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Original Article

Extraction and Purification of Anticancer Thymoquinone from Seeds of *Nigella sativa* by Preparative High-performance Liquid Chromatography

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Abstract

Thymoquinone (TQ) is a monoterpene ketone which is an important bioactive substance in *Nigella sativa* L. seed. This important natural compound has many potential medical applications for cardiovascular diseases, diabetes, stroke, neurodegenerative diseases and different types of cancer. To date, no studies have focused on development of a reliable industrial method for purification of TQ from this plant as a major challenge. In this study, an efficient extraction and purification method was developed for preparation of TQ from *Nigella sativa* L. seeds using preparative high-performance liquid chromatography (HPLC). To this end, a two-step procedure was applied for the first time for extraction of TQ: first, maceration using methyl *tert*-butyl ether (MTBE) was performed; then, liquid-liquid extraction using methanol successfully removed the majority of the impurities. Next, preparative HPLC was carried out for separation and purification of TQ using a C_{18} column and the mobile phase of methanol and water containing 0.1% trifluoroacetic acid (TFA).The collected peak from preparative HPLC was analyzed by the analytical HPLC as well as GC-MS instrument. Results of HPLC analysis proved the purity of the collected TQ by 97%, while the results of GC-MS identified the main peak appeared at around 15.6 min as TQ by library searching, and determined its purity by 97% based on peak heights. Overall, this method has a potential for industrialization to prepare purified TQ for medicinal applications.

Keywords: *Nigella sativa* L., Thymoquinone, Purification, Preparative HPLC.

Introduction

Nigella sativa L. belonging to the family of Ranunculaceae [1] is an annual plant which is widespread within southeast and west Asia, southern Europe, Northern Africa, and Australia. Its geographical distribution in Iran is generally in center, east in Khorasan, northwest in Tabriz, and south in Fars [2]. The seed of *N. sativa* known as black cumin is a promising medicinal plant source possessing a wide range of pharmacological activities, including antibacterial [3], antioxidant [4], anti-inflammatory [5], anti-diabetic [6], anticancer [7] and anti-nausea [8] activities. An authentic saying of the Prophet Mohammad (Peace Be Upon Him) about black seed is quoted in Al-Bukhari as: Abu Huraira (Allah be pleased from him) narrated that Allah's Apostle (peace be upon him) said 'Use the black seed, which is a healing for all diseases except Death' [9]. Many of the mentioned pharmacological activities of this plant have been attributed to thymoquinone (TQ) from volatiles of *N. sativa* seeds [10]. TQ is a monoterpene ketone which is one of the major bioactive constituents found in *N. sativa* [11]. 2-Isopropyl-5-methylbenzo-1,4-quinone known as

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TQ (Fig. 1) was first identified by El–Dakhakhny in 1963 [12].

Keeping in mind the importance of TQ, purification of this crucial natural product seems to be of great importance for medical purposes. As a matter of the fact, the most important step for purification is selection of an appropriate method for extraction. Generally, for extraction of volatiles from *N. sativa* seeds, the method of hydrodistillation is applied. However, percentage of TQ using this procedure is usually low for preparative purposes [13]. The amount of TQ using hydrodistillation from Iranian samples in the study performed by Nickavar was 0.6% [14]. Botnick and coworkers [1] applied MTBE as the extraction solvent for solid-liquid extraction of volatiles from *N. sativa* prior to injection to GC-MS. In this study, a two-step extraction method was successfully designed for isolation of TQ: as the first step of extraction, MTBEwas employed for extraction of terpenoide compounds and next, methanol was used for the clean-up step which simplified the medium to a great extent. Also for chromatographic separation of TQ, Ghosheh and co-workers [15] developed an analytical HPLC method for quality control of *N. sativa* seed oil according to quantification of TQ. In this study, scaling up from analytical to preparative HPLC was performed with revisions and modifications, in order to prepare the highly pure TQ from *N. sativa* seed.

