Materials and Methods
CHAPTER 3
MATERIALS AND METHODS

General experimental procedure

The melting points were determined using a Boetieus microheating table or Toshiba melting point apparatus and are uncorrected. They are expressed in degree centigrade (°C). Optical rotations were measured on a Deep vision polarimeter. The UV spectra were recorded on a Systronics Double Beam Spectrophotometer-2202 instrument. The infrared (IR) spectra were recorded on a Perkin Elmer-FTIR-GX spectrophotometer and Shimadzu-8201 FT instrument as the case may be. The $^1$H NMR spectra were recorded mostly in CDCl$_3$ at 500 MHz, 400 MHz or 300 MHz on a Bruker-AMX-500, AMX-400 or Bruker-300 Fourier Transform Spectrometer with tetramethysilane (TMS) as an internal standard. The $^{13}$C NMR spectra were recorded at 125 MHz /100 MHz mostly in CDCl$_3$ with TMS as an internal reference. The chemical shifts are quoted in parts per million (ppm). The mass spectra were recorded on a Shimadzu QP-2010 instrument.

All the solvents used for chromatography were purified by distillation. All the solvents, reagents and silica gel were obtained from Merck. Petroleum ether, benzene, ethyl acetate, chloroform and methanol were used as developing solvents. Petroleum ether of boiling range 60-80°C was used. Separation and purification of the crude products was carried out using chromatographic columns packed with silica gel (60-120) mesh activated at 120°C. Thin layer chromatography was performed using glass plates coated with silica gel (100mesh) with 13% CaSO$_4$ as binder. Detection of the chromatograms of TLC was carried out under ultraviolet light at 254 and 366 nm. A chamber containing iodine vapour was also used to locate the spots. Homogeneity of the compounds was checked on TLC. All the reagents used were of analar grade. Shift reagents were prepared according to standard methods (Vogel, 2004).
The present work is the report of the phytochemical investigation of two medicinal plants - *Acalypha fruticosa* and *Aristolochia tagala*. The work involves

3.1 Isolation and characterization of secondary metabolites of *Acalypha fruticosa*.
3.2 Isolation and characterization of the essential oil from *Acalypha fruticosa*.
3.3 Isolation and characterization of secondary metabolites of *Aristolochia tagala*.
3.4 Isolation and characterization of the essential oil from *Aristolochia tagala*.
3.5 Antimicrobial studies on the plant extracts and essential oils.
3.6 Antioxidant activity studies of *Acalypha fruticosa* extract and two of its active principles.

3.1 Isolation and characterization of secondary metabolites of *Acalypha fruticosa*

3.1.1 Plant details

Name: *Acalypha fruticosa*  
Family: *Euphorbiaceae*  
It is commonly called birch-leaved *Acalypha*.  
Distribution: Throughout India particularly in Orissa, Tamilnadu, Karnataka and Kerala.

Plant description: It is a strong smelling, pubescent bushy shrub growing up to 2.5 m in height and covered with yellow and waxy glands. Leaves ovate-oblong, 25-76 mm long, glandular beneath; spikes axillary, short, usually with male flowers towards the apex and female down below; capsules tomentose with smooth seeds (The Wealth of India, 1988).

3.1.2 Plant material - Collection and identification

The plant material was collected from Palladam, Coimbatore district, Tamilnadu in March 2005 and confirmed by the Botanical Survey of India, Coimbatore.
Acalypha Fruticosa

Aristolochia tagala
Air dried aerial parts of *Acalypha fruticosa* (5 kg)

Dewaxed with n-hexane

Hexane extract (concentrated)

Waxy material (discarded)

Residue

Extracted with acetone

Acetone extract

Concentrated under reduced pressure

Dark green semisolid (330 g)

Column chromatography over silica gel

PE:EA(2%)  PE:EA(5%)  PE:EA(6%)  PE:EA(8%)  PE:EA(9%)  PE:EA(15%)  PE:EA(23%)  PE:EA 90%

AF-1  AF-2  AF-3  AF-4  AF-5  AF-6  AF-7  AF-8  AF-9

Scheme-1

The fractions obtained after removal of AF-3 consisted of a mixture of two compounds. All similar fractions were combined, solvent evaporated and the residue was subjected to recolumn chromatography when AF-4 was obtained with 8% ethyl acetate and AF-5 was obtained with petrol ether: ethyl acetate 9%. The compound AF-6 was also obtained only after recolumn chromatography.

