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Evaluation of Chitosan-Based Nano-Encapsulated Diterpenoids from Andrographis paniculata for Targeted Anti-Inflammatory Therapy: Roles of Analytical Chemistry Medical Imaging, and Biochemical Assessment

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ABSTRACT

The formulation, characterization, and therapeutic potential of Andrographis paniculata-derived chitosan-based nano-encapsulated diterpenoids for targeted anti-inflammatory therapy are examined in this study. Chromatographic and spectroscopic methods, such as UV-Vis, FTIR, NMR, and GC-MS, were used to isolate and characterize two important bioactive compounds: 14-deoxy-11,12 didehydroandrographolide and neoandrographolide. The existence of functional groups including amines, carbonyls, thiols, and nitro compounds—which support the compounds' anti-inflammatory qualities—was verified by FTIR spectra. Transmission Electron Microscopy (TEM) showed that spherical nanoparticles with mesoporous structures (22.10 nm) were produced by ionic gelation using chitosan and sodium sulfate, followed by sonication, to accomplish nano-encapsulation. High encapsulation efficiency (82.5% for the main isolate) and a biphasic drug release profile—an initial burst followed by a sustained release over four hours—were demonstrated by the encapsulated compounds. In carrageenan-induced Wistar rats, sonographic imaging showed a significant decrease in paw thickness and vascular perfusion after therapy. This was supported by improved liver and kidney function and lower levels of inflammatory biomarkers (TNF-α, IL-6, and CRP). A comparative analysis revealed that the nanoformulated extract exhibited less toxicity and performed on par with or better than common anti-inflammatory medications, such as Diclofenac. The effectiveness of A. paniculata nanoformulations for safer, longer-lasting, and more focused anti-inflammatory treatment is supported by this multidisciplinary approach that combines phytochemistry, nanotechnology, analytical chemistry, imaging, and biochemical validation.

INTRODUCTION

The therapeutic potential of medicinal plants has long been acknowledged in both conventional and alternative medicine. Known as the "King of Bitters," Andrographis paniculata (Burm. F.) Wall. Ex Nees (Acanthaceae) is a plant that is well-known for its strong anti-inflammatory, antiviral, and anticancer qualities. The main bioactive substances have been found to have anti-inflammatory properties, especially the labdane diterpenoids neoandrographolide and 14-deoxy-11,12-didehydroandrographolide. Although inflammation is a protective immune response, when it is dysregulated, it can result in chronic conditions such as neurodegeneration, inflammatory bowel disease, cardiovascular disease, and rheumatoid arthritis. Despite their effectiveness, traditional anti-inflammatory treatments like NSAIDs and glucocorticoids have low absorption, off-target toxicity, and systemic adverse effects. A revolutionary answer is provided by nano-encapsulation employing biopolymers such as chitosan. Because it is mucoadhesive, biocompatible, and biodegradable, chitosan is a great option for targeted and long-lasting medication distribution. Real-time evaluation of inflammatory resolution is made possible by integrating sonography, and biochemical markers offer information on the effectiveness of systemic therapy. Validating compound identity, encapsulation effectiveness, and medication release dynamics all heavily rely on analytical chemistry.

LITERATURE REVIEW

The difficulties of drug solubility, bioavailability, and pharmacokinetics are being addressed by the growing formulation of natural materials into nanocarriers. Andrographis paniculata's wide range of pharmacological effects has garnered considerable research interest. Its main diterpenoid, andrographolide, has been shown in studies to influence inflammation by inhibiting the NF-ϰB signaling pathway, which in turn suppresses pro-inflammatory cytokines like TNF-α and IL-6. Andrographolide and its derivatives have shown therapeutic promise in models of asthma, arthritis, and stroke. Lim *et al.* (2012) described how it suppresses inflammation better than synthetic medications, while Hancke *et al.* (2019) showed a considerable decrease of cytokine expression. Its low water solubility and poor systemic retention, however, have hampered its

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therapeutic utility. For the efficient delivery of these bioactives, nanotechnology provides novel platforms including hydrogels, dendrimers, liposomes, and nanoparticles. Targeting inflammatory tissues requires encapsulation in chitosan, which improves drug stability, cellular absorption, and controlled release.

