METHODOLOGY

MATERIALS AND METHODS

I. Ethno botanical studies on Kuknas and Soligas

Field trips

Survey of ethnic knowledge has been undertaken on Kuknas living in Waghai forest during the years 2011 – 2013. Observations were made during three different field trips. 1st July – 15 th July 2011; 1st January – 15th January 2012, 27th February to 12th March 2013. There are nearly 8 villages covered namely Koilipada, Jhavda,Vanzat amba, Chichipada, Dungarda, Waghai, Borigowda and Takiamba of Dangs district of Gujarat.

In order to document the utilisation of indigenous medicinal plants by Soliga tribes, extensive surveys were carried out in Talamalai, which is situated in Sathyamangalam taluk of Erode district, Tamil Nadu, during different field trips from the year 2011 – 2013. Observations were made during three different periods of 1st April – 15th April 2011, 1st August – 10 th August, 2012 and 15 th February – 28th February 2013. During these field trips, interviews were carried out in 7 villages. The Soliga tribes inhabiting villages include Ittarai, Talamalai, Bejalatti, Balapadugai, Allapurathoddi, Kodampalli and Doddapuram.

Interview with informants

Information on the medicinal properties of plants used by the Kuknas and Soligas was collected randomly by interview methods using questionnaire (Appendix-I).The informations were gathered from 160 tribals of 70 families including the village headman, herbalists and the village people.Waghai forest area. In Talamalai forest area the informations were gathered from 130 tribals of 50 families from the village headman, herbalists and the village people of Waghai forest area.

The questionnaire used in this study allowed descriptive responses on the plant prescribed, such as part of the plant used, medicinal uses, mode of preparation (i.e.,

decoction, extract, gel, infusion, paste, juice, etc.), administration, form of usage either fresh or dried and mixtures of other plants used as ingredients and complications, if any. During the survey, two interview methods were followed. The first was the 'specimen display' method (Bhattarai *et al*., 2006), in which the Plant species were shown to the traditional healers and households in order to elicit any medicinal information. The same plant was shown to the different informants to confirm the accuracy of the results. Secondly, an interview with the concerned village tribals has made it possible to get both plant identification and detailed information.

Data analysis

The data includes list of the plants used as medicine by the Kuknas and Soligas. Other species are not considered in the present investigation. The results were given in tables.

Necessary instruments taken for field trips were

Camera, Laptop, Field note and Plant collecting wooden equipment.

Identification of plants

All the plants species recorded during this study were identified with the help of following floras.

1. Flora of the Presidency of Madras (Gamble and Fischer, 1957)
2. Flora of Tamil Nadu Carnatic (Matthew, 1983)
3. An Excursion flora of Central Tamil Nadu (Matthew, 1991).

Herbarium

All plant species collected were enrolled in a field note with field number, locality, habit and description of the plant species and vernacular name of the plant species were noted. Then they were poisoned with 0.1% $HgCl_2$ dissolved in absolute

alcohol and dried. The dried specimens were mounted with glue on a standard herbarium sheet following the method of Jain and Rao (1970). The details noted in the field note were transfered to the herbarium sheets and were submitted in the Department of Botany, PSGR Krishnammal College for Women, Coimbatore, Tamil Nadu.

Ethnopharmaco-Statistical study

Informant Consensus Factor (ICF)

Informant Consensus Factor (ICF) (Trotter and Logan, 1986; Heinrich *et al*., 1998) was used to find out whether there was correlation in the use of plants and the ailment categories. The informant consensus factor (ICF_j) for ailment category 'j' was calculated by using the following formula.

$$ICF_j = \frac{Nur_j - Nt_j}{Nur_j - 1}$$

Nur_j - is the number of use-reports in each ailment category j

Nt_j- is the total number of taxon used in each ailment category j by all informants.

