in sterile demineralised water and 96% ethanol for 30 sec. The plant material was subsequently rinsed in sterile demineralised water. The surface sterilized plant materials were cut into small segments (leaf: 5 × 5 mm size and stems: 10 mm length) and placed on potato dextrose medium incorporated with streptomycin sulphate (250 mgL^{-1}) and incubated at 28±1 °C with 12 h photoperiod for 3-4 days to few weeks till the growth initiated.

Individual hyphal tips that emerged from the edges of each treated plant bits were transferred separately onto fresh PDA plates, and incubated in a light chamber under near ultraviolet (UV) for at least 10~15 days. Each fungal culture was checked for purity and transferred to agar slants by the hyphal tip and single spore isolation methods (Ahmad, 1991, Strobel et al., 1996). Pure cultures of the isolates were maintained for their identification.

Morphological identification and taxonomy of fungal endophytes

The cultures were examined periodically and identified when isolates sporulated. The morphological characteristics such as growth pattern, hyphae, colour of colony on the medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics of the spore (Barnett and Hunter, 1998) were considered. Fungal mycelium was stained in cotton blue and mounted in polyvinyl lactic acid glycerol (PVLG) by heating at 65°C for two to three days and observed under microscope. An Olympus CX13 with interference contrast was employed for examination by light microscopy.

The identification of the isolates was also confirmed by expert taxonomists at the Agarkhar Research Institute, Pune, Maharashtra, India. All the endophytic isolates were identified and placed in appropriate genera and species of fungi using standard taxonomic keys and monographs. Authoritative monographs were referred for identification of endophytes [Leslie and Summerell, 2006; Bhat, 2010 (Hyphomycetes); Nag Raj, 1993; Rai 2000 (Coelomycetes); Manoharachary, 1994; Hanlin, 1990; Pande, 2008 (Ascomycetes)]. In addition, other taxonomic papers relating to particular genera and species of endophytes were also referred. For colour
Molecular and Methods

differentiation of cultures ‘Methuen Handbook of Colour’ (Kornerup and Wanscher 1978) was also referred.

Molecular phylogenetic identification of endophytic fungi

The identity of the certain interesting/non-sporulating strains was confirmed using molecular tool i.e., analysis of 18S rRNA gene sequence.

Genomic DNA extraction

Each endophytic fungus was cultured in potato dextrose broth at 25°C with constant shaking for 7 days. The fungal mycelia were freeze-dried and the genomic DNA was extracted by the CTAB (Cetyl trimethylammonium bromide) method (Ausubel et al. 1994). Briefly, 500 mg of fungal mycelia were vigorously crushed in liquid nitrogen to make a fine powder. The cells were lysed in 10 ml of extraction buffer (50 mM Tris–HCl pH 8.0, 50 mM EDTA, 0.7 M NaCl, 2% cetrimide, 1% SDS and 50 μl β-mercaptoethanol), mixed thoroughly and incubated at 65°C for 30 min with continuous shaking. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 10,000×g for 10 min at 4°C. The aqueous phase was transferred to a sterile tube; the genomic DNA was precipitated in a 2×volume of chilled isopropanol and centrifuged at 4°C for 10 min at 10,000×g. The resulting pellet was washed twice with 70% ethanol, air dried and dissolved in 20 μl of sterile Millipore water.

PCR (polymerase chain reaction) amplification, partial ITS1-5.8S-ITS2 ribosomal gene sequencing

Molecular characterization of the endophytes was carried out by the acquisition of ITS ribosomal gene sequencing. Protocol outlined by White et al., (1990) was followed for polymerase chain reaction in a CG Palm Cycler (Corbett Research, Mortlake, NSW, Australia). The ITS regions of the fungi were amplified with the universal ITS primers, ITS1 (5’ TCCGTAGGTGAACCTGCGG 3’) and ITS4 (5’ TCCTCCGCTTATTGATATGC 3’), using the polymerase chain reaction (PCR). The amplified fragments include ITS1, 5.8S and ITS2 region of rDNA. The PCR reaction mixture (50 μl) was prepared as follows: 50 ng of template DNA, 10X
PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl₂, 2.5 mM each dNTP, 200 ng/μl of each primer, and 2U/μl of Taq DNA polymerase (Genei, Banglore) and MilliQ was added to complete the final volume of the reaction. The amplified products (5 μl) were visualized on 1% (w/v) agarose gel to confirm the presence of a single amplified band.

