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Fabrication and *in vitro* antifungal potential of nanoencapsulated eugenol against fungal contaminants of *Calocybe indica*

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Fungal diseases pose a significant threat to mushroom crops, and the standard approach to controlling these diseases on farms globally often relies on fungicides. However, issues such as the development of pathogen resistance to fungicides due to frequent use and the sensitivity of mushrooms to these chemicals present serious challenges. To address these problems, incorporating herbal agents for disease prevention could contribute to a more sustainable mushroom industry. This study aimed to develop nanoencapsulated eugenol and assess its prolonged antifungal effectiveness. Four fungal competitors *i.e*., two *Trichoderma spp*, *Penicillium spp* and *Aspergillus spp* were isolated from *Calocybe indica* cultivating industries and identified based on morphological and molecular characteristics. Carboxymethyl cellulose-based nonencapsulated eugenol was synthesized and its physical and chemical properties were determined by Field Emission Scanning Electron Microscopy (FESEM), Zeta sizer, High-Performance Liquid Chromatography (HPLC) and Fourier-transform Infrared (FTIR) spectroscopy. Nanocapsules were found to be nearly spherical in shape with sizes ranging from 139.8 nm to 273.8 nm and possessed encapsulation efficiency of 90.6%. The conical flask paper cone method determined the minimum inhibitory concentration (MIC) of encapsulated eugenol required per centimeter cube. MIC values were recorded as 4.02 µL/cm^3 and 5.0 µL/cm^3 against *Trichoderma spp*, *Penicillium spp* and *Aspergillus spp* respectively. FTIR reports evidenced successful encapsulation of eugenol which might have interacted with CMC *via* intermolecular hydrogen bonding. Further, *in silico* molecular modeling studies also showed CMC-eugenol complex formation by the interaction between the hydrogen atom of hydroxy group of eugenol with the oxygen atom of CMC. The binding energy of the docked structure was calculated to be -2.57 kcal/mol. Molecular docking results well supported the findings of FTIR spectroscopic analysis. The outcome of this study will help the mushroom cultivators prevent economic losses caused by fungal contamination.

Keywords: Molecular docking, Essential oils, Carboxymethyl cellulose, *Trichoderma*, FTIR analysis, Fungal competitors, Sustained release, Conical flask paper cone method, Milky mushroom

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Urbanization and drought in recent years have reduced the area of cultivation and resources for irrigation. On the other hand, the growing population led to food scarcity[1]. In this scenario, the gap can be efficiently bridged by focusing on alternate beneficial food sources like mushrooms, single-cell protein, *etc*. Variety of mushrooms are widely cultivated and consumed all over the world[2]. According to FAOSTAT (2023), world mushroom production has reached 44 million tonnes whereas Asia (95%) produced a major share of it. Indian mushroom industry is producing 0.31 million tonnes of mushrooms and holds 5th rank in global mushroom production[3]. *Calocybe indica, commonly called milky* mushrooms, *is the first i*ndigenous mushroom *species commercialized in India.* It is one of the finest edible mushrooms known for its nutritional and pharmaceutical functionalities. It is also referred to as white summer mushroom due to its adaptability to warm and humid climatic conditions. It can be easily cultivated in tropical and subtropical areas round the year[4]. India′s humid tropical climate widely favours the growth of these mushrooms and hence, milky mushrooms occupy the third position among commercially grown mushroom species in India. This mushroom variety comprises 3% of total world mushroom production[5]. Besides India, these are cultivated and consumed in other countries like Indonesia, Africa, Malaysia, Singapore and China[6]. Since mushrooms are rich in their nutritional content, they are highly prone to microbial attacks. Management of microbial contaminants during mushroom crop growth and preservation is a major challenge in the mushroom industry. Fungal contaminants like *Trichoderma spp., Mycogone spp., Lecanicillium spp., Cladobotryum spp., Coprinus spp., Sependonium spp., Sclerotium rolfsii* and *Cephalothecum roseum,* are known to infect mushroom crops at different stages from spawn run period to maturation of fruiting bodies[7]. They degrade the mushroom quality during pre- and post-harvest stages causing huge economic losses. Repeated use of conventional fungicides like dithiocarbonates and methyl benzimidazole carbamates has led to the

evolution of fungicide resistance[8]. Also, awareness about the ill effects of fungicides among consumers has highlighted the need to research effective and safe alternatives for managing fungal contaminants in mushroom cultivation[9].

