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Fabrication, physico-chemical characterization, and bioactivity evaluation of chitosan-linalool composite nano-matrix as innovative controlled release delivery system for food preservation

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ABSTRACT

The aim of the present study was to encapsulate linalool into chitosan nanocomposite (Nm-linalool) for developing novel controlled release delivery system in order to protect stored rice against fungal infestation, aflatoxin B_1 (AFB_1) contamination, and lipid peroxidation. The chitosan-linalool nanocomposite showed spherical shapes, smooth surface with monomodal distribution as revealed by SEM and AFM investigation. FTIR and XRD represented peak shifting and changes in degree of crystallinity after incorporation of linalool into chitosan nanocomposite. Nanoencapsulation of linalool showed higher zeta potential and lowered polydispersity index. TGA analysis reflected the stability of Nm-linalool with reduced weight loss at varying temperatures. Biphasic pattern, with initial rapid release followed by sustained release illustrated controlled delivery of linalool from chitosan nanocomposite, a prerequisite for shelf-life enhancement of stored food products. Chitosan nanocomposite incorporating linalool displayed prominent antifungal and antiaflatoxigenic activity during *in vitro* as well as *in situ* investigation in rice with improved antioxidant potentiality. Further, Nm-linalool displayed considerable reduction of lipid peroxidation in rice without exerting any adverse impact on organoleptic attributes. In conclusion, the investigation strengthens the application of chitosan-linalool nanocomposite as an innovative controlled nano-delivery system for its practical application as novel environmentally friendly eco-smart preservative in food and agricultural industries.

1. Introduction

Contamination of stored food commodities with fungi and their associated mycotoxins leading to oxidative biodeterioration and the safety challenges is a burning issue for food industries today [1]. United Nation Food and Agricultural Organization has reported the approximate wastage of 1.3 billion tons of food during preharvest and post-harvest conditions, resulting into huge loss of world agriculture economy ($2.6 trillion) every year. Rice (*Oryza sativa* L.) is considered as one of the prime staple food for two-third of the world's population and an important source of carbohydrates, minerals, fatty acids and dietary fibers, having key regulatory roles in cellular metabolism and promote several advantageous effects on health [2]. However, in the tropical and subtropical regions, the rice is severely contaminated with numbers of toxigenic fungi and mycotoxins during storage by increasing the free fatty acid contents and thereby, reducing the germination potentiality of seeds [3]. Among different species of food contaminating fungi, *Aspergillus flavus* has been paid immense importance due to its potentiality to secrete aflatoxin B_1 (AFB_1) which has been categorized under class 1 human carcinogen by International Agency for Research on Cancer (IARC) with widespread toxicity. Furthermore, the AFB_1 possesses broad spectrum toxico-pathological symptoms with genotoxicity, hepatotoxicity, carcinogenicity, immunotoxicity and teratogenicity, representing a potential threat and manifestation of cellular anomalies [4]. Therefore, the demand of innovative technologies has been currently forecasted to food industries to preserve the nutritional quality and improvement in shelf life of stored food products.

Application of synthetic preservatives has reflected multiple evidences to inhibit the fungal and mycotoxin contamination in stored foods, however, their practical utilization in the current times is discouraged due to broad-scale carcinogenicity, enhancement in reactive oxygen species (ROS) mediated damages of cellular biomolecules,

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development of resistant fungal strains, rapid migration into food components and negative impacts on environmental sustainability [5]. In this scenario, the utilization of plant products is reported as safer alternatives for improving the longevity of shelved food with outstanding antimicrobial, antimycotoxigenic, antioxidant and lipid oxidation suppressing potentiality. Among the diverse pool of higher plant secondary metabolites, essential oils and bioactive components exhibit advantageous bioefficacy against different food contaminating microbes in its vapor phase and makes them more attractive to the consumers based on their exemption of toxicity data from Environmental Protection Agency (EPA) and inclusion under Generally Recognized as Safe (GRAS) status [6]. Being complex mixtures of different components, the bioefficacy of essential oil is commonly regulated by synergistic and additive effects. However, the bioactive components of essential oils have renewed the attention towards food industries on the basis of their stable nature, promising antimicrobial efficacy and a common option for laboratory synthesis [7].

However, the direct application of essential oils and bioactive components as food preservative is practically not economical due to a) hydrophobic characters b) easy degradation in presence of temperature, air and light, c) less solubility, and d) high volatility [8] rendering decrease in long term bioefficacy for protection of stored food commodities. As a matter of the fact, there is an urgent need of viable strategy to protect the essential oil and control delivery with improvement in overall bioefficacy. In this context, nanoencapsulation of essential oil seems to be a novel and attractive approach for stored food preservation against the infestation of fungi and aflatoxin contamination with desirable significance including ease of handling, improved water solubility, enhanced bioavailability and reduced toxicity in food system [9]. Plenty of research has reported the application of different biopolymer *viz.* cellulose, agar, zein, starch, chitosan, casein, gelatin, xanthan, pullulan and polyhydroxy alkanoates (PHA) for encapsulation of essential oil components, however, the important point to select the biopolymer is reliable source, abundant nature and cost effectiveness together with advantageous effects on human health [10]. Among different inexhaustible biopolymeric matrix efficient for encapsulation of bioactive components, chitosan (deacetylation product of chitin) is largely sound to the food and pharmaceutical industries due to favorable characteristics such as biodegradability, biocompatibility, non-toxicity, inherent cationic nature, abundant in origin, low cost and extends the possibility for several polymeric conformations *viz.* powders, fibers, gels, capsules and films, a primordial requirement for efficient encapsulation [11]. A number of researches have been done for encapsulation of essential oil components in an appropriate delivery system, but, currently increasing attention on ionic gelation based formation of nanocomposite through energy intensive high-speed homogenization supported ionic interaction containing small oil droplets dispersed into continuous aqueous phase is due to better physical stability, controlled delivery and increase in antifungal activity [12]. Two different methods *viz.* high energy and low energy have been employed for development of nanocomposite, however, high energy method by applying high speed homogenization have been the major attraction to the food industries because of intense disruptive forces containing a number of oil droplets into dispersion medium with better stability, functional performance and low requirement [13].

Linalool (3,7-dimethyl-1,6-octadien-3-ol, $C_{10}H_{18}O$), an alcohol of monoterpene origin with tertiary and acyclic structures (linalyl alcohol), is recognized as major bioactive component of *Coriandrum. sativum*, *Lavandula angustifolia*, *Thymus vulgaris* and *Cinnamomum camphora* essential oils with significant anti-inflammatory, antimicrobial, analgesic, anti-hyperlipidemia, anticancer, antidepressant and neuroprotective activities [14]. Moreover, linalool has been successfully used in therapeutic experiments as neurotransmitter receptor having promising avenue in drug delivery [15]. Nevertheless, fragmentary literature is available on antifungal activity of linalool, however, up to now no research has been performed on encapsulation of linalool into chitosan biopolymer, analyses of physico-chemical characteristics, and enhancement in overall antifungal and antiaflatoxigenic bioefficacy for postharvest management of rice (the model food system) biodeterioration with special emphasis to preserve the nutritional quality and protection against lipid peroxidation. The idea conceptualized in the present investigation differs from previous reports whether entrapment of linalool into chitosan nanocomposite would be helpful in (a) development of controlled delivery system for protection of rice under storage conditions against fungal infestation, AFB_1 contamination, and reactive oxygen species (ROS) mediated lipid peroxidation and, (b) improving the bioactivity of encapsulated linalool.

Hence, in the present study, the objective was designed as encapsulation of linalool into chitosan biopolymer (Nm-linalool) to develop stable colloidal nanocomposite system, detailed physico-chemical characterization and bioactivity investigation of generated colloidal system in order to control the aflatoxigenic fungal infestation and AFB_1 secretion in stored rice (the model food system). Physico-chemical characterization of Nm-linalool nanocomposite was done through SEM, AFM, XRD, FTIR and TGA analyses. *In vitro* release study was performed to facilitate its usefulness in food protection by controlled volatilization for longer periods of time. Moreover, the efficacy of linalool entrapped into chitosan nanocomposite for *in situ* preservation of rice seeds against fungal proliferation, AFB_1 biosynthesis and lipid peroxidation in rice has also been explored. The result of the study would be helpful in developing novel controlled release delivery system of colloidal nature for utilization as nano-green eco-smart food preservative.

2. Materials and methods

2.1. Solvents and chemicals

Chitosan (CAS: 9012-76-4; molecular weight 200–300 KDa), methanol, toluene, Tween-80, Tween-20, chloroform, sucrose, isoamyl alcohol, yeast extract, $MgSO_4{\cdot}7H_2O$, KNO_3, HCl, sodium tripolyphosphate (S-TPP), thiobarbituric acid (TBA), acetonitrile, sodium hypochlorite (NaOCl), trichloroacetic acid (TCA), dichloromethane (DCM), ethyl acetate, potato dextrose agar, glacial acetic acid, DPPH and ABTS were purchased from Sisco Research Laboratories and HiMedia laboratories, Mumbai, India. Chemicals were not further purified before experiments. Milli q water was prepared in laboratory after passing the double distilled water through a filter (0.22 μm) to avoid microbial contamination.

