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DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD BASED ON HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY FOR THE DETERMINATION OF PIPRETENONE IN VOLATILE OIL AND DIFFERENT EXTRACTS OF *M. LONGIFOLIA (HABAK)*

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ABSTRACT

Our TLC study of the volatile oil isolated from *Mentha longifolia* showed a major UV active spot with R_f value higher than menthol. As the components of the oil differ quantitatively due to environmental conditions we isolated and identified that spot by spectroscopic means. The data indicated that the spot corresponding to pipretenone. Pipretenone was separated on aluminum-backed silica gel 60 F_{254} plates with *n*-hexane/ethyl acetate 8:2 (%, *v*/*v*) as mobile phase. A compact band was obtained for pipretenone at R_f value of 0.32±0.04. The calibration plot was linear in the range of 50-700 ng/spot of pipretenone and the correlation coefficient of 0.9985 was indicative of good linear dependence of peak area on concentration. In this paper we determine the exact amount of pipretenone in volatile oil and different extracts of *M. longifolia* (Habak) by densitometric HPTLC validated method.

Keywords: *Mentha longifolia*, Volatile Oil, Pipretenone, Quantitative, HPTLC

INTRODUCTION

The family Labiatae, or the mint family (recently named Lamiaceae) consist of about 200 genus and between 2000 or 5000 species of fragrant plant and short shrubs having a large diversity and around 22 species are considered to be medicinal plants **[1, 2, 3]**. *Mentha* spp. have been used as a folk remedy for treatment bronchitis, flatulence, anorexia, ulcerative colitis and liver complaints due to their antiinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue and anticatharral activities **[4]**.

Three polyphenolic compounds; gallic acid, catechin and caffeic acid were identified by HPLC-DAD from *M. longifolia* **[5]**. Three luteolin glycosides; luteolin 7-*O*-glucoside, luteolin 7-*O*-rutinoside and luteolin 7-*O*-glucuronide were also reported **[6]**. In another study the antimicrobial activity of the ethanol extract of *M. longifolia* was attributed to the flavonoids derivatives luteolin-7-O-glycoside, luteolin-7, 3'-O-diglycoside, apigenin, quercetin-3-O-glycoside and kaempferol-3-O-glycoside **[7]**. Another study involved Irania collection of the plant reveal that the methanol extract posses antibacterial, antifungal and cytotoxic properties **[8]**. A double-blind, randomized, placebo-controlled, multicenter study rvealed that *M. longifolia* syrup is a safe, well-tolerated, and effective choice in inducing and maintaining regular bleeding in women with secondary amenorrhea and oligomenorrhea **[9]**.

As a member of the mint family the volatile oil of *M. longifolia* was subjected to several studies by GC-MS. Three populations collected from different places in south-central Tajikistan were qualitatively similar, but showed quantitative differences. The major components and their percentage of the oil were *cis*-piperitone epoxide (7.8-77.6%), piperitenone oxide (1.5-49.1%), carvone (0.0 21.5%), pulegone (0.3-5.4%), menthone (0.0-16.6%), thymol (1.5-4.2%), β-thujone (0.2-3.2%), carvacrol (0.0-2.7%), and (*E*)-caryophyllene (0.9- 2.5%) [10]. The oil obtained from a Serbian collection showed *trans* and *cis*-dihydrocarvone (23.64% and 15.68%), piperitone (17.33%), 1,8-cineole (8.18%) and neoisodihydrocarveol (7.87%) as the major components. This oil showed both antifungal and antioxidant activities **[11]**. Oil obtained from Bosnia and Herzegovina showed antibacterial and antioxidant activities and its major components were piperitone oxide (63.58%), 1.8-cineole (12.03%) caryophyllene oxide (4.33%), trans-caryophyllene (2.98%) and cis-caryophyllene (0.82%) **[12]**. Antibacterial, antifungal and antioxidant activates were reported for oil obtained from Tunisian collection with pulegone (54.41%) as a major component followed by isomenthone (12.02%), 1,8-cineole (7.41%), borneol (6.85%), and piperitenone oxide (3.19%) **[13]**. Insecticidal activities were reported for Irania collection of the plant with major components piperitenon (43.9%), tripal (14.3%), oxathiane (9.3%), piperiton oxide (5.9%), and dlimonene (4.3%) **[14]**. Turkish samples with major components *cis*-piperitone epoxide (26.52 %), piperitenone oxide (26.40 %), pulegone (15.66 %) and piperitenone (13.57 %) showed good protection against oxidative stress caused by a single dose of Aflatoxin B_1 in the human lymphocyte culture **[15]**.

