Indian Journal of Natural Products and Resources
Vol. 14(1), March 2023, pp. 81-88
DOI: 10.56042/ijnpr.v14i1.1130

Synthesis, characterization and antibiotic capabilities of microspheres loaded with essential oils

Choudhary Asha*, Rani Usha, Salar Raj Kumar and Thakur Rajesh
Department of Biotechnology, Chaudhary Devi Lal University, Sirsa, Haryana 125055, India

Received 15 November 2022; revised received 10 February 2023; accepted 23 February 2023

The purpose of the current work was to synthesize, describe, and assess the antimicrobial properties of microspheres against diverse microorganisms such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Aspergillus niger*. The production of polymeric microspheres using essential oils, as well as testing the impact of such microspheres on several biological properties was carried out. The ionic gelation approach was used in the laboratory to create CEO-loaded polymeric microspheres, which were then improved for use in subsequent experiments and characterized by Particle Size Analyzer, Scanning Electron Microscopy, and Transmission Electron Microscopy. The DPPH Assay and antibacterial activities were used to enhance the study of polymeric microspheres loaded with essential oils. Additionally, to observe the per cent encapsulation efficacy, synthetic microspheres have been used. Microspheres obtained during the experimental process were in size ranging from 80 to 290 nm, depending on the amount of drugs and polymers contained. Microspheres could be spherical in shape and aggregations at specific sites, according to SEM and transmission electron microscopy. The range of encapsulation efficiency for various concentrations was 6 to 25%. By using the DPPH assay, it was observed that the antioxidant activity lies between 28 to 64%. When anti-microbial activities were performed it was observed that the incorporation of essential oils into polymers showed tremendous potential against microbes. In the current research, the essential oils-loaded polymeric microspheres showed a significant impact on a variety of microorganisms. Additionally, these microspheres exhibited noticeable radical scavenging activities. In summary, it may be claimed that certain physiochemical alterations can be used to leverage the diverse activities of these microspheres for experimental uses in the future.

Keywords: DPPH Assay, Essential oil, Fumigant toxicity, Microspheres

IPC code; Int. cl. (2021.01)-A61K 36/00, A61K 36/23, A61P 31/00, A61P 39/00

Introduction

As the world's population grew, so did the demand for food. The preservation of stored grains is necessary for survival as well as to reduce food scarcity. Around, 10-40% of stored grains are destroyed due to insect damage and microbial contamination. Fungi and pests primarily destroy storage grains or cause them to lose weight, nutritional value, or aesthetic value, all of which are directly related to health[1,2]. In Asian and African countries, storage insects destroy around one-third of agricultural production[3]. *Sitophilus oryzae* L., the rice weevil, is one of the most destructive pests found in stored grains (mainly rice) and legumes and it is thought to be that this pest is indigenous to India[4,5].

Synthetic pesticides such as methyl bromide, phosphine, and sulfuryl fluoride are used to control pests (insects, fungi, rodents), but these can lead to a variety of adverse impacts on human health and the environment, increased resistance to insects, and pollute the groundwater and soil. To resolve these concerns, plant-based pesticides have been developed to control pests[6-9.]

Plant essential oils and their active compounds showed significant antibacterial, fungal, and insect action. Plant essential oil-based pesticides are used because essential oils are hydrophobic in nature and contain several complex compounds such as monoterpenes, sesquiterpenes, phenylpropenes, terpenoids, terpenes, phenylpropanoids, oxygenated terpenes, and aromatic components such as ketones, esters, alcohols, etc. which are primarily extracted from aromatic or higher plants through distillation methods[6,8].

The essential oil extracted from cumin seeds contains significant amounts of the antibacterial and antioxidant compounds β-terpinene, *p*-cymene, pinene, cumin aldehyde, and cuminal[10]. Cumin (*Cuminum cyminum* L.) is an aromatic herb of the Apiaceae family with

*Correspondent author
Email: choudharyasha260@gmail.com

special properties including medicinal, therapeutic, pharmacological, antioxidant, antibacterial, antifungal etc., is edible as well, and because of these qualities cumin has a high economic value[11].