Eugenol is a natural compound that is well known for its antifungal activity[10]. Recent research reports recommend the application of eugenol in food preservation, especially for minimizing post-harvest contaminations in fruits and vegetables[11]. In our previous study, eugenol fumigation was found to effectively control the black mold of onion caused by *Aspergillus niger*[12]. Although eugenol is well known for its antifungal activity, the high volatility and burst-effect release pattern of this compound severely handicap its utilization due to the short timelines for antibiosis[13]. Hence, the present study is aimed to overcome this problem by fabrication of eugenol-loaded nanoparticles for slow and sustained fumigation to check the growth of fungal contaminants of mushroom cultivation.

Materials and Methods

Isolation and identification of fungal contaminants in mushroom cultivation

Fungal contaminants of mushroom cultivation were isolated from various mushroom growing industries located in industrial area, Pragati Nagar, Hyderabad, Telangana, India. Briefly, bags with various stages of milky mushroom (*Calocybe indica*) growth were observed for fungal contamination and samples were collected. These samples were brought to the laboratory, cut into several small bits with a sterile scalpel and placed on potato dextrose agar (PDA) medium. These petri plates were incubated for 5-7 days at 25°C.

Isolated fungal contaminants were identified by cultural, micromorphological, and molecular characterization. All the fungal isolates were raised individually in potato dextrose broth (PDB) for 7 days at 25±1°C and DNA was isolated following the CTAB method[14]. The ribosomal ITS1-5.8S rRNA gene was amplified by using forward and reverse primers, ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) respectively. The reaction mixture for PCR amplification contained 40 pmol of each primer, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1.0 U of *Taq* DNA polymerase (Merck Genei) and 10 ng of template DNA. Forty PCR cycles were amplified by the Carbet PCR master cycler (Germany). Each cycle was programmed with a denaturation step for 15s at 94ºC, an annealing step for 30s at 55°C followed by an extension step for 60 s at 72°C. The PCR product was purified using Qiagen PCR Purification kit and both strands were sequenced by using Sanger dideoxy sequencing method (Heredity biosciences, Orissa). All the sequences were aligned using clustalW software. Phylogenetic relationships were identified by tree construction using NCBI.

Synthesis of nanoencapsulated eugenol

The encapsulation of eugenol was performed using water-in-oil emulsion method[15]. Water phase containing 0.5% tween 80 and 0.5% carboxymethyl cellulose in distilled water. While oil phase is composed of 5.0% pure coconut oil and 1% eugenol (99.9% pure, Sigma) in distilled water. Oil phase was taken in beaker and aqueous phase was added gradually while stirring (500 rpm) for 30 min using a magnetic stirrer. This allows partial encapsulation of eugenol in CMC. This mixture was subjected to sonication for 3 min at frequency of 30 kHz with 13 mm probe (cycles of 5s on/10s off). This process entraps eugenol completely in a shell of nano CMC.

Characterization of nanoencapsulated eugenol

The morphology and size distribution of nanocapsules was determined by Field emission scanning electron microscopy (FE-SEM; QUANTA, FEG 250, Japan). The sample was prepared by spray drying it on an aluminium sample stub. Then the sample is vented, pumped & vacuumed (high & low). Then the sample stub is coated with an electrically conductive thin layer of gold. This stub is then placed inside the FESEM specimen holder. Once the preliminary observation is done the beam is switched on at a set voltage. The beam of electrons after scattering gets ejected by primary electrons producing secondary and back scattered electrons that form the image of the inserted sample which can be adjusted using the monitoring system. The droplet diameter and polydispersity index were measured in a Zeta sizer (ZS 90) (fixed angle of 90°). The chemical characteristics of nanoparticles was done by FTIR Spectroscopy analysis.

Determination of encapsulation efficiency (EE)

To determine the EE of nanoparticles, free eugenol (unentrapped eugenol) was filtered using a centrifugal filter device (Amicon Ultra-7K). Further, separated eugenol was quantified by HPLC by employing a C18 column, UV detector and mobile phase of methanol:

water (65:35) with a constant flow rate of 1mL per minute[11]. The EE in percentage was calculated using the following equation

$$EE\ \% = \frac{\text{Total amount of eugenol} - \text{free amount of eugenol}}{\text{total amount of eugenol}} \times 100$$