To our knowledge, no reports on HPTLC analysis of piperitenone in different extracts and volatile oil of *M. longifolia* utilizing normal phase silica gel plates have been mentioned in the literature. In the present study we have proposed new validated simple HPTLC for the determination piperitenone in different extracts and volatile oil of *M. longifolia*. The proposed method was validated as per ICH guidelines.

MATERIAL AND METHODS

General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded on a UltraShield Plus 500MHz (Bruker) (NMR Unite at the College of Pharmacy, Salman Bin Abdulaziz University) spectrometer operating at 500 MHz for proton and 125 MHz for carbon, respectively. The chemical shift values are reported in δ (ppm) relative to the internal standard TMS or residual solvent peak, the coupling constants (*J*) are reported in Hertz (Hz). 2D-NMR experiments (COSY, HSQC, HMBC and NOESY) were obtained using standard *Bruker* program. ESIMS were measured using an Agilent Technologies model 6410 Triple quadrupole LC/MS system. Centrifugal preparative TLC (CPTLC) was performed on a Chromatotron (Harrison Research Inc. model 7924): 1 mm silica gel P254 disc. Silica gel 60/230–400 mesh (EM Science) was used for column chromatography, while silica gel 60 F254 (Merck) was used for TLC.

Chemicals

Piperitenone was purchased from Nippon Terpene Chemicals. All the solvents were of HPLC grade and other chemicals used were of analytical reagent (AR) grade.

Plant Materials

The aerial parts of *Mentha longifolia* L. was purchased from the local markets at Al-Kharj city, Kingdom of Saudi Arabia. The plants were identified by Dr. Mohammed Yusuf, Taxonomist at the Research Center of Medicinal, Aromatic and Poisonous plants, a voucher specimen (#14219) was deposited in the Herbarium of the Department of Pharmacognosy, College of Pharmacy, Salman bin Abdulaziz University for future reference.

Extractions Procedure

The dried leaves (5 g) were extracted by percolation at room temperature with MeOH till exhaustion. The solvent was evaporated under reduced pressure and the residue was dissolved in methanol using 25 mL volumetric flask. This solution was used as the test solutions in the TLC densitometric analysis. Fresh aerial parts (10 gm) were extracted with MeOH till exhaustion. The solvent was evaporated under reduced pressure and the residue was dissolved in methanol using 50 mL volumetric flask. Two patches of fresh aerial parts were soaked in 800 mL hot water. Four tea bags were added to one patch. Water extraction was repeated with 175 gm of dry plant. After cooling the plant materials were filtered and the water layer was extracted with CH_2Cl_2 (3 X 300). After concentration of the organic layer, extracts were transferred to 50 mL volumetric flasks. Then 2 mL was transferred to 10 mL volumetric flask and completed to volume with CH_2Cl_2.

Preparation of the volatile oil

The volatile oil of *M. longifolia* were prepared by hydrodistillation method (Egyptian Pharmacopoeia, 1984). Accurately weighed 200 gm of *M. longifolia* to Clevenger trap apparatus for oils lighter than water, 1000 mL of water were added and distillations were continued for 8 hr. The oil layer and water in the trap was extracted with dichloromethane (3X50 mL) and the organic layer was concentrated using rotary vacuum evaporator to afford 3.83 g. The resulting volatile oil was stored at 4°C prior to further analyses.

