Quantitative estimation of pulegone in *Mentha longifolia* growing in Saudi Arabia. Is it safe to use?

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Abstract: Our TLC study of the volatile oil isolated from *Mentha longifolia* showed a major UV active spot with higher R_f value than menthol. Based on the fact that the components of the oil from same plant differ quantitatively due to environmental conditions, the major spot was isolated using different chromatographic techniques and identified by spectroscopic means as pulegone. The presence of pulegone in *M. longifolia*, a plant widely used in Saudi Arabia, raised a hot debate due to its known toxicity. The Scientific Committee on Food, Health & Consumer Protection Directorate-General, European Commission set a limit for the presence of pulegone in foodstuffs and beverages. In this paper we attempted to determine the exact amount of pulegone in different extracts, volatile oil as well as tea flavoured with *M. longifolia* (Habak) by densitometric HPTLC validated methods using normal phase (Method I) and reverse phase (Method II) TLC plates. The study indicated that the style of use of Habak in Saudi Arabia resulted in much less amount of pulegone than the allowed limit.

Keywords: *Mentha longifolia*; pulegone; volatile oil; quantitative; HPTLC.

INTRODUCTION

The family Labiatae (Lamiaceae) consist of about 200 genus and up to 5000 species of aromatic plant and short shrubs. Around 22 species are considered to be medicinal plants (Ronald, 1974; Heyood, 1988; Hay *et al.*, 1993). Members of the genus *Mentha* have been used in folk medicine for treatment bronchitis, flatulence, anorexia, ulcerative colitis and liver complaints. These effects are due to the antiinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue and anticatharral activities (Gulluce *et al.*, 2007).

Three polyphenolic compounds; gallic acid, catechin and caffeic acid were identified by HPLC-DAD from *M. longifolia* (Najafian and Rowshan, 2013). Three luteolin glycosides; luteolin 7-*O*-glucoside, luteolin 7-*O*-rutinoside and luteolin 7-*O*-glucuronide were also reported (Orhan *et al.*, 2012). 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 (Akroum *et al.*, 2009). Another study involved Irania collection of the plant reveal that the methanol extract posses antibacterial, antifungal and cytotoxic properties (Razavi *et al.*, 2012). A double-blind, randomized, placebo-controlled, multicenter study revealed 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 (Mokaberinejad *et al.*, 2012).

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%) (Sharopov *et al.*, 2012). 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 (Dzamic *et al.*, 2010). 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%) (Niksic *et al.*, 2012). Antibacterial, antifungal and antioxidant activates were reported for oil obtained from Tunisian collection. The analyses of this collection revealed that pulegone (54.41%) was the major component followed by isomenthone (12.02%), 1,8-cineole (7.41%), borneol (6.85%), and piperitenone oxide (3.19%) (Mkaddem *et al.*, 2009). 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 d-limonene

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(4.3%) (Khani and Asghari, 2012). 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 followed a single exposure to Aflatoxin B_1 in the human lymphocyte culture (Ceker *et al*., 2013). *M. longifolia* is widely used in Saudi Arabia with the common name "Habak". A hot debate was erupted due to the presence of pulegone in the popular plant regarding its safety for use (Riyadh News paper, 2009; Shams News paper, 2009; Aledtisadiah news paper, 2009).

To the best our knowledge, quantitative analysis of pulegone in volatile oil and different extracts of *M. longifolia* by HPTLC was not reported. In the present study we have proposed new validated simple HPTLC for the quantitative analysis of pulegone using normal and reversed phase silica gel plates. The amount of pulegone in different extracts and volatile oil of *M. longifolia* was determined and the values were compared with the allowed limits set by the official European bodies.

MATERIALS AND METHODS

General experimental procedures

Ultraviolet absorption spectra were obtained on a Jasco UV-Visible V-630 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured using Ultra Shield 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 residual solvent peaks, the coupling constants (*J*) are reported in Hertz (Hz). 2D-NMR experiments (COSY, HSQC and HMBC) were recorded using *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): 1mm silica gel P254 disc. Silica gel 60/230-400 mesh (EM Science) was used as adsorbent for column chromatography, while silica gel 60 F254 (Merck) was used as coating material for TLC.

