

ORIGINAL RESEARCH ARTICLE

Preparation and physicochemical characterization of chitosan–sodium tripolyphosphate nanoparticles encapsulating methanol *Rosmarinus officinalis* extract for controlled bioactive delivery

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Abstract

Rosmarinus officinalis exhibits significant antioxidant activity, though its poor bioavailability limits its practical use. In this study, *R. officinalis* was extracted with methanol and encapsulated in chitosan–sodium tripolyphosphate nanoparticles (RCSN) at ratios of 1:0, 1:1, 1:2, and 1:3 (RCSN1–RCSN4) to improve efficacy. Thus, the nanoencapsulated extracts were characterized and evaluated for encapsulation efficiency (EE%), differential scanning calorimetry (DSC), Fourier-transform infrared (FTIR), particle-size analysis, *in vitro* bioactive extract release, and *in vivo* tests. Flavonoids and phenolics were the highest phytoconstituents. EE (%) ranged from 51% to 85%. FTIR spectrum and DSC depicted functional group consistency and decreased crystallinity, respectively. The particle size distribution indicates particle homogeneity and uniformity, with a polydispersity index of 0.434. The *in vitro* study demonstrated a controlled-release profile, with the highest release of 60% (RCSN2). The *in vivo* study of the RCSN showed higher antioxidant activity (10.814 IU/L) compared with the other extracts. The RCSN formulation exhibited sustained release and promising antioxidant potential.

Keywords: Antioxidant; *Rosmarinus officinalis*; Nanoparticles; Chitosan; Sodium tripolyphosphate

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1. Introduction

Plants have always been a source of nourishment and are wholesome ingredients that promote human well-being. Recently, the use of plant extracts as additives in the food industry has gained significant importance due to the abundance of bioactive constituents. *Rosmarinus officinalis* L (Carl Linnaeus) is a perennial, evergreen shrub belonging to the Lamiaceae family, native to the Mediterranean region. It is a globally recognized medicinal plant noted for its rich profile of secondary metabolites, including phenolic diterpenes (carnosol, carnosic acid) and flavonoids (rosmarinic acid).^{1,2} These compounds possess well-documented antioxidant, anti-inflammatory, antimicrobial, and hepatoprotective properties, positioning them as valuable agents in both the food and pharmaceutical industries.^{3,4}

Despite its therapeutic potential, the clinical application of *R. officinalis* extract is mostly hindered by its physicochemical limitations. Plant extracts are highly susceptible to degradation under environmental stressors such as light, temperature, and pH fluctuations, thereby undermining their efficacy.⁵ Moreover, many of these bioactive phenolics exhibit poor water solubility and limited bioavailability when administered in conventional dosage forms.⁶ These factors reduce the effective amount at the site of action, demanding the development of advanced delivery strategies to stabilize the extract and control its release.

Novel bioactive extract delivery systems, particularly nanoparticle-based technologies, have emerged as an important solution to these therapeutic hurdles.^{7,8} This technology enables the manipulation of materials at the molecular scale^{9,10}, enhancing the stability, solubility, and drug-targeting ability while reducing potential side effects.¹¹ Among these, some polymers, such as chitosan, which is biodegradable and biocompatible, have gained significant attention for forming stable nanoparticle matrices with cross-linking agents such as sodium tripolyphosphate via the ionic gelation method.¹²

The preparation of chitosan nanoparticles via the ionic gelation method is a highly efficient, eco-friendly, and simple approach for encapsulating plant extracts.¹³ This technology relies on electrostatic interactions between the positively charged amino groups of chitosan and the negatively charged phosphate groups of sodium tripolyphosphate.¹⁴ By manipulating the chitosan-to-sodium tripolyphosphate ratio, researchers can precisely control the physicochemical characteristics of the lipid nanoparticles.¹⁵ An optimized nanoparticle formulation not only protects sensitive phenolic compounds from the gastric environment but also promotes a controlled release profile, ensuring prolonged therapeutic action.¹⁶

Despite the growing body of literature on *R. officinalis* and nanotechnology, there remains a notable gap in studies that systematically optimize the encapsulation of full-spectrum *R. officinalis* extracts specifically for antioxidant delivery. Many studies focus on isolated single compounds, which may lack the synergistic efficacy present in the crude, multi-component extract. Additionally, there is a lack of standardization in the development of nano-based delivery systems for this particular species. This study addresses critical needs by exploring the formulation and characterization of chitosan–sodium tripolyphosphate nanoparticles loaded with *R. officinalis* extract (RCSN).