Sustained release of eugenol and MIC determination

The antifungal activity of eugenol and encapsulated eugenol against *Trichoderma* was evaluated by the conical flask paper cone method[12]. Briefly, sterile conical flasks containing PDA with a tincture of streptomycin were inoculated with 7 days old fungal discs of 6 mm diameter (disc cut from the edge of the growing colony that possessed spores and mycelium) was carefully transferred to the center of conical flask using sterile forceps and varied volumes of eugenol (1µL to 20µL) or nanoencapsulated eugenol (50µL to 250µL) were spotted on sterilized paper cones (Whatman No 1 filter paper was cut in the shape of a cone) that were hung from the top of conical flask. The mouth of the conical flasks was sealed by parafilm to prevent the escape of eugenol. Paper cone should be hanged at a distance of one inch above the surface of the medium. MIC of volatile compound required per centimetre cube of the conical flask is calculated as follows:

$$\text{MIC of eugenol /nano encapsulated eugenol (per centimetre cube)} = \frac{\text{Minimum amount of compound required to inhibit fungal growth}}{\text{Volume occupied by the compound}}$$

To prove the sustainable release of entrapped eugenol, paper cones (with eugenol/ encapsulated eugenol) from flasks with no fungal growth were aseptically transferred to a new set of conical flasks inoculated with the fungal disc. These flasks were incubated at 25±1°C and observed regularly for 15 days.

Assessment of stability

The effect of temperature on the stability of encapsulated eugenol was investigated at various time intervals (24, 48, 72, 96 and 120 h). Samples of eugenol/nanoencapsulated eugenol were stored in glass vials (10 mL) at different temperatures (4°C, 25°C and 35°C) and entrapment efficiency was assessed at predetermined time intervals. Triplicates were maintained and all data represents the mean values.

Docking Studies

To determine the interaction between CMC and eugenol, docking analysis was performed using Autodock Vina. The structure of eugenol was obtained from PubChem (CID of eugenol is 3314). The structure of CMC was modeled using the molecular constructor HyperChem (https://hyperchem.software.informer.com) and optimized by the AMBER 99 force field. The center of the molecule and box parameters were set manually, ensuring that the molecule was completely inside the computational space domain. Further, standard procedure of Autodock Vina was performed to dock CMC and eugenol. Conformational freedom and flexible rotation of all single bonds and functional groups were allowed.

Statistical analysis

Three replicates were carried out for each experiment and data were presented as mean ± standard error. The statistical significance of the data was confirmed by *t-test* for difference and one way analysis of variance (ANOVA). SPSS program version 16.0 and Microsoft Excel were used for statistical analysis.

Results and Discussion

Identification of Fungal contaminants

Four fungal isolates were recovered from contaminated mushroom mycelia based on morphological and growth characteristics on PDA medium. After 7 days of incubation, sporulated fungal isolates, *i.e*., *Trichoderma* ss1, *Trichoderma* ss2, *Penicillium* ss3 and *Aspergillus* ss4 appeared in olive green, light green, pale green and black colours, respectively. The sequences of these isolates were submitted to GenBank and accession numbers were obtained. Molecular level identification was done by performing sequence similarity search using NCBI BLASTn and a phylogenetic tree was constructed with reference sequences (Fig. 1). BLASTn analysis indicated that the fungal contaminants are members of the genus *Trichoderma, Penicillium* and *Aspergillus*. The phylogenetic analysis revealed close relationships of fungal isolates with *Trichoderma atroviride* (NR077207.1), *Trichoderma longibrachiatum* (MF422166.1)*, Penicillium crustosum* (NG069876.1) *and Aspergillus niger* (MT550028.1). The present report aligns with those of Abdullah & Maha (2022)[16], who isolated and characterized fungal competitors, *T. longibrachiatum, T. pleuroticola, R. arrhizus, M. hiemalis* and *P. glabrum,* grown on oyster mushroom culture media. In a similar study, five fungal pathogens *Trichoderma koningiopsis*, *Phomopsis* sp., *Mucor circinelloides*, and *Cladosporium bruhnei* that caused severe damage to the production yield of *Pleurotus eryngii, were*

