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## Research Article

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**Green Fabrication of *Acacia ferruginea* Based Silver Nanoparticles Targeting TNF- $\alpha$  and AMPK Receptors for the Management of Haemorrhoids**

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## Abstract

**Introduction:** Silver nanoparticles synthesized through environmentally friendly plant-based methods have attracted increasing interest due to their potential biomedical applications. The present study aimed to synthesize silver nanoparticles using the hydroalcoholic leaf extract of *Acacia ferruginea*, perform comprehensive physicochemical characterization, evaluate antioxidant and anti-hemorrhoidal efficacy using statistically validated models, and explore plausible molecular mechanisms using supportive computational approaches. **Method:** Silver nanoparticles were synthesized through a green reduction method and characterized using UV spectroscopy, FTIR, DLS, TEM, and energy-dispersive X-ray spectroscopy, with complete instrument specifications provided. Phytochemical constituents associated with nanoparticle stabilization were identified by LCMS. Antioxidant activity was evaluated using a time and concentration dependent DPPH assay. Anti-hemorrhoidal efficacy was assessed in a croton oil induced rat model through measurement of the recto-anal coefficient and histopathological examination. **Results:** The synthesized nanoparticles exhibited a characteristic surface plasmon resonance peak at 423 nm and predominantly spherical morphology with an average particle size of approximately 20 nm. Identified phenolic and flavonoid compounds contributed to nanoparticle stabilization. The nanoparticles demonstrated significantly enhanced antioxidant activity compared with the plant extract alone. In vivo studies showed a significant reduction in recto-anal inflammation and tissue damage following topical nanoparticle treatment. Molecular docking analysis suggested favourable interactions of selected phytoconstituents with TNF- $\alpha$  and AMPK as supportive, hypothesis-generating evidence. **Conclusion:** This study provides a statistically validated and mechanistically discussed evaluation of *Acacia ferruginea* derived silver nanoparticles, demonstrating advancement beyond conventional green nanoparticle synthesis studies.

**Keywords:** *Acacia ferruginea*, silver nanoparticles, green synthesis, antioxidant activity, anti-hemorrhoidal activity, molecular docking.

# **Green Fabrication of *Acacia ferruginea* Based Silver Nanoparticles Targeting TNF- $\alpha$ and AMPK Receptors for the Management of Haemorrhoids**

## **1. INTRODUCTION:**

Haemorrhoids is a prevalent anorectal medical condition characterised by painful expansion and distal displacement of the anal cushions [1]. It is one of the most common inflammatory disorders, characterized by changes in the vasculature of the anal canal, which includes muscles, supporting tissues, blood vessels, and elastic fibres. Several factors are believed to contribute to the development of haemorrhoids, including age related weakening of the anal supporting tissues, chronic constipation, prolonged straining during defecation, irregular bowel habits, diarrhoea, pregnancy, prolonged sitting posture, and increased intra-abdominal pressure leading to venous congestion. Additionally, abnormalities of the internal anal sphincter are some of the etiologic factors thought to contribute to the pathologic changes in the vascular cushions [2]. This condition is most prevalent among individuals aged between 45 to 65 years [3]. Haemorrhoidal disease is also frequently observed in individuals with spinal cord injuries, chronic constipation, persistent diarrhoea, or poor bowel habits, such as delaying defecation [4]. Dietary and lifestyle factors play a significant role as well particularly a low fibre diet, excessive intake of spicy foods, and alcohol consumption, all of which have been identified as potential risk factors. Common symptoms include rectal bleeding, mucosal prolapse. Haemorrhoids can be treated therapeutically from major surgery to dietary and lifestyle changes, depending on the severity and degree of symptoms [5]. Medical treatment includes oral flavonoids and oral calcium dobesilate. Non-operative treatment such as sclerotherapy and rubber band ligation [6]. Problem with oral flavonoids are poor bioavailability, gastrointestinal side effects, allergic reactions and bleeding risk. Topical treatments that help locally reduce inflammation and stop bleeding and swelling are highly beneficial in treating haemorrhoids. Although topical ointments

and lotions can temporarily relieve a patient's discomfort and irritation, their beneficial effects take time to show up [7].

Nanotechnology is an emerging area of biomedical research in 21st century which is rapidly growing worldwide [8]. Silver nanoparticles (SNPs) distinct physicochemical characteristics make them extremely important in the fields of biology and medicine [9]. SNPs have found extensive use across diverse fields such as nanomedicine, drug delivery, cosmetics, electronics, food technology, agriculture, and environmental protection. Their growing popularity stems from their exceptional features, including long term stability, enhanced surface resonance scattering, unique optical properties, excellent electrical conductivity, and strong thermal, chemical, nonlinear, and catalytic stability. Typically, SNPs can be synthesized through physical, chemical or biological approaches [10]. In recent decades, green synthesis using plant extracts has gained considerable attention. This eco-friendly method not only minimizes the use of hazardous chemicals but also produces nanoparticles with superior physical and biological characteristics compared to those obtained through conventional methods [11]. Topical treatment with SNPs can be more effective for soothing inflammation and pain, provide a longer lasting protective barrier, easy for application and painless. Silver nanoparticle ointment offers superior antibacterial, anti-inflammatory, and wound healing properties compared to normal ointments, promoting faster relief and recovery in haemorrhoids. In contrast, normal ointments mainly provide symptomatic relief without significant antimicrobial or tissue-regenerative effects. Silver nanoparticles synthesized through green routes have emerged as promising therapeutic agents due to their enhanced bioactivity and reduced toxicity. However, most published studies focus on synthesis and basic antimicrobial screening, with limited disease-specific validation or mechanistic insight. *Acacia ferruginea*, a medicinal plant rich in phenolic and flavonoid compounds, has not been previously explored for AgNP-mediated anti-hemorrhoidal therapy.

