Chapter 5

Standardization of ethanolic extract of *Ficusreligiosa* L. extract

5.1.Introduction

Ethanolic extract obtained from the *Ficusreligosa*L. was used for the study. In general, extract from any plant contains one or more phytochemicals. The presence of several phytochemicals makes the qualitative and quantitative analyses very difficult and the need of reference compound of each phytochemical present in the extract increases cost of the experiment. To avoid the complexity associated with analyzing each phytochemical present in the extract and to avoid time consumption, this research focused on analyzing the marker compound present in ethanolic extract of *Ficusreligiosa*L. For analyzing the marker compound, both TLC and LCMS techniques were used to isolate the marker compound and further analyses were carried out by FTIR and NMR techniques. Further, quantitative analysis for the marker compound was done by using RP-HPLC method.

5.2.Methods

5.2.1. **Isolation of ethanolic extract of *Ficusreligiosa*L.**

The stem barks of *Ficusreligiosa*L.were collected in the month of November from the locality of Banaras Hindu University (Varanasi, India) and authenticated by Prof. R. S. Upadhyay, Department of Botany, Banaras Hindu University, Varanasi, India. Stem barks of *Ficusreligiosa*L. were dried under sun for 15 days and powdered finely. 50 grams of this powder was taken into the porous container of soxhlet apparatus. 500 ml of ethanol was used for extraction; 250 ml of ethanol was taken in distilling pot and remaining ethanol was poured to porous container. Temperature of 40°C was maintained and soxhleted for 48 hours. Then the solvent was recovered and extract was dried. Traces of organic solvent were completely removed at 70°C using rotary evaporator (IKA RV 10) by nitrogen gas purging. Dried ethanolic extract was used for the study.

5.2.2. TLC

TLC was performed by using SilicagelG as adsorbent and n-butanol : acetic acid : water (4:0.5:5) as mobile phase. Plate was prepared by pouring silica gel slurry on glass plate and activated by heating at 110°C for 30 minutes. The spots were detected using vanillin reagent in sulphuric acid and R_f values were calculated. Preparative TLC was carried out as per the method of Yadav et al. (Yadav et al. 2012) by partitioning 25 grams of ethanolic extract with 50 ml of petroleum ether under reflux on a water bath (at about 70°C). This extract was then concentrated and used for isolating marker compound. Silica gel 60F254 pre-coated TLC plates, 20 X 20 cm (Merck) were developed using toluene : ethanol in the ration of 9 : 1. The band corresponding to lupeol was scraped and eluted with ethanol. The identity of lupeol was confirmed by LCMS, FTIR and NMR studies.

5.2.3. LC-MS analysis of ethanolic extract of *Ficusreligiosa*L.

For qualitative analysis of ethanolic extract of *Ficusreligiosa*L., LCMS was carried out. Mass spectroscopic analysis was performed using quadrapole time-of-flight spectrometer (G6520B, Agilent technologies) equipped with a electrospray

ionisationsource in both positive and negative modes. Minimum range and maximum range of 50 and 1500, respectively were used. Other parameters such as scan rate of 1, gas temperature of 300 °C, gas flow of10.0 l/minute and nebulizer pressure of 32 psi were used.

5.2.4. FTIR analysis of marker compound

FTIR analysis of marker compound was done by using fourier transform-infrared spectroscopy (FTIR) (FTIR-8400S, Shimadzu) by conventional KBr disc/pellet method. The sample was prepared by grinding with anhydrous KBr powder and compressed into pellets. An FTIR spectrum was measured over the range of 4000 - 400 cm^{-1} with resolution of 4 cm^{-1} for 50 scans.

5.2.5. NMR analysis of marker compound

Structural elucidation of marker compound was obtained from ^{1}H NMR and ^{13}C NMR analyses. For ^{1}H NMR analysis (Joel-FT NMR-AL300, Japan), marker compound was dissolved in dimethylsulphoxide and degassed using liquid nitrogen. ^{1}H NMR analysis was carried out at 500 mega hertz using tetramethylsilane as internal standard.