3.1.6 Purification and identification of compounds

All compounds obtained from the column were thoroughly washed with small amounts of solvents of increasing polarity in order to remove trace impurities and then finally recrystallised. The purity and homogeneity were checked on TLC plates.
Identification of the compounds involved physical data measurements, chemical analysis and spectral analysis. Melting point, mixed melting point determination and measurement of optical rotation confirmed the identity of known compounds. Chemical tests for the various classes of compounds were carried out (Krishnaswamy, N.R., 2003). Complete identification was possible through spectral methods which included UV, IR, $^1$H NMR, $^{13}$C NMR, COSY, HETCOR, DEPT and mass spectral methods. Shift reagents were added to the ethanolic solution of the compounds to study the shift in $\lambda_{\text{max}}$ in UV spectrum and correlate the position of the hydroxyl groups.

### 3.1.6.1 Compound AF-1

The compound AF-1 was obtained from the column on elution with 2% ethyl acetate in petroleum ether and crystallized from ethanol.

Melting point: 165°C; Yield: 50 mg

### 3.1.6.2 Compound AF-2

The petroleum ether: ethyl acetate eluate (95:5) afforded AF-2 which was recrystallised from methanol.

Melting point: 260-263°C; Yield: 20 mg

### 3.1.6.3 Compound AF-3

Compound AF-3 was eluted with 6% ethyl acetate in petroleum ether and crystallized from methanol as colourless crystalline solid.

Melting point: 252°C; Yield: 30 mg

### 3.1.6.4 Compound AF-4

The compound AF-4 was obtained as a pale yellow powder after recolumn chromatography with petroleum ether: ethyl acetate (92: 8).

Melting point: 203°C; Yield: 15 mg
3.1.6.5 Compound AF-5

Recolumn chromatography with petrol ether: ethyl acetate (91: 9) afforded a yellow solid which after repeated washings and recrystallisation from ethanol gave yellow needles.
Melting point: 123°C; Yield: 25 mg

3.2.6.6 Compound AF-6

Further elution with 15% ethyl acetate in petrol ether gave compound AF-6 which was recrystallised from methanol to give yellow shiny needles.
Melting point: 163°C; Yield: 50 mg

3.1.6.7 Compound AF-7

Repeated elutions resulted in a white solid with petrol ether: ethyl acetate (77: 23), which was washed thoroughly with various polarity grades of solvent and recrystallised from methanol to give white needles.
Melting point: 207°C; Yield: 30 mg

3.1.6.8 Compound AF-8

The compound AF-8 was obtained as a colourless crystalline solid from the column on elution with petrol ether: ethyl acetate (1:1).
Melting point: 250°C; Yield: 20 mg

3.1.6.9 Compound AF-9

Elution with 90% ethyl acetate afforded a white powder which was recrystallised from chloroform- methanol mixture.
Melting point: 230°C; Yield: 25 mg
3.2 Isolation of essential oil from the leaves of *Acalypha fruticosa*

3.2.1 Collection of plant material

Fresh leaves (660 g) were collected from Palladam, Coimbatore district, Tamilnadu during March 2005.

3.2.2 Extraction of the essential oil

The fresh leaves were hydrodistilled in Clevenger-type apparatus for 3 hours. The pleasant smelling yellowish brown coloured oil obtained was purified and dried over anhydrous sodium sulphate to yield 9.3 g (1.4% w/w) of oil. It was stored at 4°C until analysed.

3.2.3 Qualitative and quantitative analysis of the oil

**GC Analysis**

GC analysis was performed on a Hewlett Packard HP 6890 equipped with a split / splitless injector (280°C) a split ratio 1:10 using a HP-5 capillary column (25 m x 0.25 mm, film thickness 0.25 μm). The temperature program was 50°C (5 min.) rising to 300°C at rate of 5°C / min. Helium was used as the carrier gas at flow rate of 1.1 mL/min. The injection of each sample consisted of 1.0 μL of oil diluted to 10% v/v with acetone.

**GC/MS Analysis**

GC/MS analysis was performed on a Hewlett Packard 5973/6890 system operating in EI mode (70 eV), equipped with a split/ splitless injector (280°C), a split ratio 1:10 using two different columns, a fused silica HP –5 MS capillary column (25 m x 0.25 mm, film thickness 0.25 μm) and a HP- Innowax capillary column (60 m x 0.25 mm, film thickness 0.25 μm). The temperature program for the HP-5 MS column, 50°C
(5 min) rising to 300°C at a rate of 5°C/min and for the HP-Innowax column, 50 - 250°C at a rate of 5°C/min. Helium was used as the carrier gas at a flow rate of 1.1 mL/min.

