QUANTITATIVE ESTIMATION OF THYMOQUINONE *IN NIGELLA SATIVA LINN.* VIA HPTLC: IMPLICATIONS FOR IMMUNOMODULATORY POTENTIAL

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ABSTRACT

Nigella sativa Linn., commonly known as black seed or black cumin, is a widely used medicinal plant in traditional systems of medicine across Asia, the Middle East, and Africa. Its seeds possess a rich pharmacological profile attributed primarily to its bioactive constituent, thymoquinone. Thymoquinone has been reported to exhibit diverse biological activities, including antioxidant, anti-inflammatory, anticancer, antimicrobial, and immunomodulatory. Given the growing interest in natural immunomodulators for managing immune-related disorders and enhancing immune defense, thymoquinone has emerged as a promising phytochemical candidate. However, scientific validation of its therapeutic efficacy requires accurate standardization and quantification in herbal formulations. High-Performance Thin Layer Chromatography (HPTLC) has proven to be a reliable, reproducible, and cost-effective method for the qualitative and quantitative analysis of phytoconstituents. This study aims to develop and validate an HPTLC method for the quantitative estimation of thymoquinone in *Nigella sativa* seeds and to correlate its content with potential immunomodulatory applications. The establishment of such a method not only supports quality control of herbal preparations containing *Nigella sativa* but also provides a scientific basis for further pharmacological and clinical investigations into its immune-regulating potential.

Keywords: High-Performance Thin Layer Chromatography, immunomodulatory, *Nigella sativa*, Thymoquinone.

INTRODUCTION

Nigella sativa Linn., commonly known as black seed or black cumin, is a widely used medicinal plant with a rich history in traditional medicine systems, particularly in Unani, Ayurveda, and Siddha. It is reputed for a broad spectrum of pharmacological properties, including antioxidant, anti-inflammatory, anticancer, antimicrobial, and notably, immunomodulatory activities. The bioactive phytoconstituent most attributed to these effects is thymoquinone (TQ), a monoterpene Quinone predominantly found in the essential oil fraction of

the seeds. (Ahmad *et al.*,2013; Tembhure *et al.*, 2014; Paarakh, 2010) Quantitative analysis of thymoquinone is critical for standardization, quality control, and dose optimization of *N. sativa*-based formulations.

The seeds of *N. sativa* are known to contain a variety of constituents such as alkaloids, flavonoids, saponins, tannins and essential oils, among which thymoquinone (TQ) is considered the principal bioactive marker compound. Newman, D. J.and G. M. Cragg (2007) Thymoquinone is a monoterpene benzoquinone predominantly present in the volatile oil fraction of *N. sativa*. The accurate and reproducible quantification of thymoquinone is crucial for evaluating the quality, efficacy, and safety of *N. sativa*-based herbal preparations. Phytochemical standardisation of herbal drugs is a critical step in ensuring consistency and therapeutic reliability Harbon J B.(1998). This study focuses on the quantitative estimation of thymoquinone in *Nigella sativa* seeds using HPTLC, aiming to establish a validated analytical method for standardization. This study aims to develop and validate an HPTLC method for the quantitative estimation of thymoquinone in *Nigella sativa* seeds. It further explores the implications of thymoquinone content to the plant's immunomodulatory potential, thereby contributing to the scientific foundation for standardized phytopharmaceutical development. (Singhal *et al.,* 2024).

MATERIALS AND METHODS OF EXTRACTION

Preparation of Plant Material Dry *Nigella sativa* seeds under shade. Pulverized into a moderately coarse powder to increase the surface area. Conventional Techniques Moistening (Imbibition)was used to Moisten the powder with a small quantity of selected solvent (ethanol). Allow it to stand for 4–6 hours in a closed container to allow uniform swelling. Raaman (2006),

Preparation of the extract

Nigella sativa seeds were shade-dried, ground fine with an electric grinder, weighed and then put in a stoppered flask to be completely dissolved in ethanol. The flask was shaken hourly for the first twelve hours, following which it was set aside and shaken once again after twenty-four hours. This process was repeated for 3 days and then the extract was filtered. The final extract thus obtained was then subjected to a basic phytochemical test and HPTLC analysis.

HPTLC Conditions:

- Stationary phase: Silica gel 60 F254 plates
- Mobile phase example for thymoquinone: Toluene: Ethyl acetate: Formic acid (7:3:0.5)
- Application: Apply samples and standards using a CAMAG Linomat 5 (band width ~6 mm)
- Development: In a twin trough chamber, pre-saturated for 20 mins
- Detection: Scan under UV at 254 nm and 366 nm (or use derivatisation for better visibility)
- Derivatisation (if needed): Spray plate with anisaldehyde-sulfuric acid and heat at 10

Quantitative Phytochemical Analysis of *Nigella sativa* by HPTLC Method

High-Performance Thin Layer Chromatography (HPTLC) has emerged as a valuable analytical technique for the qualitative and quantitative estimation of chemical constituents in plant materials (Sethi, 1996). In the present study, HPTLC was employed to evaluate the presence of specific biomarkers, particularly thymoquinone, in the ethanolic extract of *Nigella sativa* seeds.

