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Quantification of Active Phytoconstituents in Ethanolic Extract of *Mentha piperita* by Modern Analytical Tools

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Abstract

The present study investigates the qualitative and quantitative analysis of the bioactive constituents mostly preservative of medicinally important selected plant. The quantitative method for ethanolic extract of *Mentha Piperita* (Extract 3A) was developed using spectroscopic methods such as UV spectroscopy, IR spectroscopy, GCMS, also chromatographic methods such as TLC, HPTLC. The present active phytoconstituents in *Mentha piperita* that possess antimicrobial and antifungal activity are *Menthol* and *Pulegone.* The modern tools like gas chromatography and mass spectroscopy are excellently work to analyses phytoconstituents from natural herb extract. Using this technique, it is very easy to achieve separation of both active constituent with full m/z range. In GC method the experiment was done on thermofischer instrument using chromeleon software. The column DB-1 (25m×0.32mm×1.0µm) was used as stationary phase and mobile phase was helium is used with optimized flow rate. In GCMS method Stationary phase was Rxi-5ms, Restek (30 m x 0.25 mm x 0.25 µm) and mobile phase was helium with 1.4ml/minute flow rate. The validation key parameters were also performed. After quantification by modern analytical tool the Extract 3A shows presence of active phytochemical in ratio of 1:10 equivalency with that of standard drug.

***Key words*:** Quantitative analysis, Chromatography, Spectroscopy, Analysis of phytoconstituents, Hyphenated techniques, Herbal formulation

Natural active ingredients or phytoconstituents are extracted nowadays using modern sophisticated extraction methods. These active phytoconstituents possess medicinal importance and need to be incorporated in formulation in food and pharmaceutical industry. The potential and acceptance of these formulation are gaining importance over chemically synthesized drug formulation [1]. The active chemical constituent which are having antimicrobial and antioxidant properties are utilized in herbal formulation as preservatives. Thus, utilization most of commonly used natural spices like clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylancium* Nees), black pepper (*Piper nigrum* L.), garlic (*Allium sativum* L.), have been added in formulation as preservatives [2]. Preliminary examination of extract often furnishes important information, which simplifies further course of analysis. Although these tests are not conclusive but sometimes, they give quite important clues for the presence of phytoconstituents meant for formulation [3]. Gas chromatography technique is cost effective while GCMS is the hyphenated technique with chromatographic and spectroscopic application gives accurate quantification of present active chemical constituents [4]. To attain separation with all other impurities can be done with gas chromatography, thus this technique also has application of qualitative analysis in herbal formulation industry [5-10].

Several commonly used natural spices like clove, cinnamon, black pepper, and garlic, known for their antimicrobial and antioxidant properties, are being added to formulations as preservatives. These natural additives offer potential benefits and are preferred over synthetic preservatives [11]. Preliminary examination of extracts provides valuable information for further analysis. While these tests may not be conclusive, they often offer important clues regarding the presence of phytoconstituents suitable for formulation [12]. Gas chromatography (GC) is a cost-effective technique used for separation, while gas chromatography-mass spectrometry (GC-MS) is a hyphenated technique combining chromatographic and spectroscopic applications, providing accurate quantification of active chemical constituents [13-15]. GC also finds application in qualitative analysis within the herbal formulation industry, aiding in separating active constituents from impurities [16-20]. Overall, these modern analytical techniques contribute to the development and quality assurance of natural formulations in the food and pharmaceutical sectors.

MATERIALS AND METHODS

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UV method

Weigh accurately 100mg of purified ethanolic extract (Extract 3A) in 100ml volumetric flask and dilute with ethanol (diluent) up to the mark to obtain stock solution. Withdraw 1ml of resulting solution and dilute with 10ml of diluent to prepare 100 ppm sample solution. Scan the solution on UV visible spectrophotometer using diluent as blank at UV range (400-200nm). Prepare series of concentration and perform validation key parameters.

IR method

Sample preparation: Mount 2-3 drops of ethanolic extract (Extract 3A) on NaCl plates and scan plates on FTIR instrument using IR solution software.