A recent study reported phytosterol-loaded silver nanoparticles (PLSNPs) that showed anti-haemorrhoidal activity in experimental models, with reduction of haemorrhoidal scores, improvements in biochemical or antioxidant status, and favourable histology. This demonstrates that PLSNPs can have measurable anti-haemorrhoidal effects in preclinical testing [12].

*Acacia ferruginea* is a small, deciduous tree that belonging to family Mimosoideae [13]. Alkaloids, flavonoids, triterpenoids, saponins, tannins, phenolic compounds, sugars, gums, mucilage's, proteins, and amino acids are some of the chemical components present in the plant. Some of the pharmacological uses are cytotoxicity, hepatoprotective, antitumor, anticancer, antifungal, antidiarrheal, anti-anxiety, antioxidant, antimicrobial, antibacterial, and anti-inflammatory, antidiabetic, antihyperglycemic, antiulcer, and free radical scavenging properties. Different portions of the plant have been used to treat diabetes, leukoderma, piles, itching, and skin infections.

*A. ferruginea* contains flavonoids, tannins, phenolics and saponins that may add anti-inflammatory, astringent and antioxidant actions when present with SNPs potentially improving symptom control (bleeding, swelling, pain) and healing versus SNPs alone. Natural products have long been used to manage hemorrhoids owing to their antioxidant and anti-inflammatory effects. Among them, *Acacia ferruginea* (Mimosoideae) has attracted attention for its potent pharmacological profile [14]. Reported that the hydroalcoholic bark extract of *A. ferruginea* significantly reduced recto anal inflammation in rats.

## **2. Materials and Methods**

### **2.1 Materials**

*Acacia ferruginea* leaves were collected from local region of Challakere, were authenticated by Avitha Marihal, Department of Botany, Akkamahadevi Women's Science College, Bagalkot. Silver nitrate and Polyethylene glycol (PEG) 400 from SDFCL, Mumbai, Polyethylene glycol (PEG) 4000 from Loba Chemie pvt.ltd Mumbai, Methyl paraben from NR

Chem Mumbai and Propyl paraben from Genuine Chemicals Mumbai, all the chemicals used for study are of analytical grade. Throughout the study double distilled water was used.

## **2.2 Methods**

### **2.2.1 Preparation of *Acacia ferruginea* Hydroalcoholic extract (AFHAE)**

The leaves were collected freshly and washed thrice with double distilled water to get rid of impurities, air dried at room temperature and powdered by using mortar and pestle. 10gm of powder used for extraction using 70ml of ethanol and 30ml of water, then kept for maceration in dark place for three consecutive days. Followed by filtration using musclin cloth and whatmann filter paper No.1, then the filtrate obtained was collected at centrifuged at 4000 rpm for 15min and supernant layer was collected and stored at 4°C for the further synthesis of SNPs [15].

### **2.2.2 Phytochemical Analysis of AFHAE**

Phytochemical analysis of the *AFHAE* was performed by following the procedures in Khandelwal et al. [16].

### **2.2.3 Quantitative phytochemical evaluation of AFHAE**

#### **a) Determination of total phenolic content (TPC)**

Folin- Ciocaltecu method was used to determine the TPC of the AFHAE. Extract of about 1ml was mixed with folin-ciocalteu reagent 5ml (diluted tenfold) followed by the addition of Na<sub>2</sub>CO<sub>3</sub> (4ml). Then the resultant solution was kept for 30min at room temperature, at 765nm UV absorbance was taken. Gallic acid was used as reference standard and by using following formula TPC was calculated [17].

$$C = C1 \times v/m$$

Where,

C = total phenolic content in mg/g, in GAE (Gallic acid equivalent),

C1= concentration of Gallic acid established from the calibration curve in mg/ml,

V = volume of extract in ml, and

m = the weight of the plant extract in g.

#### **b) Determination of total flavonoid content (TFC)**

By the formation flavonoid aluminum complex which exhibits absorbance at 430nm TFC was determined. 200 $\mu$ L of extract (0.5mg/ml) was mixed with 50 $\mu$ l of 5% (w/v). Sodium nitrite solution and distilled water of 1ml, then the resultant solution was kept at room temperature for 6min. Addition of AlCl<sub>3</sub> (10%w/v) and incubated for 5min, followed by addition of 1mM NaOH(100 $\mu$ l) and absorbance was measured at 430nm. Rutin was used as standard and TFC was determined by using the following formula [18].

$$C = C1 \times v/m$$

Where,

C = flavonoid content in mg/g, in rutin (Rutin equivalent),

C1= concentration of rutin established from the calibration curve in mg/ml,

V = volume of extract in ml, and

m = the weight of the plant extract in g.

