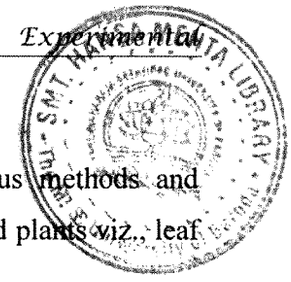
Chapter 2

Experimental



2. EXPERIMENTAL

The present section deals with the detailed description of various methods and techniques employed for carrying out different studies on the selected plants viz., leaf galls of *Pistacia integerrima* and rhizomes of *Hedychiuum spicatum*.

2.1 Pharmacognostic studies

Pharmacognostic evaluation is the initial step to confirm the identity and to assess the quality and purity of the crude drug. The selected plant drugs were subjected to pharmacognostic evaluation.

2.1.1 Collection and identification of plant material

Leaf galls of *Pistacia integerrima* were purchased from the local market and were authenticated from Regional Research Institute (Ayurveda), Pune. Authenticated rhizomes of *Hedychium spicatum* were obtained from Indian herbs Research & supply co. Ltd, Saharanpur as gift sample.

2.1.2 Macroscopic examination

The galls of *P. integerrima* and rhizomes of *H. spicatum* were subjected to macroscopic examinations using reported methods in standard text (Wallis, 1985) and the results were compared with the reported monographs (Kirtikar and Basu, 1983; Wealth of India, 1966; Ayurvdic Pharmacopoeia, 2001)

2.1.3 Microscopic examinations

A. Microscopy of whole drug

Microscopic evaluation of selected plant drugs was carried out by taking transverse sections. 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.

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.

2.1.4 Preparation of powdered material

The selected parts of the plant were properly cleaned and dried first in open and then in hot air oven at temperature not exceeding 50°C. The dried plant materials were then subjected to size reduction to coarse powder and this powder was then used for further investigations.

2.2 Proximate analysis.

Proximate analysis of crude drugs was carried out as per WHO guidelines. Following determinations were made-

1. Foreign organic matter
2. Ash value
3. Extractive value
4. Water content
5. Volatile oil content
6. Bitterness value
7. Haemolytic activity
8. Swelling index
9. Foaming index
10. Heavy metal content
11. Microbial content

Determination of foreign organic matter: 50g of sample of crude drugs was weighed and spread in thin layer. Foreign matter was sorted by visual inspection.

Determination of ash value: 2-4 g of drug was weighed and ignited by increasing the temperature gradually to 500-600°C until it is white, indicating absence of carbon.

Determination of acid insoluble ash: To the crucible containing total ash, 25 ml of hydrochloric acid was added and boiled gently for 5 minutes. The insoluble matter was collected on ash less filer paper. The filter paper containing insoluble residue was ignited to constant weight.

Determination of water soluble ash: To the crucible containing total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble residue was collected in a sintered glass crucible or on ash less filter paper and ignited for 15 min at temperature not exceeding 450°C.

Determination of water content: It is done by azeotropic method and loss on drying method.

Determination of volatile oil: Volatile oil was determined by hydro distillation method. The distillation is carried out in specially designed distillation assembly. Specific quantity of drug material is introduced in the flask and distillation was carried out. Volatile oil was collected in the graduating tube and measured.

Determination of bitterness value: The stock solution for individual plant material was prepared as specified and further dilutions were made. Solution of 0.100g of quinine hydrochloride was prepared in safe drinking water. After rinsing mouth with safe drinking water, 10 ml of test solution was swirled in mouth for about 30 seconds. If bitter sensation is no longer felt, the next higher dilution was tested after 10 minutes.

Determination of Haemolytic value: The extract of the plant material and the dilutions were prepared as per recommended in the procedures and added to the blood suspension (2%). Haemolytic activity is calculated by the formula1000x (a/b), where 1000 is defined haemolytic activity of saponins R(standard), 'a' is quantity of saponins R, that produces total haemolysis and 'b' is quantity of plant material that produces total haemolysis.

Determination of swelling index: Swelling index is the volume of water in ml taken up by the swelling of 1 g of plant material. The material is shaken repeatedly using a glass stopper measuring cylinder, for 1 h and then allowed to stand. The volume in ml was recorded.

Determination of foaming index: 1 g of powdered material was boiled with water, cooled and filtered. This decoction was poured into test tubes in successive proportions of 1 ml, 2 ml, 3 ml up to 10ml and volume was adjusted to 10ml. The tubes were shaken lengthwise and allowed to stand for 15 min. height of he foam was measured.

Determination of heavy metal content: Contamination of medicinal plant materials with arsenic and heavy metals can be attributed to many causes including pollution and traces of pesticides. Heavy metal content was determined by atomic absorbance spectroscopy.

