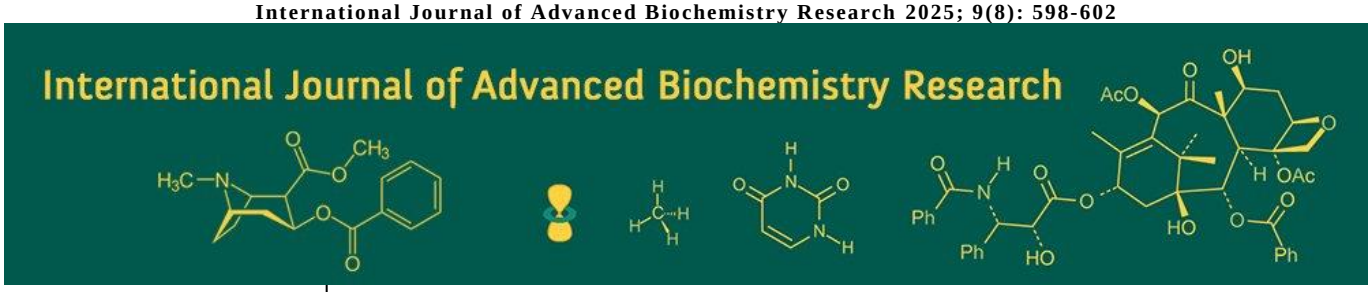

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Synthesis, characterization, and release behavior of chitosan-coated liposomes carvacrol and cinnamaldehyde in simulated gastrointestinal fluids

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Abstract
This study aimed to synthesize chitosan coated liposomes for the encapsulation of carvacrol and cinnamaldehyde, for enhanced stability and sustained release in gastrointestinal fluids. Firstly, the liposomes encapsulated carvacrol and cinnamaldehyde were prepared using the thin-film hydration method and subsequently coated with chitosan to improve their acid-resistant properties. Particle size and encapsulation effectiveness were used to describe the formulations. To determine the stability and release of both compounds under gastrointestinal conditions, their release pattern was assessed in simulated gastric fluid (SGF) and simulated intestinal fluid, simultaneously. The prepared formulation enables these phytoingradients suitable for oral delivery with enhanced stability and sustained release profiles.

Keywords: Carvacrol, cinnamaldehyde, liposome

Introduction
Essential oils (EOs) are fragrance molecules that occur naturally and have a variety of biological effects (Asbahani *et al*., 2015) [6]. EOs have been utilized as antibacterial, antiviral, antifungal properties, insecticidal, antioxidant, anti-inflammatory, anti-allergic, and anticancer agents, as well as flavouring additives, medications, and cosmetics (Burt, 2004) [2]. Thymol, carvacrol, γ-terpinene, p-cymene, sabinene, caryophyllene, germacrene, and spathulenol are among the terpenoids and phenolic compounds found in the plant's essential oil, which contribute to a number of its characteristics. Carvacrol is a monoterpenoid alcohol obtained principally from *Origanum vulgare* with a boiling point of 236–237 °C and a density of 0.976 g/mL at 20 °C. According to Yadav and Kamble (2009) [24], it is insoluble in water but highly soluble in ethanol, acetone, and diethyl ether. In addition to its antimicrobial qualities, this compound exhibits a variety of activities including antioxidant, immunomodulatory, anti-inflammatory, anticancer, analgesic, anticonvulsant, antidiabetic, hepatoprotective, and anti-obesity qualities (Khazdair *et al*., 2022) [10]. Cinnamaldehyde, which ranges from 55% to 78%, is the main flavour component extracted from the *Cinnamomum verum* bark, and eugenol, which ranges from 59% to 78%, is the main flavour compound extracted from the leaves antibacterial, anti-inflammatory and anti-cancer qualities (Wang *et al*., 2018) [23]. EOs having several disadvantages including their highly volatile nature, poor water solubility, low intestinal absorption, and sensitivity to environmental conditions (Movahedi *et al*., 2024) [13]. One technique for overcoming the drawbacks of essential oils is nanoencapsulation, which protects the compounds, improves their compatibility, solubility, and vectorization, increases the release of volatile and non-volatile components that provide antioxidant and antimicrobial qualities, and allows for controlled release (Ojeda-Piedra *et al*., 2022) [18].
Liposomes are lipid-based molecular assemblies that resemble micro- or nanospheres and contain one or more lipid bilayers surrounding an aqueous medium (Cuomo *et al*., 2013) [5]. Due to their structure, liposomes can contain both hydrophilic and hydrophobic molecules; the latter are trapped in the lipid bilayer, while the former are loaded into the water core (Schwendener & Schott, 2010) [21].

