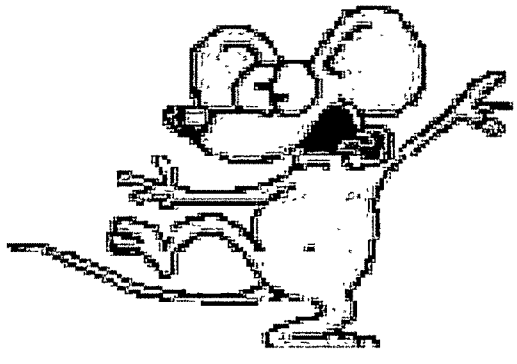

EXPERIMENTAL

The present section deals with the detailed description of methods employed for carrying out different studies, categorized into following headings.

2.1 PHARMACOGNOSTIC STUDIES

2.1.1 Collection and identification of plant material

Flower-heads of ***Sphaeranthus indicus*** Linn. and roots of ***Cissampelos pareira*** Linn. were collected from outfield of Baroda city and rhizomes of ***Curculigo orchioides*** Gaertn. were collected from local market. All the selected plant materials were identified in Botany department of the M.S.University.

2.1.2 Macroscopical examination

Macroscopical examinations of the selected parts of the plants were carried out using the reported methods in standard texts (Wallis, 1985).

2.1.3 Microscopical examinations

A. Microscopy of whole drug:

For microscopical examination of whole parts of selected drugs, transverse sections were taken. The sections were treated with various reagents before examining for the various components

1. *Lignified elements:* For staining the lignin several drops of phloroglucinol and a drop of concentrated hydrochloric acid were added to the section on a slide and drained off and the section was mounted in glycerin-water mixture. Lignified elements were colored pink.
2. *Starch:* Starch was detected in drugs by treating the section of the drug or powder with iodine solution. Starch grains stain blue.

B. Microscopy of powdered crude material:

For examining the characters of powder, little amount of powder in 5% potassium hydroxide solution was taken in test tube and warmed for

a short period. Presence of different elements was examined by treating the powder with different reagents as explained in the above section.

2.1.4 Preparation of powdered material

The selected parts of the plants were properly cleaned and dried first in open and then artificially in an oven at 60°C for approximately 4 hours. The dried plant materials were then subjected to size reduction to coarse powder and this powder was then used in further investigations.

2.1.5 Proximate analysis

Proximate analysis of crude drugs was carried out using reported methods (Indian Pharmacopoeia, 1996). Following determinations were made-

1. Loss on drying
2. Total ash
3. Acid insoluble ash
4. Water soluble ash
5. Alcohol soluble extractives
6. Water soluble extractives

2.2 PHYTOCHEMICAL STUDIES:

2.2.1 Preliminary phytoprofiles:

The powder of the air dried drug, weighing about 50 gm was taken and extracted in soxhlet apparatus with the solvents of increasing polarities as follows:

a) Petroleum ether (60-80)
b) Benzene
c) Chloroform
d) Ethyl acetate / acetone
e) Methanol
f) Water

All the extracts were concentrated in vacuum and the extracts were preserved. Consistency, color, appearance of the extracts and

percentage yield were noted. Each time before extracting with the next solvent, the material was dried in hot air oven below 50°C.

2.2.2 Qualitative chemical tests:

The extracts obtained in the successive extraction process were then subjected to various qualitative chemical tests to determine the presence of various phytoconstituents by using reported methods (Kokate, 1991; Evans, 1996).

2.2.3 TLC profile of the extracts obtained by successive solvent extraction:

All the extracts of selected plants were subjected to TLC studies using various solvent systems to determine presence of various phytoconstituents. The R_f values of observed compounds were noted for all the extracts.

2.2.4 Preparation of selective extracts and /or fractions:

2.2.4.1 *Sphaeranthus indicus:*

2.2.4.1.1 *Preparation of methanol extract and its fractions*

Air dried flowerheads were first subjected to methanol extract using soxhlet extractor for 48 hours. Methanol extract so obtained was then fractionated into different polarity solvents viz., petroleum ether (60-80), benzene, chloroform and methanol, by macerating with these solvents for few hours. Fractions so obtained were concentrated under reduced pressure and vacuum, weighed and then kept in vacuum desiccator.