Material and Methods

Sample Preparation

According to our previous work, it was revealed that *N. sativa* seeds collected from Ardi, Abarkooh, Yazdat geographical coordinates of 31°02'47.59"N and 53 °20 '23.55 "E, and 1487 m of height had the highest content of TQ from different geographical regions all over Iran [16]. For extraction of volatiles, the seeds were ground with a mortar and pestle, followed by addition of a 3 to 1 ratio mixture (v/w) of MTBE. After a short vortex, sample was shaken for 120 min/800 rpm in refrigerator. Then, the mixture was centrifuged at 6000 rpm for 4 min/4 °C and the clear supernatant was collected in a glass vial. The oil color seemed as yellowish green. Since TQ is unstable in aqueous media, especially under basic pH, and is extremely light sensitive [11], all the extraction steps were carried out in sealed dark vials, at low temperatures. Next, the sample was pre-concentrated using pure N_2 gas and subsequently, liquid-liquid extraction using methanol was performed by the ratio of 5: 1 v/v of supernatant. At this stage, the sample was consisted of two phases. After centrifugation at 6000 rpm for 2 min/4 °C, clear supernatant was collected and passed through a 0.45 μ filter in order to be prepared for injection to the analytical HPLC.

Analytical HPLC Condition

For the HPLC analysis, the Knauer (Germany) equipped with binary HPLC pumps, diode array detector, column oven and C_{18} column (Erusphher, 250 × 4.6 mm, 5 μm, 100 Å) was used. Mobile phase was consisted of methanol containing 0.1% trifluoroacetic acid (TFA) as solvent A and water containing 0.1% TFA as solvent B. Gradient program initialized with 5% solvent A and raised to 50% for 20 min, maintained for 20 minin 50%, and then changed to the initial condition in the next 2 min, at a flow rate of 1 Ml min^{-1}. The wave length used for detection of HPLC analyses was set at 254 nm and the injection volume was 10 μl.

Preparative HPLC Condition

Reversed-phase preparative HPLC were carried out on a Knauer liquid chromatography system equipped with a UV detector. The chromatographic separation was performed on a C_{18} column (Eurospher, 120 × 16 mm, 5 μm, 100 Å). UV detection was carried out at 254nm with injection volume of 58.07 μl. Mobile phase was consisted of methanol containing 0.1% TFA as solvent A and water containing 0.1% TFA as solvent B. Gradient program started with 5% solvent A and increased to 50% in 10 min, maintained for 10 min in 50%, and then reached to 100% solvent A in 5 min, holding at this percentage for 5 min, and eventually returned totheinitial condition in5min, at flow rate of 12 mLmin^{-1}.

Result and Discussion

Extraction Procedure

Extraction of diterpenoidres in acids from plant tissues is generally performed using relatively polar solvent mixtures (e.g. methanol), whereas the isolation of monoterpenes is carried out using steam distillation or extraction with relatively non-

polar solvents such as pentane or diethyl ether [17,18].

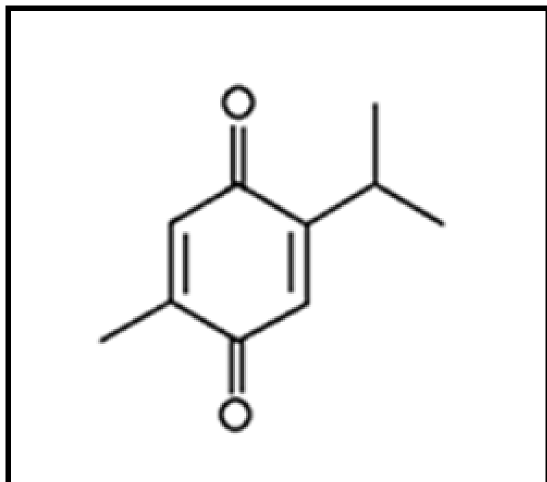


Fig. 1 Chemical structure of thymoquinone

Since Voss and Rapsomatiotis had shown MTBE was an excellent solvent for extraction of resin acids from paper mill waste [19], Lewinsohn tested this solvent for simultaneous extraction of diterpenoid resin acids and monoterpene olefins from fresh lodgepole pine stems [20]. In 2012, Botnick and coworkers applied similar extraction procedure using MTBE for extraction of volatile compounds [1]. In present study, seed powder of *N. sativa* was macerated in MTBE and volatile extract was obtained. Then, in order to clean-up the complex medium of the plant extract and elimination of monoterpene hydrocarbons and other non polar components, liquid-liquid extraction using methanol was performed. After extraction of TQ, supernatant and underlying phase were analyzed by analytical HPLC to estimate the presence of TQ (Fig 2). The identity of TQ peak was confirmed by spiking with standard and determination of retention times.

