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EVALUATION OF ANTI-CANDIDAL EFFICACY OF FREE AND MICROSPHERE ENCAPSULATED THYMOQUINONE

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ABSTRACT

Nigella sativa, an annual medicinal plant is traditionally used in different parts of world for many centuries due to its potential health benefits. Thymoquinone (TQ) is the bio-active component of *Nigella sativa* (*Ranunculaceae*) seeds exhibits various beneficial properties. The present study explores the extraction of thymoquinone from seeds of *Nigella sativa*, purified and characterized. The purified compound was then encapsulated in chitosan microspheres and the encapsulated TQ was characterized using SEM, DLS, AFM and FTIR instrumental techniques. The process of encapsulation followed in the present study does not require glutaraldehyde for stabilization of microspheres. Anti-candidal activity of both free and encapsulated TQ was studied using standard protocol. Minimum inhibitory concentration of free TQ was determined as 1500 µg and it was 500µg for encapsulated TQ. The kinetics of release studies followed zero order.

KEYWORDS: Thymoquinone**,** Antifungal activity, Antimicrobial agents, Microspheres, *Nigella sativa.*

INTRODUCTION

Generally, drugs are administered as formulated preparations instead as pure chemical substances. And the major objective of the formulation is to have the desired concentration of the drug in blood or tissue, which is therapeutically effective and non-toxic for prolonged period or entire duration of the treatment. Despite varies formulations, microspheres have

been considered as a potential formulation to deliver drugs in a controlled fashion.[1] Due to its small particle size and wide systemic distribution, effectively improves the drug absorption.[2] However, the polymeric materials employed in the formulations play a major role in the drug delivery. Both gelatin and chitosan have been widely used in formulations due to their biocompatibility, biodegradability and non-toxic nature of the degraded products.[3] Further, compared to gelatin, the preference to chitosan is more due to its characteristic behavior as penetration enhancer by opening the tight epithelial junctions.[4]

In Indian system of medicine, herbs are the vital source of drugs from the ancient time.[5] In a review on *Nigella sativa*,[6] the authors concluded that the plant *Nigella sativa* is a miracle herb with wide spectrum of pharmacological potential and its black seeds have been widely used for centuries in the treatment of various ailments throughout the world.[6] The black seeds are included in the list of natural drugs of 'Tibb-e-Nabavi' or "Medicine of Prophet (Muhammed)" and stated that the seed can heal every disease except death.[7] *Nigella sativa* has a potential anti-inflammatory, anti-microbial, anti-fungal, anti-parasitic and anti-cancer activity.[8] The seeds were found to contain numerous esters and traces of two different alkaloids (nigellimin & nigellimin-N-Oxide and nigellidin & nigellicin). In the essential oil, thymoquinone was identified as the major component (up to 50%). The proximate analysis of seed suggested that it contains 37% Oil, 4.1% Ash, 16-19.9% Proteins, 33-34% carbohydrates, 4.5-6.6 % fiber, 0.013% Saponins, 5-7% Moisture.[5]

With regard to yeast infections, candidal infections in infants always need high attention. Combating candidal infections through various medicinal herbs are in reports. The detailed study on controlling candidal infections in oral and vagina through active constituent of *Acorus calamus* in animal models has already been in public domain. [6] In all these reports, it has been found that the infection was controlled at initial stage of medication and reoccurred once the medication was stopped. Thus, controlled delivery system is always acceptable in most fungal and bacterial infections. Though, anti-candidal activity of the active constituent (TQ) of *Nigella sativa*, has already been studied, the potency of formulated TQ in the form of microsphere has not been in reports. Alam *et al*., (2012),[7] studied the nanoformualtion of TQ with chitosan polymers and reported the delivery of drugs to target brain.

Thus, the present study has been taken up to evaluate the anti-candidal efficacy of microsphere-formulated thymoquinone and comparing the efficacy with free TQ. In detail,

the study summarizes the extraction of TQ, characterization of TQ, formulation of TQ in the form of microspheres, anti-candidal efficacy of free and microsphere encapsulated TQ, determination of minimum inhibitory concentration, various instrumentation techniques to ascertain the active constituent and its encapsulation. Further, the study also detailed the new method of formulations without using toxic chemical for stabilization of microspheres.