Previous studies have highlighted the antioxidant potential of *R. officinalis*; however, there remains a critical need for formulation strategies that bridge the gap between

crude extraction and standardized, bioavailable delivery of bioactive extracts. This study addresses this need by formulating and characterizing *R. officinalis* extract RCSN. The effects of varying chitosan:sodium tripolyphosphate ratios on encapsulation efficiency (EE%), particle morphology, and structural stability were evaluated using Fourier-transform infrared (FTIR) and differential scanning calorimetry (DSC) analyses. Furthermore, the *in vitro* bioactive extract release profiles in simulated gastric and intestinal fluids and the *in vivo* antioxidant efficacy of the developed nanoparticles were investigated, providing a pathway for utilizing *R. officinalis* as a stable, controlled-release therapeutic agent.

2. Materials and methods

2.1. Materials

Rosmarinus officinalis was obtained from the Nsukka market in Enugu State. Rats ($n = 24$) were procured from the Department of Zoology and Environmental Biology, UNN. Other materials included chitosan (ChitoLytic, United States); sodium tripolyphosphate (Tri-Chem Industries, United States); glacial acetic acid (Alfa Chemicals, India); vitamin C (Randox, United Kingdom); malonaldehyde (MDA; Randox, United Kingdom); catalase (CAT; Randox, United Kingdom); and superoxide dismutase (SOD; Randox, United Kingdom). Other reagents were of analytical grade.

2.2. Preparation of *Rosmarinus officinalis* extract and phytochemical tests

Dried *R. officinalis* leaves (450 g) with voucher No. UNN/13330 were milled using an electric blender. The powdered sample was then weighed and recorded. The sample was loaded in a Soxhlet extractor and extracted using about 3 L of organic solvent (methanol). Thereafter, the extract was air-dried to allow complete evaporation of the solvent. The percentage yield was determined, and phytochemical screening was performed to identify the quantitative ($n = 3$) and major phytoconstituents present in the extract according to the previously described method.¹⁷ Subsequently, the extract was stored in a desiccator until use.

2.3. Preparation of *Rosmarinus officinalis* nanoparticles

Rosmarinus officinalis chitosan–sodium tripolyphosphate nanoparticles were formulated using the ionic gelation method (Table 1). Different chitosan:sodium tripolyphosphate ratios (1:0, 1:1, 1:2, and 1:3) were prepared using an appropriate amount of the extract (1 g) and denoted as RCSN1–RCSN4. A solution of chitosan

Table 1. Composition of *Rosmarinus* chitosan–sodium tripolyphosphate nanoparticles

Sample	C: S	ROE (mg)	Chitosan (mg)	S (mg)	Acetic acid (mL)	H ₂ O (mL)
RCSN1	1: 0	1000	500	-	1	99
RCSN2	1: 1	1000	500	500	1	99
RCSN3	1: 2	1000	500	1000	1	99
RCSN4	1:3	1000	500	1500	1	99

Abbreviations: C:S: Chitosan:sodium tripolyphosphate; RCSN: Nanoparticles containing chitosan:sodium tripolyphosphate ratio; ROE: *Rosmarinus officinale* extract; S: Sodium tripolyphosphate.

in acetic acid was prepared by dissolving 0.5 g of chitosan in a solution of 1 mL acetic acid in 99 mL of water. Approximately 0.1 g of *R. officinalis* extract was dissolved in 10 mL of methanol and added to the chitosan solution to give a viscous, homogeneous solution. The sodium tripolyphosphate solution containing 0.5 g in 100 mL of aqueous phase was added to the chitosan extract solution and mixed thoroughly for about 10 minutes. The resultant suspension was centrifuged at 4,000 rpm for 10 minutes, decanted, and air-dried.

2.4. Characterization of the nanoparticles

2.4.1. Encapsulation efficiency

The EE% of the nanoparticle was determined by centrifugation, as previously reported.¹⁸ A 1 mL volume of the bioactive extract-loaded nanoparticles of different concentrations was centrifuged at 4,000 rpm for 10 minutes. Then, the supernatant was withdrawn and analyzed for free bioactive extract using an ultraviolet–visible spectrophotometer (Spectrumlab 752, Netherlands) at 370 nm. The bioactive extract EE% was determined.

2.4.2. Particle size analysis

Particle sizes of the dried methanol extract of *R. officinalis*, excipients, and RCSN were determined by computerized image analysis on a photo microscope (PSA-900, Scitek Global, China). A 5 mg sample of the dried methanol extract of *R. officinalis* was dispersed in a small amount of distilled water on a microscope slide, covered with a coverslip, and observed under a light microscope. The diameter of the particle of each material was determined at a magnification of 400×. The particle morphology was observed and captured using a photomicrograph.