Isolation of Pipretenone from M. longifolia volatile oil

The volatile oil (1.2 g) obtained from hydrodistillation and hexane as solvent was chromatographed on Silica gel column (50 gm x 1cm i.d.) eluted with *n*-hexane. Twenty fractions 25 ml each were collected, screened by TLC and similar fractions were pooled. Fractions 3-4 showed single spot with R_f value corresponding to the major component of the oil. The fractions were further purified by CPTLC (hexane: ethyl acetate, 9: 1) to afford 11 mg of pure oily material (**1**).

Preparation of standard solutions

Accurately weighed 10 mg of standard piperitenone was dissolved in MeOH in a 100 mL volumetric flask to gives concentration of 100 μg/mL. This solution was used as a reference solution (stock solution) for piperitenone.

HPTLC method and instrumental conditions

HPTLC densitometric analysis was performed on 10 × 20 cm aluminium-backed plates coated with 0.2 mm layers of silica gel 60 F_{254} (E-Merck, Germany). Samples were applied to the TLC plates as 6 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microlitre syringe. A constant application rate of 150 nl/s was used. Linear ascending development of the plates to a distance of 80 mm was performed with hexane: ethyl acetate 8:2 (%, *v/v*) as mobile phase in a Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapour for 30 min at 22°C. After development, the plates were scanned at 289 nm using a Camag TLC scanner in absorbance mode, using the deuterium lamp. The slit dimensions were 4.00 × 0.45 mm and the scanning speed was 20 mm/s.

Method validation

The proposed HPTLC densitometric method was validated according to the guidelines of international conference on harmonization **[16]**. The linearity of piperitenone was checked between 50-700 ng/spot. Graph was plotted between concentration and peak area for linearity. Linearity data was statistically treated using least square linear regression analysis.

Accuracy

Accuracy was determined by standard addition method. The preanalyzed sample of piperitenone (300 ng/spot) was spiked with the extra 0, 50, 100 and 150 % of the standard piperitenone and the solutions were reanalyzed in six replicates by the proposed method. The % recovery and percent relative standard deviation (% RSD) were calculated at each concentration level.

Precision

Precision of the proposed method was determined at two levels i.e. repeatability and intermediate precision. Repeatability was determined as intraday precision whereas intermediate precision was determined by carrying out inter-day variation for the determination of piperitenone at three different concentration levels of 300, 400 and 500 ng/spot in six replicates.

Robustness

Robustness of the proposed HPTLC method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of piperitenone. Robustness was determined by changing the polarity of the mobile phase.

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined by standard deviation (SD) method. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

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

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

Specificity

Specificity of the proposed TLC densitometric was confirmed by analyzing and comparing the R_f values and spectra of the spot for piperitenone in the samples with that of the standards.

Quantification of pipretenone in volatile oil and different extracts of *M. longifolia*

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard piperitenone. The area of the peak corresponding to the R_f value of piperitenone standards were recorded and the amount present were calculated from the regression equation obtained from the calibration plot.

RESULTS AND DISCUSSION

Method development

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of piperitenone. The mobile phase *n*-hexane: ethyl acetate 8:2 (%, *v*/*v*) resulted in a sharp, symmetrical, and well resolved peak at R_f value of 0.32 (Figure 1). UV spectra measured for the bands showed maximum absorbance at approximately 289 nm.

Calibration curve

The calibration plot of peak area against amount of piperitenone was linear in the range 50-700 ng/spot. Linear regression data for the plot confirmed the good linear relationship **(Table 1)**. The correlation coefficient (R^2) was 0.9985 which was highly significant ($P<0.05$). The linear regression equation was $Y = 8.8894x + 531.41$, where Y is response and X is amount of pipretenone **(Figure 2)**.

Method validation

Precision

The accuracy of the method, as recovery, was 98.39-99.16 %, with RSD values in the range 0.58-1.35. These results indicated the method was accurate (Table 2). Results from determination of repeatability and intermediate precision, expressed as SD (%) are shown in **Table 3**. RSD was in the range 0.66-0.82 for repeatability and 0.79–0.98 for intermediate precision **(Table 3)**. These low values indicate the method is precise.

Robustness of the Method

Results of robustness are shown in **Table 4**. Low values of % RSD (0.78-1.05) were obtained after introducing small deliberate change into the densitometric TLC procedure proved the robustness of the proposed HPTLC method.

