Chapter -4

4. SEPARATION AND IDENTIFICATION OF COMPOUNDS FROM BIOACTIVE FRACTION

Therapeutic importance of 'Brahmadandi' is described in many traditional texts but very little information is available on its chemical constituents. During the investigations on the chemical constituents of the plants (*E. echinatus* and *T. glaberrima*) supplied as source of Brahmadandi the presence of terpenoids and alkaloids, as the important constituents in *E. echinatus* and *T. glaberrima* respectively, was revealed. The bioactivity studies of the extracts also showed that terpenoids are the active constituents in *E. echinatus*. Hence the bioactive extract/fractions were subjected to co-TLC with the available terpenoidal markers in our laboratory. Incidentally the presence of lupeol was detected in the extracts of both the plants. In *E. echinatus* the content was considerably high while in case of *T. glaberrima* the content was very low. Lupeol isolated from benzene extract of *Alstonia scholaris* was reported to induce antifertility in male rats (Gupta et al., 2005).

Therefore, detailed studies on the separation, identification and quantification of possible compounds from the bioactive extracts and fractions were undertaken.

4.1. Preparation of the fraction

A coarse powder (500 g) of the shade-dried roots was extracted with petroleum ether (60 – 80°) in a Soxhlet apparatus. The extract was concentrated on a rotary vacuum evaporator (BUCHI Rotavapor R 200) and dried under vacuum (yield = 4.96% w/w). Vacuum-dried petroleum ether extract was subjected to saponification with 20% alcoholic KOH for 2 hours, which was then subjected to distillation to remove all traces of ethanol, the lost volume being made up with water throughout the distillation. The unsaponified material was then collected by extraction with solvent ether, it was washed with small quantity of water and dried under vacuum (25% w/w)

(Indian Pharmacopoeia, 1996). Vacuum-dried terpenoid rich fraction of petroleum ether extract (TRFPE) was then subjected to column chromatography.

4.2. Column chromatography

Chromatographic conditions:

Adsorbent : Silica gel (60 – 120 mesh)

Weight of the sample : 2.0 g

Column was prepared in hexane.

Column was subjected to gradient elution starting with 100 % hexane; 2% and 5% chloroform in hexane. Eight fractions (1-8) each of 50 ml were collected using hexane (100%) and two fractions (9-10) each of 50 ml were collected for 2% and two fractions (11-12) each of 50 ml were collected for 4% chloroform in hexane.

Each elute collected was monitored continuously by thin layer chromatography (TLC) (Silica-gel G; petroleum ether: benzene (3:7)), visualized with anisaldehyde-sulphuric acid reagent.

Elute 1 gave single band just below the solvent front in the TLC which becomes pink and turns to deep purple upon treating with anisaldehyde-sulphuric acid followed by heating at 105 °C. Upon concentration it gave a thin film of white flakes on the watch glass (**EEPE-2**; 9.8 mg).

Elutes 2 and 3 contained mixture of different compounds, elutes 4 to 6 were found to contain single band on TLC plate (approx R_f = 0.18) which is invisible under UV light and gives pink color with anisaldehyde sulphuric acid. Upon concentration, elutes 4 to 6 gave a cream colored granular powder (**EEPE-1**; 22 mg).

Elutes 7 to 12 were found to be mixture of different compounds they were combined and concentrated to get a cream colored granular powder (300 mg) which was then subjected to preparative thin layer chromatography.

4.3. Preparative TLC of the fraction obtained from the column

Chromatographic conditions:

Adsorbant : Silica gel G

Solvent system : Petroleum ether: ether (9:0.4)

Detection : spray with anisaldehyde sulphuric acid and heat for 10 minutes.

The fraction was subjected to preparative TLC under the above said conditions. It showed the presence of 5 bands, of which two intense pink color bands; one at R_f 0.08 (which separates just above the point of application) and second at R_f 0.65 were found to be in major concentration, which were then isolated and purified (45 mg and 22 mg respectively). Comparative TLC showed that the compound with R_f 0.08 is similar to EEPE-1; and the compound with R_f 0.65 is similar to EEPE-2. Homogeneity of both the compounds was checked on TLC using different solvent systems (petroleum ether: benzene (3:7); petroleum ether: ether (9:0.4); Toluene: chloroform: ethyl acetate (2:0.4:0.2)). The compounds were then subjected to spectral studies. Other fractions were found to be in very low concentration and hence were not processed further.

4.4. Results

4.4.1. Isolation of compound EEPE-1

It is a cream colored granular powder, soluble in hexane, benzene, chloroform.

Melting point: 212-213 °C.

