MATERIAL & METHODS

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3.1. Procurement and authentication of plant material

Leaves and roots of *Leonotis nepetaefolia* (LN) and whole plant of *Oxalis corniculata* (OC) were collected in the flowering season from Vadodara, India. Plant parts were authenticated from Prof. M. Daniel, Botany Department and voucher specimen no. PHR/07-08/AT/LN and PHR/07-08/AT/OC respectively, were deposited at herbarium, Pharmacy Department, The M S University of Baroda, Vadodara, India for future reference.

3.2. Morphological evaluation

The macroscopic features of the fresh plant *L. nepetaefolia* and *O. corniculata* were determined using the methods described in standard texts by Evans [189]. Leaves of LN were dried under shade for 10-15 days until free of moisture while aerial parts of OC and roots of LN first dried in sun light for few days then kept under shade until free of moisture.

3.3. Determination of foreign matter

Medicinal plant material was subjected to determination of any contamination by moulds or insects, and other animal products. The determinations of odour, discoloration, slime or signs of deterioration by visual and morphological examination were also done. The plant parts that were not useful in the formulations were also considered as foreign matter [190].

3.4. Microscopy study

Anatomical sections, surface preparations of the fresh leaves and powdered samples for the microscopy and chemo-microscopy were carried out according to methods mentioned by Evans [189] and Brain & Turner [191].

3.4.1. Quantitative microscopic evaluation

Surface preparations of leaf of LN and OC were prepared by standard methods mentioned by Brain & Turner [191]. Evaluation of these preparations was carried out by determining stomatal index, palisade ratio, vein islet & vein termination no & stomatal no by reported methods [192].

3.5. Assessment of quality of plant material

The dried plant material was subjected to size reduction to get fine powder (# 40) and the powdered plant material was store in an air tight container for further investigation. The quality of the plant material was assessed as per WHO guidelines [190] and procedures stated in Ayurvedic pharmacopoeia of India [193].

3.6. Proximate analysis

Following determinations were performed on the plant material using reported methods [190].

- Loss on drying (LOD)
- Total ash
- Acid insoluble ash
- Water soluble ash
- Alcohol soluble extractives
- Water soluble extractives
- Haemolytic index
- Foaming index
- Swelling index
- Elemental and heavy metal analysis

3.6.1. Determination of loss on drying (LOD)

About 5 g of air- dried material was weighed into previously dried & tarred weighing bottle & dried in an oven at 105° C for 5 h. Dried until two consecutive weighing was not differ by more than 5 mg, unless otherwise specified in the test procedure. Then the loss of weight in mg/g of the air dried plant material before and after drying was noted [190].

3.6.2. Determination of ash values

3.6.2.1. Total ash

The total ash method is designed to measure the inorganic content of crude material. Place about 4 g of ground air dried material, accurately weighed, in a previously ignited and tarred silica crucible. Spread the material in an even layer and ignite it by gradually increasing the heat upto

500 – 600° C until it is white, indicating the absence of carbon. Cool in desiccator and weigh. Calculate the content of the total ash in mg/g of air dried material [190].

3.6.2.2. Acid insoluble ash

To the crucible containing total ash, add 25 mL of dil. hydrochloric acid, cover with watch glass and boil gently for five minute. Rinse the watch glass with 5 mL of hot water; add this liquid to the crucible. Collect the insoluble matter on ash less filter paper and wash with hot water till filtrate is neutral. Transfer the filter paper containing insoluble matter to the original crucible, and ignite to get a constant weight. Allow the residue to cool in a desiccator for 30 min, and then weigh without delay. Calculate the content of acid insoluble ash in mg/g of air dried material [190].

3.6.2.3. Water soluble ash

To the crucible containing total ash, add 25 mL of water and boil gently for 5 min. Collect the insoluble matter on ashless filter paper, transfer the filter paper containing insoluble matter to the original crucible and ignite in a crucible for 15 mins at a temperature not exceeding 450° C. Allow the residue to cool in a desiccator for 30 mins, and then weigh without delay. Subtract the weight of this residue in mg from the weight of total ash. Calculate the content of water soluble ash in mg/g of air-dried material [190].

3.6.3 Determination of extractive values

3.6.3.1. Alcohol soluble extractive

Weigh accurately 4 g of coarsely powdered air-dried material and transfer to a glass-stopper conical flask. Macerate with 100 mL of the alcohol for 6 hrs, shaking frequently, and then allow standing for 18 hrs. Filter rapidly taking care not to lose any solvent, transfer 25 mL of the filtrate to a tarred flat-bottom dish and evaporate to dryness on a water-bath. Dry at 105° C for 6 hours, cool in a desiccator for 30 min and weigh without delay. Calculate the content of extractable matter in mg/g of air dried material [190].

3.6.3.2. Water soluble extractive

Weigh accurately 4 g of coarsely powdered air-dried material and transfer to a glass-stopper conical flask. Macerate with 100 mL of the water for 6 h, shaking frequently, and then allow standing for 18 h. Filter rapidly taking care not to lose any solvent, transfer 25 mL of the filtrate

to a tarred flat-bottom dish and evaporate to dryness on a water-bath. Dry at 105°C for 6 h, cool in a desiccator for 30 min and weigh without delay. Calculate the content of extractable matter in mg/g of air dried material [190].

3.6.4. Determination of haemolytic activity

The haemolytic activities of methanol and aqueous extracts of plant material were determined by comparison with that of a reference material, saponin R, which has a haemolytic activity of 1000 U/g. a suspension of erythrocytes, is mixed with equal volumes of a serial dilution of the plant material extract. The lowest concentration to cause complete haemolysis is determined out simultaneously with saponin R. Examine the tubes and record the dilution at which total haemolysis has occurred, indicated by a clear, red solution without any desposit of erythrocytes.

To prepare the erythrocyte suspension fill a glass-stopper flask to one tenth of its volume with sodium citrate (36.5 g/L), swirling to ensure that the inside of the flask is thoroughly moistened. Introduce a sufficient volume of blood freshly collected from a healthy ox and shake immediately. Place with phosphate buffer pH 7.4 and carefully make up the volume. To prepare the reference solution, transfer about 10 mg of saponin R, accurately 100 mL. Serial dilutions of the methanol and water extract of formulations were calculated using the following formula [190]

$$1000 \text{ X } a/b$$

Where 1000= the defined haemolytic activity of saponin R in relation to ox blood,

a= quantity of saponin R that produces total haemolysis (g),

b= quantity of formulation that produces total haemolysis (g)

3.6.5. Determination of foaming index

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials is measured in terms of foaming index. Reduce about 1 g of the plant material to coarse powder (sieve size no. #40), weigh accurately and transfer to a 500 mL conical flask containing 100 mL of boiling water. Boil at moderate temperature for 30 min. Cool and filter into a 100 mL volumetric flask and add sufficient water through the filter to dilute to volume. Pour the

decoction into 10 stoppered test-tubes ml, and adjust the volume of the liquid in each tube with water to 10 mL. Stopper the tubes and shake them in a lengthwise motion for 15 sec, two shakes per second. Allow to stand for 15 min and measure the height of the foam [190].

Foaming index: 1000/a

Where, a is the volume in mL of the decoction used for preparing the dilution in the tube where foaming to height of 1 cm is observed.

3.6.6. Determination of swelling index

In 25 mL glass stoppard measuring cylinder 1 g of plant material was introduced & 25 mL of water was added. The material was shaken for every 10 min for 1 h & stand for 3 h. The volume occupied by plant material was measured [190].