2.4.3. Fourier-transform infrared analysis

The FTIR spectra of dried methanol extract of *R. officinalis*, excipients, and RCSN formulation were analyzed in the range of 4,000–650 cm⁻¹ at a resolution of 4 cm⁻¹.¹⁹ First, the potassium bromide plate was cleaned with an organic solvent prior to baseline scanning. A 10 mg sample of the

dried extract of *R. officinalis* was placed on the plate and pressed to make a pellet. The formed pellet was placed in the spectrophotometer's light path, and an infrared spectrum was obtained using an Agilent Technologies FTIR-Cary 630 (United States).

2.4.4. Differential scanning calorimetry

The DSC thermograms of the dried methanol extract, excipients, and the RCSN sample were measured using a DSC instrument (DSC 20481, Netzsch, Germany). The samples were analyzed by measuring a portion of each sample inside an aluminum crucible with a pierced lid at 30–180 °C and a heating rate of 10 °C/min. The DSC was performed to assess the thermal properties and crystallinity of the samples and to observe any chemical interaction between the constituents.

2.5. In vitro release of the bioactive extract and release kinetics studies

An *in vitro* bioactive extract release study of *R. officinalis* nanoparticles was conducted in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8) using a dialysis membrane (MW 6,000–8,000). Approximately 10 mg of *R. officinalis* was enclosed in a dialysis membrane, hermetically sealed at its ends, and placed in a 500 mL beaker containing 500 mL of dissolution medium, maintained at 37 ± 1 °C and agitated at 100 rpm by a magnetic stirrer. At predetermined time intervals, a 5 mL aliquot of the dissolution medium was withdrawn and immediately replaced with 5 mL of fresh medium for five hours. The withdrawn sample medium was appropriately diluted and analyzed for bioactive extract content spectrophotometrically (Spectrumlab 752, Netherlands) at 370 nm; the amount of *R. officinalis* released each time was determined in triplicate.

The release mechanisms of the RCSN formulation, obtained from the release data, were fitted to different kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas equations. The goodness of fit of each model was evaluated using the coefficient of

determination (R^2), and the model with the highest R^2 value was considered the best fit to describe the release behavior.

2.6. In vivo study

The animal experiment was conducted according to the guidelines established by the Research Ethical Committee of the Faculty of Pharmaceutical Science, University of Nigeria, Nsukka, with the approval code: FPSRE/UNN/23/00023, which adhered to the Animal Science Association and the European Federation of Animal Science guidelines for the use of experimental animals (2010/63/EU). Twenty-four female rats weighing 50–180 g were used for this study. They were labelled and grouped into four groups, with each group containing six rats: (i) Group A received nanoparticles (RCSN); (ii) Group B received dispersions of the pure extract; (iii) Group C was the control group (no treatment); and (iv) Group D received syrup of vitamin C. About 10 mg/kg of the nanoparticles, pure extract, and vitamin C were administered orally according to the weight of each rat. The stock solutions to be ingested orally by the rats were prepared by dissolving the weighed sample in 3% Tween 80. The experimental animals were fasted for 24 hours prior to the administration of the bioactive extract. Adequate doses of the bioactive extract (about 10 mg/kg) were administered orally daily for seven days, after which samples of blood were collected from each rat through the medial canthus of the eye. These blood samples were then centrifuged at 3,000 rpm for 5 minutes to separate plasma and serum, tested for antioxidant activity by determining the levels of SOD, CAT, and MDA using spectrophotometry, and compared with a standard antioxidant drug, vitamin C syrup.

2.7. Statistical analysis

Results were expressed as mean $\pm$ standard deviation. One-way analysis of variance with Tukey's post-hoc test was used to assess significance at $p < 0.05$.

3. Results and discussion

3.1. Phytochemical results of *Rosmarinus officinalis*

The phytochemical screening results presented in Table 2 indicated that the plant extract contained alkaloids, flavonoids, saponins, glycosides, steroids, terpenoids, and phenolics. This indicates the quality of the *R. officinalis* extract and provides insight into the potential therapeutic and pharmacological activity it may exert. Further quantification of these phytochemicals (Table 2) showed that phenolics and flavonoids had the highest concentrations in the plant extract. This explains the high

antioxidant and antibacterial activity of *R. officinalis*. The percentage yield of the *R. officinalis* extract obtained was 22.42%. The percentage yield was not very high, possibly due to transfer losses and impurities in the raw ground sample.

