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RESEARCH PAPER

TITLE:

**ECO-FRIENDLY SYNTHESIS OF *ACHYRANTHES ASPERA*-
DERIVED CHITOSAN NANOPARTICLES AND
BIOANALYTICAL CHARACTERIZATION.**

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ECO-FRIENDLY SYNTHESIS OF *ACHYRANTHES ASPERA*-DERIVED CHITOSAN NANOPARTICLES AND BIOANALYTICAL CHARACTERIZATION.

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ABSTRACT

Achyranthes aspera is known as a versatile plant due to its therapeutic properties. The current study was designed to measure the efficacy of *Achyranthes aspera* nanoparticles as compared to the solvent extracts. Anti-radical, alpha amylase inhibition, phyto-toxicity, and inflammation inhibition potentials were performed to characterize the nanoparticles. The nanoparticles were nearly 116.77nm in size and were positively charged. The methanolic extract exhibited significantly higher phenolic contents and alpha amylase inhibitory activity. While nanoparticles showed maximal flavonoids and anti-inflammatory activity. Almost analogous antioxidant activities were observed in the methanol extract and nanoparticles. All the samples were safer, non-toxic, as evident in the hemolytic assessment. Fourier transform infrared spectroscopy indicated different functional groups in extracts (amides, alkanes, amines, ethers, esters, carboxylic acid, alcohol and anhydrides). The presence of p-coumaric acid and chlorogenic acid was confirmed by high-performance liquid chromatography. These functional groups were responsible for the bioactivities and also for the polymerization of chitosan. It is established that some bioactivities of nanoparticles were higher than those of the extracts. Additional studies

to streamline the process in animal models are justified to identify possible applications.

Keywords: *Achyranthes aspera*; Nanoparticles; Antioxidant; Antidiabetic, Hemolytic

INTRODUCTION

Worldwide, plant-based remedies are a vital part of contemporary medicine. Due to lesser side effects, lower cost and accessibility, medicinal plants are considered a safe and economical substitute for synthetic drugs. Perhaps the biggest benefit is that these treatments are compatible with nature and there are no age or gender restrictions on the usage of natural product therapies. Additionally, a broad range of biological activities attributed to medicinal plants has enhanced their global consumption in both developed and developing nations (Hai-Anh et al., 2024).

Achyranthes aspera, a popular ethnomedical herb, possesses diverse pharmacological properties. This herbaceous plant, which is extensively grown in tropical and subtropical climates, is a member of the Amaranthaceae family. Latest research studies have highlighted antioxidative, antimicrobial, anti-ageing, immune-regulatory, cardioprotective and anticancer efficacies of *Achyranthes aspera* (Luo et al., 2025).

Nanotechnology is a preferred option in medical sciences due to multifaceted applications, including tailored medication administration, enhanced pharmacokinetic profile, and large surface area to volume ratio (Saeed et al., 2025).

The field of biodegradable nanomedicines is flourishing as it is an environmentally friendly approach. *A.aspera* nanoformulations have shown promising therapeutic insights (Kalaiselvi et al., 2018; Afsheen et al., 2022).

The research on chitosan-based nanoparticles of *Achyranthes Aspera* is limited (Dinakarkumar et al., 2025), especially since data are scarce on the comparison of both crude extract and nanoformulations. As the documentation of *A.aspera* in multiple traditional medicine systems intimates its further exploration, therefore, the current study was designed to measure the efficacy of *Achyranthes aspera* nanoparticles as compared to the solvent extracts. For this purpose, antioxidant contents and activity, antidiabetic, cytotoxic and anti-inflammatory activities of *Achyranthes Aspera* extracts in different solvents and nanoparticles were measured. Structural characterization was done by using a zeta analyser (size, charge), Fourier transform Infrared (FTIR) spectroscopy and high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Sample Preparation