Liposomes continue to be a valuable tool in spite of their brief circulation half-life and susceptibility to oxidation and hydrolysis, which may cause the loaded cargo molecules to be lost. The largest obstacle for liposomal delivery systems is gastrointestinal tract destruction caused by bile salts, pancreatic lipase, and pH. The gastrointestinal tract's pH, bile salts, and enzymes can all cause the liposome membrane to become unstable. In fact, the stomach's acidic pH and lipases hydrolyze the ester bonds of phospholipids that make up liposomes. Along with this, bile salts solubilize liposome membranes by acting as surfactants. Certain polymers have been developed to cover the liposome's surface in order to get around these issues. By delivering drugs in a targeted and delayed manner, stealth liposomes improved the efficacy of the drug being transported and decreased its adverse effects. Modified liposomes, like those composed of pH-sensitive materials or coated with polymers like chitosan or polyethylene glycol, may exhibit improved stability in acidic environments and controlled or delayed drug release.

A natural polymer, chitosan formed by deacetylation of chitin in shellfish, a cationic, biocompatible, and biodegradable linear polymer has a wide range of antimicrobial properties, is highly safe, and has good biocompatibility (Nilsen-Nygaard *et al*., 2015) [16]. Cationic chitosan forms a protective polyelectrolyte layer over anionic liposomes due to electrostatic interactions (Nagamoto *et al*., 2004) [14]. EOs have been encapsulated using various polymers because encapsulation increase their solubility and bioavailability, protect them, and regulate their release (Ortan *et al*., 2009; Samperio *et al*., 2010) [19, 20]. Additionally, chitosan, a naturally derived biopolymer, exhibits significant potential for antibacterial applications (Chandrasekaran *et al*., 2020) [3].

In the present study, different combinations of chitosan liposome coated carvacrol and cinnamaldehyde formulations were prepared and their encapsulation efficiency, size, and release in simulated gastrointestinal fluids were determined.

Materials and Methods

Materials

The primary ingredients for preparation cinnamaldehyde and carvacrol, the bioactive components of cinnamon and ajwain essential oils, respectively were procured from Sigma-Aldrich (USA) and Redox Pharmachem Pvt. Ltd. Other materials used was in the study included soya phosphatidylcholine (Sigma-Aldrich), cholesterol (KEM Labs Pvt. Ltd.), sodium tripolyphosphate (STPP) and chitosan (Sigma Aldrich) and Tween-80 (Sisco Research Laboratories Pvt. Ltd.), as well as analytical-grade solvents such as chloroform, ethanol, methanol and phosphate-buffered saline (PBS).

Preparation of chitosan coated liposome formulations

The thin film hydration method was used to prepare the liposomes, and a modified method given by Hasan *et al.*, (2016) [9] used for coating liposome with chitosan. Soy phosphatidylcholine, the active ingredient of oils, and cholesterol dissolved in methanol-chloroform 1:2 were combined in a round-bottomed flask that was completely dry. A thin lipid film developed on the flask wall after organic solvents were fully evaporated using a rotary evaporator (Fig 1). 10 ml of distilled water were used to suspend the thin film, which was then heated to 60℃ for 20 minutes. In an ice bath, the suspension was subjected to five minutes of 40 kHz sonication (30 sec on, 10 sec off) in the probe sonicator. To maximize encapsulation efficiency and optimize the preparation, a number of combinations of cholesterol and soy phosphatidylcholine were tested.

