Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(12); 1425-1428
doi: 10.25258/phyto.v9i11.11186
ISSN: 0975-4873

Research Article

Comparative Assessment of Extraction Methods and Quantitative Estimation of Thymoquinone in the Seeds of *Nigella sativa* L By HPLC

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Received: 8th Jul, 17; Revised 27th Nov, 17, Accepted: 8th Dec, 17; Available Online:25th Dec, 17

ABSTRACT

The objective of the present study was to find out the best organic solvent and extraction method for the isolation of thymoquinone from *Nigella sativa* L. seeds. The seeds of *N. sativa* were authenticated by a taxonomist, washed, dried and powdered. Different extraction techniques such as maceration, soxhlet, reflux and ultrasound assisted extraction were employed for extraction of thymoquinone from seeds by using three solvents of different polarity like petroleum ether, hexane and methanol. Thymoquinone was quantified in each sample extract of seeds by simple high performance liquid chromatography technique. The content of thymoquinone was found to be higher in methanolic extract among the various techniques and solvents tried. Ultrasound assisted extraction technique was observed the best for extraction of thymoquinone. The calibration curve of standard thymoquinone demonstrated linear relationship in the concentration range of 1 to 0.0625 µg/ml with correlation coefficient, r^2 of 0.9976. It can be concluded on the basis of HPLC finding that ultrasound assisted extraction technique is the best for extraction of thymoquinone . This method will be preffered in industy or academia for extraction of thymoquine from *N. sativa* seeds.

Keywords: Kalonji, Thymoquinone

INTRODUCTION

Nigella sativa L. commonly known as "black seed" is a remedy for various diseases. It is used as bitter astringent, stimulant and diuretic. The plant finds its use in diseases like jaundice, intermittent fever, dyspepsia, paralysis, piles, skin diseases, gastrointestinal problems, conjunctivitis, rheumatism, diabetes, hypertension, intrinsic hemorrhage, amenorrhea,asthma, cough, bronchitis, headache, influenza and eczema and many more[1]. Some other pharmacological activities has also been reported recently like anti-oxidant, anti-inflammatory, anti-bacterial, anti-cancer, anti-microbial, anti- fungal and many more[2]. Most activities of the herb is due to presence of phenolic compounds of which main is thymoquinone. Thymoquinone is chemically 2-isopropyl-5-methyl- 1, 4- benzoquinone and is known to show anti-oxidant, anti-inflammatory and anti-tumour activity[3]. It's anti-timour activity is through induction of apoptosis in tumor cells by suppression of nuclear factor Kappa-Beta (NF-κB), Akt activation, and extracellular signal-regulated kinase signaling pathways and also inhibition of tumor angiogenesis[3]. It can be derived from the essential oil of the seed but is present in small amount. Thus, it's quantitative analysis becomes important. The study was aimed to determine the most suitable solvent to optimize the extraction method for the isolation of thymoquinone from the seeds of *N.sativa* L. by quantitative determination of thymoquinone in different solvent extracts through high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Plant material

The seeds of *Nigella sativa* L. were purchased from Yucca Enterprises,Wadala, Mumbai, India. The seeds were authenticated by a taxonomist Department of Botany,Faculty of Science, Jamia Hamdard and a specimen is retained in School of Pharmaceutical Education & Research, Jamia Hamdard.

Chemicals

Standard thymoquinone was purchased from Yucca Enterprises,Wadala, Mumbai, India. HPLC grade acetonitrile and water were purchased from S.D Fine Chemicals, India. All other reagents used were of analytical grade and purchased from S.D. Fine Chemicals, India.

Extraction of thymoquinone

The seeds were nicely cleaned and air dried .The dried samples were then powdered with the help of grinder (Sujata Supermix, 900W).The powdered sample was kept in an air tight container away from light until use. Extraction of thymoquinone was done through different modes like maceration, hot solvent extraction by reflux technique, hot solvent extraction by soxhlet technique and ultrasound assisted extraction (UAE). Methanol,

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Table 1: Thymoquinone content in different extracts of *N.sativa* L.