#### **2.2.4 Biosynthesis of SNPs from AFHAE**

Green synthesis of SNPs was prepared by using different volume of extract (2,3,4,5 and 6ml) with 2mM AgNO<sub>3</sub> solution, and volume was adjusted to 100ml. Then the above solution was kept for incubation in a dark place for 3hrs. After 3hrs the colour changes from pale green to brick brown signifies the successful formation of SNPs. Then the colloidal solution was subjected for centrifugation at 12000 rpm for 15min. Then pellets obtained was rinsed thrice with distilled water to get rid of remaining plant residues and unreacted ions. Finally, the purified SNPs was kept in oven at 60°C for drying and obtained SNPs was kept for further characterization [19].

## **2.2.5 Characterization of SNPs**

### **2.2.5.1 UV-Vis spectrophotometer analysis**

SNPs synthesized was confirmed by taking absorbance in the range of 200-800nm by using 2mM AgNO<sub>3</sub> as blank, all the measurements were taken at room temperature. On the basis of surface plasma resonance which reflect optical properties of metallic nanoparticles [20].

### **2.2.5.2 FTIR**

The SNPs and AFHAE were dried in room temperature and then finely blended with KBr (potassium bromide) to obtain uniform mixture. This mixture was compressed into thin pellets, as KBr remains transparent within the infrared region. The FTIR spectra were obtained in transmittance mode in the wavelength range of 400–4000 cm<sup>-1</sup> [21].

### **2.2.5.3 Dynamic light scattering analysis of SNPs**

The SNPs average particle size and zeta potential (surface charge) were recorded using DLS. 1mL of the SNPs suspension was mixed with 10mL of double distilled water and transferred to cuvette. The laser light scattering was recorded at fixed angle of 90°, and the resulting data were collected [22].

### **2.2.5.4 Transmission electron microscopy (TEM) & EDAX**

Green Synthesized SNPs structural morphology was examined using TEM. Prior to imaging, the SNPs suspension was sonicated to reduce particle aggregation. A small droplet of the well dispersed sample was carefully added to a TEM grid and allowed for air-dry before observation. Analysis was carried out at a potential of 200kV to obtain high resolution images of the SNPs. Furthermore, the elemental composition was evaluated using EDAX (Energy Dispersive X-ray spectroscopy) performed under standard operating conditions [23].

#### **2.2.5.5 Anti-oxidant studies**

DPPH free radical scavenging method was used to determine the antioxidant property of green synthesized SNPs. In this method, varying concentrations of the AFHAE (10, 20, 30, 40, 50, 75 and 100µg/mL) were prepared by mixing with 0.1 mM DPPH solution in ethanol. Above solutions were incubated at room temperature for 30min, at 517nm absorbance was measured. Ascorbic acid was used as standard. The % Scavenging activity was determined by using the below mentioned formula [24].

$$\text{Percentage of DPPH radical scavenging activity} = \frac{Ac - As}{Ac} \times 100$$

Where, Ac is the absorbance of control and

As is the absorbance of test samples

#### **2.2.5.6 LCMS**

Identification of phytoconstituents that capped by silver ion or analysis of capping of phytoconstituents by LC-MS/MS. LC-MS analysis of the green synthesized SNPs was carried out in Sophisticated Analytical Instrumental Facility (SAIF) Indian Institutes of Technology Bombay. An Agilent G6550 Q-TOF mass spectrometer (Agilent Technologies, USA) fitted with an electrospray ionization (ESI) source was utilized in both positive and negative ionization modes. Separation of the compounds was done on a reverse phase C18 column maintained at 40°C, with a constant flow rate of 0.3 mL/min. A 5 µL aliquot of each sample was injected for analysis. The mobile phase comprised a binary solvent system: water with formic acid of 0.1% (mobile phase A) and acetonitrile with formic acid of 0.1% (mobile phase B) [25].

#### **2.2.6 Preparation of ointment using plant extract (5%)**

The ointment containing extract was prepared by a fusion method. The specified amount of PEG 4000 (53.3gm) was melted in a china dish over a boiling water bath. Then coconut oil (5gm) and PEG 400(33.3gm) were added to the melted PEG 4000. The mixture was then removed from heat and stirred. Methyl and propyl paraben were dissolved in

remaining quantity of PEG 400 and added to above mixture. Subsequently, 5 gm of AFHAE was dissolved in water and incorporated into the PEG mixture, followed by stirring until the mixture solidified.

### **2.2.7 Preparation of ointment using SNPs**

Silver nanoparticle ointment strength 0.02% were prepared by taking 68 mg of SNPs which is equivalent to 0.02% respectively. The specified amount of PEG 4000 (53.3gm) was melted in a china dish over a boiling water bath. Coconut oil (5gm) and PEG 400 (33.3gm) were added to the melted PEG 4000. The mixture was then removed from heat and stirred. In the remaining quantity of PEG 400, Propyl paraben and Methyl paraben were dissolved and added to the above mixture. Then, green synthesized SNPs were dispersed in water and added to above PEGs mixture and stirred until congealing [26].

