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HPTLC assay Of Thymoquinone In Black Seed And Black Seed Oil (Nigella Sativa Linn) And Identification Of Thymoquinone Conversion With Uv-Vis.

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ABSTRACT:

A reliable and simplified quantitative high performance thin-layer chromatography (HPTLC) method for the determination of the bioactive constituent of the commercially available black seed and black seed oil by using a scanning densitometer is described. The identification of bioactive thymoquinone spot obtained from 80% aq. MeOH extract of the seed is confirmed by Nuclear Magnetic Spectroscopy (NMR). A solvent system consisting of hexane and dichloromethane (1:1) was used and all spots were visualised and quantitated at 254 nm. The thymoquinone content of freshly pressed black seed oil was 1.3%, while that of the seed was 1%. Furthermore UV-Vis measurement was mainly used for quick relative comparison of thymoquinone levels in black seed oils and to follow the transformation of thymoquinone when the oil is exposed to sunlight. Considering the close values of thymoquinone levels in the seeds (1.0%) versus in the pressed oil (1.3%) obtained in this study, it can be concluded that ingesting in turn comparable amounts of the seeds and oil for therapeutic purposes may confer similar benefits.

Keywords*:* Nigella sativa; black seed; black cumin; black cumin oil; thymoquinone.

Nigella sativa L. (Ranunculaceae), commonly known as black seed or black cumin is an erect, usually profusely branched herb growing up to 0.7 m tall. It is cultivated as a crop for its black seeds at altitudes between 1500-2500 m, in East Africa, the Middle East, Mediterranean countries, Russia, South Asia etc. *Nigella sativa* whole seed (**NSWS**), when physically pressed gives 36 - 38% *Nigella sativa* pressed oil (**NSPO**) and when steam distilled 0.4 - 2.5% essential oil (Ali 2003). In Ethiopia black seed is known as Tiqur Azmud (Amh), where it is used to flavor bread and as additive in the preparation of the hot spice, Berbere. Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation (Boskabady 2010). The oil from plants grown in Ethiopia is held in greatest esteem due to high content of thymoquinone, the most important bioactive component of the seed (Houghton1995).

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In folk medicine of many countries black cumin seeds and the expressed oil are used as medicines. These products have been found to have very low degree of toxicity, as manifested by high LD50 value, key hepatic enzyme stability and organ integrity, all suggesting a wide margin of safety for therapeutic use (Zaoui 2002). Biological activities of black cumin include analgesic, ant-inflammatory (Hajhashemi 2004), antifungal, anti-asthmatic (Khan 2003), immunosuppressive, cytotoxic (Islam 2004), anti-allergic (Kallus 2003), anticonvulsant (Hosseinzadeh 2004), and hypoglycemic (El-Dakhakhny 2002) properties. In addition, acute and chronic toxicity (Mariod 2009) and anti-oxidant activities (Zaoui 2009) of *N. sativa* have been reported.

Most of the biological activities of black seed are due to thymoquinone (**TQ**), the principal secondary metabolite present both in the seeds as well as in NSPO. The present study compares the levels of TQ in the seeds and the expressed oil.

EXPERIMENTAL

Plant material and pressed oil

Nigella sativa whole seed (NSWS) was purchased from market in Addis Ababa, Ethiopia. NSPO (Nigella sativa pressed oil) was obtained by pressing NSWS and also by purchasing oil from local commercial producers.

Isolation of TQ

Powdered seeds of black cumin (10 g) were soaked in 80% aq. MeOH (70 ml), placed on a shaker for 4 h and filtered. The marc was extracted one more time with the same solvent. Water was added to the combined filtrates to make the solution 50% aq. MeOH. The resulting filtrate was then extracted twice using 50 ml $CHCl_3$. The lower organic layer was separated, dried with anhyd Na_2SO_4, and concd to give dark brown gummy extract, which was then subjected to CC on silica gel. Elution was carried out using hexane/CH_2Cl_2 (7:3), monitoring

the separation of TQ by TLC (1:1, hexane/ CH_2Cl_2), which yielded pure TQ, confirmed by its 1H, C-13, DEPT-135 and UV spectra.