2.2. Fungal strains

AFB_1 secreting strain of *Aspergillus flavus* (AF LHP R14) and other food biodeteriorating fungi *viz. A. luchuensis*, *A. candidus*, *A. versicolor*, *A. niger*, *A. repens*, Mycelia sterilia, *Fusarium oxysporum*, *Fusarium poae*, *Alternaria alternata*, *A. humicola* and *Cladosporium herbarum* infesting stored rice grains [16] were selected as the test fungal strains. Spore suspension of AF LHP R14 strain was prepared in double distilled water by using Tween-80 (0.1%) as surfactant.

2.3. Synthesis of linalool loaded chitosan colloidal nanocomposite (Nm-linalool)

Ionic gelation protocol optimized by Das et al. [17] was used for fabrication of linalool loaded chitosan colloidal nanocomposite (Nm-linalool). Solution of chitosan (1.5%) was developed in 1% glacial acetic acid by mechanical shaking overnight. Dropwise addition of Tween-80 was done to form the homogeneous solution and kept for 2 h over magnetic stirrer at 45 °C. Different amounts of linalool (0.00, 0.06, 0.12, 0.24, 0.48 and 0.60 g) mixed with 4 mL of DCM was added dropwise during high speed homogenization (IKA, T18ED, homogenizer, Germany) of the prepared solution at 12,000 ×*g* for 15 min. Thereafter,

0.4% S-TPP was added for facilitating the ionic cross-linking with chitosan to achieve nanocomposite followed by agitation in rotatory shaker (40 min). Further, centrifugation of prepared nanocomposite was done at 12,000 ×*g* for 30 min (4 °C) followed by collection of pellet. Subsequently, 10 mL distilled water was added into pellet to dissolve it properly followed by ultrasonication (total 4 min with 1 s pulse on and 1 s pulse off) (Sonics, Vibra Cell). Chitosan nanocomposite was prepared by the similar process without the addition of linalool. Immediately, the chitosan nanoparticle and Nm-linalool nanoparticle were developed after lyophilization (Lyophilizer, Alpha 1-2LD, Plus model, Sydney, Australia) of chitosan nanocomposite and Nm-linalool nanocomposite at −76 °C (96 h) for further physico-chemical characterizations. Nm-linalool nanocomposite was used for further bioefficacy testing.

2.4. Characterization of Nm-linalool nanocomposite

2.4.1. Particle size, zeta potential and polydispersity index

Particle size, zeta potential and polydispersity index of different ratios of chitosan to linalool were determined by dynamic light scattering (DLS) based zeta sizer (Zeta sizer, Malvern Instrument, UK).

2.4.2. Scanning electron microscopy (SEM)

Briefly, 0.5 mg of chitosan nanocomposite and Nm-linalool nanocomposite was suspended into 5 mL of milli q water. Five microlitres of the suspended particles were spread onto coverslip in front of sterile air. Coating of the samples was done in thin layer of gold by gold sputter and subjected to analysis in SEM (EVO-18 researcher, Zeiss).

2.4.3. Atomic force microscopy

0.5 mg of chitosan nanocomposite and Nm-linalool nanocomposite was mixed into 5 mL of milli q water and one drop of the prepared suspension was spread over small glass surface preparing a film at 25 ± 2 °C. The image was observed in atomic force microscope (CSM Instrument, ANTON PARR Tritec) having silicon probe cantilever and scan rate of 1 Hz.

2.4.4. ATR-Fourier transform infrared spectroscopy (FTIR) analysis

Samples *viz.* linalool, chitosan, chitosan nanocomposite and Nm-linalool nanocomposite for FTIR analysis were grinded within KBr and pressed them to develop disk. Analysis of the samples was done in Perkin Elmer FTIR between the wave number 400–4000 cm^{-1} with 16 scans and 4 cm^{-1} resolution.

2.4.5. X-ray diffraction (XRD) assay

XRD analyses of linalool, chitosan, chitosan nanocomposite and Nm-linalool nanocomposite were performed in Bruker D8 Advanced X-ray Diffraction Expert in between the 2θ range 5–50° having 0.02° min^{-1} step angle and scan speed of 5° min^{-1}.

2.4.6. Thermogravimetric assay (TGA)

Briefly, 10 mg of lyophilized samples were kept in TGA furnace containing platinum pan and analysis was done by Q50 TGA analyzer (TA, USA). Measurement was done between 20 and 600 °C with rate of heating 10 °C min^{-1}.

2.5. Estimation of loading capacity (LC), encapsulation efficiency (EE) and encapsulation yield (EY) of Nm-linalool

LC, EE and EY of Nm-linalool were determined spectrophotometrically based on the procedure of Chaudhari et al. [18] with slight changes. 500 μL of Nm-linalool was mixed with 5000 μL of ethyl acetate and centrifuged at 12,000 ×*g* for 14 min. The upper supernatant layer was filtered and absorbance of linalool was recorded at 274 nm. Amount was determined from standard curve of linalool ($Y = 0.0373X + 0.0245$, $R^2 = 0.9954$, $\lambda_{max} = 274$ nm) prepared in ethyl acetate. LC, EE and EY of Nm-linalool were quantified by the equations given below.

$$\%LC = \frac{M_{linalool}}{M_0} \times 100.$$

$M_{linalool}$ = total amount of loaded linalool
M_o = weight of nanocomposite

$$\%EE = \frac{M_{linalool}}{I_{linalool}}$$

$M_{linalool}$ = total amount of loaded linalool
$I_{linalool}$ = initial amount of linalool

$$\%EY = \frac{W_1}{W_2} \times 100$$

W_1 = weight of linalool loaded nanocomposite
W_2 = sum total of all the individual contents

2.6. In vitro release assay of Nm-linalool

Phosphate buffer saline (PBS, pH 7.4) and ethanol mixture was used for the determination of release of Nm-linalool. 300 μL of Nm-linalool was added into ethanol (3 mL) mixed PBS (2 mL) solution and vortexed at ambient temperature (2 min). At specific time intervals (0–168 h), Nm-linalool mixed ethanol and PBS was centrifuged (12,000 ×*g*, 8 min) followed by collection of supernatant (200 μL). Final volume of ethanol mixed PBS was maintained by addition of fresh solution mixture at each time interval. Amount of released linalool was measured spectrophotometrically at 274 nm under ambient temperature condition by using the calibration curve. Determination of cumulative release of linalool was done by the equation below.

$$\text{Cumulative release of linalool } (\%) = \sum_{t=0}^{t} \frac{M_0}{M_t} \times 100$$

M_0 = release of linalool at each sampling time
M_t = initial weight of linalool loaded into sample

2.7. In vitro testing of linalool and Nm-linalool for fungitoxic and AFB_1 inhibitory action

In vitro fungitoxicity assay of linalool and Nm-linalool was determined by adopting the procedure of Das et al. [19]. Briefly, different concentrations of linalool (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 μL/mL) and Nm-linalool (0.01, 0.02, 0.03, 0.04 and 0.05 μL/mL) were maintained into 24.5 mL of SMKY liquid medium containing 0.025 mL of AF LHP R14 spore suspension. Controls were prepared without the addition of linalool. Control for linalool was made by addition of 5% Tween-20 (500 μL) and SMKY media (25 mL), whereas, control for Nm-linalool was prepared by mixing chitosan nanocomposite (containing maximum concentration of chitosan) and 25 mL SMKY media. Control and treatment sets were left in B.O.D. incubator at 25 ± 2 °C for 10 days. Concentration of linalool and Nm-linalool representing maximum fungal growth inhibition was recorded as minimum inhibitory concentration (MIC). Broad range fungitoxicity of linalool and Nm-linalool against other biodeteriorating fungi such as *A. candidus*, *A. repens*, *A. versicolor*, *A. luchuensis*, *A. niger*, Mycelia sterilia, *Fusarium oxysporum*, *F. poae*, *Alternaria alternata*, *A. humicola* and *Cladosporium herbarum* was tested at MIC doses (0.7 and 0.05 μL/mL).

To test the AFB_1 suppressing potentiality, the media of control and treatment sets were extracted into chloroform (25 mL) followed by evaporation at 70 °C (4 h) over water bath. 1 mL of cold methanol was added into left material and 100 μL was spotted in thin layer chromatographic plate followed by development in solution mixture having

toluene (90 mL), methanol (32 mL) and isoamyl alcohol (2 mL) solution. The fluorescent spots as observed in trans-illuminator were dispensed with 10 mL of cold methanol and further centrifuged (3000 ×*g* for 5 min). Absorbance of the supernatant was recorded at 360 nm. Content of AFB_1 was calculated by the equation below.

$$AFB_1 \text{ content } (\mu g/mL) = \frac{D \times M}{E \times L} \times 1000$$

D = absorbance of sample at 360 nm; M = Molecular weight of AFB_1 (312);
E = molar extinction coefficient (21,800); L = Path length (1 cm)

Concentration of linalool and Nm-linalool representing maximum suppression (100%) of AFB_1 production were presented as minimum aflatoxin inhibitory concentration (MAIC).

