3.1 Materials

Chemicals and Reagents:

The solvents: petroleum ether (pet ether), toluene, chloroform, ethyl acetate and methanol procured from Rankem Laboratory reagents, New Delhi; reagents: phloroglucinol, safranin solution, iodine solution, hydrochloric acid, glycerin, sodium hydroxide, potassium hydroxide, vanillin hydrochloric acid reagent, Mayer's reagent, Hager's reagent, Wagner reagent, Dragendorff's reagent, Molisch's reagent, Lieberman-Buchard reagent, Millon's reagent, ferric chloride reagent, lead acetate solution and zinc dust procured from Hi-Media Laboratories Pvt. Ltd. Mumbai; and chemicals: citric acid, aluminum hydroxide, ovalbumin, DPPH, ascorbic acid, MTT, Con A and lipopolysaccharide procured from Sigma Aldrich, USA had been used. All other chemicals were of analytical grade.

Equipments:

Trianocular microscope attached to camera from Radical Microscope, Model RXL-5T; UV-visible double beam spectrophotometer from Systronic India Pvt. Ltd.; Rota evaporator from Ika Pvt. Ltd; TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m X 0.25mm, 0.25µm) column and S/SL Injector; Perspex chamber equipped with an ultrasonic nebulizer from INCO Ltd., Ambala, India and ELISA Reader from Bio-tek Instruments, USA were used.

3.2 Methods

3.2.1 Collection, authentication and preparation of plant material

The fresh rhizomes of *H. spicatum* had collected in wild from Habban, District Sirmour, Himachal Pradesh, India with GPS location coordinates 30°54'56"N 77°19'29"E (See Figure 3.1). The plant had taxonomically identified and authenticated as *Hedychium spicatum var. acuminatum* Linn. by Dr. Kulwant Rai Sharma, Senior Scientist, Prof & Head, Medicinal Plants, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan, HP. The herbarium sheet of the said sample had linked to UHF-Herbarium with field book number 13457. The rhizomes had duly washed under running tap water to draw

it free from adherents, without any other pretreatment and had shade dried. The dried plant material had pulverized to make a coarse powder using electric grinder and kept in an airtight container.

Figure 3.1: Collection of fresh rhizomes of *Hedychium spicatum var. acuminatum*

Leaf galls of *P. integerrima* had collected in both wild and cultivated trees from Narag, District Sirmour, Himachal Pradesh, India with GPS location coordinates 30°49'3"N 77°11'15"E. The leaf galls had taxonomically identified and authenticated as *Pistacia integerrima* J. L. Stewart ex Brandis by Dr. Bhupender Dutt Senior Scientist,

Associate Professor, Medicinal Plants, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan, HP. The herbarium sheet of the said sample had linked to UHF-Herbarium with field book number 13507. The plant material had dried in shade till constant weight. The dried plant material had pulverized to coarse powder using electric grinder and packed in air tight containers till further use.

3.2.2 Pharmacognostical evaluation

- **Macroscopic evaluation**

(Harborne, 1998; API, 1999b; Khandelwal, 2008; Trease and Evans, 2002, Kokate et al., 2006)

The macroscopic identity of a medicinal plant material is based on the visual appearance with naked eyes and external characters observed like shape, size, color, taste, apex, surface, base, margin, venation, texture, fracture and odor.

Size: A graduated ruler in millimeters had used for the measurement of length, width and thickness of crude drugs. Plant part had measured by aligning 10 of them on a sheet of calibrated paper, with 1 mm spacing between lines and dividing the results by 10.

Color: The untreated sample had examined under diffused daylight, if necessary, an artificial light source with wavelength similar to those of daylight was used. The color of the sample had compared with that of the reference sample.

Surface characteristics, fracture and texture characteristics: Sample had examined under magnifying lens (6x to 10x). Wetting with water or reagents had required to observe the characteristics of a cut surface. The material had touched to determine if it is soft or hard. It had then bend and replaced to obtain information on brittleness and the appearance of the fracture plane- whether it had fibrous, smooth, rough or granular, etc.

Odor: If the material had expected to be innocuous, place a small portion of the sample in palm of hand or a beaker of suitable size, and slowly and repeatedly inhaled the material. If the distinct odor was perceptible, crushed the sample between the thumb and index finger or between the palms of hands using gentle pressure. If the material had hard to break, then

crushed by mechanical means and then pour a small quantity of boiling water onto the crushed sample in a beaker. First, determined the strength of the odor (none, weak, distinct and strong) and then the odor sensation (aromatic, fruity, musty, mouldy and rancid, etc.). **Taste:** The taste of powdered drug had evaluated by placing a pinch of powdered drug on the tongue which was carried to all regions of tongue which have specialized taste buds corresponding to different types of taste such as sweet, bitter, astringent and acrid etc. Prior to and after the test, mouth had duly rinsed with potable drinking water.

- **Microscopic evaluation**

(Trease and Evans, 2002; Harborne, 1998; API, 1999b; Khandelwal, 2008)

Cytomorphology or microscopic studies had the examination of cell form and arrangement of different cells in a crude drug. The plant drugs are generally used in a powdered or combined form where the macro morphology gets destroyed, so that the evaluation of microscopic cell characters is essential. Cytomorphological characters play a major role in drug identification.

A free hand and thin sections of fresh rhizomes had cut using a sharp blade. The finest section from all the sections had placed on slide and drops of chloral hydrate had poured on it. It had allowed to boil so as to remove coloring matter for 5 to 10 min and then cooled for 5 min. The section had stained using phloroglucinol and hydrochloric acid (1:1) or safaranine. A thin layer glycerine had put on a section to moisten it and section had covered with a cover slip avoiding air bubbles in it. The sections had examined under the trianocular Radical Microscope (Model RXL-5T) at magnification 10 X and 40 X. After attaining a fine resolution of the section, the photomicrography using the camera (model number: B-7692) had done.

Transverse sections of galls had prepared by breaking it into pieces of about 2-3 cm long and 0.5-1.5 cm wide and boiled within a test tube for 30 min. Straightened the soft pieces then with a scalpel expose exact transverse or longitudinal direction and pieces of the galls had cut into fine sections by using pith (potato or carrot), then removed and placed the fine sections with a brush on the slide. For testing of lignin present in the cell structures, added

several drops of phloroglucinol and a drop of concentrated hydrochloric acid to the section on a slide, then dained off the excess of liquid, immersed the section in chloral hydrate solution and cover with a cover slip. The lignified elements had colored red or crimson. The excessive stain had washed out using acidified alcohol (API 1999b). Finally glycerine had put on the section to moisten it. The section had covered with a cover slip avoiding the air bubbles in it. The sections had then observed under the trianocular Radical Microscope (Model RXL-5T) and photomicrography had done using the camera (model number: B-7692) at resolution 10 X.

3.2.3 Physicochemical evaluation

(WHO/QCMMPM guidelines, 1998; Harbone, 1998b, API, 1999b.; Trease and Evans, 2002; Khandelwal, 2008)

- **Foreign matter:** Foreign matter is the matter consisting of any or all of the following material:
  - Parts of the medicinal plant material or materials and, other than those named with the limits specified for the plant material concerned.
  - Any organism as whole, parts or products of an organism, other than those named in the specification and description of the plant concerned.
  - Mineral admixed not adhering to the medicinal plant material, such as soil, stones, sand and dust.

Procedure:

250g of plant material had weighed and spread in a thin layer. The foreign matter had then sorted into groups either by visual inspection, using magnifying lens (6x to 10x) or with the aid of suitable sieve. The remaining sample had sifted through sieve no. 250; the dust had regarded as mineral admixture. Separated and weighed it and calculated the percentage present. The values had obtained as the average of triplicate studies.