Table 2. Qualitative and quantitative phytochemicals of *R. officinalis*

Metabolite	Qualitative	Quantitative (mg/100 g $\pm$ standard deviation)
Alkaloids	+	716.00 $\pm$ 81.62
Flavonoids	+	1,078.26 $\pm$ 22.59
Saponins	+	3.81 $\pm$ 0.98
Steroids	+	18.08 $\pm$ 2.82
Terpenoids	+	762.29 $\pm$ 61.23
Phenolics	+	1,213.13 $\pm$ 58.68
Tannins	+	173.59 $\pm$ 6.7

Key: + = Present; - = Absent; quantitative result as mean $\pm$ standard deviation; $n = 3$.

3.2. Encapsulation efficiency

Encapsulation efficiency helps us determine how much of the bioactive extract is loaded into a nanoparticle and how much it can carry. The EE% of the RCSN were $70 \pm 0.10\%$, $51 \pm 0.10\%$, $85 \pm 0.01\%$, and $64 \pm 0.02\%$ ($n = 3$), respectively for RCSN1–4. RCSN3 (containing chitosan:sodium tripolyphosphate at 1:3) showed the highest EE%.

3.3. Fourier-transform infrared analysis

Fourier transform infrared technique is a spectroscopic method that detects alterations in the overall composition of biomolecules by assessing variations in functional groups. FTIR measures the vibrational and rotational motions of molecules induced by infrared radiation at specific wavelengths, thereby identifying structural differences in molecular binding between entities. This approach provides insights into the presence of interactions and offers details about their nature. The FTIR (Figure 1) results for chitosan and sodium tripolyphosphate showed their highest peaks at $3,347.1 \text{ cm}^{-1}$ and $3,630.4 \text{ cm}^{-1}$, respectively, indicating the presence of the O–H functional group, which is associated with their high solubility. The FTIR results for the methanol extract of *R. officinalis* and the nanoparticle formulation (RCSN) showed broad peaks at $3,291.2 \text{ cm}^{-1}$ and $3,242.8 \text{ cm}^{-1}$, respectively, indicating the presence of the O–H functional group and thus increasing the solubility profiles of both the extract and the nanoparticle. The peaks identified in the wavenumber spectrum at $2,944.6 \text{ cm}^{-1}$ and $2,922.2 \text{ cm}^{-1}$ indicate the

presence of the C–H bond in methylene. The spectral results indicate that the formulation is compatible with each of the substances it interacted with and that there were no abnormal chemical interactions, thereby improving its stability and bioavailability.

3.4. Differential scanning calorimetry

The thermograms of the methanol extract of *R. officinalis*, chitosan, sodium tripolyphosphate, and RCSN (Figure 2) showed 76 °C, 150 °C, 105 °C, and 96 °C, respectively. The broad endothermic peaks observed in the nanoparticles