Limit of detection and quantification

LOD and LOQ of the proposed method was found to be 11.04 and 22.12 ng/spot, for pipretenone, which indicated that the proposed method can be used in wide range for detection and quantification of piperitenone effectively.

Specificity

The peak purity of piperitenone was assessed by comparing the overlaid spectra at peak start, peak apex and peak end position of the spot. The overlaid spectra of piperitenone standards and external analgesic formulations was given in **Figure 5**.

Quantification of piperitenone in volatile oil and different extract of *M. longifolia*

The method proposed for quantitative estimation of piperitenone showed good linearity (r^2= 0.9985) in the range of 50-700 ng/spot. The solvent system hexane: ethyl acetate (8:2, *v/v*) gives compact spot and sharp peak of piperitenone at R_f = 0.32 **(Figure 1)**. The regression equation obtained was Y = 8.8894x + 531.41, which was used for the quantification of total piperitenone content in volatile oil (22.34 %) **(Figure 2)**, methanolic extract of dry plant (1.91 %) **(Figure 3)**, methanolic extract of fresh plant (1.25 %) **(Figure 4)**. All spots were scanned at 289 nm, the maxima of piperitenone UV absorption **(Figure 5**), using a Camag TLC scanner in absorbance mode, using the deuterium lamp.

CONCLUSION

The HPTLC method developed for quantitation of piperitenone was found to be simple, accurate, reproducible and sensitive and is applicable to the analysis of a wide variety of habak-containing products. The proposed method for quantification of piperitenone is the first validated HPTLC method to the best of my knowledge. Statistical data proves that the method is reproducible and selective for the analysis of piperitenone with added advantages of short time, minimal sample preparation, in addition to the low cost.

CONFLICT OF INTEREST

The author reports no declaration of interest. The author alone is responsible for the content and writing of this paper.

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Table 1: Linear regression data for the calibration curve of pipretenone (n=6)

Linearity range (ng/spot)	**50-700**
Regression equation	**Y = 8.8894x + 531.41**
Correlation coefficient	**0.9985**
Slope ± SD	**8.880 ± 0.08475**
Intercept ± SD	**537.0 ± 35.49**
Standard error of slope	**5.12**
Standard error of intercept	**310.04**
95% confidence interval of slope	**8.705 - 9.056**
95% confidence interval of intercept	**463.4 –610.6**

Table 2: Accuracy of the proposed method (n=3)

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) ± SD	% Recovery	% RSD
0	100	295.17 ± 3.97	98.39	1.35
50	150	445.83 ± 3.49	99.07	0.78
100	200	592.50 ± 7.40	98.75	1.25
150	250	743.67 ± 4.32	99.16	0.58

Table 3: Precision of the proposed method

Conc. (ng/spot)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Avg Conc. ± SD (n = 3)	Standard error	% RSD	Avg Conc. ± SD (n = 3)	Standard error	% RSD
300	3240.00 ± 24.69	10.08	0.76	3235.50 ± 31.87	13.01	0.98
400	4166.00 ± 34.32	14.01	0.82	4154.00 ± 35.87	14.65	0.86
500	5053.67 ± 33.39	13.63	0.66	5035.33 ± 39.70	16.21	0.79

Table 4: Robustness of the proposed HPTLC method

Conc. (ng/spot)	Mobile phase composition (Hexane: ethyl acetate)			Results		
	Original	Used		Area ± SD (n = 3)	% RSD	R_f
		8:2.1	-0.1	5039 ± 39.34	0.78	0.33
500	8:2	8:2	0.0	5042 ± 53.02	1.05	0.32
		8:1.9	+0.1	5055 ± 45.67	0.90	0.30