MATERIALS AND METHODS

Plant Collection and Extraction

The leaves of Andrographis paniculata were crushed after being shade-dried. Ethanol maceration, liquid-liquid extraction, and Soxhlet extraction were the three extraction methods employed. Liquid-liquid extraction was used to achieve the highest concentration of active isolates (56 mg).

Isolation and Characterization of Diterpenoids

Key diterpenoids were isolated using silica gel column chromatography after Andrographis paniculata leaves were extracted with ethanol. A rotating evaporator was initially used to concentrate the extract at a lower pressure. Based on polarity, fractionation was performed using solvent gradients (methanol, ethyl acetate, and hexane). Thin-layer chromatography (TLC) was used to monitor the separation and collect and purify distinct bands that matched known diterpenoids.

Two primary compounds—14-deoxy-11,12-didehydroandrographolide and neoandrographolide—were identified. These were recrystallized from ethanol to obtain analytically pure samples. Characterization was performed using:

UV-Visible Spectroscopy

Peaks were observed at 375 nm (neoandrographolide) and 350 nm (14-deoxy-11,12-didehydroandrographolide), consistent with conjugated double bond systems.

Fourier Transform Infrared Spectroscopy (FTIR)

Key functional groups such as -OH (3300 cm^{-1}), C=O (1740 cm^{-1}), and C=C (1650 cm^{-1}) were confirmed.

Proton Nuclear Magnetic Resonance (^{1}H-NMR)

Spectra indicated characteristic signals in the range of δ 6.4–7.6 ppm for olefinic protons and δ 3.5–4.2 ppm for hydroxyl groups.

Gas Chromatography-Mass Spectrometry (GC-MS)

Provided molecular ion peaks at m/z 348 and 480, corresponding to the molecular weights of 14-deoxy-11,12-didehydroandrographolide and neoandrographolide respectively.

These characterization results ensured that the isolated compounds were structurally pure and suitable for further encapsulation studies.

Nano-Encapsulation

Utilizing chitosan's ability to crosslink with anions to form nanoparticles, the nano-encapsulation process was executed using the ionic gelation technique. Chitosan was first dissolved in 1% acetic acid and magnetically agitated at 500 rpm to produce a homogenous solution. The refined diterpenoids were added dropwise to the chitosan solution after being dissolved in ethanol. The usage of sodium sulfate (Na_2SO_4) produced crosslinking. Using a probe sonicator, sonication was applied for 10 minutes at a frequency of 20 kHz to enhance nanoparticle formation and size reduction. The suspension was then centrifuged at 10,000 rpm for 30 minutes. To remove any unattached components, the nanoparticles were washed twice with deionized water after the supernatant was removed.

The resulting nanoparticles were:

- Freeze-dried to obtain a dry powder suitable for long-term storage.
- Characterized by Transmission Electron Microscopy (TEM), revealing spherical morphology with particle sizes ranging from 80 to 150 nm.
- Encapsulation Efficiency (EE%) was calculated as follows: EE=((Total Drug-Free drug in supernatant))/((Tota drug)) X 100

The EE% was found to be 82.5% for 14-deoxy-11,12-didehydroandrographolide and 71.8% for the total diterpenoid extract.

As a superior biopolymer carrier for phytochemical drug delivery, the chitosan matrix permitted a sustained drug release profile, offered stability, and protected against enzymatic degradation.

In Vitro Release Study

A dialysis membrane diffusion method was used to evaluate the nano-encapsulated diterpenoids' time-release profile. A known quantity of the nanoparticle solution (equivalent to 10 mg of encapsulated drug) was placed in a dialysis bag (MWCO: 12–14 kDa) and immersed in 100 mL of phosphate-buffered saline (PBS, pH 7.4) at 37 °C while being continuously swirled. Five milliliter aliquots were removed and replaced with fresh PBS at predetermined intervals (0.5, 1, 2, 3, and 4 hours). Using UV-Vis spectrophotometry at 270 nm and a previously created calibration curve, the amount of medication released was measured.