The isolated compound **EEPE-1**was expected to be lupeol due to following reasons:

In preliminary analysis the compound was confirmed by its melting point (Lit. 215 °C), and by running a Co-TLC with authentic sample of lupeol.

IR ν_{max} (KBr) cm^{-1}: The IR spectrum of the **EEPE-1** (Figure 4.1) showed characteristic absorption at 3406.1 (due to the stretching vibration of -OH bond) and 2937.4 and 2854.5 (due to stretching vibration of –CH in steroid skeleton), 1639.4 (due to stretching vibration of C=C).

The proton nuclear magnetic resonance spectra of EEPE1 (Figure 4.2) was recorded using Bruker Avance II 400 NMR spectrometer. Due to hyperfine splitting of 20 protons in cyclohexane ring and mixed coupling constants no singlet, doublet or triplet can be seen in that region. Therefore several multiplets corresponding to cyclohexane protons are observed in the spectra. The proton signals obtained were compared with the earlier reported values (Nicollier et al., 1979; Ito and Lai, 1978).

^{1}H-NMR: 0.70 (1H, m, C-5 αH), 0.76 (3H, s, C-23H), 0.79 (3H, s, C-28H), 0.83 (3H, s, C-25H), 0.95 (3H, s, C-27H), 0.98 (3H, s, C-24H), 1.026 (3H, s, C-26H), 1.22 (1H, m, C-21H), 1.25 (1H, m, C-9 αH), 1.35 (1H, m, C-18 αH), 1.38 (2H, m, 7H), 1.58 (2H, m, C-2H), 1.66 (5H, m, C-1H, C-12H, C-13βH), 2.45 (1H, m, C-19βH), 3.2 (1H, dd, C-3βH), 4.6 (1H, spl.d, C-29H).

The elemental analysis of **EEPE-1** confirms the proposed structure (Figure 4.3). The observed elemental composition C: 82.48% and H: 12.04% complied with that of calculated value, C: 84.43% and H: 11.81%, of molecular formula $C_{30}H_{50}O$, lupeol.

4.4.2. Isolation of EEPE-2

It is a cream colored granular powder, soluble in hexane, benzene, chloroform.

Melting point: 213-215 °C.

IR ν_{max} (KBr) cm^{-1}: The IR spectrum of the EEPE-2 (Figure 4.4) showed characteristic absorption at 2916.2 and 2850.6 (due to stretching vibration