Determination of microorganisms: The plant drugs were evaluated for microbial contamination by *E. coli* and *Salmonella* sp.

2.3 Phytochemical studies

2.3.1 Successive solvent extraction.

The presence of different chemical constituents in crude drugs can be detected by subjecting them to successive solvent extraction using solvents in the order of increasing polarity and subjecting to the extracts so obtained to qualitative tests for various chemical constituents. The selected drug samples in the present study were subjected to hydro distillation for removal of volatile oil. Then the crude drug was air dried and then dried in oven at temperature not exceeding 50°C. It was then subjected to successive extraction followed by qualitative chemical tests in order to know the phytoprofiles on preliminary basis.

Preliminary phytoprofiles

The powder of the air dried drug after removing volatile oil, weighing about 50gm was taken and extracted using soxhlet apparatus with the solvents of increasing polarity as follows:

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

Each time before extracting with the next solvent, the material was dried in hot air oven at temperature not exceeding 50°C. Finally the marc was macerated with chloroform water for 24 h to obtain aqueous extract. All the extracts were concentrated in vacuum and the extracts were preserved. Consistency, color, appearance of the extracts and percentage yield were noted.

2.3.2 Qualitative evaluation of successive extracts

The successive extracts so obtained were then subjected to various qualitative chemical tests to determine the presence of various phytoconstituents (Kokate, 1991; Evans, 1996). Hydro distillation procedure was used to detect presence of volatile oil.

2.3.3 TLC profile of extracts obtained by successive solvent extraction

All the successive extracts of selected crude drugs were subjected to thin layer chromatographic studies using Silica Gel 60F $_{254}$ pre coated plates (Wagner and Balt, 1996) to confirm presence of various constituents as described above. The R_f values of observed compounds were noted for all the successive extracts.

2.4 Preparation of extracts of selected crude drugs

The phytochemical profile of Methanol extract revealed the presence of flavonoid and related compounds, the extract was screened for its bio activity. The aqueous extract generally used in the formulations of traditional medicine has shown presence of phenolics compounds, therefore was subjected to screening of bioactivity. These were prepared in the following manner.

2.4.1 Preparation of extracts of leaf galls of *P. integerrima*

About 500 g, of coarse powder of leaf galls of *P. integerrima* were first subjected to hydro distillation and after assuring for absence of volatile oil; the marc was dried and then extracted using 2 l of methanol using soxhlet extractor. The aqueous extract was separately prepared extracting 250g of coarse powder using chloroform water. Both the methanol and aqueous extracts were then concentrated under vacuum and stored in desiccator for further studies.

2.4.2 Preparation of extracts of rhizomes of *H. spicatum*

About 500 g of coarse powder of rhizomes of *H. spicatum* was subjected in similar manner as mentioned in case of *P .integerrima* and dried marc devoid of volatile oil was extracted using 2 l of methanol in soxhlet extractor. The aqueous extract was prepared by taking 250g of powder using chloroform water. Both the methanol and aqueous extracts were then concentrated under vacuum and stored in desiccator for further studies.

2.5 Fractionation of selective extracts

The methanol and aqueous extracts of both the drugs were then subjected to fractionation using different solvents for screening and identification of the fraction containing active constituents.

Both Methanol and Aqueous extracts of leaf galls of *P. integerrima* and Methanol extract Rhizomes of *H. spicatum* were fractionated by loading these on silica column (60-120mesh). The Methanol extract was eluted in Chloroform, Acetone and Ethyl acetate while the Aqueous extract was eluted in n-Butanol, Ethyl acetate and Methanol. The fractions so obtained were concentrated under reduced pressure and vacuum, weighed and then stored in vacuum desiccator.

2.6 Estimation of phenolic and flavonoid content of different extracts of *P. integerrima*

The qualitative evaluation and TLC profile of the extracts and fractions of *P. integerrima* revealed presence of phenolic and flavonoid compounds. Therefore,

different extracts as well as the fractions were subjected to quantitative determination of phenolic and flavonoid content.

2.6.1 Estimation of total phenolic content.

The phenolic content of different extracts of *P. integerrima* was determined by the method of Folin Ciocalteu method (Singleton and Rossi, 1965). The detailed procedure was as follows:

Stock solution of sample was prepared by dissolving 10 mg of test extracts in 10 ml of methanol. Folin Ciocalteu reagent was prepared by 1: 2 dilution of the reagent with distilled water. 20 gm of anhydrous sodium carbonate was dissolved in 100 ml of distilled water.1 ml of the sample was taken in 25 ml volumetric flask. To this, 10 of water and 1.5 ml of Folin Ciocalteu reagent was added. The above mixture was kept for 5 min and then 4ml of 20% sodium carbonate solution was added and the volume was made up to 25 ml with distilled water. This mixture was kept for 30 min and the absorbance of the blue color developed was measured at 165 nm. From the stock solution of standard 0.5, 0.75,1,1.25,1.5,1.75 and 2ml were taken which gave 50,75,100,125,150,200µg/ ml concentration respectively. Percentage of total phenolics was calculated from calibration curve of gallic acid plotted by using the above procedure and total phenolics were expressed as % of gallic acid.