Preparation of standard reference TQ solution [**1**]

10 mg of pure TQ obtained from the CC was dissolved in methanol in 10 ml volumetric flask (1 mg/ml or 1 µg/µl).

Extraction of powdered black seed [**2**]

To 50 mg of seed placed in 10 ml volumetric flask was added 10 ml methanol and sonicated for 20 min followed by shaking for 4 h and the resulting extract was then kept for analysis.

Preparation of NSPO solution [3]

To 50 mg of freshly pressed oil placed in 10 ml volumetric flask was added 10 ml methanol and sonicated for 30 min.

Working procedure for Camag HPTLC Analysis

From the above standard TQ Reference Solution [**1**], 2 µl, 4 µl, 6 µl were applied respectively on tracks 1-3 of the **TLC** plate using Camag Linomat 5 Sample Applicator. On tracks 4 and 5, 60 µl and 80 µl of the methanol extract of the seeds [**2**] were applied respectively. From the NSPO solution [**3**] 60 µl was applied on track 6. The TLC plate was then developed using solvent system (1:1 CH_2Cl_2/hexane), and the optical density of the unknown concentration scanned with the scanning densitometer of the **CAMAG HPTLC** instrument.

Calibration curve with TQ

2 µl, 4 µl, 6 µl and 12 µl of the above TQ Reference Solution were applied on 4 tracks of the TLC plate, and then developed using the above solvent system and the optical density scanned.

Relative comparison of TQ levels in different NSPO samples using UV-Vis spectrometry:

NSPO (10 mg) was dissolved in 10 ml hexane: ethanol (1:9) and using the same solvent mixture serially diluted to 0.01 mg/ml, and its **UV-Vis** spectrum measured.

Following transformation of TQ in sunlight by UV-VIS

NSPO (200 mg) was placed in sun-light and 10 mg withdrawn after 15 minutes then dissolved in (1 ml hexane: 19 ml ethanol), an aliquot was taken and transferred to the measuring cell and the **UV-Vis** measurement was recorded.

Recovery of TQ in black seed

A separate methanol extract of black cumin seed and NSPO 50 mg was spiked with 10 mg internal TQ standard. The spiked sample was extracted with methanol (10 ml) to give 1 µg/µl internal standard. From this 10 µl was spotted on one **TLC** plate corresponding to the application of 1.05 µg/µl and 1.065 µg/µl internal standard plus TQ in the methanol extract of the black cumin seed and in the methanol extract of NSPO respectively. To obtain the recovery value, the spiked sample was analyzed three times during one day and by dividing experimental value by the theoretical value.

Apparatus and instrumental conditions

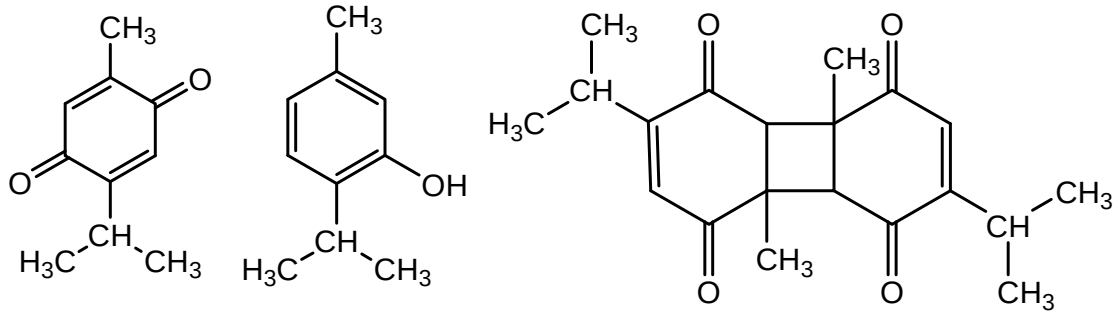
The measurements were carried out by using a **CAMAG HPTLC** (Switzerland) model Scanner III and Linomat 5, equipped with deuterium, mercury, and tungsten lamps. The quantification was done using densitometeric evaluation in the absorption mode at 520 nm. Parameters such as slit size, scanning speed, and data resolution were adjusted to 5.0 x 0.45 mm, 20 mm/s, and 100 µm/step were adjusted respectively. Other parameters such as, scan start position, scan end position, distance between tracks were monitored using win CATS software.