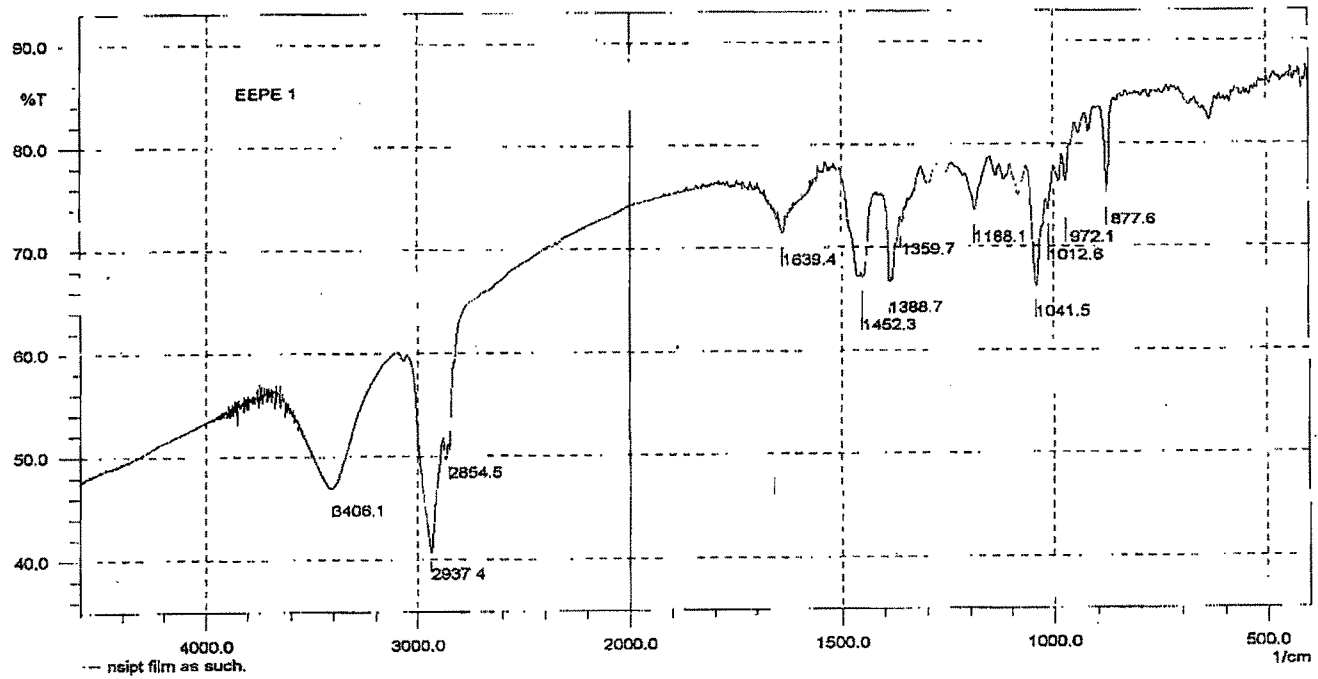

Figure 4.1. IR spectrum of the compound EEPE-1

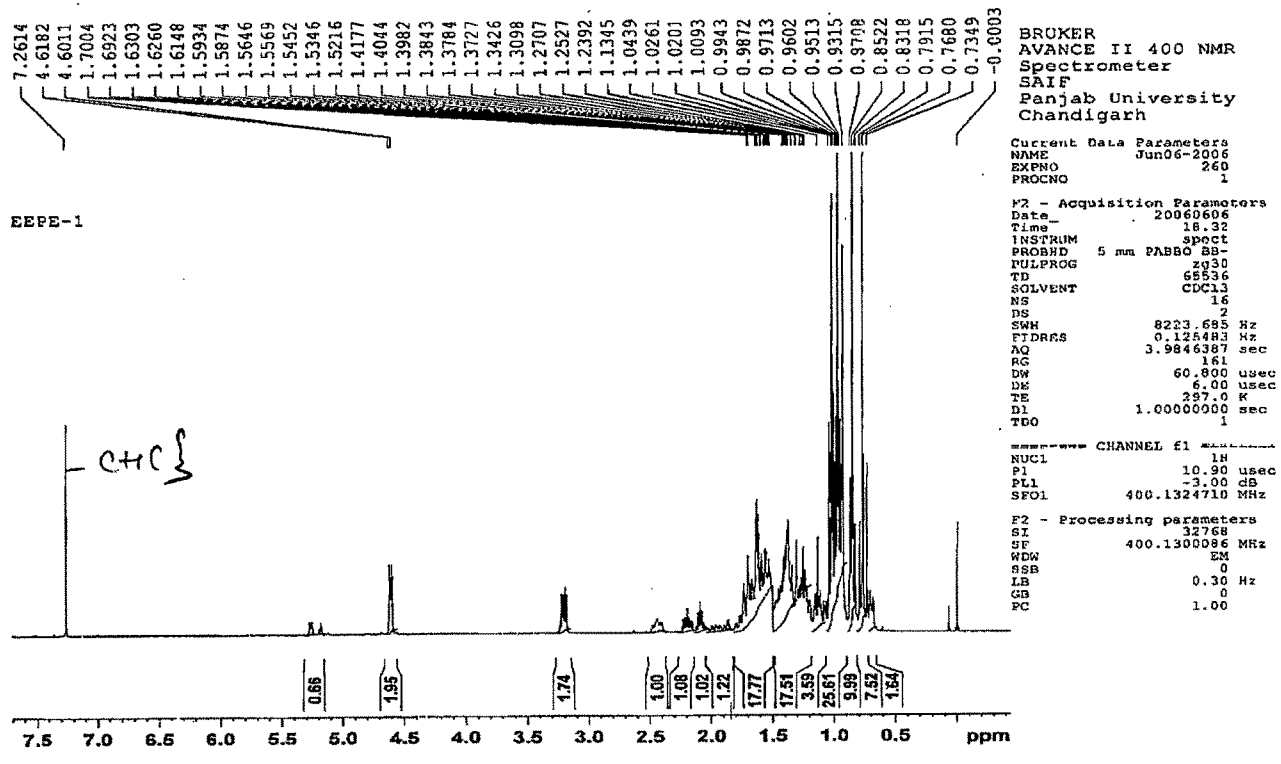

Figure 4.2. ^{1}H-NMR spectrum of the compound EEPE-1

of –CH in steroid skeleton), 1728.1 (due to stretching vibration of C=O), 1637 (due to stretching vibration of C=C).

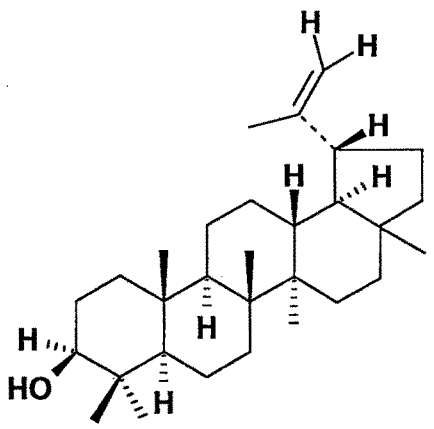

Figure 4.3

The proton nuclear magnetic resonance spectrum of EEPE-2 (Figure 4.5) was recorded using Bruker Avance II 400 NMR spectrometer. The obtained proton signals are mentioned below.