2.6.2 Estimation of total flavonoid content

The total flavonoid content was determined by two methods (Chang and Yang, 2002). In both the methods, the flavonoids content was measured in the different extracts in *P. integerrima*. The sample solution of 1.5 mg/ ml and 5mg/ ml was prepared for estimation of total flavonoid content by Aluminum chloride colorimetric method and 2, 4- dinitrophenyl hydrazine colorimetric method respectively.

A) Aluminum chloride colorimetric method-

In this method Quercetin was used as standard. A stock solution of 1mg/ ml of Quercetin was prepared in ethanol. 10g of Aluminum chloride was dissolved in 10 ml of distilled water to prepare 10% Aluminum chloride 9.814 g of potassium acetate was dissolved in 100 ml distilled water to prepare 1 M potassium acetate. Quercetin was used to make the calibration curve. For the stock solution of standard 0.1, 0.2, 0.3, 0.4 and 0.5 ml were taken which gave 10, 20, 30, 40 and 50 µg concentrations respectively. The standard solutions were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of aluminum chloride, 0.1 ml 1 M potassium acetate and 2.8 ml of distilled water. After incubation at R. T. for 30 min, the absorbance of reaction

mixture was measured at 415 nm with Shimadzu UV- 1601 spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 1.5 mg/ ml of the sample solution were reacted with aluminum chloride for determination of flavonoid content as described in above procedure.

B) 2, 4- dinitro phenyl hydrazine colorimetric method-

In this method Naringin was used as standard. A stock solution, 10 mg/ ml of Naringin was prepared in distilled water.1% 2, 4- dinitro phenyl hydrazine reagent- 1 gm of 2, 4- dinitro phenyl hydrazine was dissolved in 2 ml 96% H_2SO_4 and then diluted up to 100 ml with distilled water.1 % potassium hydroxide was dissolved in 100 ml of 70% methanol. Naringin was used to make the calibration curve. Required ml of standard stock solution were taken and diluted to give 250, 500, 1000, 2000µg/ ml conc. respectively.1 ml of each standard solution were separately mixed with 2 ml of 1% 2, 4- dinitro phenyl hydrazine reagent and 2 ml of methanol and then kept at 50°C for 10 min. After cooling to R.T., the reaction mixture were mixed with 5 ml of 1% potassium hydroxide in 70% methanol and incubated at R.T for 2 min.

Then 1 ml of the solution was mixed with 5 ml of methanol and centrifuged at 1000 rpm for 10 min to remove precipitate. The supernatant was collected and adjusted to 2.5 ml. The absorbance was measured at 195 nm. Similarly, 5mg/ ml sample solution was reacted with 2, 4- dinitro phenyl hydrazine for determination of flavonoid content as described in the above procedure.

2.7 TLC studies on the extracts and their different fractions

The active extracts and their fractions were then subjected to TLC studies in order to detect separation of various types of Phytoconstituents in different solvents using specific detecting reagents (Wagner and Blatt, 1996). The R_f values of observed spots were noted for the selective extracts and their fractions.

2.8 HPTLC fingerprint profile of active extracts and fractions

Aqueous and Methanol extracts of leaf galls of *P. integerrima* and their fractions were subjected to HPTLC fingerprinting. Similarly Methanol extract and fractions of Methanol extract of rhizomes of *H. spicatum* were subjected to HPTLC fingerprinting using different solvent systems of different polarity and scanned at three different wavelengths.

The solvent systems used for extracts of leaf galls of *P. integerrima* were Toluene: Ethyl acetate (7:3), Chloroform: Methanol (1:1), Hexane: Chloroform: Methanol (4:5:6) and

Ethyl acetate: Methanol: Water (10:1.35:1). The solvent systems used for extracts of rhizomes of *H. spicatum* were Toluene: ethyl acetate (7:3), Ethyl acetate: Chloroform: Methanol (4:5:1) and Chloroform: Methanol (9:1).