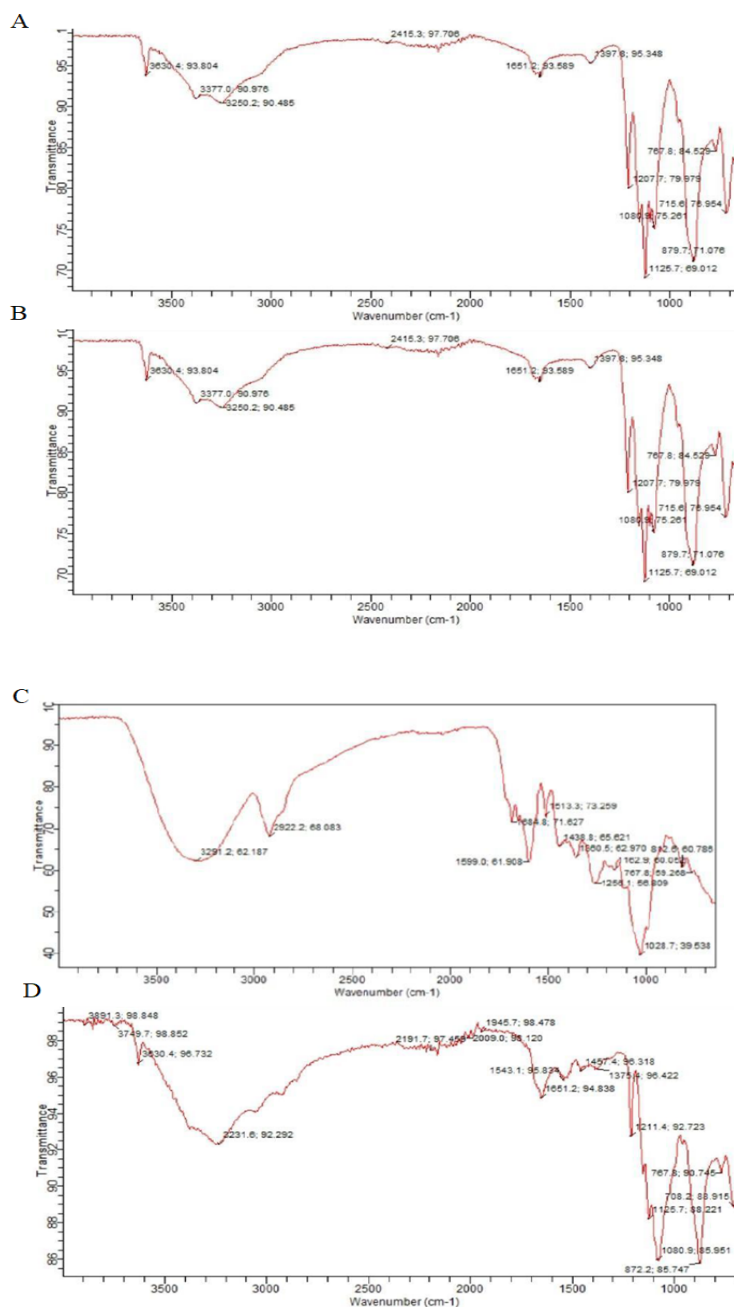


Figure 1. Fourier-transform infrared analysis. (A) Chitosan, (B) sodium tripolyphosphate, (C) *Rosmarinus officinalis* extract, and (D) chitosan nanoparticles of *Rosmarinus officinalis*.

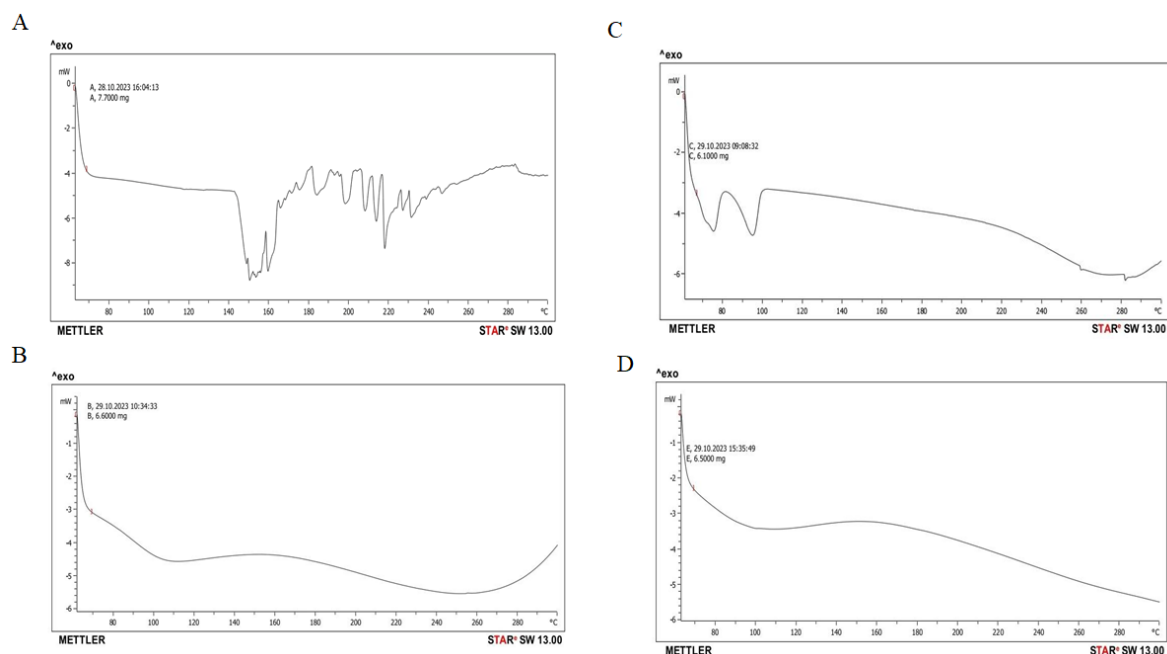


Figure 2. Differential scanning calorimetry of (A) chitosan, (B) sodium tripolyphosphate, (C) *Rosmarinus officinalis* extract, and (D) chitosan nanoparticles of *Rosmarinus officinalis*