3.6.7. Elemental and heavy metal analysis

About 5 g of the powdered plant material was ignited in muffle furnace to obtain ash. A 100 mg of the ash was dissolved in 10 mL of 1 N HCl, the solution was filtered, filtrate was diluted to 50 mL with deionised water and used for analysis of heavy metals by atomic absorption spectroscopy [190].

3.7. Qualitative phytochemical screening of plant material

3.7.1. Successive solvent extraction

The presence of the different phytoconstituents in the plant material can be detected by subjecting to successive solvent extraction with increasing polarity sovents (petroleum ether, benzene, chloroform, ethyl acetate, methanol and water successively). The plant material (100 g) were subjected to soxhlet extraction with different solvents (400 mL each) successively, plant material was dried in air before subjecting to further extraction. Then the extracts were concentrated & air-dried % yield, colour, consistency of air dried extract was recorded and extracts were kept in dessicator for further experiments. The prepared extracts were analysed by qualitative chemical tests to ascertain different phytoconstituents [194].

3.7.2. Identification of phytoconstituent by chemical test

The successive extracts were subjected to chemical test for the presence of specific class of phytoconstituents like carbohydrates, alkaloids, antraquinones, saponins, phytosterols, phenolics, tannins, flavonoids & proteins using reported methods. Alkaloids were tested by Dragendorff, Wagner, Hager and Mayer reagents. Phenolics and flavonoids were tested by $FeCl_3$, shinoda, alkali and lead acetate test. Carr-piece reagent and salkowiski reaction were used for the detection of terpenoids. Steroids and sterols were detected by vanillin sulphuric test and libermann-burchard test. Carbohydrates were detected by Molisch's reagent and Fehling's tests. Amino acid and proteins were confirmed by ninhydrin, biuret and millon's tests [195].

3.7.3. Confirmation of phytoconstituent by thin layer chromatographic studies

Various extracts obtained by successive extraction were subjected to thin layer chromatographic studies using silica gel GF_{254} precoated plates, by reported methods to confirm presence of various phytoconstituents. TLC parameters are described in the following table. These results were compared with results obtained in the qualitative chemical test [196].

Table 3.1: Chromatographic parameters for TLC profile of extracts

Phytoconstituent	Solvent System (*L .nepetaefolia*)	Solvent System (*O. corniculata*)	Detection
Alkaloids	Et Ac: MeOH: $CHCl_3$: NH_3 (3:2:2:0.1)	Tol:EA:DEA (7:3:1)	Dragendroff's reagent.
Volatile Oils	Toleune: ethyl acetate. 93:07	Toleune: ethyl acetate. 93:07	Vanillin sulphuric acid reagent.
Carbohydrates and glycosides.	Butanol: GAA: H_2O (4:1:5)	Butanol: GAA: H_2O (4:1:5)	Anisaldehyde sulphuric acid Reagent
Terpenoids	Benzene : Ether (2:3)	Benzene : Ether (2:3)	20 % $SbCl_3$ in $CHCl_3$
Phytosterols	Pet Eth: Et Ac (4:1)	Pet Eth: Et Ac (4:1)	Leibermann burchard's reagent
Flavonoids	Ethyl acetate: gl. acetic acid: formic acid: water (100:11:11:26)	Ethyl acetate: gl. acetic acid: formic acid: water (100:11:11:26)	Natural product-PEG reagent

3.8. Determination of secondary metabolites

3.8.1. Determination of total phenolics

Reagents

Preparation of test sample: Stock solution of sample was prepared by dissolving 10 mg of methanol extract in 10 mL of methanol.

Preparation of reagent: Folin ciocalteu reagent: 1: 2 dilution of the reagent with distilled water was prepared.

20 % sodium carbonate solution: 20 g of anhydrous sodium carbonate was dissolved in 100 mL of distilled water.

Procedure: Total phenolic contents in the various extracts were determined by the modified Folin-Ciocalteu method [197]. An aliquot of the extracts was mixed with 5 mL Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL of 20% sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using the Shimadzu UV1800 UV-VIS spectrophotometer. From the stock solution of 1 mg/mL of standard 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 mL were taken which gave 50, 75, 100, 125, 150, 175, and 200 µg/mL concentrations, respectively. Total phenolic content was expressed as mg/g gallic acid equivalent using the following equation based on the calibration curve: $y = 0.098x + 0.057$, $R^2 = 0.984$, where x was the absorbance and y was the gallic acid equivalent (mg/g).

3.8.2. Determination of total flavonoids

The total flavonoid content was determined by sum of two methods aluminum chloride colorimetric method and 2, 4 – dinitrophenyl hydrazine colorimetric method [198]. The sample solutions of 1.5 mg/mL and 5 mg/mL of various extracts of selected plant material were prepared for estimation of total flavonoids content.

3.8.2.1. Aluminum chloride colorimetric method

Reagents

Preparation of standard solution: A stock solution of 1 mg/10 mL of quercetin was prepared in methanol.

Preparation of reagents: 10 % aluminum chloride: 10.0 g of aluminum chloride was dissolved in 100 mL of distilled water.

1 M Potassium acetate: 9.814 g of potassium acetate were dissolved in 100 mL distilled water.

Procedure: From the stock solution of standard 0.1, 0.2, 0.3, 0.4 and 0.5 mL were taken and diluted upto 10 mL with methanol which gave 10, 20, 30, 40 and 50 µg concentrations respectively. The standard solution were separately mixed with 1.5 mL of 95 % methanol, 0.1 mL of 10 % aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min. the absorbance of reaction mixture was measured at 415 nm. The amount of 10 % aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 1.5 mg/ mL (1 mL) of the sample solution were reacted with aluminum chloride for determination of flavonoid content as described in above procedure.

3.8.2.2. 2, 4- Dinitro phenyl hydrazine colorimetric method

Reagents

Preparation of standard solution: A stock solution, 20 mg/ 10 mL of Naringin was prepared in distilled water.

Preparation of reagents: 1% 2, 4 -dinitrophenyl hydrazine reagent: 1 g of 2, 4- DNPH was dissolved in to the 100 mL of distilled water.

1 % potassium hydroxide: 1 g of potassium hydroxide was dissolved in 100 mL of 70 % methanol.

Procedure: From stock solution of standard 1.25, 2.5, 5, 7.5 & 1 mL were taken and diluted up to 10 mL with distilled water which give 250, 500, 1000, 1500, 2000 µg/mL concentrations respectively. Each standard solution (1 mL) were separately mixed with 2 mL of 1 % 2, 4-dinitro phenyl hydrazine reagent and 2 mL of methanol, and then kept at 50^0 C for 50 min. After cooling to room temperature, the reaction mixtures were mixed with 5 mL of 1 % potassium hydroxide in 70 % methanol and incubated at room temperature for 2 min. Then 1 mL of the mixture was taken, mixed with 5 mL of methanol and centrifuged at 1000 rpm for 10 mins to remove the precipitate. The supernatant was collected and adjusted to 25 mL. The absorbance of the supernatant was measured at 495 nm. Similarly, 5 mg /mL sample solution were reacted with 2, 4-dinto phenyl hydrazine for determination of flavonoid content as described in the above procedure.

3.8.3. Determination of total flavonols

Total flavonols in the plant extracts were estimated using the method of Kumaran and Karunakaran [199]. To 2.0 mL of sample (standard), 2.0 mL of 2% $AlCl_3$ ethanol and 3.0 mL (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h at 20°C. Extract samples were evaluated at a final concentration of 0.1 mg/ mL. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y = 0.005x - 0.122$, $R^2 = 0.979$, where x was the absorbance and was the quercetin equivalent (mg/g).