### **2.2.8 Anti-Hemorrhoidal study**

For evaluation of Anti-hemorrhoidal potential of green synthesized SNPs, Albino wistar rats of female sex weighed 150-200gm were used. The animals were kept with free access to standard rat chow and water to adapt to the surrounding environment for one week. Then for performing the experiment ethical clearance was granted from the IAEC (Institutional animal ethical committee) before the commencement of project work. Protocol number IAEC/HSKCOP/July 2024/ PG19.

### **2.2.9 Induction of hemorrhoids**

For induction of hemorrhoids, croton oil preparation (COP) was obtained by combining the croton oil, diethyl ether, pyridine and deionized water in the ratio of 10:5:4:1. Approximately 100  $\mu$ L of this solution was used to wet a sterile cotton swab (4 mm in diameter), which was gently inserted 20 mm into the anal aperture of overnight-fasted experimental animals, reaching the recto-anal region, and held in place for 10 seconds. The animals were monitored for a period of 7–8 hours to assess the progression of edema. After induction, the hemorrhoidal condition was

evaluated based on its severity and further analyzed through histological examinations [27].

Group I: Control group treated with placebo formulation.

Group II: Group treated with ointment containing AFHAE (5%), applied twice daily.

Group III: Group treated with ointment containing SNPs (0.02%), applied twice daily.

Group IV: Group treated with marketed standard ointment, applied twice daily.

#### **2.2.9.1 Evaluation of Anti-hemorrhoidal activity**

##### **a) Recto anal Coefficient (RAC):**

After anesthesia, the animals were sacrificed, and a 20 mm section of anal tissue was excised and weighed. Subsequently, the RAC was determined by the following formula [28].

$$\text{RAC} = \frac{\text{weight of rectoanal tissue (gm)}}{\text{Body weight (gm)}}$$

##### **b) Histopathological assessment**

Recto anal tissue specimens were collected from rats and immediately immersed in neutral buffered formalin (10%) for 24 hours to ensure proper fixation. The preserved samples were subsequently processed using a standard paraffin embedding technique. Serial fragments of 5 µm thickness have been separated with a rotary microtome and placed on glass slides. The resulting sections were deparaffinized, rehydrated with ethanol in a graded series, and smudged with haematoxylin and eosin (H&E) for histological examination. The stained slides were observed under a microscope to evaluate structural and pathological changes related with hemorrhoidal formation in the recto anal area [29- 30].

#### **2.10 Network Pharmacology**

##### **Bioactive and their targets:**

The reported phytoconstituents from *Acacia ferruginea* leaves, LCMS reports and previous literature studies. For each compound, the canonical SMILES, molecular weight and molecular formula were retrieved from PubChem database. Aromatic and heterocyclic

compounds are selected to ADMET profile and drug likeness.

#### **Drug likeness and ADMET profile:**

Drug likeness score for each compound was assessed using MolSoft according to "Lipinski's Rule of Five" model. In addition, ADMET characteristics of individual phytoconstituent was evaluated using PKCSM multiple regression models. Selection of phytoconstituents and chemical components are based on their calculated scores.

#### **Target prediction:**

Each plant phytoconstituents which are less or no toxic effect subjected to Superpred or Swiss target prediction, strong binder and addition prediction targets are exported to the excel sheet and uniprot ID are collected. Uniport IDs of each phytoconstituent are imported to Uniport ID mapping and final data exported to excel sheet. Genes are separated into column.

#### **Gene set enrichment analysis:**

Using the STRING database, proteins associated with the pathogenesis of hemorrhoids were predicted, and the relevant biological pathways were characterized with reference to the KEGG pathway database.

#### **Network construction:**

Using Cytoscape 3.6.1, a network was generated to visualize interactions among phytoconstituents, their target proteins, and related pathways. The "edge count" function in the Network Analyzer tool was used for analysis, with node size indicating the strength of connections between phytoconstituents, proteins, and pathways [31].

## **2.11 Molecular docking**

Based on network pharmacology the receptors TNF- $\alpha$  (PDB ID: 2AZ5) and AMPK (PDB ID: 4CFF) were prepared in AutoDock Tools 1.5.7 by removing water molecules, co-crystal ligands, ions, and repairing missing residues. Kollman charges and polar hydrogens were added, and receptor files were saved in PDBQT format. Ligands gyrocyanin and gravelliferone were drawn in ChemDraw, converted to 3D structures using Avogadro, and energy minimized in AutoDock with Gasteiger charge addition before saving as PDBQT files. Molecular docking was carried out using AutoDock Vina via command line execution with defined grid coordinates, resulting in output and log files. The docked complexes were imaged and analyzed in Discovery Studio 2021 to confirm the 2D and 3D ligand receptor interactions [32].

## **Results and discussion**

### **3.1 Phytochemical analysis of AFHAE**

On phytochemical examination of AFHAE showed the existence of many naturally occurring constituents such as saponins, flavonoids, alkaloids, tannins, phenolic compounds, and steroids [33].