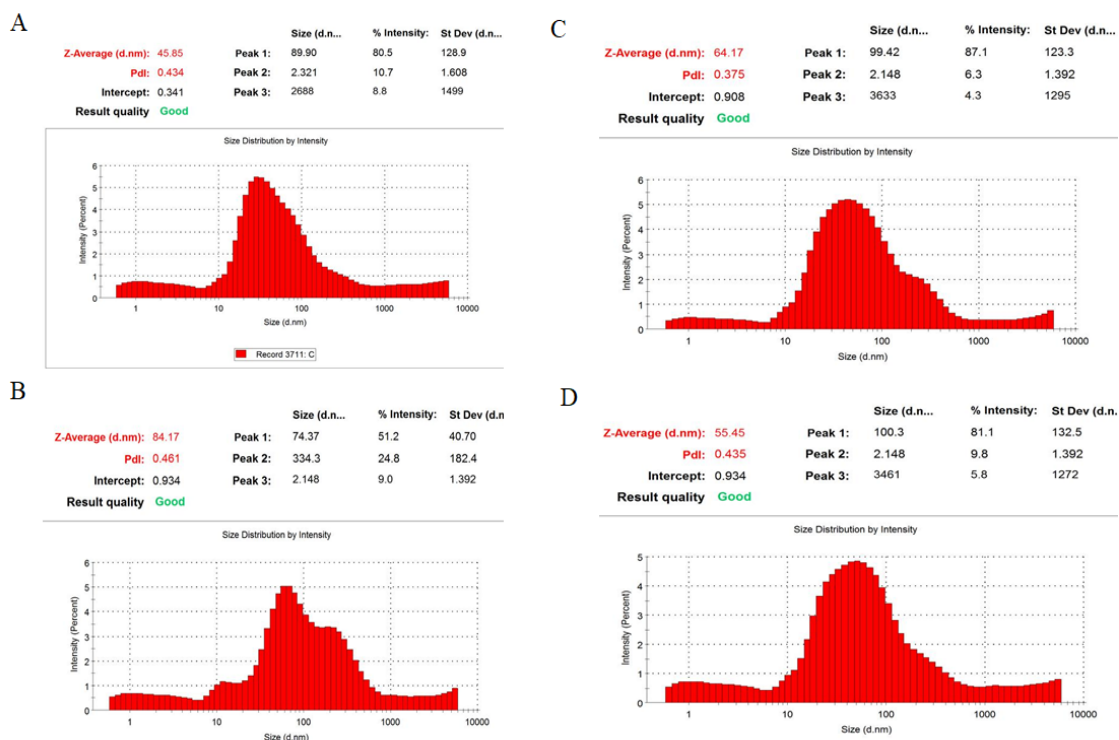


Figure 3. Particle sizes with polydispersity index (PDI) of (A) chitosan, (B) sodium tripolyphosphate, (C) *Rosmarinus officinalis* extract, and (D) chitosan nanoparticles of *Rosmarinus officinalis*

indicated the amorphous nature of the formulation and showed good solubilization of excipients in the matrices.

3.5. Particle size analysis

The results for the particle size and polydispersity index (PDI) of the chitosan, sodium tripolyphosphate, methanol extract of *R. officinalis*, and RCSN (Figure 3) were 84.17, 64.17, 45.845, and 39.60 nm, respectively. The particle sizes were all in the nanoscale range, with the RCSN having the smallest nano size. This indicated that the formulation technique employed was a good choice. The PDI was used to estimate the average uniformity of a particle solution, and higher PDI values indicate a broader size distribution in the particle sample. A sample is considered monodisperse when the PDI value is less than 0.1.²⁰ The numerical value of PDI ranges from 0.0 (for a perfectly uniform sample with respect to the particle size) to 1.0 (for a highly polydisperse sample with multiple particle size populations). Values of 0.2 and below are most commonly deemed acceptable in practice for polymer-based nanoparticle material.²¹ From the results obtained, the PDI values for chitosan, sodium tripolyphosphate, the methanol extract of *R. officinalis*, and the chitosan nanoparticle formulation were 0.461, 0.375, 0.434, and 0.501, respectively. This indicates that the formulation had a narrow particle size distribution.