MATERIALS AND METHODS

Chemicals

The solvents, Ethyl acetate, Acetone, Dimethyl sulfoxide (DMSO) were of analytical reagent grade, purchased from S.D. Fine chemicals Ltd. Sabouraud dextrose agar and 1X Phosphate buffer saline (PBS), were purchased from HiMedia Pvt Ltd. Vegetable oil, Span 80, Sebacic acid, Potassium bromide, Chitosan (low molecular weight), Thymoquinone were procured from Sigma-aldrich, USA**.**

Fungal Strain

The fungal strain, *Candida albicans* 10231, used in the present study was obtained from the American type culture collection (ATCC).

Collection of Plant Material

Nigella sativa seeds were purchased from local markets of Perumbakkam, Chennai, Tamilnadu, India and authenticated by Dr. T. Anandhan, Assistant Director (Retd.), Siddha Central Research Institute, Chennai.

Extract preparation

The seed sample was shade dried and then pulverized to a fine powder. The active metabolite was extracted using water as solvent (hot continuous extraction using Soxhlet apparatus).

Anti-candidal activity of aqueous extract

Initial screening on Anti-candidal activity was carried out using standard procedure as summarized in CLSI (1990). Sabouraud Dextrose agar (SDA) was used for assessing the anti-candidal activity. In brief, pre-sterilized Sabouraud Dextrose agar (SDA agar) was poured aseptically in a petri dish and allowed to solidify. A lawn of 24hrs old *Candida albicans* 10231 was made. Wells were created using well borers of size 10mm and it was loaded with 100, 200, 300 and 400µl of aqueous extract (5mg/ml) and incubated at 37ºC for 48hrs. Wells indented with sterile water served as a blank. Fluconazole (1mg/ml) was used as a positive

control. Followed by incubation, the diameter of the zone of inhibition was measured and expressed in millimeter (mm).

Purification

The crude extract was subjected to purification in glass column (20 X 250mm) packed with activated silica gel (60-120 mesh) using Hexane: Ethyl acetate at 9:1 ratio as mobile phase. The fractions were collected and monitored by Thin layer chromatography.[7]

Thin layer Chromatography

The fractions obtained and the reference (standard thymoquinone) spotted individually on pre-coated silica gel G60 F254 TLC plates. The plates were then developed with Hexane: Ethyl acetate (9:1) as a solvent system. The spots were visualized in the presence of iodine and the retention factor was calculated from the distance travelled by the solute and the distance travelled by the solvent.[8]

Bioautography studies

The developed TLC plate was placed in sterile petri dish and overlaid with molten SDA agar. After solidification, *Candida albicans* culture lawn was made. Microbial growth inhibition will appear as a clear zone over the agar surface.[9]

Microsphere formulation

The active component which was identified as Thymoquinone (TQ) from the TLC Bioautography plate was scrapped and extracted with Hexane: Ethyl acetate (9:1) and formulated into microsphere according to the procedure reported by Roy *et al.*,(2009),[10] with slight modifications. The polymer solution was prepared by dissolving 1% (w/v) of chitosan with 0.5% (w/v) sebacic acid and kept under constant stirring. The ratio of chitosan and cross linking agent was optimized by preliminary studies. To the homogenized solution 1 ml of purified TQ was added dropwise 0.5% vegetable oil and again homogenized at 6000 rpm for 2-4hrs for uniform mixing, proper cross linking and stabilization. The obtained sebacic acid cross-linked chitosan microsphere was recovered as a suspension in oily phase and washed several times with acetone to remove the residual oil and then freeze dried and stored at 4º C until further applications. In the whole preparation procedures followed in the present study completely avoided the use of acetic acid in solubilization of chitosan and use of glutaraldehyde for stabilization of chitosan microspheres.