Chemicals

Standard pulegone was purchased from Sigma Aldrich. All the used solvents were of HPLC grade and other chemicals used (Sigma Aldrich) 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 plant was identified by Dr. Mohammed Yusuf, Taxonomist at the RCMAP at King Saud University, a voucher specimen (#14219) was deposited in the Herbarium of the Department of Pharmacognosy, College of Pharmacy, Prince Sattam bin Abdulaziz University for future reference.

Extractions procedure

The dried leaves (5g) were extracted at room temperature with MeOH till exhaustion. The solvent was evaporated using rotary vacuum evaporator and the residue was dissolved in methanol using 25mL volumetric flask. This solution was used as the test solutions in the TLC densitometric analysis. Fresh aerial parts (10gm) were extracted with MeOH till exhaustion. The solvent was evaporated using rotary vacuum evaporator and the residue was dissolved in methanol using 50mL volumetric flask. Two aliquots of fresh aerial parts, 100gm each, were extracted with 800mL boiling water to obtain infusion. Four dry tea bags (Lipton) were added to one aliquot. Water extraction was repeated with 175gm of dry plant. The infusions were cooled, filtered and the water layer was extracted three times with 300mL CH_2Cl_2. The CH_2Cl_2 layers were then separately concentrated and the resulted extracts were transferred to 50mL volumetric flasks. Then 2mL was transferred to 10mL volumetric flask and completed to volume with CH_2Cl_2.

Preparation of the volatile oil

The volatile oil of *M. longifolia* was prepared by hydro distillation method (Egyptian Pharmacopoeia, 1984). Accurately weighed 200gm of the fresh plants of *M. longifolia* to Clevenger trap apparatus for oils lighter than water, 1000mL of water were added and distillations were continued for 8 hr. The oil layer and water in the trap was extracted with dichloromethane (3X50mL) and the organic layer was concentrated using rotary vacuum evaporator to afford 3.83g oil. The resulting volatile oil was stored at 4°C prior to further analyses.

Isolation of pulegone from M. longifolia volatile oil

The volatile oil of *M. longifolia* (1.2g) obtained from hydro distillation was chromatographed on Silica gel column (50gm x 1cm i.d.) eluted with *n*-hexane. Twenty fractions 25ml each were collected, screened by TLC and similar fractions were combined. 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, 9.5:0.5) to afford 65 mg of pure oily material (1).

Preparation of standard solutions

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

HPTLC method and instrumental conditions

HPTLC densitometric analysis was performed on glass-backed plates 10×20cm coated with 0.2mm layers of silica gel 60 F254 (E-Merck, Germany). Samples were

Table 1: Linear regression data for the calibration curve of Method I and Method II (n=6).

Parameters	**Method I**	**Method II**
Linearity range (ng/spot	50-700	50-700
Regression equation	Y=9.5748x+1153.5	Y=9.1402x+475.52
Correlation coefficient	0.9982	0.9988
Slope ± SD	9.586±0.2078	9.111±0.1333
Intercept ± SD	1166±75.99	494.0±55.80
Standard error of slope	3.914	3.720
Standard error of intercept	476.112	201.715
95% confidence interval of slope	9.103 to 9.991	8.785 to 9.437
95% confidence interval of intercept	980.0 to 1352	357.5 to 630.5
P value	<0.0001	<0.0001

Table 2: Accuracy of the proposed method (n=6).

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. Found (ng) ± SD	% Recovery	% RSD
Method I				
0	300	296.00±4.05	98.67	1.37
50	450	446.17±3.76	99.15	0.84
100	600	593.67±5.75	98.94	0.97
150	750	741.83±6.68	98.91	0.90
Method II				
0	300	294.83±3.87	98.28	1.31
50	450	445.50±3.45	99.00	0.77
100	600	593.67±6.02	98.94	1.01
150	750	744.33±4.68	99.24	0.63

Table 3: Precision of the proposed method of method I and method II.

