5. MATERIALS AND METHODS

5.1. Reagents and chemicals used:

Methanol, acetonitrile, petroleum ether, ethyl acetate, chloroform, ethanol, diethylamine, formic acid, ammonium acetate, orthophosphoric acid, potassium dihydrogen phosphate, hydrochloric acid, hydrogen peroxide, perchloric acid and sodium hydroxide were supplied by Qualigen fine chemicals and S.D. Fine chemicals. Water (HPLC grade) was obtained from Milli Q RO system. All the reagents and chemicals used were of HPLC and analytical grade.

Working standards of Apigenin (97.8%) and Luteolin (98.1%) were procured from Natural Remedies Ltd., Bangalore, India. Quercetin (98%) and Rutin (99%) were procured from Sigma Aldrich, Bangalore, India.

Formulations used:

Commercially available Achillea millefolium in the form syrup and tablet Liv-52 for analysis were procured commercially from the local market, Ooty, Tamilnadu, India.

5.2. Instruments used:

1. Sartorius single pan balance (BS R223S)
2. Systronics pH meter
3. Sonicator (Bandelin Electronics, Berlin)
4. Buchi Rota-Vacuum evaporator
5. Liquid chromatography coupled with high resolution MS
   - System: Agilent Technologies, USA 1290 infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel QTOF, Direct infusion mass with ESI & APCI (Positive & Negative mode ionization, UHPLC PDA Detector –Mass Spectrometer.
   - Mass Range: 50-3200 amu
   - Resolution: 40000 FWHM
   - High Mass Accuracy (Typically less than 1ppm)
   - High Sensitivity.
6. Shimadzu liquid chromatographic system equipped with LC – 10 AT VP solvent delivery system (pump), SPD M-10A photodiode array detector and rheodyne 7725i injector with 20 µl loop volume. Class VP 6.01 data station for data collection and processing (Shimadzu technologies, Japan).

7. Water's liquid chromatographic system equipped with waters 1515 isocratic solvent delivery system with a waters 2487 dual wavelength UV absorbance detector and rheodyne 7725i injector with 50 µl loop volume. Breeze 3.3 data station was used for data collection and processing.

8. Shimadzu 1700 UV-VIS spectrophotometer

9. Analytical column such as,
   - Phenomenex Luna C18 (250 x 4.6 mm i.d., 5µ)
   - Princeton C18 (150 x 4.6 mm i.d., 5µ)
   - Princeton C8 (150 x 4.6 mm i.d., 5µ)
   - Hibar C8 (150 x 4.6 mm i.d., 5µ)
   - Hibar C18 (250 x 4.6 mm i.d., 5µ)

5.3. EXTRACTION AND PHYTOCHEMICAL STUDIES

5.3.1. Collection and authentication

The whole plant of *Achillea millefolium* Linn. was collected during the month of February 2012, from Emerald, Ooty, The Nilgiris district. The whole plant of *Aganosma dichotoma* was collected during the month of March 2013, from the Western Ghats, Kottayam district, Kerala. Both the plants were botanically identified, confirmed and authenticated by Dr. S. Rajan, Field botanist, Medicinal plant survey and collection unit, Department of Ayush, Ministry of Health and Family welfare, Govt. of India, Emerald, Ooty, where voucher specimens were retained for future reference.

5.3.2. Extraction method

The whole plant of *Achillea millefolium* and *Aganosma dichotoma* were shade dried, powdered and extracted (50 gm) successively with each of petroleum ether,
chloroform, ethyl acetate, methanol and aqueous ethanol in a soxhlet extractor for 18-20 h. All the extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50°C) in a rotavapor apparatus. The nature and yields of the extracts were noted. All the extracts were stored in a refrigerator at 4°C till further use.

5.3.3. Phytochemical screening

A systematic and complete study of plant extracts includes a complete investigation of both primary and secondary metabolites derived from plant metabolism. The different qualitative chemical tests were performed for establishing profiles of given extracts for their nature of chemical composition. All the extracts obtained as above were tested for the following qualitative chemical tests for the identification of various phytocconstituents and shown in Table 2-3 such as:

Alkaloids: Dragendroff, Mayer and Hager, Wagner tests.
Steroids: Salkowski test, Libermann-Burchard test.
Glycosides: Legal test, Keller-Kiliani test.
Flavanoids: Shinoda test.
Tannins: Lead acetate test, Ferric chloride test.
Carbohydrates: Molisch test and
Saponins: Foam test.