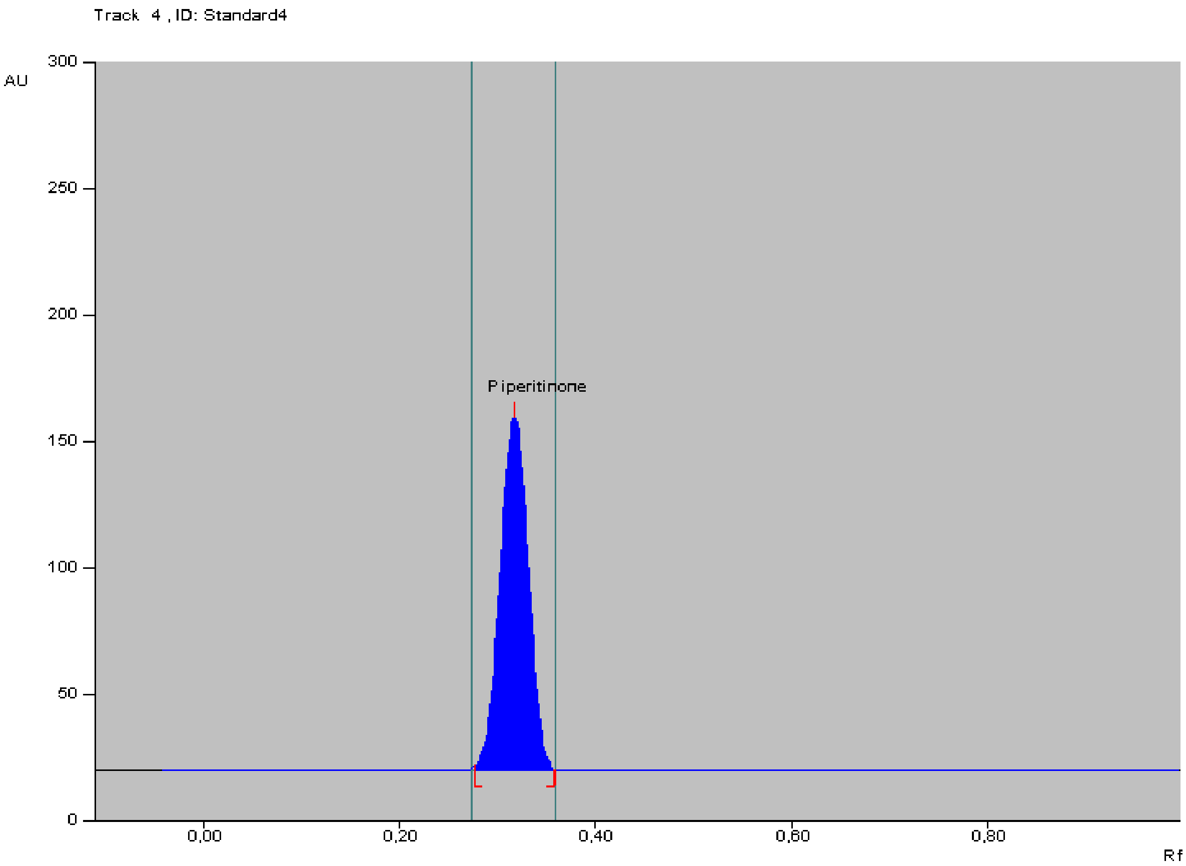


Figure 1: HPTLC chromatogram of standard Piperitenone

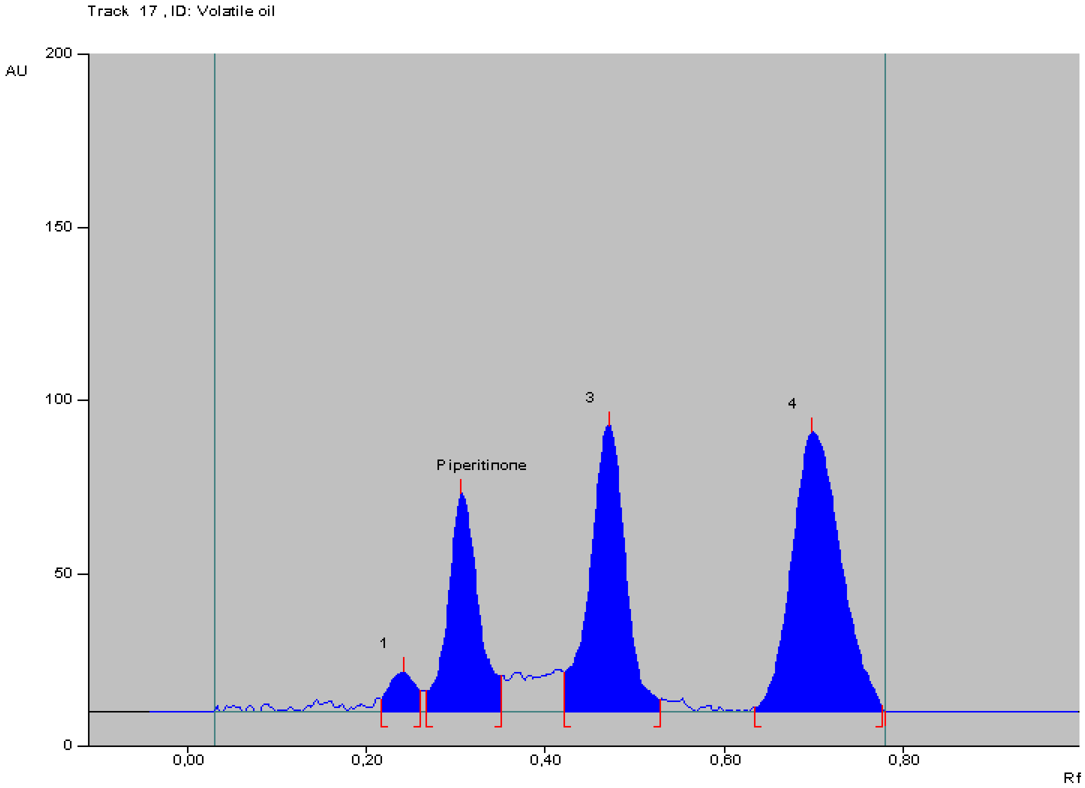