HPTLC analysis was performed using a CAMAG HPTLC system operated by Wincats Planar Chromatography software. A standard solution of thymoquinone (1.00 µg/mL) was prepared and used as the reference standard. The cold-pressed ethanolic extract of *Nigella sativa* seeds was also prepared at a concentration of 1.00 µg/mL. Aliquots of 10 µL of the standard and test extract were applied on a Silica gel 60 F_{254} pre-coated plate (10×10 cm) as 0.7 cm bands, positioned 1.5 cm from the bottom edge and 0.7 cm from the lateral edges, on two separate tracks. The TLC plate was developed in a twin-trough chamber using a mobile phase of toluene: methanol (50:50 v/v). After development, the plate was dried and scanned using a CAMAG scanner. Integration and quantification were done at 254 nm.
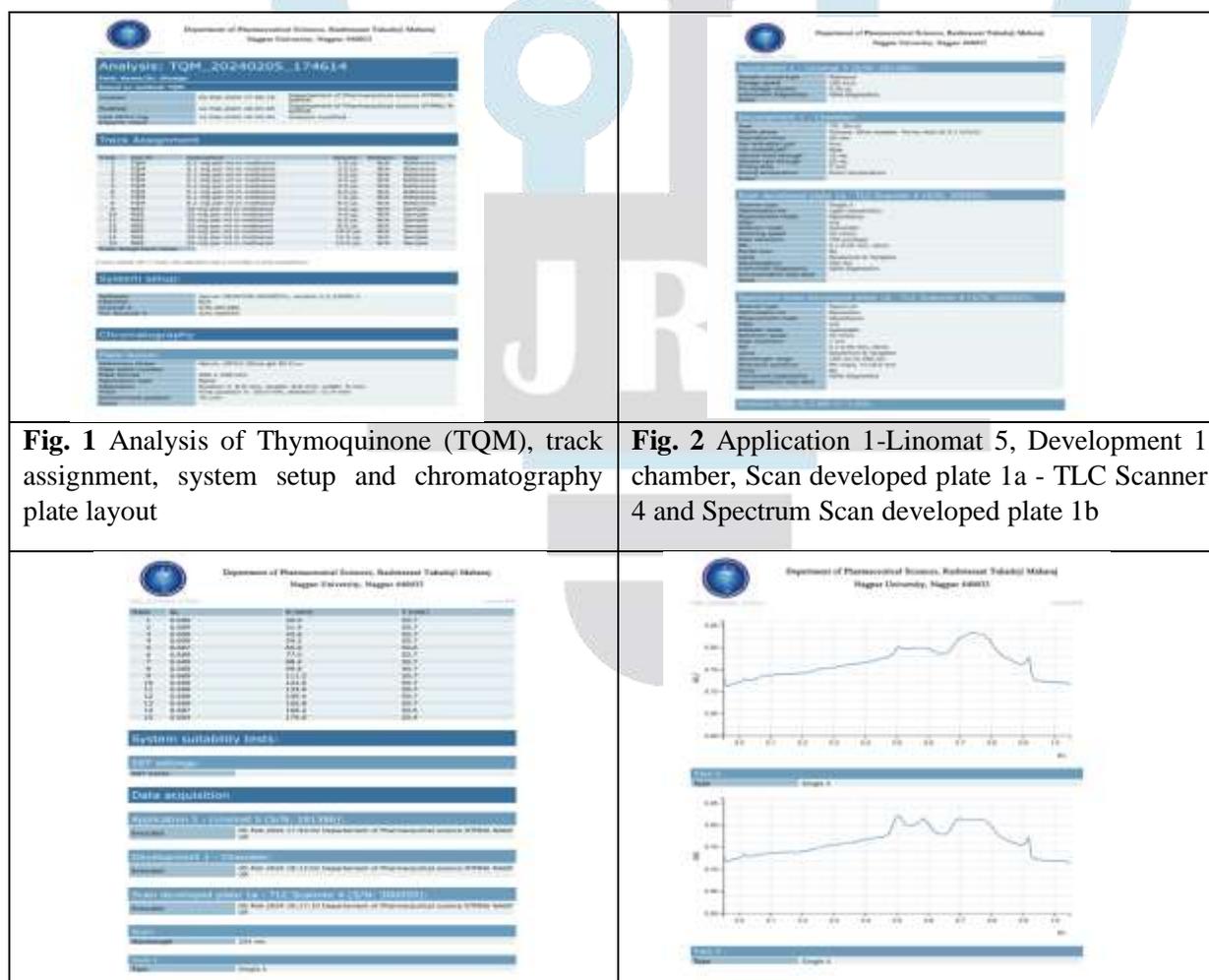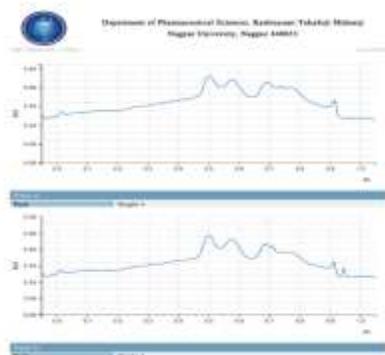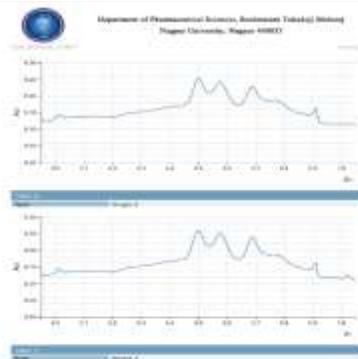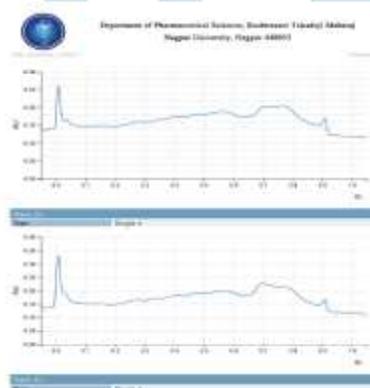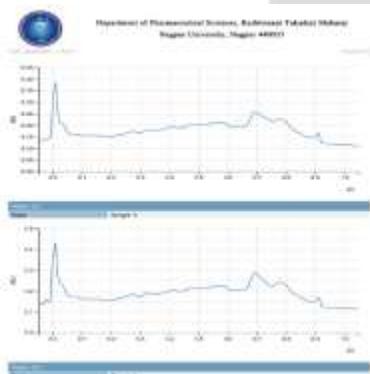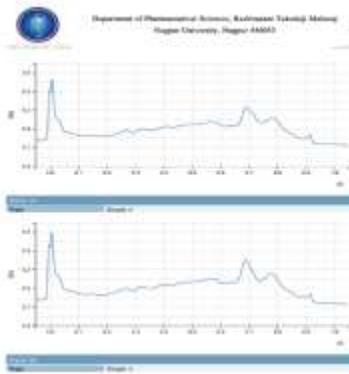
RESULTS AND DISCUSSION