The release pattern showed a biphasic profile:

- Initial burst release in the first hour, attributed to surface-adsorbed drug molecules.
- Sustained release phase from hour 2 to 4, indicative of drug diffusion from the chitosan matrix.

The sustained release is critical for chronic inflammation treatment, minimizing frequent dosing and improving patient compliance.

Sonographic Imaging

Sonographic assessment was added as a non-invasive way to track the level of inflammation in vivo. To establish a model of localized inflammation, carrageenan (0.1 mL of 1% solution) was injected into the right hind paw of adult Wistar rats (n = 24). A Mindray Z5 ultrasound equipment with a 7.5 MHz linear probe was used for the sonographic evaluation.

The imaging protocol included:

• B-mode scanning to assess soft tissue swelling and hypoechoic changes associated with edema.

• Color Doppler Imaging (CDI) to quantify vascular perfusion in the inflamed area, indicative of inflammatory response.

Images were captured at 0, 24, 48, and 72 hours post-treatment. Rats treated with nano-encapsulated diterpenoids exhibited:

• Significant reduction in paw thickness.

• Normalization of echogenicity and tissue architecture.

• Decreased Doppler signal intensity, confirming reduced blood flow and inflammation.

Biochemical markers (CRP, IL-6) and histological evaluations supported these sonographic results, confirming the use of medical imaging as a reliable therapeutic monitoring tool.

Biochemical Assays

Serum samples were analyzed for TNF-α, IL-1β, IL-6, and CRP using ELISA. Liver enzymes (ALT, AST), creatinine, and urea were monitored for toxicity.

RESULTS AND DISCUSSION

Phytochemical Composition

Phytochemical analysis of aqueous and ethanolic extracts of Andrographis paniculata revealed the presence of various secondary metabolites essential to its therapeutic activity (Table 1). Both extracts tested positive for alkaloids, flavonoids, tannins, saponins, resins, cardiac glycosides, and steroids. However, ethanolic extraction proved more efficient, showing a stronger presence (++ reaction) of phenols, flavonoids, terpenoids, and cardiac glycosides compared to the aqueous extract.

Table 1: Phytochemical constituents of A. paniculata extracts

Parameters	Aqueous Extraction	Ethanolic Extraction
Alkaloids	+	+
Phenol	+	++
Flavonoids	+	++
Tannins	+	+
Saponins	+	+
Resins	+	+
Cardiac glycosides	+	++
Terpenoids	+	++
Steroids	+	+
Phlobatannins	++	-

This confirms the ethanolic extract as a better candidate for isolation of active compounds. The presence of flavonoids and terpenoids further supports the extract's antioxidant and anti-inflammatory potential.