Figure 2: HPTLC chromatogram of volatile oil

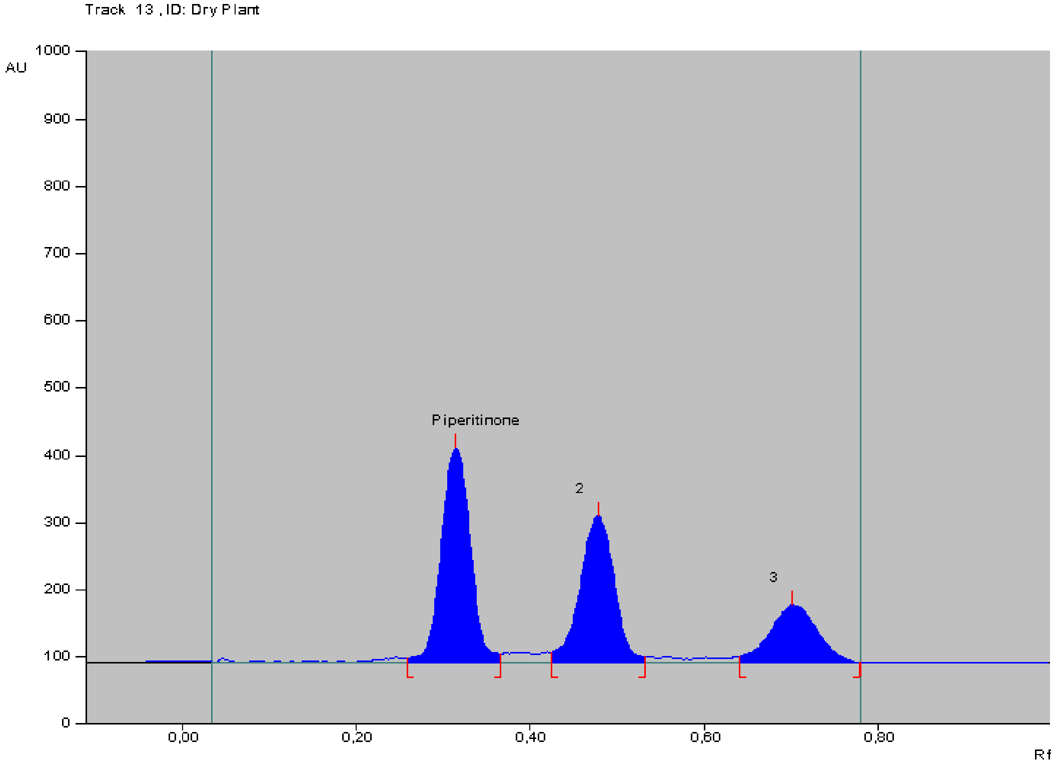


Figure 3: HPTLC