2.2.4.1.2 *Petroleum ether extract*

Air dried flowerheads were subjected to extraction with petroleum ether (60-80) using soxhlet extractor for 48 hours. Extract was filtered and concentrated under reduced pressure and vacuum, weighed and kept in vacuum desiccator.

2.2.4.2 *Cissampelos pareira:*

2.2.4.2.1 *Preparation of methanol extract*

Air dried roots were subjected to extraction with methanol using soxhlet extraction for 48 hours. Extract was filtered and concentrated under reduced pressure and vacuum kept in vacuum desiccator.

2.2.4.2.2 *Preparation of alkaloidal fraction*

Air dried roots were subjected to extraction with methanol by maceration for 48 hours. This methanol extract was utilized for preparation of alkaloidal fraction. Methanol extract was acidified with 150 ml of 5% hydrochloric acid and left overnight at room temperature. The insoluble non-alkaloid material was removed by filtration. The filtrate was subjected to ethyl ether extractions to eliminate the rest of the non-alkaloid substances. The residues obtained after evaporation of ether extracts were added to non-alkaloid fraction. The acidic solution thus purified was then alkalized with 25% ammonium hydroxide and extracted with chloroform (6x 200 ml). The evaporation of the combined chloroform extracts gave alkaloidal fraction.

2.2.4.3 *Curculigo orchioides*

2.2.4.3.1 *Preparation of methanol extract and its fractions*

Air dried rhizomes were subjected to extraction with methanol using soxhlet extraction for 48 hours. Extract was filtered and concentrated under reduced pressure and vacuum kept in vacuum desiccator. Methanol extract so obtained was fractionated by macerating into different solvents like hexane, chloroform, ethyl acetate and methanol.

Above selective extracts were utilized for biological screening and evaluation of *in vitro* antioxidant activity. Active extracts were then prepared on large scale and utilized for isolation of phytoconstituents.

2.3. BIOLOGICAL SCREENING

Animals

Swiss albino mice of either sex, weighing 20-25 g, household in standard conditions of temperature, humidity and light were used. They were fed with standard rodent diet and water ad libitum. Albino rats of either sex, weighing 150-180 g, for preparation of liver homogenate were used.

Preparation of Test samples

Weighed quantities of test extracts were suspended in 1% sodium carboxy methylcellulose to prepare suitable dosage form. The control animals were given an equivalent volume of sodium carboxy methylcellulose vehicle.

Carbon ink suspension:

Pelican AG, Germany, ink was diluted eight times with saline and used for carbon clearance test in a dose of 10µl/g body weight of mice.

Antigen (Sheep red blood cells -SRBCs)

Sheep red blood cells (SRBCs) were washed with three times in normal saline and adjusted to a concentration 20% for immunization and 1% for challenge as Antigen.

Preparation of standard immunosuppressant

Cyclophosphamide was used as a standard drug to induced immunosuppression and myelosuppression in mice. It was dissolved in distilled water and administered at dose 30 mg/kg and 50 mg/kg for inducing myelosuppression and immunosuppression respectively.

2.3.1 Immunomodulatory activity in normal animals

Selected extracts and /or their fractions as mentioned below were subjected to screening immunomodulatory activity utilizing different models in mice.

Sphaeranthus indicus

1. Methanol extract
2. Petroleum ether fraction
3. Benzene fraction
4. Chloroform fraction
5. Residual methanol fraction
6. Water extract
7. n-Butanol fraction of the aqueous extract
8. Residual fraction of aqueous extract
9. Petroleum ether extract

Above mentioned extracts and /or fractions were subjected for immunomodulatory activity at two dose levels viz., 100 mg/kg and 200 mg/kg, to identify the bioactive extract and /or fraction. Only petroleum ether extract subjected to screening at five dose levels (50, 100, 200, 300 and 400 mg/kg).

Cissampelos pareira

1. Methanol extract
2. Alkaloidal fraction

Methanol extract subjected for immunomodulatory activity at five dose levels viz., 50, 100, 200, 400 and 800 mg/kg, whereas alkaloidal fraction screened at four dose levels 25, 50, 75 and 100 mg/kg.

Curculigo orchioides

Methanol extract of *Curculigo orchioides* subjected for immunomodulatory activity at five dose levels 50, 100, 200, 400 and 800 mg/kg.

All the extracts of selected plants mentioned above were screened for immunomodulatory activity in normal animals using following methods.