In each case if a plant was mentioned by an informant as 'used' then it was considered as one 'use-report' and it was recorded in the table. If one informant used a plant to treat more than one ailment in the same category, we considered it as one use-report. Thus, a plant species could be listed in several ailment categories of indigenous uses but in terms of use-reports, each plant species was considered only once per informant in a single ailment category as mentioned by Treyvaud Amiguet *et al*., (2005). The ICF provides a range of 0–1, where high values (close to 1) are obtained when only one or a few plant species are reported to be used by a high proportion of informants to treat a particular ailment. High ICF thus means that there is a narrow well-defined group of species used to cure a particular ailment category and/or that information is exchanged between informants. On the other hand, low ICF values (close to zero) indicate that

informants disagree over which plant to use due to random choosing or lack of exchange of information about use among informants (Gazzaneo *et al*., 2005).

II. Phytochemical studies on the root of *Cissus quadrangularis*

Preliminary phytochemical screening of *Cissus quadrangularis*

Plant collection

The mature root of *Cissus quadrangularis* was freshly collected from wild conditions in different localities of the study area. The plant materials were brought to the laboratory using the sealed poly bags.

Preparation of extracts

The collected plant materials were chopped into pieces and were shade dried at room temperature for about 15 days and made into coarse powder by using Pestle and Mortar. The powders obtained were passed through sieve no: 20 and then used for successive crude extraction by different solvent such as methanol, ethanol, ethyl acetate, hexane, petroleum ether and chloroform using soxhlet extractor for 6 hours. (Kokate, 1994; Kokate *et al*., 1999).

Test for alkaloids

The following qualitative tests were done for confirmation of alkaloids and the results were recorded.

Dragendroff's test

2 ml of the extract is treated with 1 ml of Dragendroff's reagent (potassium iodide solution) and the formation of brownish orange precipitate confirms the presence of alkaloids

Wagner's test

To 2 ml of the extract, 1 ml of Wagner's test solution (Iodine) is added and formation of a reddish brown precipitate confirms the presence of alkaloids.

Mayer's test

To 2 ml of the extract, addition of 1 ml Mayer's test reagent (a mixture of mercuric chloride and potassium iodide) results in a yellowish white precipitate which indicates the presence of alkaloids.

Test for glycosides

Baljet test

To 2 ml of the extract, addition of 1 ml of sodium picrate reagent shows yellowish orange colour indicating the presence of glycosides.

Test for carbohydrates

About 2 ml of extract was dissolved in 10 ml of water. To this, 2 ml of 10% dilute HCl is added and heated for 5 minutes to hydrolyse the glycosides to break them into glycon and aglycon parts. Then this hydrolysed extract was taken for the carbohydrate analysis.

Fehling's test

About 2 ml of the hydrolysed extract boiled with 2 ml of Fehling's solution resulted in a brick red colour, which indicates the presence of carbohydrates.

Test for Saponins

Foam test

About 2ml of the extract is shaken with 5 ml of water in a test tube and a resistant froth formation confirms the presence of saponins.

Test for Steroids

Libermann Burchard test

A small quantity of the extract when treated with 0.2 ml of concentrated sulphuric acid, 4 ml of acetic anhydride and 2 ml of chloroform, the solution turns blue in colour and finally becomes green, which indicates the presence of steroids.

Test for Tannins

Ferric chloride test

To 1 ml of the extract, a few drops of 10% ferric chloride solution is added. A blue colour is observed showing the presence of tannins.

Test for Proteins

Biuret test

2 ml of extract is heated with 2 ml of Biuret reagent, results in the formation of violet colour, confirms the presence of proteins.

Test for flavonoids

5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4. A yellow colouration observed in each extract indicates the presence of flavonoids. The yellow colouration disappears on standing.

Test for triterpenoids

Salkowski test

5 ml of each extract was mixed in 2 ml of Chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of Triterpenoids.

Test for Phenols

Equal volumes of extracts and $FeCl_3$ were mixed. A deep bluish green solution indicates the presence of Phenol.

Test for Thiols (Ahluwalia, 2000)

Dissolve the plant sample (0.1g) in ammonium hydroxide and then add sodium nitroprusside solution (2-3 drops).A purple colouration indicates the presence of Thiol.