Standard preparation: Weigh 1mg of standard menthol crystal and 100mg of KBr powder (previously dried in oven at 105°C for 1hour). Triturate both using mortar and pestle. Mount this preparation in dies and scan in FTIR to obtain IR spectra.

Mount 2-3 drops of standard R (±) Pulegone on NaCl plates and scan plates on FTIR instrument using IR solution software to obtain IR spectra.

TLC method

Sample preparation: Weigh accurately 100mg of purified ethanolic extract (Extract 3A) in 100ml volumetric flask and dilute with ethanol (diluent) up to the mark to obtain 1000ppm

Standard preparation: Weigh 1mg of standard menthol crystal dilute it with ethanol (diluent) up to 10ml to obtain 100ppm standard solution A

Weigh 1mg of standard R (±) Pulegone dilute it with ethanol (diluent) up to 10ml to obtain 100ppm standard solution B. Apply 2µl sample preparation and 1µl standard preparation A and B on TLC plate and run mobile phase to optimize solvent system.

HPTLC method

Sample and Standard preparations were prepared using TLC method. HPTLC was performed on Linomat (CAMAG scanner). Initially development was done using 4 track test (Extract 3A) and both standard drug applications. The spots were visible in UV 254nm chamber. Perform validation key parameter to obtain reproducible results.

GC method

Standard solution: It was prepared by dissolving 25mg of standard R (±) Pulegone and menthol in diluent (ethanol) 25ml. From this stock solution withdraw 1ml solution and dilute up to 10ml of diluent to obtain 100ppm standard solution for each.

Sample solution: By considering concentration to be utilize in formulation weigh 25mg of purified extract of *Mentha peperita* (previously extracted in rectified spirit) in 25 ml volumetric flask and dilute this extract with diluent (ethanol) up to marking to obtain 1000ppm solution.

GC parameter: In method injection of 2µl with split ratio 100:1 given. Inlet and oven temperature set at 240°C at initial temperature 100°C for 2min with increment of 10° C per minute for 20 minutes. This program run up to 300°C to transfer line 300°C. Stationary phase was Agilent DB-1 (30 m × 0.32 mm × 1 µm) and mobile phase was helium with 25ml/minute flow rate.

Inject blank with diluent (ethanol) and record chromatogram. Then inject standard and sample solution in sequence by column conditioning with nitrogen for 30 min after each sequence. Repeat the procedure using above GC parameters in six replicates to record sequence of chromatograms.

GC-MS method

The extract was analyzed by GC-TOFMS (GCMS-QP2010 Shimadzu model) by preparing 1% of extract in ethanol. In method injection of 1µl with split ratio 100:1 given. Inlet and oven temperature set at 250°C at initial temperature 40°C of 10° C increment per minute. This program run up to 280°C to transfer line 300°C. Ion source temperature for mass spectrometer was 250°C and mass range was 33-500 m/z. with acquisition rate 10spectra per second. Stationary phase was Rxi-5ms, Restek (30 m × 0.25 mm × 0.25 µm) and mobile phase was helium with 1.4ml/minute flow rate.

Sample solution: weigh accurately 25mg of purified extract of *Mentha peperita* (previously extracted in rectified spirit) in 25 ml volumetric flask and dilute this extract with diluent (ethanol) up to marking to obtain 1000ppm solution.

RESULTS AND DISCUSSION

UV method

UV graph of extract 3A shows absorbance at approx. 280 and 330nm. The method was precise and gives reproducible results. This qualitative test indicates presence of active preservative in purified extract of *Mentha piperita*. The method was precise and gives reproducible results shown in (Table 1).

Table 1 Interpretation of UV data

Sr. No.	Standard *Menthol* (283nm)		Standard R (±) *Pulegone* (334nm)		Extract 3A		
	Concentration (ppm)	Absorbance	Concentration (ppm)	Absorbance	Concentration (ppm)	Absorbance at 283nm	Absorbance at 234nm
1	10	0.262	10	0.278	100	0.243	0.263
2	8	0.219	8	0.225	80	0.196	0.211
3	6	0.155	6	0.171	60	0.147	0.159
4	4	0.106	4	0.113	40	0.098	0.107
5	2	0.051	2	0.057	20	0.051	0.054

IR method

Data interpretation shows the functional groups in sample (Extract 3A) were matches ranges with standard graph. Although overlay indicate 50% purity of sample solution all the transmittance peaks in finger print region matches bands with both standard preparations [21-25].