2.3.1.1 Humoral antibody (HA) titre

The method described by Puri et al., (1994) was adopted. Animals were divided into groups of six animals each. The control group received

1.0 % sodium carboxy methylcellulose solution only as vehicle; while animals in the treatment groups were given the test extracts orally in 1.0 % sodium carboxy methyl cellulose daily for 7 days. The animals were immunized by injecting 0.1 ml of 20% of fresh sheep red blood cells suspension intraperitonially on 0 day. Blood samples were collected in micro centrifuge tubes from individual animal by retro-orbital plexus on 7th day to obtain serum. Antibody levels were determined by haemagglutination technique. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 µl volumes of normal saline in microtitration plate and to it added 25 µl of 1% suspension of sheep red blood cells in saline. After mixing, the plates were incubated at room temperature for 1 hr. and examined for haemagglutination under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

2.3.1.2 Carbon clearance test

Phagocytic index was determined as per the method reported by Gonda et al.., (1990). Mice were divided into groups of six animals each. The control group received 1.0 % sodium carboxy methylcellulose solution only as vehicle; while animals of the treatment groups were given test extracts orally in 1.0 % sodium carboxy methyl cellulose daily for 5 days. Carbon ink suspension was injected via tail vein to each mouse after 48 hours of 5 days treatment. Blood samples were drawn from orbital vein at 0 and 15min. Blood (25-µl) was mixed with 0.1 % sodium carbonate (2 ml) and subjected for determination optical densities at 660 nm.

The phagocytic index K was calculated by using following equation:

$$K = (\ln OD_1 - \ln OD_2) / (t_2 - t_1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively.

2.3.1.3 Delayed type hypersensitivity (DTH) response

The method described by Puri et al., (1994) was adopted. Animals were divided into groups of six animals each. The control group received 1.0 % sodium carboxy methylcellulose solution only as vehicle; while animals in the treatment groups were given the test extracts orally in 1.0 % sodium carboxy methyl cellulose daily for 7 days. The animals were immunized by injecting 0.1 ml of 20% of fresh sheep red blood cells suspension intraperitonially on 0 day.

On 7th day, the thickness of the right hind footpad was measured using digital vernier caliper. The mice were then challenged by injection of 20µl of 1% SRBCs in right hind footpad. Foot thickness was again measured after 24 hrs of this challenge. The difference between the pre and post challenge foot thickness express in mm was taken as a measure of delayed type hypersensitivity (DTH).

2.3.1.4 Cyclophosphamide induced Myelosuppression

Cyclophosphamide induced myelosuppression was studied according to the method described by Manjarekar et al., (2001). Animals were divided into groups of six animals each. The control group and cyclophosphamide group received 1.0 % sodium carboxy methylcellulose solution only as vehicle daily for 13 days while animals in treatment groups were given the test extracts (100 and 200 mg/kg, p.o.) in 1.0 % sodium carboxy methyl cellulose daily for 13 days. On days 11, 12, 13 all the animals except in the control group were injected with cyclophosphamide (30mg/kg, i.p.) 1 hour after administration of the extracts. Blood samples were collected on day 14 and total white blood cell (WBC) count was determined.

2.3.2 Immunomodulatory activity in immunosuppressed animals

Active extracts from each plant were selected and screened for immunomodulatory activity in cyclophosphamide induced immunosuppression in mice.

Following extracts were selected-

1. **Residual methanol fraction of *S.indicus* (Bioactive fraction)**
2. **Methanol extract of *C.pareira***
3. **Methanol extract of *C.orchioides***

Two pretreatment schedules were selected for this study (Sharma, 1996).

2.3.2.1 Effect of test extracts and cyclophosphamide on HA titre and DTH response using SRBCs as an antigen in mice-7 days pretreatment.

Animals were divided into 7 groups of six animals each. Animals in treatment groups were given the test extracts (50-800 mg/kg, p.o.) in 1.0 % sodium carboxy methyl cellulose daily for 7 days. Cyclophosphamide was administered on day 4 to 6 (50 mg/kg).