INSTRUMENTAL ANALYSES OF MICROSPHERES

Scanning electron microscope (SEM)

Surface morphology of chitosan microspheres was analyzed by using scanning electron microscope. The dried microspheres in the form of powder was mounted onto a separate, adhesive coated 12.5 mm diameter aluminium stubs. Excess powder was removed by tapping the stubs sharply and then gently blowing a jet of particle free compressed gas across each other. The SEM micrographs were taken using JEOL-JEL-5300 Scanning microscope.

Atomic force microscopy (AFM)

The microspheres were dispersed in acetone and spread over the cover slip. The cover slip was then placed in the AFM stage for analysis using MFP-3D AFM (Asylum Research, CA).

Fourier Transform Infrared spectroscopy (FTIR).

Fourier Transform Infrared (FT-IR) spectrum was recorded in spectrum-I FT-IR spectrophotometer (Perkin-Elmer Co, USA.). A 5µg of Chitosan, Thymoquinone (Standard) and microspheres encapsulated with TQ were mixed thoroughly with 200µg of Potassium Bromide (Sigma, USA) and pelletized using hydraulic press. FT-IR spectrum was measured in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}. All the measurements were of 20 scans with potassium bromide as a background reference.

Dynamic Light Scattering (DLS)

The particle size, particle size distribution, PDI, and zeta potential of TQ encapsulated microspheres were determined through DLS. The sample volume used for the analysis was kept constant as 1 mL. The particles exhibited Brownian motion, which causes the intensity of light to scatter from particles, which is then detected as a change in intensity with suitable optics and a photo multiplier (Malvern, Autoszer 4700 Series).

Characterization of thymoquinone microspheres

Percentage Yield

The yield of microspheres was calculated based on the quantity of polymer and drug used in microsphere according to Chandrakala *et al.* [11]

$$\% \text{ Yield} = \frac{practical\ value}{theoritical\ value} * 100$$

Swelling Index /Drug loading

Swelling index of the TQ encapsulated microspheres was assessed according to the method summarized by Chandrakala *et al.*, (2013).[11] In brief, a known amount of microspheres was soaked in phosphate buffered saline for 24 hrs. The increase in volume of the microspheres was measured followed by measuring the increase in weight of the microspheres. The degree of swelling was calculated according to the equation summarized below

Degree of swelling = [(Wt – Wo) / Wo] × 100

Where, **Wt** is weight of the microspheres at time t; **Wo** is initial weight of the microspheres.

Thymoquinone entrapment efficiency

Drug entrapment efficiency of the TQ encapsulated microsphere was calculated according to the procedures summarized by Suman *et al.*,(2006) [12] with slight modifications. A known amount of microsphere was soaked in DMSO for 24hrs and then centrifuged at 5000 rpm. The absorbance of the supernatant was measured at 260 nm. Drug entrapment efficiency was calculated from the following equation:

$$\textbf{Drug entrapment efficiency} = \frac{\text{Total amount of drug} - \text{free drug}}{\text{total amount of drug}} * 100$$

***In vitro* Thymoquinone release studies**

In vitro TQ release study was carried out as per the protocol summarized by Ibezim *et al.*,(2011).[13] In detail, a known amount of microsphere was dissolved in 10ml of PBS (pH 7.4) and kept under stirring at 100 rpm speed in magnetic stirrer. For every 1hr, 1ml of the sample was removed and centrifuged at 10,000 rpm for 5-10 min and the supernatant was analyzed by UV spectroscopy at 260nm. The volume of the reaction mixture kept constant by replacing with 1ml of fresh PBS. Thymoquinone release kinetics determined using kinetic models.

RESULTS AND DISCUSSSION

In order to increase the bioavailability and also to have sustainable release, drug formulations in various forms have been admitted, which received number of protocols and methods to satisfy the goal. In the present study, an approach has been made with the microsphere formulation of the active constituent of the seeds of the medicinal plant *Nigella Sativa* and studied the anti-candidal efficacy under *in vitro* conditions. Further, the study focuses the green method of microsphere formulation, in which no toxic constituents are involved during the formulation. The formulation thus claims the novelty of the study.