Extraction technique	Solvent	Extract yield (%w/w)	Thymoquinone content (%w/w)
Maceration	Methanol	34.8±9.8	4.27±1.1
	Petroleum ether	15.7±5.1	2.9±0.89
	Hexane	23.2±6.3	3.67±0.988
Reflux	Methanol	47.95 ±10.5	5.89±1.1
	Petroleum ether	23.3±6.2	2.44±0.84
	Hexane	17.45±5.4	3.45±0.95
Soxhlet	Methanol	40.1±8.5	6 .77±1.2
	Petroleum ether	32.7±5.1	3.66±1.43
	Hexane	32.2±32.2	4.81±1.8
UAE	Methanol	8.0±1.8	14.89±2.6
	Petroleum ether	12.4±2.4	8.270±2.7
	Hexane	9.8±1.67	10.56±3.5

petroleum ether and hexane were chosen for extraction to ensure extraction of thymoquinone in solvents of varying polarity.

Chemical tests for flavonoid

After determination of extract yield, Shinoda test was used as indicator to check the presence or absence of flavonoids in each extract[3]

Extraction via maceration

1 g of powdered drug was soaked in 10 ml respective solvents (Drug to solvent ratio- 1:10 g/mL) The extracts were evaporated to dryness under inert atmosphere using rotary vacuum evaporator (HAHN SHIN, HS-2005V-N) at 40°C .The residue was weighed and appropriate dilutions were made for quantitative estimation of thymoquinone in the extract.

Extraction via reflux

Hot solvent extraction was done using a reflux apparatus for 6 h at 40 °C using drug to solvent ratio of 1:10 g/mL. Further processing of the extracts was done in the same manner as discussed above in the case of maceration.

Extraction through soxhelation

Continuous hot solvent extraction was done using a soxhlet apparatus for 6 h at 40 °C using drug to solvent ratio of 1:10 g/mL. Further processing of the extracts was done in the same manner as described earlier.

Extraction through UAE

UAE was done for 20 min with solvent t o drug ratio of 10:1 mL/g at 40 °C in a sonicator (TOSCHON, SW7). Further processing of the extracts was done in the similar manner discussed above.

Quantitative analysis of thymoquinone by HPLC

Preparation of standard & sample solutions

Stock solution of thymoquinone was prepared in HPLC grade methanol at a concentration of 1 mg/mL. Then working solutions of 100, 125, 175, 200 and 500 µg/mL were prepared in HPLC grade methanol and stored at -20 °C. Each solution was then filtered through 0.2 µm membrane filter (Axiva) and then subjected to HPLC analysis. Peak height was obtained at a static retention time for each standard solution and calibration plot was then made for concentration (µg/mL) versus peak area.

Ten milligram of each extract was dissolved in HPLC grade methanol to obtain a final concentration of 1 mg/mL. The solution was then filtered through 0.2 µm membrane filter (Axiva) and 20 µL of the resulting solution was subjected to HPLC analysis. Final concentration of thymoquinone in the extracts was calculated by using linear equation obtained through calibration plot of standard thymoquinone.

Chromatographic conditions

HPLC analysis of standard as well as extracts was done on Quaternary System (Shimadzu, Japan) which comprised of LC10AT VP pumps (Shimadzu, Japan), a single wavelength programmable UV-visible detector, and a system controller. Rheodyne injector with a 20 µL fixed loop was employed for introducing samples into the system. A column of 25x 4.6 mm with particle size 5 µm Lichrospher C_{18} reverse phase column (Merck, Germany) was used to achieve separation. Mobile phase consisted of a solution of acetonotrile and water -55:45 v/v [5] running at a flow rate of 1mL/min at 30°C. Detection was done at a wavelength of 254nm.

RESULTS

Phytochemical testing

The prepared extracts including methanol, petroleum ether and hexane showed presence of flavonoids.

Extraction of thymoquinone by various extraction modes

Thymoquinone was extracted by four different extraction techniques - maceration, soxhlet, reflux and UAE using hexane, petroleum ether and methanol as solvents. During the experiment, extraction time, extraction temperature and drug: solvent ratio was kept constant for all the solvents. Different extraction yields (%w/w) were observed in different solvents. For all extraction techniques, methanol extract showed maximum thymoquinone content, followed by hexane. Highest thymoquinone content was found in methanolic extract of ultrasound assisted extraction technique (14.89±2.6 %w/w) while the lowest quantity of thymoquinone was seen in petroleum ether extract obtained by reflux technique i.e. 2.44±0.84 %w/w.In general, the yields thymoquinone content was found more in extract obtained by modern extraction technique (UAE) as compared to the conventional modes of extraction.