- **Determination of ash values:**

The ash remaining after ignition of medicinal plant material can be determined by 3 different methods which measures:

- Total ash value
- Acid insoluble ash
- Water soluble ash

The total ash method is method to measure the total amount of material remaining after ignition this includes both ‘‘physiological ash’’ (derived from the plant tissue itself) and ‘‘non- physiological’’ ash (residue of the extraneous matter e.g. sand and soil) adhering to the plant surface. Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and further igniting the remaining insoluble matter which measures the amount of silica present. Water soluble ash is the measured as difference in weight between the total ash and residue after the treatment of total ash with water.

Procedure:

Total ash value: Previously ignited and tarred platinum or silica crucible had used to incinerate about 3 g accurately weighed, of the ground drug at a temperature not exceeding 450ºC until free from carbon, cooled and weighed. If a carbon free ash wasn’t being obtained in this way, exhausted the charred mass with hot water, collected the residue on an ashless filter paper, incinerated the residue and filter paper, added the filtrate, evaporated to dryness, and ignited at a temperature not exceeding 450ºC. It had then cooled in a desiccator and weighed. Calculated the total ash as percentage with reference to the air-dried drug. The values had obtained as the average of triplicate studies.

Acid insoluble ash: To the crucible containing the total ash, 25ml of hydrochloric acid had added, covered with a watch glass and boiled gently for 5min. The watch glass had then rinsed with 5ml of hot water and this liquid had added to the crucible. Collected the insoluble matter on an ash less filter paper, further washed with hot water until the filtrate became neutral. The filter paper containing the insoluble matter had transferred to the original crucible, dried on a hot plate and ignited again to constant weight. The residue had allowed to cool in a desiccator for 30 min, and then weighed. Calculated the acid-insoluble

ash as percentage with reference to the air dried drug. The values had obtained as the average of triplicate studies.

Water soluble ash: To the crucible containing total ash, 25ml of water had added and boiled for 5 minutes. Collected the insoluble matter on an ash less filter paper, further washed with hot water and ignited again in a crucible for 15 min at a temperature not exceeding 450º C. The subtracted weight of insoluble matter from the weight of the ash; difference in weight represented the water-soluble ash. Calculated the water-soluble ash as percentage of with reference to the air-dried drug. The values had obtained as the average of triplicate studies.

- **Loss on drying:** This method helps in determining the amount of volatile matter (water drying off). For substances containing water as the major volatile constituent, this procedure is appropriately used.

Procedure:

Placed about 10 g of accurately weighed (within 0.01 g) drug in a tared evaporating dish. For underground or whole drug, prepare about 10 g of the drug by cutting shredding so that the parts had about 3 mm in thickness. The use of high speed mills should be avoided in preparing the samples, and milling should be done with care such that no appreciable amount of moisture is lost during preparation and that the portion taken is representative of the official sample. The sample had distributed evenly in a tarred evaporating dish and had placed in the drying chamber (oven). After placing the above said amount of the drug in the tared evaporating dish dried at 105ºC for 5 hours, and weighed. Continued the drying and followed by weighing at one hour interval until difference between two successive weighing corresponds to not more than 0.25 per cent. Constant weight had reached when two consecutive weighing after drying for 30 min and cooling for 30 min in a desiccator, don't show more than 0.01 g difference. The values had obtained as average of triplicate studies.

- **Determination of extractable matter:**

Extractable matter is helpful in evaluating the constituents of crude drug, which cannot be determined by any other means. It also indicates the nature of the constituent present in the drug. This method determines the amount of constituents that can be extracted with solvents (pet ether, methanol and water etc.) from a specific amount of medicinal plant material.

Hot extraction:

Determination of alcohol soluble extractive: About 5g of coarsely powdered air dried material accurately weighed had placed in a continuous hot extraction apparatus (Soxhlet apparatus Figure 3.2). 100 ml of ethyl alcohol had added in a round bottom flask, continuous extraction in Soxhlet extractor had carried out for 8 hours. Towards the end filtered the extract into a tared evaporating dish and evaporated off the solvent on a water bath. Dried the residue to dryness on a water bath at 105º C for 6 hours, cooled in a desiccator for 30 min and finally weighed without delay. The content of extractable matter had calculated in mg per g of air dried material. Calculated the alcohol soluble extractive as percentage with reference to the air-dried drug.

Determination of water soluble extractive: This method had been **p**roceeded as method directed for the determination of alcohol-soluble extractive, using chloroform water (90:10) instead of ethanol alone. The content of extractable matter had calculated in mg per g of air dried material. Calculated the water soluble extractive as percentage with reference to the air-dried drug.

Determination of ether soluble extractive: Transferred 5g of the air dried, crushed drug to an extraction thimble, followed by extraction with solvent ether (or pet ether, b.p. 40ºC to 60ºC) in a continuous extraction apparatus (Soxhlet apparatus Figure 3.2) for 6 hours. Filtered the extract into a tarred evaporating dish and evaporated off the solvent on a water bath. Dried the residue at 105ºC to constant weight. Calculated the ether soluble extractive as percentage of with reference to the air-dried drug.

Cold maceration:

Determination of alcohol soluble extractive: Macerated 5 g of accurately weighed, crushed and coarsely powdered air dried drug, with 100 ml of ethyl alcohol of the specified strength in a closed flask or vessels for twenty-four hours. Shaked frequently during first six hours and allowing standing for eighteen hours. Rapidly filtered taking precautions against loss of solvent. Evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed dish at 105ºC, dried to constant weight and finally weighed. Calculated the alcohol soluble extractive as percentage of with reference to the air-dried drug.

Determination of water soluble extractive: Proceeded as per method for determination of alcohol soluble extractive, using chloroform water (90:10) replacing ethanol. Calculated the water soluble extractive as percentage of with reference to the air-dried drug.

Determination of ether soluble extractive: Proceeded as per method for the determination of alcohol-soluble extractive using solvent ether (or pet ether, b.p. 40ºC to 60ºC) in place of ethanol. Calculated the ether soluble extractive as percentage with reference to the air-dried drug.

3.2.3 Phytochemical evaluation

3.2.3.1 Successive solvent extraction of plant drug (Kokate, 1991; Khandelwal *et al*., 2008).

Procedure:

Pet ether extract: 5g coarsely powdered plant material had extracted with pet ether via hot extraction process (Soxhlet apparatus Figure 3.2) for 4 hrs. After completion of extraction, the solvent had removed by distillation and concentrated in vacuum.

Chloroform extract: The marc left after pet ether extraction had dried in air and extracted with $CHCl_3$ by hot extraction process (Soxhlet apparatus) for 4 hrs. After completion of extraction, the solvents had removed by distillation and concentrated in vacuum.

Figure 3.2: Soxhlet apparatus

Ethyl acetate: The marc left after chloroform extraction had dried in air and extracted with ethyl acetate by hot extraction process (Soxhlet apparatus) for 4 hrs. After completion of extraction, the solvents had removed by distillation and concentrated in vacuum.

Methanol extract: The marc left after ethyl acetate extraction had dried in air and extracted with methanol by hot extraction process (Soxhlet apparatus) for 4 hrs. After completion of extraction, the solvents had removed by distillation and concentrate in vacuum.

Aqueous extract: The marc left after methanol extraction had dried in air and extracted with chloroform water by maceration process. After completion of extraction, the solvent had removed by distillation and concentrated in vacuum. Color, consistency and yield of the extracts had noted. The extracts had preserved under vacuum for further phytochemical studies.