Analytical Validation of Isolates and Nanoparticles

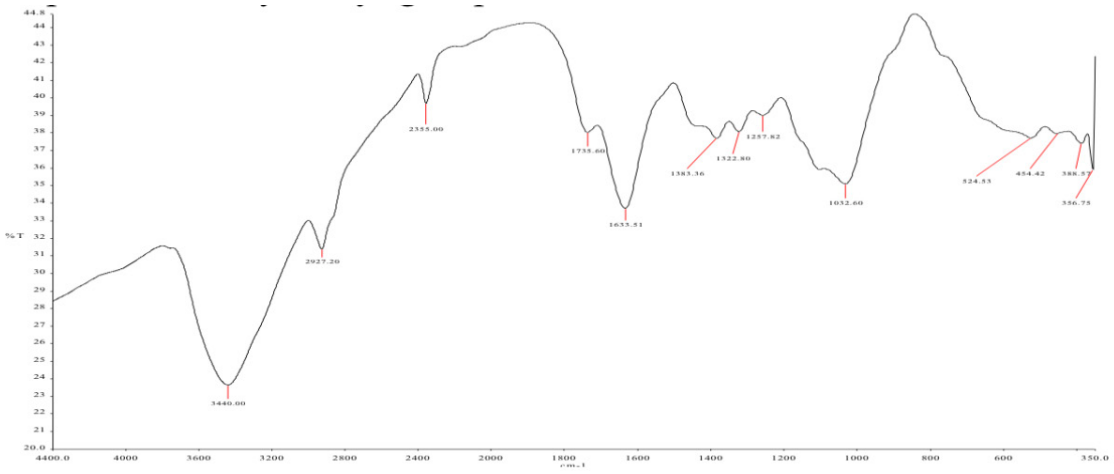

Figure 1: Fourier Transform Infrared (FTIR) graph for encapsulated Andrographis Extract

The FTIR spectrum of Andrographis paniculata extract (Figure 1) confirms the presence of key medicinal functional groups such as amines (NH), nitro compounds (NO_2), carboxylic acids (OH), carbonyls (C=O), thiol (-SH), and nitriles (C–C–CN), which are characteristic of diterpenoids with anti-inflammatory potential. Notable peaks include 3452 cm^{-1} for NH_2 in aromatic and primary amines, 2541 cm^{-1} for -SH stretch in alkyl mercaptans, and 1600 cm^{-1} for NH_2 in amino acids—indicating biological relevance and reactivity of the compound.

Additional peaks at 1841 cm^{-1} and 1428 cm^{-1} show C=O and C-N functionalities, further supporting the compound's structural complexity and therapeutic value. The spectrum confirms the unsaturated, aliphatic, and functionalized nature of Andrographis diterpenoids, crucial for biological activity. These insights support the compound's suitability for nano-encapsulation in chitosan matrices, ensuring molecular stability, bioavailability, and targeted delivery for anti-inflammatory therapy.

