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Entrapment of Seed Extract of *Nigella sativa* into Thermosensitive (NIPAAm–Co–VP) Co-Polymeric Micelles and its Antibacterial Activity

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ABSTRACT
Thermosensitive N-Isopropyl acryl amide-N-Vinyl 2-pyrollidone (NIPAAm-VP) co-polymeric micelle synthesized by radical copolymerization and the extract of *Nigella sativa* is entrapped in this polymeric system to check the release of the bioactive compound and evaluated its antibacterial activity. The size of nanoparticles was measured 75-110 nm and 1 % drug is entrapped. In this work, the seed extract has been loaded into the polymeric micelle and its effectiveness has been evaluated against gram positive strain of *Staphylococcus aureus. Bacillus subtilis* and a gram negative strain of *Escherichia coli*. *Nigella sativa* loaded polymeric micelles found hundred times efficient in comparison of the naked one. Drug release from the polymeric micellar system at 37-42^{o}C in different time intervals *i.e.* 30 min - 4 weeks is measured 7.14-66.66%. The above findings revealed that this thermosensitive polymeric system would more effectively release the drug into the body when infectious states are functional *i.e.* higher temperature and may have possibility to use as a novel drug delivery system for more herbal drugs for patient compliance.

Keywords: NIPAAm-VP co-polymeric nanoparticles, Thermosensitive micelles, *Nigella sativa*, *Staphylococcus aureus, Bacillus subtilis* antibacterial activity.

INTRODUCTION

Staphylococcus aureus, Bacillus subtilis and *Escherichia coli* are the most common cause of infections. These are Gram-positive and Gram-negative spherical bacteria and frequently found in the nose, skin and intestine of a person. It can cause a range of illnesses from minor skin infections, such as pimples, boils, cellulitis folliculitis, furuncles, carbuncles, nausea, diarrhea, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis endocarditis, Toxic shock syndrome (TSS), and septicemia. These infections that are not antibiotic resistant can be treated in about a month (depending on severity) using antibiotics.

For the decades herbal drugs have been believed to be providential for number of ailments and have been documented by physicians and patients for their improved therapeutic values as they have smaller number of adverse effects as compared with modern medicines. [1] It has previously been studied that *Nigella sativa* L. "black cumin", family Ranunculaceae, seeds has antimicrobial action and its activity against wild strains of *S. aureus*. [2] *Nigella sativa* has a pungent bitter taste and a faint smell of strawberries and is an annual herbaceous plant indigenous to Mediterranean region. It is used primarily in candies and liquors. In herbal medicine, *Nigella sativa* has anti-hypertensive, carminative, and anthelminthic properties.

It contains over 100 valuable nutrients including 21 % protein, 38 % carbohydrates and 35 % plant fats and oils. The active constituents of black seed are Thymoquinone, Nigellone, and fixed oils. Other ingredients comprise Linoleic acid, Oleic acid, Calcium, Potassium, Iron, Zinc, Magnesium, Selenium, Vitamin A, vitamin B, vitamin B2, Niacin, and Vitamin C. [3] Thin Layer Chromatography (TLC) screening of the oil samples from seeds have shown the presence of four main components, viz. thymoquinone, carvacrol, tanethole and 4-terpineol, which demonstrated radical scavenging property. These four constituents and the essential oil possessed variable antioxidant activity. [4]

Nigella sativa seeds contain 36-38 % fixed oils, proteins, alkaloids, saponins and 0.4-2.5 % essential oil. [5-6] The antioxidant, antibacterial and antifungal activities of spices

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and their derivatives have been investigated by some researchers. [7-8] Many bioactive properties have been attributed to black cumin seed, fixed oil and/or essential oil, including antibacterial [9], antifungal. [10]

There have been numerous ongoing researches on the effects of Black seed since 1959. [11-12] Glut of pharmacological actions of the crude extract have been reported against inflammation, allergy, asthma, infection, [13-14] nephrotoxicity, hepatotoxicity (induced by either disease or chemicals), [15] cancer [16] and diabetes. [17] Seeds of *Nigella sativa* have a long history of use for food and medicinal purposes. When given as oil no adverse or side effects have been reported if used within the recommended dosage, although dermatitis has been reported. In 1992 at the Department of Pharmacy, University of Dhaka, Bangladesh, scientists conducted a study in which the antibacterial activity of the volatile oil of black seed was compared with five antibiotics: ampicillin, tetracycline, cotrimoxazole, gentamicin, and nalidixic acid. The oil proved to be effective against many strains of bacteria, including those known to be highly resistant to drugs: *V. cholera, S. aureus*, *E. coli* (a common infectious agent found in undercooked meats).