Fig. 1 Analysis of Thymoquinone (TQM), track assignment, system setup and chromatography plate layout

Fig. 2 Application 1-Linomat 5, Development 1 chamber, Scan developed plate 1a - TLC Scanner 4 and Spectrum Scan developed plate 1b

| **Fig. 3** System suitability tests and data acquisition for HPTLC Analysis of Thymoquinone (TQM)	**Fig. 4** HPTLC chromatogram of standard Thymoquinone (TQM)
Fig. 5 HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 1 and 2.	**Fig. 6** HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 3 and 4.
Fig.7 HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 5 and 6.	**Fig. 8** HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 7 and 8.
Fig.9 HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 9 and 10.	**Fig.10** HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 11 and 12.
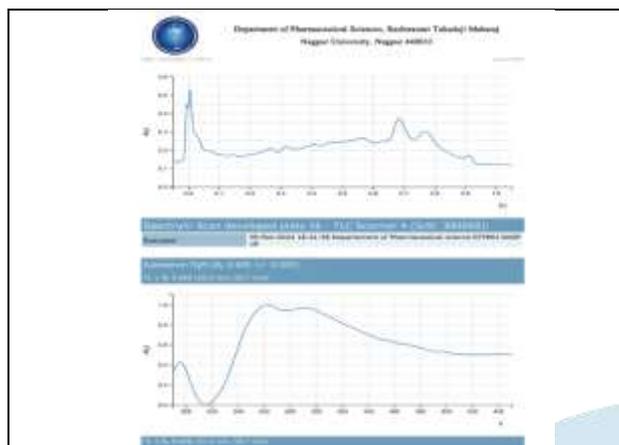
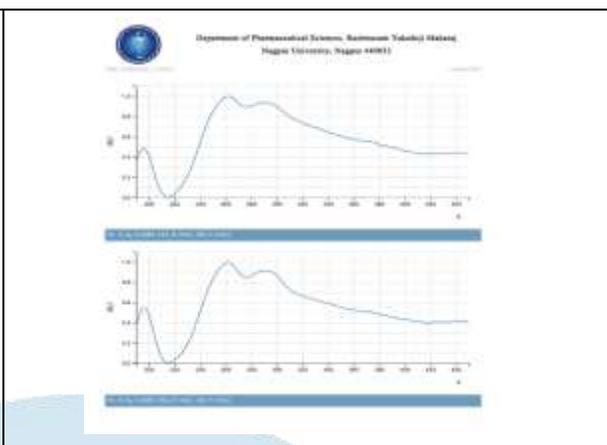**Fig.11** HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Tracks 13 and 14.