Method I						
	Repeatability (Intraday precision)			Intermediate precision (Interday)		
Conc. (ng/spot)	Avg Conc. ±SD (n=6)	Standard error	% RSD	Avg Conc. ± SD (n=6)	Standard error	% RSD
300	4156.50±37.00	15.11	0.89	4152.67±41.80	17.07	1.01
400	5048.83±39.97	16.32	0.79	5050.33±41.42	16.91	0.82
500	6045.17±40.14	16.39	0.66	6050.17±45.28	18.49	0.75
Method II						
300	3236.67±28.64	11.69	0.88	3234.33±33.72	13.77	1.04
400	4167.00±35.17	14.36	0.84	4152.50±38.10	15.56	0.92
500	5055.17±34.97	14.28	0.69	5045.67±35.27	14.40	0.70

Table 4: Robustness of the proposed HPTLC method of method I and method II.

Method I						
Mobile phase composition (hexane: ethyl acetate)						
Conc. (ng/spot)	Original	Used		Area ± SD (n = 3)	% RSD	Rf
		7.9:2.1	-0.1, +0.1	6029.40±54.70	0.91	0.49
500	8:2	8:2	0.0	6027.20±43.20	0.72	0.48
		8.1:1.9	+0.1, -0.1	6019.60±48.39	0.80	0.46
Method II						
Mobile phase composition (methanol: water)						
		7.9:2.1	-0.1, +0.1	5043.20±36.24	0.72	0.31
500	8:2	8:2	0.0	5054.80±47.86	0.95	0.34
		8.1:1.9	+0.1, -0.1	5030.60±48.26	0.96	0.36

application was performed using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microlitre syringe as 6 mm bands on TLC plates. Application rate of 150nl/s was used. The plates were developed to a distance of 80mm using Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapour for 30min at 22°C. The development was performed with hexane: ethyl acetate (8:2v/v) and methanol: water (8:2v/v) for Method I and Method II respectively as mobile phase in a. The developed plates were dried and scanned at 265nm using a Camag TLC scanner in absorbance mode, using the deuterium lamp. The slit dimensions were 4.00×0.45mm and the scanning speed was 20mm/s.

Table 5: ^{1}H- and ^{13}C-NMR data of pulegone in $CDCl_3$ (d ppm, *J* values in parentheses in Hz)[a].

Pos.	^{1}H	^{13}C
1	1.80 (Obscured by CH3 signal)	31.4
2	1.82 bd (11), 2.29 dd (11, 1.9)	50.7
3	-	203.7
4	-	131.6
5	2.05 t, (15.5), 2.53 dt (4, 15.5)	28.5
6	1.68 m, 1.41 m	32.6
7	0.81 d, (6.3)	21.6
8	-	141.6
9	1.59 s	21.9
10	1.79 s	22.8

Method validation

The proposed HPTLC densitometric method was validated according to the guidelines of international conference on harmonization (ICH guidelines 1996). The linearity of pulegone was checked between 50-700 ng/spot for method I and II. Graph was constructed between concentration and peak area for linearity. Linearity data was statistically treated using least square linear regression analysis (table 1).

Accuracy

Accuracy was determined by standard addition method. The preanalyzed sample of pulegone (300ng/spot) was spiked with the extra 0, 50, 100 and 150% of the standard pulegone and the solutions were reanalyzed in six replicates by the proposed methods I and II. The % recovery and % relative standard deviation (% RSD) were calculated at each concentration level (table 2).

Precision

Precision of the proposed methods were 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 pulegone at three different concentration levels of 300, 400 and 500ng/spot in six replicates for methods I and II (table 3).

Robustness

Robustness of the proposed HPTLC methods were determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of pulegone. Robustness was determined by changing the polarity of the mobile phase for methods I and II (table 4).

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:

LOD=3.3× SD /S

LOQ=10× SD /S

Specificity

Specificity of the proposed TLC densitometric methods were confirmed by analyzing and comparing the R_f values and spectra of the spot for pulegone in the samples with that of the standards for methods I and II.