^{1}H and ^{13}C NMR spectral measurements were recorded on Bruker ACQ 400 AVANCE Spectrometer operating at 400 MHZ equipped with 5 mm multinuclear probe and running Topspin 2.1 software at 298K.

The **UV-Vis** spectral experiment was done using T 60 U spectrophotometer (PG instruments, UK) which is equipped with deuterium and tungsten lamp. The running parameters controlled by **UV-win** software.

RESULTS AND DISCUSSION

It is well known that the substance which is most responsible for the diverse biologically activity of black cumin seeds and its pressed oil is TQ. Three previous reports dealing with TQ level determinations of black seed oils were conducted on oil samples from commercial sources and reported levels of 0.2% or less. Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation (Hosseinzadeh 2004). To the best of our knowledge there is no report on the level of TQ in NSWS. We have used a simplified extraction procedure and an efficient and reliable quantitative **CAMAG HPTLC** method (Fig. 1) to determine level of TQ in NSWS and NSPO on one **TLC** plate.



Thymoquinone (TQ) Thymol Dithymoquinone (DTQ)

Scheme 1. Structures of TQ found in *N. sativa* and its conversion products

Extraction of 10 g of black seeds as described above gave 1.5 g, 15%, of dark brown gum, which when subjected to CC gave 60 mg (0.6%) of pure TQ (R_f = 0.3, 1:1, hexane/CH_2Cl_2), ^{1}H NMR and ^{13}C spectra consistent with the structure of TQ with a UV below, UV λ_{max} = 253. This sample was used as standard reference for the determination of the unknown extract.

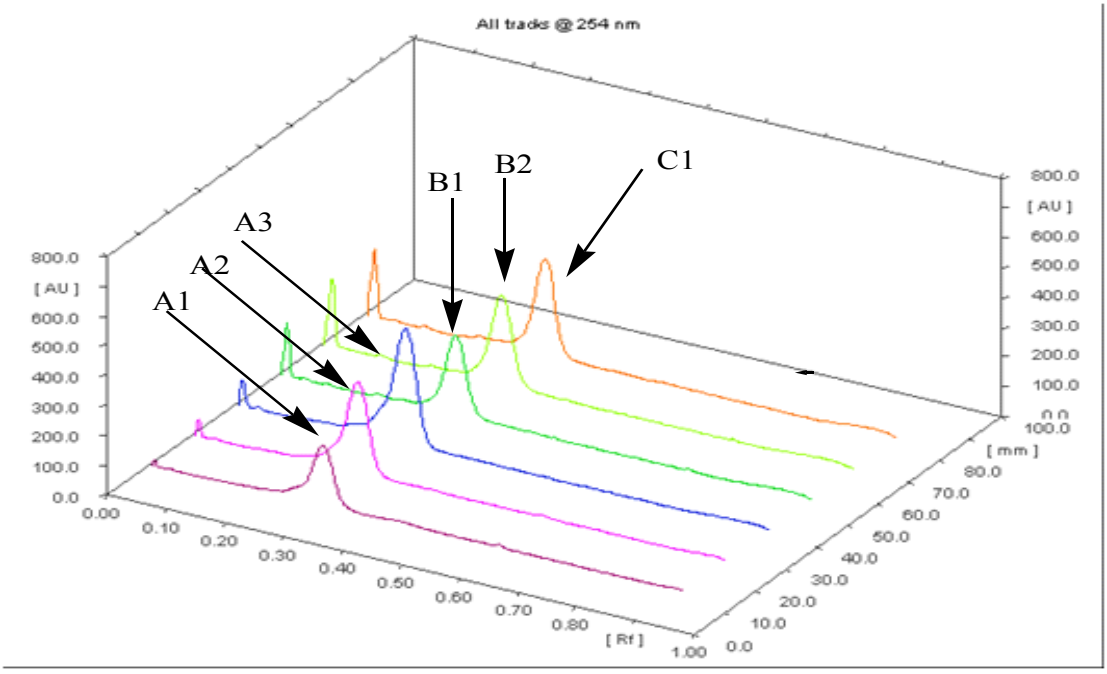


Figure 1.