5.2.6. RP HPLC method development

RP HPLC (Waters, 5.5, USA) setup comprising of binary pumps and PDA 2998 detector was used. Different proportions of mobile phase (acetonitrile, methanol and water) were tried. The mobile phase was filtered through 0.45 μm nylon filters (Millipore, USA). 20 μl volume samples were injected at the flow rate of 1 ml/min and analyzed at λ_{max} of 211 nm. The chromatographic peak of marker compound (reference standard) and extract were confirmed by comparing with their retention time. Quantification was carried out by integrating the peaks related to marker compound using external standard method. Stock solutions of lupeol were prepared separately in methanol at the concentration of 1 mg/ml. Stock was stored at 4°C. The standard solutions were prepared by diluting stock

solution with mobile phase to obtain a series of concentration ranging from 1 ng/ml to 1,000 µg/ml.

5.2.6.1.System suitability test

The system suitability parameters were performed with six replicate analyses of five standard solutions of lupeol in the concentration range of 100-500 µg/ml. The mean values of six injections were used to evaluate retention time, peak area. The percent relative standard deviation (% RSD) values were used for the evaluation of precision for lupeol (criteria ≤ 2.0%).

5.2.6.2.Linearity

The linear range of calibration curve was determined by using lower concentration (1 ng/ml) to higher concentration (1000 µg/ml). Standard solutions were prepared by dissolving in methanol. After suitable dilutions, 20 µl aliquots of solution were injected onto the HPLC system, in triplicate, and calibration curves were obtained in relation to the mass of lupeol injected, in two consecutive days. Curves obtained in two consecutive days were statically compared. The y-intercept, slope, and correlation coefficient (r^2)of lupeol were calculated.

5.2.6.3.Identity and specificity

The specificity of developed method was examined by comparing the chromatographic profile of standard compound with that of chromatograms obtained for extract sample at different points in terms of retention time, UV spectrum and lambda max (λ_{max}).

5.2.6.4.Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) for lupeol were calculated based on standard deviation (SD) and slope (S) of calibration curve constructed for assessing linearity based on the following formulas

$$\text{LOD} = \frac{3.3 \times \text{SD}}{S}$$

$$\text{LOQ} = \frac{10 \times \text{SD}}{S}$$

where, SD – standard deviation and S – slope obtained from the curve.

5.2.6.5.Precision and Accuracy

Precision was evaluated at two levels: repeatability and intermediate precision. Repeatability (intra-day) and intermediate precision (inter-day) were determined by injectingthe extract sample at three different concentrations i.e. low, medium, and high concentrations (50, 100, and 150 µg/ml, respectively) in triplicate. In case of intermediate precision, the determination was carried out by the same analyst over a period of two days.

The accuracy of developed method was estimated through the analyte recovery test in triplicate. Standard concentrations of about 50, 100 and 150 mg/ml of lupeol were added to the sample solution (extract) separately. After spiking, the recovery of lupeol was calculated after discounting analyte area. The solutions were prepared in triplicate and injected after filtration through a 0.45 µm modified PTFE membrane. The accuracy of the sample was calculated using the following formula:

$$\text{Accuracy} = \%\ \text{recovery} = \frac{\text{Actual concentration of analyte}}{\text{Theoretical concentration of analyte}} \times 100$$

5.2.6.6.Robustness

The robustness of the developed method was performed by evaluating small variations in pH of mobile phase (pH 3.0, 4.0, 5.0), mobile phase flow (0.9, 1.0, 1.1 ml min^{-1}) and stability of analytical solutions over the period of 96 hours.

5.2.6.7.Quantification of lupeol

The concentration of lupeol present in ethanolic extract of *Ficusreligiosa*L. was analysed using the peak area. The following formula was used:

$$\text{Lupeol concentration (\%)} = \frac{Ps}{Pst} \times \frac{M1}{M2} \times 100$$

where, Ps = peak area of the sample, Pst = peak area of the standard, M1 = mass of sample in mg and M2 = mass of standard in mg.

5.3.Results and discussion

5.3.1. TLC and preparative TLC

Preliminary TLC showed the presence of lupeol in the stem bark of *Ficusreligiosa*L. at the R_f value of 0.63 (Figure 5.1). From the preparative TLC, the spot of lupeol from *Ficusreligisosa*L. was isolated as colorless powder. The isolated compound was further confirmed from the studies of LCMS, FTIR, ^{1}H NMR and ^{13}C NMR.