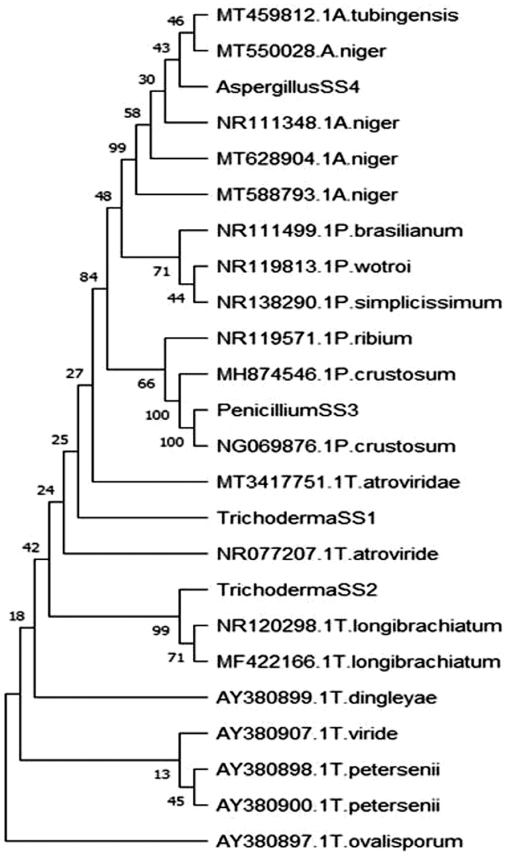

Fig. 1 — BLASTn Phylogenetic tree of Trichoderma ss1, Trichoderma ss2, Penicillium ss3 and Aspergillus ss4 isolates inferred by Neighbour Joining analysis of internal transcribed spacer (ITS) region in MEGA 4.0.

isolated from commercial farms located in Gyeongnam province, Korea[17]. In the same way, Choi *et al*. (2010)[18] identified *Aspergillus spp., Mucor spp., Penicillium spp*., *T. harzianum, T. pleuroticola, T. longibrachiatum,* and *T. atroviride* that contaminated *Agrocybe aegerita*, an important mushroom variety cultivated in Korea. Very recently four fungal pathogens *viz*., *Cylindrodendrum alicantinum, Hypomyces aurantius, Hypomyces rosellus,* and *Trichothecium roseum* causing damage to *Morchella* cultivation in china were identified for the first time[19].

***In vitro* Antifungal activity**

The antifungal potential of eugenol fumigation (free eugenol or nanoencapsulated eugenol) was analysed by conical flask paper cone method. The MIC of free eugenol and its nanoencapsulated form were determined for each of four fungal contaminants (*Trichoderma atroviride, Trichoderma longibrachiatum* and *Penicillium crustosum* growth was completely inhibited with 1200 µL of encapsulated eugenol and the MIC value was recorded as 4.02 µL/cm^3. In the case of *Aspergillus spp*., it was noticed that 1500 µL of encapsulated eugenol was required for inhibition of fungal growth and MIC was calculated as 5.0 µL/cm^3. The MIC of free eugenol was calculated as 0.028 and 0.033 µL/cm^3 against *Trichoderma spp*., *Penicillium spp*. and *Aspergillus spp*., respectively[12]. In addition to antifungal activity, nanoemulsions were known to supress biosynthesis of mycotoxins. Chitosan-encapsulated *Laurus nobilis* essential oil was proved to exhibit broad spectrum fungitoxicity and also reduced production of methylglyoxal (MG), an inducing substrate of Aflatoxin, leading to suppression of Aflatoxin B1 secretion[20]. Interestingly, in the present study, it was noticed that sporulation of all the fungal isolates was prevented by nanoencapsulated eugenol at concentration below MIC values. Further, no fungal growth was observed in the second set of conical flasks that received paper cones loaded with encapsulated eugenol from flasks (first set) with no fungal growth. This observation evidenced sustained release of eugenol from nanocapsules. However, second set of flasks with paper cones loaded with free eugenol (transferred from the first set) did not inhibit fungal growth, which proved burst release effect of nonencapsulated (crude) essential oil. From the results of the antifungal activity study, it was noticed that MIC of encapsulated eugenol was higher than nonencapsulated eugenol which can be attributed to slow release of eugenol in its encapsulated form. This observation is consistent with previous literature that has reported higher MIC or MBC values for nanoencapsulated antibiotics. Nanoantibiotics formulated by encapsulating ciprofloxacin and levofloxacin into polymer-based nanocarriers possessed equivalent or increased MIC than those of their free forms. In case of nanoencapsulated forms, incomplete release of drug molecules can result in inadequate concentration of free antibiotic molecules that exert action was believed to cause increased MIC values[21]. A study by Radovic-Moreno[22] demonstrated that MIC of free vancomycin (20 µg/mL) is much lower than its nanoencapsulated form (250 µg/mL) tested against *S. aureus*. In a similar study, lower antibacterial activity of ciprofloxacin-encapsulated poly (dl-lactide-co-glycolide) copolymer

nanostructures compared to free antibiotic was due to slow and sustained release property of nanocapsules[23].