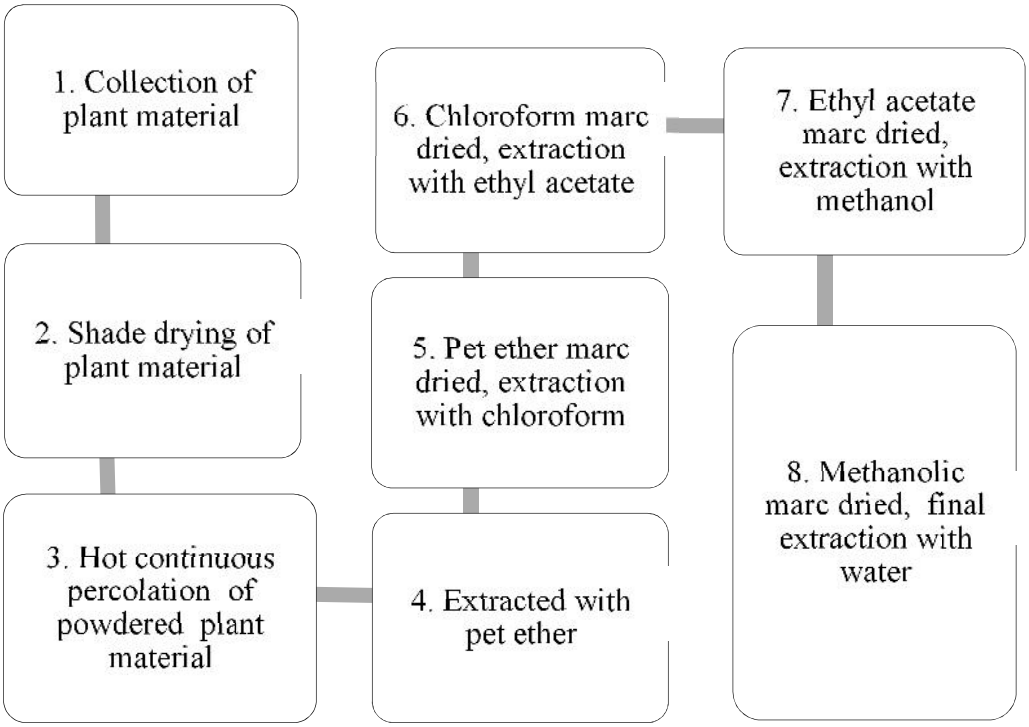

Figure 3.3: Scheme for the Successive Solvent Extraction

3.2.5.2 Preliminary phytochemical screening:

All the six extracts obtained by successive solvent extraction i.e. pet ether, toluene, chloroform, ethyl acetate, methanol and water had subjected to preliminary phytochemical screening for the detection of various phytoconstituents such as alkaloids, glycosides, tannins, phenolic compounds, flavonoids, steroids, saponins, proteins, amino acids, carbohydrates and triterpenoids using following phytochemical tests. (WHO/QCMMPM guidelines, 1998; Harbone, 1998a; Kokate et al., 2006; Khandelwal, 2008)

- **Test for alkaloids:**

- **Mayer's test:** Extract on treatment with Mayer's reagent (Potassium mercuric iodide solution) gave cream colored precipitate if alkaloids had indicated presence.
- **Dragendorff's test:** Extract on treatment with Dragendorff's reagent (Potassium bismuth iodide solution) produced reddish brown precipitate if alkaloids had indicated presence.
- **Wagner's test:** Extract on treatment with Wagner's reagent (Solution of iodine in potassium iodide) produced reddish brown precipitate if alkaloids had indicated presence.

- **Hager's test:** Extract on treatment with Hager's reagent (saturated solution of Picric acid) gave yellow precipitate if alkaloids had shown presence.
- **Tannic acid test:** Extract on treatment with 10% tannic acid solution gave buff colored precipitate if alkaloids had indicated presence.

- **Test for glycosides:**

a) General test for the presence of glycosides:

- **Part A:** Extract on warming in a test tube with 5ml of dilute (10%) sulfuric acid on a water bath at 100°C for 2min had filtered and pipetted off supernatant or filtrate. The acid extract had neutralized with 5% solution of sodium hydroxide. Further 0.l ml of Fehling's solution A and then B had added until alkaline (test with pH paper) and heated on a water bath for 2min. The red precipitate formed had noted and compared.
- **Part B:** Extract treated with 5ml of water instead of sulfuric acid. After boiling equal volume of water had added to the volume of NaOH used in the above test. Then 0.lml of Fehling's solution A and B had added until alkaline (test with pH paper) and heated on water bath for 2min. The quantity of red precipitate formed had noted and compared with the quantity of precipitate formed in Part-A. If the precipitate in Part-A were greater than in Part-B then glycoside were present. Since Part-B represented the free reducing sugar already present in the crude extract. Whereas Part-A represented free reducing sugar plus those related on acid hydrolysis of any sides.

b) Chemical tests for specific glycosides:

- **Test for Saponin glycosides:**
  - **Froth Test:** 1ml solution of drug had placed in water in a semi- micro tube, shaken well and the stable froth had noted.
- **Test for Anthraquinone glycosides:**
  - **Borntrager's test:** Extract had boiled with 1ml of dil. sulfuric acid in a test tube for 5min (with acid treatment anthracene glycosides were hydrolyzed to aglycone and sugars)

centrifuged or filtered while hot (if centrifuged hot, the plant material can be removed while anthracene aglycones were still sufficiently soluble in hot water, these were, however insoluble in cold water), pipetted out the supernatant or filtrate, cooled and shaken with an equal volume of dichloromethane (the aglycones dissolve preferably in dichloromethane). The lower dichloromethane layer had separated and shaken with half its volume with dilute ammonia. A rose pink to red color had produced in the ammonical layer (aglycones based on anthraquinones give the red color in the presence of alkali) if anthraquinone had indicated presence.

- **Modified Borntrager's test:** 200mg of the extract added with 2ml of dil. sulfuric acid; 2ml of aqueous ferric chloride (5% solution) had boiled for 5min and the test had continued as above. As some plant contains anthracene aglycone in a reduced form, ferric chloride used during the extraction caused their oxidation to anthraquinones, which confirmed Borntrager's test if anthraquinone had indicated presence.

c) Test for cardiac Glycosides:

- **Kedde's test:** Extract with one drop of 90% alcohol and 2 drops of 2% 3,5-dinitro benzoic acid (3,5-dinitro benzene carboxylic acid-Kedde's reagent) had added in 90% alcohol. It had made alkaline with 20% sodium hydroxide solution. Purple color produced had shown the color reaction with 3, 5-diinitrobenzoic acids depends upon the presence of α, β-unsaturated-o lactones in the aglycone.
- **Keller-killiani test (deoxy sugars):** Extract in chloroform had evaporated to dryness. Added 0.4ml of glacial acetic acid containing a trace amount of ferric chloride had to it. It had transferred to a small test tube and added 0.5ml of concentrated sulfuric acid had carefully by the side of the test tube, blue color appeared in the acetic acid layer if cardiac glycosides had indicated the presence.

- **Test for Tannins & Phenolic compounds:**

- **Ferric chloride test:** Extract had given blue green color with ferric chloride if phenols had indicated presence.
- **Vanillin Hydrochloride test**: When extract had treated with a few drops of vanillin hydrochloride reagent had given purplish red color if the test had positive results. Extract when treated with heavy metals, tannins precipitated out.

- **Test for flavonoids:**

- **Shinoda test (Magnesium Hydrochloride reduction test):** To the extract, few fragments of Magnesium ribbon and conc. Hydrochloric acid had added drop wise. Pink scarlet, crimson or red color appeared after few min if flavonoids had indicated presence.
- **Alkaline reagent test:** To the extract, had added a few drops of sodium hydroxide solution; production of an intense yellow color, which turns to colorless on the addition of a few drops of dil. HCl had indicated the presence of flavonoids.

- **Test for Proteins & Amino acids:**

- **Millon's test:** Extract when treated with 2ml of Millon's reagent had given a white precipitate which turns red upon gentle heating. Which had indicated the presence of proteins and amino acids.
- **Ninhydrin test:** When the extract boiled with 0.2% solution of Ninhydrin reagent (Indane 1, 2, 3- trione hydrate), it had given violet color if proteins and amino acids had indicated presence.