Procedure

HPTLC fingerprint profiles were established for selective extracts. A stock solution (1 mg/ ml) was prepared in respective solvents. Suitably diluted stock solution was spotted on pre-coated silica gel G 60 F 254 TLC plates using CAMAG Linomat V Automatic sample spotter and the plates were developed in solvent systems of different polarities to resolve polar and nonpolar components of the bioactive fraction. The plates were scanned using TLC scanner 3 (CAMAG) at 254 nm (absorbance/ reflectance mode), 366 nm (fluorescence/ reflectance mode) and 540 nm (absorbance/ reflectance mode). The R_f values, spectra, λ max and peak areas of resolved bands were recorded. Relative percentage area of each band was calculated from peak areas.

2.9 Isolation of compounds from extract of *P. intigerrema* and *H. spicatum*

Extracts and fractions which were found to be biologically activity were identified at the initial stage from the selected plants. These extracts were subjected to column chromatography for isolation of the compounds.

2.9.1 Isolation of compounds from ethyl acetate fraction of methanol extract of *P. intigerrema*

Methanol extract of *P. intigerrema* was fractionated using ethyl acetate and loaded on column to isolate the compounds.

Column chromatography of ethyl acetate fraction of Methanol extract:

Conditions for column chromatography:

Adsorbent: Silica (60-120 mesh)

Weight of sample: 3.0 g

Column was prepared in hexane and eluted with Hexane: ethyl acetate (98:2, 90:10, 80:20) which yielded fraction 1, 2 and 3. Fractions were collected in the quantity of 100 ml. Fractions 1, 2, 3 gave similar pattern on TLC. These fractions were mixed. It gave a brown colored sticky mass. Fractions 4 and 5 which were obtained after elution with Hexane: Ethyl acetate (70:30) gave a similar pattern on TLC. Fractions 6-11 obtained on elution with Hexane: Ethyl acetate (50:50) gave a similar pattern on TLC. When concentrated it gave a yellow colored powder around 200 mg. This fraction was further isolated by preparative TLC. Fractions 14-25 obtained by solvent Ethyl acetate: Chloroform (70:30) when concentrated gave light brown colored

powder around 150 mg. This fraction was subjected to preparative TLC. All the fractions were subjected to HPTLC fingerprint.

2.9.2 Isolation of PI-1and PI-2 from the flavonoid fraction of ethyl acetate fraction of methanol extract of *P. integerrima*

Hexane: Ethyl acetate fraction of Methanol extract was concentrated which afforded to give yellow colored powder (200 mg). This fraction was subjected to TLC and further HPTLC to give three compounds. It was further subjected to preparative TLC for isolation of two flavonoids.

2.9.3 Isolation of PI-3 from the phenolic fraction of ethyl acetate fraction of methanol extract of *P. integerrima*

The Ethyl acetate: Chloroform (70:30) fraction afforded to give brown colored solid powder. It was further subjected to TLC and HPTLC. One compound was isolated by subjecting this fraction to preparative TLC.

2.9.4 Isolation of compounds from methanol extract of *H. spicatum* by column chromatography (Sharma, 1976 and Sharma, 1975)

Methanol extract of *H. spicatum* was found to contain more nonpolar compounds like terpenes and hence loaded on column to isolate the compounds to isolate diterpenes.

Column chromatography of Methanol extract:

Conditions for column chromatography:

Adsorbent: Silica (60-120 mesh)

Weight of sample: 5.0 g

Column was prepared in n-hexane and eluted with Hexane: Benzene (9:1, 8:2, 7:3) which yielded fractions 1-10 gave similar pattern on TLC. These fractions were mixed and concentrated. They yielded white colored oily nonstick product. Fractions 11-35 which were obtained by Benzene: Ethyl acetate (6:4, 5:5 and 4:6) gave similar pattern on TLC and mixed together. It yielded light yellow colored oily nonstick product. Fractions 36-49 gave similar pattern on TLC with Benzene: Ethyl acetate (9:1-5:5). These fractions were mixed together and yielded dark brown colored liquid on concentrating. Fractions 50-54 obtained by Benzene: Ethyl acetate (5:5) were mixed together as they showed a similar pattern on TLC. They gave a brown colored semisolid mass. Ethyl acetate fractions 55-64 were mixed together as they gave similar pattern on TLC. All the fractions were subjected to HPTLC fingerprinting using n-hexane: ethyl acetate (7:3) solvent system. No fraction yielded single compound. The major fraction (11-33) was further isolated by preparative TLC. The

compounds obtained by preparative TLC were again subjected to HPTLC fingerprinting. The Hexane benzene fraction showed presence of single compound

2.9.5 Isolation of HS from the methanol extract of *Hedychium spicatum*

Methanol extract of *Hedychium spicatum* was subjected to column chromatography by hexane- benzene, benzene, benzene- ethyl acetate and ethyl acetate for isolation of diterpenes. TLC and HPTLC pattern showed presence of major amount of diterpenes in hexane- benzene fraction. This fraction was brown semisolid in nature. It was subjected to preparative TLC to yield single compound which was confirmed by HPTLC. Remaining fractions were not sufficient to carry out further isolation.