### **3.2 Quantitative phytochemical evaluation of AFHAE**

AFHAE was used in the current investigation to evaluate various components. The results of this study showed that AFHAE contained higher levels of flavonoids. The AFHAE contained  $73.27 \pm 1.39$  mg/g of flavonoids and  $10.77 \pm 0.03$  mg/g of polyphenols [34].

### **3.3 Characterization of SNPs**

#### **3.3.1 UV-Vis spectrophotometer analysis**

Green synthesized SNPs was demonstrated by a change of color from pale green to brick brown within three hours. The subsequent increase in colour intensity was produced by the activation of electrons in the SNPs, which resulted in surface plasmon resonance. The green

synthesized SNPs were then investigated using UV-Vis spectrophotometry. Figure No1 shows the spectra of SNPs. The bio generated SNPs revealed an absorbance maximum at 423 nm [35].

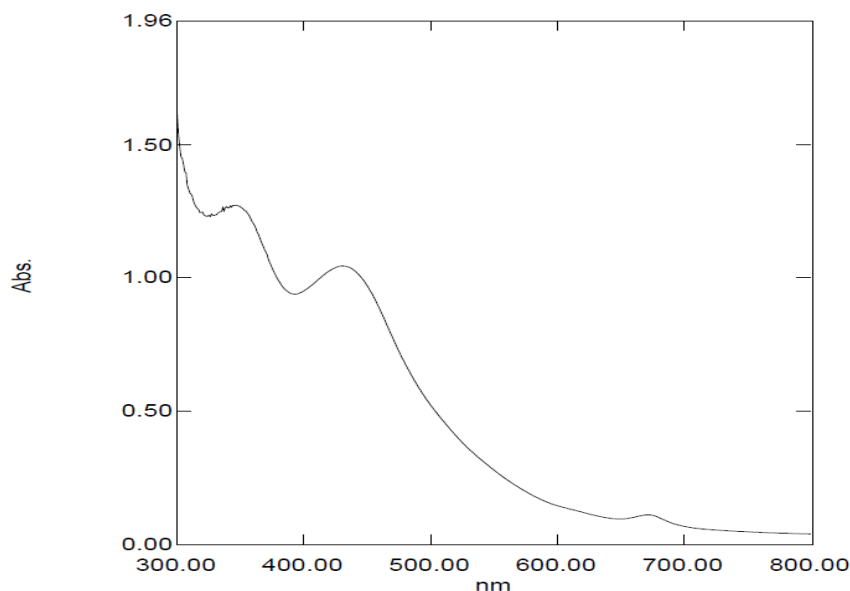


**Figure No: 1 UV-Vis spectroscopy of SNP**

### 3.3.2 FTIR

The functional groups which are responsible for reducing silver ions ( $\text{Ag}^+$ ), capping, and stabilizing green synthesized SNPs was identified by FTIR. The spectra of AFHAE (Figure No: 2a) showed prominent absorption bands at 3301, 2926, 2854, 1708, 1605, 1537, 1512, 1443, 1201, and 1030  $\text{cm}^{-1}$ , which correspond to hydroxyl (O-H), carbonyl (C=O), amine (N-H), aromatic rings, and ether (C-O-C) functional groups. These peaks show that the extract contains bioactive substances such polyphenols, flavonoids, tannins, and proteins. These biomolecules are known for their antioxidant and metal-chelating characteristics, making them useful in the biosynthesis process. In comparison, the FTIR spectrum of the synthesized silver nanoparticles (SNP1) (Figure No:2b) revealed distinct peaks at 3270, 2918, 2853, 1728, 1651, 1613, 1512, 1443, 1345, 1201, 1143, 1034, and 519  $\text{cm}^{-1}$ . The broad peak at 3270  $\text{cm}^{-1}$  is due to O-H stretching, showing the role of phenolic chemicals in the reduction and stability of silver ions. The peaks at 2918 and 2853  $\text{cm}^{-1}$  are associated with aliphatic C-H stretching, while the strong absorption at

1728  $\text{cm}^{-1}$  (C=O stretching) and amide-related peaks at 1651 and 1613  $\text{cm}^{-1}$  confirm the involvement of carbonyl and protein groups in nanoparticle formation. The emergence of a new peak at 519  $\text{cm}^{-1}$  is attributed to Ag–O or Ag–N bonding, signifying successful nanoparticle synthesis and biomolecule interaction with silver. The slight shifts in peak positions and intensity changes between AFHAE and SNP confirm that the plant biomolecules act as both reducing and capping agents, enabling an eco-friendly and sustainable approach to nanoparticle synthesis [36].