Anti-candidal activity of aqueous extract

Preliminary screening on anti-candidal efficacy of aqueous extract of *Nigella sativa* seeds with the different volume of crude aqueous extract ranging from 100, 200, 300 and 400 µl which corresponds to the concentration of 500, 1000, 1500 and 2000µg suggested that the maximum zone of inhibition was 12 – 13 mm with 1500 and 2000 µg and the minimum zone of 2-3 mm with 500-1000 µg (Fig. 1). Though increase in volume/concentration of extract increases the zone size, but the results are insignificant at high concentrations. When compared to standard drug Fluconazole at 1 mg/ml concentration, anti-candidal efficacy of the crude aqueous extract of *N. sativa* was less. The observation corroborates well with the observation made by Ahmed *et al.*,(2013),[14] in which the authors reported that the aqueous extract of *Nigella Sativa* did not showed anti-candidal activity.

Further, anti-candidal efficacy of thymoquinone encapsulated microspheres was assessed for different concentration ranging from 500-2000 µg. The maximum zone of inhibition of 18mm was observed with 1500 and 2000 µg and the minimum zone of 12-15 mm was observed with 500 and 1000 µg (Fig. 1).

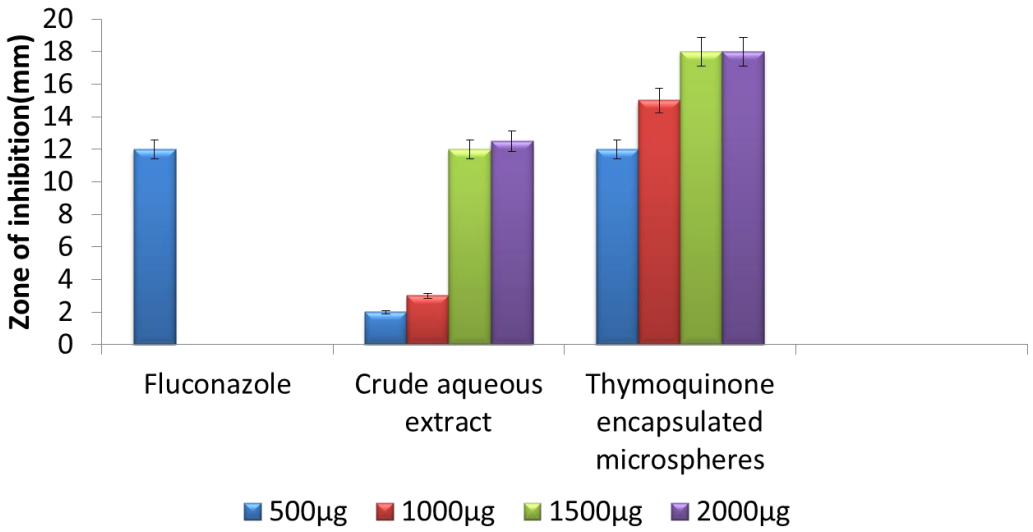

Fig. 1 Anti-candidal activity of Control drug (fluconazole), Crude aqueous extract, thymoquinone encapsulated microspheres.

Thin layer chromatography (TLC)

Fig. 2a depicts the thin layer chromatogram of aqueous extract and the standard Thymoquinone compound, developed using Hexane and Ethyl acetate solvent system. The Rf value of Standard thymoquinone was 0.74, and the active component from *N.sativa* aqueous

extract showed R_f value of 0.72. Similar value for TQ of crude extract was observed by Suthar *et al.*,(2010).[15]

Fig. 2a Thin Layer Chromatogram of aqueous extract of N.sativa seeds in comparison with the standard Thymoquinone (T- Standard Thymoquinone and A- Crude Aqueous extract)

Bioautography

In order to confirm the anti-candidal efficacy of the active component of crude extract, bioautography study was conducted. Figure 2b illustrates the growth inhibition of the *candida albicans* culture in and around the spot of the active compound in the thin layer chromatogram developed, which corroborates well with the zone of inhibition exerted by the crude extract.