As it is illustrated in Fig 2, all of TQ compound was extracted by methanol in supernatant and the underlying phase was devoid of this compound. Therefore, supernatant was used for purification of TQ using preparative HPLC. Before performing a large-scale preparative HPLC run which is a costly and expensive procedure, the information obtained from analytical separation scan be used for prediction of the preparative conditions. The small-scale systems hold has as many parameters as possible in common with large-scale system. Equation (1) can be used for calculating different preparative parameters [21].

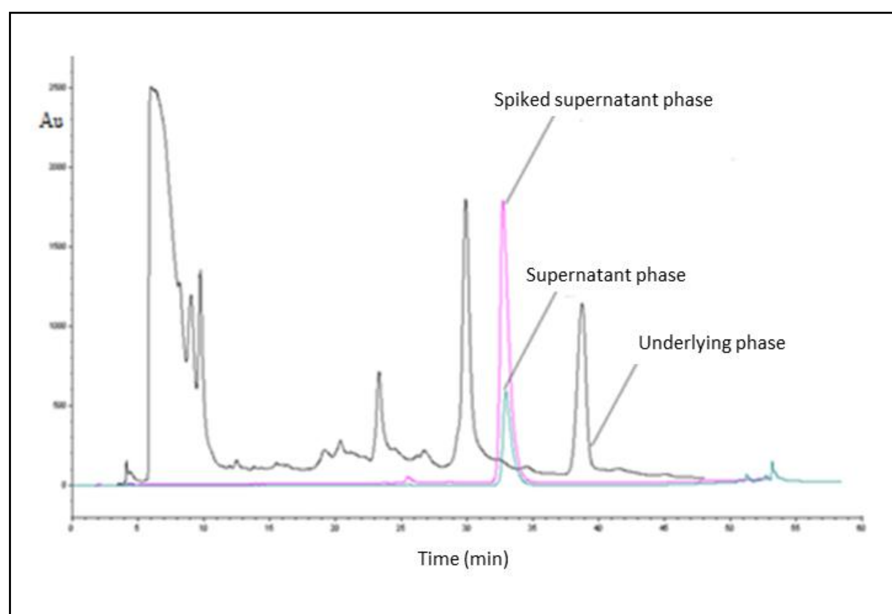


Fig. 2 HPLC chromatogram of methanolic extract: supernatant, underlying phase, and spiked supernatant phase; (detection was carried out at λ_{254}, flow rate of 1mL min^{-1}, C_{18}column: Eruspher, 250× 4.6 mm, 5µm, 100Å).

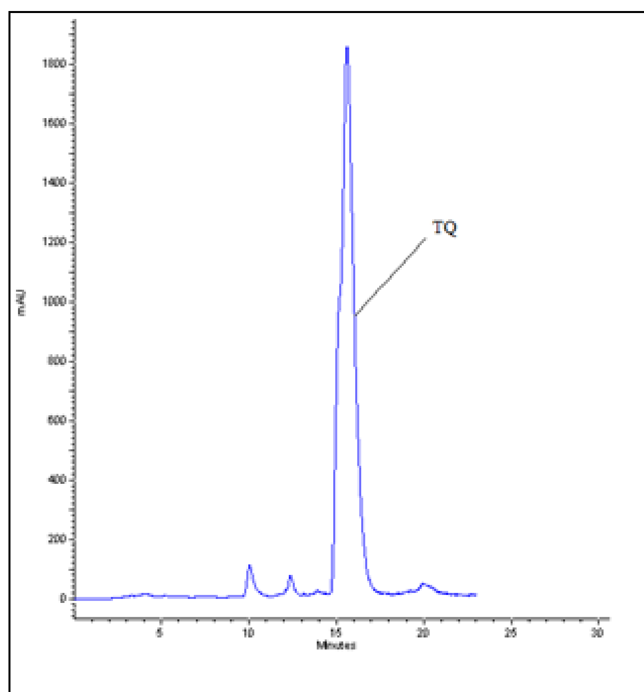


Fig. 3 preparative HPLC chromatogram of the supernatant of methanolic extract; appeared peak at 16 min represents the peak of TQ (detection was carried out at λ_{254}, flow rate of 12 mL min^{-1}, C_{18} column: Eruspher, 120 × 16 mm, 5 µm, 100 Å).