Animals in control group received equal amount of vehicle only. The animals were immunized by injecting 0.1 ml of 20% of fresh sheep red blood cells suspension intraperitoneally on 0 day. Blood samples, collected in micro centrifuge tubes from individual animal by retro-orbital plexus on 7th day were centrifuged to obtain serum. Antibody levels were determined by haemagglutination technique described by Puri et al. (1994). The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

On day 7, the thickness of the right hind foot pad was measured using digital vernier calipers and mice were then challenged by injection of 20µl of 1% SRBCs in right hind foot pad. 24 hrs after this challenge foot thickness was again measured. The difference between the pre and post challenge foot thickness expressed in mm was taken as a measure of delayed type hypersensitivity (DTH).

2.3.2.2 Effect of test extracts on HA titre and DTH response using SRBCs as an antigen in mice- 15 days pretreatment.

Mice were divided into 6 groups, each group containing 6 mice. Animals in treatment groups were given the bioactive fraction (50-800

mg/kg, p.o.), methanol extract of *C. pareira* (50-800 mg/kg, p.o.) and methanol extract of *C. orchioides* (50-800 mg/kg, p.o.) in 1.0 % sodium carboxy methyl cellulose. The pretreatment time was 15 days based on the method described by Sharma et al., (1996). Schedule for drug administration was 7 days prior to immunization (days -6,-5,-4,-3,-2,-1, 0) and 7 days after immunization (+1, +2, +3, +4, +5, +6,+7). The procedure as described in earlier method was followed for remaining determinations.

2.4 *IN VITRO* ANTIOXIDANT STUDIES

The free radical scavenging properties of selective extracts and /or fractions of drugs under investigations were examined *in vitro* in five systems, namely radical scavenging activity by DPPH reduction (DPPH assay), superoxide radical scavenging activity in riboflavin/light/NBT system, nitric oxide scavenging activity, reducing power and inhibition of lipid peroxidation induced by Iron/ADP/Ascorbate system in rat liver homogenate.

2.4.1 DPPH assay

Principle

DPPH assay was used as a rapid method to provide an evaluation of antioxidant activity due to free radical scavenging. Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of colored DPPH brought about by the samples. 1, 1-diphenyl-1-picrylhydrazyl radical (DPPH) a purple colored stable free radical is reduced into the yellow colored diphenylpicryl hydrazine (Vani et al., 1997; Blois, 1958).

Reagents

1. *DPPH stock solution*

   1.3 mg of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was dissolved in 1ml of methanol.

Procedure

100µl of suitably diluted stock solution of methanolic extracts of the drugs were mixed with 3ml of methanol. 75µl of DPPH solution was added and decrease in the absorbance was noted after 15 minutes at 516nm against methanol as blank. Curcumin was used as positive control. The percent inhibition by sample exposure was determined by comparison with a methanol-treated control group.

The obtained data was used to determine the concentration of the sample required to scavenge 50% of the DPPH free radicals (IC_{50}). The percent inhibition was plotted against the concentration and the IC_{50} was obtained. A lower IC_{50} denotes a more potent antioxidant. The results were expressed as the mean ± SEM of three replicates. IC_{50} was calculated from % inhibition.

2.4.2 Assay for superoxide radical scavenging activity

Principle

The assay was based on the capacity of the drug to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971).

Reagents

1. *Phosphate Buffer (50mM, pH 7.6)*

(a) 2.72gms of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 60 ml of distilled water. The volume was then made upto 100ml with distilled water.

(b) 800mg of sodium hydroxide (NaOH) was dissolved in 60 ml of distilled water. The volume was then made upto 100ml with distilled water.

50ml of (a) was mixed with 42.4ml of (b). The pH of the mixture was adjusted to 7.6 with 1N hydrochloric acid (HCl). The volume was then made upto 200ml with distilled water.

2. *Riboflavin*

5mg of riboflavin was dissolved in 25ml of phosphate buffer (50mM, pH 7.6).

3. *EDTA (12mM)*

402mg of EDTA was dissolved in 5ml of buffer. The volume was then made upto 10ml with phosphate buffer.

4. *Nitro blue tetrazolium (NBT) (0.1%)*

5mg of NBT was dissolved in 2ml of buffer. The volume was then made upto 5ml with phosphate buffer.

Procedure

The reaction mixture contained 2.5ml buffer, 100µl riboflavin solution, 200µl of EDTA solution, 100µl of diluted methanolic extract of drug and 100µl of NBT solution, added in the mentioned sequence. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract in UV light for 5 minutes. Immediately after illumination the absorbance was measured at 290nm. Ascorbic acid was used as positive control. The percent inhibition by sample exposure was determined by comparison with a methanol-treated control group.