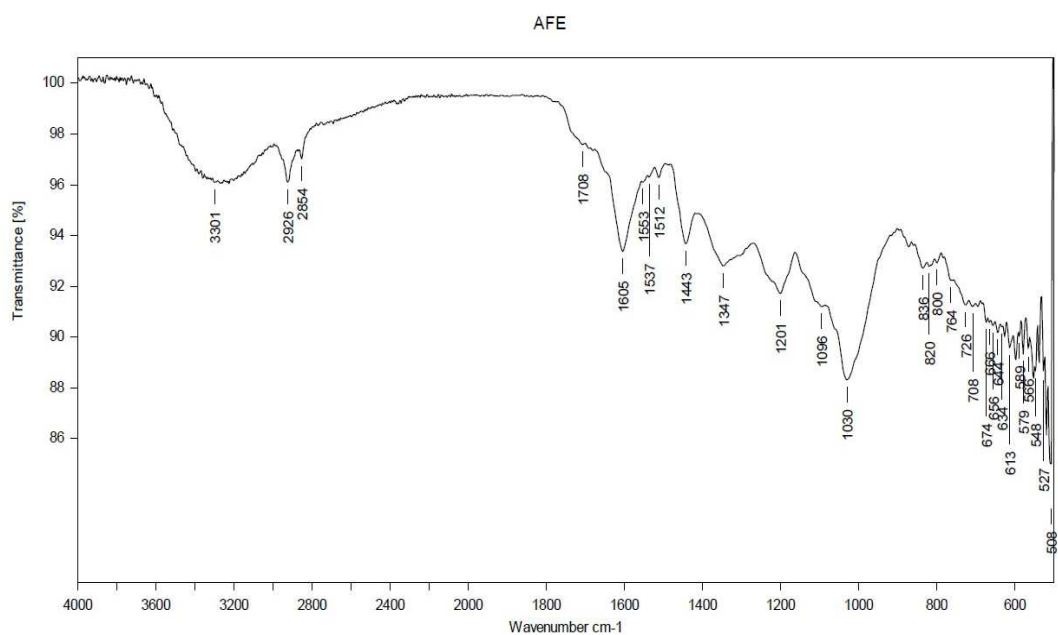


**Figure No- 2a: FTIR spectra of AFHAE**

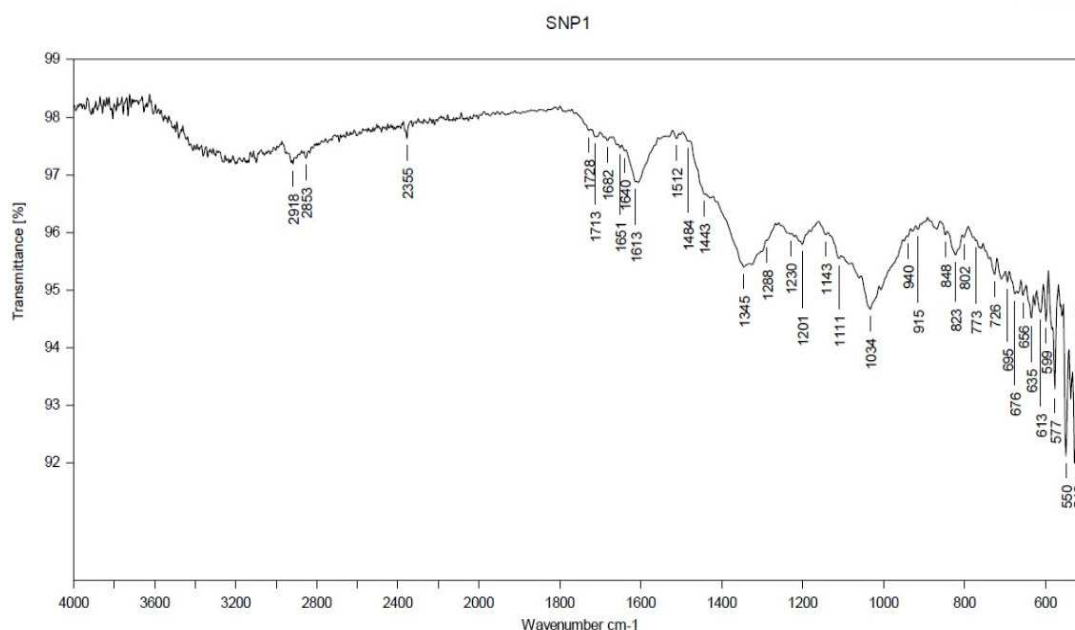


**Figure No- 2b FTT-IR spectra of SNP**

### 3.3.3 Dynamic light scattering analysis of SNPs

Using DLS, the average particle size of SNPs was investigated. The particle size of all five formulations ranged from 226.1 to 415.4 nm, with a zeta potential of -22.6 to -12.5mv. The mean particle size of SNP1 was 226.1 nm with zeta potential -22.6mv, SNP2 with mean particle size of 267.8nm and zeta potential -20.9mv, SNP3 the mean particle size of 278.4nm with zeta potential -17.6mv, SNP4 the mean particle size of 330.7nm with zeta potential of -15.3mv and SNP5 the mean particle size of 415.4nm with zeta potential of -12.5mv. Particle size was increased as the concentration of extract was increased, SNP1 formulation demonstrated the least average particle size, hence it was selected for further characterisation, anti-oxidant efficacy, and anti-hemorrhoidal studies [37].