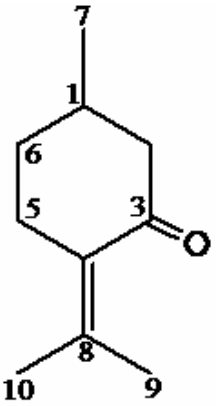

Fig. 1: Chemical structure of pulegone.

Quantification of pulegone in volatile oil, methanol, water extracts of dry, fresh and fresh plant with tea of M. longifolia

The test samples were applied to the TLC and chromatograms were obtained under the same conditions as for analysis of standard pulegone. The area of the peak corresponding to the R_f value of pulegone standards were measured and the concentrations were calculated from the regression equation obtained from the calibration plot.

RESULTS

Pulegone (1): $C_{10}H_{16}O$, Pale yellow oil; UV l_{max}: 265nm.^{1}HNMR and ^{13}CNMR: see table 5; ESIMS *m/z* 153 (100, $[M + H]^+$), 152 (45, M^+).

The results of methods validation are presented in tables 1-4. Chromatograms for determination of pulegone in volatile oil, different extracts of fresh and dry plants are presented in figs. 4- 9.

DISCUSSION

The ^{1}H-NMR data of 1 (table 5) showed the presence of two methyl singlets at d_H 1.59, 1.79 and a methyl doublet at δ_H 0.81ppm. ^{13}C-NMR (table 5) showed 10 carbon signals including a carbonyl group at δ_C 203.7ppm. The spectrum also showed two quaternary olefinic carbon signals at δ_C 131.6 and 141.6ppm indicating the presence of one double bond. Both the chemical shift of the carbonyl group and the UV λ_{max} at 265 nm indicated the presence of α, β-unsaturated ketone. The obtained NMR data of **1** were consistent with the data publish for pulegone. The assignment of the protons and carbons was achieved via various 2D-NMR experiments (Pouchert and Behnke, 1992; Lee *et al*., 2008).

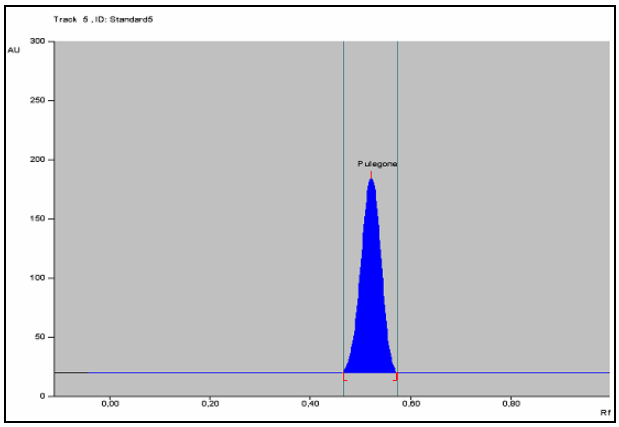

Fig. 2: HPTLC chromatogram of standard pulegone in Method I.

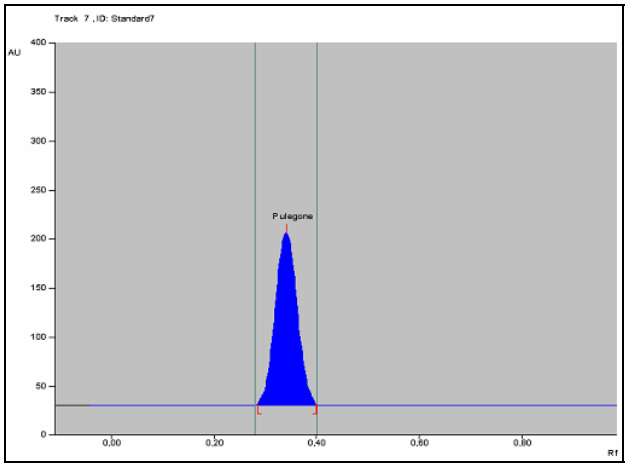

Fig. 3: HPTLC chromatogram of standard pulegone in Method II.