The plant sample (aerial part) was obtained from a local retail market in Faisalabad, Pakistan. The voucher specimen (number 110-1-123) was deposited at the herbarium after authentication. The dried powdered sample was used to prepare aqueous,

methanol and *n*-hexane extracts by microwave-assisted method. The percentage yield was calculated and the semisolid extracts were used for further analysis by Hannan et al. (2023). Chitosan-based nanoparticles were synthesized using the ionic gelation method. Briefly, in 100 mL acidified water (3 mL acetic acid), 50 mg chitosan was added and thoroughly mixed using a magnetic stirrer at 25-40°C at 900 rpm. Tripolyphosphate (TPP) was added when chitosan was properly dissolved and then placed on a magnetic hot plate to obtain a clear solution. Then, 50 mg of the methanolic extract was added drop-wise using an insulin syringe. The whole process was done at a stirrer so the temperature was kept 40-65°C at 1500rpm to obtain a thick consistency. By centrifuging for 10-15minutes (14000rpm), the pellet was obtained. The mixture of pellet and distilled water was sonicated to have a clear homogeneous solution (Noreen et al., 2020).

Antioxidant Profile

For the estimation of Total Phenolic Content (TPC), a mixture of 75 µL Na₂CO₃, 15 µL sample and 100 µL Folin reagent was incubated (120 minutes). The absorbance at 750 nm was used to calculate TPC (µg GAE/mL). In the assay for Total Flavonoid Contents (TFC) measurement, 20 µL sample was mixed with 70 µL methanol and incubated for 10 minutes at ambient temperature. After the addition of 4 µL AlCl₃ and 4 µL (1M) potassium acetate, it was again incubated (10 minutes), and absorbance was noted at 415 nm to calculate TFC (µg CE/mL). The antioxidant activity was determined by the ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH). After

incubating a mixture of 150 μ L DPPH solution and 1.5 μ L sample for 30 minutes, the absorbance was taken at 570nm **Hussain *et al.*, (2021)**. The control was ascorbic acid and antioxidant activity was measured as: $[A(\text{control}) - A(\text{sample}) / A(\text{control})] \times 100$.

Antidiabetic Activity

The mixture of 50 μ L sample and μ L alpha amylase (source: *Bacillus subtilis*, EC number 3.2.1.1 by Sigma Aldrich) was incubated (20 minutes) at room temperature. The substrate (1% starch) was added and incubated (30 minutes) to complete the catalysis. Then, 1% HCl was added to stop the enzyme activity, as shown by **Hussain *et al.* (2021)**. Acarbose was the positive control and absorbance (Abs) at 570 nm was used to calculate % enzyme inhibition as: $[Abs(\text{control}) - Abs(\text{sample}) / Abs(\text{control})] \times 100$.

Cytotoxicity Activity - Hemolytic Assay

From a volunteer, 3 mL of blood was taken after informed consent and centrifuged at 1200 rpm for 4 minutes. The red blood cells (RBCs) were washed with phosphate buffer saline, pH of 7.4. Then, 150 μ L cell suspension and 25 μ L sample were incubated (20 minutes). To 80 μ L supernatant, 950 μ L phosphate buffer saline was added and absorbance was checked (576 nm). The saline solution was a blank control. Triton X-100

was the reference standard (**Shahzadi *et al.*, 2019**).

% Haemolysis: $[Abs. \text{ Sample} - Abs \text{ blank control} / Abs \text{ reference standard}] \times 100$

Anti-inflammatory Activity

The bovine serum albumin (25 μ L) and sample (25 μ L) were placed at 72°C for 5 minutes in a water bath (Williams *et al.*, 2008). The reference blank was phosphate buffer saline and the reference standard was 0.001% diclofenac sodium for absorbance measurement (255 nm). The inhibition (%): $100 \times [Abs(\text{Control}) - Abs(\text{Sample}) / Abs(\text{Control})]$.