2.8. Antioxidant activity

Antioxidant activity of linalool and Nm-linalool was performed through DPPH• and $ABTS^{\bullet+}$ assay. For determination of DPPH• radical sequestration activity, different amounts of linalool and Nm-linalool was incorporated with 5 mL 0.004% methanolic DPPH solution and kept at 25 ± 2 °C for 30 min in dark. The absorbance of the reaction mixture was recorded at 517 nm. Percent antioxidant activity (IC_{50}, 50% radical scavenging potentiality) was quantified by using the equation below.

$$\%\text{Antioxidant activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

$A_{control}$ = absorbance of methanolic DPPH solution;
A_{sample} = absorbance of the sample

For $ABTS^{\bullet+}$ assay, the different amounts of samples were dissolved into 1900 μL of $ABTS^{\bullet+}$ reaction mixture containing 7000 μM $ABTS^{\bullet+}$ and 140,000 μM $K_2S_2O_8$ and kept in dark for 6 min (ambient temperature). Fresh DPPH• and $ABTS^{\bullet+}$ solution served as control for the experiment. Absorbance of the reaction mixture was measured at 734 nm. IC_{50} was determined by using the same equation of DPPH assay.

2.9. *In situ* evaluation of linalool and Nm-linalool as antifungal and AFB_1 suppressor in food system: rice (*Oryza sativa L.*) as model food

In the present course of investigation, rice was selected as the model food system to study the *in situ* efficacy because rice is as one of the important staple foods throughout the world and possesses variety of components *viz.* carbohydrates, fats, proteins, fatty acids, minerals and macronutrients [20] which provide frequent contamination by food spoilage fungi (*A. flavus* has excessive infesting potentiality) resulting into secretion of aflatoxins, which have been recognized hazardous for animals and humans and constitute a prime factor for declining the economy of food products [21]. Moreover, AFB_1 contamination in rice induces the secretion of aflatoxin M_1 (AFM_1) representing severe poisoning of baby foods with resultant teratogenicity, carcinogenicity and hepatotoxicity. Therefore, the *in situ* investigation of linalool and Nm-linalool in stored rice seeds is considered to be a prime parameter of the present study. 200 g of rice seeds were stored in plastic container and fumigated with MIC and 2 MIC doses of linalool and Nm-linalool. Control was prepared without linalool and Nm-linalool fumigation. Two different types of control and fumigated sets *viz.* inoculated (addition of AF LHP R14 spore suspension) and uninoculated (without AF LHP R14 spore suspension) were prepared. Both the sets were kept in storage chamber at 25–27 °C and RH ~ 70%. Thereafter, the efficacy of linalool and Nm-linalool was tested for suppression of fungal association and AFB_1 synthesis.

For the assessment of *in situ* efficacy, surface sterilization of rice seeds was done in 1% NaOCl followed by washing of seeds with distilled water. One gram of grinded rice seeds (control and differently fumigated) was mixed with 5000 μL of milli q water followed by serial dilution in potato dextrose agar medium for mycoflora analysis. Inhibition efficiency of linalool and Nm-linalool against fungal proliferation was determined using the equation below.

$$\%\text{Protection} = \frac{F_c - F_t}{F_c} \times 100$$

F_c = number of fungal colonies in control;
F_t = number of fungal colonies in treatment

Determination of AFB_1 inhibitory efficacy of linalool and Nm-linalool in rice was based on the HPLC mediated protocol of Sheijooni-Fumani et al. [22]. 5 g of well milled rice seeds were added into aqueous methanolic solution (8:10 *v*/v) followed by incubation in mechanical shaker at 400 ×*g* for 30 min. Then, seed samples were centrifuged (5000 ×*g* for 10 min) and the supernatant was subjected to mixing with KBr mixed water (6 mL) and chloroform followed by centrifugation again. Collected lower portion was evaporated in water bath under steam of nitrogen. 1 mL of methanol was mixed with settled portion followed by injection of 5 μL into the HPLC system containing C-18 reverse phase column (250 mm × 4.6 mm i.d. × 5 μm) using the solvent mixture of methanol, acetonitrile and water (17:19:64 *v*/v/v) having the flow rate of 1.2 mL/min. Calibration curve of AFB_1 was developed in between the range of 12.5–500 ng/50 μL. Amount of AFB_1 in rice was measured through photo diode array (PDA) detector and expressed as μg/kg of rice.

2.10. Determination of lipid peroxidation in rice

Briefly, one gram of rice seeds (both fumigated and non-fumigated) was digested in TBA reagent containing 20% TCA, 2.5 N HCl and 0.5% TBA followed by heating over waterbath (85 °C for 30 min). Thereafter, the samples were cooled by running water, centrifuged at 2000 ×*g* for 5 min and collection of supernatant was performed. Absorbance of the supernatant was recorded at 532 and 600 nm. Control was prepared without addition of rice samples. MDA equivalents of rice samples were calculated through molar extinction coefficient 1.56 × 10^5 M^{-1} cm^{-1} and expressed in terms of μM/g FW of rice.

2.11. Sensory analysis

Sensory analysis of rice seeds for four different organoleptic properties *viz.* color, odor, texture and flavor were done after linalool and Nm-linalool fumigation by using the hedonic test. Five point hedonic scale (1; dislike extremely, 2; dislike moderately, 3; neither like nor dislike, 4; moderately like, 5; extremely like) was applied to judge the organoleptic attributes by 10 different unskilled panelists of Botany Department, Banaras Hindu University, India and scored the overall acceptance.

2.12. Statistical analyses

The experiments were performed in the form of three biological replications followed by representation of data sets as mean ± standard error format. One way analysis of variance (ANOVA) was accomplished with 95% confidence level ($P < 0.05$) using SPSS 23.0 (SPSS., Inc., Chicago, IL, USA) software.

3. Results and discussion

3.1. Fabrication and characterization of chitosan encapsulated linalool colloidal nanocomposite (Nm-linalool)

In the present investigation, encompassment of linalool into chitosan nanomatrix was performed by ionic gelation mediated electrostatic interaction between cationic amino groups of chitosan and anionic phosphate group of S-TPP. Chitosan has the proven merits to be used as polymeric matrix for effective encapsulation of essential oil components based on its biodegradability, biocompatibility, controlled delivery, ability to form small size particles, high absorptive capacity, ease of application and low cost [23]. In addition, the lack of toxicity and inclusion under GRAS category (both linalool and chitosan) develop a frontier avenue for practical applications of chitosan entrapped nano-encapsulated linalool in food and agricultural industries. Although, the chitosan may suffer significant alteration in its chemical structure during interaction with S-TPP and linalool molecule and use of Tween-80 as surfactant had the purpose to stabilize the composite particles and achieve small sized homogeneous nanocomposite after subsequent high speed homogenization and sonication. Different ratios of linalool and chitosan (1:0, 1:0.2, 1:0.4, 1:0.6, 1:0.8, 1:1) were prepared to optimize the higher encapsulation efficiency and loading capacity with smaller size nanocomposite particles and greater system stability for applications in food and allied sectors.

3.1.1. Particle size, zeta potential and polydispersity index (PDI)

Particle size, zeta potential and polydispersity index of prepared nanocomposite of different chitosan to linalool (w/v) ratio was measured through DLS based zeta sizer. Average particle diameter of chitosan nanocomposite was 22.2 nm (Table 1), while the particle size was increased at higher ratios of chitosan to linalool which may be ascribed to interaction of linalool with chitosan biopolymer and entrapment as core material within polymer matrix. Our observations are in corroboration with Zhang et al. [24] during incorporation of *Paulownia tomentosa* essential oil into chitosan nanoparticle. They demonstrated the average size of the essential oil loaded nanoparticle as 185 nm, however, our result illustrated very small particle size ranging between 22.2 and 80.1 nm (Table 1). Small sized Nm-linalool nanocomposite have greater surface to volume ratio and facilitate the targeted as well as controlled delivery with desired application in food system by checking the biodeterioration process.