2.10 Identification and characterization of isolated compounds

The isolated compounds were subjected to following studies.

2.10.1 Physico-chemical characterization

The isolated compounds were subjected to physical characterization by studying their state, melting point, solubility and λmax.

2.10.2 Characterization by spectral analysis

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

2.10.3 Assessment of % purity of isolated compounds by HPLC

All the isolated compounds were subjected to HPLC for determination of % purity.

Reagents: Acetonitrile, methanol, water (HPLC grade)

Preparation of sample: Sample solutions were prepared by dissolving 1 mg/ml of isolated compounds in methanol. Further dilution was made in mobile phase i.e. Acetonitrile: methanol: water (80:15:5).

Chromatographic conditions: The chromatographic separation was achieved on C_{18} Phenomenex column (250mmX 4.6mm, 5 µm). The mobile phase was filtered through nylon filter 0.2 µm and degassed before use. The flow rate was 1ml/min.

2.11 Estimation of identified compounds by HPTLC

2.11.1 Quantification of Kaempferol *H. spicatum*

Preparation of sample: Plant tissue was hydrolyzed with 2M HCl for 30-40 min at 100°C. The cooled solution was extracted twice with ethyl acetate and combined extracts were taken to dryness and the residue was taken up in a small volume of ethanol. Further this extract was subjected to TLC using ethyl acetate: methanol: water (100:13.5:10) solvent system and developed using NP-PEG reagent which gave

yellow fluorescence. In Ethyl acetate: Methanol: Water: Formic acid (3.5: 2.5: 0.5: 0.1) using aluminum chloride showed yellow fluorescence. This indicated presence of Kaempferol in the drug. This was further confirmed by subjecting the test sample to TLC using Benzene: Pyridine: Formic acid (36: 9: 5). Reagents which were used for development were 1% Aluminum chloride and 1% sodium carbonate. The spots were tallow in day light and reagents showed yellow green color. This indicated presence of Kaempferol which was confirmed by using standard markers (Harborne, 1973, Karel, 1972).

Preparation of Standard stock solution: standard stock solution containing Kaempferol 1mg/ml was prepared in methanol.

HPTLC instrumentation: A Camag HPTLC system equipped with an automatic TLC sampler, TLC scanner 3, UV cabinet and twin trough glass tank was used for analysis

Preparation of calibration curve: A calibration curve was established using six analyte concentrations (2-12µl) representing 2-12µg of Kaempferol. Standard zones were applied by means of Linomat V automated spray-on band applicator with following settings- band length 6mm, distance between the bands 4mm, distance from the plate side edge 8mm and distance from the bottom of the plate 15mm. Plate was developed in a vapor equilibrated Camag twin trough chamber. After development, the plates were air dried for 5 min and standard zones were quantified by linear scanning at 400 nm.

2.11.2 Quantification of Quercetin from *P. integerrima* (Swaroop, 2005)

Sample preparation: Quercetin was evaluated quantitatively in Methanolic extract of *P. integerrima*. 1mg/ml sample solution was prepared

Preparation of Standard stock solution: Standard stock solution containing Quercetin. 1mg/ml was prepared in methanol.

Mobile phase: Ethyl acetate: Methanol: Water (100:13.5:10)

HPTLC instrumentation: A Camag HPTLC system equipped with an automatic TLC sampler, TLC scanner 3, UV cabinet and twin trough glass tank was used for analysis

Preparation of calibration curve: A calibration curve was established using six analyte concentrations (2-14µl) representing 2-14µg of quercetin. Standard zones were applied by means of Linomat V automated spray-on band applicator with following settings- band length 6mm, distance between the bands 4mm, distance from

the plate side edge 8mm and distance from the bottom of the plate 15mm. Plate was developed in a vapor equilibrated CAMAG twin trough chamber. After development, the plates were air dried for 5 min and standard zones were quantified by linear scanning at 254 nm.

2.11.3 Quantification of Gallic acid from *P. integerrima* (Jegannath, 2008)

Sample preparation- Gallic acid was evaluated quantitatively in methanol extract of *P. integerrima.*

Preparation of Standard stock solution: standard stock solution containing Gallic acid 1mg/ml was prepared in methanol.

HPTLC instrumentation: A Camag HPTLC system equipped with an automatic TLC sampler, TLC scanner 3, UV cabinet and twin trough glass tank was used for analysis.