**PCR reaction mixture contained – (50 μl)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA (50 ng/μl)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>PCR buffer (10 X)</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>MgCl₂ (1.5 mM)</td>
<td>3.0 μl</td>
</tr>
<tr>
<td>dNTP’s (2.5 mM)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>ITS 1 Primer (200 ng/μl)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>ITS 4 Primer (200 ng/μl)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Taq polymerase (2U/μl)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>MilliQ</td>
<td>31.0 μl</td>
</tr>
</tbody>
</table>

**PCR thermal cycler conditions**

- Initial denaturation: 95°C 5 min
- Denaturation: 94°C 1 min
- Annealing: 56°C 1 min
- Extension: 72°C 1 min
- Final extension: 72°C 7 min

The PCR products were purified using a QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR product was directly sequenced using the same primers by MWGAG Biotech (Bangalore, India). The results of sequencing obtained in FASTA format were subjected to BLASTn (http://www.ncbi.nlm.nih.gov) analysis for identity of the test fungus and then submitted to GenBank using SEQUIN program. The GenBank also provided accession numbers for submitted sequences of the respective strains.

**Phylogenetic analysis**

The ITS sequences of endophytic fungi were compared with the data in National Center for Biotechnology Information, USA (NCBI) using BLAST search.
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(http://blast.ncbi.nlm.nih.gov/Blast.cgi) to estimate the phylogenetic relationship. CLUSTAL X software (version 2.0, Conway Institute, USA) was used to generate alignment of endophytic fungi (Larkin et al., 2007). Phylogenetic analysis was carried out by the neighbor-joining method using MEGA software (version 4.0, Biodesign Institute, USA). The bootstrap was 1,000 replications to assess the reliable level to the nods of the tree (Tamura et al., 2007).

Data and Diversity analysis

The data gathered was subject to analysis. The colonization frequency (CF) was calculated as the number of plant segments colonized by a single endophyte divided by the total number of segments observed×100. Colonization rate (CR) was calculated as the total number of plant tissue segments infected by fungi divided by the total number of segments incubated. Isolation rate (IR) was determined as the number of isolates obtained from plant segments divided by the total number of segments incubated. Isolation frequency (IF) was calculated as the total number of isolates of one species divided by the total number of isolates in that sample×100. In addition, three different diversity indices such as Simpson’s diversity index, Shannon-Wiener index and Evenness index were calculated using Primer software to determine the endophytic species diversity of leaf and bark tissues isolated from Pterocarpus santalinus.

Stimulatory effect of endophyte and endomycorrhiza on plant growth and in planta production of triterpene saponins (immunomodulators) of C.asiatica

The plant derived saponins based immunomodulators are of importance in improving the immunity levels by optimizing the immune functions (Pragathi et al., 2011). The botanical immunomodulators being nontoxic and having no side effects are attracting the global attention. The chemical synthesis of immunomodulators is difficult and economically infeasible due to their complex nature. Furthermore, growing demand for natural medicines from medicinal plants is spurring interest in novel and more efficient production methods (Rai et al., 2001). To overcome this situation there is a need to adopt biological techniques which improves the in planta production and accumulation of botanical immunomodulators.
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Thus, this study seeks to explore the potential of inoculation of *Centella asiatica* with fungal endophyte and endomycorrhiza as part of a commercially viable production scheme.

**Selection of plant**

*Centella asiatica* can serve as good model plants for studying the effects of fungal endophyte colonization on secondary metabolism. It has significant economic importance, a well documented profile of triterpene saponins and thus, can be investigated whether the immunemodulating chemicals could be affected by endophytic fungi and endomycorrhizal colonization.

**Cultivation of plants - experimental design**

Nursery of *C.asiatica* was raised in ethanol disinfected plastic pots (10.2cm×25.4 cm), filled with 3.5kg of sterilized garden soil + sand (1:1 w/w). The stem cuttings with single node (2-3 cm) were surface-sterilized by dipping in 2% NaOCl solution for 10min and grown on potting media. The plantlets were planted in 4 different sets of pots and were subjected to the following treatments. These included:

(1) T1: Control (without inoculation); (2) T2: Endomycorrhiza treated (*Glomus mosseae*); (3) T3: Fungal endophyte treated (*Aspergillus aculeatinus*); (4) T4: Endomycorrhiza and fungal endophyte treated (*A.aculaetinus + G.mosseae*).

Eight experimental replicates were prepared for each treatment, so there were 32 total plants prepared (each plant was in a separate poly pot) according to a completely randomized design. The plantlets were grown in a glasshouse under natural photoperiods (23.5/18 ºC day/night, 4000-6000 lux light intensity, 16/8 h light-dark cycle, and 55/75% relative humidity) and watered daily. Harvest occurred 60 days after planting the plantlets of *C.asiatica*.

**Preparation of endomycorrhizal inoculum**

The endomycorrhiza were extracted using the wet sieving and decantation method (Sieverding, 1991; Gerdemann and Nicolson, 1963).
Isolation of VAM

Twenty five grams of root zone soil (Collected from medicinal plant garden, Department of Botany, Sri Venkateswara University, Tirupati) was suspended in 500 ml of distilled water and stirred thoroughly. The suspension was passed through a series of metal sieves of different mesh size (1 mm, 750 μm, 250 μm, 75 μm and 45 μm) arranged one below the other in descending order. The suspension containing VAM spores in the two bottom sieves were collected and transferred on to separate petri plate containing pre wetted filter paper. The petri plate was placed under dissecting microscope for counting the spores.