To make the chitosan solution, 1% chitosan was combined with a 1% w/v acetic acid solution using a magnetic stirrer (Cuomo *et al*., 2018) [5]. Dropwise, sodium tripolyphosphate solution (TPP) (0.1% w/v) was added and gently stirred for 30 minutes at room temperature (Nouri, 2019) [17].

To coat the liposome, 1% stock chitosan was diluted to 0.1%, 0.3% and 0.5% strength solution. The final pH was set at 6.0. Then, chitosan and liposomes were taken in different ratio, and the chitosan was added drop by drop while being continuously stirred at 100 rpm for an hour on a magnetic stirrer. The mixture was sonicated and filtered, then stored at 4℃ (Fathi *et al*., 2021) [7]. The final formulations encapsulation efficiency was evaluated and preparations of chitosan-coated liposomes were kept in glass bottles at 4℃ in the dark (Fig 2).

Fig 1: Thin film formation by rotary evaporator

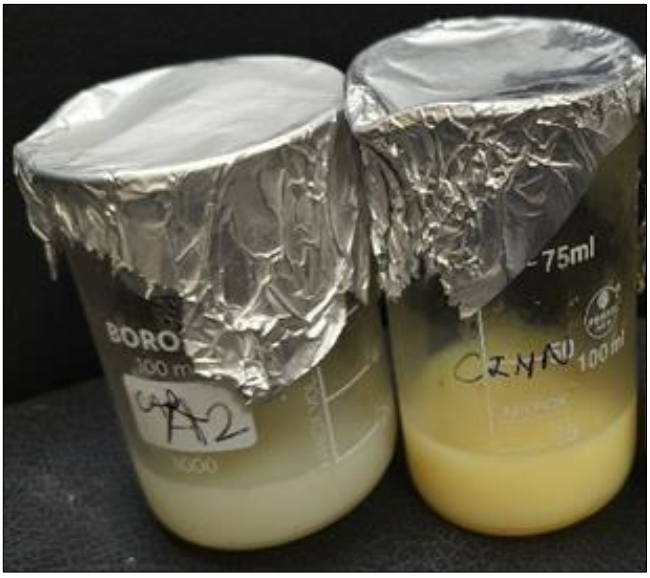

Fig 2: Liposome coated carvacrol and cinnamaldehyde

Zeta size

The average hydrodynamic diameter prepared formulations were determined by means of dynamic light scattering measurements using zeta sizer (Fig 3) (Nanotrac wave-3000).

Fig 3: Zeta sizer

Encapsulation efficiency

Entrapment efficiency of coated formulations was calculated by UV Visible Spectrophotometer, the volume of essential oils was measured at λ = 275 nm using method described with some modifications by (Fathi *et al.*, 2021) [7]. Carvacrol and cinnamaldehyde calibration curves were produced by diluting stock solutions at different concentrations and absorbance measured at 275 nm for each standard with the help of a double-beam UV-Vis spectrophotometer. Then, calibration plots of absorbance versus concentration were created on MS-Excel, and the equation and regression coefficient of the graph were noted.

The formulations were centrifuged for 20 minutes at 4°C at 10,000 rpm, and the supernatant was gathered and suitably diluted to fit the calibration ranges. The absorbance of the diluted supernatant was measured, and the concentrations of unencapsulated carvacrol and cinnamaldehyde were computed by interpolating on the corresponding calibration curves. The formulation with the highest encapsulation efficiency was identified.

EE %=[(T-F) / T] × 100;

Where, EE= Encapsulation efficiency

T= Total amount of initially added carvacrol or cinnamaldehyde

F= Free/remaining carvacrol or cinnamaldehyde in the supernatant

Simulated gastrointestinal fluids digestion

The Release experiments were performed according to Chen *et al.*, (2020) [4] description with some adjustments.

Simulated Gastric Fluid (SGF)

Around 20 mL of simulated gastric fluid (sodium chloride (NaCl) 2 mg/ml, pepsin 3.2 mg/ml, and pH adjusted at 1.5) was mixed with an appropriate amount, about 20 ml of carvacrol and 20 ml of cinnamaldehyde loaded formulations, respectively, and incubated in a shaker incubator at 37 °C for two hours. Samples were taken for analysis at different times (0, 5, 15, 30, 60, and 120 minutes).