Several issues have arisen with essential oil-based insecticides, including the transfer of bioactive substances and the effect of residual active compounds during implementation[2]. When essential oils were used as insecticides, they caused some issues mainly due to them being less soluble in water, having minimal physical durability, and fast environmental destruction. However, there are many ways to use different types of nanoparticles to solve these issues. For instance, encapsulating essential oils in polymer-based microspheres can be a useful strategy to maximise the effectiveness and stability for long-term use[12,13].

The antimicrobial effect of essential oils has recently been investigated, and it has been observed that it is due to their hydrophobic property, which allows them to permeate the bilayer membrane of various microorganisms, causing structural disruption[14].

Among polymers, chitosan has drawn a high interest as an encapsulating polymer because of its accessibility, bioactivity, nontoxicity, degradability, antimicrobial effectiveness, and consistent physicochemical properties[15]. Pectin is a natural water-soluble polysaccharide that is widely used in various industries, including pharmaceutical, cosmetic, and food, due to its outstanding properties like gelling behaviour, anionic nature, amorphous, non-toxicity, and inexpensive production costs[16,17]. It can be derived from the bio-waste of citrus fruit, sugarcane, and apples. Its chemical structure is primarily determined by poly [-(14)-D-galacturonic acid] or homogalacturonan helix strands[18].

A literature search revealed that cumin essential oil (CEO) has insecticidal activities, which has drawn the attention of researchers over the past decade as an eco-friendly potential alternative to synthetic pesticides[19]. However, no research has been conducted to assess the fumigant toxicity of CEO and its principal constituents against *S. oryzae*.

The objectives of this study were to synthesise, characterise, and determine the % RSA and encapsulation efficiency of synthesised microspheres, as well as to test antimicrobial activity and fumigant toxicity.

Currently, essential oil-based bioinsecticides are used to control insect pests of stored grains, which would be favourable for technological, commercial, as well as environmental considerations.

Materials and Methods

Chemicals

All of the chemicals such as calcium chloride, pectin, CEO, Tween 80, and Span 80 were purchased from Sigma and Hi-Media and of analytical grades.

Synthesis of CEO-loaded polymeric microspheres (CEO/P/Ms)

The ionic gelation process was used to synthesize the microspheres as the method described by Kala *et al.*, with some modifications. 0.8% aqueous solution of pectin was prepared[20]. Tween 80 (4): Span 80 (1.5) was added to the solution during the last two hours of stirring. CEO was then added to the solution and agitated for a further 15 min. Lastly, 0.4% w/v $CaCl_2$ was added dropwise to the sample.

Encapsulation efficiency of CEO/P/Ms

CEO was measured spectrophotometrically at a maximum wavelength of 209 nm. 350 µL of pectin microspheres loaded with CEO were quickly combined with 3 mL of ethyl acetate, centrifuged, and then the absorbance was measured. The absorbance of CEO forms the basis for the creation of a standard calibration curve. The following equation was used to obtain the % encapsulation efficiency (EE).

$$EE(\%) = \frac{\text{Total amount of loaded CEO}}{\text{Initial amount of CEO}} \times 100$$

Characterization of CEO/P/Ms

PSA and Zeta Potential

Using a particle analyzer, the produced microspheres' particle sizes and Zeta Potential (Malvern Instruments, Malvern, UK) were examined. Water was used as the dispersing medium to determine the size.

Scanning electron microscopy

Scanning Electron Microscopy (SEM) (7610F Plus/JEOL) was used to examine the morphology of microspheres.

Transmission electron microscopy

TECNAI (HR-TEM) Transmission Electron Microscopy was used to examine the internal structure of synthesized microspheres.

DPPH Assay

Using the 2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, the free radical scavenging activities of CEO/P/Ms, Gallic acid, and blank microspheres were analyzed. Gallic acid was used as a positive control.

$$\text{DPPH radical scavenging capacity } (\%) = [(A0 - As)/A0] \times 100$$

Here, A0= control absorbance and As=sample absorbance

Antimicrobial activity

The antibacterial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* and also antifungal activity against *Aspergillus niger* of CEO-loaded pectin microspheres were evaluated. The microbes were procured from the Bio-nanotechnology Microbiology laboratory at Guru Jambheshwar University of Science and Technology in Hisar, Haryana. The bacteria were revived in nutrient broth, while the fungi were revived in potato dextrose broth medium.