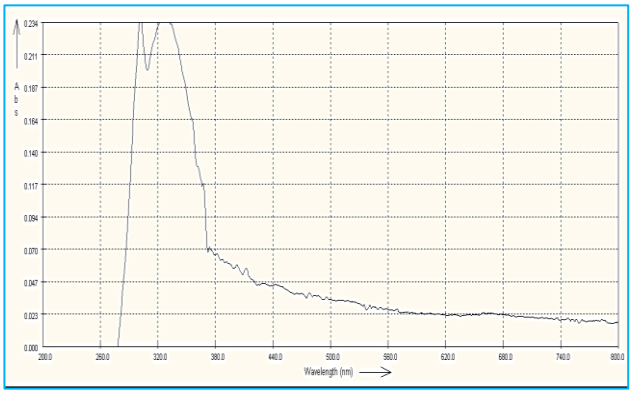

Fig 1 UV absorbance of extract 3A

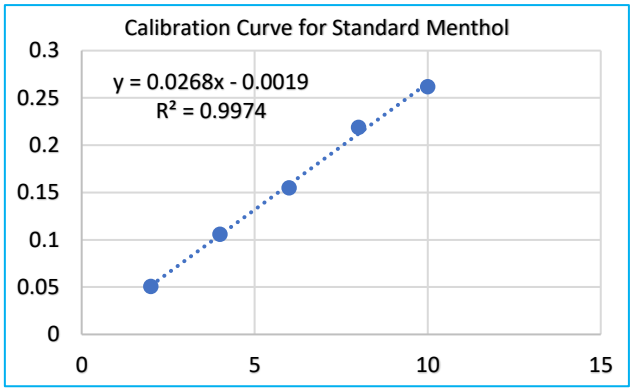

Fig 2 Linearity curve for standard *Menthol*

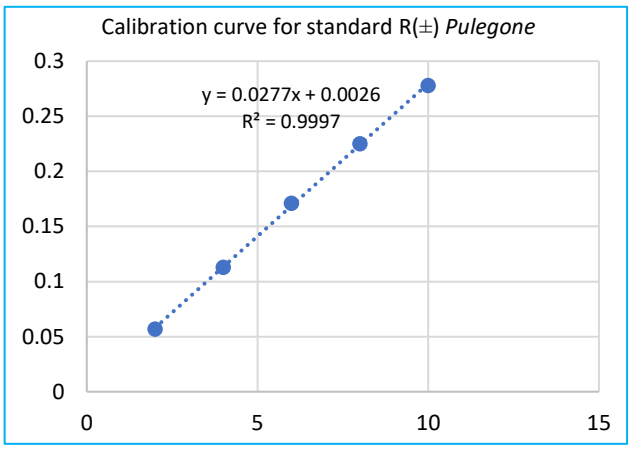

Fig 3 Linearity curve for standard R(±) *Pulegone*

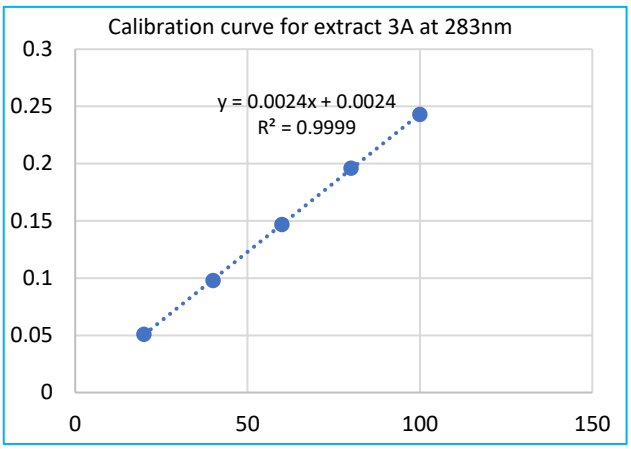

Fig 4 Linearity curve for extract 3 A at 334nm

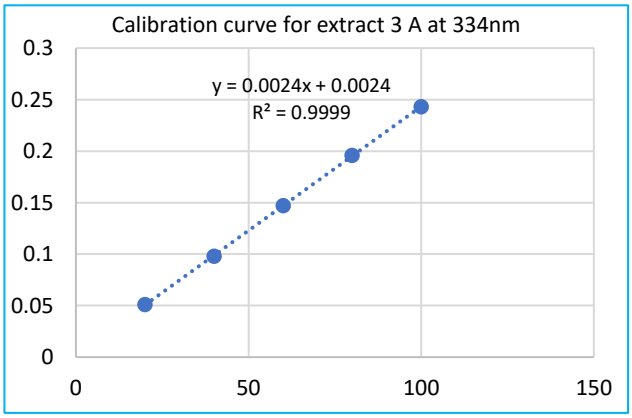

Fig 5 Linearity curve for extract 3 A at 334nm

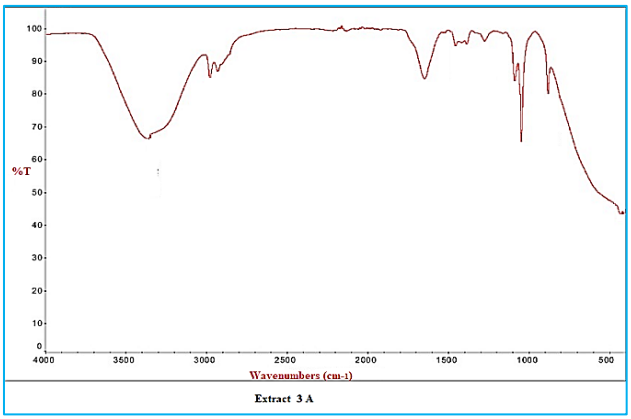

Fig 6 IR spectra of extract 3A

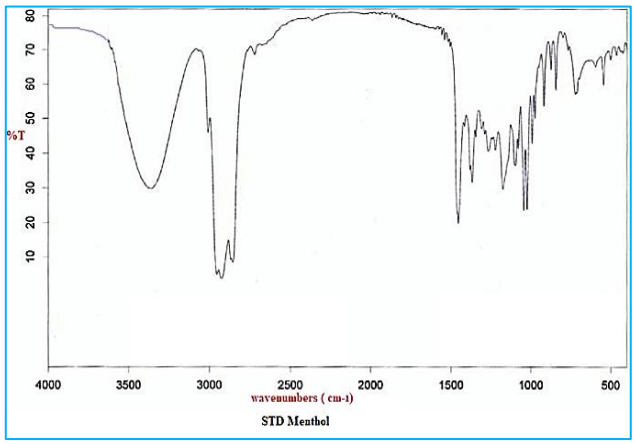

Fig 7 IR spectra of standard menthol

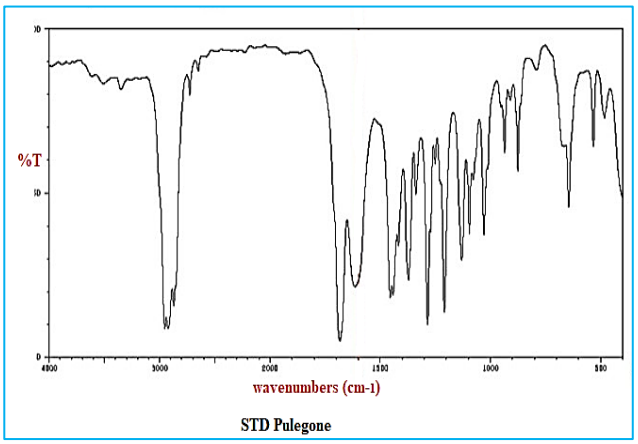

Fig 8 IR spectra of standard R(±) *Pulegone*

Table 2 Interpretation of infrared data

S. No.	Functional groups	Frequencies observed in cm^{-1}		
		Extract 3A	Standard menthol	Standard R(±)Pulegone
1	Aromatic C=O	1688.32	-	1685.12
2	C-H stretch	2952.13	2955.05	2943.73
3	OH stretch broad peak	3311.09	3310.81	-
4	C-H stretch of CH_3 substitute	1190.09	1187.11	1190.12
5	C-H stretch of aromatic ring	910.55	907.43	912.30
6	FPR rich in monoterpene bands	Seen many stretch bands	-	1700-500

TLC method

Rf value for both standard drug is identified with extract 3A at 254nm (UV cabinate) using solvent system toluene and ethyl acetate (70:30).