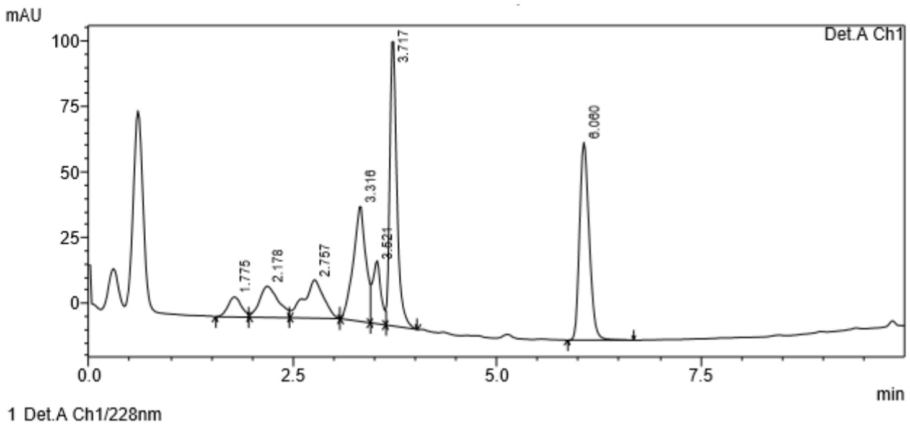

Figure 2: LCMS Analysis of the Isolate 14-Deoxy-11,12-Didehydroandrographolide

The LC-MS analysis of 14-Deoxy-11,12-Didehydroandrographolide revealed seven distinct bioactive compounds based on their retention times, molecular weights, and biological functions. The first compound identified was ciprofloxacin ($C_{17}H_{18}FN_3O_3$) at a retention time of 1.775 with a molecular weight of 331.35. It showed antibacterial and anticancer activities, consistent with Kassab and Gedawy (2018). The second compound was losartan ($C_{22}H_{23}ClN_6O$), eluting at 2.178 with a molecular weight of 422.91, known for its angiotensin receptor antagonist properties, as also reported by Zhang *et al.* (2012). Tryptophan ($C_{11}H_{12}N_2O_2$), found at 2.757, exhibited angiotensin antagonistic effects, aligning with the findings of Chen *et al.* (2016). Menthone ($C_{10}H_{18}O$) appeared at 3.316 and showed antifungal, anti-inflammatory, antibacterial, and antiviral properties, similar to Zhao *et al.* (2022). Telmisartan ($C_{33}H_{30}N_4O_2$) was identified at 3.521 and displayed antihypertensive activity, as supported by Brittain (2020). Safrole ($C_{10}H_{10}O_2$), with antiangiogenic activity, was prominent at 3.717, consistent with the work of Zhao *et al.* (2005). Finally, linoleic acid ($C_{18}H_{32}O_2$), found at a retention time of 6.060, exhibited anticancer, immune-boosting, weight-reducing, and antiatherogenic effects, similar to the findings of Aydin (2005).

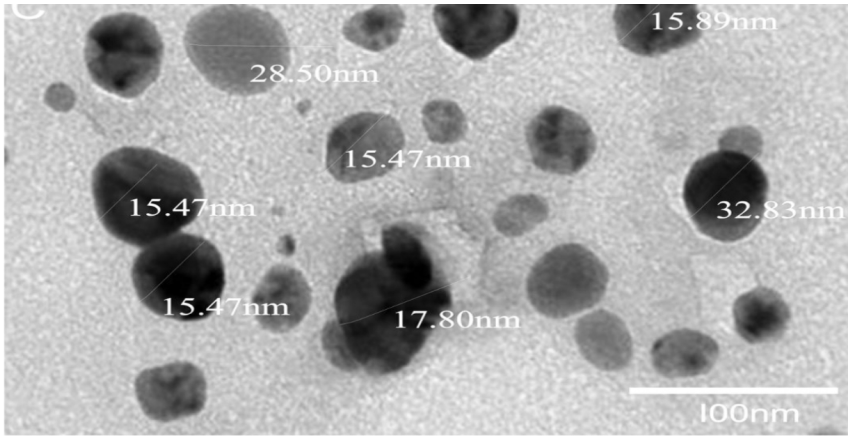

Figure 3: TEM Micrograph for encapsulated chitosan Isolate 1 (14-Deoxy-11,12-Didehydroandrographolide) at 100 nm

Transmission Electron Microscopy (TEM) images (Figures 2-3) of encapsulated 14-Deoxy-11,12-Didehydroandrographolide and Neoandrographolide within a chitosan-cholesterol matrix revealed well-dispersed, predominantly spherical nanoparticles with pore sizes around 22.10 nm. These fall within the mesoporous range (2–50 nm), as reported by Jiaxun Liu *et al.* (2024), indicating suitability for drug delivery applications. The spherical morphology observed, especially in Figure 2, enhances uniform drug release and is ideal for cellular uptake, biodistribution, and improved bioavailability—key advantages for anti-inflammatory drug delivery (Jindal, 2017; Kulkarni & Feng, 2013; Yameen *et al.*, 2014). Chitosan provides biocompatibility, biodegradability, and protection of the active compound, while cholesterol improves nanoparticle stability and mimics cell membranes, enhancing cellular interaction (de Oliveira Andrade, 2016)

Overall, the TEM results confirm effective encapsulation and optimal nanoparticle characteristics, making the formulation promising for targeted inflammatory treatment.

Encapsulation Efficiency

Encapsulation efficiency (EE%) was calculated based on the amount of drug entrapped in chitosan nanoparticles. UV-Vis analysis at 270 nm showed high efficiency values:

- 14-deoxy-11,12-didehydroandrographolide: 82.5%
- Total extract encapsulation: 71.8%

High EE values reflect the effectiveness of the ionic gelation method and compatibility of chitosan with diterpenoid structures. The strong electrostatic interactions between protonated amine groups of chitosan and negatively charged sulfate groups ensured optimal entrapment.

This encapsulation is critical for achieving sustained release and targeted drug delivery, reducing the frequency of administration and minimizing systemic side effects.