^{1}H-NMR: 0.73, 0.84, 0.85, 0.86, 0.87, 0.88, 0.89, 0.92, 0.94, 0.96, 0.97, 0.98, 0.99, 1.01, 1.02, 1.04, 1.23, 1.25, 1.28, 1.30, 1.31, 1.32, 1.33, 1.34, 1.37, 1.39, 1.40, 1.41, 1.52, 1.56, 1.58, 1.60, 1.62, 1.63, 1.65, 1.69, 2.27, 2.29, 2.30, 4.6, 4.61.

Elemental composition: C: 82.14%; H: 12.60%.

The results of the spectral studies showed that the compound EEPE-2 shows most of the properties similar to that of EEPE-1. Thus it is expected that the compound EEPE-2 could be derivative of EEPE-1. Since the yield of the compound was very low therefore no further investigations were undertaken.

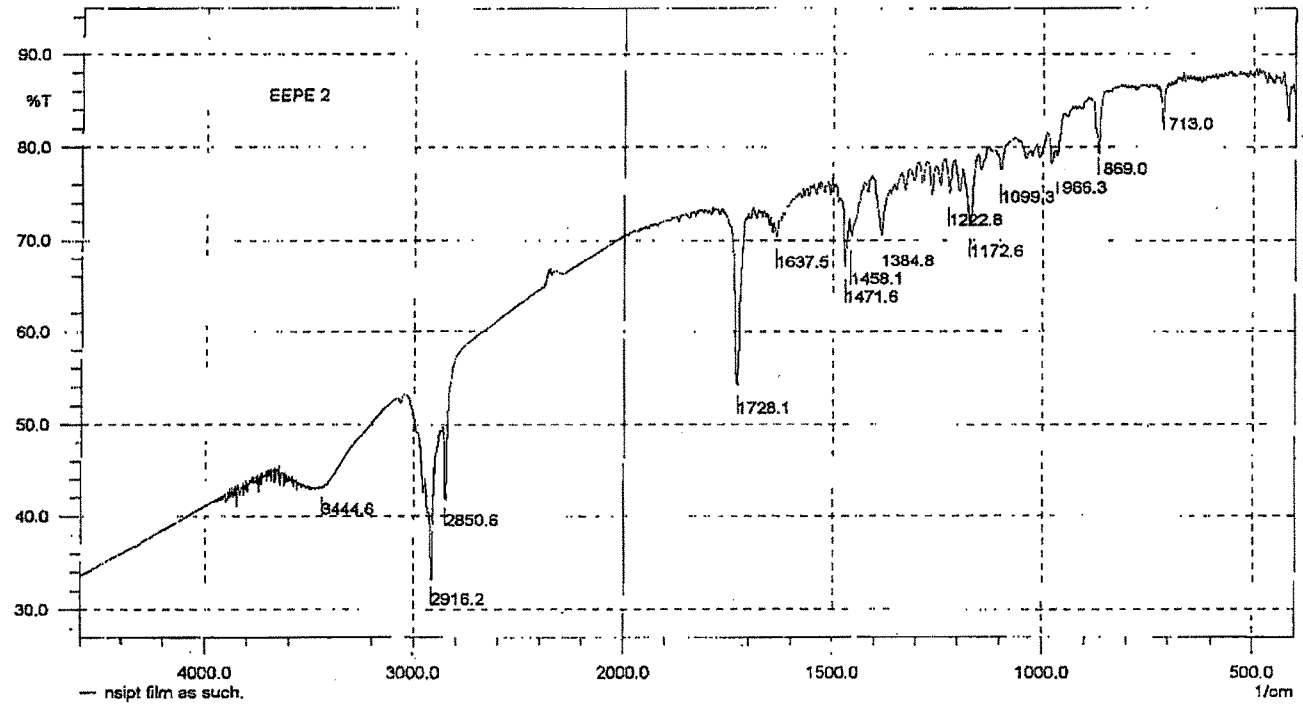

Figure 4.4. IR spectrum of the compound EEPE-2

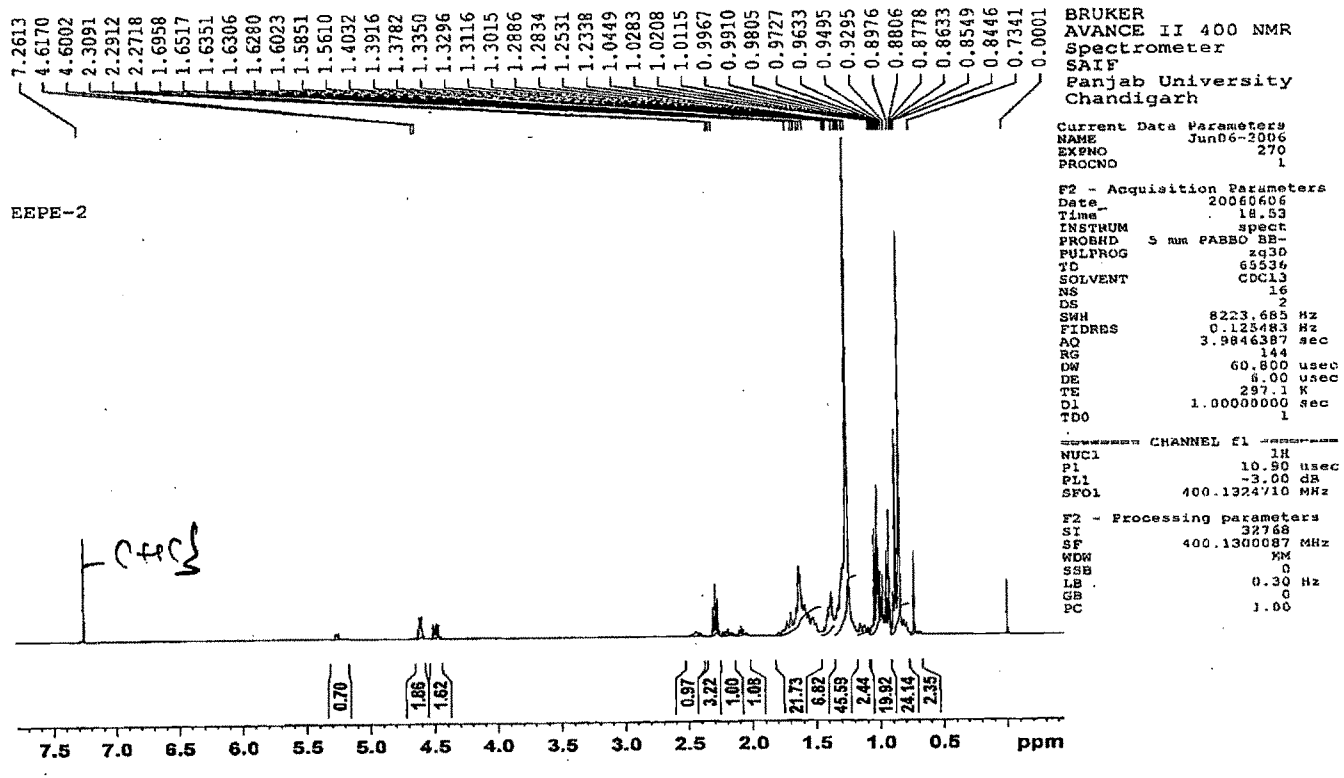

Figure 4.5. ^{1}H-NMR spectrum of the compound EEPE-2

4.5 Development of HPTLC assay method for quantification of lupeol

4.5.1. Reagents and chemicals

Pure lupeol was obtained from M/s Sigma chemicals, antimony trichloride (S. d. fine Chemicals), glacial acetic acid, etc. Other solvents and chemicals used were of analytical grade.

4.5.2. Preparation of test solutions

Accurately weighed 1.0 g of the coarse powder of *E. echinatus* roots and 2.5 g of *T. glaberrima* aerial parts were separately extracted with methanol (4 X 25 ml) under reflux (30 minutes each time) on a water bath. The combined extracts were filtered and concentrated, which were then transferred separately to 25 ml volumetric flasks and their volume was made up with methanol.

4.5.3. Preparation of standard solutions

A stock solution of the lupeol (100 µg/ml) was prepared by dissolving 5 mg of accurately weighed lupeol in methanol and making up the volume of the solution to 50 ml with methanol. Further dilutions were prepared by transferring aliquots (0.5 to 2.5 ml) of stock solution to 10 ml volumetric flask and adjusting the volume to 10 ml with methanol so that the obtained standard solutions contains 5, 10, 15, 20 and 25 µg/ml lupeol, respectively.