3.6. In vitro bioactive extract release study and release kinetics of chitosan–sodium tripolyphosphate nanoparticles loaded with *R. officinalis* extract

In vitro release of *R. officinalis* in RCSN, as presented in Figure 4, showed a maximum bioactive extract release of 23, 42, 29, and 23% in SGF (pH 1.2) and 20, 60, 40, and 50% in SIF (pH 6.8) at five hours for RCSN1, RCSN2, RCSN3, and RCSN4, respectively. The nanoparticles demonstrated sustained release of bioactive extract in both media, with a controlled release profile. This indicated the impact of the different concentrations of chitosan:sodium tripolyphosphate on the bioactive extract release profile of the nanoparticles. Formulation RCSN2 showed the highest percentage release. This may be due to the presence of chitosan and sodium tripolyphosphate in equal proportions in the nanoparticles.

The release kinetics of RCSN obtained in SGF (pH 1.2) and SIF (pH 6.8) were fitted to zero-order, first-order, Higuchi, and Ritger–Peppas models to determine the release mechanism, with the models selected based on the highest correlation coefficient (R^2).²² In SIF, all the nanoparticles showed the best fit to the Higuchi model (R^2 : 0.97–0.98), indicating diffusion-controlled release, and to the Korsmeyer–Peppas model (R^2 : 0.98; $n > 0.89$),

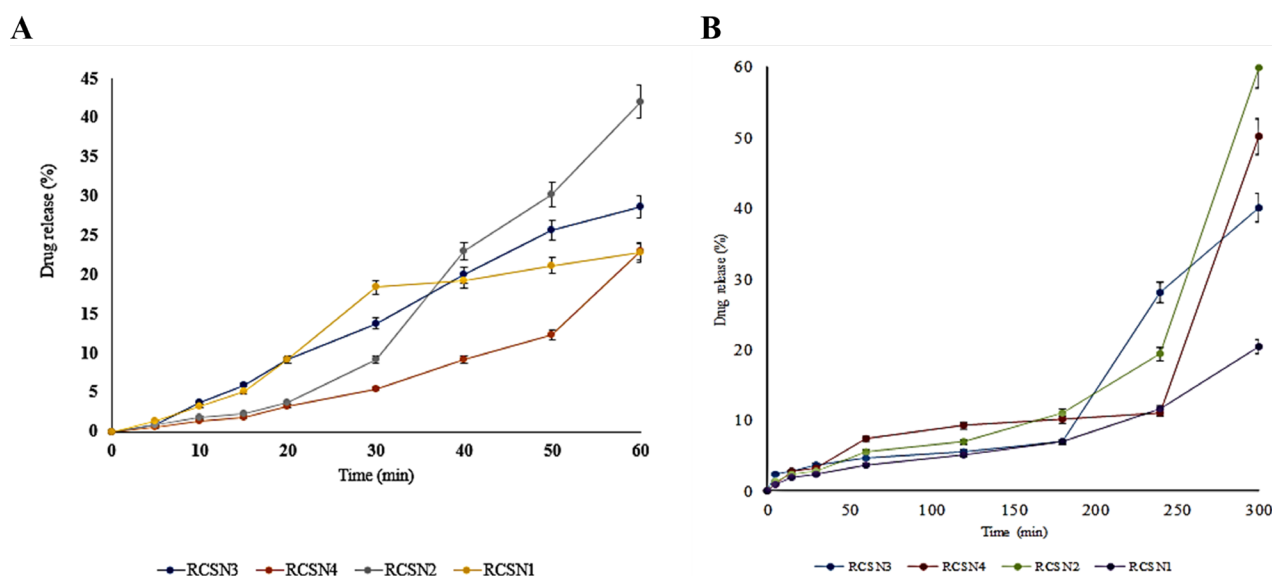


Figure 4. *In vitro* release of *Rosmarinus officinalis* nanoparticles (RCSN) in (A) simulated gastric fluid and (B) simulated intestinal fluid
Note: RCSN1–4 represent *Rosmarinus officinale* nanoparticles containing chitosan:sodium tripolyphosphate ratios 1:0, 1:1, 1:2, and 1:3, respectively.

Table 3. Release kinetic study of *Rosmarinus officinalis* nanoparticles in simulated gastric fluid

Kinetic model	Parameter	RCSN1	RCSN2	RCSN3	RCSN4
Zero-order	R^2	0.9301	0.9262	0.9945	0.8923
First-order	R^2	0.9203	0.9235	0.9959	0.8770
Higuchi	R^2	0.9662	0.9478	0.9805	0.9836
Ritger–Peppas	R^2	0.9662	0.9478	0.9805	0.9836
	n	1.1985	1.6338	1.3452	1.4718

Note: RCSN1–4 represent *Rosmarinus officinale* nanoparticles containing chitosan:sodium tripolyphosphate ratios 1:0, 1:1, 1:2, and 1:3, respectively.