Quantitative estimation of thymoquinone by HPLC

The standard solutions of thymoquinone and sample extracts were subjected to HPLC technique using

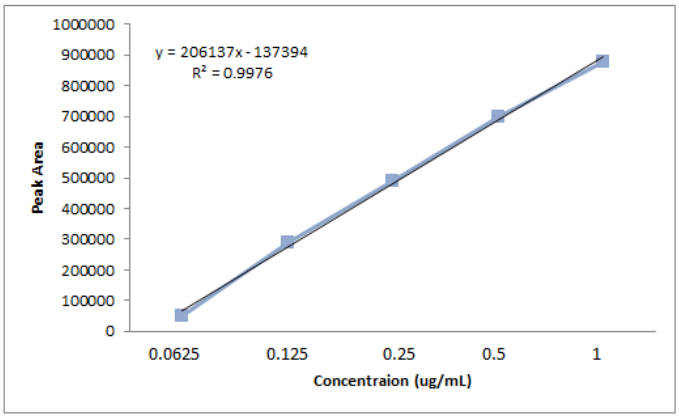

Figure 1: Calibration Curve for standard Thymoquinone with respect to peak area.

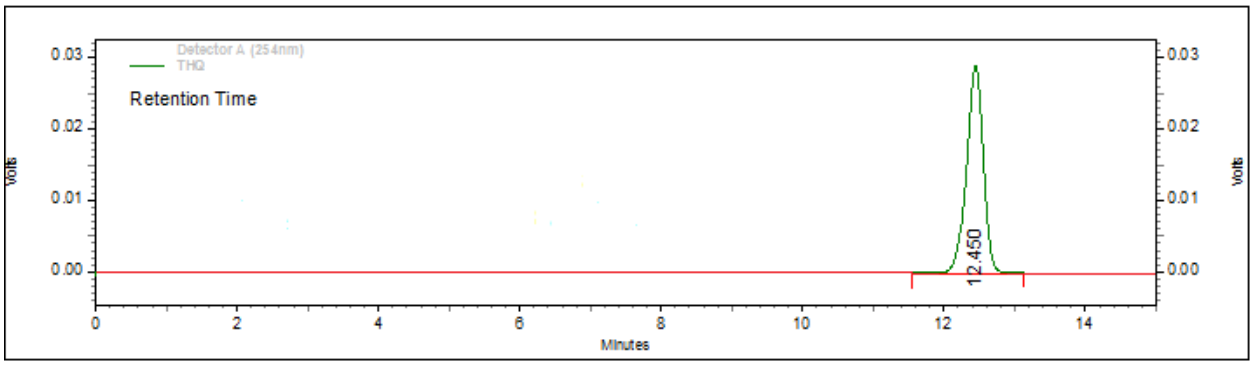

Figure 2: HPLC peak for standard thymoquinone.

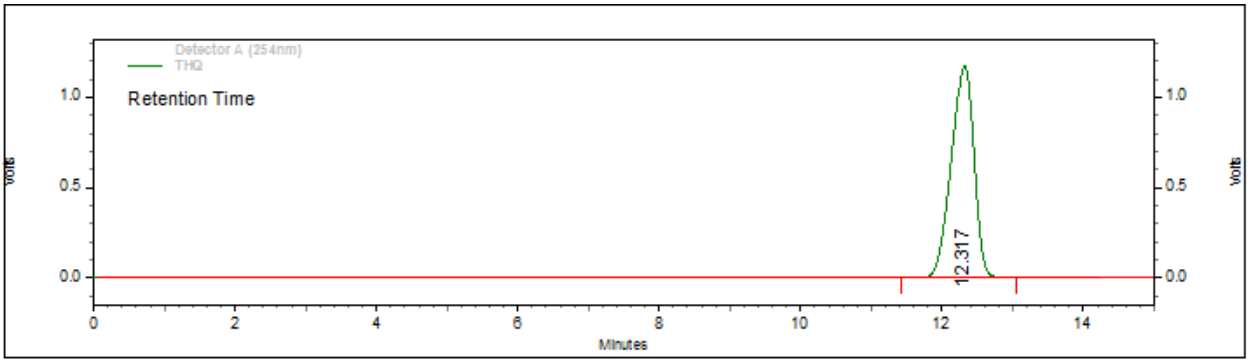

Figure 3: HPLC peak for *Nigella sativa* L. seed extract.