Agar well diffusion method

The antibacterial and antifungal activity of essential oils and essential oil-loaded microspheres was investigated by the method of Joseph and Priya[21]. To obtain a viable strain, a loop containing bacterial and fungal strains was inoculated separately in 30 mL of nutrient broth and potato dextrose agar broth in a flask for 72 and 220 h. Petri dishes were carefully poured with nutrient agar and potato dextrose agar. After the media solidified, separate wells (5 mm in diameter) were punched, and the test strains were added (20 µL) into the well-marked wells. The 20 µL of CEO and CEO/P/Ms that were added to the well plates were incubated for 2 to 5 days, respectively, at 37 and 20°C. This experiment was performed in triplicates to get better results. The diameter of the zone of inhibition was measured and compared with the control and the standard drug used which was Streptomycin and Ketoconazole.

Rearing of insects

Adults *S. oryzae* were procured from the entomology laboratory of Chaudhary Charan Singh Haryana Agricultural University Hisar and raised for further studies. *S. oryzae* was cultured in wheat flour in a Kilner jar with a muslin linen cover to enable air circulation. The container was kept at a temperature of 27°C in a culture room with a 14:10 h light: dark cycle.

Insecticidal fumigant toxicity assay

Pectin microspheres loaded with CEO were assessed for fumigant toxicity using an impregnated paper assay against adults of *S. oryzae*[1]. Various amounts of CEO-loaded pectin microspheres (20.0, 40.0, 60.0, and 80.0 µL/L) were separately dissolved in the solvent and treated to circular filter paper (Whatman No.1) before being placed into the plastic jars to achieve a total volume of 0.040, 0.80, 0.120, and 0.160 µL/mL, and acetone was used as a control in the experiment. Fifteen adults from the test insects were placed in the jar containing 80 g of cereal grains. Three duplicates were used in each experiment, along with a control system. After 24 h of exposure, the percentage mortality of the pests was calculated using Abbot's formula used by [1]:

$$\text{Mortality P (\%)} = \frac{\text{T-C}}{\text{100-C}} \times 100$$

where P=% corrected mortality, T=% kill in the treatment group, and C=% kill in the control.

Results and Discussion

Synthesis of CEO/P/Ms

Ionic gelation was used to synthesize EO/P/Ms, and calcium chloride was used as a crosslinker.

Encapsulation efficiency of CEO/P/Ms

The Encapsulation efficiency was measured by using the supernatant and the absorbance was taken at 209 nm to measure the concentration of free essential oil in the leftovers. The calibration curve was used to estimate how much essential oil was encapsulated in the synthesized nanoparticles. The results revealed that the EE (%) of CEO ranged from 6% to 54.7%.

Characterization of CEO/P/Ms

PSA and Zeta Potential

The average size was found to be 285.04 nm, while the Polydispersity Index was 0.58 as shown in Fig. 1a The zeta potential of CEO/P/Ms was 0.687, confirming the stability of the microspheres (Fig. 1b).

SEM Analysis

The synthesized microsphere was examined using scanning electron microscopy. Fig. 2a shows a mesoporous structure with particles as small as a few microns in diameter, which facilitates in the sustained release of the oil from the polymer. Fig. 2b depicts a nearly spherical microsphere with aggregations in various places.

TEM Analysis

The form and characteristic size of microspheres were indicated by Transmission Electron Microscopy (TEM), and the results are shown in Fig. 3. The TEM image clearly showed that microspheres have a tiny, spherical morphology with uniform distribution of particles.