Characterization and stability assessment of nanocapsules

Stabilization of essential oils is required to reduce their vulnerability to environmental stresses like light, heat and oxidation. Also, optimal utilization of these volatile compounds can be achieved by encapsulating them in an appropriate matrix, aiding in their controlled and targeted release. Essential oils are encapsulated in various biopolymeric-based nanocarriers. Encapsulated forms of essential oils are largely explored in several innovative food packing methods[24]. This study used CMC nanoparticles to encapsulate eugenol, a bioactive hydrophobic molecule. The size of CMC/eugenol nanometric assemblies was determined by FESEM analysis (Fig. 2). The nanocapsules were nearly spherical in shape, with sizes ranging from 139.8 nm to 273.8 nm with an average particle size of 200. The PDI value of nanocapsules were recorded as 0.282±0.04, while the average droplet diameter was 156 nm. Encapsulation efficiency that indicates the amount of eugenol entrapped in nanoshell of CMC/coconut oil was calculated as 90.6%. These results proved CMC as better nanocarrier to infuse essential oils, this statement is in agreement with Sánchez-Hernández *et al*. (2024)[25] who achieved up to 89% encapsulation efficiency with chitosan-carboxymethylcellulose-alginate (COS–CMC–ALG) nanocarriers (NCs) for loading carvacrol, a well-known essential oil. Whereas chitosan nanostructures encapsulated *Laurus nobilis* essential oil with a maximum efficiency of only 34%[26]. Besides better encapsulation efficiency, other promising features like good mechanical strength, film forming ability, viscosity, wide availability, non-toxicity and hydrophilic nature makes CMC a highly suitable nanocarrier and finds applications in diverse fields like pharmaceutical, wastewater treatment, food, paper, textile and quality maintenance of agricultural produce[27]. Various investigators employ nanoencapsulation to accomplish different purposes. In current study, prolonged release of eugenol, thereby sustained antifungal activity is achieved by nanocarrier. Targeted delivery of antibiotics through encapsulation based on charge switch mechanism[22], Prevention of *Levodopa* oxidation by nanostarch drug vehicles for better treatment of Parkinson′s disease[28] and alleviation of memory impairment, oxidative stress, and neuroinflammation by nanoencapsulated curcumin[29]. Nanoencapsulated eugenol has diverse applications in food preservation, medicine, and agriculture. Chitosan nanoparticles containing eugenol have been identified as a potent nanoherbal agent for treating autoimmune diseases like rheumatoid arthritis[30]. Encapsulated eugenol is also utilized as a natural additive for preserving meat and meat products[31]. Additionally, nanoemulsions have been shown to improve the permeation of eugenol, thereby enhancing its antibacterial properties[32].

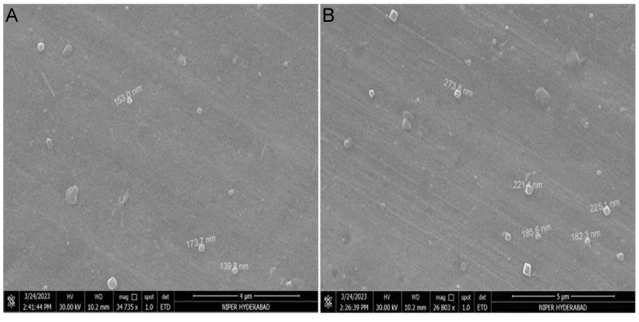

Fig. 2 — (A,B) SEM images of CMC-NP with particle sizes

FTIR analysis

FTIR spectrum of eugenol, Tween 80, coconut oil and CMC were analyzed (Table 1). In FTIR spectrum of eugenol, sharp peaks at 1514.12, 1610.56, 1637.56 cm^{-1} revealing the presence of C=C stretching of the aromatic ring structure were observed. Also, signature peaks of eugenol present in 700–1200 cm^{-1} region were recorded. The spectrum also showed OH stretching frequency at 3520.09 cm^{-1}, C-H stretching of CH_2 and CH_3 groups at 3068.75 cm^{-1}, 2908.65 cm^{-1}, C=C double bond peak at 1510.26 cm^{-1} and aromatic C=C bond carbon at 1637.56, (C=C) doubled bond

Table.1 — FTIR spectra peak points with chemical bond linkage at different peaks of Tween 80, Eugenol, Coconut oil, CMC, CMC-NP (combined mixture)