Characterization

FTIR (model: Bruker Tensor 27 FTIR spectrometer) was used to describe the structure. The plant extract and Potassium bromide were mixed and transformed into pellets using compression dye for analysis in the 400 – 4000 cm^{-1} range (**Khalid *et al.* 2023**).

In HPLC, a mixture of 0.5g dried plant material and 20 mL of HPLC-grade ethanol was prepared. In a polyethylene micro-centrifuge tube, centrifugation was done (25000rpm) for 10 minutes. The supernatant was filtered and used for analysis, as published in **Singh (2014)**. The analysis was done at the Central Hi-Tech Laboratories, University of Agriculture, Faisalabad, Pakistan.

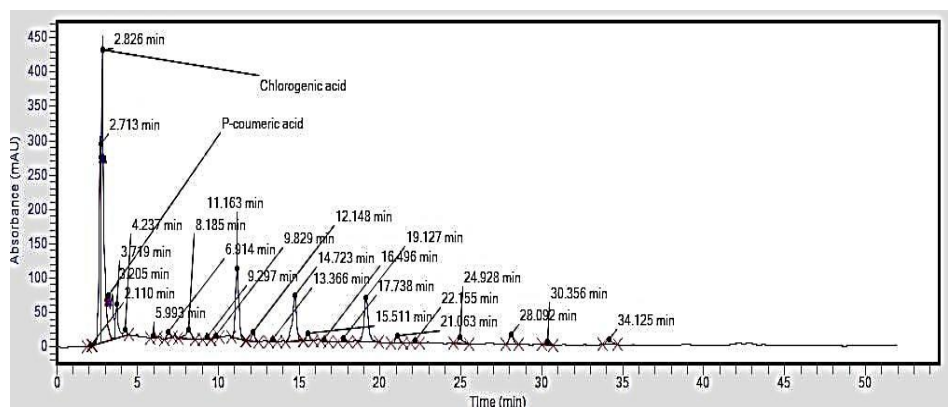


Figure 2: HPLC spectra of *A. aspera*

Structural Characterization of Nanoparticles

The key parameters like surface charge and nanoparticle size were measured by a zeta analyzer (Model: Beckman Coulter Inc.,

Delsa Nano 4C), Central Hi-Tech Laboratories, University of Agriculture, Faisalabad, Pakistan, using litesizer and Soft Imaging Viewer programs as described previously (Akhtar *et al.*, 2020).