Evaluation of DPPH Assay

DPPH Assay

Through DPPH Assay, it was observed that the gallic acid had 43.5 to 64.2% and CEO/P/Ms had 28.8 to 57.9%free radical scavenging activity as shown in

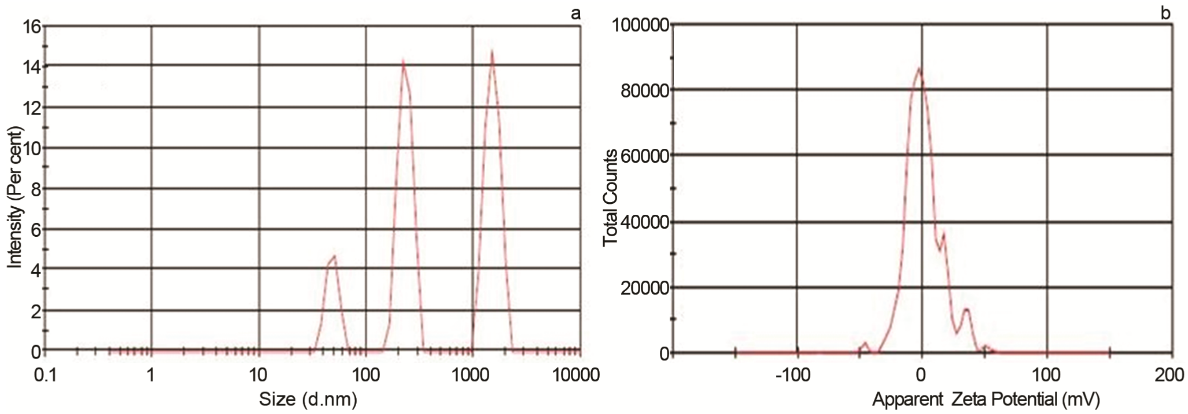

Fig. 1 — a) Dynamic light scattering; and b) Zeta potential of CEO/P/Ms.

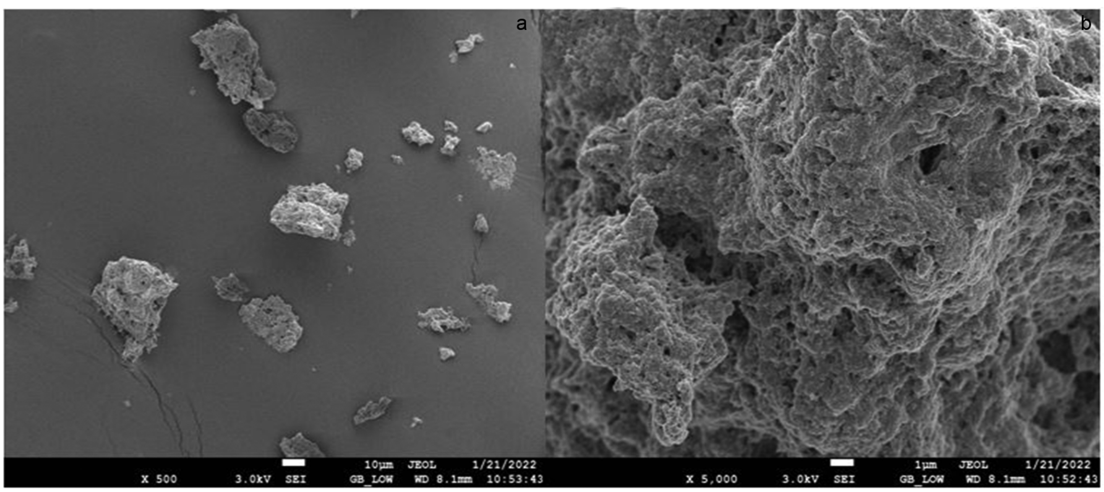

Fig. 2 — Scanning electron microscopy micrograph CEO-loaded polymeric microspheres. Scale bar is a) 10 µm; and b) 1µm

Fig. 4. CEO/P/Ms exhibited a significant difference in % radical scavenging activity as compared to blank microspheres which had P <0.001, by using three replicates (n=3) and the statistical analysis done was one way ANOVA and Tukey's multiple comparison tests.

Antimicrobial activity

The antimicrobial activities of CEO/P/Ms, CEO, and Blank microspheres (BMs) were evaluated against *B. subtilis*, *P. aeruginosa*, and *A. niger*. According to Fig. 5, the CEO/P/Ms showed the greatest antifungal inhibitory effectiveness throughout all assays. In these instances, inhibition halo (IH) values of 19, 16, and 20 mm were obtained against *B. subtilis*, *P. aeruginosa*, and *A. niger*, respectively. A stronger antibacterial activity could be achieved by fabricating microspheres using CEO with comparison to control.