Tween 80 (cm^{-1})	Eugenol (cm^{-1})	Coconut oil (cm^{-1})	CMC (cm^{-1})	Combined Mixture (cm^{-1})	Assignments
3500	3520.09		3400	3382	υ(OH)
2918.30	3068.75	2922.16	3043.67	2924	υ(C-H)
2862.36	2908.65	2852.72	2908.65	2854	
1734.01		1743.85	1680	1745	υ(C=O)
1639.49	1637.56			1640	υ (C=C)Ar
	1510.26	1514.12	1589.34		υ (C=C)
1097.50	1265.30		1326.95	1236	υ (C-O-C)

carbon at 1606.70 cm^{-1}, ether linkage (C-O-C) at 1265.30 cm^{-1}. This spectrum data was well in agreement with Nuchuchua *et al.* (2009)[33] and Pramod *et al.* (2015)[34]. In case of CMC, its characteristic peaks in the region at 1000- 2000cm-1 and 3427 cm-1 representing O-H stretching were recorded, observed as a broad peak at 3400cm^{-1}, CH_2 and CH_3 stretching frequencies are at 3043.67 cm^{-1} and 2908.65 cm^{-1}. carbonyl peak at 1680 cm^{-1}, C=C double bond peak at 1589.34 cm^{-1}, ether linkage at 1326.95 $cm^{-1.}$ This FTIR spectrum is similar to data reported by Shan *et al.* (2010)[35] and Pramod *et al.* (2015)[34]. The characteristic peaks of Tween 80 were recorded at around 1600 cm-1, 2900 cm-1 and 3600 cm^{-1} that represented HOH bending, CH_2 stretching and OH stretching respectively were recorded. Also, O-H stretching observed as a broad peak at 3500cm^{-1}, CH_2 and CH_3 stretching frequencies are at 2918.30 cm^{-1} and 2862.36 cm^{-1}. carbonyl peak at 1734.01 cm^{-1}, C=C double bond peak at 1639.49 cm^{-1}, ether linkage at 1097.50 cm^{-1}. Similar peaks were reported by Pramod *et al* (2015)[34]. In FTIR spectrum of coconut oil, sharp characteristic peaks at 1150, 1463, 1743(C=O ester), 2853 and 2921 cm^{-1} (C-H stretch) were observed. In coconut oil peaks observed at 2922.16 cm^{-1} and 2852.72 cm^{-1} due to stretching vibrations of CH_3 and CH_2 groups of aliphatic chains. Another peak observed at 1743.85 cm^{-1} due to the stretching vibrations of carbonyl (C=O), C=C double bond peak at 1514.12 cm^{-1}. These results were in agreement with FTIR data of coconut oil presented by sari *et al* (2018)[36].

FTIR spectra of nanoencapsulated eugenol was compared with the spectra of individual components *i.e.*, CMC, coconut oil, Tween 80 and eugenol to explain successful encapsulation and possible interactions of eugenol with other compounds (Fig. 3). In nanoencapsulated mixture, O-H stretching shifted towards at 3382cm^{-1}as a broad peak, CH_2 and CH_3 stretching frequencies are at 2924cm^{-1} and 2854cm^{-1}. carbonyl peak at 1745 cm^{-1}, C=C double bond peak at 1640 cm^{-1}, ether linkage at 1236 cm^{-1}. In comparison with the FTIR spectrum of eugenol, nanoencapsulated mixtures resulted in a noticeably decreased intensity of the O-H stretching peak at 3382 cm^{-1} which might be due to the formation of inter molecular hydrogen bond. This increment of O-H stretching peak indicate a successful encapsulation of eugenol into NEM 2931 cm^{-1}, The FTIR of NEM showed bands at 1236 cm^{-1}, which were absent in other components but present in eugenol, indicating successful incorporation of eugenol in the ECNP. In another study, encapsulation of flurbiprofen (FP), a non-steroidal anti-inflammatory drug was done using nanochitosan (CS). FTIR spectroscopic data of CS-FP micro-nano spheres that showed O-H and N-H stretching bands shift to lower wavenumbers (3108.4 cm^{-1}) due to H-bonding system revealed structural differences of chitosan after formation of nanocapsules and also evidenced encapsulation of flurbiprofen[37].