The three-dimensional **CAMAG HPTLC** image at 254 nm of standard TQ (**A1, A2, A3**: three concentrations 2 µg/ul, 4 µg/ul, 6 µg/ul); unknown concentrations of: the methanol extract of NSWS **B1** (60 µl), **B2** (80 µl) and NSPO **C1** (60 µl).

Using the Polynomial regression curve shown below in **Fig. 2**, the levels of TQ in NSWS and in NSPO can be determined directly from the densitometeric regression curve on the x-axis shown in **Fig 2.**

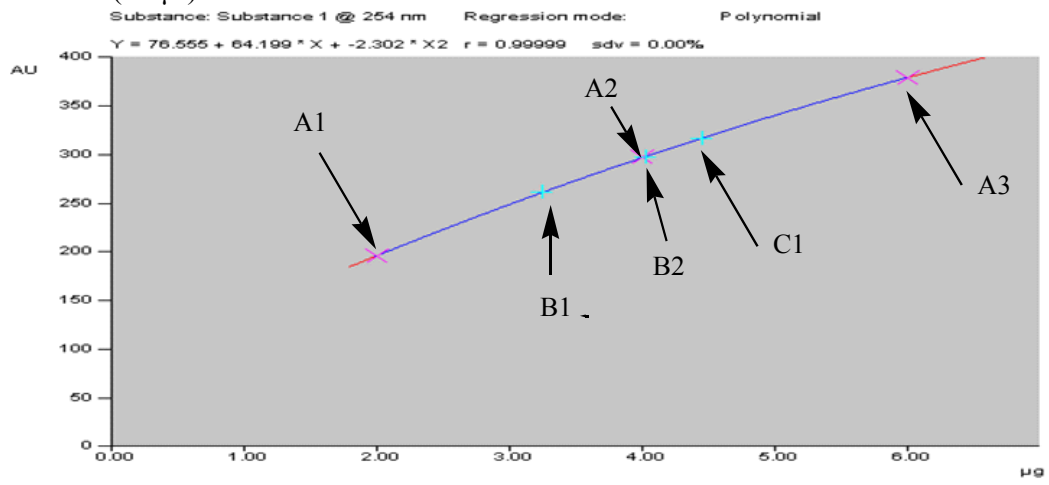


Figure 2.

Polynomial regression curve graph via height of standard reference TQ (**A1, A2, A3**: three concentrations 2 µg/ul, 4 µg/ul, 6 µg/ul). Unknown concentrations

of: the methanol extract of NSWS **B1** (60 µl), **B2** (80 µl) and NSPO **C1** (60 µl) showing the densitometeric concentration of the unknown obtained after developing the **TLC** and scanning on the **HPTLC** scanner III on the x-axis with pink color.

Summary of the results of TQ level determinations of the methanol extracts of NSWS and NSPO, showing level of TQ in the seeds and oil to be 1.0% and 1.3% respectively which is clearly shown in the polynomial regression curve via height (*Average of 3 determinations).

Table 1.