- **Test for Phytosterols & Triterpenoids:**
  - **Liebermann-Buchard test:** When had treated (boiled) the extract with few drops of acetic anhydride and cooled. Added conc. sulfuric acid from the sides of the test tube, brown ring formed at the junction of two layers and the upper layer turns green, which had indicated the presence of steroids and formation of deep red color had indicated the presence of triterpenoids.
  - **Salkowski test:** Extract treated with chloroform and few drops of conc. sulfuric acid, had shaken well and allowed to stand for some time, red color in the lower layer had indicated the presence of steroids and formation of yellow color in lower layer had indicated the presence of triterpenoids.

- **Test for carbohydrates:**
  - **Molisch's test:** The extract treated with few drops of alcoholic α-naphthol and when 0.2ml of conc. H_2SO_4 had added slowly through the side of the test tube, a purple or violet color ring formed at the junction indicated presence of carbohydrates.
  - **Benedict's test:** The extract added with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath if reddish brown precipitate had formed reducing sugars had indicated presence.

- **Barfoed's test:** It is a general test for monosaccharide. Heated the test tube containing 1ml of reagent and 1ml of extract fraction in a beaker of boiling water; if red cuprous oxide had formed within 2 min., monosaccharide had shown presence. Disaccharide on prolonged heating (about 10min) may also had caused reduction, owing to partial hydrolysis to monosaccharides.
- **Fehling's test:** Equal volume of Fehling's solution A (Copper sulfate in distilled water) and Fehling's solution B (Potassium tartarate and Sodium hydroxide in distilled water) had mixed and added few drops of extract and boiled, a brick red precipitate of cuprous oxide formed, if reducing sugars had indicated presence.

- **Tests for fats & fixed oils:**
  - **Spot test:** The small quantity of extract had pressed between two filter papers; the spot on 1st filter paper had indicated the presence of fixed oils.
  - **Saponification test:** Added few drops of 0.5N of alcoholic potassium hydroxide to small quantities of various extracts along with a drop of phenolphthalein separately and heated in a water bath for 1 -2 hrs. The soap formation or partial neutralization of alkali had indicated the presence of fixed oils and fats.
- **Tests for Terpenoids:** 0.2 g of the each extract had mixed with 2 ml of chloroform and concentrated H_2SO_4 (3ml) had carefully added form a layer. A reddish brown coloration of the interface had formed to indicate positive results for the presence of terpenoids.

3.2.5 Isolation and physicochemical characterization of volatile oil (Clevenger, 1928; API, 1999b)

3.2.5.1 Isolation of volatile oil: The determination of volatile oil from the plant drugs had made by distilling the drugs, collecting the distillate in a graduated tube. The aqueous portion of the distillate got automatically separated out from the mixture and returned to the distilling flask, and lastly had measured the volume of the oil.

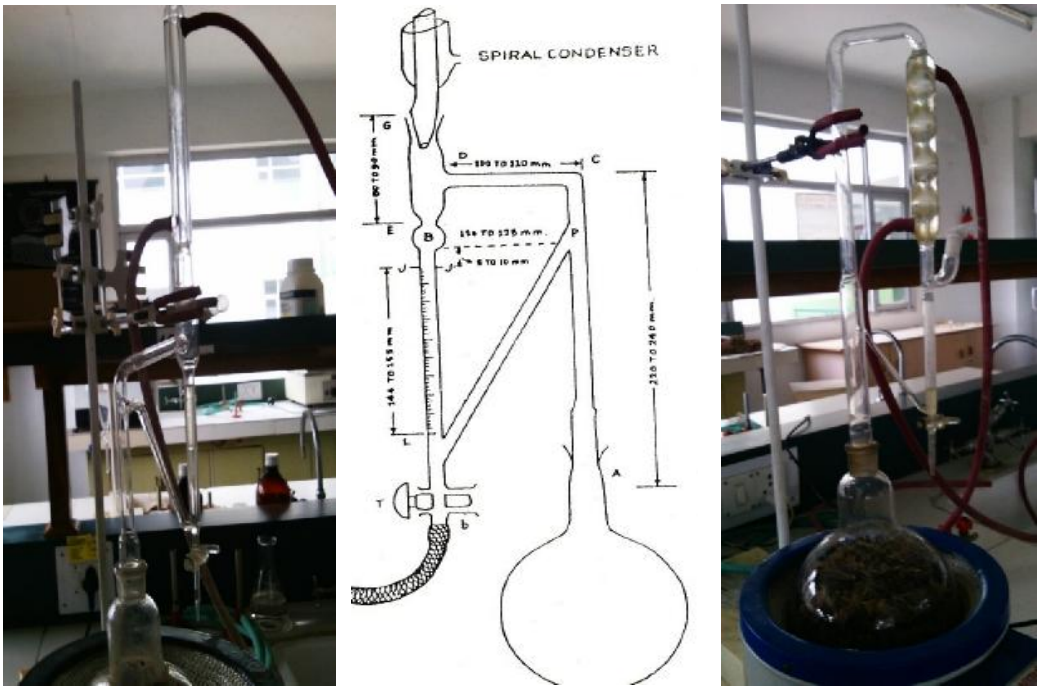

Figure 3.4: Clevenger apparatus

Apparatus: The Clevenger apparatus consists of the following parts (See Figure 3.4). The suitable dimensions of apparatus may be used provided that it permits complete distillation of the volatile oil. The dimensions of the apparatus constituting of following parts

- **Distilling flask –**A spherical flask, 2,000 ml capacity with ground neck, taper of ground socket 1 in 10.
- **Still head –**graduated measuring tube, and return flow tube made in one piece, in accordance with the following specifications.
- **Tube AC,** length –220 to 240 mm, internal diameter –13 to 15 mm.
- **Bulb CD,** length –100 to 110 mm, internal diameter –13 to 15 mm.
- **Spiral condenser –**Ground joint accurately fitting in the ground neck of the tube, taper 1 in 10. The distance between B and P is 120 to 125 mm.
- **Measuring tube JL –**Length of the graduated portion 144 to 155 mm capacity 2 milliliters graduated into fifths and fiftieths of a milliliter.
- **Tube PL** –Return flow tube –Internal diameter –7 to 8 mm. Leveling tube, length –450 to 500 mm. internal diameter 10 to 12 mm tapering at the lower end with a

wide top (20 to 25 mm diameter). Rubber tubing a-b length 450 to 500 mm and internal diameter 5 to 8 mm.

- **Heating mantle and sensitive regulative tap:** Heating mantle which can constantly provide heat to distilling flask and sensitive regulative tap continuous water.
- **Stand –**An asbestos covered ring retort stand and carrying clamp metal. Tubing connected by a short length of rubber tubing with the water inlet tube of the condenser jacket.

Procedure:

- The apparatus had cleaned before each distillation by washing successively with acetone and water, then inverting it, filling it with chromic acid and sulfuric acid mixture, after closing the open end, and allowing standing, and finally rinsing with water. A 500g of the coarsely powdered of each i.e. rhizomes of *H. spicatum* and galls *P. integerrima* dried drug together with 75 ml of glycerin for entrapment of the volatile constituents. 1000 ml of water had added in the 2 L distilling flask, along with a few pieces of porous earthen ware and one filter paper 15 cm cut into small strips, 7 to 12 mm wide in the distilling flask, which then had then connected to the still head.
- Before attachment with the condenser, water had ran into the graduated receiver, keeping the tap open until the water overflows from it. Any air bubbles found in the rubber tubing had carefully removed by pressing the tube with tap closed. Water tap had opened after the condenser attached to it. The contents of the flask had heated and frequent stirred by agitation until ebullition had commenced. The distillation had continued at a rate which keeps the lower end of the condenser cooler. The flask had occasionally rotated to wash down any material that adhering to its sides.
- Discontinued at the end of the 8 hours of heating. The apparatus had allowed to cool for 10 min and the tap had opened; as soon as the layer of the oil completely enters into the graduated part of the receiver the tap had closed and the volume had read.