The obtained data was used to determine the concentration of the sample required to scavenge 50% of the superoxide radicals (IC_{50}) as mentioned above.

2.4.3 Assay for nitric oxide radical scavenging activity

Principle

Assay was based on the inhibition of nitric oxide which was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions which can be estimated by use of Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Sreejayan and Rao, 1997).

Reagents

1. Phosphate buffer saline (pH-7.4) –

Dissolve 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 ml. Adjust the pH if necessary.

2. Sodium nitroprusside (5 mM) –

Dissolve 149 mg of sodium nitroprusside in 10 ml of phosphate buffered saline and make up the volume to produce 100 ml.

3. Griess reagent-

It is comprised of 1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylehylenediamine dihydrochloride

Procedure

Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with different concentrations of test extracts dissolved in methanol and incubated at room temperature for 150 minutes. Blank without test extract but equivalent amount of methanol was conducted in an identical manner. After incubation solutions were removed and equal amount of Griess reagent. The absorbance of the chromophore formed was read at 546nm. EC_{50} was calculated as 50% reduction in absorbance brought about by sample compared with blank. Standard used was Curcumin.

2.4.4 Reducing power determination

Principle

The measurement of reductive ability was based on the Fe3+-Fe2+ transformation in the presence of sample.

Reagents

1. *Phosphate buffer (2M, pH 6.6)*
2. *Potassium ferricyanide (1%)*

   Accurately weighed 1 g of potassium ferricyanide was dissolved in distilled water and volume was made up to 100 ml.
3. *Trichloroacetic acid (10%)*

   Accurately weighed 10 g of trichloroacetic acid was dissolved in distilled water and volume was made up to 100 ml.

4. *Ferric chloride (0.1%)*

   Accurately weighed 100 mg of ferric chloride was dissolved in distilled water and volume was made up to 100 ml.

Procedure

The reducing power was determined according to the method of Oyaizu (1986). Samples were mixed with 5 ml phosphate buffer and 5 ml potassium ferricyanide, the mixture was then incubated at 50^0 C for 20 minutes, 5 ml trichloroacetic acid was added an the mixture was centrifuged at 4000 rev./ min. The upper 5 ml solution was then mixed with 5 ml distilled water and 1 ml ferric chloride. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid (0.3 mg) was used as standard.

2.4.5 Measurement of effect drugs on lipid peroxidation on rat liver homogenate

Principle- Assay was based on the inhibition of extent of lipid peroxidation into the rat liver homogenate induced by the complex, iron-ADP-Ascorbate. In presence of iron and ADP, ascorbic acid was acts as a prooxidant and induces lipid peroxidation in rat liver homogenate.

Reagents

1. *Tris hydrochloride buffer (10mM, pH 7.4)*

   1.21gm tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. *Ferric chloride (100 µM)*

   1.62 mg ferric chloride was dissolved in distilled water and volume made up to 100 ml.

3. *Adenine-di-Phosphate (1.7 µM)*

   10.63 mg dissolved in 10 ml of distilled water.

4. *Ascorbic acid (500 μM)*

8.81 mg of ascorbic acid was dissolved in distilled water and volume was made up to 100 ml.

5.*Thiobarbituric acid (0.67% w/v)*

0.67gm of thiobarbituric acid was dissolved in 50ml of hot distilled water and the final volume was made upto 100ml with hot distilled water.

6. *Trichloroacetic acid (10% w/v)*

10gms of trichloroacetic acid was dissolved in 60ml of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

Rat liver homogenate was prepared by homogenizing the tissue in chilled Tris buffer at a concentration of 10% w/v; peroxidation was induced in liver tissue by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted 0.5 ml of the liver homogenate (10% w/v), 100 μM $FeCl_3$, 1.7 μM ADP, 500 μM of ascorbate and different concentrations of samples in 2 ml of total incubation medium. The medium was incubated for 20 min. at 37°C. Extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) content. Briefly 2.0ml of the incubation medium was added to 2.0ml of freshly prepared trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0ml of clear supernatant solution was mixed with 2.0ml of freshly prepared thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The color developed was measured at 532nm against reagent blank (Slater and Sawyer, 1971).

Results were expressed in terms of decrease in MDA formation by the sample extract. Ascorbic acid was used as positive control.