One of the major problems that any drug faces is its nonspecific distribution throughout the body and creation of toxic effects. However, phytotherapeutics needs a scientific approach to deliver the components in a sustained manner to increase patient compliance and avoid repeated administration. This can be achieved by designing novel drug delivery systems for herbal constituents. The uncontrolled distribution and rapid clearance will diminish the total available concentration of drugs at their site of action reducing the chance of successful and effective treatment without causing undesired toxic effects. [18] However, modern phytopharmaceutical research solves the scientific needs for herbal medicines as in modern medicine, which gives way for developing novel formulations such as nanoparticles, microemulsion, matrix systems, solid dispersions, liposomes, solid lipid nanoparticles, and so on. [19] This article summarizes various drug delivery technologies for herbal actives, which are gaining more attention for better therapeutic response.

Developing a suitable delivery system for such an extract will cause more of the active constituent to be deposited into infectious state. Smaller size will increase its surface area for absorption. [20-21] More of the drug is expected to get into the blood in shorter period of time. [22] Nano sized delivery system, compared with those of micrometer size, can penetrate much deeper into tissue through the fine capillaries, crossing the fenestration present in the epithelial lining and generally taken up more efficiently by cells. [23-24] Nano sized delivery system was selected because of the minimum in the side effects, decrease in the dose of the drug and the concentration of the drug seems to persist at the sites for the longer periods. [25]

To overcome this problem, attempt has been made to formulate its controlled release formulation, to have supplementary effect on its properties. Targeted drug delivery with polymeric nanoparticles and hydrogels has been proven. [26-28] Poly (NIPAAm) has one of the interesting features among the polymers that it precipitates above lower critical solution temperature (LCST) at 32^{o}C. By addition of other co-monomers and surfactant, LCST of the NIPAAm becomes change and it precipitates at higher temperature work as a trigger to deliver the drug at infectious stage. [29-31] The most exciting opportunity in regulated drug delivery systems come through the way of stimuli responsive drug delivery systems which may help to deliver a drug *via.* implantable devices. [32-33]

One of Kalonji's outsized numbers of properties which has been exploited includes its antibacterial nature. In this work, the seed extract had been loaded into a thermosensitive polymeric micelle system [34] which was prepared keeping in mind its effectiveness during fever which is encountered due to pyrogenic effect of microorganisms and therefore control in the release of the drug when required. The drug was incorporated inside the hydrophobic core of the polymeric micelles and the formulation was then tested for its antibacterial properties.

EXPERIMENTAL PROTOCOL

General remarks

All chemicals and solvents used were of reagent grade. Solvents were dried and distilled before use according to the standard procedure. Redistilled and deionized water was used wherever it was needed. Methanol and chloroform were purchased from Merck Company. Ampicillin was purchased from Hi media (India). N-isopropylacrylamide (NIPAAm) from Across Organics (USA) re-crystallized with N-hexane at 40^{o} C and dried under vacuum, stored at 4^{o}C. N-vinyl 2-pyrrolidone (VP) was purchased from Across Organics (USA) and freshly distilled before use. N-N' methylene bis-acrylamide (MBA) was bought from Sigma (USA) and used directly. Ammonium per sulphate (APS) was bought from Hi media and ferrous ammonium sulphate (FAS) from Qualigens (India) of analytical grade and used directly. Sterile filter paper discs were purchased from Hi media (India). Yeast extract, glucose, tryptone and NaCl were purchased from Hi media.

Seeds of *Nigella sativa* (locally known as Kalonji) were procured locally.

Controlled strain of *Staphylococcus aureus* used in this study provided in the form of pure bacterial stock culture and was collected from the Department of pathology, Majeedia hospital, Hamdard Nagar, New Delhi, India. *Escherichia coli* DM 4100 *Bacillus subtilis* NCIM 2708 were bought from MTCC, Chandigarh, India. Bacterial isolates were maintained containing 15 % (vol/vol) glycerol at -20^{o}C then cultured and sub cultured in a modified Luria Bertani broth at 37^{o}C until the desired cell population was obtained.

Dynamic Light Scattering (DLS) And Zeta Potential- The particle size distribution and poly dispersion coefficients were determined by photon Correlation spectroscopy (PCS) in a Zeta sizer III (Malvern Instruments, Malvern, UK). At least four different batches were analyzed to give an average value and standard deviation for the particle diameter. Zeta potential was also determined in order to know the stability of the formulation so formed. In vitro release studies were performed at temperature of about 37±5°C.

Transmission Electron Microscopy- For the particle size and morphology determination TEM (Transmission electron microscope) study was also carried out in Phillips EM300 instrument in AIIMS, New Delhi. The NIPAAm-VP co-polymeric powder was dispersed in methanol and mounted on a carbon coated grid and after drying at room temperature loaded on the TEM.

