



FORMULATION AND EVALUATION OF ACETOGENIN-LOADED GELLAN GUM POLYMERIC NANOPARTICLES FOR POTENTIAL COLORECTAL CANCER APPLICATION

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ABSTRACT

Annonaceous acetogenins obtained from *Annona muricata* L. have attracted considerable interest because of their reported biological and anticancer potential; however, their pharmaceutical application may be limited by physicochemical and drug-delivery-related challenges. The present study aimed to extract and characterize an acetogenin-containing fraction from *A. muricata* leaves and formulate it into gellan gum polymeric nanoparticles for potential application in colorectal cancer research. The dried leaves were extracted with ethanol using Soxhlet extraction, and the acetogenin-containing fraction was preliminarily characterized using Kedde's reagent test, thin-layer chromatography (TLC), silica-gel column chromatography, UV-Visible spectroscopy and high-performance liquid chromatography (HPLC). The selected fraction was incorporated into gellan gum polymeric nanoparticles using a cross-emulsion technique with Aerosol OT (AOT) as surfactant and calcium chloride as cross-linking agent. Ethanolic extraction produced a yield of 15.13% w/w, while Kedde's reagent produced a characteristic purple colour indicating the presence of acetogenin-type constituents. TLC using n-hexane:acetone (1:1) produced a selected band with an R_f value of 0.44. Among the 12 fractions obtained by column chromatography, F4 and F5 showed positive Kedde reactions. UV-Visible analysis showed a maximum absorption at 229.5 nm, while HPLC analysis demonstrated a prominent peak at a retention time of 8.070 min. The developed acetogenin-loaded gellan gum nanoparticles exhibited a mean particle size of 50.3 nm, a polydispersity index of 0.198 and a zeta potential of -15.0 mV. SEM analysis revealed irregular to near-spherical nanoparticles ranging from 36.4 to 86.0 nm, with an approximate mean size of 54.4 nm. These findings demonstrate the successful preliminary enrichment and characterization of acetogenin-type constituents from *A. muricata* leaves and their incorporation into a nanosized gellan gum polymeric carrier. The developed formulation provides a promising platform for further investigation, including encapsulation efficiency, drug loading, in-vitro release, stability and cytotoxicity studies against colorectal cancer cell lines, before its therapeutic potential can be established.

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1. INTRODUCTION:

Colorectal cancer (CRC) is a malignant disease originating primarily from the epithelial cells of the colon or rectum and represents a major global health burden. According to GLOBOCAN 2022 estimates, colorectal cancer accounted for approximately 1.93 million new cases and 904,000 deaths worldwide, representing about 9.6% of all newly diagnosed cancers and 9.3% of global cancer-related deaths [1]. The development of CRC is a multistep process involving the progressive accumulation of genetic and epigenetic alterations that can transform normal colorectal epithelium through precursor lesions into invasive and metastatic malignancy. Both non-modifiable and modifiable factors contribute to CRC risk, including increasing age, hereditary predisposition, family history, inflammatory bowel disease, obesity, physical inactivity, dietary factors, smoking and alcohol consumption [1,2].

Current management of CRC involves surgery, chemotherapy, radiotherapy, targeted therapies and immunotherapy, selected according to tumor location, molecular characteristics and disease stage. Cytotoxic agents such as 5-fluorouracil, oxaliplatin and irinotecan remain important components of systemic treatment. Despite advances in treatment, therapeutic efficacy can be restricted by systemic toxicity, inadequate drug accumulation at tumor sites and the emergence of drug resistance. These limitations have stimulated interest in alternative bioactive compounds and drug-delivery strategies capable of improving the therapeutic index of anticancer agents [2,3].

Medicinal plants constitute an important source of structurally diverse bioactive compounds and continue to attract attention in anticancer drug discovery. *Annona muricata* L., belonging to the family Annonaceae and commonly known as soursop, graviola or guanabana, is an evergreen tropical plant traditionally used for a variety of medicinal purposes. Phytochemical investigations of its leaves, bark, roots, fruits and seeds have identified numerous secondary metabolites, particularly alkaloids, phenolic compounds, flavonoids and annonaceous acetogenins. Among these constituents, annonaceous acetogenins have received particular attention because of their potent biological and cytotoxic activities [4–6]. More than 100 acetogenins have been reported from different parts of *A. muricata*, with the leaves representing an important source of these compounds [4].

Annonaceous acetogenins are a distinctive group of polyketide-derived, long-chain fatty-acid derivatives typically containing a terminal α,β -unsaturated γ -lactone together with one or more tetrahydrofuran (THF) rings and hydroxyl substituents [7,8]. Their biological activity has been associated particularly with inhibition of mitochondrial NADH oxidoreductase (complex I), resulting in disruption of mitochondrial electron transport and depletion of cellular ATP [7,9]. Because cancer cells have substantial metabolic requirements, interference with mitochondrial energy production represents one proposed mechanism contributing to the cytotoxic activity of these compounds. Nevertheless, the biological effects of individual acetogenins depend on their molecular structure, concentration and experimental model, and their potential toxicity must also be considered [6–9].