3.9. Extraction, fractionation and isolation of phytoconstituents

Plant material from both the plants were extracted by using various solvents and further fractionated to isolate individual secondary metabolites as marker, both for chemical and biological evaluation. All the fractions were also analysed by HPTLC fingerprinting for the presence of chemical constituents.

3.9.1. Preparation of extracts and sub-fractions of *Leonotis nepetaefolia* (LN)

Dried leaf and root powder (2 kg, separately) was extracted in methanol using soxhlet extraction method, while aqueous extract was prepared by decoction, resulting in total methanol extract of leaf (TMLNL- 615 g), root (TMLNR-511 g) and total water extract of leaf (TWLNL-728 g) and root (TWLNR- 489 g). Total methanol extract was then diluted with hot distilled water and successively fractionated [200] to diethyl ether (ET of TMLNL-82.5 g and ET of TMLNR- 76.4 g), ethyl acetate (EA of TMLNL-78.5 g and EA of TMLNR- 67.5 g) and n-butanol (n BuOH of TMLNL- 102 g and nBuOH of TMLNR 45 g) fractions as per the given scheme (Figure) and the residual methanol extract (RMLNL-84.4 g and RMLNR- 63.3 g) was collected. All the extracts, were then dried in vacuum and subjected to TLC for detection of phytoconstituents [201].

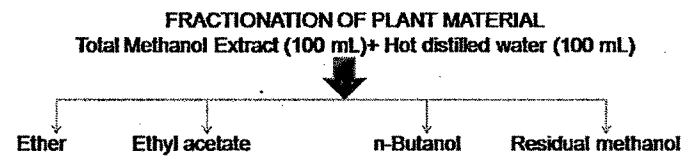

Figure 3.1: Fractionation of hydroalchoholic extract of LNL and LNR

3.9.2. Total flavonoid fraction of LNL

The dried leaf powder (1 kg) is extracted exhaustively in methanol by soxhlet extractor for 7 days. Methanol extract is concentrated and shaken with equal quantity of dichloromethane and methanol. Lower layer is discarded and resulting extract is concentrated [195]. This is subjected to column chromatography by using alumina as stationary phase and various mixtures of ethyl acetate and methanol (10, 25, 50, 75 and 100%) are used as mobile phase to collect flavonoid rich fraction. Presence of flavonoids was ascertained by TLC with NPPEG reagent as derivative agent [196]. Fractions F2 to F30 were combined to get flavonoids rich fractions.

3.9.3. Terpene fraction

Leaf powder and root powder (1 kg each) were extracted separately with dichloromethane in soxhlet extractor exhaustively, dichloromethane extract is concentrated and dissolved in equal volume of methanol and 4% lead acetate. Solution is filtered and filtrate is concentrated. This is extracted with dichloromethane and the extract is dried and monitored on TLC [195]. The terpene rich fraction was stored in a dessicator for further studies. A part of this extract (100 g) was subjected for column chromatography for isolation of compound by using silica gel (for column) as stationary phase and required quantities of petroleum ether and ethyl acetate as eluant (10, 25, 50 and 80 %). Fractions were monitored by TLC and compound LN-02 isolated from fraction F15 to F25 and purified by repetitive preparative TLC by using pet ether: ethyl acetate (4:1) as mobile phase. LN-02 was separated at Rf 0.53 and detected by spraying with 10% sulphuric acid.

3.9.4. Alkaloidal fraction

Leaf powder was soaked in 10% ammonium hydroxide overnight and extracted exhaustively with methanol. Methanol extract (100 g) of leaves is partitioned between ethyl acetate and 5% sulphuric acid (100 mL each). Aqueous acidic layer is basified with ammonium hydroxide to pH 9 and alkaloid is extracted in chloroform (each time quantity of 100 mL) until free of alkaloid. Chloroform layer is combined, evaporated in vacuum to dryness and stored in a dessicator for further studies [202].

Isolation of LNLAL-01: Alkaloid was isolated from alkaloidal fraction by column chromatography. The fraction is adsorbed on silica gel column and eluted with various proportions of toluene, ethyl acetate and methanol (Toluene: ethyl acetate and ethyl acetate: methanol-10, 20, 30, 50, 80 and 100% v/v) and 20 mL elute was collected each time. LNLAL-01 was found in mixture of toluene and ethyl acetate (10-30%) F4 to F20 and purified by repetitive preparative TLC by using ethyl acetate: chloroform: methanol (3:1.5:0.5 v/v) as mobile phase and detected in UV chamber.

3.9.5. Preparation of extracts and sub-fractions from whole plant of *Oxalis corniculata* (OC)

Whole plant of *O. corniculata* was subjected to solvent extraction for preparation of following fractions [200]. Dried Whole plant powder (2 kg) was extracted in methanol using soxhlet extraction method, while aqueous extract was prepared by decoction, resulting in total methanol extract (TMOC-585 g) and total aqueous extract (TAOC-734 g). Total methanol extract was then diluted with hot distilled water and successively fractionated to diethyl ether (DETMOC-45.5 g), ethyl acetate (EATMOC-68 g) and n-butanol (274 g) fractions as per the given scheme (Figure 3.2) and the residual methanol extract (RMOC-73 g) was collected. All the extracts, were then dried in vacuum and subjected to TLC for detection of phytoconstituents [196].

3.9.6. Isolation of compound (OC-01) from flavonoids rich fraction (OCFF)

N-butanol fraction of total methanol extract was further treated with dilute acid and dilute base to get flavonoid rich fraction which was then subjected to HPTLC and HPLC [203]. The fraction (OCFF) was found rich in the content of flavonoids by thin layer chromatography (TLC) studies giving four spots of flavonoids when sprayed with natural product reagent and observed under UV 366 nm [196]. OCFF (25 g) was subjected to column chromatography using silica gel as stationary phase and ethyl acetate: methanol (9:1) as an eluant. Eluates, about 1 to 80 in number, each of 25 mL, were collected as fractions and monitored for phytoconstituents by TLC. Fraction number 12 to 35, indicating presence of flavonoids on TLC, were combined and evaporated in a vacuum giving yellow brown residue. Compound OC-01 was then isolated by repetitive preparative TLC using silica gel HF_{254} as stationary phase and ethyl acetate: methanol: water (4:0.5:0.5) as mobile phase. The isolated compound OC-01 was then purified by subjecting it to column chromatography using Sephadex LH-20, with 25% methanol as the eluant, which

afforded as yellow powder (470 mg). Identification was made on the basis of Co-TLC and spectral characteristics [204]. The purity of compound was determined by HPLC (Shimadzu LC AT20, Japan) using an Excil C-18 column (ODS, 25 X 4.6 mm, 5 μm) and acetonitrile: water (1:3) as mobile phase.