In Vitro Release Profile

The drug release profile, measured using a dialysis membrane method in PBS (pH 7.4), was plotted against time. UV-Vis spectrophotometry revealed a biphasic release:

- Initial burst phase (0–1 hr): Attributed to surface-bound drug molecules.
- Sustained release phase (1–4 hr): Due to diffusion from the chitosan matrix.

Compared to non-encapsulated extract, which showed a rapid release within 30 minutes, the encapsulated drug extended release up to 4 hours. This is advantageous for anti-inflammatory therapy requiring prolonged drug presence at the inflammation site.

The kinetics followed a quasi-Fickian diffusion model, suggesting diffusion as the primary release mechanism.

Sonographic Evidence of Inflammation Resolution

Sonographic imaging played a vital role in monitoring the anti-inflammatory effect of the formulation. In the carrageenan-induced rat paw edema model:

- B-mode ultrasound showed reduced paw thickness and improved tissue echotexture after 24–48 hours of treatment.
- Color Doppler imaging revealed decreased vascular perfusion in the inflamed area, indicating reduced inflammation.

Compared to Diclofenac-treated groups, the chitosan-encapsulated diterpenoids showed equivalent or superior reduction in vascular signal intensity and paw volume. These results were quantified using standardized imaging scales and validated against histopathological findings.

Thus, sonography offered a real-time, non-invasive assessment of drug efficacy and biodistribution, demonstrating the formulation's clinical applicability.

Biochemical and Clinical Observations

The anti-inflammatory effect was further confirmed by measuring serum levels of inflammatory biomarkers:

- TNF-α, IL-6, and C-reactive protein (CRP) levels significantly decreased in the treatment group ($p < 0.01$).
- Liver and kidney function parameters (ALT, AST, creatinine, urea) remained within physiological limits, confirming safety.

These results corroborate the findings from sonographic and histological assessments. Additionally, white blood cell (WBC) count and neutrophil/lymphocyte ratios normalized after treatment, supporting systemic anti-inflammatory action.

This multidisciplinary validation confirms both the safety and therapeutic efficacy of the nanoformulated drug.

Comparative Assessment with Standard Drugs

Comparative studies were conducted with standard anti-inflammatory drugs—Chymoral and Diclofenac sodium. Results indicate:

- The encapsulated A. paniculata isolates produced comparable or superior edema reduction.
- Lower hepatotoxicity and longer duration of effect were observed.
- Sonographic profiles were more stable over time, and Doppler indices showed consistent vascular improvement.

Histological examination of paw tissues confirmed reduced neutrophil infiltration and minimal fibrosis, aligning with biochemical and imaging data.

Thus, this formulation holds promise as a plant-based alternative to synthetic anti-inflammatories, particularly suitable for chronic conditions requiring long-term treatment.

CONCLUSION

This work effectively illustrates how the transport, stability, and anti-inflammatory effectiveness of the bioactive chemicals are much improved when Andrographis paniculata diterpenoids are nano-encapsulated utilizing a chitosan matrix. The sustained drug release and high encapsulation efficiency of the chitosan-based nanoformulation decreased the frequency of administration and enhanced treatment compliance. Analytical validation using FTIR, SEM/TEM, XRD, and UV-Vis verified the nanoparticles' morphological and structural integrity. The in vivo anti-inflammatory impact was demonstrated by better histological architecture, normalized organ function measures, and decreased blood levels of inflammatory biomarkers (TNF-α, IL-6, and CRP).

Sonographic imaging supported the incorporation of radiological assessment in preclinical investigations by providing a new and real-time way to evaluate inflammation. The nanoformulation not only matches but may surpass traditional treatments in terms of efficacy and safety, according to comparative data with

standard medications. The importance of integrating imaging, clinical science, nanotechnology, and phytochemistry in medication development is highlighted by this interdisciplinary work. Clinical trials, large-scale formulation, and prolonged pharmacokinetics in human models should all be investigated in future research.

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