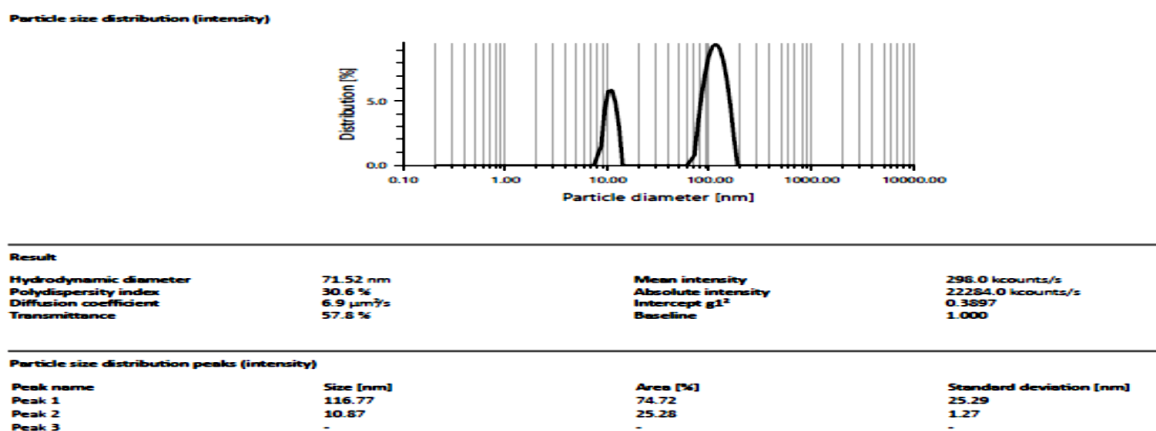


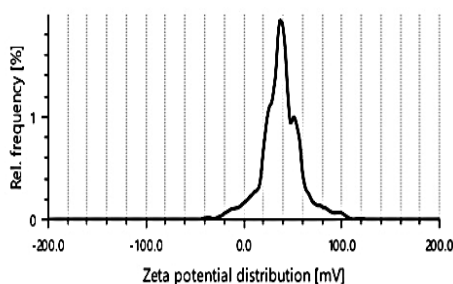
Figure 3: Results of the zeta sizer

Statistical analysis

Analysis of variance with a level of significance $P < 0.05$ was used to compare

treatments. Data analysis was done by Minitab (version 21).

Zeta potential distribution



Result

Mean zeta potential	31.6 mV	Mean intensity	492.1 kcounts/s
Standard deviation	0.8 mV	Filter optical density	2.5047
Distribution peak	38.3 mV	Conductivity	0.119 mS/cm
Electrophoretic Mobility	2.4650 $\mu\text{m}^2\text{cm/Vs}$	Transmittance	48.2 %

Figure. 4: Results of zeta potential

RESULT AND DISCUSSION

Following the extraction process, the percentage yield was determined. The best yield (12.57%) was recorded in the aqueous sample. The yields of *n*-hexane and methanol

extracts were 2.38% and 7.28%, respectively. In this study, the phytochemical content and biological activities of *Achyranthes aspera* varied notably between solvent extracts and the nanoparticle formulation (Table 1).

Table 1: Bioanalytical Characterization

Activities	Treatment				
	Control	<i>n</i> -hexane	Aqueous	Methanol	Nanoparticles
Anti-radical Activity					
TPC ($\mu\text{g GAE/mL}$)	-	128.39 $\pm$ 0.58	136.71 $\pm$ 0.78	164.13 $\pm$ 0.58*	8.71 $\pm$ 0.00
TFC ($\mu\text{g CE/mL}$)	-	114.22 $\pm$ 1.35	123.56 $\pm$ 1.17	140.67 $\pm$ 0.88	273.11 $\pm$ 0.19*
DPPH (%)	97.80 $\pm$ 0.05	40.46 $\pm$ 0.35	39.13 $\pm$ 0.31	57.07 $\pm$ 0.13	57.88 $\pm$ 0.18*
Alpha-amylase Inhibition					
Inhibition (%)	88.31 $\pm$ 2.03	46.37 $\pm$ 0.03	64.92 $\pm$ 0.11	78.37 $\pm$ 0.80*	62.12 $\pm$ 1.27
Cytotoxicity					
Activity (%)	97.68 $\pm$ 0.17	1.42 $\pm$ 0.60	3.67 $\pm$ 0.50	5.13 $\pm$ 0.67	6.74 $\pm$ 0.35
Anti-inflammatory Activity					

Inhibition (%)	83.78 ±2.70	35.29±2.94	37.65 ± 2.94	52.17± 8.70	65.22±4.35*
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Data presented as mean+/- S.D. (n=3). *Significant P < 0.05.

The total phenolic content (TPC) of the extracts ranged from $128.39 \pm 0.58 \mu\text{g GAE/mL}$ in the *n*-hexane extract to $136.71 \pm 0.78 \mu\text{g GAE/mL}$ in the aqueous extract and reached $164.13 \pm 0.58 \mu\text{g GAE/mL}$ in the methanol extract. In contrast, the nanoparticle formulation had a significantly lower TPC of $8.71 \pm 0.00 \mu\text{g GAE/mL}$. This significant decrease implies that the majority of phenolic compounds are encapsulated or bonded to the surface of the nanoparticles during their production, resulting in a reduced detectable quantity. Previous research has likewise shown that solvent polarity significantly affects the efficiency of phenolic extraction. Chaudhary (2025) reported TPC values of 15.863 mg GAE/g for hexane, 10.991 mg GAE/g for chloroform, and 2.358 mg GAE/g for methanol extracts. In contrast, **Awasthi et al. (2021)** recorded substantially higher TPC levels of $362.74 \pm 10.13 \text{ mg/g}$ in methanolic extracts. These differences indicate that solvent choice and plant part used have a significant impact on extraction. Nanoparticle samples generally show reduced TPC because the synthesis process typically encapsulates phenolic compounds, making them less measurable as free molecules. An important advantage of this approach is that a nanoparticle system with surface-holding flavonoid molecules provides enhanced protection against oxidation and degradation. This solvent-related effect agrees with observations from prior research.