Zeta potential is a measure of surface charge density, electrostatic attraction/repulsion in nanocomposite system and thus ensures the stability of the prepared nanocomposite. Maximum positive value of zeta potential (+51.2) was observed for chitosan nanocomposite. In case of Nm-linalool nanocomposite, the zeta potential ranged between +45.8 to +12.8 (Table 1). With increasing concentrations of linalool, the zeta potential value was decreased significantly. Zeta potential value ± 30 mV has been considered as stable nanocomposite because of less sensitivity to destabilization and agglomeration reactions such as Brownian motion, Van der Waal's forces and particle interactions [25]. In this study, strong electrostatic repulsion between the dispersed composite particles represented the greater positive value of zeta potential with better stability. Higher positive value of chitosan nanocomposite might be associated with protonation of amino groups of chitosan. Decline in zeta potential of Nm-linalool nanocomposite may be due to armor effect of positive charges and adsorption of linalool on the surface of chitosan nanocomposite ensured the masking of chitosan free amino group [9]. The positive zeta potential value has also been demonstrated by Sotelo-Boyás et al. [26] and López-Meneses et al. [27] while working on encapsulation of lime and *Schinus molle* essential oil into chitosan nanoparticles. The chemical profile of essential oil, characteristics of polymeric matrix and fabrication process may be a responsible factor for variation in zeta potential values. Greater positive zeta potential demonstrated the application of small size Nm-linalool nanocomposite in food system with effective stability.

PDI demonstrates the distribution of particle size in nanocomposite system. Lower value of PDI suggests homogeneity in particle distribution and reflects the desirable uniformity in diameter. Results displayed PDI of nanocomposite in between 0.413 and 0.213 (Table 1) indicating stable as well as mono-dispersed particles having uniformity in distribution. Similar result in PDI values between 0.117 and 0.337 has been recently reported by Hadidi et al. [28]. Hence, the uniform size and stability of Nm-linalool nanocomposite maximize their application as nano-based smart green preservative against fungal and aflatoxin mediated biodeterioration of stored food commodities.

Table 1
Particle size, zeta potential and polydispersity index of Nm-linalool nanocomposite.

Chitosan/linalool (w/v)	Particle size (nm)	Zeta potential (mV)	Polydispersity index
1:0.0	22.2 ± 2.6^{a}	$+51.2 \pm 7.2^{a}$	0.413 ± 0.087^{a}
1:0.2	39.8 ± 3.9^{b}	$+45.8 \pm 10.6^{b}$	0.389 ± 0.064^{ab}
1:0.4	51.4 ± 7.1^{c}	$+39.6 \pm 8.2^{c}$	0.348 ± 0.094^{b}
1:0.6	67.8 ± 5.6^{d}	$+27.2 \pm 4.9^{d}$	0.287 ± 0.026^{c}
1:0.8	72.2 ± 10.8^{e}	$+19.6 \pm 3.2^{e}$	0.249 ± 0.051^{d}
1:1	80.1 ± 11.3^{f}	$+12.8 \pm 2.8^{f}$	0.213 ± 0.011^{e}

Note: - = not determined, values are mean (n = 3) ± SE, the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

3.1.2. SEM analysis

SEM analyses of chitosan nanocomposite and Nm-linalool nanocomposite exhibited spherical shapes, smooth topology with monomodal distribution. Size of chitosan nanocomposite ranged between 26.20 and 37.06 nm, however, expansion in the size (52.59–83.44 nm) was observed after loading of linalool into the chitosan nanomatrix (Fig. 1A, B). Similar ranges of colloidal particle size were confirmed by DLS based zeta sizer analysis. Somewhat differences in the size range may be attributed to the sensitivity of different instruments. Higher particle size as observed in SEM as compared to the DLS analysis may be due to the loss of stability of nanocomposite after lyophilization and somewhat particle aggregation, whereas in case of DLS, analysis of finely diluted composite particle samples were done for preventing their agglomeration or interactions [29]. Enhancement in size of Nm-linalool nanocomposite might be associated with entrapment of linalool within the chitosan matrix reinforced by nanostructural compaction. Our result is consistent with the previous studies of Kavaz et al. [30] and Hosseini et al. [31] with expansion in size of nanoparticles after loading of *Cyperus articulatus* and *Origanum vulgare* essential oil into chitosan biopolymer. However, it was critically observed in our study that the hydrodynamic diameter of Nm-linalool nanocomposite was smaller in size than the previous investigation suggesting the successfulness of the utilized process and ensured the application of Nm-linalool nanocomposite as novel innovative nano-delivery system in the phases and inter-phases of the stored food items where the fungal cells are easily proliferated and secrete aflatoxins.

3.1.3. AFM observation

AFM microphotograph displays the two and three dimensional image of chitosan nanocomposite and Nm-linalool nanocomposite. The mere spherical shape, smooth surface and nano range size of both chitosan nanocomposite and Nm-linalool nanocomposite was confirmed by AFM (Fig. 1 C, D, E, F). The distribution of size was in the range of 25–75 nm. The Nm-linalool nanocomposite was larger in size than the chitosan nanocomposite which may be due to the efficient entrapment of linalool within chitosan nanomatrix. Variation in size of nanocomposite particles could be associated with the differential sensitivity of the instruments and dry state of samples for analysis, whereas in DLS, aqueous solution was observed. Our result is consistent with the previous investigation of

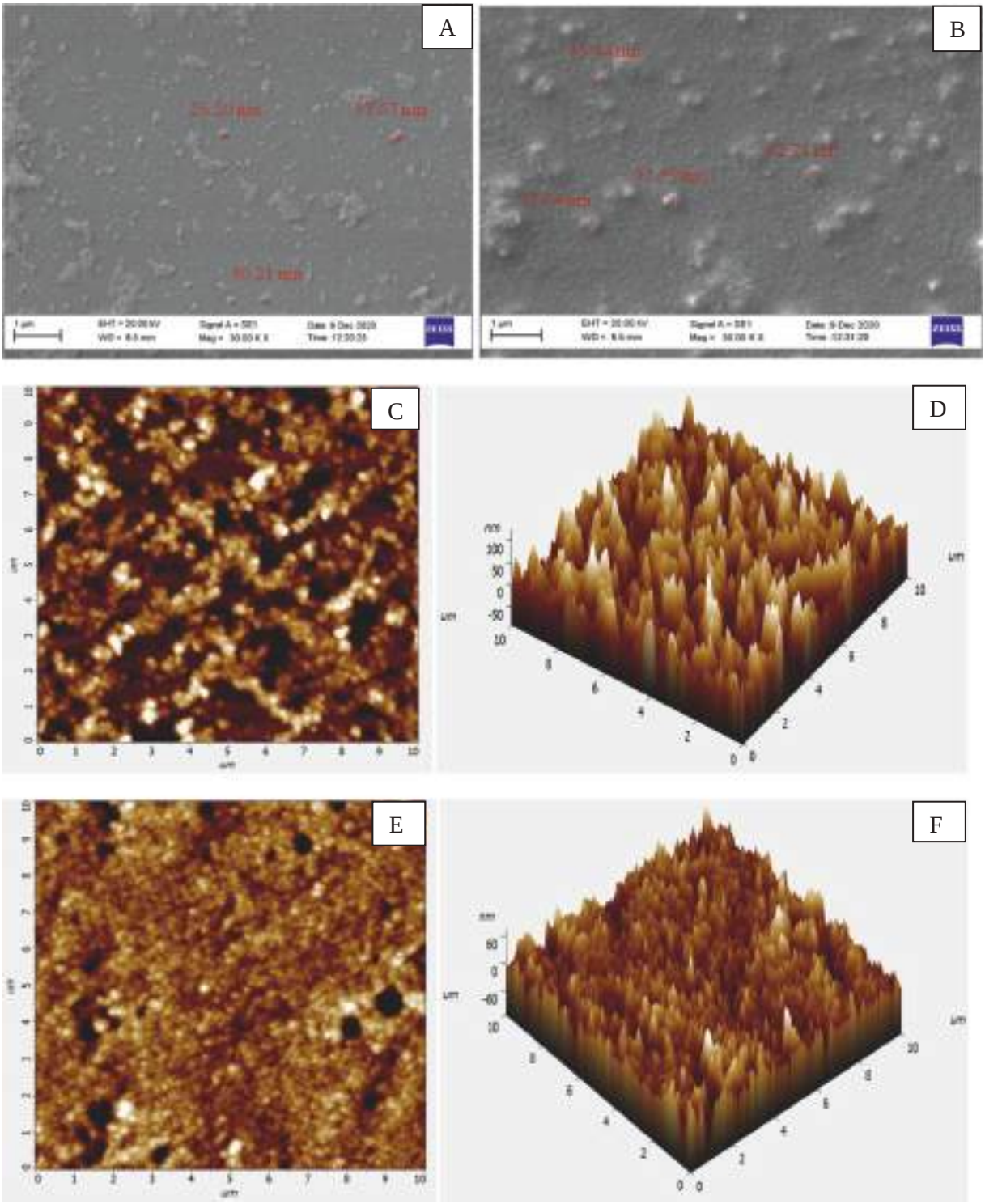

Fig. 1. (A) SEM analyses of chitosan nanocomposite (B) SEM analysis of Nm-linalool nanocomposite (C) 2D AFM image of chitosan nanocomposite (D) 3D AFM image of chitosan nanocomposite (E) 2D AFM image of Nm-linalool nanocomposite (F) 3D AFM image of Nm-linalool nanocomposite (G) FTIR analyses of chitosan, chitosan nanocomposite, linalool and Nm-linalool nanocomposite (H) XRD analyses of chitosan, chitosan nanocomposite, and Nm-linalool nanocomposite (I) TGA analyses of chitosan, linalool and Nm-linalool nanocomposite.