### 3.3.4 TEM & EDAX

To analyse the morphology of produced SNPs TEM was used. Analysis of the TEM image (Figure No: 3) showed that the SNPs were mainly in spherical shape and exhibited a polydispersed distribution. As observed in Figure No: 3, the SNPs were surrounded by a faint,

thin coating and organic capping layer derived from AFHAE. To determine the average particle size, TEM images were processed using ImageJ software. The size distribution data were plotted using GraphPad Prism software, showing an average particle size of approximately 19.688 nm (Figure No: 3). The organic capping layer plays a crucial role in preventing nanoparticle aggregation. Furthermore, EDAX showed the presence of silver in the green synthesized SNPs (Figure No-4), with elemental composition indicating 29.02% silver concentration [38].

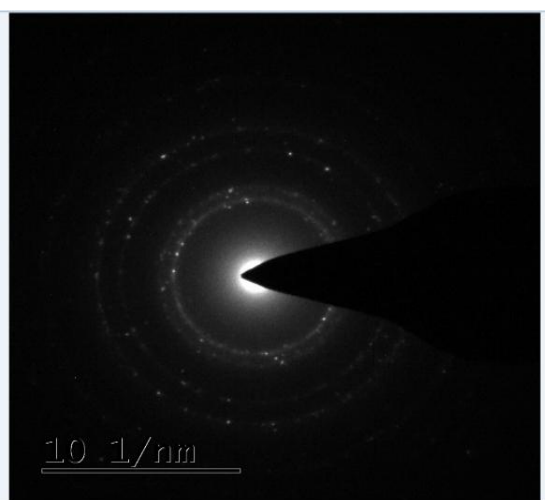


**Figure No-3a**

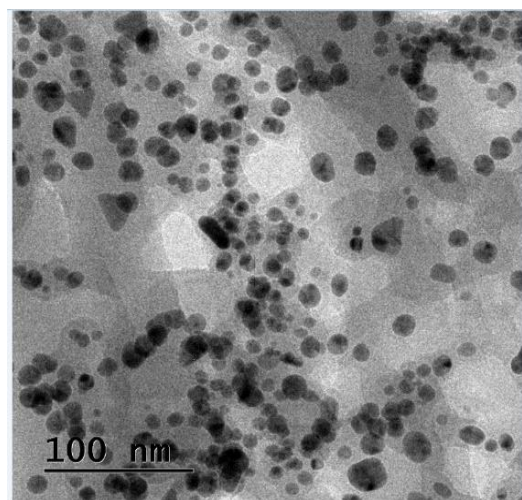


**Figure No-3b**

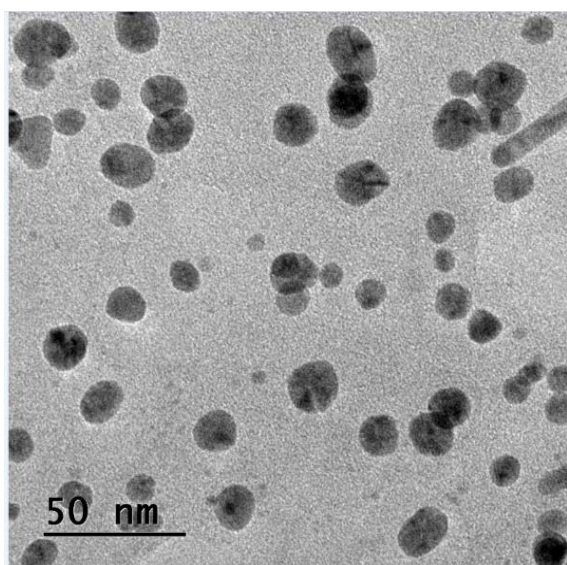


**Figure No-3c**

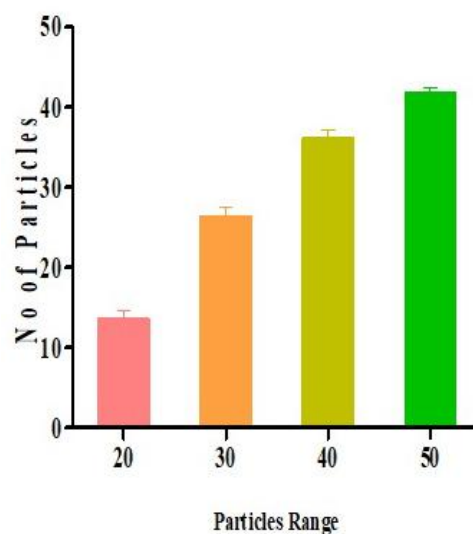


**Figure No-3d**

**Figure No 3: Particle size analysis using Image J software average particle size distribution histogramy using Prism graph pad software**