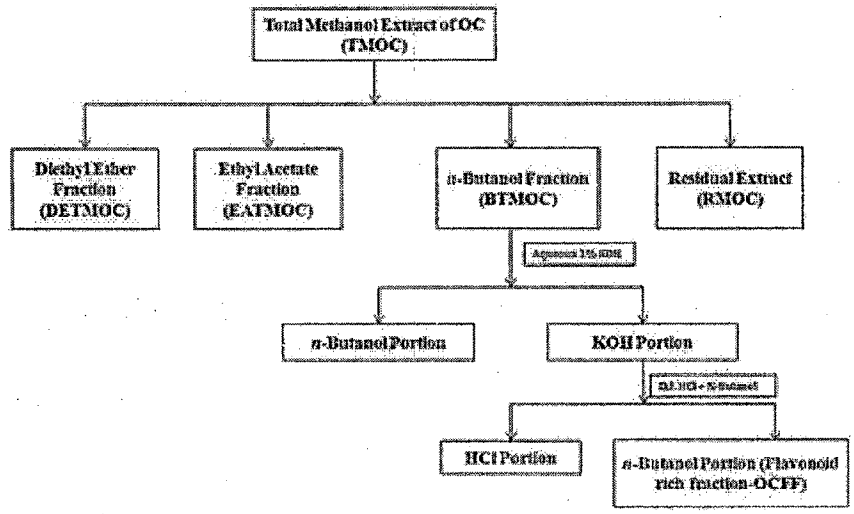

Figure 3.2: Preparations of extracts and its fractions of *O. corniculata*

3.9.7. Unsaponifiable fraction of LN and OC

Plant powder (leaf, root of LN and whole plant OC, each 800 g) is exhaustively extracted separately with petroleum ether in a soxhlet apparatus. Petroleum extract is concentrated and dried. This is refluxed with 5% w/v alcoholic KOH for 2 h. This is then filtered and diluted with hot water. This is taken in a separating funnel and extracted with ether three times. Ether extract is combined and washed with water in separating funnel. Lower layer is rejected and upper layer is shaken with 3% w/v alcoholic KOH three times. Wash ether layer with two 50 mL quantities of water. Ether layer is collected and dried [205]. The fraction was analysed by TLC and stored in a dessicator for further studies.

3.10. HPTLC studies

HPTLC is the most simple separation technique available today which give better precision and accuracy with extreme flexibility for various steps (stationary phase, mobile phase, development technique and detection) [206].

3.10.1. Materials required

Selection of plate & adsorbent : 20X10 cm, 20X20 cm or 10X10 cm TLC plate of silica gel 60 F_{254} of Merck was used for detection.

Chemicals: Standards were obtained from Sigma and all the solvents used were of analytical grade from sd fine ltd.

Test samples

Whatman filter paper (No. 41)

3.10.2. Instruments

Sample applicator: Liomet V, Hamilton glass syringe (100 µL)

Solvent chamber: CAMAG twin trough (20X20, 20X10 or 10X10)

TLC scanner: CAMAG 3

UV cabinet (254 and 365 nm)

CAMAG Dipping chamber, plate dryer

3.10.3. HPTLC fingerprinting

TLC densitometry analysis of various extracts/fractions was done on silica gel GF_{254} prepared plates. Extract was separated in three different solvent systems of varying polarity for separation of different phytoconstituents and these were analysed using CAMAG HPTLC system with Linomet V applicator. Different proportions of hexane, toluene, chloroform, ethyl acetate, methanol and water were tried, among these Petroleum ether: ethyl acetate (3:1 v/v), Ethyl acetate: formic acid: methanol (4:0.25:0.5 v/v), Toluene: ethyl acetate: methanol: ammonia (1:1.5:2:0.25 v/v) mobile phases were found most suitable for separation of non polar, medium polar and polar compounds of methanolic extract of leaf of *L. nepetaefolia*. Hexane: ethyl acetate (3:1 v/v), Benzene: diethyl ether (2:3 v/v) and ethyl acetate: methanol: formic acid: water (2:1:0.25:0.5 v/v) were found to be most suitable solvent system for separation of phytoconstituents of *L. nepetaefolia* root extract. Hexane: Ethyl acetate (3:1 v/v), Ethyl acetate: chloroform: methanol (4:0.5:0.5), Toluene: ethyl acetate: formic acid: water (20:100:10:10 v/v)

mobile phases were found most suitable for separation of non polar, medium polar and polar compounds from extracts of *O. corniculata.* Developed plates were scanned in CAMAG scanner at absorption maxima of 254 nm and 366 nm. Plates were than sprayed with anisaldehyde sulphuric reagent, heated in an oven at 105°C for 10 min and scanned at 540 nm for presence of various phytoconstituents [196].

Chromatographic conditions

- Stationary phase: 20X10/10X10 cm precoated silica gel 60 F_{254} TLC plates from Merck
- Chamber saturation time: 20 min.
- Solvent run: 85 mm
- Detection: UV 254, 366
- Derivatization: anisaldehyde sulphuric reagent

3.10.4. HPTLC quantification of marker compounds

3.10.4.1. Quantitative estimation of LNLAL-01 in *L. nepetaefolia* leaves by HPTLC

3.10.4.1.1. Calibration curve for LNLAL-01

Standard solution: Stock solution of LNLAL-01 (0.1 mg/mL) was prepared by dissolving 10 mg of accurately weighed LNLAL-01 in chloroform:methanol (1:1 v/v) and making up the volume of the solution to 100 ml with methanol. The aliquots (1 to5 mL) of stock solution were transferred to 100 mL volumetric flasks and the volume of each was adjusted to 100 mL with methanol to obtain standard solutions containing 1, 2, 3, 4 and 5 µg/mL LNLAL-01 respectively. Ten µL each of standard solutions of LNLAL-01 were applied using Linomat-5 applicator, with the band width of 8 mm, between the bands 5 mm in triplicate on separate precoated silica gel 60 F254 TLC plates.

Test solution: The stock solution (1 mg/mL) was prepared by dissolving 10 mg each of alkaloid fraction and chloroform extracts of *L .nepetaefolia* in 10 mL of methanol separately. Ten µL each sample solutions were applied in triplicate on a precoated silica gel 60 F_{254} TLC plate with band width of 8 mm.

3.10.4.1.2. Development

The plate was developed in a twin trough chamber (CAMAG) using chloroform: ethyl acetate: methanol (3.5:1.0:0.5 v/v) as solvent system.

3.10.4.1.3. Detection and quantification

After developing the plates were dried and scanned at 290 nm using a TLC SCANNER-3 (CAMAG). Peak areas for each band were recorded. Calibration curve was obtained by plotting peak area vs concentration of LNLAL-01. A spectrum of LNLAL-01 in test samples was confirmed by overlaying the spectra of LNLAL-01.

3.10.4.2. Quantitative estimation of β-sitosterol in *L. nepetaefolia* by HPTLC

3.10.4.2.1. Calibration curve for β-sitosterol

Stock solution of β-sitosterol (100 µg/ mL) was prepared in methanol and different amounts (100– 600 ng/spot) were applied on a TLC plate, using Linomat V for preparing six point calibration graph of peak area versus concentration.

3.10.4.2.2. Quantification of β-sitosterol in test sample

Petroleum ether extract of LNL and LNR, and unsaponifiable fractions of LNL and LNR were used as test samples (1 mg/mL). Five µL of sample solution was applied in triplicate on a TLC plate, developed and scanned as above. Peak areas were recorded and the amount of β-sitosterol was calculated using the calibration plot.

3.10.4.2.3. Development

The linear ascending development was carried out in a CAMAG twin trough chamber (20 cm X10 cm) which was presaturated with 20 mL mobile phase toluene: chloroform: methanol (4:4:1 v/v) for 20 min at room temperature (25±2 °C and 40% relative humidity). The length of the chromatogram run was 8 cm.