Methods

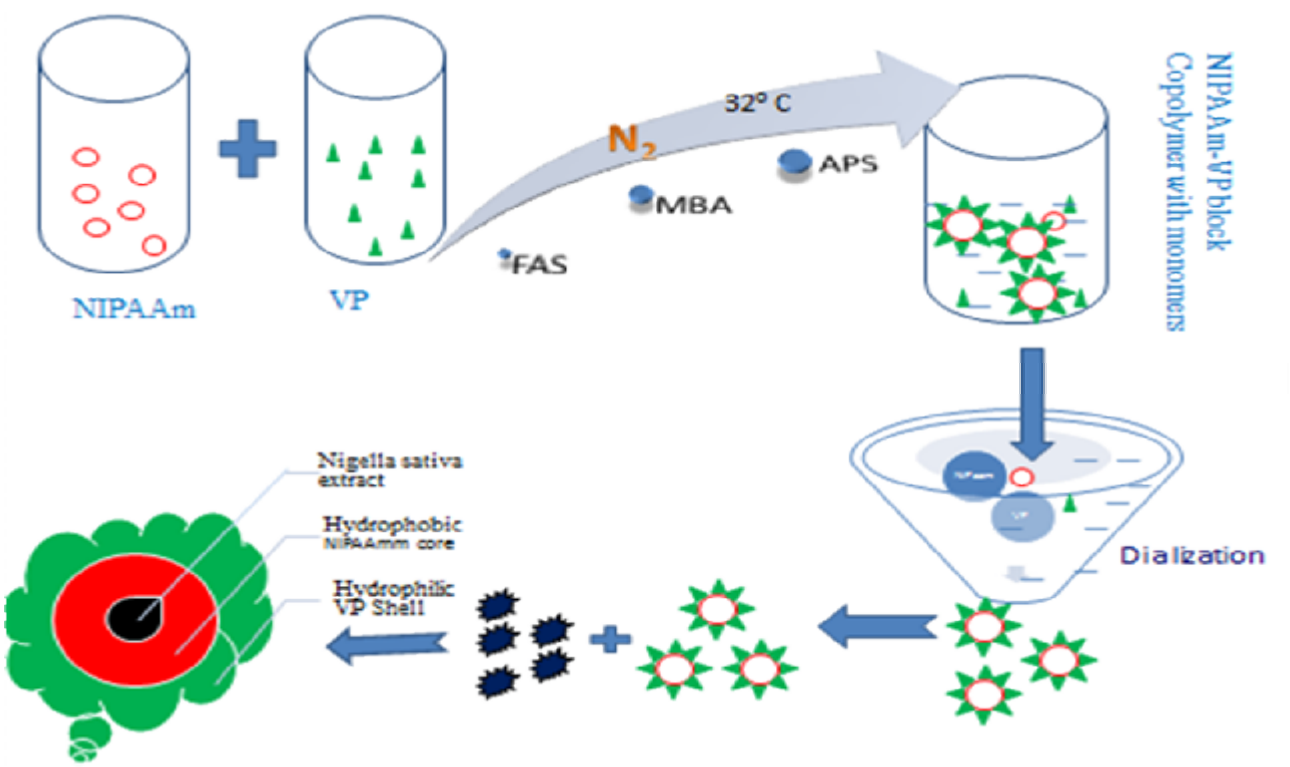

Fig. 1: Schematic representation of the synthesis of NIPAAm-VP co-polymeric micelles