The maximum absorbance of pulegone was determined at approximately 265 nm for both method I and II (fig. 10). The mobile phases were optimized in order to reach accurate TLC densitometric methods for analysis of pulegone. The application of mobile phases composed of hexane: ethyl acetate (8:2 v/v) and methanol: water (8:2 v/v) resulted in symmetrical and well resolved peaks with sharp apexes at R_f value of 0.48 (fig. 2) and R_f value of 0.34 (fig. 3) for methods I and II respectively.

The linearity range of the standard pulegone covers the rang between 50-700ng/spot as shown in table 1 for methods I and II. The regression equation were Y= 7.3826x + 347.19 and Y=6.178x + 236.1 with correlation coefficients (R^2) of 0.9982 and 0.9988 for methods I and II (table 1).

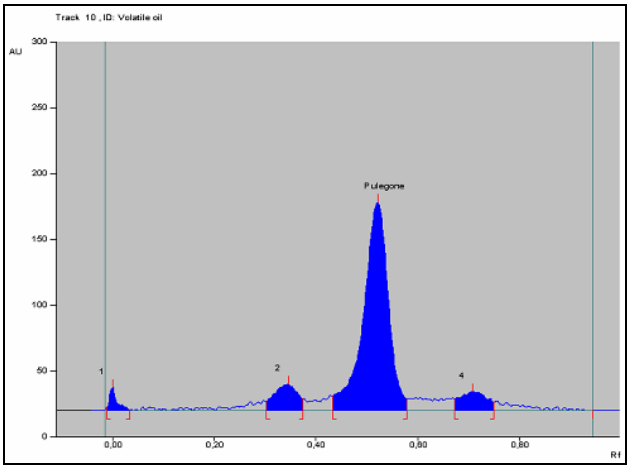

Fig. 4: HPTLC chromatogram of volatile oil in method I.

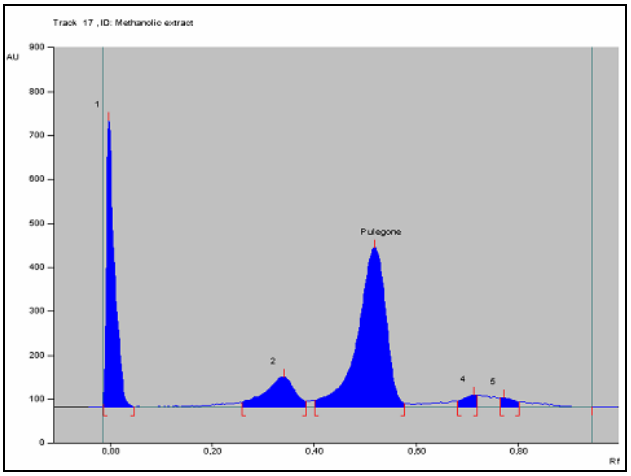

Fig. 5: HPTLC chromatogram of MeOH extract of dry plant method I.

The accuracy of the proposed method was calculated by recovery analysis, which afforded recovery of 98.67-99.15% and 98.28-99.24% for methods I and II after spiking the additional standard drug solution to the previously analyzed test solution. The values of % recovery and % RSD are presented in table 2. The low values of % RSD (0.84-1.37) and (0.63-1.31) for methods I and II was a indication for the accuracy of the proposed methods.

Results from determination of repeatability and intermediate precision, expressed as SD (%) are presented in table 3. RSD was in the range 0.66-0.89 and 0.69-0.88 for repeatability, 0.75-1.01 and 0.70-1.04 for intermediate precision for methods I and method II respectively. The obtained low values are mathematic reflection for methods precision.

Robustness results are shown in table 4. After introducing small deliberate changes into the densitometric TLC procedure low values of % RSD (0.72-0.91) and (0.72-0.96) for methods I and II were obtained. These results proved the robustness of the proposed HPTLC method.