chromatogram of MeOH extract of dry plant

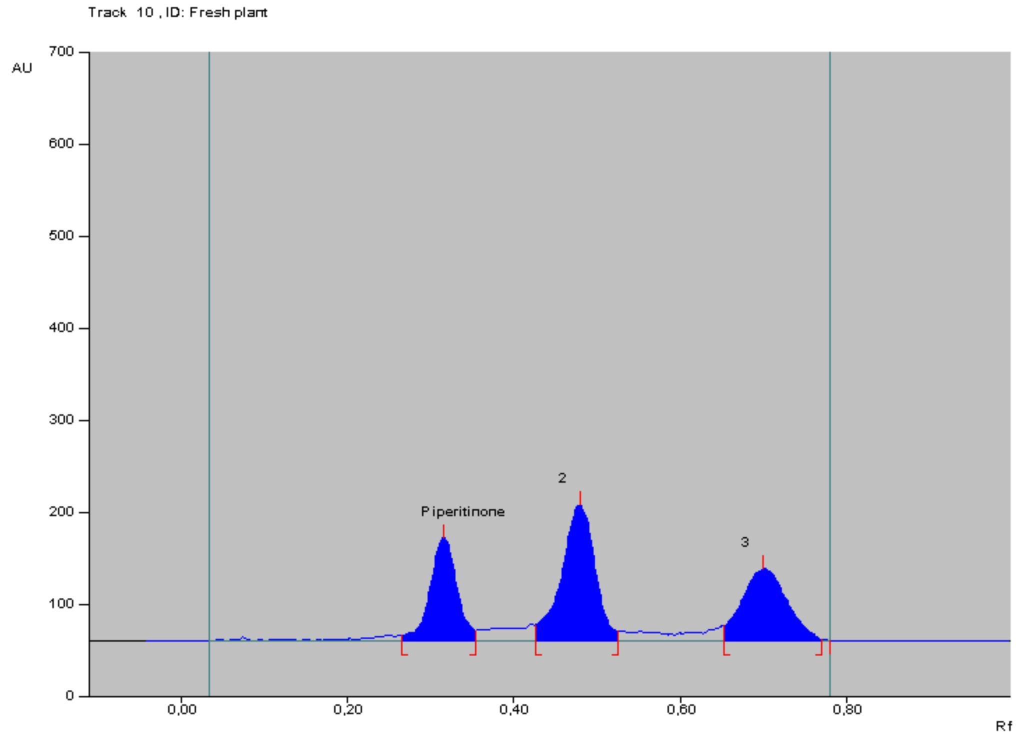
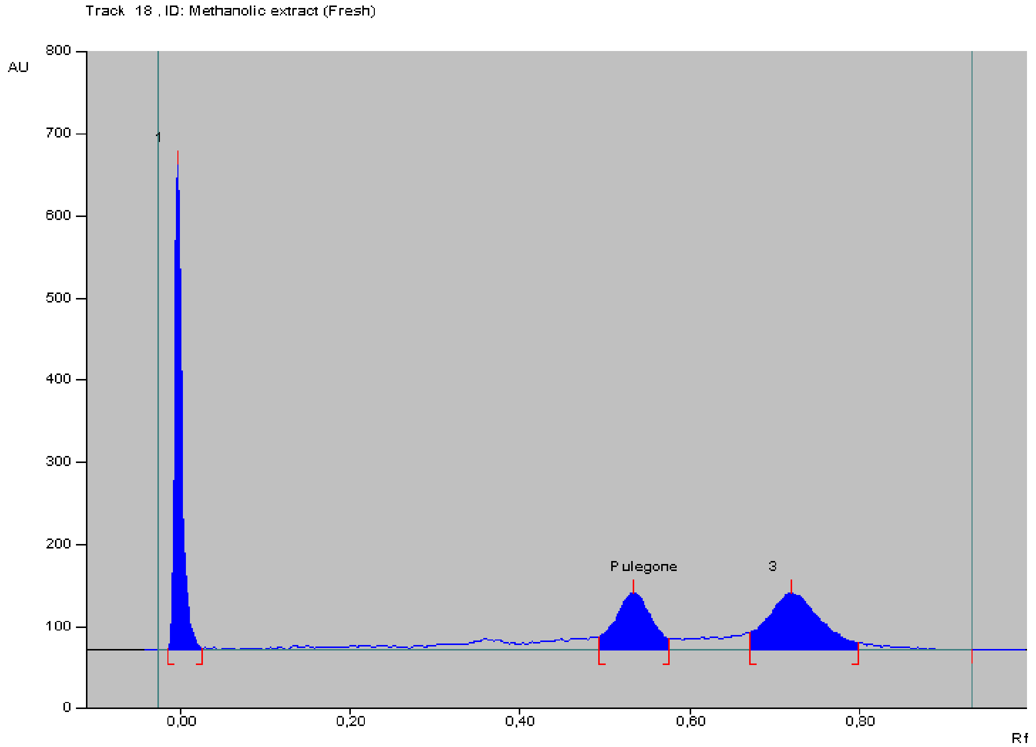