5.4. Method development and optimization of chromatographic conditions for HPLC and LC-MS methods

Proper selection of the chromatographic methods depends upon the nature of the sample (ionic or neutral molecule), its molecular weight and solubility. For the present study, reverse phase HPLC and LC-MS methods were considered as they are more suitable for the estimation of active constituents present in the selected herbal plant extracts, because they are extremely specific, linear, precise, accurate, sensitive and rapid methods.

Chromatographic conditions for HPLC and LC-MS like

- Selection of detection wavelength/mass range (LC-MS)
MATERIALS AND METHODS

- Nature of stationary phase
- Nature and ratio of mobile phase were optimized.

5.4.1. Selection of detection wavelength/mass range:

The sensitivity of the HPLC method that uses UV/Visible detection depends upon the proper selection of the wavelength. An ideal wavelength is one that gives good response for all the components to be detected.

UV spectrums of 10 µg/ml of standard herbal components in selected solvents were recorded individually. The spectrums were superimposed to get overlay spectrum. From this overlain spectrum the detection wavelength was fixed at which selected herbal constituents show good absorbance.

The mass range of the selected constituent was determined in SCAN mode. 10 µg/ml of standard herbal drug in selected solvents were injected into to the MS system and scanned for the fragmentation pattern. The peak corresponding to m/z ratio equivalent to (M+H/M-H) was selected for further analysis in SIM mode.

5.4.2. Nature of stationary phase:

Different reverse phase stationary phases of various dimensions (C8 and C18) were used for the separation of the constituents in herbal standards by HPLC and LC-MS methods. Based on the retention time and peak shape, Hibar Lichrospher C8 (150 x 4.6 mm i.d., particle size 5 µ), Phenomenex Luna C18 (250 x 4.6 mm i.d., particle size 5 µ) were used for the separation of the herbal constituents of selected plants such as apigenin, luteolin, quercetin, rutin and their formulations by HPLC and for LC-MS methods.

5.4.3. Nature of mobile phase:

Based on sample solubility, stability and suitability various mobile phase compositions were tried to achieve good separation and resolution with sharp peaks for HPLC and LC-MS methods. Acetonitrile was used for the initial separation conditions. When acetonitrile was substituted with other solvents, the solvents to buffer ratios were calculated using solvent strength. Various ratios of water or buffers and different organic solvents like acetonitrile and methanol were tried to get good separation and peak resolution. The different chromatographic conditions were maintained for herbal extracts such as mobile phase pH, solvent strength, addition of peak modifiers, flow rate and solvent ratio on the peak resolution and symmetry.
were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetry factor, resolution and column efficiency were calculated. The conditions that gave good resolution, symmetry and efficiency were selected for the estimation of herbal constituents. Volatile buffers at very low concentrations were used for LC-MS methods to achieve proper separation and resolution.

5.5. FORCED DEGRADATION STUDIES

The specificity of the method can be demonstrated through force degradation studies conducted on the sample using acid, alkaline, neutral, oxidative and UV degradations. The sample was exposed to these conditions and the marker compound peak was studied for the peak purity, which will indicate the effective separation of the selected herbal constituents from the degradation products. In order to establish whether the analytical method and the assay were stability indicating, the selected plant extracts were stressed under various conditions to conduct forced degradation studies.

As the constituents are freely soluble and stable in methanol, methanol was used as a co-solvent in all the forced degradation studies. The solutions were prepared by dissolving the herbal constituents in methanol and further the degradation was carried out in various degradation medias viz., hydrochloric acid (acidic hydrolysis), sodium hydroxide (basic hydrolysis), hydrogen peroxide (oxidation), distilled water (neutral degradation) and UV light (photolysis). The resultant solution was analyzed every day.

5.6. Optimized chromatographic conditions:

Based on the above studies, the following optimized chromatographic conditions were selected for the simultaneous estimation of selected herbal plant extracts and selected formulations.
5.6.1. HPLC chromatographic conditions for simultaneous estimation of apigenin and luteolin

Stationary Phase : Hibar Lichrospher C8 (150 x 4.6mm i.d., 5µ)
Mobile phase : Solvent A: Methanol
Solvent B: 0.5% trifluoroacetic acid
Solvent ratio : 80:20 %v/v
Detection : 269 nm
Flow rate : 1.0 ml/min
Sample size : 20 µl
Run time : 10 min
Retention time : Apigenin (2.9 min) Luteolin (3.6 min)
Instrument used : Water’s liquid chromatographic system.

Preparation of standard solution

The standard stock solution (1 mg/ml) of apigenin and luteolin were prepared in methanol. 10 mg of each apigenin and luteolin was taken into 10 ml volumetric flasks. To this, 5 ml of methanol was added and sonicated for 10 min until a clear solution was obtained. The resulting solution was made up to 10 ml with acetonitrile. These stock solutions were stored in light resistant containers. Aliquots of apigenin and luteolin (1-5 µg/ml) were prepared in the mobile phase for the analysis.