2.4 Preparation of HPTLC fingerprint profile of active extracts and /or fractions:

Different active extracts from selected plants were subjected to preparation of HPTLC fingerprint profiles. HPTLC fingerprint profiles were established for following extracts and /or fractions.

S. indicus

- Methanol extract
- Petroleum ether fraction
- Benzene fraction
- Chloroform fraction
- Residual methanol fraction
- Petroleum ether extract

C. pareira

- Methanol extract
- Alkaloidal fraction

C. orchioides

- Methanol extract

Procedure:

HPTLC finger print profiles were established for selective extracts. A stock solution (1mg/ml) was prepared in respective solvents. Suitably diluted stock solution was spotted on pre-coated Silica gel G60 F254 TLC plates using CAMAG Linomat V Automatic Sample Spotter and the plates were developed in solvent systems of different polarities to resolve polar and non-polar components of the bioactive fraction. The plates were scanned using TLC Scanner 3 (CAMAG) at 254 nm (absorbance/reflectance mode) and 366 nm (fluorescence/reflectance mode) and Rf values, spectra, λ_{max} and peak areas of resolved bands were recorded. Relative percentage area of each of bands was calculated from peak areas. Table 2.1, shows different solvent systems utilized for the resolution of different compounds from extracts and /or fractions.

Table 2.1: Solvent systems used for preparation of HPTLC profiles of different extracts.

Extract/ Fraction	Solvent system 1	Solvent system 2	Solvent system 3
S. indicus			
Methanol extract	A	B	C
Petroleum ether fraction	D	A	-
Benzene fraction	D	A	-
Chloroform fraction	D	A	-
Residual methanol fraction (Bioactive fraction)	A	B	C
Petroleum ether extract	D	A	-
C. pareira			
Methanol extract	E	C	-
Alkaloidal fraction	E	C	-
C. orchioides			
Methanol extract	A	B	C

A Toluene: ethyl acetate (7:3)

B Ethyl acetate: methanol: water (100:13.5:10)

C n-butanol: glacial acetic acid (6:2:2)

D Hexane: ethyl acetate (9:1)

E Hexane: Chloroform: Methanol (4:5:1)

2.5 Isolation of compounds from active extracts and /or fractions:

Extracts and/or fractions possessing immunomodulatory activity and antioxidant activity were identified at initial stages from the selected plants. These extracts and/ or fractions were screened for presence of different phytoconstituents and then utilized for isolation of the compounds.

2.5.1 Isolation of compounds (S-1, S-2) from petroleum ether extract of *S.indicus*.

Petroleum ether extract screened at five different dose levels exhibited noted immunomodulatory activity, hence it was thought worthwhile to undertake some isolation from this extract. It has mainly revealed presence of terpenoids, sesquiterpenoids, steroids and some phenolics. Isolation from this extract was carried out through unsaponifiable matter and subsequent column chromatography or fractionation of the extracts.

Isolation by separating Unsaponifiable matter:

Procedure: Petroleum ether extract (60 g) was subjected to separation of unsaponifiable matter as per the procedure given in Indian Pharmacopoeia (1985). Briefly, petroleum ether extract was refluxed with 5% alcoholic KOH for 6 hrs, then it was mixed with hot distilled water and extracted with diethyl ether continuously to extract unsaponifiable matter.

All diethyl ether portions were mixed and reduced the volume. This diethyl ether extract was shaken with distilled water and washings checked with phenolphthalein for the presence of alkali. Diethyl ether extract was washed until the washings gave negative reaction to phenolphthalein. Diethyl ether extract then evaporated to dryness at room temperature and weighed. Remaining

Total unsaponifiable matter obtained was 18.00 g. This unsaponifiable matter was then subjected to fractionation into benzene, chloroform and ethyl acetate and methanol. Fractions obtained were as: Benzene- 13.00g; Chloroform- 1.2g; Ethyl acetate- 2.2g; Methanol- 1.6g.

Compounds from benzene fraction:

Acetone added in benzene fraction to give acetone soluble (10.0 g) and acetone insoluble (3.0 g) parts.

Acetone soluble portion of the benzene fraction upon standing overnight gave white sticky powder **S-1** (30 mg) separated. It was removed and continuously washed with acetone and weighed. Homogeneity of the compound was checked on TLC plate by applying different solvent systems. Different developing reagents (Iodine, UV-365, Anisaldehyde sulfuric acid reagent) used to check the presence of different compounds.