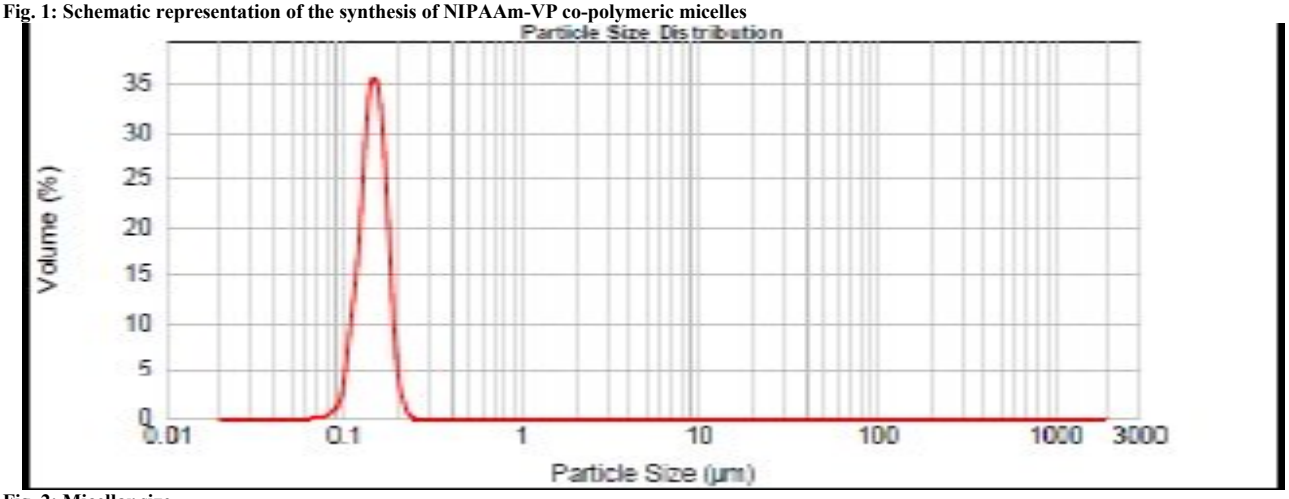

Fig. 2: Micellar size

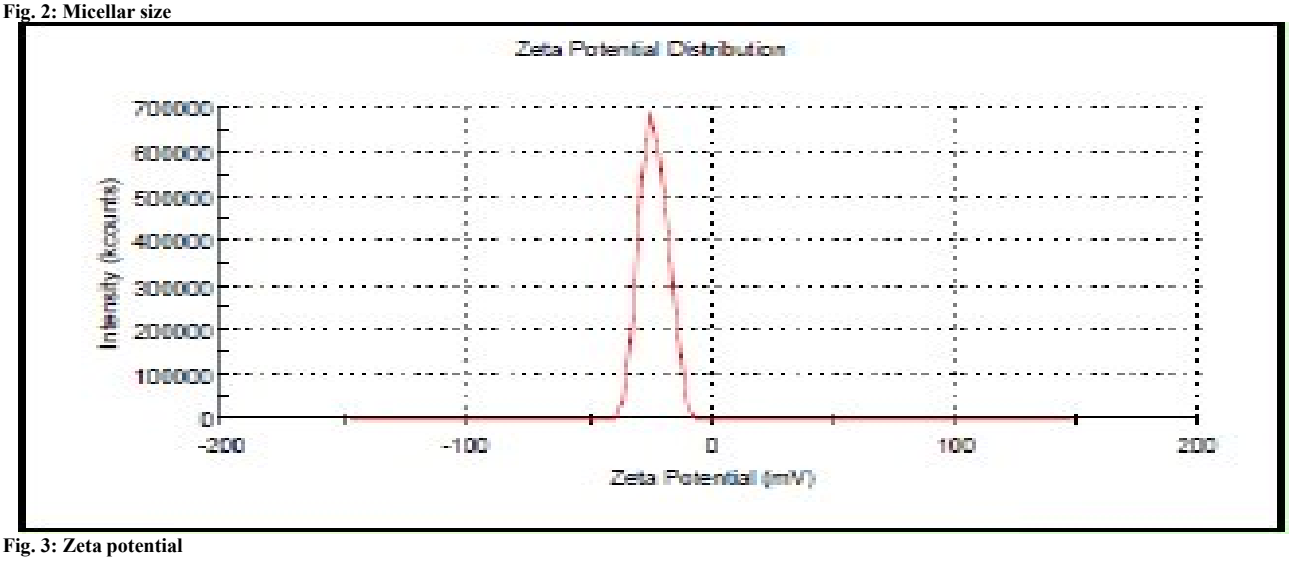

Fig. 3: Zeta potential

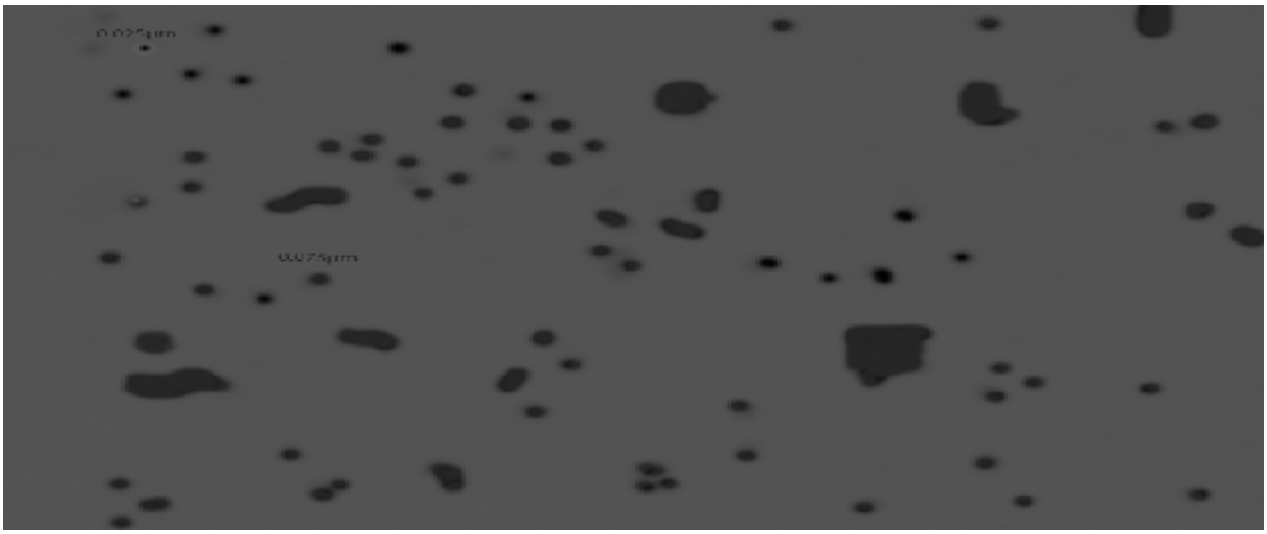

Fig. 4: Particle characterization by TEM before entrapping of extract

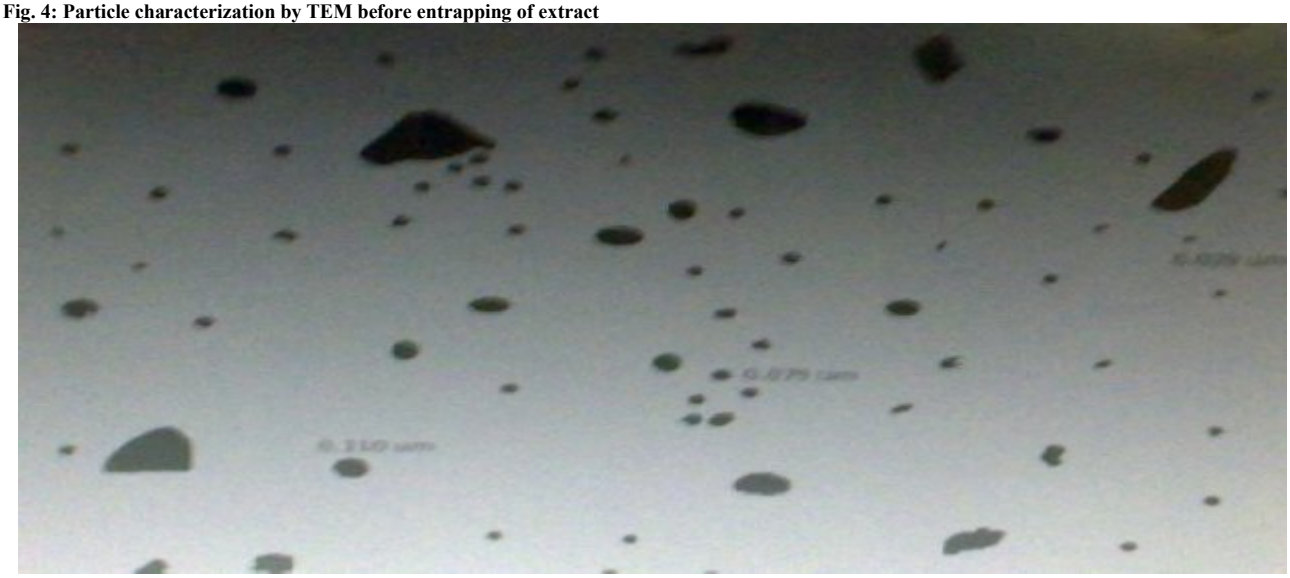

Fig. 5: Particle characterization by TEM after entrapping extract

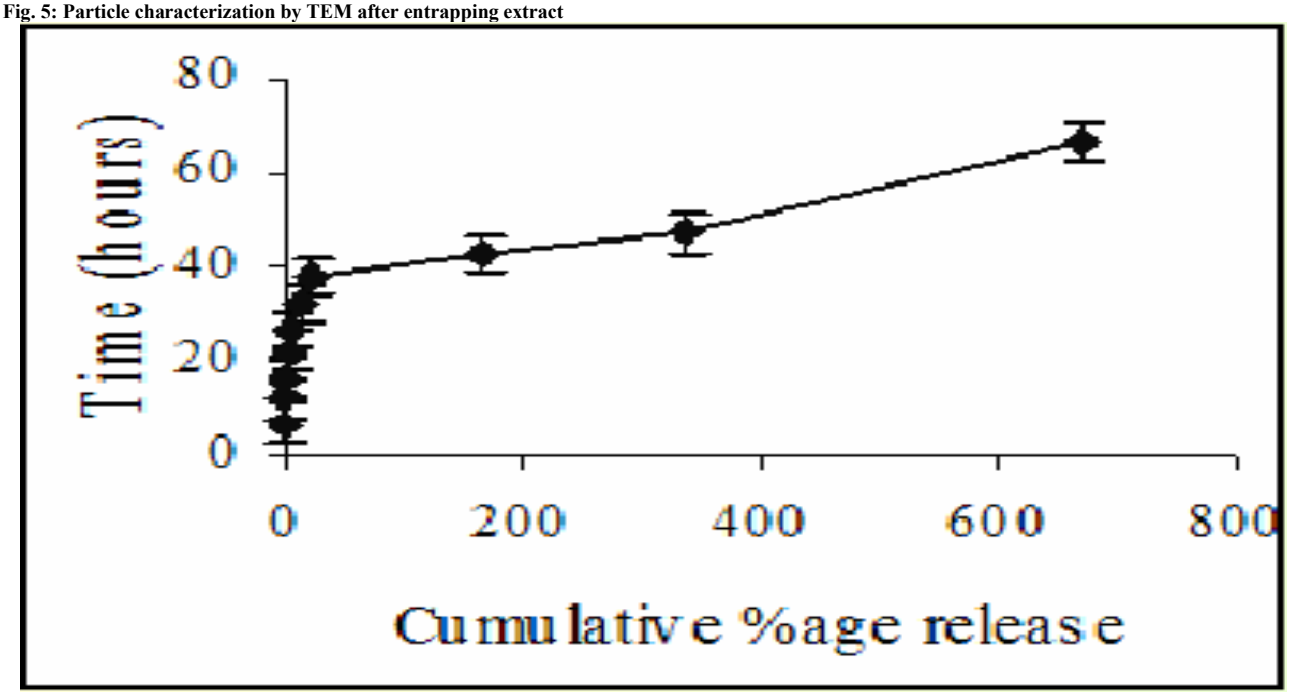

Fig. 6: *In vitro* release studies from the polymeric micelles

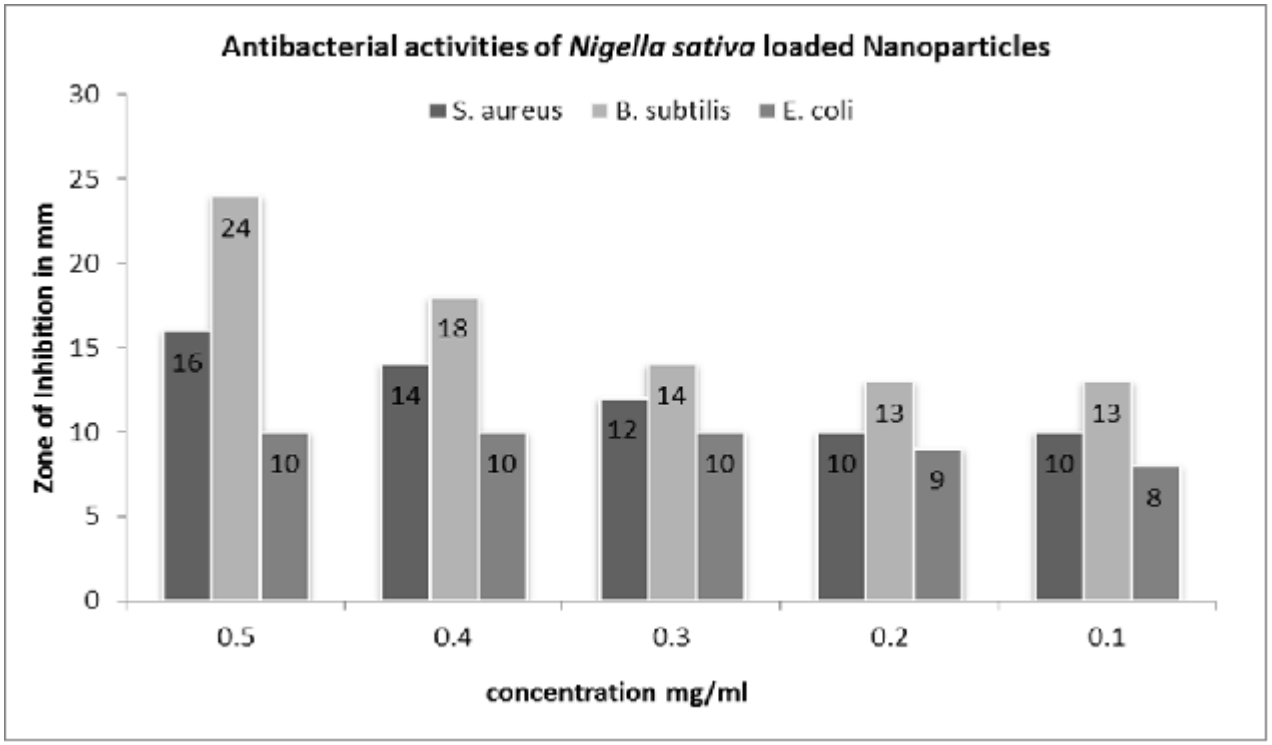

Fig. 7: Antibacterial activities of *Nigella sativa* loaded nano particles

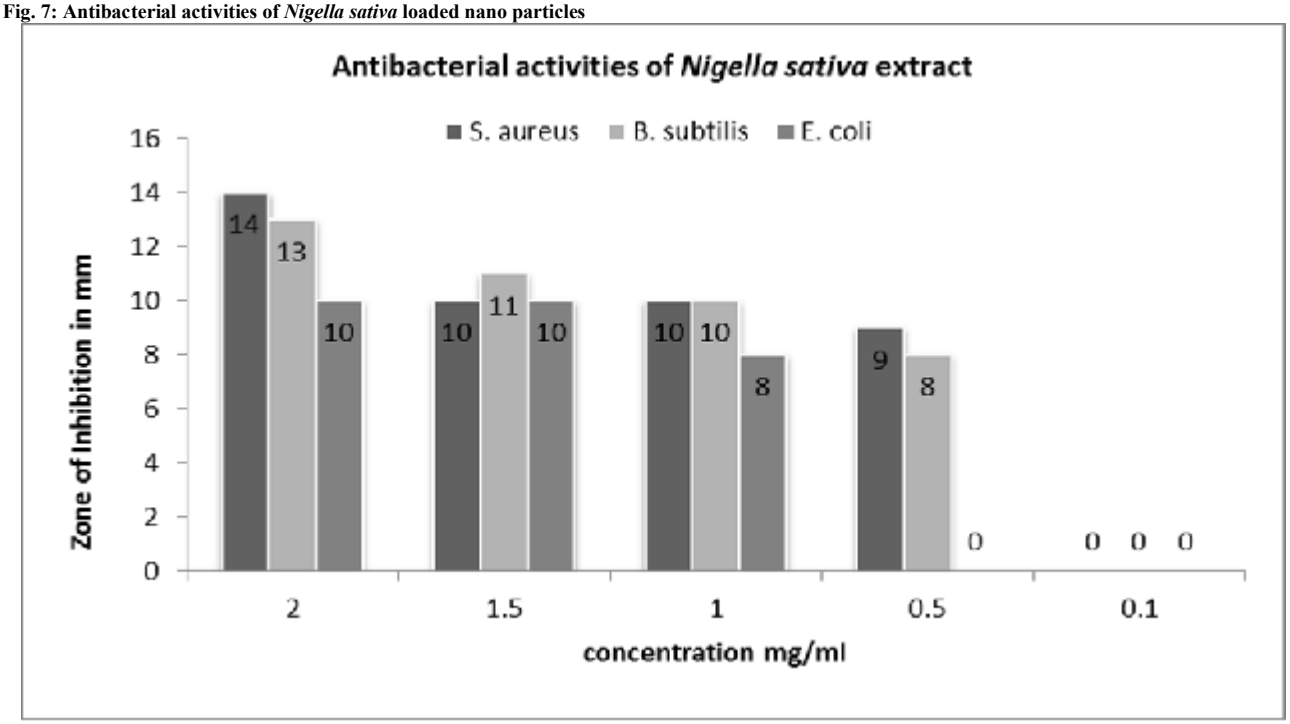

Fig. 8: Antibacterial activities of *Nigella sativa* extract