Ghaderi-Ghahfarokhi et al. [32] for encapsulation of cinnamon essential oil into chitosan nanobiopolymer. They suggested the average size of the synthesized nanoparticles as 235.6 nm, however, our investigation demonstrated very small size of nanocomposite particle suggesting higher intake of bioactive component into chitosan nano-carrier with greater surface area to volume ratio, a prerequisite for controlled volatilization and targeted delivery in stored food system.

3.1.4. Molecular interaction by ATR-FTIR analysis

FTIR analyses of chitosan, chitosan nanocomposite, linalool and Nm-

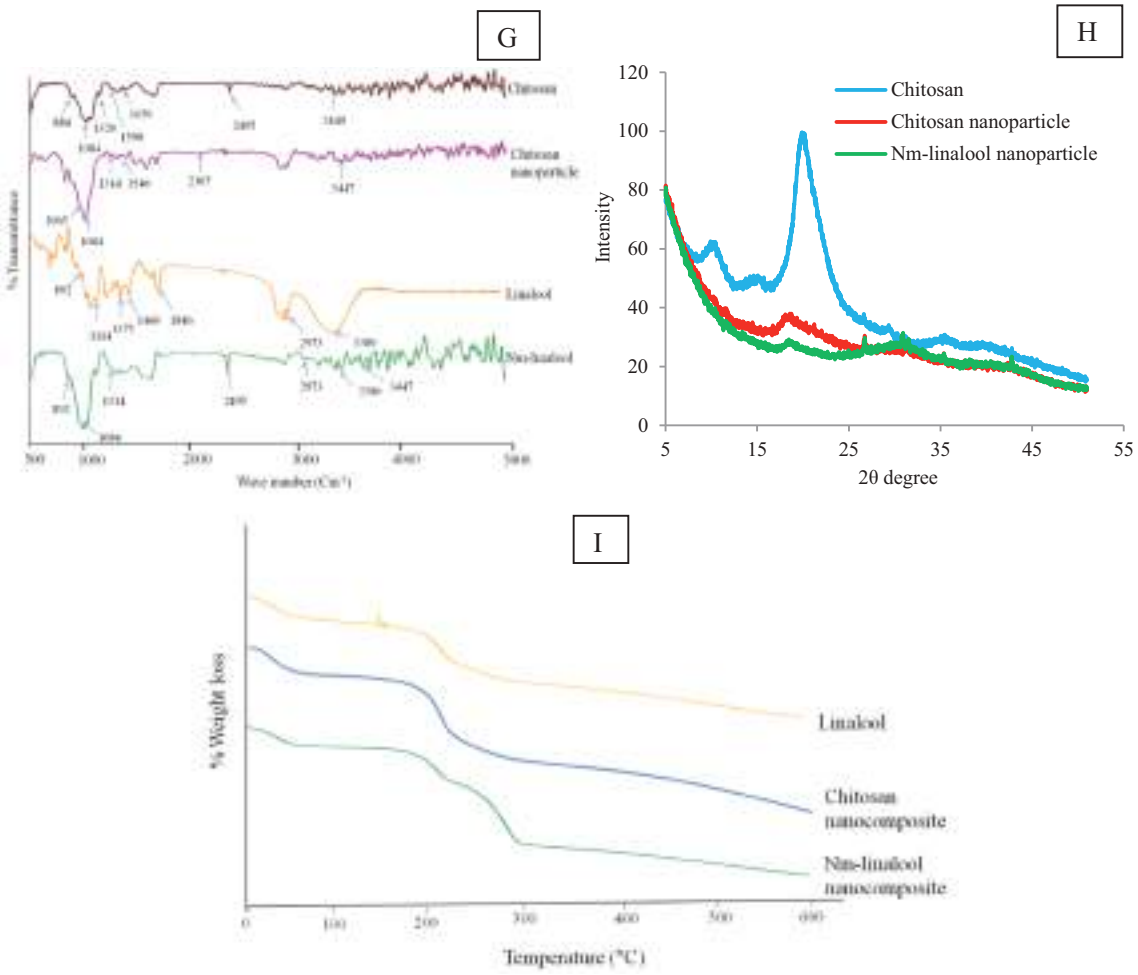

Fig. 1. (*continued*).

linalool nanocomposite are presented in Fig. 1 G. Arrangement of peaks, their intensity and rearrangements as evident through FTIR indicated the effective encapsulation of linalool into chitosan nanocomposite and possible molecular interaction. Chitosan displayed specific peaks at 884 (pyranoside ring stretching), 1084 (C—O—C stretching), 1320 (amide III), 1380 (C—O—H, H—C—H), 1580 (curvature of —NH_2), 1590 (—NH bending and amide II), 1650 (amide II), 2870 (C—H), and 3440 (—OH and —NH stretching) cm^{-1} [33]. In case of chitosan nanocomposite, absorption band at 1314 (P═O), 1084 (C—O—C stretching), 1540 (amide II), 2165 (—CH stretching) and 3447 (—OH stretching) cm^{-1} were observed. Band of 1580 cm^{-1} was less evident in chitosan nanocomposite which was related to the utilization of —NH_2 curvature during cross linking with tripolyphosphate of S-TPP as evident by formation of new peak at 1314 (P═O). Linalool exhibited a number absorption bands at 3380 (alcohol), 2973 (—C—H stretch), 2390 (—C—H alkane group), 1846 (—C—H aromatic groups), 1460, 1375 (—CH group) and 1114 (secondary alcohol group C—O) cm^{-1} and this is in corroboration with the previous investigation of Jabir et al. [34]. Accordingly, the entrapment of linalool into chitosan nanocomposite caused small change in peak intensity, shifting and flattening in absorption bands that may be the resultant of noncovalent and electrostatic interaction of linalool and chitosan *via* hydrogen bonding, hydrophobic and Van der Waal's forces [35].

3.1.5. XRD investigation

Fig. 1 H represents the XRD analyses of chitosan, chitosan nanocomposite and Nm-linalool nanocomposite. XRD analysis determines the changes in crystallinity after entrapment of linalool which was reflected in diffractogram. The diffractogram of chitosan powder displayed strong reflection peak at 2θ value 10.7 and 21.5°, which were associated with the hydrated as well as regular crystal of the chitosan [36,37]. The peak intensity was almost diminished in case of chitosan nanocomposite suggesting reduction in crystallinity and significant disarray in the alignment of nanocomposite particles which may be due to ionic interaction of cationic amino group of chitosan with anionic polyphosphate group of S-TPP during ionic gelation process. It was observed that incorporation of linalool into nanostructured chitosan particle had more specific decreasing effects on crystallinity of Nm-linalool nanocomposite particles with widening of peak area. Reduction in crystallinity has been correlated with increasing amorphousness of Nm-linalool particles by diminishing the peak intensity. Significant increase in amorphousness may correspond to the newer interaction of chitosan with functional groups of linalool and destroy the original crystallinity of chitosan. Our result is in corroboration with the previous study of Bagheri et al. [38] with reduction in crystallinity after incorporation of *Urtica dioica* essential oil into chitosan nanoparticle. Efficient interaction of linalool with chitosan having dense network of nanostructural confirmations facilitates the application of Nm-linalool nanocomposite particles as innovative smart nano-delivery vehicles against fungal and aflatoxin mediated biodeterioration of food commodities.

3.1.6. TGA assay

TGA represents the weight loss at varying temperatures for assessing the thermal stability of prepared nanocomposite. TGA was used to verify the stability of linalool and chitosan nanocomposite at different

temperatures which may be encountered during processing of some food products. This result could suggest whether the linalool incorporated into chitosan nanocomposite are stable at high temperature if encountered during storage or processing. Moreover, TGA of linalool entrapped into chitosan nanocomposite also demonstrates their application not only for protection of rice, but also for other foods encountering high temperature processing and storage. The temperature presenting the maximum decomposition or weight loss was determined as decomposition temperature (T_d) and was displayed through thermogram. Linalool exhibited two step mass losses from 60 to 110 °C and 205–300 °C (Fig. 1I). Similar to linalool, chitosan nanocomposite also showed loss of weight in two different steps at peak 70–100 °C and 180–275 °C (Fig. 1I) which might be associated with moisture evaporation, chitosan decomposition or may represent the T_d of chitosan as well as TPP cross linked with chitosan. However, incorporation of linalool into chitosan nanobiopolymer (Nm-linalool) manifested three step weight loss at temperatures 80–110 °C, 195–230 °C and 295–350 °C (Fig. 1I) which demonstrated the loss of free linalool and encapsulated linalool. The result reflected the achievement of linalool entrapment into chitosan nanocomposite with improved thermal stability. This also implied that the whole linalool might be entrapped into chitosan nanobiopolymer and significant nanostructure compaction may provide the higher thermal stability. Our finding is in agreement with the investigation of Hosseini et al. [31] and Yilmaz et al. [39] suggesting greater thermal stability of oregano essential oil after entrapment into chitosan nanomatrix. However, the variation in thermal stability may also depend on nature of concentration of polymeric matrix used, particle aggregation and electrostatic as well as hydrogen bond interaction during encapsulation process [40].