Experimental evidence provides a specific rationale for investigating *A. muricata* acetogenins in colorectal cancer. An ethyl acetate fraction of *A. muricata* leaves demonstrated cytotoxic effects against human HT-29 and HCT-116 colon cancer cells, accompanied by G1-phase cell-cycle arrest, increased reactive oxygen species, loss of mitochondrial membrane potential, cytochrome-c release, caspase activation, up-regulation of Bax and down-regulation of Bcl-2 [10]. In another bioassay-guided investigation, anomuricin E isolated from *A. muricata* leaves inhibited HT-29 colon cancer cell growth and induced mitochondria-mediated apoptosis; the corresponding leaf fraction also reduced azoxymethane-induced aberrant crypt foci in an experimental rat model [11]. Earlier phytochemical investigations have similarly reported acetogenins isolated from *A. muricata* with cytotoxic activity against HT-29 colon adenocarcinoma cells [12]. Collectively, these findings support further investigation of acetogenin-rich fractions as potential candidates for colorectal-cancer-oriented drug-delivery research, while clinical efficacy in humans remains to be established.

Despite their promising biological activity, translation of plant-derived bioactive constituents into pharmaceutical products can be limited by physicochemical and biopharmaceutical challenges, including poor aqueous solubility, instability, variable absorption, limited bioavailability and inadequate delivery to the desired site. Nanotechnology-based drug-delivery systems

provide an opportunity to address some of these limitations. Polymeric nanoparticles are submicron carrier systems capable of incorporating bioactive compounds within a polymeric matrix or associating them with the nanoparticle surface. Such systems can protect incorporated compounds from degradation, alter their release profile and modify their interaction with biological environments [3]. Polymeric nanoparticles have consequently attracted considerable interest for anticancer drug delivery, including approaches directed toward colorectal and colon-specific therapy [3].

Natural polysaccharides are particularly attractive materials for pharmaceutical nanocarriers because of properties such as biodegradability, biocompatibility and the possibility of modifying their physicochemical characteristics. Gellan gum is a high-molecular-weight anionic exopolysaccharide produced by bacterial fermentation and possesses the ability to undergo ion-mediated gelation. Its capacity to form cross-linked matrices in the presence of suitable cations has enabled its investigation in hydrogels, beads, films and nanoparticulate drug-delivery systems [13]. Recent literature has also highlighted the potential of gellan-gum-based carriers for controlled and anticancer drug delivery because of their adaptable physicochemical characteristics, biodegradability and capacity to form stable drug-containing matrices [13].

In this context, incorporation of acetogenin-containing fractions into gellan-gum-based polymeric nanoparticles may provide a formulation strategy for improving the pharmaceutical characteristics of these plant-derived bioactive constituents. However, information concerning gellan-gum nanoparticulate delivery of acetogenins obtained from *A. muricata* leaves for colorectal-cancer-related applications remains limited. Development and physicochemical characterization of such a formulation are therefore important preliminary steps before its biological efficacy and safety can be established.

Accordingly, the present study was undertaken to extract and characterize an acetogenin-containing fraction from *Annona muricata* leaves and to formulate it into gellan gum polymeric nanoparticles using a cross-emulsion technique. The acetogenin-containing fraction was investigated using Kedde's reagent test, thin-layer chromatography (TLC), column chromatography, UV-Visible spectroscopy and high-performance liquid chromatography

(HPLC). The prepared nanoparticles were subsequently characterized for particle size, polydispersity, zeta potential and surface morphology using scanning electron microscopy. The study was intended to establish the physicochemical characteristics of an acetogenin-loaded gellan gum nanoparticulate system as a preliminary platform for subsequent investigation in colorectal cancer.

To formulate and evaluate acetogenin-loaded gellan gum polymeric nanoparticles as a potential drug-delivery system for colorectal-cancer-related applications. The objectives of the study were to extract an acetogenin-containing fraction from *Annona muricata* leaves; characterize the fraction using appropriate phytochemical and chromatographic techniques; formulate acetogenin-loaded gellan gum polymeric nanoparticles by the cross-emulsion method; and characterize the developed nanoparticles with respect to particle size, polydispersity, zeta potential and surface morphology.

2. MATERIALS AND METHODS

2.1. Materials

Fresh leaves of *Annona muricata* L. were used as the plant material for the preparation of an acetogenin-containing fraction. Ethanol was used as the extraction solvent. Gellan gum was employed as the polymer for nanoparticle preparation, Aerosol OT (AOT; sodium bis(2-ethylhexyl) sulfosuccinate) as the surfactant, calcium chloride (CaCl_2) as the cross-linking agent, and methylene chloride as the organic phase. Silica gel was used as the stationary phase for thin-layer chromatography (TLC) and column chromatography. Analytical-grade chemicals and solvents were used for extraction, phytochemical screening and nanoparticle preparation, while HPLC-grade solvents were used for chromatographic analysis.

Kedde's reagent was employed for the preliminary detection of annonaceous acetogenins. The reaction is useful for detecting the α,β -unsaturated γ -lactone functionality characteristic of many annonaceous acetogenins and produces a characteristic pink-to-purple coloration [7,14].

2.2. Collection and Authentication of Plant Material

Fresh and healthy leaves of *Annona muricata* L. were collected from Tiruvannamalai, Tamil Nadu, India. The leaves were cleaned thoroughly to remove adhering dust and extraneous matter. The plant material was authenticated by a qualified

botanist from Kalaingar Karunanidhi Government Arts College, Tiruvannamalai, Tamil Nadu. A voucher specimen was prepared and deposited for future reference under voucher specimen number 2026-204.

The authenticated leaves were shade-dried at room temperature until sufficiently dry and subsequently pulverized using a mechanical grinder to obtain a coarse powder. The powdered material was transferred to a clean, dry and airtight container and protected from moisture and direct light until extraction.