Mobile phase: Toluene: Ethyl acetate: Formic acid: methanol (3:3:0.8:0.2)

Preparation of calibration curve: A calibration curve was established using six analyte concentrations (2-14µl) representing 2-14µg of gallic acid. Standard zones were applied by means of Linomat V automated spray-on band applicator with following settings- band length 6mm, distance between the bands 4mm, distance from the plate side edge 8mm and distance from the bottom of the plate 15mm. Plate was developed in a vapor equilibrated CAMAG twin trough chamber. After development, the plates were air dried for 5 min and standard zones were quantified by linear at 288 nm.

2.12 In vitro antioxidant activity of extracts and fractions of *P. integerrima*

From the qualitative examinations, *P. integerrima* was found to be rich in phenolic and flavonoid content, therefore was subjected to in vitro antioxidant activity by following methods.

2.12.1 DPPH free radical scavenging activity (Sharma, 2008)

Free radical scavenging potential was tested against a methanolic solution of DPPH. 0.1 mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 3 ml of different concentrations (5-400 µg/ml) sample solution in methanol. It was incubated at room temperature for 30 min and absorbance was measured at 517 nm against the corresponding blank solution. Ascorbic acid was taken as reference. The inhibition of DPPH free radical was calculated by following equation

% scavenging activity = [(Ac- As)/ Ac] x 100

Ac is absorbance of control reaction, As is the sample. The antioxidant activity is expressed as IC_{50}.

2.12.2 Reducing power assay (Sharma, 2008)

The different concentration of P1 and P2 (5-60 µg/ml) in 1 ml deionized water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5ml). the mixture was incubated at 50°C for 20 min. Trichloro acetic acid (2.5 ml, 10%) was added . it was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%)and absorbance was measured at 700 nm. Ascorbic acid was taken as standard.

2.12.3 Scavenging of hydrogen peroxide (Ruch, 1989)

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by recording the absorbance at 230 nm. Different concentrations of samples (5-100 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance was measured at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The % scavenging was calculated by

% scavenging activity = [(Ac- As)/ Ac] x 100

Ac is absorbance of control reaction, As is absorbance of sample.

2.12.4 Hydroxyl radical scavenging activity (Hallowell, 1987)

The assay was performed by adding 0.1 ml EDTA, 0.1ml hydrogen peroxide, 0.36 ml deoxyribose, 1 ml test solution (10-100µg/ml) dissolved in distilled water, 0.33 ml, of phosphate buffer (50mM, pH 7.4) and 0.1 ml ascorbic acid in sequence. The mixture was incubated at 37° C for 1hr. A 1 ml portion of the incubated mixture was mixed with 1 ml of 10% trichloroacetic acid and 1.0 ml of 0.5% thiobarbituric acid to develop pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity was reported as % inhibition of deoxyribose degradation and calculated as

% scavenging activity = [(Ac- As)/ Ac] x 100

Ac is absorbance of control reaction, As is absorbance of sample.

2.13 Biological screening of extracts and fractions.

Aqueous and Methanol extracts of both the plant drugs and their fractions were subjected to acute toxicity studies prior to assessment of biological activity. After assessing safety of the extracts and fractions, they were subjected to biological activity.

2.13.1 Acute toxicity studies

Acute toxicity studies were performed for selective extracts and their fractions according to the acute toxic classic method as per guidelines 423 prescribed by OECD (OECD, 1996). Female albino rats were used for acute toxicity study. The animals were kept fasting for overnight providing only water. Theses were divided into groups of each containing three animals. Each of these groups was then administered with Aqueous and Methanol extracts of *P. integerrima* and *H. spicatum* and their fractions at the dose of 300mg/kg p.o. The animals were observed for 30 min and then periodically for first 24 h with special attention during first 4 h and thereafter daily for 14 days. The observations like sedation, convulsions, tremors, lethargy, death etc were systematically recorded with individual records of each animal. Since no mortality was seen at the dose level 300 mg/kg, the procedure was repeated with higher dose of 2000 mg/kg p.o .in fresh animals.

2.13.2 Adaptogenic activity

The usage of the plants as tonic and stimulant was mentioned in Ayurveda. It has become therefore an important task to evaluate these drugs in scientific manner. The activity was performed in following manner.

A. Animals

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

B. Preparation of test samples

Weighed quantities of test extracts were suspended in 1.0% 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.

Preparation of *E. coli* suspension

1x 10^8 cells of *E. coli* was prepared in nutrient broth and used for *E. coli* induced abdominal sepsis and given in dose of 0.2 ml *i.p.*

Preparation of standard immunosuppressant

Cyclophosphamide was used as a standard drug to induce myelosuppression in mice. It was dissolved in distilled water and administered in dose 250mg/kg for inducing myelosuppression in mice.

2.13.2 Adaptogenic activity in vivo

Selected extracts and their fractions mentioned below were subjected to screening adaptogenic activity utilizing different models in animals.