Retention indices for all compounds were determined according to the Van den Dool approach (Van den Dool, 1963). The identification of the components was based on comparison of their mass spectra with those of Wiley (McLafferty, 1989) and Adams (Adams, 2001) and Joulain (Joulain, 1998) libraries as well as by comparison of their retention indices with literature data. The compositional data is given in Table-12.

3.3 Isolation and characterization of compounds from *Aristolochia tagala*

3.3.1 Plant details

Name : *Aristolochia tagala* Cham.

Synonym : *Aristolochia roxburghiana* Klotzsch.

It is commonly called Oval leaf Dutchman’s pipe or India Birthwort.

Family : *Aristolochiaceae*.

Distribution : Distributed in tropical and temperate regions. Found in Himalayas from Nepal eastwards to Sikkim, from Bihar to Assam, and in the Deccan peninsula on the Western Ghats.

Plant description: It is a large climbing shrub with stout, grooved stems, leaves ovate, acuminate, deeply cordate at base, flowers numerous, in loose slightly-hairy racemes, greenish yellow, perianth upto 7 cm long, pale green with a globose base and curved tube, oblique mouth and straight lip; capsules 2.5 cm long, oblong ellipsoid, transversely ridged, glabrous.

3.3.2 Plant material - Collection and identification

This endangered species was collected from Wyanad of Western Ghats of Kerala, South India in March 2004 and confirmed by the Botanical Survey of India, Coimbatore.
3.3.3 Pretreatment of the plant material - Dewaxing

The aerial parts of *Aristolochia tagala* was air dried at room temperature for a week. The dried material (3 Kg) was chopped and powdered and extracted with n-hexane with occasional shaking. The contents were left overnight and then filtered. The marc left behind was further treated with hexane and the process repeated thrice. The hexane extracts were combined and concentrated. The residual waxy material was discarded.

3.3.4 Extraction of the plant material

The marc left behind after hexane extractions were dried in air for three hours to remove the residual solvent. It was then extracted with methanol (4 x 2.5 L) in a soxhlet apparatus for a week. The methanol extract on concentration on a rotary evaporator gave a dark brown gummy mass (500 g). TLC of this concentrated extract showed a complex mixture of compounds, some of them showing fluorescence under UV. So it was decided to fractionate the mixture to reduce the complexity. The solid mass was partitioned between water and chloroform and the chloroform extract was concentrated (300 g) and fed into a column of silica gel and eluted with petroleum ether, petroleum ether: ethyl acetate mixtures and ethyl acetate. This led to the isolation of three compounds, AT-1, AT-2 and AT-3 all of which are isolated for the first time from this plant. The chart (Scheme-2) below illustrates the isolation technique.
Aerial parts of *Aristolochia tagala* (3Kg)

Hexane extract

Concentrated

Waxy material (discarded)

Residue

Extracted with methanol

Methanol extract (500g)

Concentrated under reduced pressure

Dark brown pasty mass

Partitioned between water and chloroform

Aqueous layer

Chloroform soluble fraction (300g)

Solvent evaporated

Viscous brown pasty mass

Column chromatography over silica gel

PE:EA(2.4%) PE:EA(10%) PE:EA(14%)

AT-1 AT-2 AT-3

Scheme-2
3.3.4.1 Compound AT-1

The compound AT-1 was obtained from the column on elution with 2.4% ethyl acetate in petroleum ether and crystallized from ethanol. 
Melting point: 168°C; Yield: 40 mg

3.3.4.2 Compound AT-2

Elution with 10% of ethyl acetate in petroleum ether afforded a white solid which was recrystallised from methanol to give a white crystalline solid. 
Melting point: 160°C; Yield: 30 mg

3.3.4.3 Compound AT-3

From the petrol ether: ethyl acetate (86: 14) eluate, compound AT-3 was isolated. It was washed with petrol ether and recrystallised from methanol as colourless needles. 
Melting point: 218°C; Yield: 30 mg

3.4 Isolation and characterization of the essential oil from Aristolochia tagala

3.4.1 Collection of the plant material

The aerial parts (570 g) of Aristolochia tagala were collected from the dense forests of Wyanad in Kerala during May 2005.