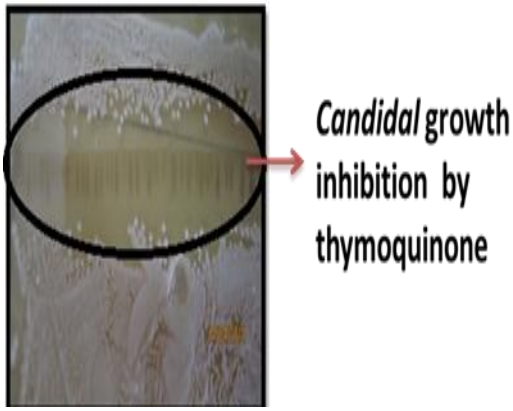

Fig. 2b Bioautography of crude extract against *Candida albicans.*

Formulation studies

Further to have the pure active component, the TLC spot was scraped and the compound was extracted with Dimethyl sulfoxide (DMSO) and formulated to microspheres by

emulsification method as described in the materials methods section. Fig. 3 depicts the morphology of the chitosan microspheres containing thymoquinone. The microspheres looks like grains and have free flowing nature.

Fig. 3a Morphology of Thymoquinone encapsulated chitosan microspheres

Instrumental Analysis of Microspheres

Scanning electron microscope (SEM)

The surface morphology of the microspheres was investigated by Scanning electron microscope (Fig. 3b)**.** The size of the microspheres was in the range of 8.36µm. The spheres have very smooth surface and spherical geometry due to high cross linking efficiency and it was homogeneously distributed. The SEM image does not show any aggregation of particle and there was no grafting of polymer in chitosan microspheres. This evidently proved that the thymoquinone loaded chitosan microspheres were suitable for controlled release drug delivery.

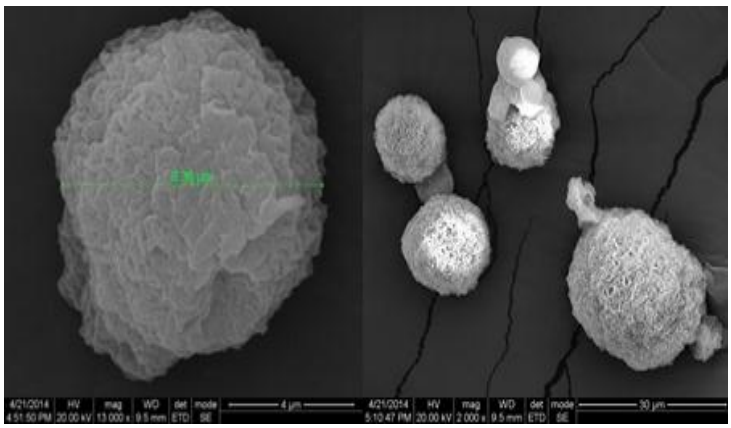

Fig. 3b SEM image of Thymoquinone encapsulated microspheres

Atomic force microscopy (AFM)

The surface morphology of Thymoquinone loaded microspheres were studied using AFM technique. Fig. 3c shows that the surface of the chitosan microspheres was smooth and in the 3D image, the peaks are evenly spread throughout the surface area.