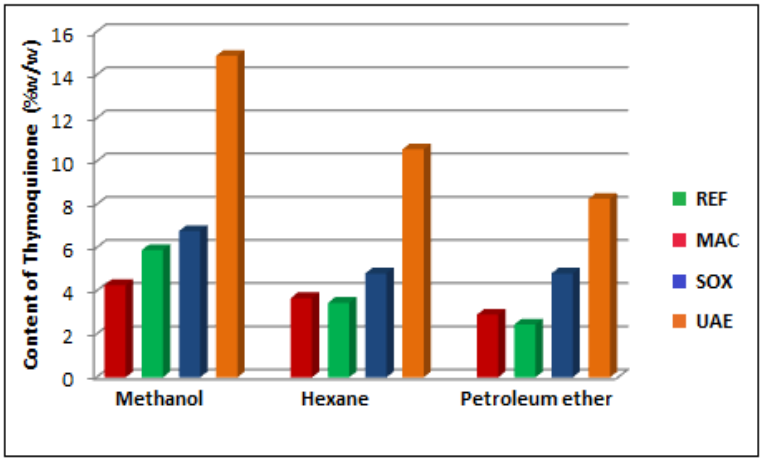

Figure 4: Comparative chart of different techniques and solvents used for Thymoquinone extraction.

acetonotrile and water (55:45 v/v) as mobile phase, in isocratic mode. The retention time of thymoquinone in HPLC chromatogram was observed at 12.1 minutes.Calibration curve with respect to peak area for standard thymoquinone for made five dilutions with concentration ranging fom 20 to 100 µg/ml. The linear regression equation was obtained in the form of y=mx+c, where y and x corresponds to peak area and concentration respectively (Figure 2). The regression equation obtained was y=206137x-137394 with a correlation coefficient of r^2=0.9976. The relative quantity of thymoquinone in the prepared extracts was then calculated from this equation and the results are shown in Table 1.

Quantification of different extracts of *N.sativa* L. through HPLC revealed that methanol is the best solvent for the extraction of thymoquinone. Thymoquinone content in methanol extract obtained through maceration, reflux, soxhelation, and UAE techniques was found to be 4.27±1.1%, 5.89±1.1%, 6 .77±1.2% and 14.89±2.6 respectively (Table 1).

Thus, it was observed that among various techniques employed for extraction and isolation of thymoquinone, UAE technique was found to be the most efficient. Clearly, the results of our study indicated that methanol is the most suitable solvent for thymoquinone extraction.

CONCLUSION

Certain studies report that the extraction and isolation techniques employed effects the biological activities of the phytocompounds being extracted. Henceforth, it becomes indispensible to select a method that is least affected by interfering substances[6].

Thymoquinone possess plethora of pharmacological properties making it an important phytocompound. Thus, its content in the plant must be known. It is present in very minute quantity, making HPLC technique most suitable for its quantification.

The extract yields varied with the solvents and/or extraction techniques but the maximum thymoquinone yield was obtained with UAE. It can be inferred that modern non-thermal extraction method are better choice for extract preparation from *N.sativa* L. seeds as compared to thermal methods. Also, HPLC analysis results showed that thymoquinone content was the highest in methanol extracts irrespective of the extraction technique employed, which implies that methanol is a favourable choice of solvent for the extraction of thymoquinone.The study concludes that extraction mode as well as solvent affects the isolation of thymoquinone from *N.sativa* L. seeds. The end result of the study will help the further researchers in selecting the solvent as well as technique of extraction for thymoquine from *N.sativa* L. seeds.

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