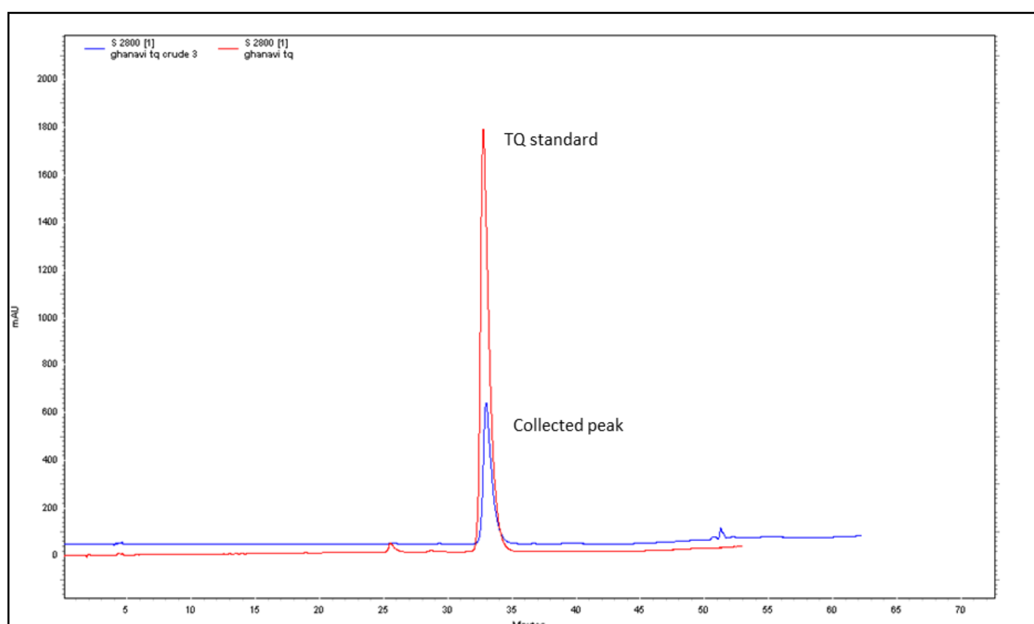


Fig. 4 Analytical HPLC chromatogram of the collected peak from preparative HPLC; appeared peak at 34 min represents the peak of TQ (detection was carried out at λ_{254}, flow rate of 1 mL min^{-1}, C_{18} column: Eruspher, 250 × 4.6 mm, 5 µm, 100 Å); concentration of TQ standard was 500 ppm.

Equation 1:

$$\frac{Fprep}{Fanal} = \frac{Lprep}{Lanal} \times \left(\frac{Dprep}{Danal}\right)^2$$

Where F, L and D stand for the flow rate, column length and internal diameter, respectively.

According to the obtained results from analytical HPLC, gradient technique was employed forpreparative HPLC and apreparative column was selected which was similar to the analytical column, but differed only in length and diameter.

According the equation 1, flow rate of preparative separation was calculated by approximately 6 mL min^{-1}. Under these conditions, corresponding peak of TQ was appeared at around 34 min. However,

since one of the important factors in preparative HPLC which plays a main role in overall cost of the method is the total run time, we duplicated the flow rate of preparative procedure to 12 mL min^{-1}, which resulted the peak of TQ to be appeared at 16 min. Fig. 3 illustrates preparative HPLC chromatogram of the supernatant of methanolic extract; appeared peak at 16 min, representing the peak of TQ was collected.

In order to determine the purity of the collected peak from preparative HPLC, it was injected to the analytical HPLC and GC-MS instruments. Result of HPLC analysis proved the purity of the obtained TQ by 97% (Fig. 4). Also, the results of GC-MS analysis are illustrated in Fig. 5. As is shown in upper figure, the main peak within the GC chromatogram appears at around 15.6 min, whose mass spectrum is depicted in lower figure. The parent mass of 164 within MS spectrum accompanied by its fragmentations indicated that this compound is TQ.