4.5.4. Preparation of calibration curve for lupeol

10 µl each of the standard solutions (50, 100, 150, 200 and 250 ng per spot) were applied (band width: 6mm) on a TLC plate. The plate was developed in twin trough chamber (previously equilibrated with mobile phase for not less than 20 minutes) using a solvent system of toluene-chloroform-ethyl acetate-glacial acetic acid (10:2:1:0.03 v/v) to up to a distance of 8 cm. After development the plate was dried in air (2 minutes) and then treated with freshly prepared antimony trichloride reagent (Wagner and Bladt, 1996) in a derivatization chamber. After treatment the plate was dried in air and then heated in oven at 105 °C for 10 minutes before densitometric scanning.

Densitometric scanning was performed in fluorescence mode at 366 nm. The peak areas were recorded. Calibration curve of lupeol was obtained by plotting peak area versus concentration of lupeol applied.

4.5.6. Quantification of lupeol in test samples

10 µl each of the sample solutions were applied in triplicate on a TLC plate with Linomat-V applicator. The plate was developed and scanned as mentioned above. Peak areas were recorded and the amount of lupeol in both the samples was calculated using the calibration curve of lupeol.

4.5.7. Method validation

The method was validated for precision, repeatability and accuracy (Anonymous, 2005). Instrumental precision was checked by repeated scanning of the same spot of lupeol (150 ng) seven times and was expressed as coefficient of variance (%RSD). Repeatability of the method was affirmed by multiple measurements (n = 5) of lupeol after application on TLC plate (150 ng per spot) under the same analytical procedure and laboratory conditions and was expressed as %RSD. Variability of the method was studied by analyzing aliquots of standard solution of lupeol (100, 150, 200, 250 ng per spot) on the same day and on different days (inter-day precision) and the results were expressed as %RSD.

Accuracy of the method was tested by performing the recovery studies at three levels. To 0.5 g quantities of powdered roots of *E. echinatus* known amounts of lupeol (100, 200 and 300 µg) were added, extracted and estimated as described above. The percentage recovery as well as average percentage recovery was calculated.

4.6 Results

Of the various solvent systems tried the one with toluene-chloroform-ethyl acetate-glacial acetic acid (10:2:1:0.03 v/v) gave the best resolution of free lupeol (R_f = 0.40) in the presence of other compounds in the extract. Identity of the band of lupeol in the sample extracts was confirmed by overlaying its UV absorption spectrum with that of the standard lupeol (Figure 4.7).

Purity of lupeol in the sample extract was confirmed by comparing the absorption spectra at start, middle and end position of the band. The method was validated in terms of precision, repeatability and accuracy (Table 4.1; Table 4.2). The relationship between the concentration of lupeol and the peak response was linear within the concentration range of 50 - 250 ng/spot with a correlation coefficient of 0.999 (Figure 4.6). The percentage recovery at three different levels was found to be 99.79%, 101.74% and 101.02% with an average of 100.85% (Table 4.3).

The free lupeol content in the roots of *E. echinatus* and aerial parts of *T. glaberrima* was estimated by the proposed method and was found to be 0.0355 and 0.0051 % w/w respectively (Table 4.4; Figure 4.7).

4.7 Quantitative estimation of lupeol content in TRFPE of the roots of *E. echinatus* by HPTLC

4.7.1. Preparation of TRFPE from the roots of *E. echinatus*

See chapter 4; section 4.1

4.7.2. HPTLC conditions

The HPTLC system (CAMAG) consisting of a LINOMAT-IV applicator and TLC Scanner-III, precoated plates of silica gel G60 F254 (E-Merck) and a mobile phase containing toluene-chloroform-ethyl acetate-acetic acid (10:2:1:0.1) were used in the studies. Detection was done using antimony trichloride

Table 4.1. Method validation parameters for quantification of lupeol using proposed HPTLC method.

Sl. No.	Parameter	Results
1	Precision (%RSD, $n = 7$)	0.56
2	Repeatability (%RSD, $n = 5$)	2.87
3	Accuracy (average % recovery)	100.85
4	Limit of detection	10 ng
5	Limit of quantification	50 ng
6	Linearity (correlation coefficient)	0.999
7	Dynamic range (ng per spot)	50-250

Table 4.2. Intra and inter day precision study

Sl. No.	Concentration (ng spot^{-1})	Intra day Precision*	Inter day Precision*
1	150	2.02	1.91
2	200	1.22	2.02
3	250	0.71	1.03

* Mean (n = 3)

Table 4.3. Recovery studies of lupeol

Sl. No.	Amount of lupeol present in the sample (µg)	Amount of lupeol added (µg)	Amount of lupeol found* (µg)	Recovery* (%)	Average recovery (%)
1	177.5	90	266.95 ± 3.72	99.79	
2	177.5	180	363.75 ± 0.72	101.74	100.85
3	177.5	270	452.07 ± 3.15	101.02	

* Mean ± SD (n = 3)

Table 4.4. Quantification of lupeol content in two plant materials using the HPTLC method described.