Stability and sustained release

Stability of CMC nano systems and sustained release of eugenol from them was investigated by incubating up to 120 h at various temperatures (4°C, 20°C, 35°C). The encapsulation efficiency was assessed by quantifying the amount of free eugenol at different time periods (Fig. 4). Non-capsulated eugenol was used as control. In nonencapsulated solution, residual eugenol content at various temperatures varied significantly. However, no significant variation was observed among retained eugenol content in case of encapsulated solution stored at various temperatures. Nanocapsules retained considerable amount of eugenol even after 120 h of incubation, with residual eugenol of 83%, 74% and 50% at temperatures 4°C, 20°C, 35°C respectively. This clearly shows slow trend in the release of

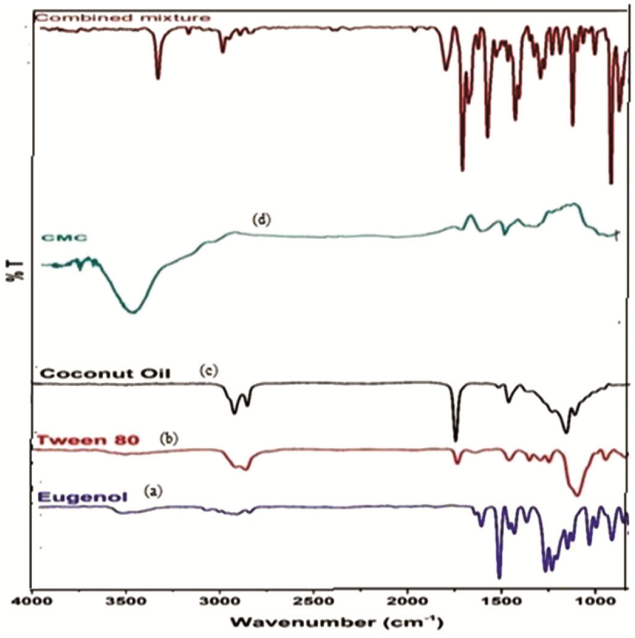

Fig. 3 — Fourier transform infrared (FT-IR) spectra of (A) Eugenol (B) Tween 80 (C) Coconut oil (D)CMC (E)CMC-NE

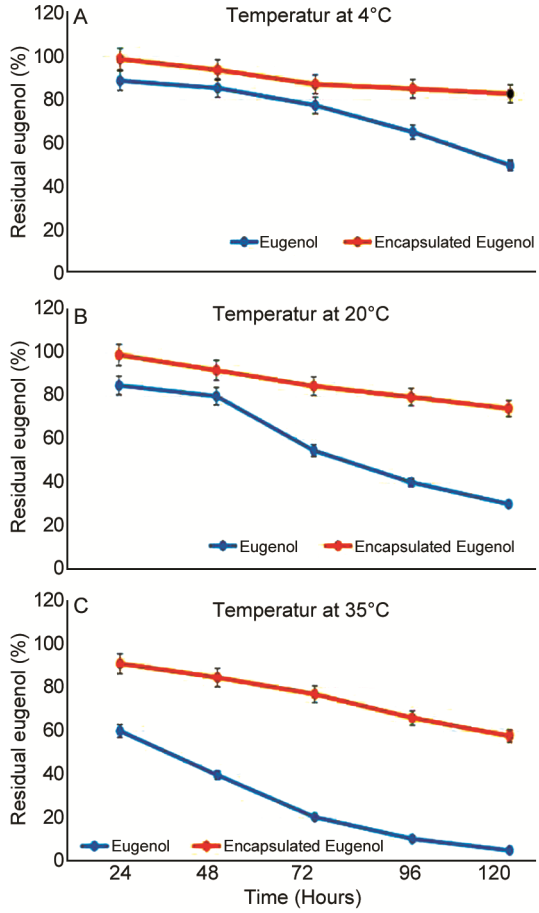

Fig. 4 — Encapsulation efficiency of free eugenol assessed at different time intervals in hrs (on X- AXIS) & % residual eugenol on Y- AXIS at different temperatures i.e. 4˚ C (A), 20˚ C (B), 35˚ C (C) respectively