2.3. Preparation of Ethanolic Extract of *Annona muricata* Leaves

A quantity of 35 g of powdered *A. muricata* leaves was extracted with 200 mL of ethanol using a Soxhlet apparatus for 24 h. Ethanolic extraction has been employed in investigations of *A. muricata* phytoconstituents, including studies involving acetogenin-containing fractions [4,15].

After completion of extraction, the extract was filtered to remove insoluble plant material. The filtrate was concentrated under reduced pressure using a rotary evaporator at a controlled temperature, preferably not exceeding approximately 40–45 °C, to minimize thermal degradation of the phytoconstituents. The concentrated material was further dried to obtain the ethanolic extract.

The percentage extraction yield was calculated using the following equation:

$$\text{Percentage yield (\%)} = (\text{Weight of dried extract} / \text{Weight of dried plant material}) \times 100$$

The dried extract was transferred into an airtight amber-coloured container and maintained under refrigerated conditions until further investigation.

2.4. Preliminary Phytochemical Screening

The ethanolic leaf extract of *A. muricata* was subjected to qualitative phytochemical screening for major groups of secondary metabolites, including alkaloids, flavonoids, phenolic compounds, tannins, saponins, glycosides and terpenoid/steroidal constituents using standard phytochemical procedures.

Particular attention was given to annonaceous acetogenins because *A. muricata* is a well-established source of this structurally distinctive class of secondary metabolites [5,7].

2.5. Detection of Acetogenins by Kedde's Reagent Test

The ethanolic extract was screened for acetogenins using Kedde's reagent. A small quantity of the

extract was dissolved in a suitable solvent and treated with freshly prepared Kedde's reagent. The appearance of a pink, rose, magenta or purple coloration was regarded as a positive reaction for compounds possessing the α,β -unsaturated γ -lactone moiety characteristic of many annonaceous acetogenins.

Kedde's reagent-based visualization has been extensively employed for the detection of annonaceous acetogenins during TLC and chromatographic fractionation [14,16]. Therefore, the Kedde-positive reaction was used as preliminary evidence for the presence of acetogenin-type compounds rather than as definitive structural identification.

2.6. Thin-Layer Chromatography

TLC was performed for preliminary chromatographic profiling of the ethanolic extract and identification of fractions showing a positive reaction for acetogenins. Silica gel 60 F₂₅₄ precoated TLC plates were used as the stationary phase.

An appropriate quantity of the extract was dissolved in methanol and applied onto the TLC plate. The chromatogram was developed in a previously saturated chromatographic chamber using *n*-hexane:acetone (1:1, v/v) as the mobile phase.

After development, the plate was removed from the chamber, the solvent front was immediately marked and the chromatogram was allowed to air-dry. The developed plate was examined under UV light where applicable and subsequently treated with Kedde's reagent. Pink-to-purple bands produced after treatment with Kedde's reagent were considered indicative of acetogenin-containing regions. Silica-gel TLC followed by Kedde-reagent visualization is an established approach for screening acetogenins in *Annona* extracts [14,16].

The retention factor (R_f) was calculated as:

$$R_f = \text{Distance travelled by the compound} / \text{Distance travelled by the solvent front}$$

Fractions exhibiting Kedde-positive chromatographic bands were selected for subsequent fractionation.

2.7. Isolation of the Acetogenin-Containing Fraction by Column Chromatography

The ethanolic extract was subjected to silica-gel column chromatography to obtain an acetogenin-enriched fraction. A glass chromatographic column was packed with silica gel using an appropriate solvent. The crude extract was adsorbed onto a small quantity of silica gel, dried to obtain a free-flowing material and carefully applied to the packed column.

Elution was carried out using solvents of progressively increasing polarity. The eluates were collected as individual fractions and concentrated where required. Each collected fraction was monitored by TLC followed by Kedde-reagent visualization.

Fractions exhibiting similar chromatographic behaviour and Kedde-positive bands were combined to obtain an acetogenin-containing/enriched fraction. Comparable approaches involving silica-gel column chromatography, TLC monitoring and Kedde-reagent detection have been employed for the isolation and enrichment of annonaceous acetogenins [14,16]. The selected fraction was concentrated and stored in an airtight amber-coloured container under refrigerated conditions until further characterization and nanoparticle formulation.

2.8. UV–Visible Spectroscopic Characterization

The selected acetogenin-containing fraction was subjected to UV–Visible spectroscopic analysis. An appropriate quantity of the fraction was dissolved in a suitable spectroscopic-grade solvent and scanned over the selected wavelength range using a UV–Visible spectrophotometer.

The absorption spectrum was recorded and the wavelength of maximum absorption (λ_{max}) was determined. The UV spectrum was used as supportive characterization in combination with Kedde's reaction, TLC and chromatographic analysis.

Annonaceous acetogenins generally possess relatively weak UV chromophores. Published structural investigations have reported absorption in the low-UV region associated with the unsaturated γ -lactone moiety; for example, an acetogenin isolated from *A. muricata* fruit pulp exhibited a λ_{max} of approximately 209 nm [14]. Consequently, UV–Visible spectroscopy alone was not regarded as sufficient for definitive identification of an individual acetogenin.

2.9. HPLC Analysis of the Acetogenin-Containing Fraction

High-performance liquid chromatography was performed for chromatographic characterization of the selected acetogenin-containing fraction. The fraction was dissolved in HPLC-grade methanol and filtered through a 0.22 μm membrane filter before injection.

Chromatographic separation was performed using a reverse-phase C18 column. The mobile phase consisted of methanol and distilled/deionized water,

delivered at a flow rate of 1.0 mL/min. The injection volume was 200 μL , and detection was performed at 240 nm under the experimental conditions used in the present study.