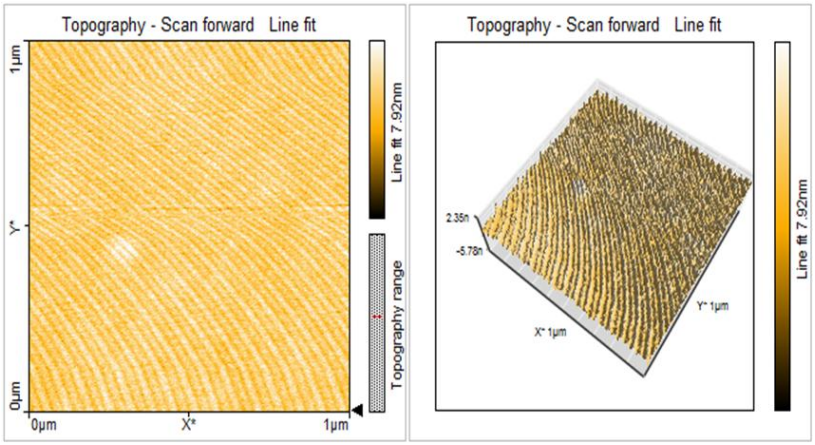

Fig. 3c Surface Morphology of single microsphere

FTIR- Fourier Transform Infrared spectroscopy

Fig. 4 depicts the FTIR spectrum of chitosan polymer, thymoquinone alone and the encapsulated thymoquinone microsphere. The peak values obtained for all the three samples shown in Table 1 for comparison. It has been observed that the IR spectrum of thymoquinone obtained in the present study matches with the spectrum reported by Pagola *et al.*, (2004)[16]. And the spectrum of TQ encapsulated microspheres showed the presence of functional groups responsible for C=O and CH_3 stretching vibrations. In addition the cyclic C-H stretching vibrations were also observed in the spectrum of chitosan microspheres. All these observations suggested the presence of TQ in the prepared chitosan microspheres.

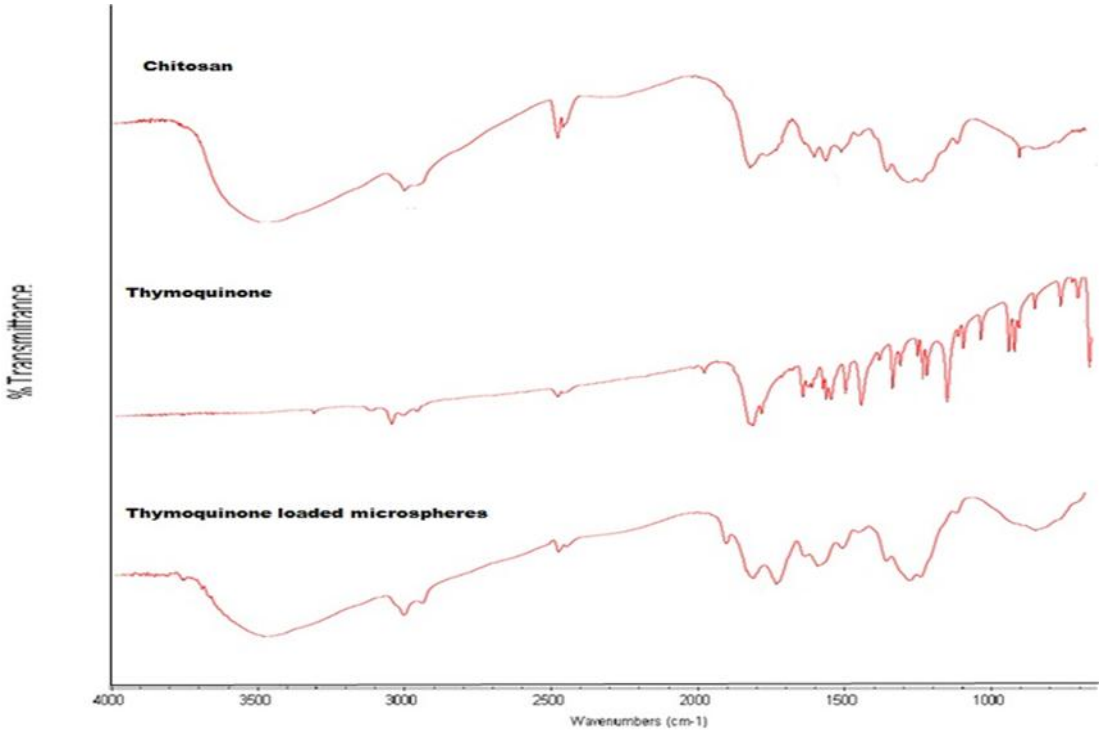

Fig. 4 FTIR spectrum of Chitosan alone, Thymoquinone alone and Thymoquinone encapsulated microspheres.