3.2. Encapsulation efficiency (EE), loading capacity (LC) and encapsulation yield (EY) of Nm-linalool

EE and LC of Nm-linalool were measured spectrophotometrically at 274 nm. EE determines the degree of dispersion of linalool within chitosan matrix, while LC is a measure of weight of linalool possessing the capability of chitosan matrix based on its concentration used. EE of Nm-linalool ranged between 22.19 and 88.69% (Table 2). It was possible to observe the directly proportional relationship between the content of linalool and increase in EE up to 1:0.6 ratio (chitosan/linalool; w/v). However, inverse pattern representing decrement in EE at higher linalool concentration was marked at 1:0.8 and 1:1 ratio. Similar results for decrement in EE were reported by Hosseini et al. [31] and Amiri et al. [41] during encapsulation of oregano and cumin essential oil into chitosan nanoparticles. LC of Nm-linalool was found in the range of 0.65–4.01% (Table 2). Initial increment of LC was found as an increasing function of linalool and highest value was achieved at 1:0.6 ratio followed by rapid decline in LC value. Reduced EE and LC at higher linalool to chitosan ratios (w/v) may be due to the feeble adsorption of linalool onto the surface of chitosan matrix or disintegration of chitosan-TPP interacting bonds which may facilitate the opening of chitosan chains, thereby release of some of the linalool content which was further separated during centrifugation [42]. The present finding is in agreement with the recent observation of Karimirad et al. [8] having the maximum EE and LC (15.33 and 5.89%, respectively) during encapsulation of bitter orange essential oil within chitosan nanobiopolymer. Our result exhibited superior EE and LC for encapsulation of linalool into chitosan nanomatrix suggesting greater holding potentiality and efficient entrapment in nanostructured system, thereby suggesting their application in protection of stored food commodities from fungal infestation and aflatoxin contamination. The EY of Nm-linalool at highest EE and LC values (1:0.6 ratio) was observed as 20.69%. Similar result of EY has been presented by Hasheminejad et al. [12] during encapsulation of clove essential oil into chitosan nanoparticle. Some differences in EY may be attributed to the variability in nature of encapsulant used and process utilized [17]. Higher EY ensured the stable nature of Nm-linalool nanocomposite strengthening their application in long term food protection.

Table 2
Encapsulation efficiency, loading capacity and encapsulation yield of Nm-linalool nanocomposite.

Chitosan/ linalool (w/v)	Encapsulation efficiency (%)	Loading capacity (%)	Encapsulation yield (%)
1:0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	–
1:0.2	22.19 ± 1.87^{b}	0.65 ± 0.11^{b}	–
1:0.4	61.59 ± 2.92^{c}	2.08 ± 0.34^{c}	–
1:0.6	88.69 ± 3.61^{d}	4.01 ± 0.96^{d}	20.69
1:0.8	76.54 ± 4.11^{e}	$3.51 \pm 1.02e$	–
1:1	68.02 ± 3.18^{f}	2.97 ± 0.89^{f}	–

Note: - = not determined, values are mean (n = 3) ± SE, the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

3.3. In vitro release of Nm-linalool

For, further understanding of long term stability of linalool incorporated chitosan nanocomposite, release behavior was tested in alcoholic aqueous solution at pH 7.4, since the system simulates similar conditions for majority of stored food products. *In vitro* release of Nm-linalool was observed in PBS and ethanol mixture at 274 nm and is shown in Fig. 2. The release profile suggests the successfulness of linalool encapsulation within chitosan biopolymer and application for controlled as well as targeted release. It was evident that Nm-linalool exhibited two step biphasic release profile *i.e.* initial rapid release followed by sustained release with respect to times. Initial rapid release (30.67%) of Nm-linalool was observed within 4 h, while after 8 h the release was found to be 21.74% followed by 20.76% release within 12–24 h. The release rate in between 24 and 48 h was 2.4%. After 96 h, the release rate was represented with plateau suggesting its long term sustained release pattern offering new insight for the development of gradual antifungal and antiaflatoxigenic efficacy in stored food system. The reason of initial burst phase of release may be due to the release of linalool molecules adsorbed at the surface of the chitosan nanocomposite including diffusion, surface erosion, decomposition and desorption [33]. Sustained and gradual release of linalool from nanostructured chitosan after 96 h may be due to the release of linalool through pores on the surface of the hydrocarbon portions of the chitosan matrix and specific intermolecular interactions between core components with wall matrix. The *in vitro* release characteristics of linalool may affect the crucial factors responsible for vapor phase targeted migration of linalool molecule in the food system [43] leading to enhancement in antifungal efficacy with the resultant shelf life extension.

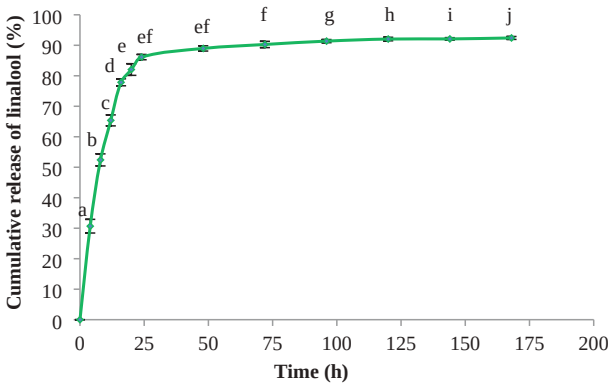

Fig. 2. *In vitro* release profile of Nm-linalool.

3.4. In vitro testing of linalool and Nm-linalool nanocomposite for fungitoxic and AFB_1 inhibitory action

In vitro inhibitory efficacy of linalool and Nm-linalool against fungal infestation and AFB_1 biosynthesis was determined by MIC and MAIC assay and results are summarized in Table 3. MIC of linalool for inhibition of AF LHP R14 cells was found to be 0.7 μL/mL. Nm-linalool nanocomposite exhibited better performance for inhibition of AF LHP R14 cells at lower doses (0.05 μL/mL). MAIC of linalool was achieved at 0.4 μL/mL, while Nm-linalool represented superior efficacy for mitigation of AFB_1 biosynthesis at 0.04 μL/mL. Additionally, the linalool and Nm-linalool significantly inhibited the infestation of 11 different food biodeteriorating fungi *viz. A. candidus*, *A. repens*, *A. versicolor*, *A. luchuensis*, *A. niger*, Mycelia sterilia, *Fusarium oxysporum*, *F. poae*, *Alternaria alternata*, *A. humicola* and *Cladosporium herbarum* at MIC doses (0.7 and 0.05 μL/mL) (Fig. 3). Chitosan nanocomposite displayed negligible efficacy for inhibition of fungal proliferation (2.09%) and AFB_1 secretion (1.67%). Fungitoxic and AFB_1 inhibitory efficacy of linalool was found far better than previous report of Das et al. [44] representing the activity of several essential oil bioactive components such as elemicine, apiol, α-pinene, fenchone and p-cymene for inhibition of toxigenic *A. flavus* and AFB_1 secretion. Moreover, the linalool exhibited superior efficacy as compared to previously demonstrated essential oils and their bioactive components *viz. Origanum vulgare*, *Cinnamomum zeylanicum*, carvacrol and cinnamaldehyde for inhibition of *A. flavus* and AFB_1 biosynthesis [45]. The hydrophobic and lipophilic nature of linalool (non-phenolic terpenoid component) may serve as a key factor for easy interaction with plasma membrane of fungal cells enabling breakdown of important lipids, leakage of vital cellular components and inhibition of conidiogenesis as well as spore germination leading to cell death [46]. Moreover, the encapsulation of linalool into chitosan nanostructure deciphers a frontier way for low dose requirement with least wastage of component, emphasizing targeted delivery for the inhibition of broad range fungal infestation and AFB_1 contamination. Better performance of linalool and Nm-linalool for inhibition of AFB_1 secretion at lower doses than antifungal action may be due to interference in the metabolic pathways including carbohydrate catabolism during AFB_1 biosynthesis [47]. Moreover, the downregulation of *lae A* gene (global regulatory gene for sporulation and aflatoxin synthesis) by essential oils and bioactive components may be a possible reason for efficient restraining in the AFB_1 biosynthesis [48].