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Track	Volume applied per track	Peak height at 254 nm Mean* ± S.D.	Peak area at 254 nm Mean* ± S.D.	Derived Amount from the peak height of the polynomial curve Mean* ± S.D.	Derived Amount from the peak area Mean* ± S.D.	Average amount and % of TQ from peak height and area
A1	2 µl (2 µg)	137.8 ± 0.08	4995.4 ± 0.02			
A2	4 µl (4 µg)	224.8 ± 0.15	7819.4 ± 0.03			
A3	6 µl (6 µg)	317.6 ± 0.09	10418.5 ±0.04			
B1	60 µl (unknown)	221.8 ±0.16.	5948.4 ± 0.05	3.3 ± 0.05 µg	2.7± 0.07 µg	3.0 µg (1%)
B2	80 µl (unknown)	242.7 ± 0.14	6546.4±0.08	4.1±0.02 µg	3.1 ±0.05 µg	3.6 µg (1%)
C1	60 µl (unknown)	280.6 ± 0.15	6909.4 ±0.04	4.6 ±0.04µg	3.3±0.04 µg	4.0 µg (1.3%)

Recovery of TQ in NSWS and NSPO

The recovery of TQ was determined using the quantitative **CAMAG HPTLC** method by dissolving a known amount of the pure internal standard in the methanol extracts of NSWS and NSPO. In both cases 97% of the added TQ was recovered. The intra-day determination (n = 3) of TQ level in NSWS extract gave a mean value of 10.25, while that of NSPO gave 10.52. These correspond to a relative standard deviation of ±1.5% and ±2.10% for NSWS and NSPO respectively. The developed **TLC** method for quantification of TQ showed correlation coefficient, which is clearly seen in **Figure 2**, polynomial regression curve graph via height (r= 0.99999) in the concentration range of 2 to 12 µg with respect to the peak height and area.

Linearity

Polynomial regression curve via height in the calibration curves were constructed over the range of 1 µg - 12 µg with the correlation coefficient 0.99999, (n = 3). **Fig. 2** clearly shows the correlation coefficient generated directly from the quantitative **CAMAG HPTLC** instrument during the quantitative TQ determination in the methanol extracts of NSWS and NSPO. Each determination was done on one **TLC** plate where n = 3, corresponding to reference TQ and NSWS and NSPO extract samples.

UV-Vis spectrum of TQ and NSPO

The potential of using **UV-Vis** measurements for detection and relative comparison of TQ levels in different oils is evident from the following experiments. The **UV-Vis** spectra of different concentrations of reference TQ (0.002, 0.004 0.006 0.008 mg/ml), generated in ethanol ($\lambda_{max:}$ 253 nm), are shown in **Fig. 3**. A similar linear curve was obtained when spectra were generated using 0.1, 0.2 0.3 and 0.4 mg/ml of freshly prepared NSPO dissolved in hexane/EtOH (1:9).

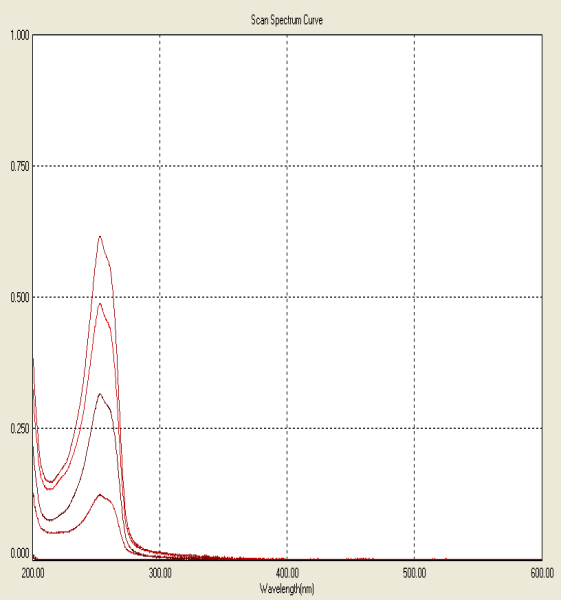

Figure 3 (TQ)