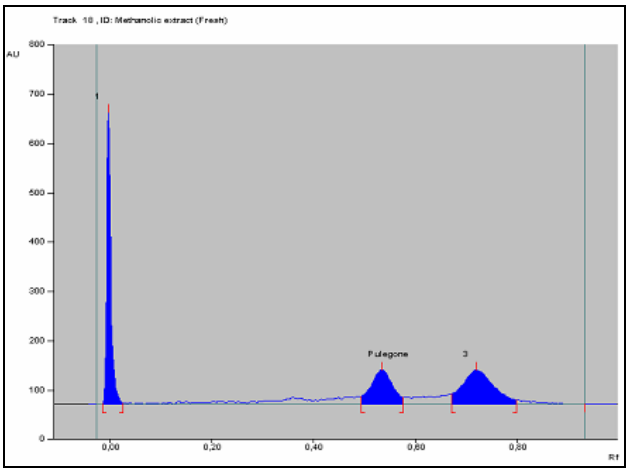

Fig. 6: HPTLC chromatogram of MeOH extract of fresh plant method I.

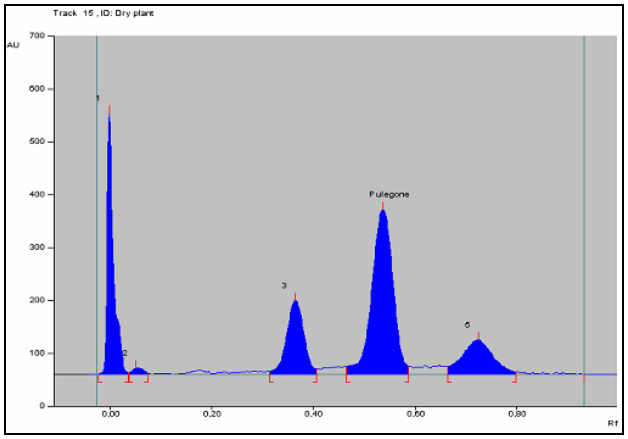

Fig. 7: HPTLC chromatogram of dry plant water extract method I.

LOD and LOQ of the proposed methods were found to be 5.78 and 17.35ng/spot and 6.12 and 18.11ng/spot for methods I and II respectively. These values indicate that the two methods can be used for detection and quantification of minute amounts of pulegone effectively.

The method proposed for quantitative estimation of pulegone showed good linearity (r^2=0.9982) and (r^2= 0.9988) in methods I and II respectively in the range of 50-700 ng/spot. The solvent system hexane: ethyl acetate (8:2, v/v) and methanol: water (8:2, v/v) gives compact spot and sharp peak of pulegone at R_f=0.48 and R_f=34 in methods I and II respectively (fig. 2, 3). The regression equation obtained was *Y*=9.5748x+1153.5 in method I which was used for the quantification of total pulegone content in volatile oil (71.3%) (fig. 4), dry plants: methanol extract (5.91%) (fig. 5), water extract (3.26%) (fig. 7); fresh plants: methanol extract (0.093%) (fig. 6), water extract (0.07%) (fig. 8) and fresh plants combined with dry tea bags water extract (0.116%) (fig. 9). The amount of pulegone content used by method II in volatile oil, methanol, water extracts in dry, fresh and fresh plant with tea of *M. longifolia* was found to be approximately same as mentioned by method I.

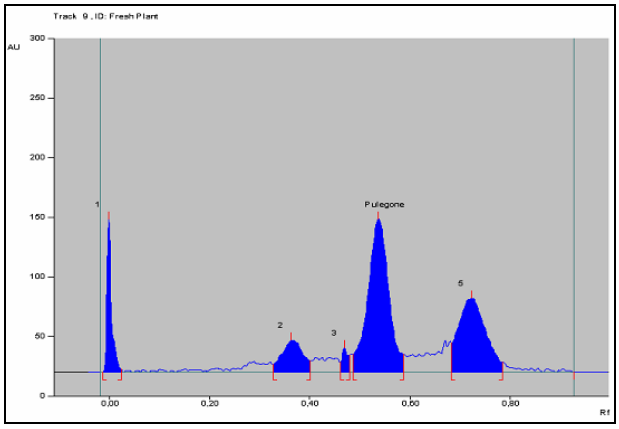

Fig. 8: HPTLC chromatogram of fresh plant water extract.