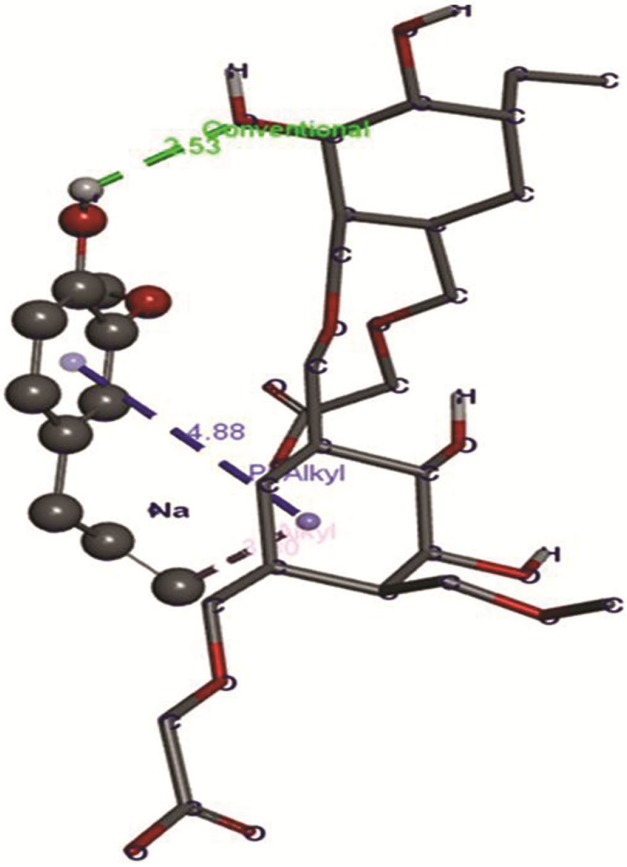

Fig. 5 — Autodocking structure of CMC-NE structure representing hydrogen & Pi-Alkyl bonding of eugenol & CMC with their bond length & lowest energy

eugenol in its nanoencapsulated form. On the other hand, eugenol was rapidly lost from nonencapsulated solution with 50% and 30% of eugenol remaining at temperatures 4°C and 20°C after 120 h of incubation. At 35°C eugenol dissipated very rapidly with only 20% remaining after 72 h of incubation which was further reduced to 5% (after 120 h). These results evidences burst effect and short-term existence of eugenol in its nonencapsulated form. Temperature, Time and pH variations can showcase profound effect on release of target molecule from its encapsulated system. In the present work, at 4°C the residual eugenol content of non-encapsulated and encapsulated eugenol did not differ significantly. On the other hand, there is a significant difference in retained eugenol contents at 20°C and 35°C indicating the effect of temperature on eugenol release and signifies the role of encapsulation in preventing burst effect of essential oils. Khambhaty & Bondada (2023)[38] studied the influence of pH of the medium on rate and amount of *in vitro* release of doxorubicin (DOX), a major chemotherapeutic drug from the DOX loaded AgNPs. Release of DOX was more pronounced at acidic pH (pH-4.6) than neutral pH level. pH dependent charge switching and release property of PLGA-PLH-PEG- vancomycin nanocapsules was explored to exert charge mediated targeted antibacterial activity[22]. Sustained release of thyme essential oil from chitosan nanocapsules is dependent on storage time and temperature, 75% of loaded thyme was retained when stored at low temperatures (4°C) and only around 60% thyme residual content remained at 25°C after storing for 2 weeks[39].

Docking results

The docking studies were conducted to investigate the possible interactions of eugenol with CMC. The binding energy of best docked CMC-eugenol complex was calculated as -2.57 kcal/mol. The binding pose of eugenol and CMC docked structures with the lowest energy are shown in Fig. 5. The

flexible docking results showed conventional hydrogen bonds between hydrogen atom of hydroxyl group of eugenol and oxygen atom of CMC (bond length 2.53), Pi-alkyl interactions (4.88) and alkyl interactions (3.10). Facilitation of encapsulation by hydrogen bonds, electrostatic interactions and weak Van der waal forces between biopolymers and pharmaceutical compounds/essential oils was evidenced by many research works. For instance, interactions between hydroxyl groups of *Rosmarinus officinalis* essential oil and those of the alginate/chitosan nanoparticles aided the retention of essential oil[40]. In another *in silico* investigation, the interaction between flurbiprofen and chitin was analyzed by docking studies, which were further compared with experimental results. The chitosan-flurbiprofen complex showed ΔG binding energy -3.90 kcal/mol, where flurbiprofen was bonded to chitosan through a new hydrogen bond between carboxyl oxygen of drug (O-174) and chitosan O-35 atoms[41].

Conclusion

Nanoencapsulated eugenol fabricated by CMC was found to exhibit sustainable *in vitro* antifungal activity against isolated fungal contaminants. FTIR spectra of the nano encapsulated mixture revealed intermolecular hydrogen bonding between eugenol with CMC which confirmed the successful entrapment of eugenol in CMC. Computational studies revealed intermolecular interactions, including conventional hydrogen bonding, alkyl and Pi-alkyl interactions between CMC and eugenol which might aid encapsulation and sustainable release of eugenol. Hence, CMC-Eu showing slow & sustainable fumigation at low concentration may be recommended as a safe novel and much more effective management strategy for preventing fungal contamination during pre & post mushroom cultivation. However, this study represents only a first step towards prospecting the potential of these nanoparticles. There is still much research to be done to study their stability in varied environmental conditions, *in vivo* activity, etc.

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Conflict of interest

The authors declare that they have no competing interests.

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