3.10.4.2.4. Detection and quantification

TLC plates were dried in current air with the help of a hair dryer. The post chromatographic derivatization was carried out in anisaldehyde-sulphuric acid followed by heating at 110 °C for 5 min. Quantitative evaluation of the plate was performed in absorption-reflection mode at 540 nm, using a slit width of 6.0X0.45 mm and data resolution 100 µm step and scanning speed 20 mm/s with a computerized Camag TLC scanner-3 integrated with winCATS 4 software. Quantification of β-sitosterol in the extract of *L. nepetaefolia* root and leaves was performed by external standard method, using pure β-sitosterol as standard

3.10.4.3. Quantitative estimation of OC-01 in *O. corniculata* by HPTLC

3.10.4.3.1. Calibration Curve for OC-01

Stock solution of OC-01 (100 µg/ mL) was prepared in methanol and different amounts (100–800 ng/spot) were applied on a TLC plate, using Linomat V for preparing six point calibration graph of peak area versus concentration.

3.10.4.3.2. Quantification of OC-01in Test Sample

Methanol extract and flavonoids rich fraction of *O. corniculata* were used as test samples (1 mg/mL). Five µL of sample solution was applied in triplicate on a TLC plate, developed and scanned as above. Peak areas were recorded and the amount of b-sitosterol was calculated using the calibration plot.

3.10.4.3.3. Development

The linear ascending development was carried out in a CAMAG twin trough chamber (20 cm X10 cm) which was presaturated with 20 mL mobile ethyl acetate: methanol: water: formic acid (4:4:0.5:0.5 v/v) for 20 min at room temperature (25±2 °C and 40% relative humidity). The length of the chromatogram run was 8 cm.

3.10.4.3.4. Detection and Quantification

TLC plates were dried in current air with the help of a hair dryer. Quantitative evaluation of the plate was performed in fluorescence-reflection mode at 338 nm, using a slit width of 6.0X0.45 mm and data resolution 100 µm step and scanning speed 20 mm/s with a computerized CAMAG TLC scanner-3 integrated with winCATS 4 software. Quantification of OC-01 in the extract of *O. corniculata* was performed by calculation of peak areas of each band. Calibration curve was obtained by plotting peak area vs concentration of OC-01. A spectrum of OC-01in test samples was confirmed by overlaying the spectra of OC-01.

3.10.4.4. Method validation

The developed methods were validated in terms of limit of detection, limit of quantification, linearity, range, accuracy and precision according to ICH guidelines [207].

3.10.4.4.1. Accuracy

The recovery studies were performed by applying the known amount of the samples and the percentage recovery of that same amount is been calculated against the theoretical values. Pre-analyzed samples were applied at three different concentration levels of the standard (80%, 100% and 120% w/w) containing LNLAL-01, β-sitosterol, OC-01 and analyzed with the instrument set up as same in case of the estimations of the sample. This was done to check the recovery of the drug at different levels in the extracts. The experiments were performed in triplicate.

3.10.4.4.2. Repeatability

Repeatability (precision) was determined by repeated analysis of standard sample using the same equipment, same analytical procedures, same laboratory and on the same plate. Repeatability of measurement was determined by spotting 10 µL of standard solution on TLC plate, after development spot was scanned six times without changing position. The % RSD was determined for LNLAL-01, β-sitosterol and OC-01.

3.10.4.4.3. Linearity and specificity

Linearity was determined by spotting various concentrations of standard and finding regression. The specificity of the method was ascertained by comparing the R_f value and the peak purity was assessed by comparing the spectrum of LNLAL-01, β-sitosterol and OC-01 with those acquired at the peak start, peak apex, and peak end positions of sample bands.

3.11. HPLC studies

3.11.1. Materials and chemicals

Test samples

Standard substance

Excil ODS C-18 column (5 µm, 4.6 X 250 mm) SGE, India

Nylon membrane filter paper (0.2 µm)

Vacuum filtration assembly

Sonicator

Solvents (HPLC grade, Qualigens, India)

Double distilled water

3.11.2. Instrument

Isocratic HPLC system, a Rheodyne model 7725 injector (20 µL) with LC-20 AT pump and SPD-20A UV/VIS detector (Prominence, Shimadzu, Japan)

3.11.3. Quantitative estimation of LNLAL-01 in *L. nepetaefolia* leaves by HPLC

The mobile phase consisted of acetonitrile-water (3.5:6.5, v/v) with 1% diethylamine. The flow rate was 0.8 mL/min. The detector was set at 290 nm and all the measurements were performed at room temperature.

Preparation of standard: A stock solution of LNLAL-01was prepared in methanol at 1 mg/mL. The stock solution was serially diluted with mobile phase to obtain the desired concentrations (1.0-200.0 µg/mL).

3.11.4. Quantitative estimation of OC-01 in *O. corniculata* by HPLC

The mobile phase consisted of acetonitrile-water (2.5:7.5, v/v) with 1% glacial acetic acid. The flow rate was 0.8 mL/min. The detector was set at 338 nm and all the measurements were performed at room temperature.

Preparation of standard: A stock solution of OC-01 was prepared in methanol at 1 mg/mL. The stock solution of OC-01 was serially diluted with mobile phase to obtain the desired concentrations (1.0-100.0 µg/mL).

3.11.5. Method Validation

The standard samples were injected onto the LC column to determine if the detector output was a linear function of concentration over the nominal concentration range of the standards (0.100-100.0 µg/mL). The linear regression of the curve for peak area ratio (y) versus concentration of standard (x) was determined by 1/x2 (the reciprocal of the square of the standard concentration). Standard samples with low and high concentrations were used to evaluate accuracy and precision of the assay. Six replicate standard samples at each concentration were analyzed in a single sequence for the within-day assessment. For the between-day assessment, six replicate standard

samples at each concentration were analyzed on three occasions. The precision of the assay was determined by calculating the RSD. The accuracy of the assay was determined by calculating the % deviation of the observed concentrations from the nominal concentrations (relative error, RE %). The lower limit of quantitation was defined as the lowest drug concentration that can be determined with an RE and precision (relative standard deviation) of less than 20%. Standard samples were analyzed to determine the recovery in test samples. Recovery was calculated by comparing the observed concentrations with the spiked concentrations [207].

3.12. Assessment of therapeutic claims

Various herbs have been identified as possessing antioxidative and anti-inflammatory properties, and some of these are currently being used to treat disorders caused by reactive oxygen species (ROS). The scope of ROS-mediated diseases is believed to be broad, and herbs that scavenge reactive oxidant chemicals before they damage tissue may prevent or slow each of these processes.

3.12.1. Acute toxicity studies

Acute toxicity studies were performed following OECD guidelines (OECD 423, Acute Toxic Class Method) [208]. Oral dose of 2000 mg/kg, 3000 mg/kg of the test extracts and 500 mg/kg, 1000 mg/kg for the test fractions were given to different groups of female rats. The animals were observed for first 4 hours of treatment to next 14 days. The evaluated parameters were mortality, signs and symptoms of toxicity, body weight, food consumption and necropsy observations. Observations were compared with normal control group.

3.12.2. *In-vitro* studies

In-vitro antioxidant activity was measure by following three methods for both the plant material

A) DPPH radical scavenging assay
B) Total antioxidant capacity by phosphomolybdenum method
C) Ferric chloride reducing power

3.12.2.1. DPPH radical scavenging assay

The free radical scavenging capacity of extract and fractions was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [209]. Various concentrations of the samples (10, 20, 40, 80 and 100 µg/mL) were prepared in methanol (0.010 mg/mL for the standard compounds) and in water. Methanolic solution of DPPH (200 mM, 0.5 mL) was mixed with different concentrations of each test compound in methanol and 0.1 M acetate buffer (pH 5.5, 1.0mL), and the absorbance change at 517 nm was measured 30 min later. The reaction solution without DPPH at 0 minute was used as a blank. Measurements were performed in duplicate, and the IC_{50} was determined graphically.

$$\% \text{ inhibition} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

where $A_{control}$ and A_{sample} are the absorbance values of the control and test sample, respectively. The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50}) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentration.