3.5. Antioxidant activity

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical sequestration assays were performed to measure the antioxidant activity of linalool and Nm-linalool and the scavenging effects were presented through IC_{50} values. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ IC_{50} values of linalool were observed at 21.96 and 17.29 μL/mL, respectively. High antioxidant activity of linalool may be due to very fast cross termination and self-termination chain reactions occurred through co-oxidation of substrate [49]. Encapsulation of linalool into chitosan matrix achieved superior radical scavenging potentiality as demonstrated by lower $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ IC_{50} values. Improvement in antioxidant activity of Nm-linalool may be related to the nanometric size of composite particles with greater surface area to volume ratio and controlled delivery of aroma which assisted in sequestration of majority of the biodeteriorating free radicals with long term effects. Chitosan nanocomposite had no significant antioxidant activity (IC_{50} ≥100 μL/mL) because of most of the side chains containing free amino group ($-NH_2$) has been utilized during the formation of stable nanocomposite system through high speed homogenization based ionotropic gelation. The present finding is in agreement with previous report of Damasceno et al. [50] for encapsulation of *Lippia origanoids* essential oil into chitosan nanogel with improved antioxidant activity. However, the present study displayed better free radical scavenging activity offering the possible practical application of Nm-linalool with controlled release as novel shelf life enhancer of stored food commodities based on antioxidant mediated diminution of fungal infestation and AFB_1 production.

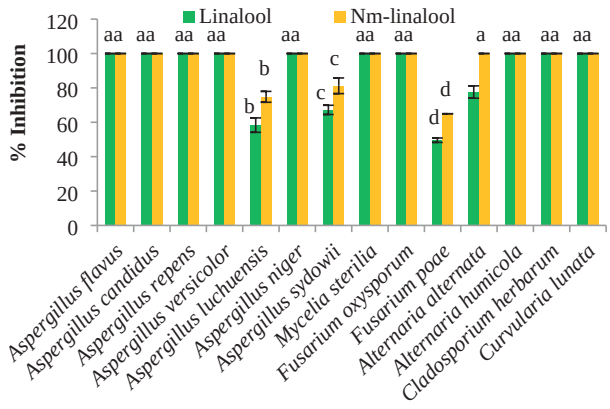

Fig. 3. Fungitoxic spectrum of linalool and Nm-linalool against 14 different food contaminating fungi.

3.6. In situ antifungal and AFB_1 inhibitory efficacy of linalool and Nm-linalool nanocomposite in food system: rice (Oryza sativa L.) as model food

In the course of present investigation, rice was selected as the model food because in the storage conditions exposure of high humidity and temperature intended to severe infestation of toxigenic *Aspergillus flavus* and secretion of AFB_1 in rice with due course of time. A report on AFB_1 contamination (154 μg/kg) of basmati rice with further development of AFM_1 metabolic byproducts (22 μg/kg) has been demonstrated by Nordkvist et al. [51]. Additionally, the Rapid Alert System for Food and Feed of European Union (http://ec.europa.eu/food/food/rapidalert/archive_2008_en.htm) recognized the heavy incidence of AFB_1 contamination in rice with progressive deterioration of the nutritional qualities.

Table 3
Efficacy of linalool and Nm-linalool nanocomposite for inhibition of mycelial weight and AFB_1 synthesis.

Linalool			Nm-linalool		
Concentration (μL/mL)	Mycelial weight (g)	% AFB_1 inhibition	Concentration (μL/mL)	Mycelial weight (g)	% AFB_1 inhibition
CNT	0.859 ± 0.050^{a}	0.00 ± 0.00^{a}	CNT	0.859 ± 0.050^{a}	0.00 ± 0.00^{a}
0.1	0.794 ± 0.023^{b}	11.68 ± 1.12^{b}	0.01	0.627 ± 0.043^{b}	21.35 ± 3.48^{b}
0.2	0.527 ± 0.031^{c}	47.84 ± 3.41^{c}	0.02	0.421 ± 0.039^{c}	51.67 ± 4.29^{c}
0.3	0.486 ± 0.019^{d}	82.03 ± 4.01^{d}	0.03	0.298 ± 0.011^{d}	76.08 ± 2.22^{d}
0.4	0.319 ± 0.026^{e}	100 ± 0.00^{e}	0.04	0.157 ± 0.020^{e}	100 ± 0.00^{e}
0.5	0.175 ± 0.041^{f}	–	0.05	0.00 ± 0.00^{f}	–
0.6	0.121 ± 0.012^{g}	–			
0.7	0.00 ± 0.00^{h}	–			

Note: - = not determined, values are mean (n = 3) ± SE, the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Hence, the evaluation of the *in situ* fungal infestation and AFB_1 contamination in stored rice and their mitigation by linalool and Nm-linalool nanocomposite is an important parameter of the present study. Moreover, the *in vitro* testing of bioactive essential oil components for inhibition of broad spectrum food contaminating fungi and AFB_1 secretion established the preliminary criteria to screen the potential bioefficacy. Under *in situ* conditions, relative humidity, temperature, available water and chemical constituents of the food system may affect the bioefficacy of the component by absorbing some of the volatile contents itself. Therefore, *in situ* efficacy in model food system (rice) fulfills the criteria to extend the frontier way for the practical applications as effective novel green food preservative with special emphasis to preserve the food nutritional attributes.

The results of *in situ* efficacy in rice were measured on the basis of percent protection against fungal association up to one year of storage. Percent protection against fungal contamination for inoculated and uninoculated treatment with MIC and 2 MIC doses of linalool was found to be 39.08, 46.39, 79.23, and 86.12%, respectively (Table 4). It has been critically observed that the MIC dose of linalool as recognized by the *in vitro* testing was not able to provide complete protection in real food system (rice). Therefore, special care should be taken during exploration of linalool from *in vitro* to *in situ* practical applications. The complex interactions of rice moisture content, water availability, pH and chemical compositions with surrounding environmental factors may influence the potential efficacy of essential oil components. Moreover, there is a chance for considerable absorption of the volatile aroma of linalool by rice during long term preservation. While, nano-encapsulation approach completely protects excessive absorbance of the volatile constituents because of controlled volatilization and targeted delivery providing a green horizon to maximize their application in food and agricultural industries. In our study, encapsulation of linalool into chitosan nanocomposite facilitated the lower dose inhibition of fungal contamination in rice. In contrast to linalool, Nm-linalool nanocomposite displayed complete inhibition of fungal proliferation in both uninoculated and inoculated treatment sets at 2 MIC dose (Table 4). Better achievement in inhibition of fungal contamination by Nm-linalool fumigation may be due to nanostructured chitosan incorporated with linalool having sub-cellular size and greater surface area leading to controlled as well as targeted delivery of components. Moreover, the colloidal Nm-linalool nanocomposite may assign to high resistance of water vapor diffusion in the stored rice ensuring the inhibition of fungal infestation and spore formation. The inhibitory efficacy of linalool against fungal contamination further extended its efficacy in AFB_1 diminution in rice. The inoculated and uninoculated control rice sample was represented with much higher level of AFB_1 (19.87 and 17.20 μg/kg) as determined through HPLC analysis, exceeding the maximum concentration limit set by European Regulatory Commission (Commission Regulation (EU) No.165/2010, 2010). However, during our *in situ* practical study, both the linalool and Nm-linalool critically inhibited the AFB_1 biosynthesis in stored rice at MIC and 2 MIC doses. Reduced solubility of essential oil components in their actual stoichiometric form has been recognized as a major limitation in exploitation of broad spectrum antimicrobial activity, because only the soluble portions of aqueous phase containing the dissolved essential oil molecules is able to easily interact with microbial cellular components [52]. It is presumed from our investigation that incorporation of linalool into chitosan nanocomposite enhanced the solubility which in turn facilitated the greater transportation of linalool into the plasma membrane of fungal cells, resulting into its disruption and further extension of the AFB_1 inhibitory activity [53]. Moreover, differential efficiency of linalool and Nm-linalool against fungal infestation and AFB_1 biosynthesis may be due to variation in inoculums density, potential and modulatory activities responsible for inhibition of different steps in carbohydrate metabolism, a prerequisite factor for aflatoxin biosynthesis [54]. The result of our investigation supports the previous observation of Wan et al. [13] confirming protection of rice grains by *Fusarium graminearum* infestation and secretion of deoxynivalenol, 15-acetyl deoxy nivalenol and 3-acetyl deoxynivalenol. Therefore, Nm-linalool nanocomposite possesses the potential application as nano-based smart delivery vehicle with long term inhibition of fungal infestation and AFB_1 biosynthesis causing prolonged shelf life of not only rice but also of other postharvest stored agri-food commodities.

Table 4
Efficacy of linalool and Nm-linalool nanocomposite against fungal infestation and AFB_1 production in rice.