Reverse-phase C18 chromatography is well established for separation of annonaceous acetogenins. Gromek et al. demonstrated separation of *A. muricata* acetogenins using a $\mu\text{Bondapak}$ C18 column with water–acetonitrile gradients and also reported successful separation using methanol:water systems ranging approximately from 90:10 to 80:20 [17]. Other structural studies have employed C18 RP-HPLC with UV detection at approximately 214 nm [14].

The detection wavelength of 240 nm in the present investigation represents the experimentally employed analytical condition and should not be interpreted as the literature-established absorption maximum of annonaceous acetogenins.

Chromatographic peaks and corresponding retention times were recorded. Because an authentic acetogenin reference standard and definitive structural techniques such as LC-MS and NMR were not employed, the chromatographic findings were interpreted as evidence for an acetogenin-containing/enriched fraction rather than definitive identification or quantification of a specific acetogenin.

2.10. Preparation of Acetogenin-Loaded Gellan Gum Polymeric Nanoparticles

Acetogenin-loaded polymeric nanoparticles were prepared using gellan gum as the polymer, AOT as the surfactant and calcium chloride as the cross-linking agent by the cross-emulsion technique employed in the present investigation.

The required quantity of gellan gum was dispersed in purified water with continuous stirring until a homogeneous aqueous polymeric phase was obtained. The selected acetogenin-containing fraction was incorporated into the formulation at the required concentration.

Separately, AOT was dissolved in methylene chloride to prepare the organic phase. The aqueous gellan-gum phase containing the acetogenin fraction was introduced into the organic phase under continuous high-speed stirring/homogenization to produce the primary emulsion.

Calcium chloride solution was subsequently added in a controlled manner to promote cross-linking of the gellan gum. Gellan gum is an anionic polysaccharide capable of undergoing ion-mediated gelation in the presence of suitable cations,

providing a basis for its application in pharmaceutical carrier systems [13].

The resulting dispersion was continuously stirred for the predetermined period to permit nanoparticle formation. The nanoparticles were subsequently separated by centrifugation at 10,000 rpm for 1 h. The recovered nanoparticle pellet was washed with purified water to remove unreacted components and residual surfactant and was subsequently redispersed or dried according to the requirements of further characterization.

2.11. Characterization of Polymeric Nanoparticles

2.11.1. Particle Size and Polydispersity Index

The hydrodynamic particle size and polydispersity index (PDI) of the prepared nanoparticles were determined by dynamic light scattering (DLS). The nanoparticle dispersion was suitably diluted with filtered distilled/deionized water and transferred to a clean measurement cuvette.

The mean particle size was expressed as the Z-average diameter in nanometres (nm), while PDI was recorded as an indicator of the width of the particle-size distribution. Lower PDI values indicate a comparatively narrower particle-size distribution, whereas higher values indicate increasing heterogeneity.

2.11.2. Zeta Potential

The surface charge of the prepared nanoparticles was evaluated by electrophoretic light scattering and expressed as zeta potential in millivolts (mV). The nanoparticle dispersion was appropriately diluted and transferred into a suitable zeta-potential measurement cell.

The magnitude and sign of the measured zeta potential were used to characterize the surface-charge properties of the nanoparticle dispersion and its potential contribution to electrostatic colloidal stability.

2.11.3. Scanning Electron Microscopy

The surface morphology of the selected acetogenin-loaded nanoparticle formulation was examined by scanning electron microscopy (SEM). A small quantity of dried nanoparticles was mounted onto an SEM specimen stub using conductive adhesive tape and coated with an appropriate conductive material where required.

The sample was examined at suitable magnifications and accelerating voltage. SEM micrographs were evaluated for particle morphology, surface characteristics and aggregation.

Particle dimensions observed by SEM were interpreted independently from DLS measurements because SEM examines nanoparticles in the dried state, whereas DLS determines their hydrodynamic diameter in dispersion.

2.12. Statistical Analysis

Quantitative experimental measurements were performed in triplicate wherever applicable and were summarized descriptively. Mean and standard deviation should be reported for measurements for which replicate raw data are available. No inferential statistical comparison is presented in the current study.

3. RESULTS AND DISCUSSION

3.1. Extraction Yield

Soxhlet extraction of 35 g of powdered *Annona muricata* leaves with ethanol produced a concentrated dried extract weighing 5.294 g. The empty china dish weighed 18.063 g, whereas the china dish containing the dried extract weighed 23.357 g. Therefore, the percentage extraction yield was calculated as:

$$\begin{aligned} \text{Weight of extract} &= 23.357 - 18.063 = 5.294 \text{ g} \\ \% \text{ extraction yield} &= \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100 \\ &= (5.294 / 35) \times 100 = 15.13\% \text{ w/w} \end{aligned}$$

Thus, the ethanolic extraction process produced an extractive yield of 15.13% w/w. The recovery indicates that ethanol extracted an appreciable proportion of soluble phytoconstituents from the leaf material. *A. muricata* leaves contain several classes of secondary metabolites, including annonaceous acetogenins, alkaloids, phenolic compounds and flavonoids [4,5]. However, the extraction yield represents the total ethanol-soluble constituents and should not be interpreted as the percentage yield of acetogenins specifically.

3.2. Preliminary Detection of Acetogenins

The ethanolic extract was subjected to Kedde's reagent test for preliminary detection of annonaceous acetogenins. Addition of freshly prepared Kedde's reagent produced a distinct **purple coloration**, indicating a positive reaction.