3.12.2.2. Total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of extract and its different fractions was evaluated by the method of Prieto et al. (1999) [210]. An aliquot of 0.1 ml of sample solution (containing 10, 20, 40, 80 and 100 µg/mL) was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Methanol (0.1 mL) was used as blank in place of sample. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min, and then cooled at room temperature. The absorbance of each solution was then measured at 695 nm against a blank in a double beam UV–Visible spectrophotometer Shimadzu UV1800.

3.12.2.3. Ferric chloride reducing power

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay et al., 2003) [211]. The reducing power can be determined by the method of Athukorala et al. (2006) [212]. One mL extract (containing 10, 20, 40, 80 and 100 µg/mL) is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 mL) is mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (6 mM) and

absorbance is measured at 700 nm. Ascorbic acid and butylated hydroxytoluene (BHT) are used as positive control for above procedures.

3.12.3. Rapid screening of antioxidant constituent by HPTLC

Different fractions and extracts that were active in antioxidant assay were semi-quantitatively visualized by HPTLC in CAMAG system. All active extracts/fractions (10 µL) were applied on TLC plate and plate was developed in a solvent system consisting of ethyl acetate: chloroform: methanol: water (3:0.5:0.5:0.25 v/v). The plate was scanned at 254 and 366 nm and then dipped in 0.2% solution of DPPH in methanol, and scanned at 445 nm. The appearance of white and yellow coloured spots on violet colour background was the indirect measure of radical scavenging components from extracts and fractions [213].

3.12.4. Evaluation of biological activity of *Leonotis nepetaefolia*

3.12.4.1. *In-vitro* evaluation of the antiproliferative effects of LN extracts/fractions

3.12.4.1.1. Cell Cultures

Human non-small lung cancer cell lines A549; purchased from NCCS, pune, India; were maintained in Dulbecco Modified Eagle Medium (DMEM) routinely supplementing with 10% heat-inactivated fetal bovine serum (FBS), 5% penicillin, 5% streptomycin and incubated at 37°C in humidified 5% CO_2 atmosphere with media replacement every 2 days. When the cell cultures reached 90% confluence they were harvested using 0.25% trypsin and 1 mM EDTA solution (Sigma) and reseeded in new flasks at a density of 10-15 × 10^3cells/cm^2 [214].

3.12.4.1.2. MTT Assay

Cell viability was evaluated by using 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for the tested extracts against the cancer cell lines. The assay depends on the cleavage of the tetrazolium salt (MTT) into formazan blue by the mitochondrial enzyme succinate dehydrogenase. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of viable cells present. Thus, the MTT assay is potentially useful for assaying antiproliferative activities of materials. For this purpose the cancer cells were seeded in complete medium in a 96-well plate at a density of $1x10^5$ cells/well. After reaching confluent, the cells were incubated for 48 hr with test samples (10, 20, 50, 100 and 200 µg/well of different extracts/fractions). DMSO and PBS (the vehicles) were used as controls. The medium was then discarded and the adherent cells were washed twice with phosphate buffer

solution (PBS), then 20 µL of MTT stock solution (5 mg/mL in PBS) were added to each well and the plates were further incubated overnight at 37 °C and 5% CO_2. DMSO (100 µL) was added to each well to solubilize the formazan crystals produced by viable cells. After complete dissolving of formazan blue, the absorbance was measured at 570 nm, using microplate reader. The percentage of cell viability was calculated according to the equation described in Moongkarndi *et al.* (2004) [215].

$$\% \text{ of cell viability} = (\text{OD of treated cells} / \text{OD of control cells}) \times 100$$

The concentrations required for inhibition of 50% of cell viability (IC_{50}) were calculated.

3.12.4.1.3. Statistics

The data were subjected to correlation coefficient by using Graph pad prism statistical analysis software. The correlation of the data was determined by Student 't' test. $P < 0.05$ was considered as statistically significant.

3.12.4.2. *In-vitro* 5-lipoxygenase (LOX) inhibition assay

LOX-inhibiting activity was measured by modifying the spectrophotometric method developed by Tappel [216]. 5-LOX type I-B (Soybean, 5-lipoxygenase) and linoleic acid were purchased from Sigma.

The final enzyme concentration was 167 U/mL. Test substances were added as DMSO solutions (final DMSO concentration of less than1.0%). The enzyme solution was stored on ice, and controls were measured at intervals throughout the experimental period to ensure that enzyme activity was constant. Baicalein, a well-known inhibitor of 5-LOX and indomethacin were employed as a positive control. The IC_{50} values were determined by linear interpolation by the measuring points closest to 50% activity.

The absorbance at 234 nm was measured with a microplate elisa reader. The percent inhibition of LOX activity was calculated as follows

$$\% \text{ inhibition} = (A-B)/A \times 100$$

where A=absorbance at 234 nm without a test sample, and B = absorbance at 234 nm with a test sample.

3.12.4.3. *In-vitro* ROS inhibitory activity

3.12.4.3.1. Isolation of human neutrophils

Heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank and neutrophils were isolated by the method of Siddiqui *et al.* (1995) [217]. Whole blood was mixed with Ficoll paque (Pharmacia) and left for the sedimentation of unwanted red blood cells. After 30 min, the buffy coat was layered on the Ficoll paque in the centrifuge tube. It was centrifuged for 30 min at 1500 rpm. After discarding the supernatant, RBCs were lysed by mixing with hypotonic ammonium chloride solution (0.83%). It was centrifuged again and the neutrophils were washed with MHS (modified Hank's solution, pH 7.4; 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.33 mM $NaHPO_4$, 20 mM HEPES, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 10 mM glucose) and resuspended at a concentration of 1 $X10^5$ cells/mL.

3.12.4.3.2. Respiratory burst inhibition assay

The respiratory burst inhibiting activity of test compounds was determined using a modified assay of Tan and Berridge (2000) [218]. This *in vitro* assay is based on the reduction of highly water-soluble tetrazolium salt (WST-1) in the presence of activated neutrophils. Respiratory burst inhibitory activity was determined in a total volume of 250 µL MHS (pH 7.4) containing 1.0 $X10^4$ neutrophils/mL, 500 µM WST-1 and various concentrations of test compounds (10, 20, 40, 80 and 100 µg/mL).

The control contained buffer, neutrophils and WST-1. All samples were equilibrated at 37 °C and the reaction was initiated by adding opsonized zymosan A (15 mg/mL) (Sigma). Absorbance was measured at 450 nm. A control test was also carried out by mixing just the test solution and WST-1 solution. Ketotifen was used as positive control which is widely used as non-steroidal antiinflammatory drugs (NSAIDs) for the treatment of several inflammatory diseases (MacDonald *et al.*, 2002; Martin *et al.*, 2002) [219, 220]. Dose dependent (50, 100, 200 and 400 µg/mL) % inhibition was studied for the fractions that found active in LOX assay. The IC_{50} values were calculated by comparison with the DMSO as blank and expressed as the % inhibition of superoxide anions produced.