HPTLC method

After development of TLC method, HPTLC was performed on Linomat (CAMAG scanner). Initially development was done using 4 track test (Extract 3A) and standard drug applications. The spots were visible in UV 254nm chamber the absorbance was seen at 220nm [26]. The overlay of test sample matches with both standard drug samples [27].

Table 3 HPTLC data interpretation

Track	Vial	Amount (µl)	Rf		Area		Remark
			Menthol	R(±)Pulegone	Menthol	R(±)Pulegone	
1	1	1	0.32	0.74	18.22	293.91	Extract 3A
2	1	1	0.32	0.74	18.62	294.37	Extract 3A
3	1	1	0.32	0.74	19.04	294.92	Extract 3A
4	1	2	0.32	0.74	32.66	580.28	Extract 3A
5	1	2	0.32	0.74	32.71	585.99	Extract 3A
6	1	2	0.32	0.74	33.28	588.13	Extract 3A
7	2	2	0.32	0.74	65.73	1261.42	Standard Spike
8	2	2	0.32	0.74	66.07	1296.36	Standard Spike
9	2	2	0.32	0.74	66.19	1340.02	Standard Spike
10	2	3	0.32	0.74	99.67	1892.80	Standard Spike
11	2	3	0.32	0.74	99.87	1897.23	Standard Spike
12	2	3	0.32	0.74	100.01	1902.35	Standard Spike
13	2	4	0.32	0.74	132.18	2539.31	Standard Spike
14	2	4	0.32	0.74	133.41	2597.01	Standard Spike
15	2	4	0.32	0.74	133.74	2630.11	Standard Spike

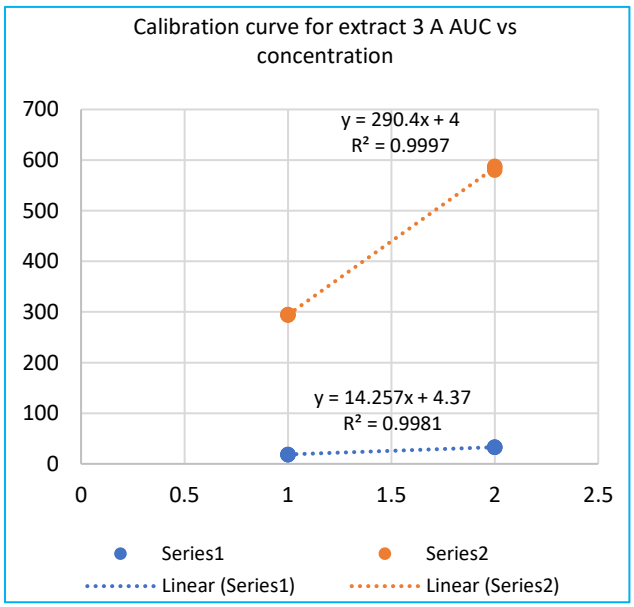

Fig 9 Calibration curve prepared by plotting the concentration of Extract 3A vs. average peak area

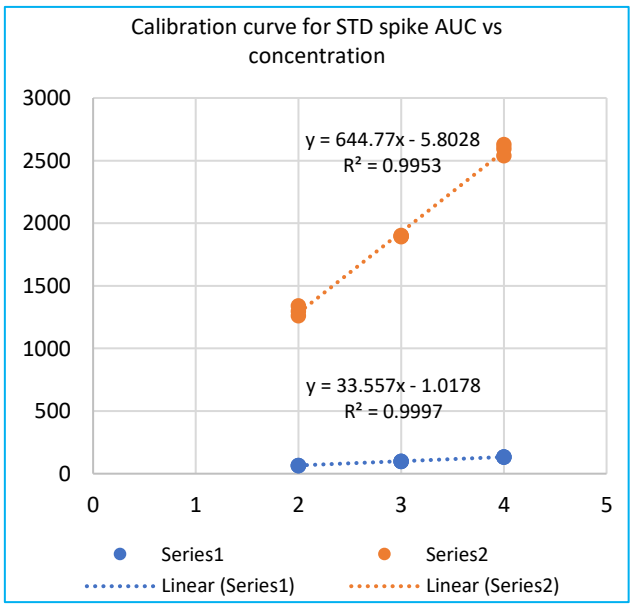

Fig 10 Calibration curve prepared by plotting the concentration of standard spike vs. average area of the peak