Table 1 Peak details of FT-IR spectra

Frequency(cm^{-1})	Chitosan (C)	Thymoquinone(TQ)	C+ TQ	Functional group
3400-3250	3423	3253	3436	N-Hstretch, secondary Primary amines,amides
3000-2850	2923	2968	2925	C-H stretch, alkanes
1760 -1690	1653	1646,1612	1643	C=Ostretch, carbonyls,carboxylic acids
1650-1580	1653	1646,1612	1643,1558	N-H bend, primary amines
1500-1400	1420	1461,1429.8	1405.5	C-C stretch, aromatics
1470 -1450		1461		C-H bend alkanes
1360 – 1290	1320	1388.1,1376.1,138.8, 1305.5	1316.8,1070. 4	N-O symmetric stretch, nitro compounds
1320-1000	1320	1305.5,1249,1181.3,1 133.9,1105.6,1041.1,1 024.5,1006.3	1316.8,1070. 4	C-O stretch alcohols, carboxylic acids, esters
950-910		933.4		O-H bend, carboxylic acid
850-550	668.4	872.6,809.3,705.8,687 .3,612	608.6	C-Cl stretch, alkyl halides
690-515		517.2		C-Br stretch, alkyl halides

Dynamic Light Scattering (DLS)

DLS analysis was carried out to determine the hydrodynamic diameter and size distribution of chitosan microspheres. The size distribution graph shown in Fig. 5 suggests the average particle size of the TQ encapsulated microsphere was in the 820.8 ± 119.5 nm. The size of the

microspheres was mainly influenced by TQ: Chitosan ratio. Minimal sized spheres were obtained in 1: 1 ratio, whereas, the sphere size increases with increase in TQ concentration. Whereas, in the nano formulation of TQ encapsulated chitosan nanoparticles the average size of the particle was reported as 150-200nm.[7]

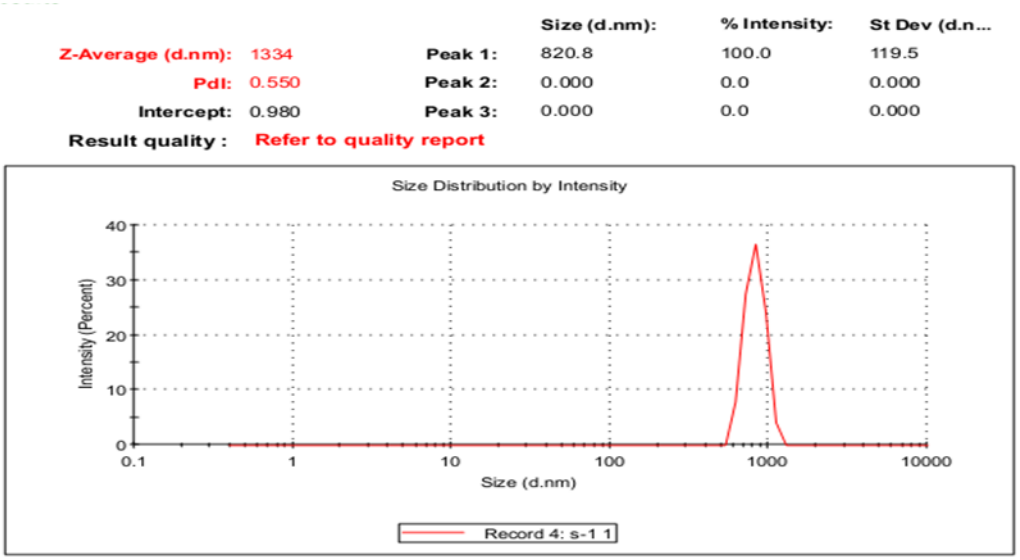

Fig. 5 Dynamic Light Scattering analysis of Thymoquinone encapsulated chitosan Microspheres