3.12.4.4. *Ex-vivo* studies of different extracts of *Leonotis nepetaefolia*

3.12.4.4.1. Experimental animals

Male and female Wistar albino rats, weighing 150-200 g and mice weighing 25-30 g, housed under controlled conditions of temperature at 25±2°C, relative humidity of 60±5% and light-dark cycle of 12-12 h for ex-vivo and in-vivo experiments. They were fed food pellets and water ad libitum. Animals were placed in polypropylene cages, each containing a maximum of 6 animals, with paddy husk as bedding. The necessary approvals were obtained for studies from the local Institutional Ethical Committee, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA/4/01), Government of India.

3.12.4.4.2. Mast Cell Degranulation by Compound 48/80

Effect of *L. nepetaefolia* on *ex-vivo* mast cell degranulation by Compound 48/80 induced allergy test was studied according to (Lee et al., 1990; Loeffler et al., 1971) [221, 222]. Mice of either sex were housed in normal experimental conditions. Animals were divided in 16 different groups (n=6). One group is normal control and another positive control, received only vehicle (10% Tween 80 in distilled water). Various extracts (200 mg/kg and 400 mg/kg), fractions (50 mg/kg and 100 mg/kg) and isolates (20 mg/kg) were given p.o. for 7 days prior to the experiment to the treatment groups. After 7 days of pretreatment with fractions/isolates, mast cells were isolated after 1 h of the dose. Normal saline containing 5 units/mL of heparin was injected in the peritoneal cavity of rats lightly anaesthetized with ether. After a gentle abdominal massage, the peritoneal fluid containing mast cells was collected in centrifuge tubes placed over ice. Peritoneal fluid was collected and centrifuged at 2000 rpm for 5 min. Supernatant solution was discarded and the cells were washed twice with saline and resuspended in 1 mL of saline. All the solutions were prepared in normal saline. The peritoneal cell suspension divided in various parts each containing 0.1 mL of cell suspension, 0.1 mL of test samples (1 mg/mL) and incubated in a constant temperature in water bath at 37°C for 15 min then 0.1 mL of compound 48/80 (10 g/mL) was added in all samples except in normal control and the suspensions were further incubated for 10 min at 37°C. The cells were then stained with 10% of toluidine blue solution and observed under the high power of light microscope. The % granulated and % degranulated mast cells were counted. Student "t" test was done to calculate significant difference in the values.

3.12.4.4.3. Milk-induced Leucocytosis

The procedure was followed as described by Brekhman *et al.*, (1969), Bhargava and Singh (1981) and Taur *et al.*, (2007) [223-225]. Animals were divided in 16 different groups (n=6). One group is normal control, received only vehicle (10% Tween80 in distilled water) and another positive control, received vehicle and milk (4 mL/kg, sc), where the animals belonging to other groups received various extracts (200 mg/kg and 400 mg/kg), fractions (50 mg/kg and 100 mg/kg) and isolates (20 mg/kg) given p.o. for 7 days prior to the experiment. After 7 days of pretreatment with fractions/isolates, blood samples were collected from retro-orbital plexus. Total leukocyte count was done in each group before drug administration and 24 h after milk injection (boiled and cooled; 4 mL/kg, sc), except for the normal control group. Blood was sucked in WBC pipette up to mark and further diluted with WBC diluting fluid. Pipette was shaken for few seconds and kept aside for 5 min. Neubaur's chamber was charged with above fluid and total leukocyte count was done. After 1 h of drug treatment each animal was injected with milk. Difference in Total leucocytes count before and 24 h after the drug administration was calculated.

3.12.4.4.4. Milk-induced Eosinophilia

The procedure was followed as described by Brekhman *et al.*, (1969), Bhargava and Singh (1981) and Taur *et al.*, (2007) [223-225]. Blood samples of the above animals were collected from retro-orbital plexus. Blood was sucked in WBC pipette up to mark with diluting fluid and further diluted with eosin solution. The eosin solution facilitates destruction of all corpuscles except eosinophil. Pipette was shaken for few seconds and kept aside for 5 min. Neubaur's chamber was charged with above fluid and eosinophil count was done. Eosinophil count was done in each group before drug administration and 24 h after milk injection (boiled and cooled; 4 ml/kg, sc). Difference in total eosinophil count before and after 24 h of drug administration was calculated.

3.12.4.4.5. Statistical analysis

The data are presented as mean ±S.E.M. One-way Analysis of Variance (ANOVA) was applied for statistical analysis with post-hoc analysis (Bonferroni Multiple Range Test) and $p<0.01$ value has been considered as statistical significance level.

3.12.4.5. In-*vivo* studies of different extracts of *Leonotis nepetaefolia*

3.12.4.5.1. Carrageenan-induced hind paw edema in rats

The acute hind paw edema was produced by injecting 0.1 mL of carrageenan (freshly prepared as 1% suspension) locally into the plantar aponeurosis of the right hind paw of rats (Winter et al., 1962) [226]. The following test samples, TMLNL: Total methanol extract (200 and 400 mg/kg), TWLNL: Total water extract (200 and 400 mg/kg), ET of TMLNL: ether sub-fraction of TMLNL (50 and 100 mg/kg), nBuOH of TMLNL: n-butanol fraction of TMLNL (50 and 100 mg/kg), TMLNR: Total methanol extract of root (50 and 100 mg/kg), TERLNR: Terpene fraction of root (50 and 100 mg/kg), LN-01: isolated alkaloid (20 mg/kg), LN-02: isolated compound from LNR (20 mg/kg), were administered to different groups while the other two groups served as normal and positive controls and received vehicle (1 mL/kg, p.o.) and standard drug, respectively. For each treatment group six animals were used. Test extracts/fractions were administered for 7 days and 1 h prior to the injection of carrageenan. A mark was made at the ankle joint of the paw of rat and pedal volume up to this point was measured using plethysmometer at 0 h (just before) and 1, 2, 3 and 4 h post-carrageenan injections. Increase in the paw edema volume was considered as the difference between 0 and 1, 2, 3 or 4 h. Percent inhibition of edema volume between treated and control groups was calculated as follows.

$$\text{Percent inhibition} = 1 - V_T/V_C \times 100$$

Where, V_C and V_T represent the mean increase in paw volume in control and treated groups, respectively.

All the animals were fasted for 12 h and deprived of water only during the experiment. The deprivation of water was ensured uniform hydration minimized variability in oedematous response.

3.12.4.5.2. Effect of *L. nepetaefolia* fractions on histamine induced bronchoconstriction in guinea pig

Animals: Guinea-pigs (500–600 g) of either sex were fed with a standard pellet diet (Lipton India) and water *ad libitum*. The animals were maintained at standard laboratory conditions. Guinea pigs were selected and randomly divided into five groups each containing six animals. The drugs were administered orally in 10% tween80 in distilled water.

Experimental bronchial asthma was induced in guinea pigs by exposing them to 0.25% histamine hydrochloride under constant pressure (1 kg/cm^2 in an aerosol chamber, Chandra, 1986; Sheth *et al.* 1972) [227, 228]. They showed progressive dyspnoea and then convulsions. The time until the appearance of the convulsions was designated the pre-convulsive time (PCT). As soon as the convulsions started, the animals were removed from the aerosol chamber and placed in fresh air and the PCT was taken as day 0. Guinea-pigs were administered with the test fractions (alkaloid fraction of LNL, terpene fraction of LNR in 10% tween80 in distilled water, each 50 and 100 mg/kg p.o.) once daily for 7 days. On day 7, 2 h after the administration of last dose of test extract, the onset of convulsions was recorded as for day 0. The % protection offered by the administration of *L. nepetaefolia* extracts was then measured as follows-

$$\% \text{ protection} = (1\text{-}Tc/Ts) \times 100$$

Where Tc= pre-convulsive time of control group, Ts= pre-convulsive time of extract group

3.12.4.5.3. Statistical analysis

The data are presented as mean ±S.E.M. In the control group, one-way Analysis of Variance (ANOVA) was applied for statistical analysis with post-hoc analysis (Bonferroni Multiple Range Test) and $p<0.01$ value has been considered as statistical significance level.