The total flavonoid content (TFC) displayed an opposite trend, where the nanoparticle

showed a sharp increase ($273.11 \pm 0.19 \mu\text{g QE/mL}$) compared to solvent extracts, which recorded $114.22 \pm 1.35 \mu\text{g QE/mL}$ in *n*-hexane, $123.56 \pm 1.17 \mu\text{g QE/mL}$ in aqueous, and $140.67 \pm 0.88 \mu\text{g QE/mL}$ in methanol. The notable enhancement involves a nanoparticle system with surface-bound flavonoid molecules, offering protection against oxidation and degradation. This solvent-related variation has also been documented in earlier studies. Chaudhary (2025) found that methanolic extracts contained only 6.41 mg QE/g of flavonoids, while substantially higher amounts—205.03 and 173.71 mg QE/g—were obtained with hexane and chloroform, respectively. Likewise, **Awasthi et al. (2021)** reported flavonoid contents of $96.33 \pm 7.67 \text{ mg/g}$ in methanolic extracts. When compared with earlier studies, the considerably higher nanoparticle value obtained in this work indicates that nanoformulation plays a crucial role in safeguarding flavonoids by diminishing their degradation and enhancing their overall retention.

The antioxidant capacity shifted clearly, as evidenced by the DPPH radical scavenging results.

While *n*-hexane extracts exhibited $40.46 \pm 0.35\%$ activity, this increased to $57.07 \pm 0.13\%$ in methanol extracts, and the nanoparticle displayed a comparable $57.88 \pm 0.18\%$. Despite having a lower phenolic content, the nanoparticle's bioactive compounds remained highly effective after nanoscale conversion, resulting in high antioxidant activity. It can be justified that

other bioactive compounds in the nanoparticles might compensate for the low phenolic content. Such a resemblance has been reported in previous studies. Awasthi et al. (2021) found that the methanolic extract of *A. aspera* was the most potent antioxidant (IC₅₀: 61.396 µg/mL), while **Raut et al. (2021)** observed significant DPPH scavenging (IC₅₀: 30.5 µg/mL). (**Sharma et al., 2025**) reported hydroalcoholic extracts with an IC₅₀ of 4.17 ± 1.73 µg/mL. The close alignment of results indicates that nanoparticles act as a protective matrix for active compounds, facilitating a controlled release that preserves, and potentially elevates their antioxidant activity.

A similar pattern was also observed in α -amylase inhibition. The solvent extracts revealed $46.37 \pm 0.03\%$, $64.92 \pm 0.11\%$, and $78.37 \pm 0.80\%$, respectively, but the nanoparticle showed $62.12 \pm 1.27\%$. The slightly lower activity of the nanoparticle compared to the methanol extract likely reflects that the inhibitory compounds are released more gradually from the nanoparticle's surface. This behaviour is in line with previous studies. For example, **Prakash and Sagar (2021)** observed $69.90 \pm 0.50\%$ inhibition with methanolic extract and $46.65 \pm 0.42\%$ with aqueous extract, while **Sharma et al. (2025)** reported an IC₅₀ of 105.4 µg/mL for the hydroalcoholic extract. In conjunction, these findings suggest that while solvent-based extracts provide rapid inhibition of enzymes, nanoparticles offer a more controlled and sustained effect over an extended period. The nanoparticle's somewhat reduced activity in the current study could be attributed to a slower diffusion or release of bioactive chemicals, indicating

a regulated and extended enzyme inhibition pattern rather than an immediate response.