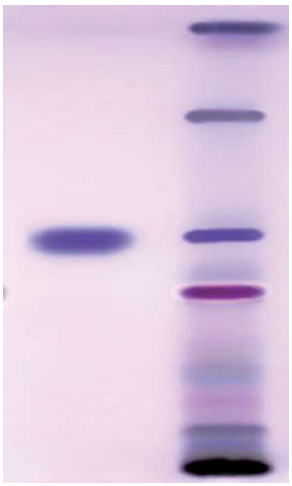

Figure 5.1: TLC finger printing of lupeol standard and *Ficusreligiosa*L. extract

5.3.2. LCMS analysis of lupeol

For qualitative analysis, LCMS was carried out. In the LCMS system, LC column first separates the compounds present in the sample and then transfers the separated compounds from the LC column into the MS ion source. Based on the molecular weight of separated compounds, compounds are identified. From the positive ion mode $(M+H)^+$ spectrum of ethanolic extract of *Ficus religiosa* L., three peaks were obtained at charge (m/z) of 166.12, 180.10 and 427.24 (Figure 5.2). The LCMS mode used for the study was positive ion mode and hence, the mass to charge of the most abundant compound is 426. MS spectrum suggested the molecular formula of $C_{30}H_{50}O$ which is the molecular weight of lupeol (Manorenjitha et al., 2014). This study shows the presence of lupeol in the ethanolic extract of *Ficus religiosa* L.

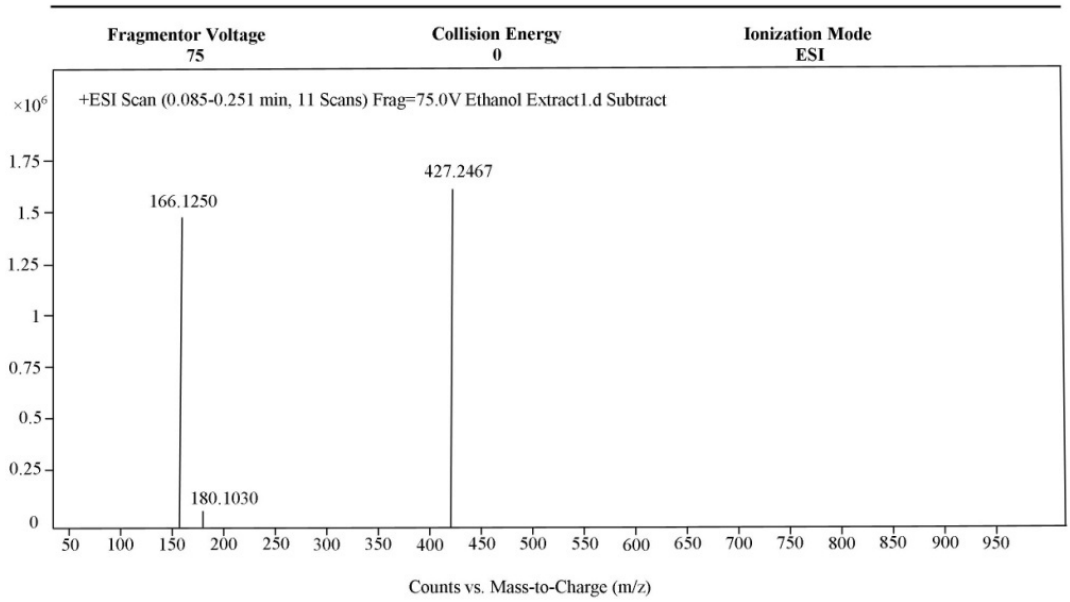

Figure 5.2: LCMS (positive mode) spectrum of ethanolic extract of *Ficus religiosa* L.