3.4.2 Extraction of the essential oil

The fresh plant parts were hydrodistilled in a Clevenger-type apparatus for 4 hours. Powerful earthy pleasant smelling oil was obtained which was purified and dried over anhydrous sodium sulphate to yield 12 g (2.1% w/w) of the oil. It was stored in a freezer till the time of analysis.
3.4.3 Characterization of the oil

GC/FID Analysis

The oil was analyzed by GC/FID using a Nucon 5765 series gas chromatograph fitted with SE-30 (10%) Chromosorb-W packed stainless steel column (2 m x 2 mm) with FID detector. Nitrogen was used as the carrier gas at flow rate of 40 mL/min. Oven program: 80°-150°C (8°C/min.), 150°-230°C (5°C/min), 230°C (10 min), injector temperature 220°C, detector temperature 250°C. Relative percentages of components were calculated from the peak area-percent report of volatiles from GC/FID data (Table-10).

GC/MS Analysis

GC/MS analysis of the oil was performed by splitless injection of 1.0 μL of the oil on a Hewlett Packard 6890 gas chromatograph fitted with a cross-linked 5% PH ME siloxane HP-5 MS capillary column, 30 m x 0.32 mm, 0.25 μm coating thickness, coupled with a model 5973 mass detector. GC/MS operation conditions: injector temperature 220°C; transfer line 290°C; oven temperature program 60°- 246°C (3°C/min); carrier gas - Heat 1.4 mL/ min. Mass spectra: Electron Impact (EI+) mode 70 eV, ion source temperature 250°C. Individual components were identified by Wiley 275 L database matching and by comparison of retention times and mass spectra of constituents with published data (Adams, 2001). Relative retention indices (RRI) of constituents were determined using n-alkanes as standards (Van den Dool, 1963).

3.5 Antimicrobial study on the plant extracts and essential oils

The antimicrobial study of the plant extract of *Acalypha fruticosa* and *Aristolochia tagala* and the essential oils from both the plants were done by the disc diffusion method (Cappuccino et al, 1998; Bershe et al, 1991) against two Gram-positive bacteria, *Staphylococcus aureus* and *albus* and two Gram-negative bacteria,
Proteosus sp. and Pseudomonas aeruginosa. The culture medium used for the bacteria was Muller- Hinton agar medium at pH 7.2. The agar medium was poured into the plates to uniform depth of 5 mm and allowed to solidify. Then the microbial suspensions were streaked over the surface of the media using a sterile cotton swab to ensure the confluent growth of the organism. Aliquots of 10 µL of samples at 1:2 dilution in acetone were aseptically applied onto the surface of the agar plates at well spaced intervals. These plates were incubated at 37°C for 24 hours and the observed growth inhibition zones were measured. The control element used was gentamycin.

3.6 Antioxidant studies

3.6.1 Determination of DPPH free radical scavenging activity

Free radical scavenging activity was done for the compounds AF-5, AF-6 and acetone extract of Acalypha fruticosa. The scavenging activity was measured by the method of Lamaison (Lamaison et al., 1991) based on the reduction of methanolic solution of the coloured DPPH. Free radical scavenging ability of the test substances added to the methanolic solution of DPPH is inversely proportional to the difference in initial and final absorption of DPPH solution at 517 nm. The drug activity is expressed as 50% inhibitory concentration (IC50). The reaction mixture contained 1x10^4 mM methanolic solution of DPPH and various concentrations of the test substances. Percentage inhibition was determined by comparing the absorbance values of test and control tubes. IC50 values were obtained from the plot drawn: concentration (µg) vs. percentage inhibition.

\[
\% \text{ Inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100
\]

\[A = \text{absorbance}\]
3.6.2 Determination of the reducing power

The reducing power of the compounds AF-5 and AF-6 was determined according to the method of Oyaizu (Oyaizu, 1986) as described by Yen (Yen et al., 1993). The samples (200-1000 µg) in 1mL methanol were mixed with phosphate buffer (5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5 mL, 1%) and the mixture was incubated at 37°C for 20 minutes. Five milliliters of trichloroacetic acid (10%) was added to the reaction mixture which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (5 mL) was mixed with distilled water (5 mL) and ferric chloride (1 mL, 1%) and the absorbance was measured at 700 nm. Increased absorbance indicated reducing power.