Table 4. Release kinetic study of *Rosmarinus officinalis* nanoparticles in simulated intestinal fluid

Kinetic model	Parameter	RCSN1	RCSN2	RCSN3	RCSN4
Zero-order	R^2	0.8958	0.7195	0.8096	0.6467
First-order	R^2	0.8774	0.5962	0.7900	0.6413
Higuchi	R^2	0.9361	0.8547	0.7208	0.8766
Ritger–Peppas	R^2	0.3961	0.8547	0.6986	0.8766
	n	0.6733	0.7846	0.492	0.7639

Note: RCSN1–4 represent *Rosmarinus officinale* nanoparticles containing chitosan:sodium tripolyphosphate ratios 1:0, 1:1, 1:2, and 1:3, respectively.

indicating that the bioactive extract release followed super case II transport (Table 3). RCSN1–3 fitted equally well to zero- and first-order kinetics, with R^2 values of 0.92–0.99, suggesting concentration-dependent and mixed-order release mechanisms from the nanoparticles. In SIF (Table 4), only the Higuchi model provides the best fit for RCSN1 (R^2 : 0.94). The overall RCSN release was governed by polymer–polymer interactions, with chitosan-rich nanoparticles majorly promoting sustained, diffusion-controlled release.

3.7. In vivo study

Superoxide dismutase and CAT are the primary enzymatic first responders that neutralize reactive oxygen species, whereas MDA serves as a direct biomarker of lipid peroxidation, which is associated with cellular membrane destruction. The *in vivo* antioxidant activity of the orally

taken nanoparticles compared to the standard (vitamin C) and other controls is shown in Table 5. MDA showed a significant ($p < 0.05$) decrease in the treated Groups A, B, and D (groups that received the nanoparticles [RCSN], the extract of *R. officinalis*, and the standard [vitamin C], respectively) compared to the untreated Group C (control, which had no treatment). This indicates that Group C (which receives no treatment) exhibited the lowest levels of protective enzymes (SOD and CAT) and the highest level of cellular damage due to MDA. This suggests that the bioactive extract exhibits antioxidative properties. Group A showed higher SOD and CAT levels ($p < 0.05$) when compared to Group B in both SOD and CAT tests ($p < 0.05$). Thus, this is consistent with a previous report that suggested that SOD and CAT are more sensitive tests than the MDA test because it specifically measures the activity of an enzyme that helps to neutralize reactive oxygen species.²³

Table 5. In vivo test results for the antioxidant activity of dried methanol extract and chitosan nanoparticle formulation of *Rosmarinus officinalis*

Group	Superoxide dismutase (IU/L $\pm$ SD)	Malondialdehyde (mg/dL $\pm$ SD)	Catalase (mg/dL $\pm$ SD)
A	10.814 $\pm$ 0.001*	2.584 $\pm$ 0.110	0.849 $\pm$ 0.211
B	9.846 $\pm$ 0.110*	3.614 $\pm$ 0.122	0.684 $\pm$ 0.321
C	8.412 $\pm$ 0.123	4.942 $\pm$ 0.144	0.246 $\pm$ 0.501
D	10.511 $\pm$ 0.101*	2.305 $\pm$ 0.200	0.795 $\pm$ 0.208

Note: Results are shown as mean $\pm$ standard deviation (SD); *asterisks $p < 0.05$.

4. Conclusion

The current research successfully demonstrated the feasibility of encapsulating RCSN via ionic gelation. The findings confirm that the physicochemical challenges associated with *R. officinalis* extract can be mitigated by this nano-delivery strategy. This technology shielded the delicate bioactive extract from premature degradation in the gastrointestinal tract. The chitosan, being mucoadhesive, enhanced steady, prolonged absorption, which, in synergy with the sodium tripolyphosphate, controlled and sustained the release of the bioactive extract. This may provide clinical benefits in the treatment of chronic oxidative stress. This research can serve as a foundation for developing standardized, bioavailable *R. officinalis* formulations, paving the way for future pharmacokinetic studies and clinical applications in managing oxidative stress-related disorders.

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Conflict of interest

The authors declare no conflict of interest.

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Supervision: Calister E. Ugwu

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Writing—review & editing: Calister E. Ugwu

Ethics approval and consent to participate

The animal experiment was conducted according to the guidelines established by the Research Ethical Committee of the Faculty of Pharmaceutical Science, University of Nigeria, Nsukka, with the approval code: FPSRE/UNN/23/00023, which adhered to the Animal Science Association and the European Federation of Animal Science guidelines for the use of experimental animals (2010/63/EU).

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request

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