5.3.3. FTIR analysis of lupeol

FTIR technique helps in identifying various functional groups present in the sample by obtaining infrared spectrum of absorption or emission of the sample. FTIR spectrum of lupeol is shown in Figure 5.3. FTIR study showed a characteristic peak at 1450 cm^{-1}; this might belong to methylenic vibration, absorption frequencies observed at 3398 cm^{-1} and 1383 cm^{-1} might be due to O-H and C-O bond vibrations, respectively; peak observed at 976 cm^{-1} might be due to an unsaturated out of plane C-H vibration; C=C vibration was shown around 1649 cm^{-1} as a weak intense band. Peak observed at 2933 cm^{-1} might be due to –COOH stretching and peak observed at 1400 cm^{-1} might be due to -CH bending vibration. These peaks are the characteristic peaks of lupeol (Darekar et al., 2008).

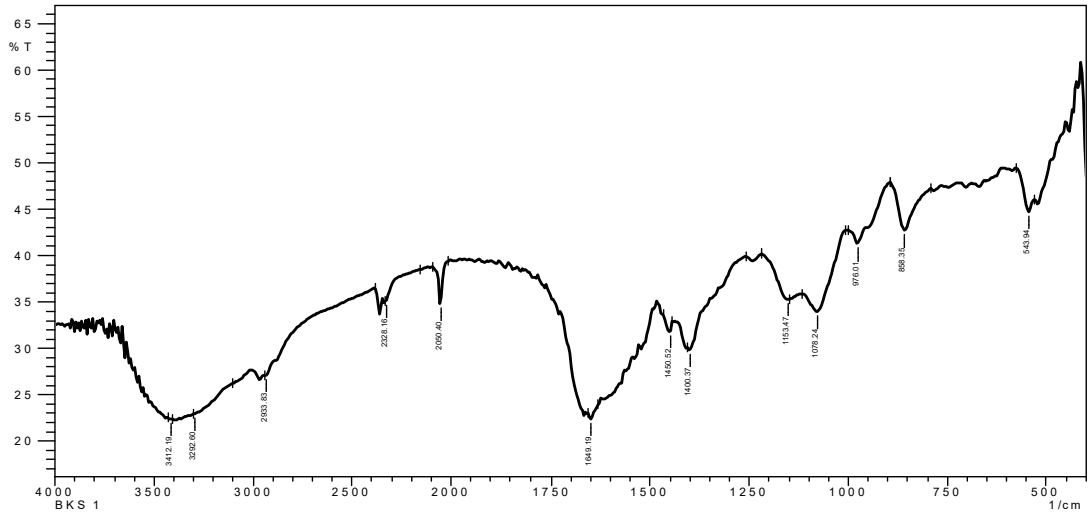

Figure 5.3: FTIR spectrum of lupeol in *Ficus religiosa* L.

5.3.4. NMR analyses of lupeol

5.3.4.1. ^{1}H NMR

Proton nuclear magnetic resonance is used to determine the structure of molecules by applying nuclear magnetic resonance with respect to hydrogen nuclei present in the molecules of a sample. ^{1}H NMR spectrum of lupeol is shown in Figure 5.4. ^{1}H NMR spectrum showed a doublet at δ 3.31 is due to the proton attached to secondary carbinol, two broad singlet's were seen at δ 4.59 and 4.65 due to the two exomethylene protons attached, hydromethine proton at δ 3.19, broad singlets at 4.73 and 4.59 were indicative of olefinic protons, 2.36 m assigned to a methynic proton, a doublet at 1.97, 1.66 triplet assignable to a methyl group on double bond, a singlet at 1.23.