Simulated Intestinal Fluid (SIF)

A time-dependent release study was carried out under mild agitation for four hours at 0, 5, 15, 30, 60, 120, 180, and 240 minutes at 37°C. The digested sample of SGF was added in a 1:1 ratio to simulated intestinal fluid (K2HPO4 6.8 mg/ml, bile salts 0.2 mg/ml, NaCl 8.8 mg/ml, pancreatin 3.2 mg/ml, and pH was adjusted at 7.4). Following a predetermined amount of shaking at different time interval, samples were collected and equal amount replaced with isopycnic water. Thereafter, the eppendorf tubes were kept immediately in ice cold water for 15 minutes to stop further hydrolysis. The tubes were centrifuged at 5000 rpm for 15 min. at 4°C, supernatant were collected and analyzed using 275 nm with a UV-visible spectrophotometer (Mahobia *et al*., 2017) [12].

Results

Encapsulation efficiency (EE)

In the final formulations, combinations of cholesterol and soy phosphatidylcholine were tested. Liposome coated carvacrol and liposome coated cinnamaldehyde had encapsulation efficiencies mean was75.62±2.03 and 95.10±2.85, respectively.

Table 1: The table represent the results from encapsulation efficiencies of formulations

Preparation	Mean±SD
Liposome coated Carvacrol	75.62±2.03
Liposome coated Cinnamaldehyde	95.10±2.85

Zeta size

Zeta size of the preparations was measured. Liposome coated carvacrol and liposome coated cinnamaldehyde zeta size mean was 63.13±11.64 and 75.67±20.50, respectively illustrated in Fig 4 & 5. Zeta size of chitosan liposome coated carvacrol and chitosan liposome coated cinnamaldehyde mean was 106.00±21.67 and 139.40±11.56, respectively.

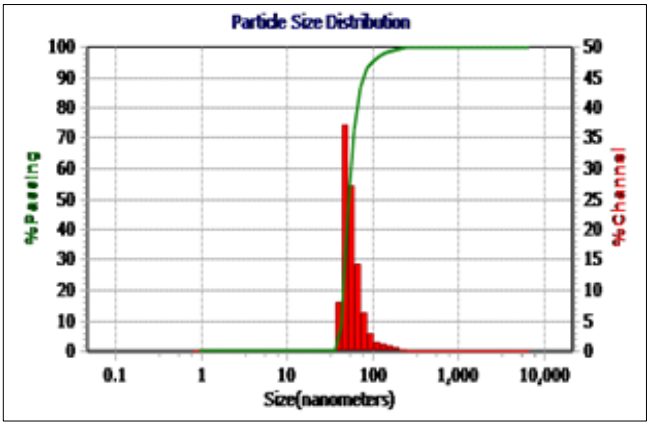

Fig 4: Zeta size of liposome coated carvacrol

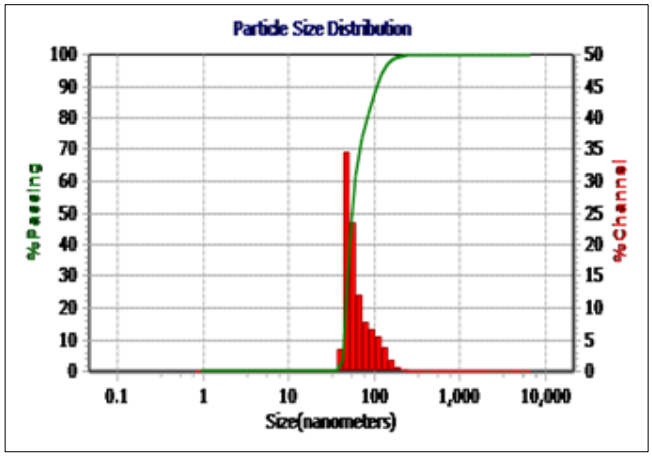

Fig 5: Zeta size of liposome coated cinnamaldehyde

Table 2: The table represent the results from zeta size of formulations

Preparation	Mean±SD
Liposome coated Carvacrol	63.13±11.64
Liposome coated Cinnamaldehyde	75.67±20.50
Chitosan liposome coated Carvacrol	106.00±21.67
Chitosan liposome coated Cinnamaldehyde	139.40±11.56