- The distillation had continued again for another hour and the volume of oil had read again, as before after cooling the apparatus. If necessary, the distillation had continued again until successive readings of the volatile oil did not differ. The measured volume of volatile oil is considered to be the content of volatile oil in the drug.

3.2.5.2 Physicochemical characteristics of oil

The volatile oil both rhizomes of *H. spicatum* and galls *P. integerrima* after isolation, collection and measurement had coded as SUPYA-HA-VO and SUPYA-PC-VO respectively. Physicochemical characteristics provide a base line for suitability of oils (Parthiban et al., 2011). The various physicochemical properties of the samples SUPYA-HA-VO and SUPYA-PC-VO had determined such as color, odor, % yield, specific gravity, iodine number and saponification value using following methods. (Akubugwo, 2007)

- **Color determination:** Color of the respective oils was determined by physical observation in day light and under ultraviolet radiation of 254 and 366 nm using ultra violet chamber (Bamgboye, 2010).

- **Odor determination:** Odor of the respective oils had determined by organoleptic evaluation following Evans (Evans, W.C., 2002).

- **Determination of percentage oil yield:** The percentage oil yield had calculated by using following relation (AOAC, 2000).

  % yield of the oil= Volume of the oil/Total weight of the material used for extraction x 100

- **Determination of specific gravity:** For the determination of specific gravity of oils, a clean 50 ml specific gravity bottle had weighted (W_0). Then the bottle had filled to the brim with water and stopper had inserted. Extra water spilled out. The water on the stopper and bottle had carefully wiped off and reweighed (W_1). Same process had repeated, but using oil samples instead of water and weighted again (W_2).

The specific gravity of the all oil samples had calculated using the following formula (AOAC, 2000).

Specific gravity of test sample = W2 −W0/ W1−W0

Where

W_0 = Weight of empty specific gravity bottle

W_1 = Weight of water + specific gravity bottle

W_2 = Weight of test sample + specific gravity bottle.

- **Iodine value determination:** 0.2 g of respective oil had weighed into a conical flask. 10 ml of carbon tetrachloride and 20 ml of the Wij's solution had added to the flask and the solution had kept in dark for 30 min at room temperature. 15 ml of 10 per cent potassium iodide solution with 100 ml of distilled water had added to the flask. The resulting solution had titrated against 0.1 M sodium thiosulphate ($Na_2S_2O_3$), using starch as indicator till the end point where the blue black coloration becomes colorless. A blank titration had carried out at the same time starting with 10 ml carbon tetrachloride. Iodine value had then calculated by the following formula (AOAC, 2000; Kyriakidis et al., 2000; Knothe, 2002; Khoddami, et al., 2011).

  Iodine number = {(B − S) × N ×12.69} /Weight of the sample

  Where

  B = 0.1 N sodium thiosulfate required (ml) by blank

  S = 0.1 N sodium thiosulfate required (ml) by sample

  N = Normality of sodium thiosulfate solution.

- **Determination of saponification value:** 2 g of each oil sample had weighted into a clean dried conical flask and 25 ml of alcoholic potassium hydroxide had added. A reflux condenser had attached to the flask and heated for an hour with periodic shaking. The appearance of clear solution indicated the completion of saponification. Then 1 ml of 1 % phenolphthalein indicator had added and the hot excess alkali had titrated with 0.5 M hydrochloric acid (HCl) until it reached the end point where it turned colorless.

A blank titration had carried out at the same time and under the same condition. The saponification value had calculated as (AOAC, 2000).

Saponification value = (b-a)/m ×8.05

Where

b = 0.5 N HCl required (ml) by the blank
a = 0.5 N HCl required (ml) by the sample.

3.2.6 Gas chromatography-mass spectroscopy

3.2.6.1 Gas chromatography-mass spectroscopy (GC-MS) of volatile oils

The volatile oils isolated from both rhizomes of *H. spicatum* and galls *P. integerrima* had transferred to Eppendroff's tube coded as SUPYA-HA-VO and SUPYA-PC-VO respectively. Then sent for analysis by gas chromatography-mass spectroscopy (GC-MS) to CIL/ SAIF Panjab University, Chandigarh, Punjab, India. The gas chromatography-mass spectroscopy had carried out on TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m X 0.25mm, 0.25µm) column and S/SL Injector. The injector temperature had kept at 280˚C and MS transfer line temperature had kept at 240˚C along with ion source temperature 240˚C. The column temperature had programmed between 60˚-210˚C at 3˚C/min using helium as carrier gas at a carrier flow rate of 1ml/min. Injection volume had 1.0µl prepared in n-hexane having Split flow 40ml/min. The mass spectra had taken at 75 eV with mass scan range from *m/z* 40-500 amu. The individual constituents had identified by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, U.S. Department of Commerce) compounds and Wiley (John Wiley & Sons Ltd) libraries (Rauf et al., 2013).

3.2.6.2 Gas chromatography-mass spectroscopy (GC-MS) of pet ether extract

The coarsely powdered drug (1Kg of each) of both selected plants material had extracted with pet ether via hot extraction process using Soxhlet apparatus (Figure 3.2) for 8 hrs. After completion of extraction, the solvent had removed by distillation and concentrated in

vacuum. Pet ether extract of rhizomes of *H. spicatum* and galls *P. integerrima* had transferred to Eppendroff's tube coded as SUPYA-HA-PE and SUPYA-PC-PE respectively and then sent for analysis by gas chromatography-mass spectroscopy (GC-MS) to CIL/ SAIF Panjab University, Chandigarh, Punjab, India. The gas chromatography-mass spectroscopy had carried out on TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m X 0.25mm, 0.25µm) column and S/SL Injector. The injector temperature had kept at 280˚C and MS transfer line temperature had kept at 240˚C along with ion source temperature 240˚C. The column temperature had programmed between 60˚-210˚C at 3˚C/min using helium as carrier gas at a carrier flow rate of 1ml/min. Injection volume had 1.0µl prepared in n-Hexane having split flow 40ml/min. The mass spectra had taken at 75 eV with mass scan range from *m/z* 40-500 amu. The individual constituents had identified by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, U.S. Department of Commerce) compounds and Wiley (John Wiley & Sons Ltd) libraries (Rauf et al., 2013).

3.2.7 Pharmacological *in-vivo* evaluations

3.2.7.1 Acute toxicity study (OECD 423 Limit test)

Animals

Wistar albino rats of either sex weighing 180-220g had procured from Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar, Haryana and had housed at Animal house facility, Shoolini University, Solan, HP (Registration no. 1541/PO/a/11/CPCSEA). The animals had acclimatized to experimental laboratory conditions for 4 to 5 days before experimentation which had maintained at temperature 25±2°C, humidity 45±5% and 12:12 hour light and dark cycles. Animals had caged in steel wire cages, fed with standard food pellets diet from Ashirwad Industries, Chandigarh and water *ad libitum*. The study had conducted after obtaining ethical clearance from Institutional Animal Ethics Committee (IAEC), Shoolini University, Bajhol, Solan vide protocol approval no. IAEC/SU-PHARM/12/023.

Procedure

Acute toxicity studies had conducted as per acute toxicity class method (Limit test) following OECD guidelines (423 a) (OECD guidelines, 2001). Animals had been divided

into 9 groups each group had 6 animals n=6 (3+3). The pet ether and hydro-ethanolic (90%) extracts had been suspended in 0.2% carboxy methyl cellulose (CMC) solution to achieve optimum consistency of suspension and to maintain uniformity of dosage. The overnight fasted rats for each dose had fed with pet ether and hydro-ethanolic extract of rhizomes *H. spicatum* and galls of *P. integerrima* each at two dose levels 2000 and 5000 mg/kg body weight *p.o*. The animals had observed on 0, 7th and 14th day for behavioral signs, toxic manifestations and mortality. The experimental protocol for acute toxicity studies is described in Table 3.1.