Preparation of sample solution

50 mg of each extract was weighed and transferred into a 50 ml volumetric flask. To this 10 ml of mobile phase solution was added and sonicated for 10 min. The resulting solution was made up to 50 ml with mobile phase and filtered through Whatmann filter paper No.42. Aliquots of the sample were prepared in the mobile phase. The standard and sample solutions were analysed by the optimized chromatographic conditions and the chromatograms were recorded (Figure 5-10).
Preparation of plant formulation

0.527 mg of finely powdered tablet and 5 mg of the syrup were taken individually and soaked in 100 ml of methanol. The solution was sonicated for 20 min until a clear solution was obtained. The resulting solution was allowed to stand for 10 min and filtered through Whatmann filter paper. These stock solutions were stored in light resistant containers. The solution was analyzed and the chromatograms were recorded.

Stress degradation studies of apigenin and luteolin

Standard stock solution: 10 mg of each apigenin and luteolin was dissolved in 10 ml methanol (1 mg/ml solution).

Stress degradation studies were performed by subjecting the standard drug solution to various degradation media such as acidic medium, basic medium, neutral medium, oxidation and photo degradation studies. Depending on the extent of degradation observed, the studies were prolonged by certain variations in the concentrations of the degradation medium. The studies were performed at room temperature and in certain cases it was extended to 24 hours at room temperature.

Acid degradation:

Degradation medium: Hydrochloric acid (0.1 N)

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 0.1 N hydrochloric acid. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 1, 2, 4, 8, 12 and 24 hours. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 10, Figure 11 – 13).

Basic degradation:

Degradation medium: Sodium hydroxide (0.1 N)

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 0.1 N sodium hydroxide. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was
diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 1, 2, 4, 8, 12 and 24 hours. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 11, Figure 14 – 16).

Degradation in neutral condition

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with water. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 1, 2, 4, 8, 12 and 24 hours. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 12, Figure 17 – 19).

Oxidation degradation

Degradation medium: Hydrogen peroxide (3%)

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with 3% hydrogen peroxide. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 1, 2, 4, 8, 12 and 24 h. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 13, Figure 20 – 22).

Photo degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with water. The solution was exposed to UV light for 24 h. 1 ml aliquots of the samples were withdrawn at 8, 12, 24 h, diluted with mobile phase and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 14, Figure 23 – 25).
Validation

Linearity

Standard solutions of 1-5 µg/ml of apigenin and luteolin were analyzed to check the linearity of response (Table 4, Figure 26 – 27).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of apigenin and luteolin in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections of apigenin and luteolin were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 5).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined by adding a known quantity of apigenin and luteolin to the placebo solution. Three levels of solutions were made having concentrations of 10, 20 and 30 µg/ml for apigenin and luteolin. The percentage recoveries obtained from each sample was calculated (Table 6).

Robustness

Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines (Table 7).

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantitation (LOD) of the developed RP HPLC method obtained (Table 8).
5.6.2. LC-MS chromatographic conditions for apigenin and luteolin

**LC conditions**
- **Stationary Phase**: C₈ column (150 x 4.6 mm i.d., 5 μ)
- **Mobile phase**: 0.1% formic acid: Acetonitrile (20: 80)
- **Flow rate**: 0.4 ml/min
- **Injection volume**: 10 µl using auto injector
- **Column oven temp**: Room Temperature

**MS Conditions**
- **System**: Agilent 1290 infinity UHPLC, ifunnel QTOF
- **Interface-Polarity**: ESI-Positive
- **Operation mode**: SCAN
- **Probe Temperature**: 350°C
- **Capillary Voltage**: 80.0 Volts
- **Nebulizer gas flow**: 2.5 L/min
- **Drying gas**: 5 l/min
- **Detection**: Luteolin – m/z 287.1; Apigenin – m/z 271.1
- **Retention time**: Luteolin-8.01 min; Apigenin-8.48 min.

**Preparation of standard solutions**
10 mg of each apigenin and luteolin was taken into 10 ml volumetric flasks. To this, 5 ml of acetonitrile was added and sonicated for 10 min until a clear solution was obtained. The resulting solution was made up to 10 ml with acetonitrile. These stock solutions were stored in light resistant containers. Aliquots of mixed standards of apigenin and luteolin (10 µg/ml) were prepared in mobile phase for analysis.