The Kedde reaction is associated with compounds containing an α,β -unsaturated γ -lactone moiety, an important structural feature of many annonaceous acetogenins [7,14]. Therefore, the positive reaction provided preliminary evidence supporting the presence of acetogenin-type constituents in the *A. muricata* leaf extract. Nevertheless, because Kedde's test is a functional-group-based color reaction rather than a definitive structural technique, the result was

interpreted together with subsequent TLC, column chromatographic, UV and HPLC findings.

3.3. Thin-Layer Chromatographic Analysis

TLC analysis of the ethanolic extract was performed using silica gel as the stationary phase and n-hexane:acetone (1:1, v/v) as the mobile phase. The acetogenin-positive band migrated 2.3 cm, while the solvent front migrated 5.2 cm.

The R_f value was calculated as:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

$$R_f = \frac{2.3}{5.2} = 0.44$$

Thus, the selected chromatographic band exhibited an R_f value of 0.44. Visualization of the separated region using Kedde's reagent provided further support for the presence of acetogenin-type compounds. Silica-gel chromatography in combination with Kedde-reagent detection has historically been used during isolation and fractionation of annonaceous acetogenins [7,14]. The observed R_f value should be regarded as specific to the chromatographic conditions employed in the present investigation because R_f is influenced by stationary phase, mobile-phase composition, chamber saturation, sample concentration and experimental conditions.

3.4. Column Chromatographic Fractionation

Silica-gel column chromatography of the ethanolic extract yielded 12 fractions (F1–F12). The individual fractions were screened using Kedde's reagent. Among the collected fractions, F4 and F5 showed a positive Kedde reaction and were therefore selected for subsequent characterization.

The selective response of F4 and F5 suggests enrichment of constituents possessing the γ -lactone functionality associated with annonaceous acetogenins. Fractionation of *Annona* extracts by silica-gel chromatography followed by screening of fractions has been extensively employed in acetogenin isolation studies [7,15]. The results therefore demonstrate that chromatographic fractionation successfully separated Kedde-positive constituents from other components of the crude ethanolic extract.



Figure 1. Column chromatographic fractions obtained from the ethanolic extract of *Annona muricata* leaves.

3.5. UV–Visible Spectroscopic Analysis

UV–Visible spectroscopic analysis of the selected Kedde-positive fraction demonstrated a prominent absorption maximum (λ_{max}) at 229.5 nm, with an absorbance of 1.7432.

Annonaceous acetogenins possess relatively weak UV chromophores, with absorption primarily associated with their unsaturated γ -lactone functionality. The observed absorption in the low-UV region was therefore considered supportive of the chromatographic and phytochemical findings [7,14]. However, the λ_{max} of 229.5 nm alone cannot establish the identity of an individual acetogenin because UV absorption lacks sufficient structural specificity.

Accordingly, the UV result was used as supportive characterization of the acetogenin-containing fraction rather than definitive confirmation of a specific acetogenin.

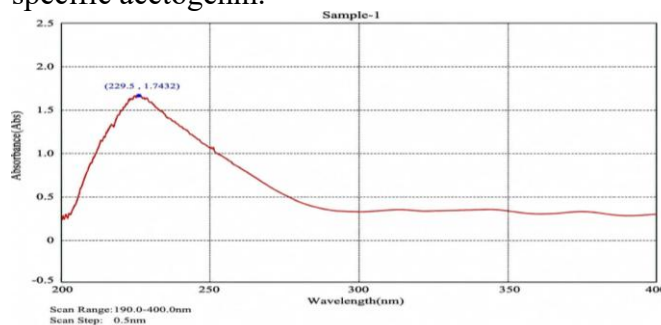


Figure 2. UV–Visible absorption spectrum of the acetogenin-containing fraction obtained from *Annona muricata* leaves.

3.6. HPLC Characterization

HPLC analysis of the selected fraction at 240 nm demonstrated a prominent major chromatographic peak at a retention time (RT) of 8.070 min. The peak exhibited an area of 7,065,085, a theoretical plate count of 2217, and a tailing factor of 1.044.

The tailing factor close to unity indicates that the principal chromatographic peak exhibited relatively good symmetry under the analytical conditions employed. Gromek et al. demonstrated the usefulness of reversed-phase C18 HPLC for separation and analysis of annonaceous acetogenins from *A. muricata*, including methanol–water and acetonitrile–water mobile-phase systems [17]. HPLC is therefore an appropriate complementary technique for chromatographic characterization of acetogenin-containing fractions.

Nevertheless, retention time is method-dependent, and a peak at 8.070 min cannot by itself establish the molecular identity of an acetogenin. In the absence of comparison with an authentic reference standard or structural confirmation by LC–MS and/or NMR, the present HPLC result should be interpreted as chromatographic evidence supporting an enriched

fraction rather than definitive identification of a particular acetogenin.

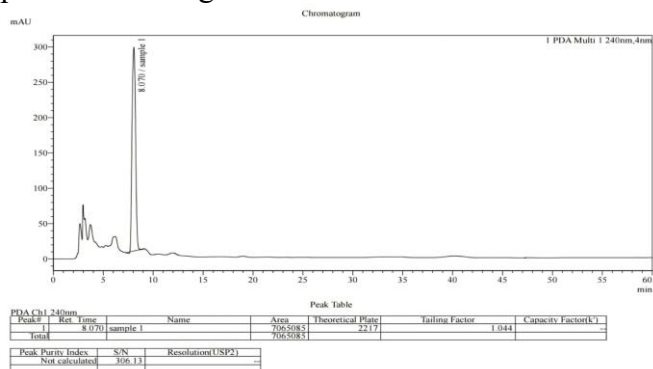


Figure 3. HPLC chromatogram of the acetogenin-containing fraction obtained from *Annona muricata* leaves.