Hemolytic activity steadily increased across the extracts ($1.42 \pm 0.60\%$, $3.67 \pm 0.50\%$, and $5.13 \pm 0.67\%$), and surged slightly higher to $6.74 \pm 0.35\%$ in the nanoparticle. Increased surface reactivity in the nanoparticles is likely the reason for the observed rise, as it promotes more robust interactions with red blood cells. Similar trends in cyto-compatibility have been reported in other formulations, where slight hemolysis is often related to nanoparticle surface charge and size. Yet the measurements remain within acceptable, safe biological limits.

In the pool of tested activities, anti-inflammatory effects showed the most pronounced improvement. The solvent extracts showed $35.29 \pm 2.94\%$, $37.65 \pm 2.94\%$, and $52.17 \pm 8.70\%$, whereas the nanoparticles showed $65.22 \pm 4.35\%$. The elevated activity observed in the nanoparticle enables a sustained release of bioactive compounds and more efficient penetration of these compounds. This trend is consistent with previous studies; for instance, **Ha-Anh et al. (2024)** reported only modest efficacy in methanol extracts, while **Samaiya et al. (2024)** demonstrated that nanogel formulations indicated significantly greater anti-inflammatory effects than their corresponding pure extracts. The current findings reinforce the idea that nanoformulation not only preserves the activity of bioactive components but can also increase their therapeutic impact and bioavailability.

All-inclusive, comparisons with earlier studies show how transforming solvent extracts into nanoparticle form can

meaningfully alter both their functional performance and chemical composition. While the nanoparticle demonstrated increased flavonoid concentration, marginally higher antioxidant activity, and a significant improvement in anti-inflammatory potential, the extracts had higher quantifiable phenolic compounds and immediate antioxidant and enzyme inhibitory effects, in line with previous solvent-based studies. These changes show that, in contrast to earlier extraction-based research, the nanoparticle formulation converts these molecules into a more stable and bioavailable form, improving therapeutic efficacy even

while conventional solvents effectively extract bioactive chemicals.

Structural characterization

Fourier-transform infrared spectroscopy

FTIR provides an infrared (IR) spectrum when compared to a standard spectrophotometer. It is applied in order to determine some functional groups and identify the structure of the sample material. The results of FTIR analysis are provided in Figure 1 and Table 2. Table 2 shows the absorption values of different compounds through FTIR analysis.

Table-2. FTIR spectra for *Achyranthes aspera*

Peaks	Compound class	Functional group
3278.2	Alcohol, amines, amides	O-H, N-H
2920.4	Alkanes	C-H
2182.4	Alkyne	C≡C
2167.4, 2202.9	Alcohols, Alkyne	O-H, C≡C
1792.8, 1768.6	Anhydride	C=O
1241.2, 1015.7	Alcohols, ethers, esters, carboxylic acids	C-O, C-N