Release kinetics of formulations in the simulated gastrointestinal fluid

To estimate the cumulative release of essential oil formulations in simulated gastrointestinal fluid several concentrations of chitosan was used. Chitosan liposome coated carvacrol release at the 1:1 ratio and chitosan concentration at 0.1%, 0.3%, and 0.5% was showed 79.75%, 77.05%, and 66.75%, respectively release in simulated gastric fluid, while in intestinal fluid at 0.1%, 0.3%, and 0.5% concentration 100% release occurred at 15 min., 30 min. and 120 min., respectively. Chitosan liposome coated cinnamaldehyde at the concentration of 0.1%, 0.3%, and 0.5% release was 64.38%, 68.47%, and 60.22%, respectively and at intestinal fluid at 240 min 100% release occurred in all formulation. There was a burst release of formulation in gastric fluid. To control this release of essential oil formulations at gastric level different ratio of chitosan and liposome were studied. Chitosan (0.5%) and liposome ratio at 3:1 for carvacrol showed 65.75% release at 120 min. and in SIF 100% release occurred at 30 min, while in ratio 3:2 at 120 min. release was 53.25% and in SIF 100% formulation released.

Chitosan (0.5%) and liposome ratio at 3:1 for cinnamaldehyde release at 120 min. was 48.77% and in SIF 100% release at 240 min, while in ratio 3:2 at 120 min. was 31.51 and in SIF 100% release at 240 min. On the basis of this study, 0.5% concentration of chitosan and 3:2 ratio for chitosan and liposome were used for further study.

Discussion

Encapsulation efficiency of different formulations was measured and results showed that EE of liposome coated carvacrol was less than cinnamaldehyde. This finding was supported by the previous study (Liolios *et al*., 2009) [11] in which lower encapsulation efficiency of carvacrol found. Reason behind that is the carvacrol may get stuck too deeply in the lipid bilayer, which could cause the membrane to pack poorly and leak or not load properly. Contrary, cinnamaldehyde, is moderately lipophilic and more polar, so it may stay more at the bilayer–aqueous interface, which could lead to better encapsulation.

The coating of chitosan layer to liposomes resulted in an increase in zeta size, in contrast to only coating with liposome. Chitosan concentration also affects the liposome size. The bridging between liposomes and chitosan was most likely the cause of the increase of size (Abdelbary, 2011) [1].

In vitro drug release rate of both liposome and chitosan liposome coated carvacrol and cinnamaldehyde, respectively was typically carried out in simulated gastrointestinal fluid at different pH by using the dialysis approach (Sebaaly *et al*., 2021) [22]. Our study found the burst release of chitosan liposome coated formulations occurred in gastric fluid which caused by the release of the drug entrapped near the surface. With the increasing concentration of chitosan 0.5% w/v drug release was delayed and more gradual compared to 0.1 and 0.3% concentrations like the study done by (Gradauer *et al*., 2013) [8]. A variable ratio of chitosan to liposomes was utilized to limit this burst release in gastric fluid; the final formulations were prepared using a 3:2 ratio, which had a considerably slower release was selected for further study. According to this study, the chitosan coating of nanoliposomes reduces membrane fluidity, which is essential for the entrapped medication's release behavior (Nguyen *et al*., 2014) [15].

Conclusion

This study has successfully demonstrated that coating of chitosan to liposomes not only improved their surface charge and stability but also had preserved their antibacterial activity and improved the release profiles in simulated gastrointestinal fluids. The findings highlighted the potential of chitosan-coated liposomes as a smart delivery system for sensitive, bioactive compounds in gut-targeted applications.

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