Preparation of Kalonji extract: The seeds of *Nigella sativa* washed thoroughly with water to remove dust, and dried in the air for one week under shade. These are grinded to fine powder. 125 gm of this powder is soaked in 125 ml of petrol in a 500 ml sterile flask, refluxed for 2 h and material so obtained is finally filtered. The product was kept in Petridis at room temperature for 4 days to allow the solvent to evaporate. The extract is ready to use.

Synthesis of polymeric micelles: In the present study, we synthesized NIPAAm-VP co-polymeric micelles using NIPAAm and VP monomers. For synthesis of co-polymeric micelles a reported methodology [35] is adopted under Nitrogen atmosphere at 32^{o}C temperature using ferrous ammonium sulfate (FAS) as initiator and ammonium per sulfate (APS) is added to activate the reaction as catalyst, respectively. Methylene bis-acrylamide (MBA) is also added to cross-link the polymer chain. After 8-10 h the reaction was terminated and synthesized solution was dialyzed using cellulose dialyzing membrane (12 kDa cut off). Finally, the polymeric solution is subjected to lyophilization and then a dry powder so obtained [Fig. 1].

Entrapment of extract into polymeric micelles

Drug was loaded into the micelles by simple dissolution. The 50 mg concentration of the particles was taken and dissolved into 10ml double distilled water. The drug 10 mg/ml was prepared in chloroform. The drug is gently added to the particles and vortexed the solution until the solution is cleared. 1 % loading is achieved.

Table 1: Cumulative percentage release of the drug with time

Time	Cumulative percentage release from micelles at temperature 37-42°C
30 Min	7.14 %
1 h	11.94 %
2 h	16.61 %
4 h	22.29 %
6 h	26.40 %
12 h	32.64 %
24 h	38.32 %
1 week	43.04 %
2 weeks	47.23 %
4 weeks	66.66 %

RESULTS AND DISCUSSION

Characterization studies

Size of the micelles so formed were estimated to be around 100-150 nm as depicted from [Fig. 2 Micellar size]. Zeta potential was determined to be about -20 mV which indicates the stability of the formulation so formed [Fig. 3 Zeta potential].