Sl. No.	Sample	Content of lupeol (% w/w)
1	Roots of *E. echinatus*	0.0355
2	Aerial parts of *T. glaberrima*	0.0051

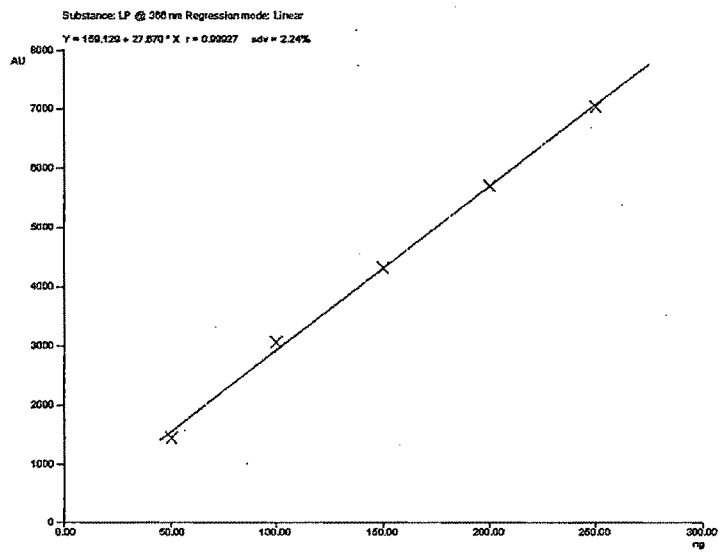

Figure 4.6. Linearity of the calibration curve for quantification of lupeol

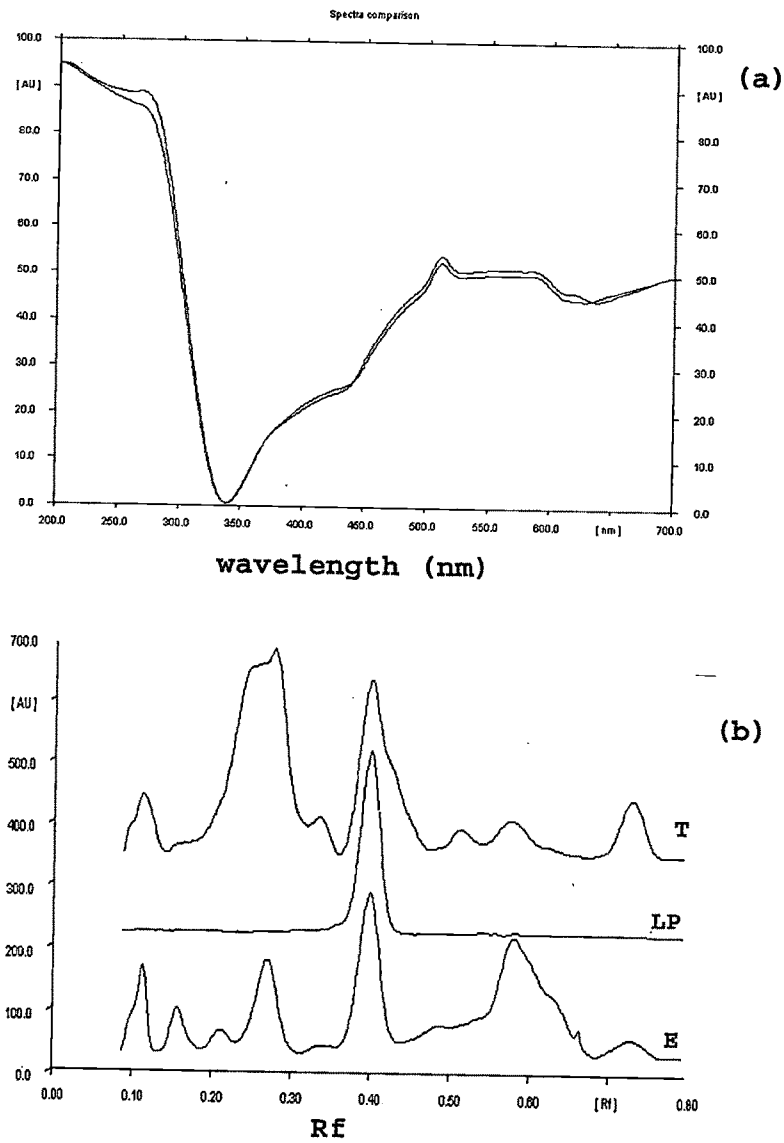

Figure 4.7. (a) Overlaid spectra of lupeol standard and sample in absorption mode in the UV range (b) Chromatogram of methanol extract of the roots of *E. echinatus* (E), aerial parts of *T. glaberrima* (T) and standard lupeol (LP), recorded at 366 nm.

reagent (Wagner and Bladt, 1996) followed by scanning under ultraviolet (UV) light at 366 nm.