1. Aqueous extract of *P. integerrima and* n Butanol fraction, Ethyl acetate fraction and Methanol fraction of Aqueous extract of *P. integerrima.*

2. Methanol extract of *P. integerrima* and Chloroform fraction, Ethyl acetate fraction, Acetone fraction, Residual fraction of methanol of Methanol extract of *P. integerrima*

3. Aqueous and Methanol extract of *H. spicatum* and Chloroform fraction, Ethyl acetate fraction and Remaining methanol fraction of methanol extract

Aqueous and methanol extracts were subjected to adaptogenic activity at three dose levels viz., 100mg/ kg, 200mg/ kg , 500mg/ kg, to identify the bioactive extracts. All the fractions of both the extracts were subjected to screening at three dose levels viz., 50mg/ kg, 100mg/ kg, 150mg/ kg

2.13.2.1 *E. coli* induced abdominal sepsis

The method described by Sangle (2004) was adopted. Animals were divided into groups of six animals each. The control received 0.1% sodium carboxy methyl cellulose solution only as vehicle; while animals in the treated groups were given the test extract orally in 1% sodium carboxy methyl cellulose for 15 days. All the animals were treated with extracts for15 days prior to bacterial challenge. On the 16^{th} day, the animals were injected with 0.2 ml suspension of *E. coli* (1x 10^8 cells) *i.p.* Animals were then observed for 16 hours to find mortality if any. Blood samples were then withdrawn from retro-orbital plexuses using heprarinized capillary tubes and by cardiac puncture in case of dead animals. Blood samples were analyzed for total and differential WBC count.

2.13.2.2 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 0.1% sodium carboxy methyl cellulose solution only as vehicle; while animals in the treated groups were given the test extract orally in 0.1% 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 15 min. Blood (25µl) was mixed with 1% sodium carbonate (2 ml) and subjected for determination of optical densities at 660nm.

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.

2.13.2.3 Cyclophosphamide induced myelosuppression

Cyclophosphamide induced myelosuppression was studied according to the method described by Pallabi et al., (1998). The animals were divided in group of six each. The control group received 0.1% sodium carboxy methyl cellulose solution only as vehicle; where as the treatment groups were given test extracts in 0.1% sodium carboxy methyl cellulose Different hematological parameters like WBC, RBC, Hb, HCT and MCV were determined using reported methods (Mukhergee, 1997). The treatment was continued for further 10 days and on the 25th day from the day of start of treatment; all groups received a single dose cyclophosphamide 250mg/kg orally. On the 26th day, blood was collected from retro orbital plexus of each animal and the hematological parameters were determined.

2.13.2.4 Anoxia tolerance test

Albino mice of either sex were divided into four groups. Stress was induced in the animals by placing individual animal in hermetic vessel of 1 L capacity. Reaction to anoxia stress was recorded as anoxia tolerance time i.e. first sign of convulsion was considered as end point. The study was conducted for 21 days and at the end point of 1st, 2nd and 3rd week anoxia stress tolerance time was recorded with all animals. (Hirumalima, 2000; Suberans, 1993)

2.13.2.5 Forced swim test

Adaptogenic activity by forced swim method was performed by Krupavaram et al., (2007). Albino rats of either sex (100-150 g) were divided groups of six animals each. The control group received 0.1% sodium carboxy methyl cellulose solution only as vehicle; where as the treatment groups were given test extracts in 0.1% sodium carboxy methyl cellulose. The rats were subjected to swimming stress by keeping them in cylindrical vessels (48 × 30 cm) filled with water to a height of 25 cm and the total swim time for each rat was noted. Extracts were given to rats once daily for 7 days. On 8th day rats were allowed to swim till complete exhaustion and the end point was taken when the animal starts drowning and the mean swimming time for each

group was calculated. The animals were killed and blood was collected by cardiac puncture to estimate biochemical parameters like serum glucose, triglycerides, cholesterol, BUN and blood cell count (RBC, WBC and DLC). The weight of organs such as liver, spleen, adrenals was recorded after washing with alcohol.

2.14 Statistical Analysis

The mean values ± SEM were calculated for each parameter. Each parameter was separately analyzed by one way analysis of variance (ANOVA) followed by Bornferonis test. The statistical analysis was done by Graphpad prizm version 3.02.

2.15 Hepatoprotective activity in vitro

The selected plant dugs are reported to be used in liver disorders in the traditional literature. The reports are available regarding the hepatoprotective activity of these plant drugs. To confirm the claims, in vitro hepatoprotective activity of the bioactive fractions and isolated compounds against paracetamol was carried out.