Identification of VAM isolates

The VAM spores recovered from the rhizosphere were collected in a watch glass. The spores were washed with chloramin-T (2 %) and streptomycin sulphate (200 ppm) to remove all debris and contaminants. Microscopic slide preparation of the spores was made using lacto glycerol as mount. The spores were identified up to species level using stereo zoom microscope and consulting the manuals, available keys and photographs for identification of VAM fungi (Morton, 1988; Schenck and Perez, 1987; Sieverding, 1991). The fungus predominantly associated was identified as *Glomus mosseae* based on the characteristics of the spores.

VAM inoculum

The inoculum of *Glomus mosseae* was developed in pots on the roots of *Sorghum vulgare* in sterilized soil. The pots were maintained in a glass house by watering to field capacity. After 4 months of growth, the mycorrhizal roots were air dried, homogenized by mixing the soil and cutting the roots into pieces less than 1 cm in length.

Inoculation of *Centella asiatica* plants

The *G.mosseae* colonised *Sorghum* root cultures (with hyphae and extraradicular spores) cultivated on 90 mm plates with growing medium are used for inoculum preparation. The month-old cultures (together with the culture media)
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from 1 or 2 plates are collected and gently mixed under sterile conditions using 30 ml of sterile water in a blender (about 3-5 seconds at low speed). This *G. mosseae* suspension, which contains about 20-25 spores/ml and colonised root fragments is directly used for inoculation, applying 20gm/pot by layer around the roots of plantlets below the surface of the soil (approx 500 spores) (Andry et al., 2006).

**Preparation of endophytic fungal inoculum**

The selected endophytic fungi, *Aspergillus aculaetinus* was grown on PDA plates (30°C, static, 14 d). The spores were scraped from the two weeks old cultures plates by the use of 2 ml sterile distilled water mixed using 0.1% Triton X-100 and the spore concentration was adjusted to $1 \times 10^8$ conidia/ml.

**Inoculation of Centella asiatica plants**

The plantlets were treated with drench inoculation method. For the inoculation, 20 ml of the spore suspension was applied to the surface of the soil in each pot (Francisco et al., 2007). Controls received sterile 0.1 % Triton X-100 applied in the same way as mentioned above.

**Combined inoculation of Centella asiatica plants**

The consortium of endomycorrhizae (*Glomus mosseae*) and fungal endophyte (*Aspergillus aculaetinus*) was made by adding equal volume of spore suspension of both strains and were inoculated same way as mentioned above, at the time of planting the plantlets.

**Analysis of biometric parameters and chlorophyll content**

**Biometric parameters**

The biometric parameters were measured on 60th day after planting in all the treatments.

**Shoot length:** The plants were uprooted carefully without damaging the root system and the shoot length of the plants was measured from the collar region to the tip of the plant, using a standard scale. The values were recorded in centimeters.
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**Root length:** After the growth period, the plants were uprooted carefully without damaging the root system. The root system was cleaned under a running tap water to eliminate the sand and soil particles adhering to it. The root length was recorded by measuring the root from its crown region to the root tip using the measuring scale and values were recorded in centimeters.

**Number of leaves:** Number of fully opened leaves in all the treatments was counted manually.

**Petiole length:** The petiole length of the leaf in all the treatments was also recorded in centimeters using the measuring scale.

**Leaf length:** The length of the leaves was measured using a standard scale and values were recorded in centimeters.

**Leaf width:** The width of the leaves was recorded using the measuring scale and values were recorded in centimeters.

**Number of nodes:** The number of nodes occurring along each primary branch in all the treatments was recorded manually.

**Fresh shoot biomass:** The shoot portion was separated from the root system and was blotted on the blotting paper to observe the moisture. The weight of the shoot was estimated using an electronic monopan balance and the values were expressed in grams.

**Fresh root biomass:** The plants were carefully uprooted and roots were washed in running tap water to clear adhering the soil particles. The washed roots were blotted on the blotting paper and the roots were weighed using monopan electronic balance. The values were expressed in grams.

**Dry shoot biomass:** After measuring the fresh shoot biomass of the plants, it was placed in a paper cover and dried in a hot air oven at 60ºC for 2 days to attain constant weight. Then dry weights of the shoots were recorded in grams.
**Dry root biomass:** To obtain the dry root biomass of the plants, the roots were placed in a paper cover and dried in a hot air oven at constant temperature of 60°C, for 48 hours and the values were expressed in grams.