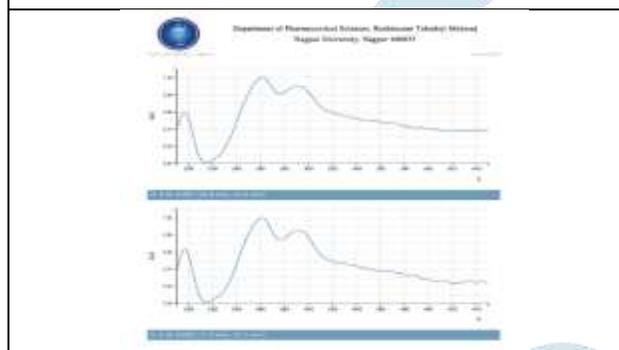**Fig.12** HPTLC chromatogram of Thymoquinone (TQM) at 254 nm with Track 15.

Fig.13 Spectrum scan developed plate 1 b TLC scanner 4 of Thymoquinone Tr-1, Rf-0.689 (254 mm, 50.7 mm)
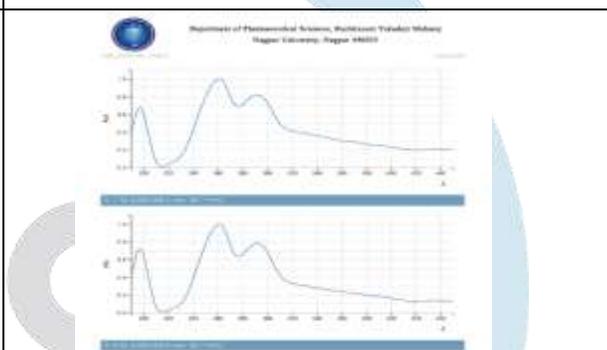

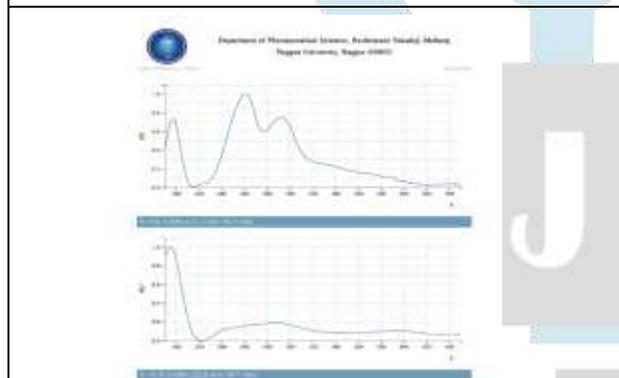**Fig.14** Spectrum scan developed plate 1 b TLC scanner 4 of Thymoquinone

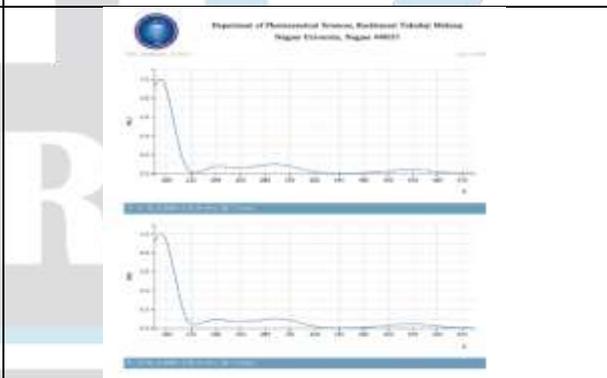**Fig.15** Spectrum scan developed plate 1 b TLC scanner 4 of Thymoquinone

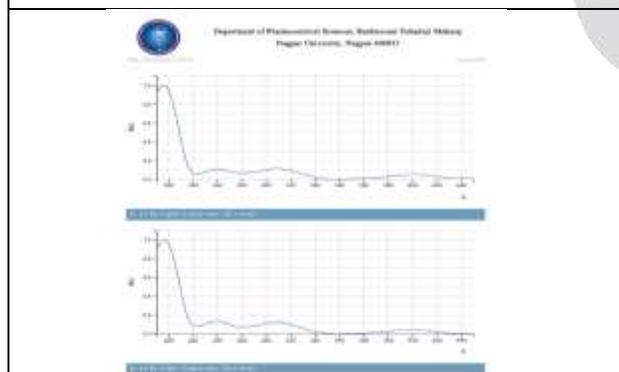**Fig.16** Spectrum scan developed plate 1 b TLC scanner 4 of Thymoquinone