Gas Chromatography- Mass Spectrometry analysis (GC-MS)

Analysis of root of *C. quadrangularis* was performed using GC-MS (Thermo GC - Trace Ultra VER: 5.0, Thermo MS DSQ II) equipped with mass selective detector and DB-5 capillary column. Helium was used as carrier gas with a flow rate of 1.0 ml/min. A linear programmed oven temperature from 70^{o}C to 260^{o}C at a rate of 6^{o}C/min was used for root. Sample was injected automatically in the split mode at split ratio of 1:100. The compounds were identified by comparison of recorded mass spectra.

Estimation of phytochemicals in root of *Cissus quadrangularis*

Preparation of extracts

The root materials were successively extracted with petroleum ether (to remove lipids and pigments), chloroform, and methanol using Soxhlet apparatus for 24 hrs. Each time before extraction with the next solvent, the material was dried in hot air oven at 40ºC. The extracts were concentrated using a rotary vacuum-evaporator (Yamato BO 410, Japan) and the solvent traces were removed by lyophilisation (VirTis Benchtop 4K, U.S.A.). The freeze-dried extract thus obtained was mixed with the relevant solvent as 1 mg/ml ratio then subjected to various investigations.

Extract recovery percent

The extract recovery in different solvents was expressed as percent of the dry matter. The amount of crude extracts recovered after successive extractions were weighed and the percentage of yield was calculated using the following formula.

$$\text{Extract Recovery Percent} = \frac{\text{(Extract + container) (g) – Empty container (g)}}{\text{Amount of plant sample (g)}} \times 100$$

Estimation of Total Phenolics and Tannins

The total phenolic content of the extracts was determined according to the method described by Siddhuraju and Becker (2003). Known concentration of each extract was taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was observed at 725 nm using against the reagent blank. Gallic acid was used as a standard. The analysis was performed in triplicate and the results were expressed as the gallic acid equivalents (GAE). Using the same extract, the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP). 100 mg of PVPP was weighed in to a 10 × 12 mm test tube and to this 1.0 ml of distilled water and then 1.0 ml of tannin containing phenolic extract were added. The content was vortexed and incubated at 4ºC for 4 h. Then the sample was centrifuged (3000xg for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured as mentioned above and expressed as the content of non-tannin phenolics on a dry matter basis. From the above results, the tannin content of the sample was calculated as the difference between total phenolics and non-tannin phenolics.

The total phenolic content was expressed in term of mg/g as gallic acid equivalent (GAE) (standard curve equation: $y = 0.607x + 21.24$, $R^2 = 0.958$) and tannin in rutin equivalent (RE) (standard curve equation: $y = 0.093x + 9.125$, $R^2 = 0.996$).

Quantification of Total Flavonoids

The flavonoid content of the sample extracts was determined by the use of a slightly modified colorimetric method described previously (Zhishen *et al.,* 1999). A 0.5

ml extract was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$was added and allowed to stand for 6 min, then 2 ml of 4% NaOH solution was added to the mixture. Immediately distilled water was added to bring the final volume to 5 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus prepared water blank. All the values were expressed as milligram of rutin equivalents (RE) per gram of extract. The total flavonoid content was also expressed in term of mg/g as gallic acid equivalent (GAE) (standard curve equation: $y = 0.353x + 15.19$, $R^2 = 0.745$).

III. Biological activity of the root of *Cissus quadrangularis*

Antioxidant activity of the root of *Cissus quadrangularis*

Antiradical activity of root of *Cissus quadrangularis* using DPPH method (Blois, 1958)

The antioxidant activity of the root extract of *C. quadrangularis* was determined in terms of hydrogen donating or radical scavenging ability using the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, according to the method of Blois (1958). Sample extract at various concentrations was taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27ºC. The absorbance of the sample was measured at 517 nm using UV-Visible spectrophotometer (SHIMADZU, Japan). Radical scavenging activity of the samples was expressed as IC_{50}.Concentration of the sample necessary to decrease initial concentration of DPPH by 50% (IC_{50}) under the experimental condition was determined. Therefore, lower value of IC_{50} indicates a higher antioxidant activity. BHT (Butylated Hydroxytoluene) and rutin were used as standard antioxidants in DPPH assay.