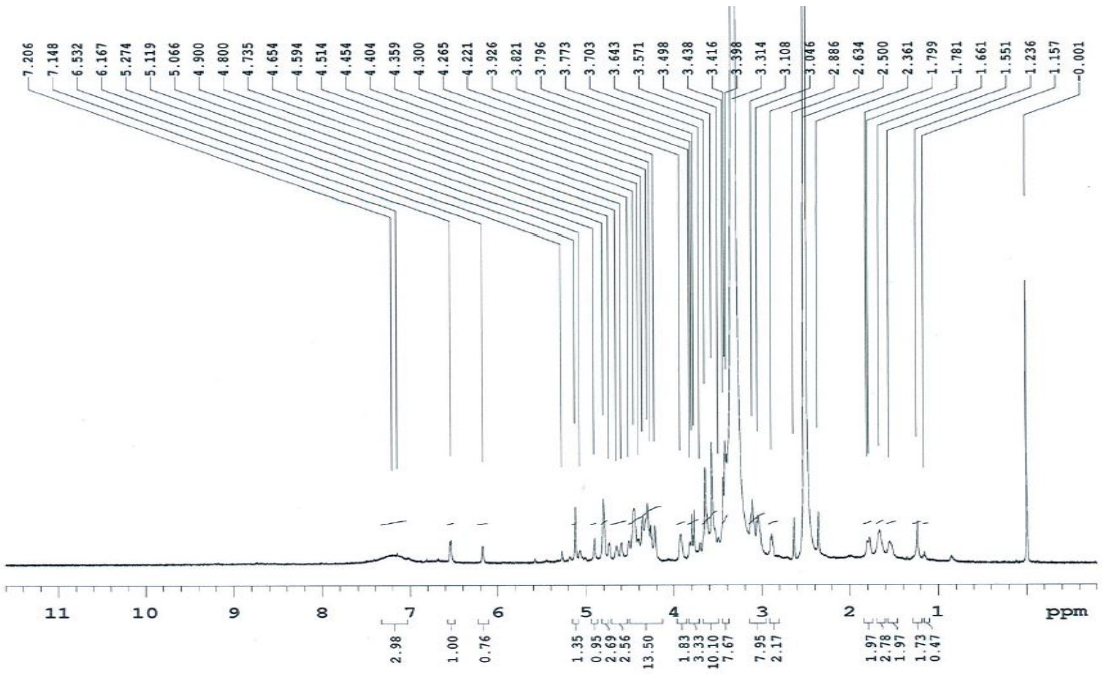

Figure 5.4:^{1}H NMR spectrum of lupeol in *Ficusreligiosa*L.

5.3.4.2. ^{13}C NMR

^{13}C NMR identifies nature of carbon atoms present in the sample. This helps in structural elucidation of unknown samples. ^{13}C NMR spectrum of lupeol is shown in Figure 5.5. ^{13}C NMR spectrum revealed seven methyl groups (at δ 38.0, 39.1, 39.3, 39.5, 39.6, 14.8 and 43.3); signals due to an exomethylene group (at δ 115.6 and 179.6); ten methylene, five methine and five quaternary carbons were observed.

From the combined results of LCMS, FTIR, ^{1}H NMR and ^{13}C NMR, the marker compound was confirmed as lupeol and the results are in agreement with the reported literature (Darekar et al., 2008). Structure of lupeol is given in Figure 5.6.

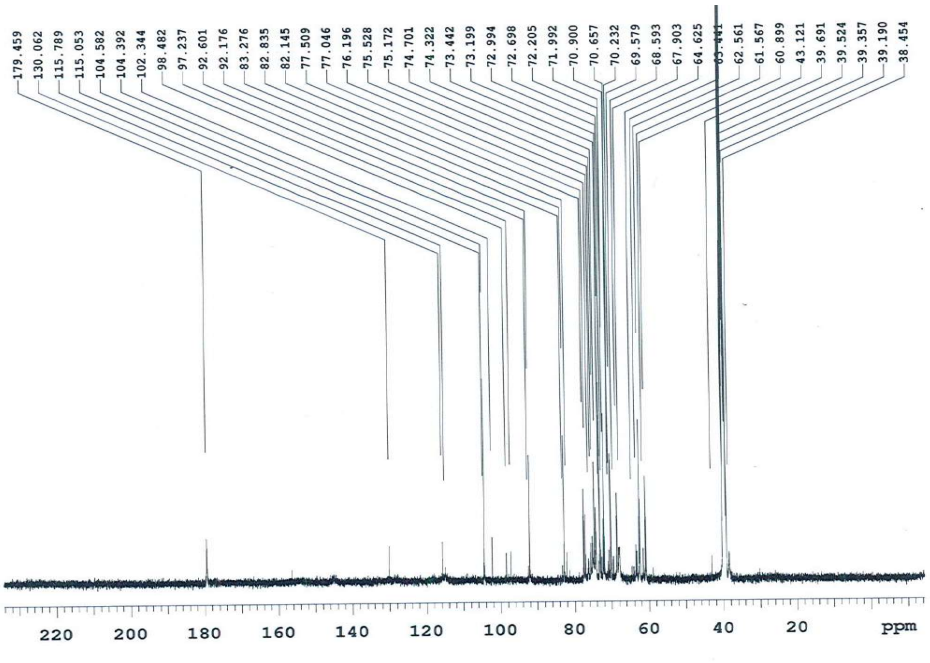

Figure.5.5: ^{13}C NMR spectrum of marker compound of *Ficusreligiosa*L.