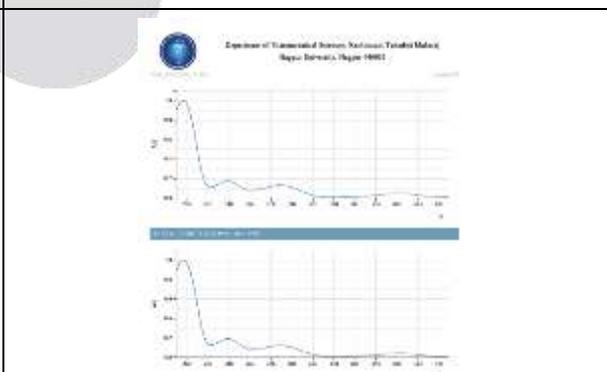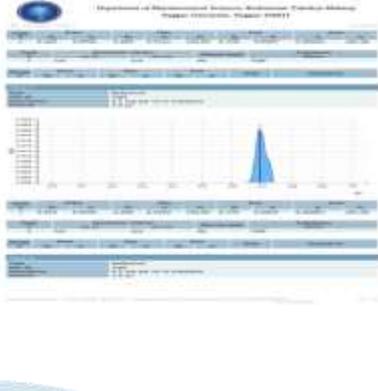**Fig. 17** Spectrum scan developed plate 1 b TLC scanner 4 of Thymoquinone

Fig. 18 Spectrum scan developed plate 1 b TLC scanner 4 of Thymoquinone

Fig. 19 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (0.1mg per ml in methanol Vol. 1.0 μL)

Fig. 20 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (0.1mg per ml in methanol Vol 3.0 μL)
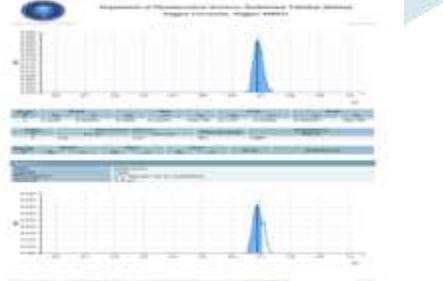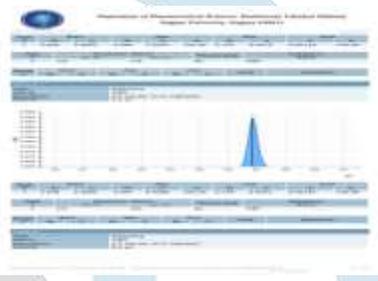
Fig. 21 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (0.1 mg/ml in methanol, Vol. 4.0) μL)

Fig. 22 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (Vol 5.0 μL)
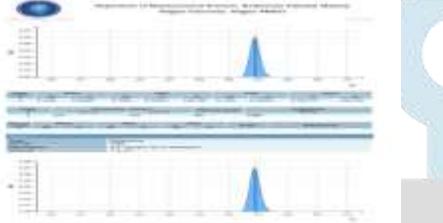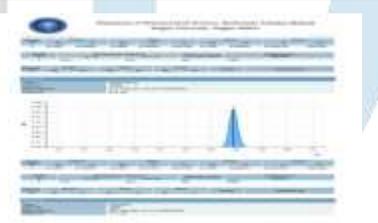
Fig. 23 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (Vol 7.0 μL)

Fig. 24 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (Vol 8.0 μL)
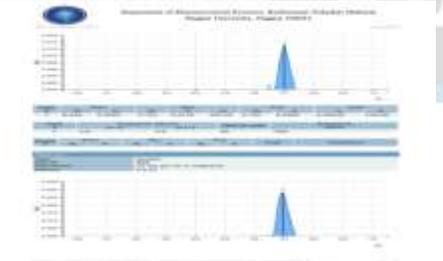
Fig. 25 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (Vol 4.0 μL)

Fig. 26 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (Vol 8.0 μL)
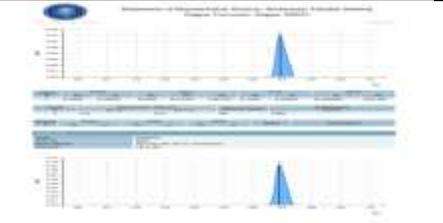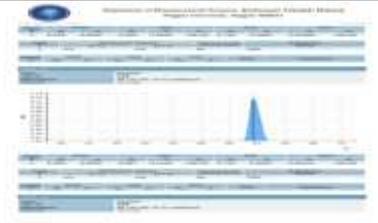
Fig. 27 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (25mg/ml methanol, Vol 10.0 and 4.0 μL)