3.12.5. *In-vivo* studies of different extracts of *Oxalis corniculata*

3.12.5.1. Effect of extracts/fractions on isoproterenol-induced myocardial infarction

Isoproterenol (ISO)-induced myocardial necrosis is a well established model of MI in rats. The activities and capacities of antioxidant systems of heart declined following ISO challenge leading to the gradual loss of prooxidant/ antioxidant balance which accumulates into oxidative damage of cardiac myocyte. Therefore, the present study evaluated the role of *O. corniculata* extracts and OC-01 in combating ISO associated macromolecular damage in the myocardium of MI rats. Extracts/fractions were administered for 21 days as per following groups.

3.12.5.2. Induction of experimental cardiotoxicity

The cardiotoxicity was induced by subcutaneous (s.c.) administration of isoproterenol (85mg/kg) to rats daily for two consecutive days at 24 h interval [229].

3.12.5.3. Experimental groups

We selected male rats for our studies, since females are shown to be protected from cardiovascular complication [230]. A total of 78 rats were used in the study. They were randomly divided into 12 groups, with 6 animals in each group.

Group 1: (vehicle-control): Rats were administered saline orally (3ml/kg/day) using intragastric tube for 21 days and on 20th and 21st day received 0.3 ml saline, s.c. at an interval of 24h.

Group 2: (ISO-control): Rats were administered saline orally (3 ml/kg/day) for 21 days along with ISO (85 mg/kg, s.c., at 24h interval) on 20th and 21st day.

Group 3: (Compound OC01+ISO): Rats were administered compound OC-01 orally (20mg/kg/day) for 21 days along with ISO (85 mg/kg, s.c., at 24h interval) on 20th and 21st day.

Groups 4 and 5: (DEFTMOC+ISO): Animals were treated with diethyl ether fraction of hydroalcoholic extract (DEFTMOC) (50 and 100 mg/kg/day) orally for a period of 21 days along with ISO (85mg/kg, s.c., at 24h interval) on 20th and 21st day.

Groups 6 and 7: (EAFTMOC+ISO): Animals were treated with ethyl acetate fraction of hydroalcoholic extract (EATMOC- 50 and 100 mg/kg/day) orally for a period of 21 days along with ISO (85mg/kg, s.c., at 24h interval) on 20th and 21st day.

Groups 8 and 9: (OCFF+ISO): Animals were treated with n-butanol fraction of hydroalcoholic extract (OCFF- 50 and 100 mg/kg/day) orally for a period of 21 days along with ISO (85mg/kg, s.c., at 24h interval) on 20th and 21st day.

Groups 10 and 11: (RMOC+ISO): Animals were treated with residual methanolic extract (RMOC- 50 and 100 mg/kg/day) orally for a period of 21 days along with ISO (85mg/kg, s.c., at 24h interval) on 20th and 21st day.

Groups 12 and 13: (TAOC+ISO): Animals were treated with total aqueous extract (TAOC - 200 and 400 mg/kg/day) orally for a period of 21 days along with ISO (85mg/kg, s.c., at 24h interval) on 20th and 21st day.

3.12.5.4. Biochemical investigation

Serum enzymes like lactate dehydrogenase (LDH), creatine phosphokinase (CPK), aspartate transaminase (AST) and alanine transaminase (ALT) were estimated by using commercially available standard enzymatic kits (Span Diagnostics Pvt. Ltd., India). Rats were sacrificed by cervical dislocation. Immediately after the sacrifice, the hearts were excised and washed in ice-cold isotonic saline and blotted with a filter paper. Subsequently, the hearts were weighed and a

portion of the tissue was homogenized in 0.1M Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 7000rpm for 15 min and an aliquot of supernatant was used for the estimation of malondialdehyde (MDA) (Okhawa et al., 1979) and reduced glutathione (GSH) (Moron et al, 1979). The activities of catalase (Aebi, 1974) and superoxide dismutase (SOD) (Marklund and Marklund, 1974) were assessed [231-234].

Malonaldehyde (MDA) level: Briefly 1.0 mL sample was mixed with 0.2 mL 4% w/v sodium dodecyl sulphate, 1.5 mL 20% acetic acid in 0.27 M HCl (pH 3.5) and 1.5 mL 0.8% thiobarbituric acid (TBA). The mixture was heated in a hot waterbath at 85°C for 1 h. The intensity of pink colour developed was read against reagent blank at 532 nm following centrifugation at 1200 g for 10 min. The amount of MDA was calculated using molar extinction coefficient 1.56×10^5 /M.m^3 and reported as nmoles of MDA/mg of protein.

Superoxide dismutase (SOD): Sample (0.1 mL) was mixed with 0.1 mL EDTA (0.0001 M), 0.5 mL of carbonate buffer (pH 9.7) and 1.0 mL of epinephrine (0.003 M). The optical density of formed adrenochrome was read at 480 nm for 3 min. at interval of 30 sec. and results were expressed as U/min/mg of protein.

Catalase activity (CAT): Sample (50 µL) was added to buffer (50 mM phosphate buffer pH 9.7 containing 30 mM H_2O_2) to make volume upto 3 mL. The decrease in absorbance was read at 240 nm for 2.5 min at interval of 25 sec. The activity was calculated using extinction coefficient of H_2O_2 0.041 /µmol/cm^2. Results were expressed as µmol of H_2O_2 utilized/min/mg of protein.

Reduced glutathione (GSH): sample (2 mL) was mixed with 10% chilled TCA. The mixture was kept in ice-bath for 30 min and centrifuged at 1000 g for 10 min at 4°C. Supernatant (0.5 mL) was mixed with 2 mL of 0.3 M disodium hydrogen phosphate and 0.25 mL of 5,5- dithiobis-2-nitrobenzoic acid (40 mg/100 mL in 1% sodium citrate) was added just before measuring the absorbance at 412 nm. Standard curve of GSH was prepared using standard glutathione. Results were expressed as ng of GSH/ mg of protein.

3.12.5.5. Histology and Histomorphometry (Light microscopic study)

Histological evaluation was performed on lower portion of the heart. The tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (4µm thick) were cut using microtome. Each section was stained with hematoxylin and eosin (H&E) to evaluate the extent of myocardial inflammation. The sections were examined under light microscope (Zeiss, Japan).

3.12.5.6. Determination of myocardial necrosis by direct staining

This was performed by using triphenyltetrazoliumchloride (TTC) dye (Lie et al., 1975) [235]. Myocardium of rat was frozen immediately after removal. When the tissue was firm, the heart was sliced into 1 mm segments and incubated at 37°C for 20 min in 1% TTC. The formazan precipitate resulting from the reaction of lactate dehydrogenase in normal and ischemic regions delineated the area at risk from the infarcted tissue.

3.12.5.7. Statistical analysis

The data are presented as mean ±S.E.M. In the isoproterenol group, one-way Analysis of Variance (ANOVA) was applied for statistical analysis with post-hoc analysis (Bonferroni Multiple Range Test) and $p<0.01$ value has been considered as statistical significance level.