Fumigant toxicity bioassay

The resultant LC50 value was found to be considerably more effective against *S. oryzae* (66.6 µL/L air) when compared to the pure CEO for *S. oryzae* (94.8 µL/L air). When compared to CEO, CEO/P/Ms exhibited 100% mortality against *S. oryzae* for the same concentration.

Discussion

Numerous eco-friendly strategies are required for insect control in agricultural and medical fields. Aromatic plant essential oils are thought to be credible alternatives to synthetic insecticides since they are harmless for the atmosphere and people's life. Moreover, the utilization of essential oils is limited because they are highly volatile and rapidly degraded when exposed to sunlight. The encapsulation of essential oil extracted from *Cuminum cyminum* into pectin polymer is an effective method to boost physicochemical characteristics and defend from

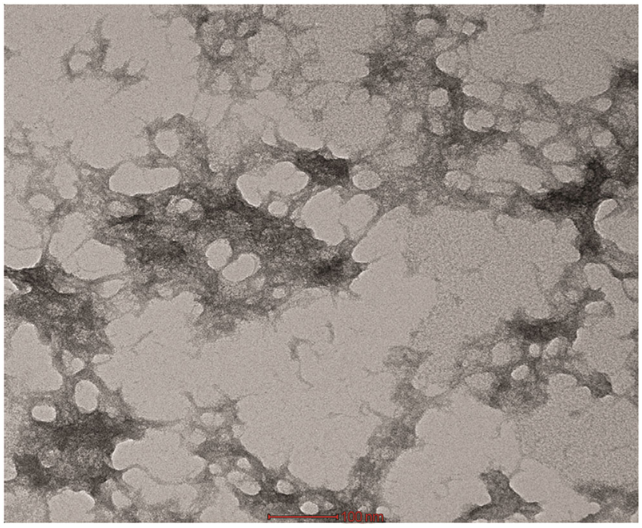

Fig. 3 — Transmission electron microscopy CEO-loaded polymeric microspheres at 100 nm scale bar.

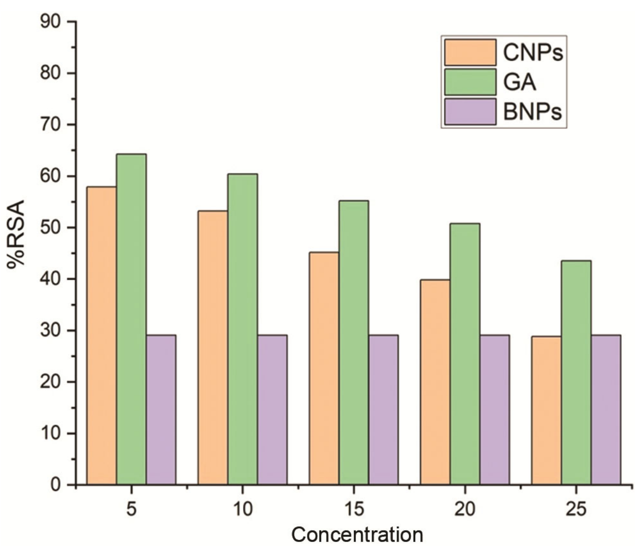

Fig. 4 — DPPH radical scavenging activity (%), CEO loaded pectin microspheres (CNPs), Gallic acid (GA), and Blank microspheres (BNPs).

decay, along with enhancing the prolonged release of the drug for better management of *S. oryzae*[4].

Therefore in the experiment, CEO/P/Ms were encapsulated using an ionic gelation method with pectin and calcium chloride. Calcium chloride is a common cross-linker used in the preparation of macroscopic gels and the synthesis of pectin nanoparticles. It has positively charged Ca^{2+} that binds with two dissociated acid groups on adjacent polymeric strands[16].

Moreover, similar studies found that the encapsulation efficiency of pectin/chitosan jasmine oil composite nanoparticles (Pec/CS/JO NPs), according to Attallah *et al.*, was 8.60% at 2.5 mg concentration and 30.30% at 10.0 mg concentration. Jasmine oil (JO), which is hydrophobic by nature, interacts with a pectin/chitosan polymer matrix, which is hydrophilic by nature. As a result, the majority of JO is confined to the particle surface, leading to the fluctuation in encapsulation efficacy[22]. Similarly, Shetta *et al.*, found that the Encapsulation Efficiency (EE%) of Green Tea oil loaded chitosan nanoparticles (CS/GTO NPs) and Peppermint oil loaded chitosan nanoparticles (CS/PO NPs) was approximately 22-81% and 78-82%, respectively[23].