Fig. 28 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (25mg/ml methanol, Vol 14.0 μL)

Fig. 29 Chromatogram of the ethanolic extract of *Nigella sativa* at 254 nm (25mg/ml methanol, Vol 14.0 μL)	**Fig. 30** HPTLC finger printing profile of *Nigella sativa* seeds
Fig. 31. Area calibration for substance Thymoquinone STD at 254 nm	**Fig. 32** Results of Phytochemicals screening of different extracts of the *Nigella sativa* seeds
Fig. 33 HPTLC Analysis of Thymoquinone Standard and *Nigella sativa* extract	
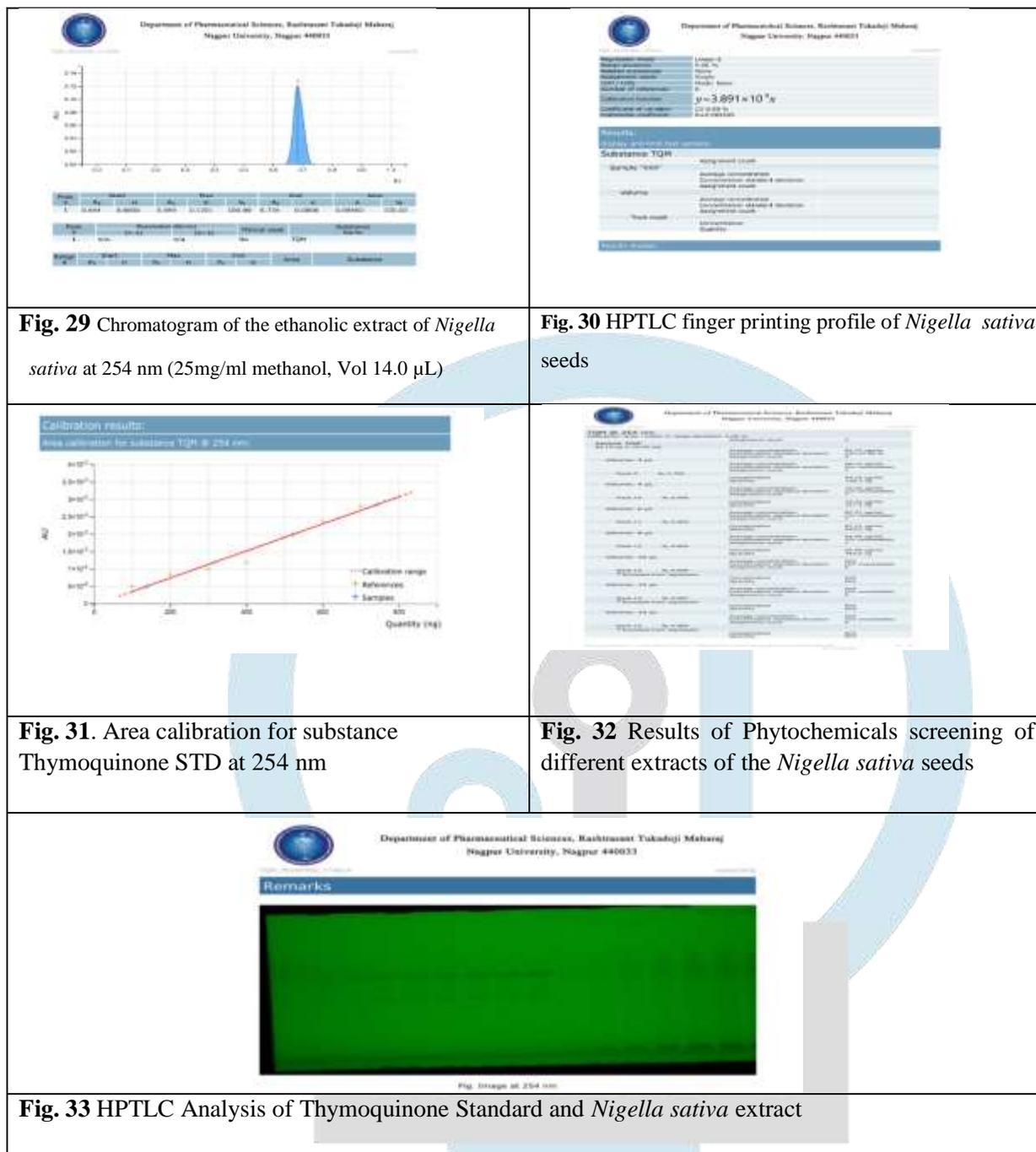
The HPTLC chromatograms confirmed the presence of thymoquinone as the principal biomarker in the ethanolic extract of *Nigella sativa* seeds. The quantification results demonstrated that thymoquinone was the most abundant compound, as indicated by the prominent peak in the chromatograph at wavelength 254 nm. The results indicate the potential of thymoquinone to contribute to the therapeutic properties of *Nigella sativa*, such as immunomodulatory, antipyretic, antiallergic, antibiotic, and antihistaminic effects.