Figure 4: HPTLC chromatogram of MeOH extract of fresh plant



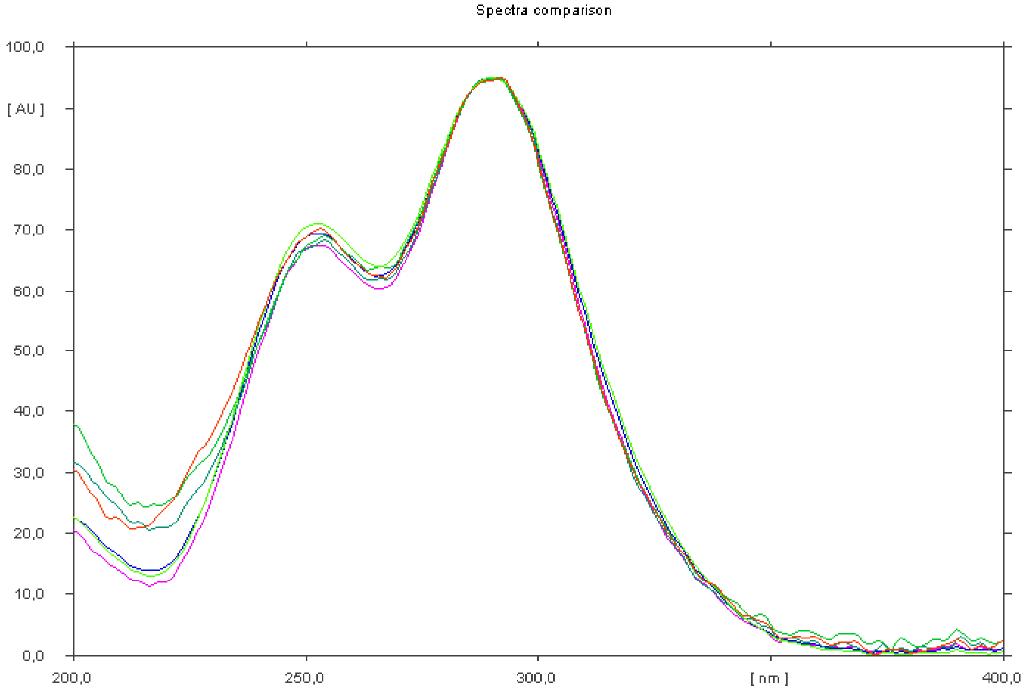


Figure 5: UV spectra of standard pipretenone and different extracts of *M. longifolia*