Acetone insoluble portion of benzene fraction was then loaded on column to separate compounds.

Column chromatography of acetone insoluble portion of benzene fraction:

Conditions of column chromatography:

Adsorbent: Silica (60-120 mesh)

Weight of sample: 3.0 g.

Column prepared in hexane.

Elution scheme-

Solvents	Ratio	Quantity	Fraction no.
Hexane	-	100 ml	1
Hexane: Ethyl acetate	98: 2	700 ml	2- 7
Hexane: Ethyl acetate	95: 5	400 ml	8- 11
Hexane: Ethyl acetate	90: 10	100 ml	12

Fractions collected in the quantity of 100 ml. Fraction 2, 3, and 4 gave similar pattern when observed on the TLC plates. These three fractions were then mixed together and concentrated. To the concentrated fractions acetone was added, which gave fluffy precipitates of white colored. Precipitates separated and dissolved in benzene and on evaporation of the solvent gave yellowish amorphous powder **S-2** (20 mg). Homogeneity of the compound was checked on TLC plate by applying different solvent systems. Different developing reagents (Iodine, UV-365, Anisaldehyde sulfuric acid reagent) used to check the presence of different compounds.

Isolation by fractionation of petroleum ether extract

Procedure: 15.0 g of petroleum ether extract was fractionated into hexane, benzene, chloroform and ethyl acetate. Hexane fraction (2.0g) was loaded on column of silica (60-120) and eluted with hexane, hexane: ethyl acetate (98:2) each 200 ml. Initial fractions upon concentration and addition of methanol gave fluffy precipitate that was filtered and dried, weighed 25 mg **(S-3)**

2.5.2 Isolation of compounds (S-4, S-5, S-6, S-7) from petroleum ether fraction of the methanol extract of *S. indicus*

Petroleum ether fraction of methanol extract was found to be one of the active fractions having immunomodulatory activity. The fraction was subjected to column chromatography for isolation of the compounds.

Conditions of column chromatography:

Adsorbent: Silica (60-120 mesh)

Weight of sample: 10.0 g.

Column prepared in hexane.

Elution scheme-

Solvents	Ratio	Quantity	Fraction no.
Hexane	-	200 ml	1
Hexane: Benzene	9: 1	100 ml	2
Hexane: Benzene	8: 2	100 ml	3
Hexane: Benzene	7: 3	200 ml	4- 5
Hexane: Benzene	6:4	500 ml	6- 10
Hexane: Benzene	5: 5	200 ml	11- 12
Hexane: Benzene	4: 6	200 ml	13- 14
Hexane: Benzene	3: 7	400 ml	15- 18
Benzene	-	500 ml	19- 23
Benzene: Chloroform	9: 1	300 ml	24- 26
Benzene: Chloroform	8: 2	200 ml	27- 28
Benzene: Chloroform	7: 3	200 ml	29- 30
Benzene: Chloroform	6: 4	200 ml	31- 32
Benzene: Chloroform	5: 5	200 ml	33- 34
Benzene: Chloroform	4: 6	100 ml	35
Benzene: Chloroform	3: 7	100 ml	36
Benzene: Chloroform	2: 8	100 ml	37
Benzene: Chloroform	1: 9	100 ml	38
Chloroform	-	100 ml	39

All fractions were checked for the presence of the compounds on TLC plates using appropriate solvent system.

Fractions 1 and 2 gave no evidence for any compound and hence discarded. Fractions 3 and 4 gave 150 mg of sticky mass, showing presence of one compound along with some minor impurities. This was loaded on a small column (1 cm diameter and 15 cm length) and eluted with hexane; initial fractions were collected and concentrated to offer a singular compound **S-4**, weighed **25** mg.

Fractions 5, 6 and 7 showed presence of three compounds. The weight of mixture was 65 mg.

Fractions 8, 9, 10, and 11 showed similar pattern with one major compound and minor impurities. These fractions were mixed together and concentrated. Addition of acetone gave white precipitate which on drying afforded **S-5, 30** mg of the compound. Homogeneity of the compound was checked on TLC plate by applying different solvent systems. Different developing reagents (Iodine, UV-365, Anisaldehyde sulfuric acid reagent) used to check the presence of different compounds.