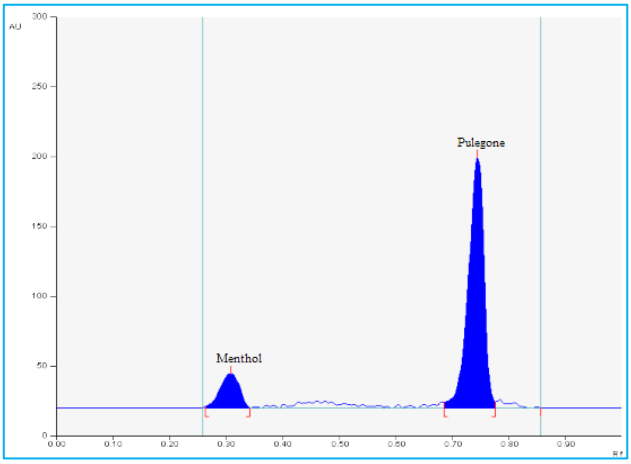

Fig 11 Rf for standard drug spike with area interpretation

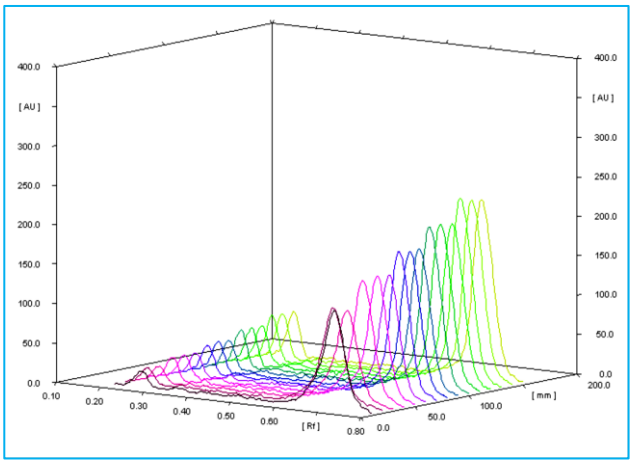

Fig 12 3D view of overlay of extract 3A with standard spike solution at 220nm

GC Method

Precise GC method was developed to shows reproducible results. The purified ethanolic extract of *Mentha piperita* containing phytoconstituents *Pulegone* and menthol are equivalent to standards in ratio of 1:10 by quantification in area calculation by GC and GCMS method. Both qualitative and quantitative methods show reproducible results [28-30]. Quantification of phytoconstituent of *Mentha piperita* with their standards keeping ethanol as blank run are as follows.