Isolated hepatocytes have become a useful model for pharmacological, toxicological, metabolic and transport studies of xenobiotics since the development of techniques for high yield isolation of rat hepatocytes (Skett, 1994). Various hepatotoxins like carbon tetrachloride, thioacetamide and paracetamol have shown reduction of viability of hepatocytes and leakage of enzymes which are considered to be the markers of cellular injury (Belinski et al, 1984; Zimmerman and Mao, 1965).

2.15.1 Isolation of rat hepatocytes

Hepatocytes were isolated from rat liver as per the reported method (Sarkar and Sil, 2006) (19) with some modifications .The liver was isolated under aseptic conditions and placed in chilled HEPES (N-2-hydroxyethylpiperazine-N- 2 ethane sulphonic acid) buffer containing HEPES (0.01M), NaCl (0.142M) and KCl (0.0067M), pH7.4. The liver pieces then incubated in a second buffer containing HEPES (0.01M), NaCl (0.142M) and KCl (0.0067M) and collagenase type IV, at pH 7.6 for about 45 min at 37°C. Hepatocytes were obtained after filtration through muslin cloth and cold centrifugation (4°C, 200rpm/min for 2 min three times) and resuspended in 4-5 ml HEPES buffer I. The viability of the hepatocytes was assessed by trypan blue exclusion method (Kiso etal, 1983).

2.15.2 Trypan blue dye exclusion method

This is the most commonly employed criterion of cellular integrity or viability. Cells with an intact plasma membrane exclude this dye whereas damaged cells become stained particularly at the nucleus. Under ordinary conditions the dye concentration of

0.2% is sufficient but staining intensity may show a considerable variation depending on components in the cell suspension or in particular batch of trypan blue. The stained hepatocytes were observed under neaubaur's chamber under optical microscope and four different fields were scanned to calculate the viability of cells.

2.15.3 Primary cultures of rat hepatocytes

The method of Tinstorm and Obrink (1989) with significant modification was used for this purpose. The freshly isolated viable hepatocytes were suspended in the culture medium RPMI- 1640 supplemented with calf serum (10%), HEPES and Gentamycin (1μg/ml). These cells approximately 1.2×10^6/ml were seeded into culture bottles and incubated at 37°C in atmosphere of 5% CO_2. The hepatocytes formed a monolayer upon incubation for 24hrs. The newly formed cells were round and mostly appeared as individual cells. These cells were 96-97% viable as confirmed by trypan blue.

The protocol (Rao and Mishra, 1998b) used for hepatic cytotoxicity testing of fractions is as given below.

Table 1: The protocol for the in vitro assessment of hepatic cytotoxicity

Group	Contents	24h
Control	0.1ml HS+0.1ml vehicle+0.8ml HEPES buffer I	
Toxicant	0.1ml HS+0.1ml toxicant+0.8ml HEPES buffer I	Estimation of hepatocytes
Sylimarine	0.1ml HS+0.1 ml Sylimarine+0.1ml toxicant+0.8ml HEPES buffer I	Viability, GOT, GPT, TP
Fractions/ Isolated compounds	0.1ml HS+0.1 ml extrct/fraction+0.1ml toxicant+0.8ml HEPES buffer I	

Vehicle: 30% DMSO, Sylimarine: 100μg/ml, Toxicant: Paracetamol: 300μg/ml, HS: Hepatocytes suspension

The protocol (Rao and Mishra, 1998b) followed for the in vitro hepatoprotective activity of various fractions against different toxicants is as given in the table 5. 24 hrs after establishment of the monolayer of the hepatocytes, the medium was decanted and the culture was washed with HEPES buffer I and finally the hepatocytes were suspended in 5ml of HEPES buffer I. The hepatic toxicity was induced with paracetamol 300μg/ml. test substances including Sylimarine were dissolved in 30% DMSO (Tasaduq et al 2003). Hepatocytes suspension (0.1ml) in triplicate were distributed into various culture plates labeled as control, toxicant, standard

(Sylimarine+ toxicants) and test (test samples+ toxicants). The control group received 0.1ml of vehicle (30% DMSO) and toxicant group received 0.01ml of respective test solutions (100,200 and 500µg/ml of extracts/ fractions dissolved in 30% DMSO) followed by 0.1ml of Sylimarine solution (100µg/ml) followed by respective hepatotoxin. The content of all the tubes were made up to 1ml with HEPES buffer I. The contents of all the plates were mixed well and incubated for 24hrs at 37°C. In test and standard groups of hepatocytes were incubated with respective solution for 30 min and then exposed to hepatotoxin. After incubation hepatocytes suspensions were collected to assess cell damage by trypan blue exclusion method (Kurma and Mishra 1998b). Hepatocytes suspensions were centrifuged at 200rpm. The leakage of enzymes GOT, GPT and Total proteins secreted outside the cells were determined from the supernatant by using standard kits for enzyme estimation.

2.14 References:

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