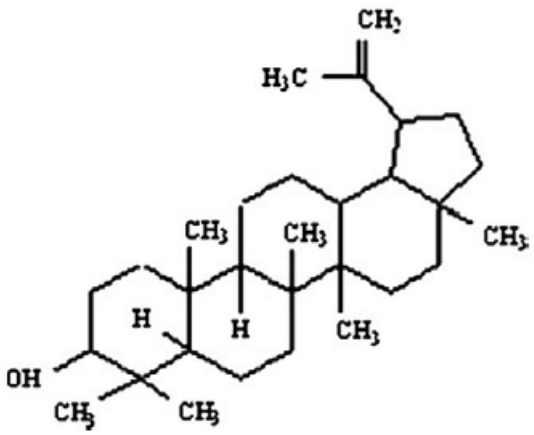

Figure 5.6: Structure of lupeol

5.3.5. RP HPLC method development of lupeol

5.3.5.1. Method development and optimization

Lupeol is a water insoluble compound. Hence, various proportions of methanol:water and acetonitrile:water system were tried as mobile phase but results were not satisfactory. Mixture of methanol:acetonitrile:water in different ratios were tried (Table 5.1). From the results, it was found that the mobile phase composition of methanol:acetonitrile:water in the ratio of 45:45:10gave better separation with reproducible results with retention time lesser than 10 minutes.

Table 5.1: Different mobile phase compositions tried

Solvent composition	**Methanol: Water**	**Acetonitrile:Water**	**Methanol:Acetonitrile :Water**
Ratio	70:30	70:30	30:30:40
	75:25	75:25	35:35:30
	80:20	80:20	40:40:20
	85:15	85:15	45:45:10
	90:10	90:10	47.5:47.5:5
	95:05	95:05	48.75:48.75:2.5

5.3.5.2.**System suitability test**

Six replicate injections of standard solution gave % RSD values of retention time and peak area within 2%, indicating low variation of the measured values (Table 5.2). This indicated that the developed HPLC condition is suitable for lupeol analysis in *Ficusreligiosa*L. extract.

Table 5.2: Chromatographic properties of lupeol

Compound	Concentration (ng/ml)	Mean ± % RSD	
		Retention time (min)	Peak area
Lupeol	20	9.1 ± 0.016	298755 ± 1.236
	40	9.0 ± 0.023	597438 ± 2.009
	60	9.0 ± 0.101	873195 ± 0.014
	80	9.1 ± 0.009	1185082 ± 0.354
	100	9.0 ± 0.048	1463613 ± 0.698

5.3.5.3.Linearity

Concentration range of 1 ng/ml to 1000 µg/ml were tried in order to cover wide range. Linear range for lupeol was found to be 20-100 ng/ml. The obtained results were directly proportional to the concentration of analyte present in the sample. Calibration curves obtained at two consecutive days, for lupeol were statically similar ($p< 0.05$).

5.3.5.4.Identity and specificity

Identity of lupeol in ethanolic extract was done by comparing the retention times of standard with that of extract (Figure 5.7). Specificity (100 µg/ml) was determined by spiking with standard in triplicate. For investigated compound, only one peak was observed at specified retention time ie. 9.0 minutes.