Fractions 12, 13, 14, 15 and 16 were mixed together and concentrated to afford 250 mg of the mixture. This was dissolved in minimal solvent and a compound was isolated from it by performing preparative TLC using hexane: ethyl acetate as a solvent system on glass plates with thick coats of Silica gel G layers. Compound **(S-6)** was repeatedly recrystallized from ethyl acetate, weighed **19** mg.

Fractions 17 and 18, 19- 23, 24- 26, 27- 28, 29- 30 exhibited the similar pattern but upon concentration none of them afforded sufficient quantities of extract to process further.

Fractions 31 and 32 afforded singular compound **(S-7)** (25 mg) on addition of methanol in the form of fluffy precipitate.

Further none of the fraction afforded singular compounds and sufficient quantities of the mixtures to work out.

2.5.3 Isolation of compound (S-8) from residual methanol fraction (Bioactive fraction) of *S. indicus*.

Procedure: Bioactive methanol fraction (15 g) was fractionated into different mixtures of chloroform: methanol, viz., 4:1, 3:1, 2:1 and 1:1. The fraction obtained with 4:1 mixture (7.0 g) was further fractionated into ethyl acetate and ethyl acetate: methanol (9:1). The ethyl acetate fraction weighed 1.62 g was concentrated and on addition of methanol gave yellowish precipitate, which was separated by filtration and upon continuous wash with methanol provided yellowish- white powder (29 mg). The compound **(S-8)** was checked for the homogeneity into different solvent systems and confirmed single.

2.5.4 Isolation of compound C-1 from methanol extract of *Cissampelos pareira*.

Procedure: Methanol extract (15.0 g) of C. pareira was loaded on silica (60-120) and subjected to extraction with ethyl acetate, mixture of chloroform: methanol (4:1) and methanol.

Ethyl acetate fraction obtained (2.5 g) was further subjected to fractionation by macerating with hexane, chloroform and ethyl acetate. Hexane fraction was concentrated and on addition of methanol gave precipitate, which afforded whitish sticky compound (40 mg). It was reacted on TLC with Anisaldehyde Sulfuric acid reagent to give dark violet color.

2.5.5 Isolation of compound C-2 from alkaloidal fraction of *Cissampelos pareira*.

Procedure: Alkaloidal fraction obtained from methanol extract by previously described method was utilized for isolation of an alkaloid. Alkaloidal fraction (1.0 g) was fractionated into hexane, benzene and chloroform by macerating with these solvents. TLC pattern showed a prominent alkaloidal spot in benzene fraction. Therefore benzene (200 mg) fraction was loaded on column (diameter 1cm and length 15 cm) prepared from silica (60-120) in chloroform. Column was eluted with mixture of chloroform: methanol (1:1), and later on methanol only.

Fractions were collected in the volume of 10 ml. Fraction showing similar compound were mixed together to give a single compound (35 mg) which was reacted with Dragendorff's reagent. Compound was found to be single when tested for homogeneity using solvent system; n-butanol: glacial acetic acid: water (6:2:2).

2.5.6 Isolation of compound O-1 from methanol extract of *C. orchioides*

Procedure: Methanol extract of *C. orchioides* was fractionated into hexane, chloroform, ethyl acetate and methanol. Ethyl acetate fraction (2.5 g) was loaded on column (Diameter 1.5 cm and length 30 cm) prepared from silica (60-120) in chloroform. Column was eluted with chloroform, chloroform: methanol (9:1, 8:2, 7:3, and 6:4) 100 ml each. Fractions were collected in the volume of 25 ml. Fraction 10- 12 on concentration afforded whitish precipitate that weighed 10 mg. It was reacted on TLC with Anisaldehyde Sulfuric acid reagent to give violet color.

2.6 Identification and characterization of isolated compounds.

The compounds isolated from these extracts were then subjected to following studies:

2.6.1 Physico-chemical characterization

The isolated components were subjected to physical characterization by studying their state, melting point, solubility and ε_{max} etc. Chemical characterization was done by quantitative elemental analysis (CDRI, Lucknow).

2.6.2 Characterization by Spectral Analysis

The isolated compounds were subjected to spectral analysis such as Mass spectroscopy, IR spectroscopy, ^{1}H-NMR spectroscopy in order to characterize these compounds.