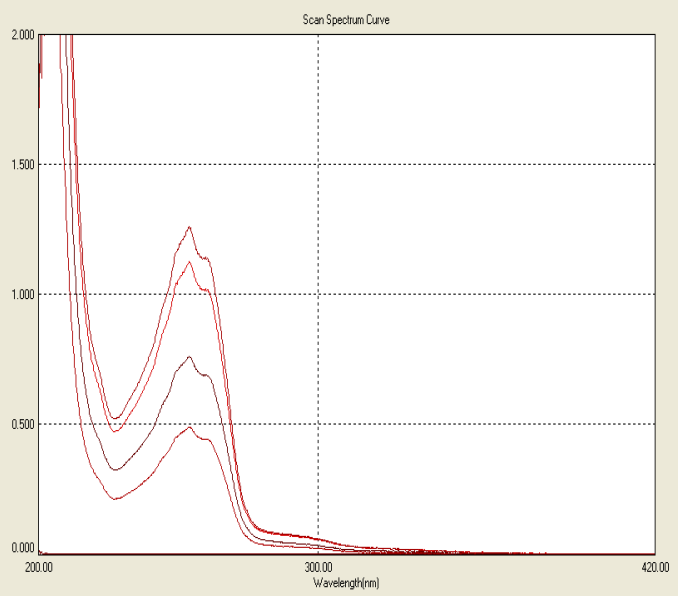

Figure 4 (NSPO)

Figure 3 and 4.

UV-Vis spectrum at different concentrations of the standard reference thymoquinone and Nigella sativa pressed oil showing the fresh TQ before exposure to sunlight.

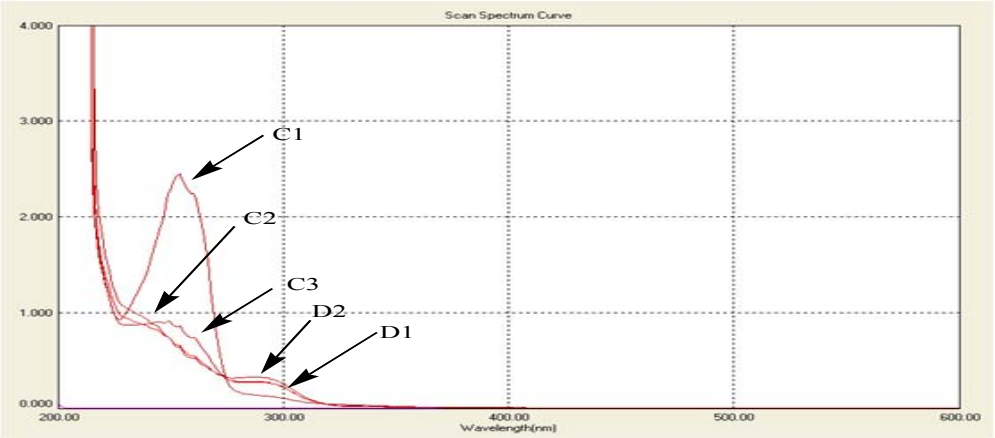

Figure 5.

Uv-Vis spectrum of NSPO after exposure to direct sun-light for 0 (**C1**), 30 (**C2**) and 45 (**C3**) minutes showing gradual decline of the starting TQ (**C1**) and appearance of **D1** and **D2** peaks is due to its conversion products to DTQ and thymol [1].

Stability study of NSPO

Fig. 5 clearly shows the quick transformation of TQ when exposed to direct sunlight. Random analysis of commercial oil samples obtained from different sources showed gross variations in the TQ levels depending on their age and storage conditions. These results indicate why previous determinations of TQ levels reported in the literature are very low (Houghton1995). Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation yielded much lower levels (ca. 0.2%) relative to our results here of 1.3% in NSPO and 1% in NSWS. Further study is required to determine storage conditions and shelf life of NSPO.

CONCLUSION

An efficient high performance thin-layer chromatography (HPTLC) method has been developed to assay the levels of thymoquinone in seeds and pressed oil of commercially available black cumin. UV-Vis spectroscopic measurements can also be used to monitor stability of TQ and also to compare its levels in different commercial black seed oil samples. It can be concluded from this study; in particular in view of the closeness of thymoquinone levels in the seed (1.0%) and pressed oil (1.3%), ingesting the seeds for therapy is almost as good as taking the oil.
The results are significant in view of the large difference in price between black seed and its oil.

Acknowledgements

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