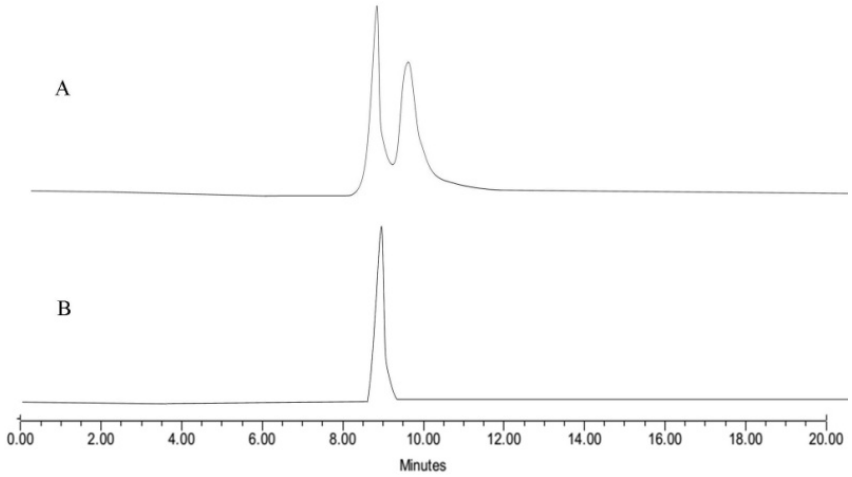

Figure 5.7: HPLC chromatograms of A – *Ficusreligiosa*L. extract and B – lupeol standard

5.3.5.5.Limit of detection and limit of quantification

Detection limits of 23 ng/ml and 65 ng/ml were obtained for lupeol. The low values of detection and quantitation limits demonstrate the high sensitivity of the developed method. The limits are consistent with the requirements for analysis of the lupeol.

5.3.5.6.Precision and Accuracy

The intraday and interday precision and accuracy for the determination of lupeol are shown in Table 5.3. In the intra-day precision analyses (n= 6), % RSD values of lupeol were 0.321 – 0.456. % RSD values found in inter-day precision (n=6) were 0.145 - 1.687 for lupeol. The obtained RSD (%) values were lower than 2.0% which showed the precision of the developed method. A mean recovery (n= 6) of 99.15-100.12 % for lupeol proved the accuracy of developed method for the determination of lupeol.

Table 5.3: Precision (% RSD) and accuracy for the determination of lupeol

Lupeol (µg/ml)	Intraday assay			Interday assay		
	Mean (µg/ml)	Precision (%RSD)	Accuracy (%)	Mean (µg/ml)	Precision (%RSD)	Accuracy (%)
50	49.65	0.365	99.38	50.01	0.145	100.02
100	100.12	0.456	100.12	99.98	0.654	99.98
150	148.73	0.321	99.15	149.64	1.687	99.76

5.3.5.7.Robustness

The developed method was unaffected by changes in chromatographic parameters such as pH of mobile phase, mobile phase flow and stability of analytical solutions over the time period of 0, 24, 48 and 96 hours. The robustness results of lupeol are shown in Table 5.4. The developed method had good robustness and it was not significantly affected by these small changes. Also, changes in chromatographic parameters had no significant impact on the specificity, resolution and peak shape of lupeol.

Table 5.4: Robustness study of developed method

Property		pH			Mobile phase flow			Stability of analytical solutions			
	Lupeol	3	4	5	0.9	1.0	1.1	0	24	48	96
RT*		9.098	9.104	9.099	9.082	9.096	9.158	9.170	9.007	9.101	9.109

*RT – retention time

5.3.5.8.Quantification of lupeol

Quantity of lupeol found in the ethanolic extract of *Ficusreligiosa*L.was found to be 13.67%.

5.4.Summary

Extraction of ethanolic extract from stem barks of *Ficusreligiosa*L. was done by soxhlet method using ethanol. LCMS study of *Ficusreligiosa*L. ethanolic extract in diluted form revealed mass to charge of the most abundant compound is 426 and molecular formula of $C_{30}H_{50}O$. in accordance with the LCMS results, TLC was done to isolate the major compound present in *Ficusreligiosa* L. extract. The spot corresponding to R_f value, 0.71 (which had the melting point of 219º C) was scrapped from preparative TLC and it was isolated as colorless powder after several washes with organic solvents and recrystallized with ethanol. From the combined results of LC-MS, TLC, FTIR and NMR, the marker compound was confirmed as lupeol. RP-HPLC method was developed for the quantification of lupeol in *Ficusreligiosa* L. extract.