3.7. Calibration Curve

The relationship between concentration and absorbance was investigated over the concentration range of 0–10 µg/mL. The experimental values are presented in Table 2.

The absorbance generally increased with increasing concentration, demonstrating a concentration-dependent spectrophotometric response. Linear regression of the complete dataset produced an approximate relationship of:

$$y = 0.0277x + 0.0251$$

with:

$$R^2 = 0.9627$$

where y represents absorbance and x represents concentration in µg/mL.

The calibration data demonstrated an increasing absorbance with concentration and the plotted regression showed $y = 0.0277x + 0.0251$ with $R^2 = 0.9627$. This curve should be regarded as preliminary because the linearity is lower than typically expected for a validated quantitative analytical method. Replicate standards and additional concentration levels are recommended before using the method for definitive quantification.

Table 1. Concentration and absorbance data for the calibration curve

Concentration (µg/mL)	Absorbance
0	0.0000
2	0.1162
4	0.1353
6	0.1806
8	0.2530
10	0.2970

Figure 5. Calibration curve showing the relationship between concentration and absorbance of the acetogenin-containing fraction.

3.8. Particle Size and Polydispersity Index

Dynamic light-scattering analysis demonstrated a mean hydrodynamic particle size of 50.3 nm for the acetogenin-loaded gellan gum nanoparticles. The formulation showed a PDI of 0.198, indicating a relatively narrow particle-size distribution.

The PDI provides information regarding the breadth of the particle-size distribution; values approaching zero represent increasingly uniform populations. The observed PDI of 0.198 therefore suggests that the prepared formulation possessed a comparatively homogeneous nanoscale population.

The particle size obtained in the present investigation is also consistent with the general size range achievable for acetogenin-containing polymeric nanocarriers. For example, acetogenin-enriched *A. muricata* leaf extract incorporated into targeted poly(glycerol adipate) nanoparticles has been reported to produce particles of approximately 100 nm with a narrow size distribution [18]. More recently, acetogenin-loaded chitosan-based nanoparticles prepared from *A. muricata* constituents were reported with average sizes of approximately 52–71 nm [19]. These reports support the feasibility of formulating acetogenin-containing materials into nanoscale polymeric carrier systems. The 50.3 nm particle size obtained in the present formulation may provide a relatively high surface-area-to-volume ratio and is suitable for further investigation as a nanoparticulate delivery platform. However, biological performance cannot be inferred from particle size alone and requires drug-release, cellular-uptake, cytotoxicity and in-vivo investigations.

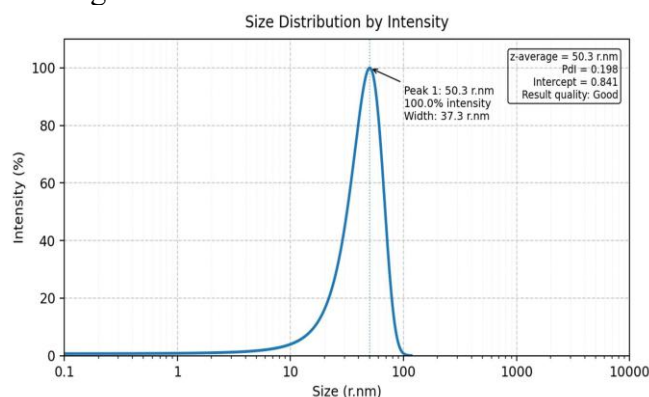


Figure 6. Particle-size distribution of acetogenin-loaded gellan gum polymeric nanoparticles determined by DLS.

3.9. Zeta Potential

The acetogenin-loaded gellan gum nanoparticles exhibited a zeta potential of -15.0 mV, demonstrating a negatively charged particle surface. The negative surface charge is consistent with the anionic nature of gellan gum, which contains ionizable carboxyl groups [13]. Gellan-gum-

containing delivery systems have also been associated with negatively charged nanoparticulate formulations, although surface charge depends strongly on nanoparticle composition and formulation conditions [20].

The magnitude of the observed zeta potential suggests that electrostatic repulsion contributes to dispersion stability; however, -15.0 mV should not be interpreted as evidence of strong electrostatic stabilization. Nanoparticle stability is influenced by several additional factors, including polymeric/steric stabilization, ionic strength, pH, concentration and storage conditions. Consequently, the physical stability of the present formulation should ultimately be established by monitoring particle size, PDI and zeta potential during a defined stability study. The result nevertheless confirms the negatively charged surface characteristics expected for a gellan-gum-containing nanoparticulate formulation.

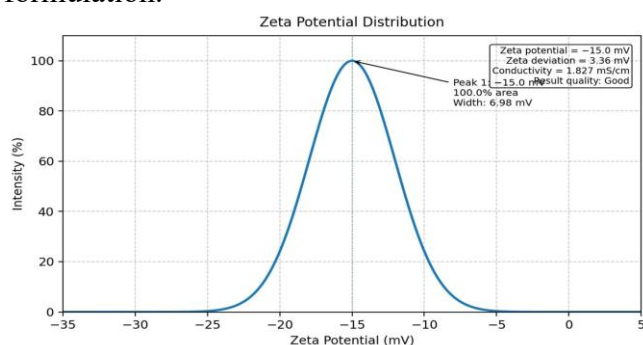


Figure 7. Zeta-potential distribution of acetogenin-loaded gellan gum polymeric nanoparticles.