HPTLC profile for standard biomarkers and hydroethanolic extract of *Nigella sativa* seeds

This work successfully developed and validated a high-performance thin-layer chromatography (HPTLC) method for quantitatively detecting thymoquinone in black cumin seeds (*Nigella sativa*). The method

was reliable for measuring thymoquinone since it demonstrated outstanding consistency, sensitivity, and specificity. All the examined black cumin seed samples had thymoquinone content, indicating the method's adaptability and durability. The study's results indicated that the UV-visible absorption spectrum provides vital information for identifying and measuring thymoquinone in the NS extract.

The measured peak demonstrated effective extraction and isolation at 254 nm, which agreed with the recognized thymoquinone absorption properties. The TLC analysis satisfactorily demonstrates the thymoquinone content by comparing the NS extract to the standard. Thymoquinone's identity is confirmed by its RF 0.66 value and fluorescence patterns corresponding to its Rf 0.68 identification, demonstrating its efficient extraction and use in pharmacological research.

The major peak in Fig. 4 to 18, around RF 0.68, represents thymoquinone. The overlay dendrogram contrasts the chromatographic profiles of the NS extract and the thymoquinone standard. The absorbance units (AU) are shown on the Y-axis, and the retention factor (RF), which ranges from 0.0 to 1.0, is shown on the X-axis. At around RF 0.68, thymoquinone is located in the centre peak. Thymoquinone is present in the (NS) extract, as indicated by the tight alignment of the standard and extract peaks with Rf 0.66. The yellow dashed lines show the expected range for Thymoquinone detection, demonstrating the chromatographic method's precision and reliability.

Table5. HPTLC profile and spectrum correlation data for a standard biomarker Thymoquinone.

Substance name	Track	RF	R(s,m)	r(e,m)	Ref. spectrum	Correlation
TQM	1	0.689	0.000000	0.000000	Tr.2, Rf 0.689, Sub,TQM	0.992309
TQM	2	0.689	0.000000	0.000000	Tr.1, Rf 0.689, Sub,TQM	0.992309
TQM	3	0.689	0.000000	0.000000	Tr.2, Rf 0.689, Sub,TQM	0.994176
TQM	4	0.689	0.000000	0.000000	Tr.3, Rf 0.689, Sub,TQM	0.993735
TQM	5	0.687	0.000000	0.000000	Tr.4, Rf 0.689, Sub,TQM	0.977850
TQM	6	0.689	0.000000	0.000000	Tr.5, Rf 0.687, Sub,TQM	0.992068
TQM	7	0.689	0.000000	0.000000	Tr.6, Rf 0.689, Sub,TQM	0.993522
TQM	8	0.689	0.000000	0.000000	Tr.7, Rf 0.689, Sub,TQM	0.994167
TQM	9	0.689	0.000000	0.000000	Tr.8, Rf 0.689, Sub,TQM	0.408890
TQM	10	0.689	0.000000	0.000000	Tr.8, Rf 0.689, Sub,TQM	0.311709
TQM	11	0.689	0.000000	0.000000	Tr.8, Rf 0.689, Sub,TQM	0.296663
TQM	12	0.689	0.000000	0.000000	Tr.8, Rf 0.689, Sub,TQM	0.287665
TQM	13	0.689	0.000000	0.000000	Tr.8, Rf 0.689, Sub,TQM	0.295479
TQM	14	0.687	0.000000	0.000000	Tr.8, Rf 0.689, Sub,TQM	0.289615

| TQM | 15 | 0.684 | 0.000000 | 0.000000 | Tr.8, Rf 0.689, Sub,TQM | 0.279895 |

*TQM represents Thymoquinone

Chromatograms were acquired at 254 nm, and peaks were integrated after each injection to locate the Biomarker (TQ) peak in the NSSE (Iqbal *et al.,* 2019). An absolute reference standard was then used to compare the retention time of the NSSE chromatograms.

The peaks' constant height and clarity demonstrate high linearity and reproducibility in thymoquinone detection.(Fig 19 to 29) This confirms the reliability of the chromatographic approach for quantitative determination of thymoquinone.