Antioxidant activity by ABTS assay (Re *et al*., 1999)

The total antioxidant activity of the root extract of *C. quadrangularis* was measured also by ABTS radical cation decolorization assay according to the method of Re *et al*. (1999). ABTS was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12-16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89, v/v) and equilibrated at 30º C to give an absorbance of 0.700 ± 0.02 at 734 nm using UV-Visible spectrophotometer. The stock solution of the sample extracts were diluted such that after introduction of 10 µl aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 ml of diluted ABTS solution to 10 µl of sample or Trolox standards (final concentration 0-15 µM) in ethanol, absorbance was measured at 30ºC exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated of the blank absorbance at 734 nm and then was plotted as a function of Trolox concentration. The unit of Trolox equivalent antioxidant capacity (TEAC) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µM/g sample extract on dry matter.

Metal chelation (MC) assay (Dinis *et al*., 1994).

The chelation of ferrous ions by the sample extracts was estimated according to the method of Dinis *et al*., (1994). 100 µl of sample extract was incubated with 20 µM Fe^{2+} (ammonium ferrous sulfate) in 5% ammonium acetate (pH 6.9). The reaction was initiated by the addition of 100 µM ferrozine and after an incubation period for 10 min, the absorbance was read at 562 nm using UV-Visible spectrophotometer. The chelating activity of the samples was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract.

Antidiabetic activity of root of *Cissus quadrangularis*

α-glucosidase activity (Miller, 1959)

The enzyme α-glucosidase inhibitory activity was determined by premixing α-glucosidase (0.07 units) with 100 – 500 µg/ml of extract. Then 3mM of p-nitrophenyl glucopyranoside was added as a substrate. This reaction mixture was incubated at 37˚C for 30 min and the reaction was terminated by addition of 2 ml of sodium carbonate. The α- glucosidase activity was determined by measuring the p-nitrophenyl release from p-nitrophenyl glucopyranoside at 400 nm using UV-Visible spectrophotometer. The percentage of α-glucosidase inhibitory activity is calculated by the following formula.

$$\text{Inhibition (\%)} = (\text{Control OD} - \text{Sample OD} / \text{Control OD}) \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of α-glucosidase activity under assay condition.

Anti-inflammatory property of root of *Cissus quadrangularis*

Inhibition of protein denaturation (Williams 2008)

The reaction mixture (0.5 ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.05 ml of the sample extract (100 – 500 µg/ml) was prepared. The pH of this mixture was adjusted to 6.3 using a small amount of 1N HCl. The samples were incubated at 37° C for 20 min and then heated at 57° C for 3 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660nm using UV-Visible spectrophotometer. For control tests 0.05 ml of distilled water was used instead of extracts while product control tests lacked bovine serum albumin. Aspirin was used as standard drug in different concentration. The percentage inhibition of protein denaturation was calculated as follows.

$$\text{Inhibition Percentage} = 100 - \left\{ \frac{\text{OD of test} - \text{OD of product control}}{\text{OD of Control}} \right\} \text{x } 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of protein denaturation under assay condition.

Proteinase inhibitory activity (Oyedepo *et al.*,1995)

The reaction mixture (2.0 ml) contained 0.06 mg of trypsin, 1.0 ml of 25mM of Tris – Hcl buffer (pH – 7.4) and 1.0 ml of aqueous solution of the sample extract (100 – 500 µg/ml) were prepared. The mixtures were incubated at 37° C for 5 min and then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 min. 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged. Absorbance of the supernatant was read at 280 nm against Tris – Hcl buffer as blank using UV-Visible spectrophotometer. Aspirin was used as standard drug in different concentration. The percentage of inhibition was calculated as follows.

$$\text{Inhibition Percentage} = 100 - \left\{ \frac{\text{OD of test} - \text{OD of product control}}{\text{OD of Control}} \right\} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of protein denaturation under assay condition.

Antimicrobial activity of the root of *Cissus quadrangularis*

Media used

Nutrient agar (NA) and Potato Dextrose Agar (PDA) mediums were used for testing the antibacterial and antifungal activity respectively and their composition were given below.

Antibacterial activity

The following strains of bacteria were used to estimate the antibacterial activity of root extracts of *C. quadrangularis*. The strains of bacteria were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, (IMTECH) Chandigarh.