3.10. Scanning Electron Microscopy

SEM analysis confirmed the formation of nanosized particles. The observed particle dimensions ranged approximately from 36.4 to 86.0 nm, with an estimated mean size of 54.4 nm. The particles exhibited predominantly irregular to near-spherical morphology with some degree of aggregation.

The approximate SEM mean particle size of 54.4 nm was broadly comparable with the DLS-derived hydrodynamic diameter of 50.3 nm. However, direct numerical equivalence between DLS and SEM should not be expected because the two analytical techniques characterize particles under fundamentally different conditions. DLS determines the hydrodynamic behaviour of particles dispersed in a liquid medium, whereas SEM examines particles after drying and sample preparation. Aggregation during drying can also influence the morphology observed by electron microscopy.

The SEM findings therefore complement the DLS results and provide morphological evidence

supporting successful formation of nanosized polymeric particles.

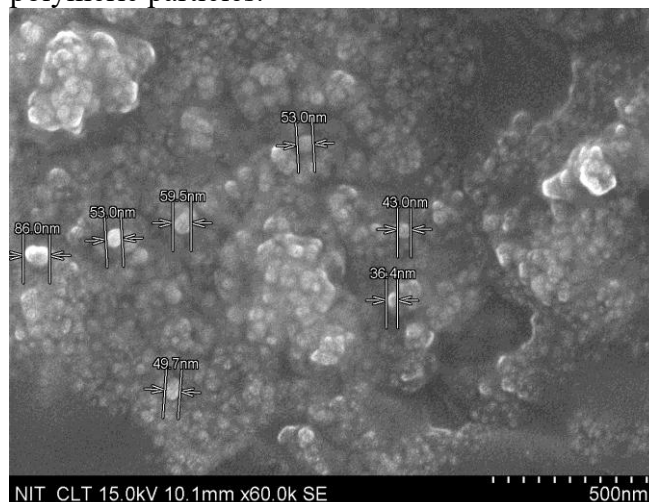


Figure 8. SEM images of acetogenin-loaded gellan gum polymeric nanoparticles showing nanoscale morphology.

3.11. Overall Interpretation of the Formulation

Taken together, the phytochemical and chromatographic findings support successful preparation of an acetogenin-containing/enriched fraction from *A. muricata* leaves. A positive Kedde reaction, TLC separation with an R_f of 0.44, isolation of Kedde-positive F4 and F5 fractions, a UV absorption maximum at 229.5 nm and the presence of a prominent HPLC peak at 8.070 min provide complementary evidence for enrichment of acetogenin-type constituents. However, these techniques do not provide sufficient structural information to assign the fraction to a particular acetogenin molecule.

The subsequent nanoparticle studies demonstrated successful formation of a gellan-gum-based nanoscale system characterized by a 50.3 nm DLS particle size, PDI of 0.198, zeta potential of -15.0 mV and approximate SEM mean size of 54.4 nm. The findings are broadly consistent with previous investigations demonstrating the feasibility of incorporating *A. muricata* acetogenin-rich extracts or isolated acetogenins into polymeric nanocarriers [18,19].

Importantly, previous work has demonstrated that acetogenin-enriched *A. muricata* extract can be incorporated into polymeric nanoparticles and evaluated for targeted cancer-cell delivery [18]. However, the present study has established physicochemical formulation feasibility rather than anticancer efficacy. No conclusion regarding colorectal-cancer treatment can therefore be made from particle characterization alone. Evaluation of encapsulation efficiency, drug loading, release behaviour and biological activity against appropriate

colorectal cancer cell lines would be required to establish the therapeutic relevance of the developed formulation.

4. CONCLUSION

The present investigation demonstrated the extraction, preliminary characterization and nanoparticle incorporation of an acetogenin-containing fraction obtained from *Annona muricata* leaves. Soxhlet extraction with ethanol produced a 15.13% w/w extractive yield. The positive Kedde reaction, TLC band at an R_f of 0.44, selection of Kedde-positive column fractions F4 and F5, UV absorption maximum at 229.5 nm, and major HPLC peak at 8.070 min collectively supported enrichment of acetogenin-type constituents.

The selected fraction was successfully incorporated into gellan gum polymeric nanoparticles using the cross-emulsion approach. DLS demonstrated a mean particle size of 50.3 nm and PDI of 0.198, while the nanoparticles exhibited a zeta potential of -15.0 mV. SEM further confirmed nanoscale particles ranging approximately from 36.4 to 86.0 nm, with an estimated mean size of 54.4 nm.

These findings establish the preliminary physicochemical feasibility of gellan gum as a carrier for an acetogenin-containing *A. muricata* fraction. The present results should not, however, be interpreted as demonstrating anti-colorectal-cancer efficacy. Further studies involving definitive acetogenin identification by LC-MS/NMR, encapsulation efficiency, drug loading, in-vitro release kinetics, formulation stability, cytotoxicity and apoptosis studies using relevant colorectal cancer cell lines such as HCT-116 and HT-29, followed where appropriate by in-vivo evaluation, are required to determine the therapeutic potential of the formulation.