**Preparation of sample solutions**
50 mg of each extract was weighed and transferred into a 50 ml volumetric flask. To this 10 ml of mobile phase solution was added and sonicated for 10 min. The resulting solution was made up to 50 ml with mobile phase and filtered through Whatmann filter paper No.42. Aliquots of the sample were prepared in the mobile phase. The standard and sample solutions were analysed by the optimized chromatographic conditions and the chromatograms were recorded (Figure 28-30).
Validation:

Linearity

Standard solutions of 10-50 ng/ml of apigenin and luteolin were analyzed to check the linearity of response (Table 15, Fig 31).

Specificity

The specificity of the method was ascertained by analysing the standards and the samples. The peaks of apigenin and luteolin in samples were confirmed by comparing the retention time and mass spectra of the standards.

Precision

Six injections of apigenin and luteolin were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 16).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined by adding a known quantity of apigenin and luteolin to the sample solution. Three levels of solutions were made having concentrations of 10, 20 and 30 ng/ml for apigenin and luteolin. The percentage recoveries obtained from each sample were shown in Table 17.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed LC-MS method obtained were shown in Table 18.

5.6.3. HPLC chromatographic conditions for quercetin and rutin

Stationary Phase : Phenomenex Luna C\textsubscript{18} (250 x 4.6mm i.d, 5\textmu)

Mobile phase : Solvent B: 20mM Amm. Acetate Buffer (Adjusted to pH 3) Solvent A: Acetonitrile

Solvent ratio : 60:40 %v/v

Detection : 259 nm

Flow rate : 1.0 ml/min
Sample injector: Auto sampling unit with 100 µl loop volume

Sample size: 20 µl

Run time: 10 minutes

Retention time: Quercetin (4.3 min) Rutin (1.71 min)

Instrument used: Shimadzu LC 2010AT VP HPLC (Auto sampler)

Preparation of standard solution

The standard stock solution (1 mg/ml) of quercetin and rutin were prepared in acetonitrile. 10 mg of each quercetin and rutin was taken into 10 ml volumetric flasks. To this, 5 ml of acetonitrile was added and sonicated for 10 min until a clear solution was obtained. The resulting solution was made up to 10 ml with acetonitrile. These stock solutions were stored in light resistant containers. Aliquots of quercetin (1 - 5 µg/ml) and rutin (0.1 - 0.5 µg/ml) were prepared in the mobile phase for the analysis.

Preparation of sample solution

50 mg of each extract was weighed and transferred into a 50 ml volumetric flask. To this 10 ml of mobile phase solution was added and sonicated for 10 min. The resulting solution was made up to 50 ml with mobile phase and filtered through Whatmann filter paper No.42. Aliquots of the sample were prepared in the mobile phase. The standard and sample solutions were analysed by the optimized chromatographic conditions and the chromatograms were recorded (Figure 32-37).

Stress degradation studies of quercetin and rutin

Standard stock solution: 10 mg of each quercetin and rutin was dissolved in 10 ml of acetonitrile (1 mg/ml solution).

Stress degradation studies were performed by subjecting the standard drug solution to various degradation media such as acidic medium, basic medium, neutral medium, oxidation and photo degradation studies. Depending on the extent of degradation observed, the studies were prolonged by certain variations in the concentrations of the degradation medium. The studies were performed at room temperature and in certain cases it was extended to 24 hours at room temperature.
Acid degradation:

**Degradation medium: Hydrochloric acid (0.1 N)**

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with 0.1 N hydrochloric acid. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 0, 2, 4, 8, 12 and 24 hours. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 26, Figure 38-40).

**Basic degradation:**

**Degradation medium: Sodium hydroxide (0.1 N)**

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with 0.1 N sodium hydroxide. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 0, 2, 4, 8, 12 and 24 hours. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 27, Figure 41-43).

**Degradation in neutral condition**

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with water. The solution was kept at room temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was diluted to 10 ml with mobile phase. The samples were injected and the chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 0, 2, 4, 8, 12 and 24 hours. The samples were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 28, Figure 44-46).

**Oxidation degradation**

**Degradation medium: Hydrogen peroxide (3%)**

1 ml of standard stock solution was taken into 10 ml volumetric flask and volumn was made up with 3% hydrogen peroxide. The solution was kept at room
temperature for 2 hours. The samples were analysed after 2 hours. 1 ml of sample was
diluted to 10 ml with mobile phase. The samples were injected and the
chromatograms were recorded. 1 ml aliquots of the samples were withdrawn at 0, 2,
4, 8, 12 and 24 hours. The samples were further diluted and analysed by the
optimised chromatographic conditions. The chromatograms were recorded (Table 29,
Figure 47-49).