1. *Klebsiella pneumonia* MTCC432

2. *Escherichia coli* MTCC739

Composition of Nutrient Agar (NA) medium (g / litre)

Peptone	: 5.0
Beef extract	: 3.0
Yeast extract	: 3.0
Sodium chloride	: 5.0
Agar	: 3.0
Distilled water	: 1000 ml
pH	: 5.0

Preparation of inoculum

The test organisms were subcultured by streaking them on Nutrient Agar medium, followed by incubation for 24 hrs at 37°C. Several colonies of each bacterial species were transferred to sterile nutrient broth. The suspensions were mixed for 15 sec and incubated for 24 hrs at 37° C on an orbital incubator shaker. Working concentration of the microbial suspension was prepared in 3 ml of sterile saline to turbidity equivalent to 0.5 McFarland scale (i.e., adjusting the optical density to 0.1 at 600 nm), yielding a cell density of 1-2 × 105 CFU/ml.

Nutrient Agar (NA) plates were seeded with 8 hr broth culture of different bacteria. In each of these plates, wells were cut out using sterile cork borer. Using sterilized dropping pipettes, different concentrations (500, 1000, 1500 and 2000 µg/well) of plant extract was carefully added into the wells and allowed to diffuse at room temperature for 2 hrs. The plates were then incubated at 37°C for 18–24 h. Gentamicin (10µg) was used as positive controls and DMSO as negative control. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

Gentamicin (µg) was used as standard control for the comparison of antibacterial activity exhibit by the plant sample.

Antifungal activity

The following strains of fungi were used to estimate the antifungal activity of the root extracts of *C. quadrangularis*. The fungal strains were procured from the Microbial Type Culture Collection (MTCC, IMTECH), Institute of Microbial Technology, Chandigarh.

1. *Candida albicans* MTCC227

2. *Malassezia furfur* MTCC1374

Composition of Potato Dextrose Agar (PDA) medium (g / litre)

Infusion from potatoes	: 200g
Dextrose	: 20gm
Agar	: 15g
Distilled water	: 1 litre
pH	: 5.6 ± 0.02

Preparation of inoculum

The fungal pathogens were cultured in Potato Dextrose Agar for 72 hrs at 27° C and the spores/cells were harvested in sterile saline using a sterile squirrel brush. Working concentration of spore suspension was prepared with sterile saline to turbidity equivalent to 0.5 McFarland scale (i.e., adjusting the optical density to 0.1 at 530 nm), yielding a cell density of 1-5 × 106 CFU/ml.

Potato Dextrose Agar plates were seeded with 100 µl of spore/cell suspension of different test organisms. In each of these plates, wells were cut out using sterile cork borer. Using sterilized dropping pipettes, different concentrations (500, 1000, 1500 and 2000 µg/well) of plant extract was carefully added into the wells and allowed to diffuse at room temperature for 2 h. The plates were then incubated at 27° C for 48 hrs. Ketoconazole (10µg)

was used as control. The antifungal activity was evaluated by measuring the diameter of inhibition zone.

Antihaemolytic activity (Naim *et al*., 1976)

Antihemolytic activity of the sample extract was assessed as described by Naim *et al*. (1976). The experimental 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 Ethical Committee (1454/po/c/11/CPCSEA). The blood samples were collected from 3 male wistar rats (180 – 220 gm) under mild anesthesia (intraperitoneal administration of ketamine (60 mg/kg) and zylazine (5mg/kg)) via cardiac puncture method into EDTA coated tubes. The erythrocytes were separated by centrifugation and washed with 0.2M phosphate buffer (pH 7.4). The erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. The sample extracts in saline buffer at various concentrations (100 – 500µg) was added to 2 ml of the erythrocyte suspension and the volume was made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in reaction mixture was adjusted to bring about 90% haemolysis of blood cells after 120 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of haemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation. The same procedure was followed by using Quercetin as standard instead of plant sample. Percentage of antihemolytic activity of the sample was calculated as follows.

Antihaemolytic activity (%) = (Control OD – Sample OD / Control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.