5. REFERENCES

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024;74(3):229–263. doi:10.3322/caac.21834.
2. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin.* 2024;74(1):12–49. doi:10.3322/caac.21820.
3. You X, Kang Y, Hollett G, Chen X, Zhao W, Gu Z, et al. Polymeric nanoparticles for colon cancer therapy: overview and perspectives. *J Mater Chem B.* 2016;4(48):7779–7792. doi:10.1039/C6TB01925K.
4. Moghadamtousi SZ, Fadaeinasab M, Nikzad S, Mohan G, Ali HM, Kadir HA. *Annona muricata* (Annonaceae): a review of its traditional uses, isolated acetogenins and biological activities. *Int J Mol Sci.* 2015;16(7):15625–15658. doi:10.3390/ijms160715625.
5. Gavamukulya Y, Wamunyokoli F, El-Shemy HA. *Annona muricata*: is the natural therapy to most disease conditions including cancer growing in our backyard? A systematic review of its research history and future prospects. *Asian Pac J Trop Med.* 2017;10(9):835–848. doi:10.1016/j.apjtm.2017.08.009.
6. Qazi AK, Siddiqui JA, Jahan R, Chaudhary S, Walker LA, Sayed Z, et al. Emerging therapeutic potential of graviola and its constituents in cancers. *Carcinogenesis.* 2018;39(4):522–533. doi:10.1093/carcin/bgy024.
7. Zeng L, Ye Q, Oberlies NH, Shi G, Gu ZM, He K, et al. Recent advances in Annonaceous acetogenins. *Nat Prod Rep.* 1996;13(4):275–306. doi:10.1039/NP9961300275.
8. Liaw CC, Wu TY, Chang FR, Wu YC. Historic perspectives on Annonaceous acetogenins from the chemical bench to preclinical trials. *Planta Med.* 2010;76(13):1390–1404. doi:10.1055/s-0030-1250006.
9. Miyoshi H, Ohshima M, Shimada H, Akagi T, Iwamura H, McLaughlin JL. Essential structural factors of annonaceous acetogenins as potent inhibitors of mitochondrial complex I. *Biochim Biophys Acta.* 1998;1365(3):443–452. doi:10.1016/S0005-2728(98)00097-8.
10. Moghadamtousi SZ, Karimian H, Rouhollahi E, Paydar M, Fadaeinasab M, Kadir HA. *Annona muricata* leaves induce G1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon cancer cells. *J Ethnopharmacol.* 2014;156:277–289. doi:10.1016/j.jep.2014.08.011.
11. Moghadamtousi SZ, Rouhollahi E, Karimian H, Fadaeinasab M, Firoozinia M, Abdulla MA, et al. The chemopotential effect of *Annona muricata* leaves against azoxymethane-induced colonic aberrant crypt foci in rats and the apoptotic effect of acetogenin annomuricin E in HT-29 cells: a bioassay-guided approach. *PLoS One.* 2015;10(4):e0122288. doi:10.1371/journal.pone.0122288.
12. Kim GS, Zeng L, Alali F, Rogers LL, Wu FE, McLaughlin JL, et al. Two new mono-tetrahydrofuran ring acetogenins, annomuricin E

- and muricapentocin, from the leaves of *Annona muricata*. *J Nat Prod*. 1998;61(4):432–436. doi:10.1021/np970534m.
13. Osmałek T, Froelich A, Tasarek S. Application of gellan gum in pharmacy and medicine. *Int J Pharm*. 2014;466(1–2):328–340. doi:10.1016/j.ijpharm.2014.03.038.
 14. Liaw CC, Chang FR, Lin CY, Chou CJ, Chiu HF, Wu MJ, et al. New cytotoxic monotetrahydrofuran Annonaceous acetogenins from *Annona muricata*. *J Nat Prod*. 2002;65(4):470–475. doi:10.1021/np0105578.
 15. Wu FE, Gu ZM, Zeng L, Zhao GX, Zhang Y, McLaughlin JL, Sastrodihardjo S. Two new cytotoxic monotetrahydrofuran Annonaceous acetogenins, annomuricins A and B, from the leaves of *Annona muricata*. *J Nat Prod*. 1995;58(6):830–836. doi:10.1021/np50120a002.
 16. Champy P, Melot A, Guérineau Eng V, Gleye C, Fall D, Höglinger GU, et al. Quantification of acetogenins in *Annona muricata* linked to atypical parkinsonism in Guadeloupe. *Mov Disord*. 2005;20(12):1629–1633. doi:10.1002/mds.20632.
 17. Gromek D, Hocquemiller R, Cavé A. Qualitative and quantitative evaluation of annonaceous acetogenins by high performance liquid chromatography. *Phytochem Anal*. 1994;5(3):133–140. doi:10.1002/pca.2800050309.
 18. Damrongrak K, Kloysawat K, Bunsupa S, Sakchasri K, Wongrakpanich A, Taresco V, et al. Delivery of acetogenin-enriched *Annona muricata* Linn leaf extract by folic acid-conjugated and triphenylphosphonium-conjugated poly(glycerol adipate) nanoparticles to enhance toxicity against ovarian cancer cells. *Int J Pharm*. 2022;618:121636. doi:10.1016/j.ijpharm.2022.121636.
 19. Mariappan V, Kumararaja G, Subban R. Folate-chitosan nanoparticles loaded with an apolar acetogenin inhibiting the proliferation of cervical cancer cells. *Int J Biol Macromol*. 2025;302:140640. doi:10.1016/j.ijbiomac.2025.140640.
 20. Posadowska U, Brzywczy-Wloch M, Pamula E. Injectable gellan gum-based nanoparticles-loaded system for the local delivery of vancomycin in osteomyelitis treatment. *J Mater Sci Mater Med*. 2016;27(1):9. doi:10.1007/s10856-015-5604-2.