4.7.3. Preparation of sample solutions

A test solution containing 1 mg/ml of the TRFPE was prepared in chloroform and methanol (1:1).

4.7.4. Preparation of calibration curve

See section 4.5.4

4.7.5. Estimation of the lupeol in TRFPE

Test solution (10 µl) was applied in triplicate on a precoated TLC plate of uniform thickness (200 ng per spot). The plate was developed in the above said solvent system and the peak area of lupeol was recorded as described for the calibration curve. The amount of lupeol present in the sample was calculated from the calibration curve of lupeol (Figure 4.6).

4.8. Results

Terpenoids in TRFPE were separated on a TLC plate and were detected by treatment with anisaldehyde sulfuric acid reagent (Figure 4.8). HPTLC chromatogram of TRFPE showed the presence of six peaks at R_f values 0.12, 0.25, 0.36, 0.52, 0.74 and 0.99, of which the peak at R_f 0.52 was found to match with that of pure lupeol (Figure 4.7). The content of lupeol in the TRFPE was found to be 38.3% w/w.

4.9. Discussion

From the work undertaken to isolate and separate the chemical constituents from TRFPE, lupeol, a known terpenoid was isolated for the first time. It was previously reported from many plants and known for its various biological activities (Agarwal and Rangari 2003; Sunitha et al., 2001).

Figure 4.8. Detection of terpenoids in TRFPE on TLC using anisaldehyde sulfuric acid reagent.

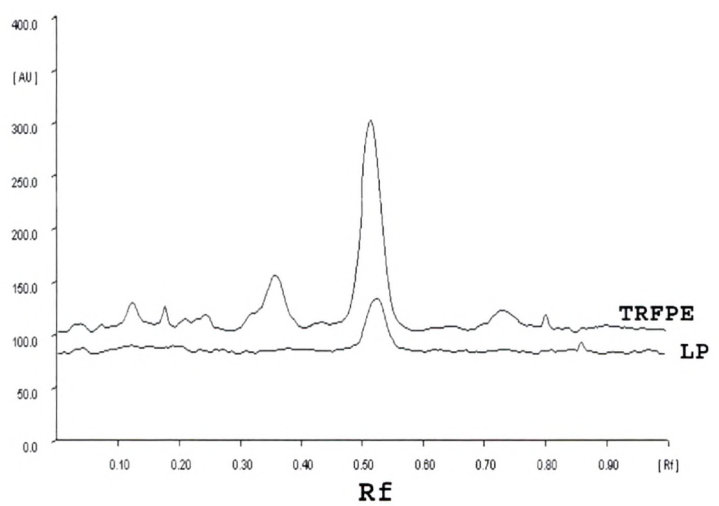

Figure 4.9. Chromatogram of TRFPE and standard lupeol (LP) recorded at 366 nm.

Marker compounds along with chromatographic profiles may be used to standardize the herbal raw materials. The exact chemical composition of a plant material depends on its growing conditions (soil and climate), harvest and post-harvest handling etc. The clinical efficacy and pharmacological effects of a plant material will depend strongly on the amounts of biologically active ingredients present, and these must be accurately measured if a plant material is to be chemically standardized. Thus for the evaluation of identity and determination of quality of a medicinal plant material a validated analytical method of analysis for the active ingredients has to be developed.

Considering the wide therapeutic applications and importance of 'Brahmadandi' a quantitative HPTLC assay for lupeol content in roots of *E. echinatus* and aerial parts of *T. glaberrima* has been developed and validated, which can be used to ensure the quality of commercial samples. The method developed in this study permits for chemical standardization of both the plant materials. The practical applicability of the proposed method for quality control of Brahmadandi was demonstrated through validation of various parameters. The method has been shown to be linear in the range of 50 - 250 ng per spot with a limit of detection of 10 ng. One of the principle requirements for any method of quantitave analysis is its specificity which ensures that no component of the samples interferes with the detection of the target compound. This has been confirmed by overlaying the spectrum of standard lupeol with that in the sample material. The developed HPTLC method for estimation of lupeol is simple, precise and accurate and can be used for the routine quality control of the commercial samples of Brahmadandi.

The content of lupeol was also determined in TRFPE by using the proposed HPTLC method (38.3% w/w). Various therapeutic applications and the biological activities associated with the use of the roots of *E. echinatus* may be endowed to the presence of lupeol and other terpenoidal compounds in it.

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