In the case of particle size and zeta potential, similar research was conducted by Nguyen & Le, who reported that the DLS range for capsules was 100–500 nm. The entire z-average diameter was 235.3 nm, although the polydispersed PDI value was substantial when related to many other reports[24]. According to Sugumar *et al.*, the size of nanoemulsions was 9.4 nm and their polydispersity index (PI) was 0.060, showing that nanoemulsions are uniform, based on a thermodynamic stability analysis[25].

While in the current investigation, encapsulation CEO/P/Ms were primarily spherical in form with agglomerations visible in SEM. Ahmadi *et al.*, also

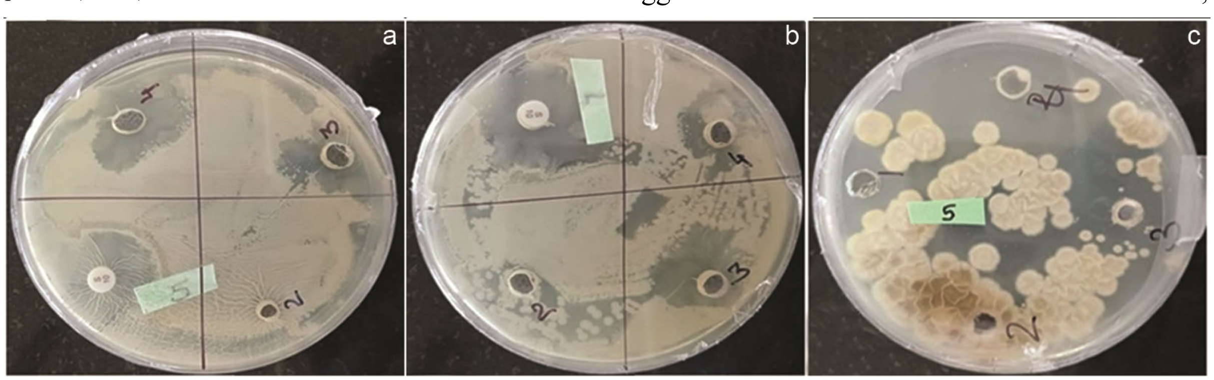

Fig. 5 — Antimicrobial activity of CEO/P/Ms against, a) *Bacillus subtilis*; b) *Pseudomonas fluorescens*; and c) *Aspergillus niger*.

noticed that the water lost during the solidification results in the *Satureja hortensis* EO-loaded chitosan nanoparticles (S.EO@NPs) spherical form and rough surface. The S.EO@NPs varied in size, which suggested that they would lose strength even during the freezing-drying process and as a result of the aggregation effect[26]. Sharma *et al.* noticed that the agrochemical loaded (spinosad and permethrin) chitosan nanoparticles had a generally spherical morphology and ranged in size from 50 to 100 nm[27]. By observing the SEM micrographs it was analyzed that the size of nanoencapsulated mace essential oil (N-24 MEO) was less than 100 nm[28].

However, Ahmadi *et al.*, state that TEM was used to examine the shape and average size of nanoparticles both before and after *Satureja hortensis* essential oil was loaded. The CS-TPP nanoparticles have a spherical form and a size of 80±10 nm, as can be seen from the TEM image. It was noticed that the essential oil of *S. hortensis* encapsulated in CS-TPP used to have a 153±20 nm sphere[26].

The IC50 values for *Homalomena aromatica* essential oil (HAEO) and HAEO that was encapsulated in chitosan nano matrix (CS-HAEO-Ne) were found to be 15.98 and 4.57 µL/mL, correspondingly, according to Tiwari *et al.*[29]. In a related study, Bagheri *et al.* observed that the inhibitory activity of free and loaded nettle essential oil for DPPH was in the ranges of 13.5-56.8% and 14.2-67.4%, accordingly[15]. According to Sundararajan *et al.*, the *Ocimum basilicum* L. leaf essential oil had excellent antioxidant activity and their IC50 value was assessed to be 13.21 µg/mL and nanoemulsion was determined to be 10.47 µg/mL, whereas the IC50 value for gallic acid was 7.90 µg/mL[30].