CHARACTERIZATION OF THYMOQUINONE MICROSPHERES

Percentage yield, drug loading efficiency, drug entrapment efficiency

The percentage yield of microspheres prepared by emulsification method was found to be 72.4%. The drug loading efficiency of the microsphere was calculated as 83% and the drug entrapment efficiency was determined as 76%. The yield of microspheres and loading efficiency was observed to be directly proportional to TQ: Chitosan ratio. The increase in polymer ratio showed a marked increase in size and yield of microspheres. Similarly, increase in TQ is reflected in low entrapment efficiency of microspheres due to the interaction of drug with polymer which hinders the attraction between the polymer and sebacic acid. This leads to increase in size and drug loading efficiency of microspheres. Alam *et al*., (2012),[7] reported that the drug entrapment and drug loading efficiency of TQ encapsulated chitosan nanoparticle was 63.3± 3.5 % and 31.23± 3.14, which was less than the percentage observed in the present study with chitosan microspheres.

***In vitro* drug release of thymoquinone microspheres**

The need of formulation is to extend the rate of release of the drug especially to the target site. There are many factors which play a key role in drug release pattern. Controlled drug release occurs when the polymer combines properly with the drug in such a way that the drug releases in a predesigned release pattern,[17] Chitosan microspheres dissolve faster in acidic media than the alkaline media.[18] Therefore, the study was carried out in Phosphate buffer (pH 7.4.) and the drug release profile shown in Fig. 6.

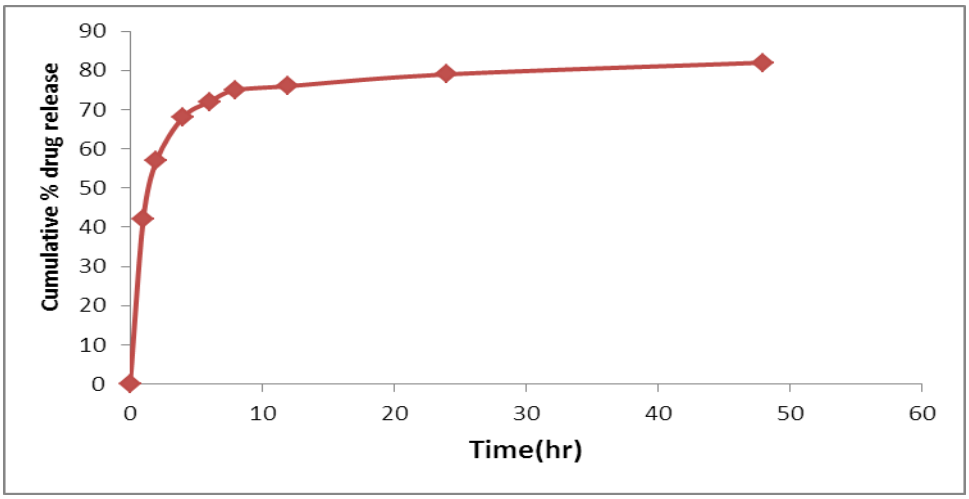

Fig. 6 *In-vitro* Thymoquinone release profile from microspheres

The release pattern of Thymoquinone from the microspheres, suggested that there was no rapid or burst release of thymoquinone initially. The release pattern was observed as slow and it was stabilized after 6hr. To investigate the mechanism of drug release, the release data was analysed using various models. Release kinetics of active compound loaded microspheres showed linearity with R^2 value near to unity (0.965) indicating the possibility of zero order kinetics.

CONCLUSIONS

The present study was designed to formulate the active component (Thymoquinone) from the crude extract of *Nigella sativa*. Formulation in the form of microspheres made using chitosan as a polymer and sebacic acid as a dissoluting agent as well as cross-linking agent. No toxic compounds are involved in the preparation of microspheres. The spheres were characterized by SEM, AFM, FTIR, DLS, mean particle size, swelling studies, Drug Entrapment efficiency, Drug loading and *in-vitro* release studies. The result of the study was satisfactory. The release profile showed slow and stable release after 6hrs which is suitable for controlled

drug release. Though nano particle formulation of TQ was in reports, the microsphere formulation presented in the study suggests the wide application of TQ in various therapeutic interventions.

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