Table 3.1: Experimental protocol for evaluation of acute toxicity study

S.No.	Groups (n=6)	Treatment
1	NC	Normal saline treated
2	SUPY-HA-PE-1	*H. spicatum* pet ether extract 2000 mg/Kg *(p.o.)*
3	SUPY-HA-PE-2	*H. spicatum* pet ether extract 5000 mg/Kg *(p.o.).*
4	SUPY-HA-HE -1	*H. spicatum* hydro-ethanolic extract 2000 mg/Kg *(p.o.).*
5	SUPY-HA-HE-2	*H. spicatum* hydro-ethanolic extract 5000 mg/Kg *(p.o.).*
6	SUPY-PC-PE-1	*P. integerrima* pet ether extract 2000 mg/Kg *(p.o.)*
7	SUPY-PC-PE-2	*P. integerrima* pet ether extract 5000 mg/Kg *(p.o.)*
8	SUPY-PC-HE-1	*P. integerrima* hydro-ethanolic extract 2000 mg/Kg *(p.o.)*
9	SUPY-PC-HE-2	*P. integerrima* hydro-ethanolic extract 5000 mg/Kg *(p.o.)*

3.2.7.2 Evaluation of anti-tussive activity

The pet ether and hydro-ethanolic extracts of rhizomes of rhizomes of *H. spicatum* and galls of *P. integerrima* had evaluated for their anti-tussive effect using citric acid induced cough model in guinea pig (Laude et al., 1994; Nepali et al., 2011).

Animals:

Male Hartley guinea pigs weighing 500-700 g had been used in present study. The animals had been procured from Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar, Haryana and had housed at Animal house facility, Shoolini University, Solan, HP (Registration no. 1541/PO/a/11/CPCSEA). The animals had divided into different groups, each group comprising 4 animals (n=4). The animals had acclimatized to experimental laboratory conditions for 4 to 5 days before experimentation which had maintained at temperature 25±2°C, humidity 45±5% and 12:12 hour light and dark cycles. Animals had caged in steel wire cages, fed with standard food pellets diet from Ashirwad Industries, Chandigarh and water *ad libitum*. The study had conducted after obtaining ethical clearance from Institutional Animal Ethics Committee (IAEC), Shoolini University, Bajhol, Solan vide protocol approval no. IAEC/SU-PHARM/12/023. The experimental protocols used for present study had carried out in accordance with guidelines of committee for the purpose of control and supervision of experiments on animals.

Screening of animals: Animals underwent a screening procedure before pre-treatment with drugs. On the first day after a 3 min acclimatization period animals had first exposed to normal saline and subsequently 5 min later to aerosolized 7.5% citric acid for a period of 10 min. Animal selection criterion had made either on the basis of number of coughs (< 7 or > 15) on exposure to aerosolized 7.5% citric acid or their tendency to cough on exposure to normal saline. Animal produced cough outside, this stated limit on exposure to of citric acid or aerosolized saline had excluded as this had taken as an indication of infection or hyper-reactivity. The success rate of screening method was 87.5% as 70 of 80 animals passed the test.

Figure 3.5: Evaluation of anti-tussive activity (Cough response)

Experimental protocol

The animals passing the selection criterion had randomly divided into 10 groups. Each group was comprised of 4 animals (n=4). The pet ether and hydro-ethanolic (90%) extracts had been suspended in 0.2% carboxy methyl cellulose (CMC) solution to achieve optimum consistency of suspension and to maintain uniformity of dosage. Codeine dissolved in Tween 80 (1%) had been administered at 10mg/kg as standard. The samples orally administered 60 min before the second challenge of aerosolized citric acid (7.5% w/v) solution. Table 3.2 represents the study design and groups assignment.

Table 3.2: Experimental protocol for evaluation of anti-tussive activity

S.No	Groups	Treatment
1	NC	Normal saline treated
2	SUPYHAPE-1	*H. spicatum* pet ether extract 200 mg/Kg *(p.o.).*
3	SUPYHAPE-2	*H. spicatum* pet ether extract 400 mg/Kg *(p.o.).*
4	SUPYHAHE -1	*H. spicatum* hydro-ethanolic extract 200 mg/Kg *(p.o.).*
5	SUPYHAHE-2	*H. spicatum* hydro-ethanolic extract 400 mg/Kg *(p.o.).*
6	SUPYPCPE-1	*P. integerrima* pet ether extract 200 mg/Kg *(p.o.).*
7	SUPYPCPE-2	*P. integerrima* pet ether extract 400 mg/Kg *(p.o.).*
8	SUPYPCHE-1	*P. integerrima* hydro-ethanolic extract 200 mg/Kg *(p.o.).*
9	SUPYPCHE-2	*P. integerrima* hydro-ethanolic extract 400 mg/Kg *(p.o.).*
10	CODE- ST	Codeine-10mg/kg (*i.p.)*

Evaluation of cough response

After 60 min of oral administration of all the drugs and extracts the cough challenge had given at the same time of day for each animal and minimum of 24 h interval had allowed between challenges to eliminate any short term prophylaxis. Animals had allowed free access to food and water up to the time of testing. Each animal had placed in a Perspex chamber, dimensions 30 cm X 20 cm X 20 cm and exposed to an aerosolized aqueous solution of 7.5% w/v citric acid for a period of 10 min. The output of the aerosolizer (INCO Laboratories, Ambala, India) had 0.25 ± 0.02 ml per min and same aerosolizer had used throughout the experiment. The animals had watched continuously by the trained observer. Coughs frequency and latency time (seconds) to initial cough response had noted refer Figure 3.5.

3.2.7.3 Evaluation of anti-asthmatic activity

The pet ether extracts of both rhizomes of rhizomes of *H. spicatum* and galls of *P. integerrima* had evaluated for had evaluated for their antiasthmatic activity in systemically antigen induced asthmatic subjects. The animals had sensitized using ovalbumin for a period of 14 days. The antiasthmatic potential had evaluated individually by the means of thorough *in vivo* experimentation **(**Bande et al., 2012; Mahajan, 2011).

Animals

Male Hartley guinea pigs weighing 500-700 g had only been used in present study to avoid hormonal complications of female which may affect the pathogenesis of asthma. The animals had been procured from Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar, Haryana and had housed at Animal house facility, Shoolini University, Solan, HP (Registration no. 1541/PO/a/11/CPCSEA). The animals had divided into different groups, each group comprising 4 animals (n=4). The animals had acclimatized to experimental laboratory conditions for 4 to 5 days before experimentation, which had maintained at temperature 25±2°C, humidity 45±5% and 12:12 hour light and dark cycles. Animals had caged in steel wire cages, fed with standard food pellets diet from Ashirwad Industries, Chandigarh and water *ad libitum*. The study had conducted after obtaining ethical clearance from Institutional Animal Ethics Committee (IAEC), Shoolini University, Bajhol, Solan vide protocol approval no. IAEC/SU-PHARM/12/023. The experimental protocols of present study had in accordance with guidelines of committee for the purpose of control and supervision of experiments on animals.

Sensitization of animals

The animals had actively sensitized by the method of Andersson et al. (1981). Briefly the intraperitoneal injection of ovalbumin 20 µg and $Al(OH)_3$ 100 mg in normal saline (0.2 ml) had administered twice in a gap of seven days. Two weeks later (on 14^{th} day) from the first day of sensitization, the animals had placed in histamine chamber equipped with an ultrasonic nebulizer (INCO Ltd., Ambala, India) and challenged with 0.5% ovalbumin aerosol to verify that the sensitization has occurred. Withdrawn the animals

from antigen exposure at the first sign of respiratory abnormality. The animals showing airway hyper-responsiveness to the inhaled antigen had referred to as sensitized animals. The success rate of sensitization method was 85 % as 67 of 80 animals showed airway abnormalities to inhaled antigen.