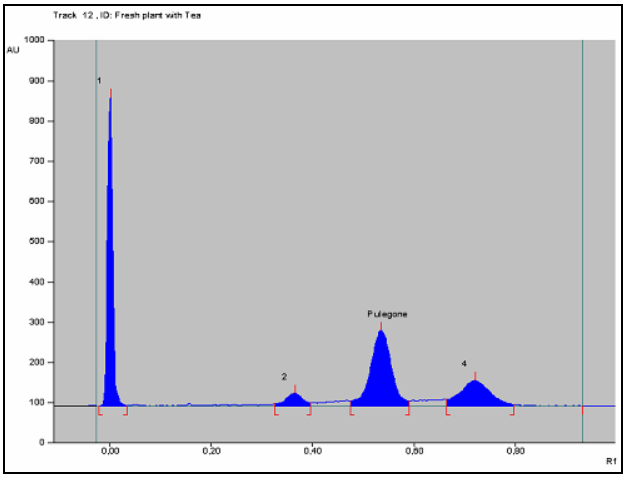

Fig. 9: HPTLC chromatogram of fresh plant with tea water extract.

The importance of this subject arises from the fact that *M. longifolia* is widely used in Saudi Arabia as flavouring agent for tea. Pulegone is a known toxic compound. It was reported that the chemical is toxic to rats if a large quantity is consumed (Thorup *et al*., 1983). The oral LD_{50} for (R)-(+)-pulegone in the rat was reported to be 470 mg/kg body weight (EEC, 1988). Comparison of the i.p.

acute toxicity of pulegone in mice indicates that the (S)-(-)-isomer is about one third as toxic as the (R)-(+)-isomer (Gordon *et al*., 1982). Pulegone is also an insecticide - the most powerful of 3 insecticides naturally occurring in many mint species (Franzios *et al*., 1997). Annex II of Directive 88/388/EEC on flavourings sets the following limites for pulegone in flavoured foodstuffs and beverages: 25mg/kg in foodstuffs, 100mg/kg in beverages, with the exception of 250mg/kg in peppermint or mint flavoured beverages and 350mg/kg in mint confectionery. Pulegone itself must not be added to foodstuffs (EEC, 1988).

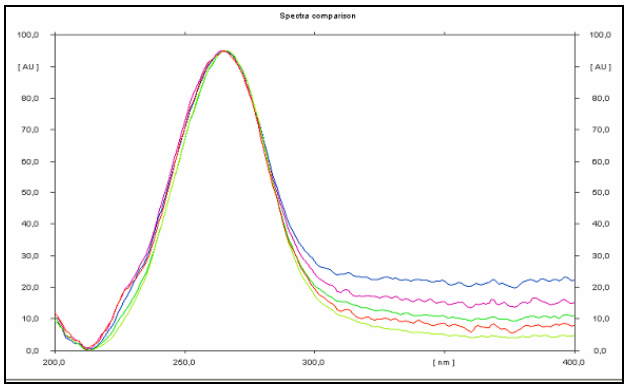

Fig. 10: UV spectra of standard pulegone and different extracts of *M. longifolia*.

CONCLUSION

In our comprehensive study of *M. longifolia* we detected the presence of the toxic terpene ketone "Pulegone". As the plant is largely consumed as flavouring agent with tea in Saudi Arabia we attempted to determine the amount of pulegone ingested with a cup of tea flavoured with this plant. Based on HPTLC estimation the amount of pulegone in fresh plant used alone was 0.07%. Using the plant with tea increased the amount to 0.116%. This effect may be due to components in tea that increase pulegone extraction with water. The average amount of fresh Habak used per cup of tea was estimated as 3gm. Our calculations indicated that these 3gm will introduce 2.1 mg of pulegone to the cup if used alone and 3.5mg when used with tea. That means the amount of pulegone is about 12mg/1L flavoured tea, which complies with the Annex II of Directive 88/388/EEC limits (EEC, 1988).

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