Thymoquinone may be reliably quantified using this approach, as demonstrated by the strong correlation coefficient and calibration data in Fig. 31. The presence and concentration of Thymoquinone in the NS extract were confirmed by the identification and quantification of the particular peaks for Thymoquinone in both the sample and the reference standard (Sayyad and Bhise, 2024).

Quantification using the Area Normalization Method showed that thymoquinol constituted approximately 6.83% of the total extract. This is a significant concentration, indicating that thymoquinol is one of the major constituents in the cold-pressed ethanoic extract. The relatively high content of thymoquinol supports the therapeutic potential of *Nigella sativa*, as thymoquinol is known for its potent antioxidant, anti-inflammatory, and potential anticancer properties.

The presence and quantification of thymoquinol in this extract are particularly important because most studies have focused on thymoquinone, a closely related compound. However, thymoquinol also exhibits promising pharmacological activities, and its identification adds value to the therapeutic profile of *Nigella sativa*.

Furthermore, the Area Normalization Method, while relatively simple, provided a reliable estimation of thymoquinol content and could serve as a useful analytical tool in quality control of *Nigella sativa* extracts.These findings underscore the relevance of *Nigella sativa* as a source of pharmacologically active compounds and justify its continued investigation in natural product-based drug development.

CONCLUSION

In conclusion, the HPTLC method developed in this study proved effective for the separation, identification, and quantification of thymoquinone and its derivatives. The findings reinforce the potential of *Nigella sativa* seed extract as a valuable source of bioactive compounds with diverse therapeutic applications. The present investigation concludes that *Nigella sativa* seeds contain a diverse array of bioactive compounds. Owing to these constituents, traditional practitioners have long utilized the seeds for the treatment of various ailments. The isolation and characterisation of individual phytochemical components may further enhance their therapeutic efficacy when subjected to specific biological assays. *Nigella sativa* seeds represent a promising source of pharmacologically active compounds with potential applications in diverse clinical settings. High-Performance Thin-Layer Chromatography (HPTLC) analysis of the ethanolic extract revealed that 9,12-

octadecadienoic acid, ethyl ester (44.137%), thymoquinone (20.538%), and hexadecanoic acid, ethyl ester (11.300%) were the principal constituents. These compounds exhibited a positive correlation with the extract's antioxidant and immunomodulatory activities. However, further studies are warranted to elucidate the precise mechanisms through which these molecules contribute to the observed biological effects of *Nigella.* The HPTLC method is thus proved to be effective for the separation, identification, and quantification of thymoquinone and related compounds in *Nigella sativa*. This method can serve as a reliable tool for routine quality control and standardisation of herbal formulations containing *Nigella sativa*.

References:

Ahmad Aftab, Asif Husain, Mohd Mujeeb, S.A.Khan, A.K.Najmi, N.A.Siddique, Z.A.

Damanhouri and Firoz Anwar (2013) A review on therapeutic potential of *Nigella sativa*: A miracle herb Asian Pacific Journal of Tropical Biomedicine ; 3(5): 337-352.

Harbon J B.(1998) Phytochemical methods: a guide to modern techniques of plant analysis. Chapman and Hall Int. New York; 488–93.

Iqbal Sehrish, Aqeel Javeed, Adeel Sattar and Rabia Tanvir (2019) Pharmacokinetics of thymoquinone in layer chickens following oral and intravenous administration Journal of Veterinary Pharmacology and Therapeutics. 42(6);707-712.

Newman, D. J.and G. M. Cragg (2007) Natural Products as Sources of New Drugs over the Last 25 Years. Journal of Natural Products, 70(3), 461–477.

Paarakh M. P.(2010) *Nigella sativa* Linn- A Comprehensive Review.Indian journal of natural product and resources,1(4):409- 429.

Raaman Nanjian (2006) Phytochemical techniques. New India Publishing.

Sayyed. R. R and K.S.Bhise (2024) Quantitative Analysis of Thymoquinone in Black Cumin Seeds using High-Performance Thin-Layer Chromatography African Journal of Biological Science. 6(12); 1514-1520

Senguttuvan .J and P.Subramaniam (2016) HPTLC fingerprints of various secondary metabolites

in the traditional medicinal herb *Hypochaeris radicata* L. J Botany;14:1–11.

Sethi P. D.(1996) HPTLC Quantitative Analysis of Pharmaceutical Formulations, 1st edition,

published by CBS Publisher and Distributor, New Delhi.

Singhal Rini, Shewta Verma & Vijendra Singh (2024) Phytochemical investigation of *Nigella sativa* seed extract by HPTLC, HPLC and GC-MS: a comparative geographical study. Natural Product Research, 1–7.

Tembhure S.V, Feroz S, More B.H and D.M.Sakarkar (2014) A review on therapeutic potential

of *Nigella sativa* (kalonji)seeds. Journal of Medicinal Plants Research,8(3):167-177.