Experimental protocol

The sensitized animals had randomly divided into 10 groups. Each group had comprised of 4 animals (n=4). All the plant extracts had suspended in 0.2% CMC solution and standard: aminophylline had dissolved Tween 80 (1%). Aminophylline 50 mg/kg had administered as standard treatment. Animals of sensitized control and normal control groups had administered with normal saline as vehicle. The plant extracts had administered orally as test drugs and standard had injected interperitoneally once a day for seven days. The administration of aminophylline through *i.p.* route had been standardized producing significant protection against asthma in most of the experiments in our laboratory (Bande et al., 2012). The detailed experimental protocol has been summarized in Table 3.3.

Table 3.3: Experimental protocol of anti-asthmatic study

S.No.	Groups	Treatment
1.	NC	Non sensitized/Normal saline treated
2.	SC	Sensitized Control/Normal saline treated
3.	SUPYHAPE-1	*H. spicatum* pet ether extract 200 mg/Kg *(p.o.)*.
4.	SUPYHAPE-2	*H. spicatum* pet ether extract 400 mg/Kg *(p.o.)*.
5.	SUPYPCPE-1	*P. integerrima* pet ether extract 200 mg/Kg *(p.o.)*.
6.	SUPYPCPE-2	*P. integerrima* pet ether extract 400 mg/Kg *(p.o.)*.
7.	AMN	Aminophylline-50mg/kg (*i.p.*)

Figure 3.6: Evaluation of respiratory hyper-reactivity

Evaluation of respiratory hyper-reactivity

Final challenge with ovalbumin aerosol (0.5% in normal saline) had performed on the seventh day of treatment, 30 min after the last dose. The guinea pigs had placed in the nebulizer and challenged with an aerosol of ovalbumin (5mg/ml in water) for 30 min. Airway abnormalities of the animals had visibly monitored by two trained observers who had blinded to the group assignment of the animals for the changes occurring in respiratory activity of the animals subjected to different treatments. Evaluation of latency time (min) for the appearance of first respiratory abnormalities assessed as the time between onset of aerosolization and the first stroke of cough and the frequency of cough till 10 min after the first cough stroke had measured refer Figure 3.6 (Gupta et al., 1968).

Collection of broncho-alveolar lavage (BAL) fluid and lung tissue homogenate preparation

Twenty four hours after aerosolized ovalbumin challenged, animals had sacrificed with sodium pentobarbitone (200 mg/kg *i.p.*). The trachea had immediately cannulated and lungs had lavaged with 5 x 4 ml aliquots of Ca^{2+} and Mg^{2+} free 0.1M phosphate buffered saline solution of pH-7.0. To standardize the lavage technique, 50% of instilled medium (the maximum volume that could be consistently recovered) had withdrawn from each animal. The collected BAL fluid had centrifuged and used for further biochemical estimations. The lung tissue had dissected out, washed in cold saline, blotted

dry and homogenized (10%) in phosphate buffer saline pH 7.4. The homogenate had centrifuged using a refrigerated centrifuge and the supernatant had used for further biochemical estimations.

Assessment of airway inflammation

Total leukocyte counts (TLC) in BAL

The BAL fluid had centrifuged at 150Xg for 10 min at 4ºC using refrigerated centrifuge (Remi CPR-24 Plus). The supernatant had discarded and the cells had resuspended in 1 ml 0.1M PBS containing 0.1 M EDTA. Total and differential leukocyte count had performed manually with a haemocytometer under DMWB Series Motic digital microscope.

Estimation of lipid peroxidation (TBARS) in BAL and lung homogenate

The TBARS levels lung tissue homogenates and BAL fluid had estimated spectrophotometrically as described by Ohkawa et al (1979). Briefly, to each test tube, 0.5 ml supernatant of 1g lung tissue homogenate in 5 ml PBS, 0.5 ml of normal saline (0.5 ml of BAL fluid in case of measurement of TBARS level in BAL fluid), 1 ml of 20% trichloroacetic acid (TCA) and 0.25 ml of TBA reagent (200 mg of thiobacbituric acid dissolved in a mixture of 30 ml distilled water and 30 ml of acetic acid) had added. The test tubes had kept for boiling at 95ºC for one hour. To each of the test tubes, 3 ml of n-butanol had added, vortexed for 5 min and centrifuged at 200Xg for 10 min. The separated butanol layer had read spectrophotometrically against reagent blank at 535 nm. TBARS concentration is expressed in terms of µmol of malondialdehyde per ml of BAL fluid and per grams of wet lung tissue (Oliveira et al., 2006; Ohkawa et al., 1979).

Estimation of NOX level in BAL and lung homogenate

The amount of stable nitrite (nitrite and nitrate), the end product of NO generation by RPE cells had determined in BAL fluid and lung tissue homogenates by a spectrophotometric assay according to the method of Green et al (1982). Briefly, 50 µl supernatant of 1g tissue homogenated in 5 ml PBS (supernatant of BAL fluid in case of determination in BAL fluid) had mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1%

naphthylethylenediamine dihydrocholoride, 5% H_3PO_4), and incubated at room temperature for 10 min. The absorbance had read at 540 nm. The amount of nitrite had calculated from a $NaNO_2$ standard curve and expressed as µmol per ml of BAL fluid and lung tissue homogenates.

Determination of glutathione content in BAL and lung homogenate

The determination of glutathione content in BAL fluid and lung tissue homogenates had measured spectrophotometrically according to the method of Ellmann et al., 1959. Briefly, 1ml supernatant of 1g tissue homogenated in 5 ml PBS (supernatant of BAL fluid in case of determination in BAL fluid) had mixed with 1 ml of protein precipitant (20% trichloro acetic acid + 0.1 mM EDTA) and centrifuged at 150Xg for 10 min. 200 µL of the supernatants obtained had mixed with 1.8 ml of Ellmann's reagent (10mM solution of 5, 5' –Dithiobis- 2-nitrobenzoic acid of pH 7.0). The resulting solutions had read spectrophotomtrically at 412 nm after 5 min against blank (1.8 ml Ellmann's reagent mixed with 200µL protein precipitant) (Ellman, 1959).

Histopathological studies

After BAL Fluid had obtained, the lung tissue had fixed in 10% neutral buffered formalin for 24 h. The lung tissue had embedded in paraffin, and then cut into 4 mm thickness sections, stained with H&E solution (haematoxylin; Sigma MHS-16 and eosin, sigma HT110-1-32). The tissue had subsequently mounted and cover-slipped with Dako-mounting medium (Dakocytomation; Denmark Carpinteria CA). The lung tissue sections had studied for Peribponchial cellular infiltration by acute and chronic inflammatory cell infiltrate comprising of neutrophils, eosinophils and lymph mononuclear cell infiltrate, widening of interalveolar septa because of vasodilatation, edema & increase in no. of macrophages, goblet cell hyperplasia and bronchodilatation.

Statistical analysis

All the values had expressed as mean±standard deviation (SD) and had statistically analyzed using one way ANOVA followed by Bonferroni's multiple comparison tests as

post hoc test. The $p<0.05$ had considered to be statistically significant. A software Graph Pad Prism (version 5) was used for statistical analysis.