By using *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Staphylococcus aureus*, and *Listeria monocytogenes*, Bagheri *et al.*, investigated the antibacterial inhibitory efficiency of pure nettle essential oil (NEO), chitosan nanoparticles prepared without NEO, and chitosan NPs prepared with NEO. The strongest antibacterial inhibitory activity was demonstrated by the NEO-loaded chitosan NPs, with the maximum zone of inhibition against *S. aureus* measuring 4.11 cm[15]. According to a study done by Rajkumar *et al.*, adults of *Sitophilus oryzae* and *Tribolium castaneum* are fumigant-toxic when exposed to *Piper nigrum* essential oil (PNO) and *P. nigrum* essential oil-loaded chitosan nanoparticles (CS/PNO NPs)[1]. According to Elsherif *et al.*, the minimum inhibitory concentration (MIC) of all Essential oils (EOs) and their nano-emulsions (NEs) was 0.78% against *Listeria monocytogenes* and 1.56% against *Shigella flexneri* whereas carvacrol EO and its NEs exhibited 0.78% for both microbes[31].

Adult Sitophilus granarius mortality was 97% for Cuminum cyminum oil-loaded nanogels (OLNs) and 18% for Cuminum cyminum essential oil after 24 h exposure[32]. Fumigant toxicity of CEO/P/Ms to *S. oryzae* revealed that insect mortality varied with concentration and it was more efficient than pure essential oil. In comparison to pure PNO for *S. oryzae* (48.97; 85.77 µL/L air) and *T. castaneum* (55.77; 97.93 µL/L air), the observed LC50 and LC90 ratios were found to be substantially efficacious in CS/PNO NPs lethality against *S. oryzae* (25.03 and 44.31 µL/L air) and *T. castaneum* (29.02; 59.13 µL/L). When compared to PNO 100 µL/L air concentration with 100% death rates observed, CS/PNO NPs displayed a remarkable presentation of 100% mortality against *S. oryzae* and *T. castaneum* at the same concentration of 75.0µ L/L air[4]. Furthermore, the fumigant effect of *Atalantia monophylla* essential oil was tested on adults of *Callosobruchus maculatus* and *S. oryzae*, with the findings, revealing that the LC50 value was 101.69 µL/L air for *C. maculatus* and 113.67 µL/L air for *S. oryzae* within a 12-hour incubation time observed Nattudurai *et al.*[5]. Tak *et al.*, observed an LC50 of 901.3 µg/mL air using a fumigant toxicity assay of lemongrass oil[33].

Pure chitosan nanoparticles suppressed aflatoxin synthesis by 21.5%, but essential oil-loaded chitosan nanoparticles (NpCS-SEO) inhibited it by up to 59%[34]. Similar study was done by Das *et al.*, and the result found that, for the safeness against mycotoxin, rice seeds observed 66.67% MIC and 73.73% 2 MIC doses of *Myristica fragrans* essential oil (MFEO). MFEO-loaded chitosan nanoemulsion demonstrated superior antifungal efficiency at MIC (84.84%) and 2 MIC (100%) concentrations[35].

Conclusion

Microsphere has a significant capability to improve pest management strategies and lower risk in pesticidal materials. Calcium chloride was used to successfully synthesize CEO/P/Ms in this investigation, as shown by analytical techniques. SEM images revealed a spherical microsphere with aggregations in various locations, and the average size was determined to be 285 0.4 nm. CEO/P/Ms exhibit the strongest antibacterial efficacy when tested against *A. niger*, *P. aeruginosa*, and *B. subtilis*. Strong insecticidal action was demonstrated by CEO/P/Ms against the stored product insect pest *S. oryzae*, which was more sensitive to CEO/P/Ms than the CEO. By releasing oil in a controlled and efficient way, polymeric pectin microspheres may enhance the pesticidal activity of essential oils and encourage the widespread use of these biopesticides in the management of storage.

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