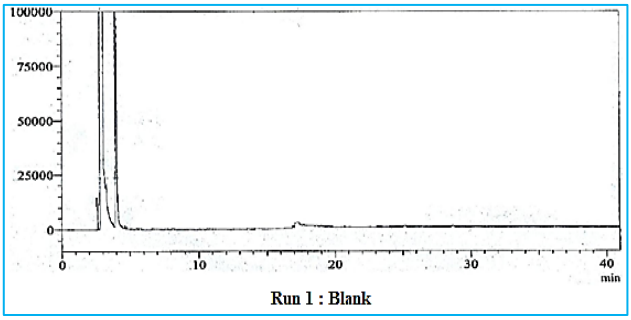

Fig 13 GC chromatogram for ethanol

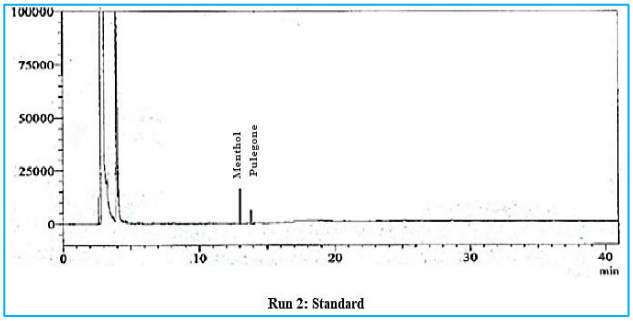

Fig 14 GC chromatogram for standard *menthol* and R (±) *pulegone* spike

Table 4 Data interpretation of GC chromatograms

S. No.	Standard Spike Run		Extract 3A Run	
	Area of menthol	Area of standard R(±)Pulegone	Area of menthol	Area of standard R(±)Pulegone
1	781.212	358.143	75.127	39.959
2	785.333	358.997	76.154	40.017
3	785.435	358.219	75.387	39.324
4	788.519	361.377	77.011	39.231
5	786.066	359.204	75.66	39.434
6	785.315	358.518	76.096	40.026
Mean	785.313333	359.076	75.9058	39.6652
SD	2.35282525	1.20279	0.67162	0.37381
RSD	0.29960338	0.33497	0.8848	0.94241

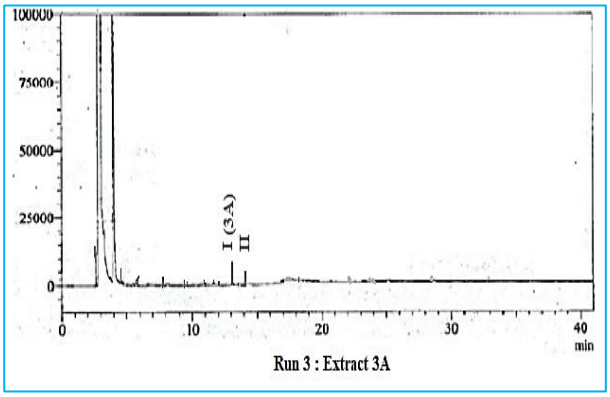

Fig 15 GC chromatogram for extract 3A

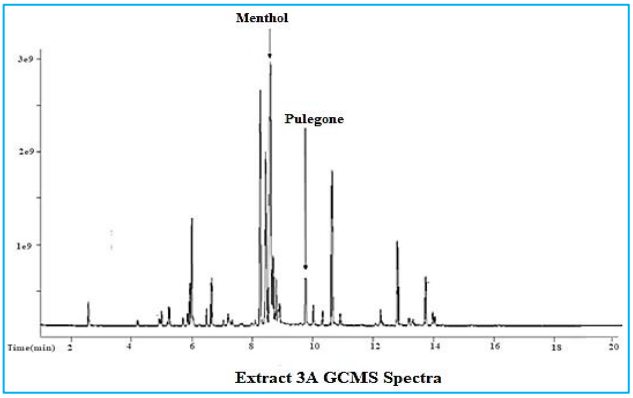

Fig 16 GC-MS spectra for extract 3A

GCMS method

The present active phytoconstituents in *Mentha piperita* that possess antimicrobial and antifungal activity are *Menthol* and *Pulegone*. The modern tools like gas chromatography and mass spectroscopy are excellently work to analyses phytoconstituents from natural herb extract [31]. Using this technique, it is very easy to achieve separation of both active constituent with full m/z range.

CONCLUSION

The qualitative test like UV, IR, TLC interprete the presence of active constituent in prepared purified extract as the values of test solution matches with standard drug solution. Also, in GC the Rt of Extract 3 A is similar to that of standard while putting the quality standard drug solution. All qualitative test gives reproducible results while performed validatory key parameters. In quantification of different concentration of Extract 3 A by AUC (Area under the Curve) calculation method HPTLC and GC gives accurate results that purified ethanolic extract of *Mentha Piperita* (Extract 3A) containing phytoconstituents *Pulegone* and *Menthol* are equivalent to standards in ratio of 1:10. Thus, the modern analytical tools are effectively found out the concentration of active phytoconstituent in herbal extract which are meant to be incorporated in pharmaceutical formulation.

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