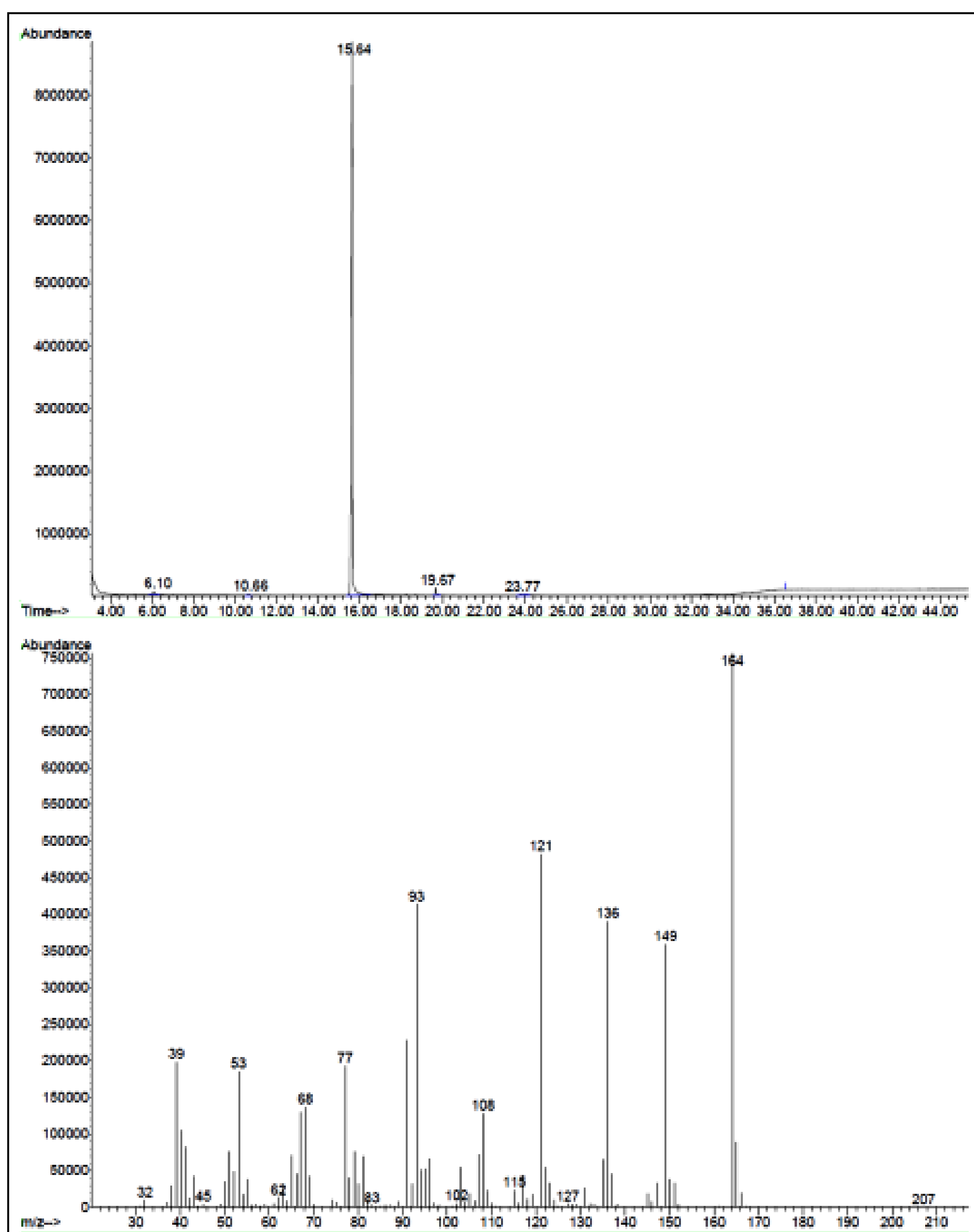
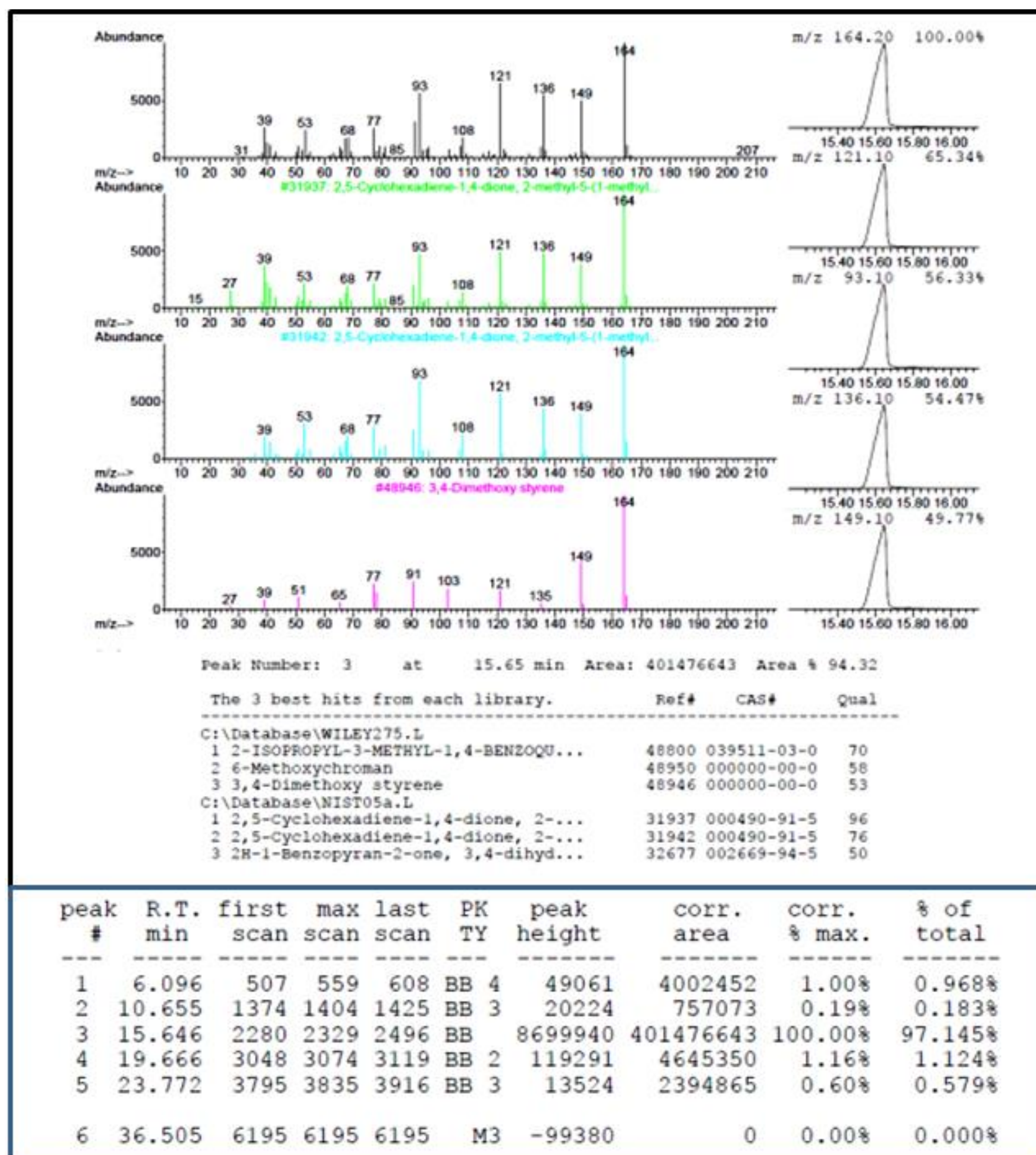