3.2.8 Pharmacological *in-vitro* evaluations

3.2.8.1 Anti-oxidative activity

Reactive oxygen species (ROS) are various forms of activated oxygen, which include free radicals such as superoxide ions, and hydroxyl radicals, as well as non-free-radical species such as hydrogen peroxide (1, 2). The antioxidant activity of the essential oil and pet ether extracts of both rhizomes of *H. spicatum* and *P. integerrima* galls had determined spectrophotometrically using the 2,2-Diphenyl-1- picrylhydrazyl (DPPH) radicals scavenging assay with slight modification. Ascorbic acid had purchased from Qualigens, India and DPPH had purchased from Sigma Aldrich, US. All the chemicals and solvents used had of analytical grade. Using serial method for dilution the standard antioxidant drug Ascorbic Acid (25 mg) had taken and dissolved in distilled methanol and diluted up to 50 ml. From this stock solution, different micrograms standard solution of 20, 40, 60, 80 and 100 µg/ml had prepared. 25 mg of each volatile oils of rhizomes of *H. spicatum* (SUPY-HA-VO) and galls of *P. integerrima* (SUPY-PC-VO) respectively, had taken and dissolved in distilled methanol and diluted up to 50 ml. From this stock solution, different micrograms solution of 20, 40, 60, 80 and 100 µg/ml had prepared by dilution method. Similarly for the pet ether extracts SUPY-HA-PE and SUPY-PC-PE of rhizomes of *H. spicatum* and galls of *P. integerrima* respectively, 25 mg of extract had taken and dissolved in distilled methanol and diluted up to 50 ml. From this stock solution, different micrograms solution of 20, 40, 60, 80 and 100 µg/ml had prepared by dilution method. 5 ml of each solution had taken in a test tube and 1 ml of 0.001 M of DPPH solution had added to it. All these solutions had kept in dark for 30 min. Also 5 ml methanol had taken and 1 ml of DPPH solution had added, for control solution. At the end of incubation period, the mixtures had examined for the antioxidant activity using UV- Visible spectrophotometer at wavelength of 517 nm. The experiments had performed with

triplicate readings. The antioxidant activity had expressed as IC_{50} using log-dose inhibition curve. (Bhatt et al., 2008; Arulmozhi et al., 2007; Uddin et al., 2012 b, c). Percent inhibition had determined using the formula as follows:

% Inhibition = Control abs - Extract abs × 100/Control

3.2.8.2 Anti-inflammatory activity

The role of nitric oxide (NO) in diseases has generated a considerable discussion over the past several years since the journal *Science* named it the molecule of the year in 1992. NO is an important bio-regulatory molecule, which has a number of physiological effects including control of blood pressure, neural signal transduction, platelet function, antimicrobial and antitumor activity. For beneficial functions low concentrations of NO are sufficient, in most cases. But during infections and inflammations, formation of NO is elevated and may bring about some undesired deleterious effects. The continuous exposure to free radicals generated from the chronic inflammation has been found to cause more cancers than environmental chemicals (Ames and Gold, 1990). NO is a short-lived (half-life 3–30 s) colorless gas that is moderately soluble in water (up to 2 mmol/L) but highly soluble in organic solvents (Ignarro et al., 1987; Nathan, 1992). It is lipophilic in nature and can diffuse between cells very easily. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS), (Moncada et al., 1991). The three main isoforms are neuronal (n) NOS, inducible (Lala, 1998) NOS, and endothelial (e) NOS. Generally, nNOS and eNOS are expressed constitutively in neurons and endothelial cells, respectively, though they can also be expressed by other cells. NO has an unpaired electron, hence is a free radical (NO). NO becomes nitrosonium cation (NO^+) or nitroxyl anion (NO^-) by donating or accepting an electron, respectively (Nathan and Xie, 1994). NOS is synthesized in a variety of cell types from multiple mammalian species and can produce consistent, high concentrations of NO upon induction with cytokines and or bacterial lipopolysaccharide (LPS) (Nathan and Xie, 1994). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of

excessive NO generation in vivo. Nitric oxide generation is evaluated by assay of nitric oxide scavenging where nitric oxide had generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (Green *et al.*, 1982; Marcoci et al., 1994a, b), which interacts with oxygen and liberated nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen which lead to reduced production of nitric oxide (Marcocci et al., 1994a, b). Sodium nitroprusside (5 mM) in phosphate-buffered saline had mixed with different concentrations of the standard, pet ether extract and volatile oil dissolved in the suitable solvent systems and carried out incubation at 25 °C for 150 min. A solution of potassium nitrite (KNO_2) had used for standard curve after preparing different dilutions 10, 20, 40, 80 and 100 µg /mL concentration. Ascorbic acid had used as positive standard. After preparing different dilutions 10, 20, 40, 80 and 100 µg /mL concentration of both pet ether extracts of rhizomes of *H. spicatum* (SUPY-HA-PE) and galls of *P. integerrima* (SUPY-PC-PE) and volatile oils of rhizomes of *H. spicatum* (SUPY-HA-VO) and galls of *P. integerrima* (SUPY-PC-VO) in dilution had reacted with Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromaphore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme had read at 546 nm and referred to the absorbance of standard solutions of Ascorbic acid treated in the same way using Griess reagent. The experiments had performed with triplicate readings (Marcocci et al., 1994a, b). The nitric oxide scavenging activity had expressed as IC_{50} using log-dose inhibition curve.

3.2.8.3 Immunomodulatory activity

Induction of immune cell proliferation is always related to the immunomodulatory property. Pet ether extracts of rhizomes of *H. spicatum* (SUPY-HA-PE) and galls of *P. integerrima* (SUPY-PC-PE) had along with volatile oils of rhizomes of *H. spicatum* (SUPY-HA-VO) and galls of *P. integerrima* (SUPYPCVO) evaluated for Immunomodulatory potential. (Yeap et al., 2007, 2010)

Isolation of human PBMC

Blood sample (20 to 25 ml) had taken from different healthy donors using 25 ml syringe and diluted with same volume of PBS-BSA-EDTA and centrifuged by the diluting blood sample and carefully layering on Ficoll-Paque Plus (Amersham Biosciences, USA). The mixture had centrifuged under at 400x g for 40 min at 18 to 20℃. The undisturbed lymphocyte layer had carefully transferred out. The lymphocyte had washed and pelleted down with three volumes of PBS-BSA-EDTA for twice and resuspended RPMI-1640 media. (Flowlab, Australia). Cell counting had performed to determine the PBMC cell number with equal volume of trypan blue.

MTT cell viability assay

The effect of the pet ether extracts SUPYHAPE and SUPYPCPE and volatile oil SUPYHAVO and SUPYPCVO on cell viability of PBMC had determined by using a colorimetric technique which is 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 100 µl of RPMI-1640 media with 10% of FBS had added into the all the well except row A in the 96 well plate (TPP, Switzerland). Then, 100 µl volatile oil and 100 µg/ml diluted extract had added into row A and row B. A series of two fold dilution of pet ether extracts (SUPYHAPE and SUPYPCPE) and volatile oil (SUPYHAVO and SUPYPCVO) had carried out down from row B until row G. Row H had left untouched and the excess solution (100 µl) from row G had discarded. Hundred µl of lymphocyte (from human peripheral blood) with cell concentration at 5×10^5 cells/ml had added into all wells in the 96 well plate to make up the final volume of 200 µl and thus diluted the extract into the concentration 25, 50, 100, 200 µg/ml. All the plates had incubated for 24 hrs at 37°C, 5% CO_2 and 90% humidity incubator. 20 µl of MTT (Sigma, USA) at 5 mg/ml had added into each well in the 96 well plates and incubated for four hours in 37°C, 5% CO_2 and 90% humidity incubator. 100 µl of medium with MTT had removed from every well and 100 µl DMSO (Fisher Scientific, UK) had added to each well to extract and solubilize the formazan crystal by incubating for 10 min in 37º C, 5% CO_2 incubator. Finally, the plate had read at 595 nm wavelength by using µ Quant ELISA

Reader (Bio-tek Instruments, USA). The results of the plant extract had compared with the result of Con A (1 µg/ml) and LPS (1 µg/ml) and control (without extract) by using same method along with control had assayed in triplicate in three independent experiments.

The percentage of proliferation had calculated by the following formula:

% age of proliferation = $(OD_{[sample]} - OD_{[control]}) / OD_{[control]} \times 100$