Treatment sets	Fungal colony number	% Fungal protection	AFB_1 content (μg/kg)	% AFB_1 protection
Inoculated control	59 ± 2.03^{a}	0.00 ± 0.00^{a}	19.87 ± 0.31^{a}	0.00 ± 0.00^{a}
Uninoculated control	52 ± 5.31^{b}	0.00 ± 0.00^{a}	17.20 ± 0.22^{b}	0.00 ± 0.00^{a}
Inoculated linalool treatment (MIC)	33 ± 6.12^{bc}	39.08 ± 2.75^{b}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Uninoculated linalool treatment (MIC)	29 ± 4.98^{c}	46.39 ± 5.28^{c}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Inoculated linalool treatment (2 MIC)	11 ± 3.87^{d}	79.23 ± 5.03^{d}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Uninoculated linalool treatment (2 MIC)	7 ± 1.89^{e}	86.12 ± 4.37^{e}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Inoculated Nm-linalool treatment (MIC)	31 ± 2.55^{f}	44.08 ± 2.59^{bc}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Uninoculated Nm-linalool treatment (MIC)	18 ± 1.69^{g}	67.02 ± 5.23^{cd}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Inoculated Nm-linalool treatment (2 MIC)	0.00 ± 0.00^{h}	100 ± 0.00^{f}	0.00 ± 0.00^{c}	100 ± 0.00^{b}
Uninoculated Nm-linalool treatment (2 MIC)	0.00 ± 0.00^{h}	100 ± 0.00^{f}	0.00 ± 0.00^{c}	100 ± 0.00^{b}

Note: values are mean (n = 3) ± SE, the means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

3.7. Efficacy of linalool and Nm-linalool nanocomposite against lipid peroxidation in rice

Lipid peroxidation is a primary factor for stored rice biodeterioration associated with free radical chain reactions and interaction of polyunsaturated fatty acids (PUFAs) with reactive oxygen species (ROS) by thermodynamic constraints. Presence of Fe in stored food commodities act as catalyst for initiation of lipid peroxidation [55]. Moreover, the fungal association together with the secretion of AFB_1 in stored rice potentially led to generation of ROS, resulting into production of lipid hydroperoxides, aldehydes and peroxides which affect the quality and nutritional value of the food ingredients. Previous investigation on induction in the level of lipids and proteins peroxidation by AFB_1 mediated reactive oxygen species generation has been demonstrated by Madhusudhanan et al. [56]. The level of lipid peroxidation in stored foods depends on composition of fatty acids, pH susceptibility, ions distribution, oxygen concentration, rheology, thickness, charge, permeability and droplet size of lipid molecules in food [57]. In the present study, extent of rice lipid peroxidation was measured through the determination of malondialdehyde (MDA) (major aldehyde produced during lipid oxidation) adducts with TBA. High MDA content was observed in both uninoculated (409.09 μM/g FW) and inoculated control (471.23 μM/g FW) rice samples. However, rice samples inoculated with toxigenic AF LHP R14 strain displayed higher MDA content as compared to uninoculated control that may be due to greater content of AFB_1 synthesis leading to higher oxidative damage in lipid molecules. The rice seeds after linalool fumigation could maintain the low MDA

content at MIC (310.97 μM/g FW) and 2 MIC concentrations (287.22 μM/g FW) (Fig. 4A). Interestingly, the Nm-linalool exhibited superior efficacy in effective diminution of MDA content (Fig. 4A) in both un-inoculated and inoculated treatment sets at MIC and 2 MIC doses as compared to unencapsulated linalool. Better inhibitory kinetics for generation of MDA adducts by Nm-linalool nanocomposite may be due to the nanometric size of composite particles having greater surface to volume ratio facilitating better suitability to check the free radical chain reactions during peroxidation of fatty acids [58]. Therefore, our investigation could be helpful in recommending the application of Nm-linalool as controlled release delivery system by suppressing the AFB_1 induced lipid peroxidation in rice and maximizing the shelf life of stored food and agri-products.

3.8. Sensory analysis

Impact of linalool and Nm-linalool fumigation on sensory attributes (color, odor, texture and flavor) of rice was determined through hedonic test and reflected the consumer's willingness, acceptance and preference for further consumption purposes. Results of sensory test are presented in Fig. 4B. All the sensory attributes of control rice samples were unacceptable due to significant influence of fungal contamination and AFB_1 secretion. Peroxidation of rice lipids and generation of free radicals led to changes in color, odor and flavor which is also consistent with higher MDA content in control rice samples as determined in our lipid peroxidation test. Higher acceptability for different sensory attributes of rice was observed after linalool fumigation at the MIC dose. However, at the 2 MIC dose of linalool the odor and flavor of rice samples were unacceptable that may be due to absorption of linalool aroma. Rice sample fumigated with Nm-linalool gave rise to the acceptability score 4–5 for all organoleptic attributes at MIC and 2 MIC doses after one year of storage. Controlled release as well as targeted delivery of linalool aroma from chitosan derived nanocomposite was suggested as a possible reason for long term preservation of organoleptic attributes of stored rice. Similar finding for preservation of organoleptic properties (color, aroma, taste and texture) of maize after fumigation with α-terpineol loaded chitosan nanoemulsion up to six months of storage periods has recently been reported by Chaudhari et al. [18]. However, our results showed superiority in preservation of color, odor, texture and flavor of rice over a period of one year, suggesting the application of Nm-linalool nanocomposite as potential nano-green, smart, and controlled release delivery vehicle against stored food biodeterioration.

4. Conclusions

Encapsulation in colloidal matrices can be useful in improving the biological activity and stability of bioactive components for application as innovative food preservatives. The conducted study aimed towards successful encapsulation of linalool into colloidal chitosan nanocomposite by homogenization based ionic gelation process. In the present investigation, we applied an economical and non-specific encapsulation strategy for encompassment of linalool producing monodispersed nanocomposite particles. The encapsulated linalool showed biphasic release with potential implications in controlled release, commonly opted for shelf life extension of stored food products. Noteworthy, the entrapment of linalool in chitosan nanobiopolymer improved the overall bioefficacy in terms of strong antifungal, antiaflatoxigenic and antioxidant activity over its unencapsulated form. Moreover, the acceptable organoleptic properties of treated rice samples and considerable reduction in rice lipid peroxidation strengthen the future possibilities in practical application of chitosan-linalool nanocomposite as eco-smart green preservative in food and allied industries.

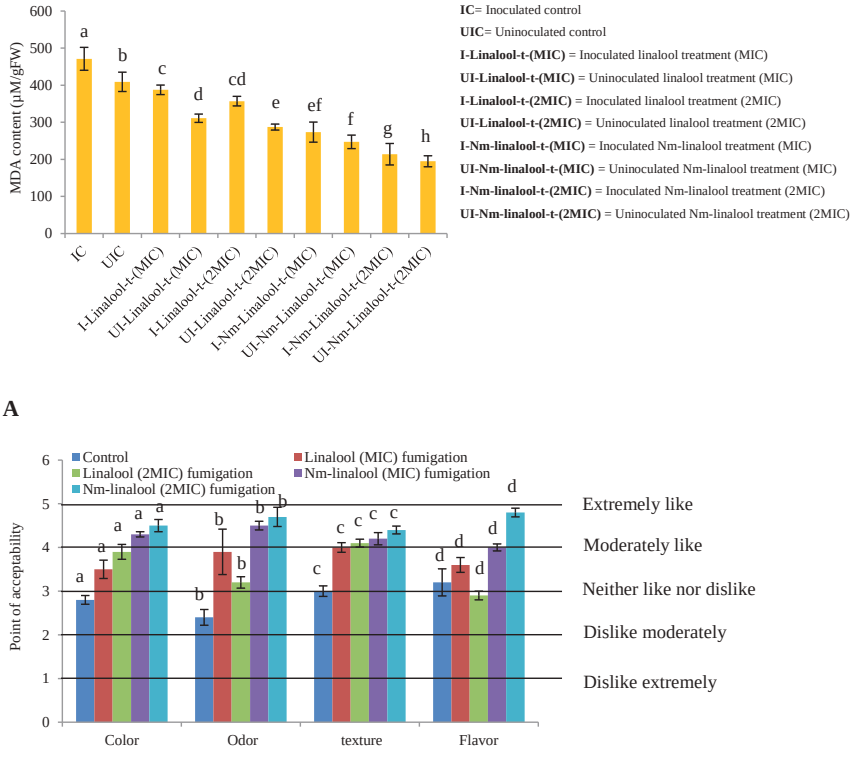

Fig. 4. A. Effect of linalool and Nm-linalool on MDA content in rice.
B. Effect of linalool and Nm-linalool on organoleptic properties (color, odor, texture and flavor) of rice.

CRediT authorship contribution statement

Somenath Das: Conceptualization; Writing - original draft; Funding acquisition. **Vipin Kumar Singh:** Review & editing. **Anand Kumar Chaudhari:** Data curation. **Abhishek Kumar Dwivedy:** Investigation; Methodology; Validation; Visualization. **Nawal Kishore Dubey:** Review & editing; Supervision.

Declaration of competing interest

Authors reported no conflict of interest.

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