The surface morphology is determined and also size is measured using the TEM. TEM is carried out before entrapping and after entrapping of the extract. The micelles are porous and average size of 75 - 110 nm shown in [Fig. 4 and 5].

Cumulative percentage release of the drug from the micelles

In-vitro release studies were carried out on micellar systems, approximately 10 mg (weighed quantity) nanoparticles after lyophilization were suspended in 1 ml of phosphate buffer saline (pH 7.4, 50mM). They were placed in a beaker in horizontal position and kept in incubator shaker for the period of study (37°C, 200rpm). At predetermined time intervals, the supernatant was collected and fresh buffer solution was replenished at each sampling time. The supernatant was used to determine the amount of the released drug by taking absorbance at 760 nm.

From the In Vitro release studies we can infer was also observed that with increase in temperature the release in the amount of the drug was higher. There was very less or insignificant release at temperature below 37°C as shown [Fig. 6] and described [Table 1].

Pharmacology

Preparation of the drug impregnated filter paper disc

The drug impregnated filter paper discs were prepared to use. Whatman paper No. 1 filter paper discs were autoclaved at 121^{o}C for 20min. The stock solution (2.0 mg/ml) of the extract in DMSO was made and diluted 2.0, 1.5, 1.0 and 0.5 mg/ml and five dilutions of 0.1-0.5 mg/ml were prepared of extract loaded nanoparticles and reference drug were initially prepared by 0.1 mg/ml in DMSO. 10µl of extract and reference drug sample were kept gently on the filter paper disc of 6mm diameter, place on the Luria Bertani (LB) agar media plate while 0.5µl of extract loaded nanoparticles were placed on filter paper disc and then plates were inoculated with bacteria as described by Bauer and Kirby. [36]

Inoculation of plates

This was done by the modified method of Pelczar *et al* [37] using flood-inoculation technique. Bacterial suspension having OD equivalent 0.5 McFarland was freshly prepared and 0.6 ml of this was transferred onto the Luria Bertani Agar plate and distributed gently over surface of medium with gentle rocking. The excess fluid was removed from the plate and the plate was kept in incubator at 37ºC for 30 minutes for drying before application of discs.

Antimicrobial susceptibility testing

Antibacterial activity of the prepared formulation was investigated against the standard gram positive strain: *Staphylococcus aureus*, *Bacillus subtilis,* and gram negative *E. coli* by disc diffusion testing. This was carried out by placing discs impregnated with test material on surface of inoculated LB agar plates. The plates were then kept in incubator at 37°C for 18 hours and diameters of zones of inhibition were measured.

Clear inhibition zones unraveled that the compounds showed the antibacterial activity of the antibiotic discs against *S. aureus, Bacillus subtilis* and *E. coli.* Each plate had five discs having different concentrations of ampicillin, crude of *Nigella sativa* and drug loaded polymeric micelles. There is graphical representation of the activity of drug loaded nanoparticles is shown in [Fig. 7 and 8].

It was observed that controlled strain of gram positive *S. aureus, Bacillus subtilis* and *E. coli* were sensitive against crude as well as nano formulated extract of *Nigella sativa*. The antibacterial assay studies reveal that inhibition zone values of the drug are higher than of the crude. Even after hundred times dilution of crude extract, no marked decrease in zone of inhibition was observed for extract loaded nanoparticles. Zone of inhibition with standard ampicillin for *S. aureus, Bacillus subtilis* and *E. coli* were 16 mm, 18 mm and 11 mm, respectively. Extract loaded nanoparticles showed the high activity than naked extract and ampicillin. Zone of inhibition of extract loaded nanoparticles for *S. aureus, Bacillus subtilis* and *E. coli* were 16 mm, 24 mm and 10 mm at concentration 500 µg/ml and 10 mm, 13 mm and 8 mm was measured at 100 µg/ml concentration, respectively. While Zone of inhibition of naked extract was measured 14 mm, 13 mm and 10 mm at 2 mg/ml concentration, respectively. It was signifying that nano formulated drug is effective at much lesser concentration as compared to the crude extract. Its hundreds of times dose effectiveness.

In this research approach radical copolymerization of NIPAAm with VP results in the thermosensitive copolymers with hydrophobic/ hydrophilic micelles to deliver the drug at high body temperature to have a better control over the pharmacodynamic properties of the drug and showed does reduction at large scale. *Nigella sativa* has innumerable number of therapeutic properties. Bioassay which screens the antibacterial activity of *Nigella sativa* loaded nanoparticles against drug resistant *S. aureus*. An essential concept of *in vitro* susceptibility testing is the measurement of zone inhibition was measured and found more effective at low does. The effectiveness of such thermosensitive formulations unbolts the locks to the pathways to overcome the adverse effects of normally used various agents.

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