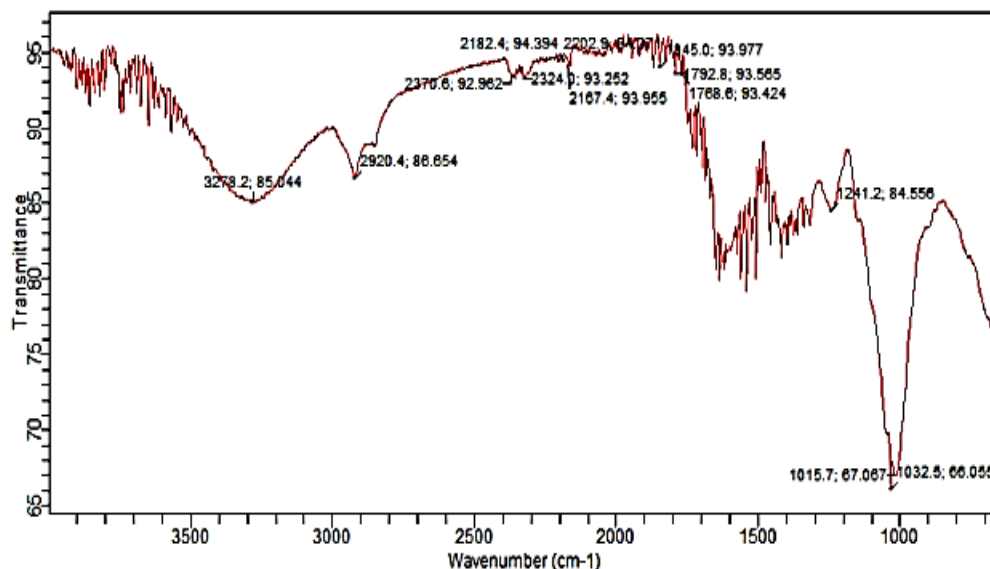


Figure. 1: FTIR spectra of *A. aspera*

According to Nadeem *et al.* (2008), the amino, carboxylic, phosphate, and carbonyl functional groups were the primary ones found on the surface that were not treated and exhibited peaks between 3500 and 3000 cm^{-1} , resulting from NH stretching and NH bending, which presented amino groups, and C O stretching between 1550 and 1500 cm^{-1} . While our results showed anhydride groups at 1792.8 and 1768.6 cm^{-1} . The spectrum showed that the samples that were not treated exhibited peaks between 3500 and 3000 cm^{-1} , attributed to NH stretching and NH bending, while our results at 3278.2 cm^{-1} showed Alcohol, H-bonded, Primary and secondary amines and amides, which presented amino groups between 1550 and 1500 cm^{-1} , and C-O stretching, which occurred at 1641 cm^{-1} . Wave numbers 1470-1365 cm^{-1} have peaks that correspond to amino-substituted alkyl groups. The peaks that arise between 3000 and 2500 cm^{-1} are caused by carboxylic groups.

According to Ashok *et al.* (2018), the FTIR (Fourier Transform Infrared) analysis of *Achyranthes aspera* revealed a variety of functional groups, indicating the presence of multiple bioactive compounds. Key functional groups identified include hydroxyl groups (O-H) around 3300 cm^{-1} , carbonyl groups (C=O) around 1700 cm^{-1} , and C-H stretching vibrations in the region of 2800-3000 cm^{-1} . These functional groups are significant in understanding the plant's pharmacological properties, including its antibacterial and antioxidant activities, while our results showed anhydride groups at 1792.8 and 1768.6 cm^{-1} and we have alkanes at 2920.4 cm^{-1} .

High Performance Liquid Chromatography

The performances are shown in Figure 2. Chlorogenic acid was exhibited at a 2.826 min retention time. The peak of p-coumaric acid properties retention time was observed at 11.163 min with 1,434,003.5 peak area.