**Chlorophyll content**

The chlorophyll content of the plants was estimated in all the treatments on 60th days after planting according to the method of Arnon, (1949). The leaves were excised from the plants and washed with distilled water and blotted dry. One gram of leaf sample was homogenized with 80 % acetone in a pre-chilled mortar. A pinch of CaCO₃ was added to facilitate easy grinding. The extract was then centrifuged at 5000 rpm for 15 minutes and the supernatant was made up to 10 ml with 80 % acetone. The supernatant was filtered through Whatmann No. 1 filter paper and the clear supernatant was transferred to 1 cm glass cuvette. The absorbance was measured using specific absorptions co-efficient for chlorophyll a and b at 645 nm and 663 nm using 80 % acetone as blank in Shimadzu (UV – 240) double beam spectrophotometer. The following simultaneous equations were setup for measuring chlorophyll concentrations.

\[
\text{Chlorophyll ‘a’} = (0.0127 \times \text{OD at 663 nm}) - (0.000269 \times \text{OD at 645 nm}).
\]
\[
\text{Chlorophyll ‘b’} = (0.0229 \times \text{OD at 645 nm}) - (0.00408 \times \text{OD at 663 nm}).
\]

**Extraction of triterpene saponins**

The triterpene saponins content in leaves of all the treatments was determined appropriately using the method described by Rafamantananana et al., (2009). The leaves of *Centella asiatica* was cut and dried in oven at 50°C for 3-4 days. Dried samples were ground into powder using a mini grinder. Solvent extracts were prepared by transferring 1g of the powder to sterile wide mouthed screw capped bottles containing the solvent. It was allowed to soak for 24 hours at room temperature then heated for an hour at 100°C. The mixture was then centrifuged at 2000 rpm for 10 minutes at 4°C. The supernatants were filtered through a sterile funnel containing sterile Whatman filter paper no.1 and then filter sterilized using syringe filter containing 0.2μ cellulose acetate membrane (Krishnan et al., 2009).
The filtrate was defatted by using petroleum ether 3 x 1 h at 40 °C. After filtering the petroleum ether, the sample was extracted with methanol 3 x 1 h with mild heating. The combined methanol extract was concentrated and the methanol extract was dissolved in methanol-acetone mixture (1:5 v/v) to precipitate the saponins (Krishnan et al., 2009). The precipitate was dried under vacuum, turning to a whitish amorphous powder named as crude extract (CE). The CE was loaded on silica gel-60 (230-400 mesh, Merck) chromatography column and eluted with chloroform-methanol-water (70:30:10) (Krishnan et al., 2009). The first fraction collected was evaporated under reduced temperature; the resultant residue was called pure saponin fraction.

**High performance liquid chromatography analysis of triterpene saponins**

The dried saponin extract was dissolved in 10 ml of methanol. Triterpene standards (purchased from APExBIO, Houston, TX 77054, UK) were prepared in three different concentrations. A stock solution (2.5 mg/ml) of asiatic acid diluted with HPLC grade methanol to obtain a concentration of 0.25, 1.0, and 2.5 mg/ml. A stock solution (5.0 mg/ml) of madecassoside and asiaticoside were diluted to obtain a concentration of 0.5, 2.5, and 5.0 mg/ml respectively.

The extract solution was diluted with methanol (1:5 v/v). Prior to injection, the samples and standards were filtered through a 0.45 filter (Whatman). The HPLC Waters separation module (Waters, Milford, MA, USA) used consisted of a pump (501), an autoinjector (U6K), a UV-VIS spectrophotometric detector (484), all controlled by a personal computer with Empower chromatography data software.

**Table 2. Gradient conditions for HPLC**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pump A, water (%)</th>
<th>Pump B, acetonitrile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>35</td>
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<td>35</td>
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<td>40</td>
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<tr>
<td>45</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>55</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>
Materials and Methods

The chromatographic separation was performed with a reversed phase RP-18 LiChroCART® column (250 mm×4mm I.D.; particle size: 5µm). Mobile phase was a gradient of acetonitrile/water (Table 2), a flow rate of 1 ml/min and detection at 206 nm. The injection volume was 20 μl with three injections being performed for each samples and standards. The concentrations of triterpenes in the samples were estimated from the standard curve that has been prepared by plotting peak area against concentration of standards: madecassoside, asiaticoside and asiatic acid with coefficient ($r^2$) greater than 0.99 and the values are expressed in mg/ml.

Structures of triterpene saponins of *Centella asiatica*

![Structures of triterpene saponins](image)

- Madecassoside: $R_1=\text{OH}$, $R_2=\text{Glu-Glu-Rha}$
- Asiaticoside: $R_1=\text{H}$, $R_2=\text{Glu-Glu-Rha}$
- Asiatic acid: $R_1=\text{H}$, $R_2=\text{H}$

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using statistical package for the Social Science (SPSS) software, version 11.5. Results were expressed as mean±S.D. P values < 0.05 were considered significant.