Fig. 5 GC-MS analysis of the collected peak from preparative HPLC; upper: GC chromatogram of the sample; lower: mass spectrum of the base peak (peak with abundance of 97%).



peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	6.096	507	559	608	BB 4	49061	4002452	1.00%	0.968%
2	10.655	1374	1404	1425	BB 3	20224	757073	0.19%	0.183%
3	15.646	2280	2329	2496	BB	8699940	401476643	100.00%	97.145%
4	19.666	3048	3074	3119	BB 2	119291	4645350	1.16%	1.124%
5	23.772	3795	3835	3916	BB 3	13524	2394865	0.60%	0.579%
6	36.505	6195	6195	6195	M3	-99380	0	0.00%	0.000%

Fig. 6 Identification and purity determination of the collected TQ; upper: results of library searching; lower: peak heights and frequencies

Moreover, the instrument library search was performed using two existing libraries on the device (WILEY275 and NIST05a) which both identified the compound as TQ (Fig. 6A).

It should be noted that libraries have reported and identified the IUPAC name of TQ. Fig. 6B indicates the frequency of the base peak appeared at 15.6 min by 97%. Eventually, in order to obtain TQ powder, solvent of the sample was evaporated by purging N_2 gas until dryness.

Conclusion

In conclusion, *N. sativa* seed sample of Abarkooh was used for preparative purification of TQ which is an important potential anticancer medicine. In this favor, a combination of the maceration technique using MTBE and liquid-liquid extraction using methanol was employed as a new and efficient extraction method accompanied by preparative HPLC for large-scale production of TQ. Using the proposed method, TQ was purified at a high purity of over 97%. This method is simple and highly efficient, and has been demonstrated to be effective for the preparation and purification of TQ from *N. sativa* L. for medical applications.

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