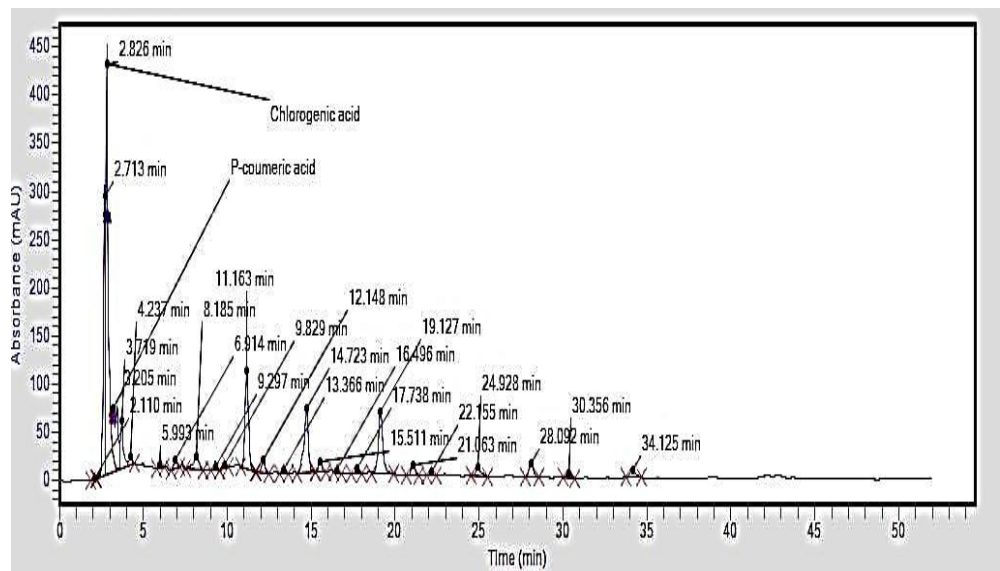


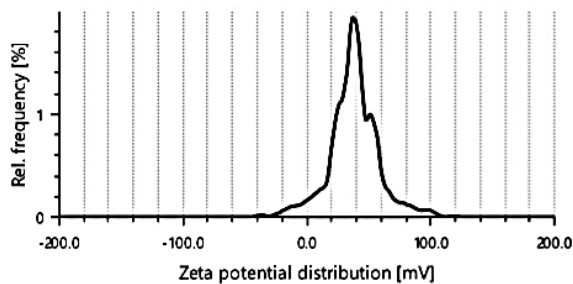
Figure. 2: HPLC spectra of *A. aspera*

Characterization of nanoparticles

The characterization of nanoparticles was done using a Litesizer 500 in terms of zeta

potential. The size of the nanoparticles was 116.77nm (Figure 3), and the nanoparticles were positively charged with a potential of 31.6 mV (Figure 4).

Zeta potential distribution



Result

Mean zeta potential	31.6 mV	Mean intensity	492.1 kcounts/s
Standard deviation	0.8 mV	Filter optical density	2.5047
Distribution peak	38.3 mV	Conductivity	0.119 mS/cm
Electrophoretic Mobility	2.4650 $\mu\text{m}^2\text{cm/Vs}$	Transmittance	48.2 %

Figure .4: Results of zeta potential

The nanoparticles were characterized for size and surface charge using a well-established protocol and research focused on comparative bioactivities of crude extract and nanoparticles instead of drug-release profile, target efficiency, or toxicity. Therefore, the polydispersity index was not crucial for the research objectives. **Agarwal et al. (2018)** found that nanoparticles showed a zeta potential of 50mV, while our results showed 31.6 mV. This positive zeta potential suggests that the chitosan nanoparticles are well-suited for various biomedical applications due to their ability to remain stable in colloidal suspensions. For *Achyranthes aspera*, nanoparticle size was observed to be normal and showed three peaks and showed sizes of 116.77nm, 10.87nm and 10.87nm while their standard deviation was observed to be 25.29nm, 1.27nm and 0.00 nm, respectively. **Agarwal et al. (2018)** observed that the size of nanoparticles was 216 nm, while our results showed a 116.77nm value, which represents good results and better efficiency than previous results. According to **Hannan et al. (2025)**, the prepared chitosan nanoparticles showed a diameter of 83.9 nm by zeta sizer, while our nanoparticles showed a diameter of 116.77nm, which is higher than previous results but has value in a normal range.

CONCLUSION

It is established that some bioactivities of nanoparticles were higher than those of the extracts. Additional studies to streamline the process in animal models are justified to identify possible applications. There is a need to study it regarding in vivo testing, toxicity, and pharmacokinetic analysis to facilitate its clinical use in the future. At enhanced

validation, *A. aspera* would present itself as a strong exhibitor of new herbal drug development.

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