**Photo degradation**

1 ml of standard stock solution was taken into 10 ml volumetric flask and
volume was made up with water. The solution was exposed to UV light for 12 hours.
1 ml aliquots of the samples were withdrawn at 0, 8, 12 hours, diluted with mobile
phase and analysed by the optimised chromatographic conditions. The chromatograms were recorded (Table 30, Figure 50 – 52).

**Validation**

**Linearity**

Standard solutions of 1-5 µg/ml of quercetin and 0.1-0.5 µg/ml of rutin were
analyzed to check the linearity of response (Table 20, Figure 53 - 54).

**Specificity**

The specificity of the method was ascertained by analyzing the standards and
the samples. The peaks of quercetin and rutin in samples were confirmed by
comparing the retention time and spectra of the standards.

**Precision**

Six injections of quercetin and rutin were made and analyzed to examine the
precision of the method. The mean peak area, standard deviation and % RSD were
calculated (Table 21).

**Accuracy**

Accuracy of the method was determined by recovery experiments. The
recovery of the method was determined by adding a known quantity of quercetin
and rutin to the sample solution. Three levels of solutions were made having
concentrations of 1, 2 and 3 µg/ml for quercetin and 0.1, 0.2 and 0.3 mg/ml for rutin.
The percentage recoveries obtained from each sample were shown in Table 22.
**Robustness**

Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines. The results were shown in Table 23.

**Limit of detection and Limit of quantification**

The limit of detection (LOD) and the limit of quantitation (LOD) of the developed RP HPLC method obtained were shown in Table 24.

**LC-MS chromatographic conditions for quercetin and rutin**

**LC conditions**
- **Stationary Phase**: Phenomenex C18 (150 x 4.6 mm i.d., 5 μ)
- **Mobile phase**: 10mM Amm. Acetate: methanol (20: 80)
- **Flow rate**: 0.4 ml/min
- **Injection volume**: 10 μl using auto injector
- **Column oven temp**: 30°C

**MS Conditions**
- **System**: Agilent 1290 infinity UHPLC, ifunnel QTOF
- **Interface-Polarity**: ESI-Positive (Quercetin-Positive mode; Rutin-Negative mode)
- **Operation mode**: SCAN
- **Probe Temperature**: 350°C
- **Capillary Voltage**: 80.0 Volts
- **Nebulizer gas flow**: 2.5 l/min
- **Drying gas**: 5 l/min
- **Detection**: Quercetin – m/z 303.0; Rutin-m/z 609
- **Retention time**: Quercetin – 4.091 min; Rutin – 3.317 min.

**Preparation of standard solution**

The standard stock solution (1 mg/ml) of quercetin and rutin were prepared in acetonitrile. 10 mg of each quercetin and rutin was taken into 10 ml volumetric flasks. To this, 5 ml of acetonitrile was added and sonicated for 10 min until a clear
solution was obtained. The resulting solution was made up to 10 ml with acetonitrile. These stock solutions were stored in light resistant containers. Aliquots of quercetin (1 - 5 µg/ml) and rutin (0.1 - 0.5 µg/ml) were prepared in the mobile phase for the analysis.

**Preparation of sample solution**

50 mg of each extract was weighed and transferred into a 50 ml volumetric flask. To this 10 ml of mobile phase solution was added and sonicated for 10 min. The resulting solution was made up to 50 ml with mobile phase and filtered through Whatmann filter paper No.42. Aliquots of the sample were prepared in the mobile phase. The standard and sample solutions were analysed by the optimized chromatographic conditions and the chromatograms were recorded and were shown in Figure 55 - 57.

**Validation:**

**Linearity**

A standard solution of 1 – 5 ng/ml of quercetin and 10 – 50 ng/ml of rutin were analyzed to check the linearity of response. (Table 31, Figure 58 - 59).

**Specificity**

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of quercetin and rutin were confirmed by comparing the retention time and mass spectra of the standards.

**Precision**

Six injections at three different concentration of quercetin (1, 3, 5 ng/ml) and rutin (10, 30, 50 ng/ml) were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 32).

**Accuracy**

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined by adding a known quantity of quercetin and rutin to the sample solution. Three levels of solutions were made having
concentrations of 1, 3 and 5 ng/ml for quercetin and 10, 30 and 50 ng/ml for luteolin. The percentage recoveries obtained from each sample were shown in Table 33.

Limit of Detection and Limit of Quantification
The limit of detection (LOD) and the limit of quantification (LOQ) of the developed LC-MS method obtained were shown in Table 34.