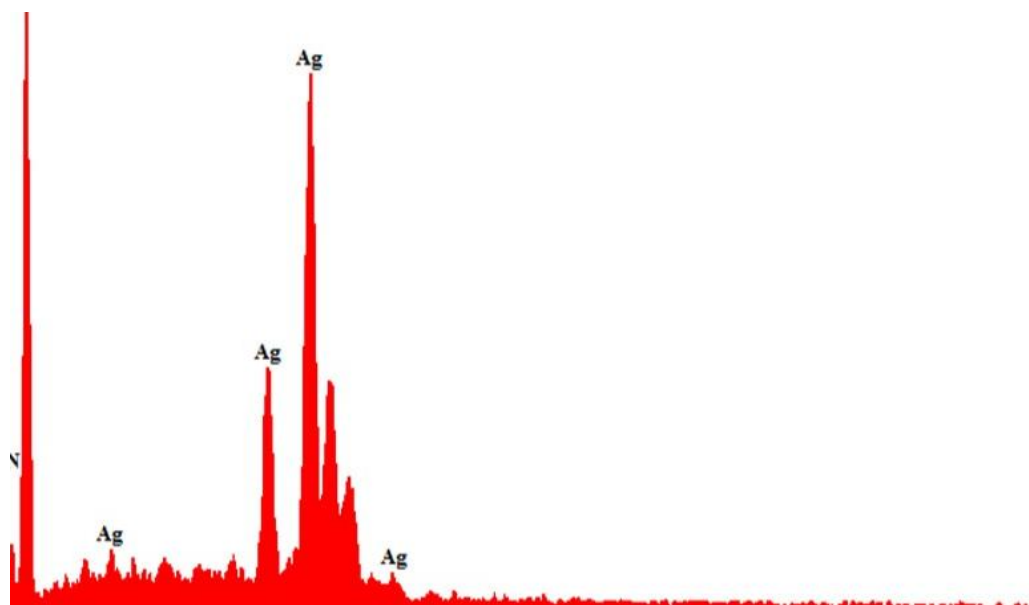


**Figure No-4 Energy dispersive X-ray analysis of SNP**

### **3.3.5 Anti-oxidant studies**

DPPH free radical scavenging test was used to evaluate the antioxidant property of AFHAE and SNPs. DPPH, a stable and widely recognized free radical, functions by accepting hydrogen or electrons from donor molecules. On the basis of observed colour change the antioxidant efficacy of SNPs was found out, as the control sample exhibited no such change. Upon the addition of SNPs to the DPPH solution, a noticeable colour shift occurred, indicating DPPH scavenging through hydrogen atom donation that stabilizes the DPPH radical, corresponding to an absorbance at 517 nm. The antioxidant efficiency of SNPs is likely due to the presence of bioactive functional groups from AFHAE attached to the nanoparticle surface. The  $IC_{50}$  values were determined as 62.54  $\mu\text{g/mL}$  for AFHAE, 51.28  $\mu\text{g/mL}$  for SNP1, and 45.29  $\mu\text{g/mL}$  for the standard ascorbic acid. The results demonstrated antioxidant activities of  $71.25\% \pm 0.234$  for AFHAE,  $83.66\% \pm 0.234$  for SNP1, and  $94.02\% \pm 0.92$  for the standard. Antioxidants block pro-

inflammatory enzymes like COX and TNF- $\alpha$ , which reduce cytokine release, relieve pain and itching, and promote tissue regeneration [39].

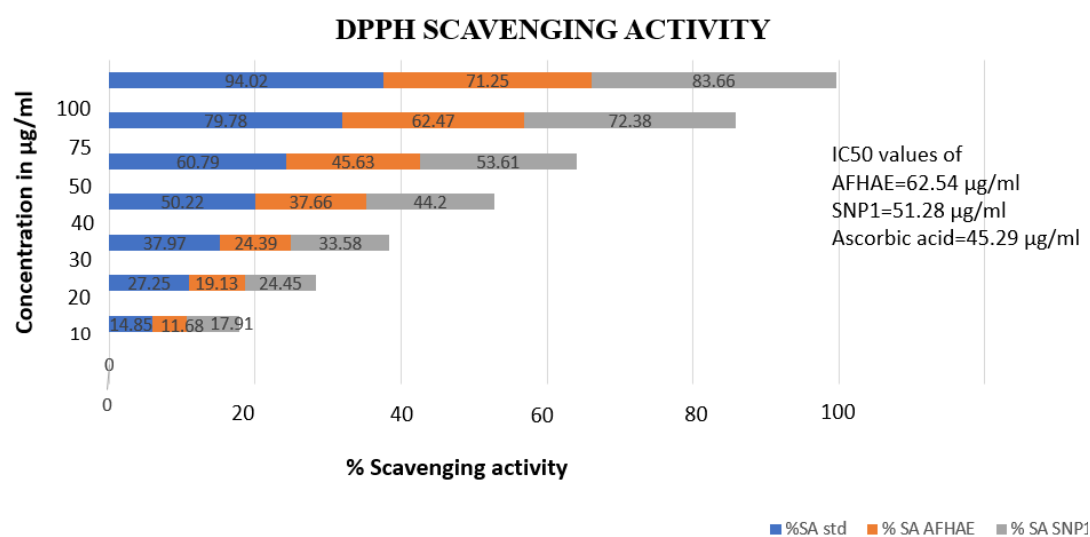


**Figure No-5 Antioxidant activity by DPPH scavenging method**

### 3.3.6 LCMS

The LC-MS/MS-QTOF analysis in negative ionization mode (Figure No-6) identified several bioactive phytoconstituents with potential anti-hemorrhoidal activity, including apimaysin, gyrocyanin, gravelliferone, and piceatannol 4'-galloylglucoside. Apimaysin ( $C_{27}H_{28}O_{13}$ ; MW 560.15 Da, RT 10.20–11.22 min) showed characteristic flavonoid glycoside fragments and is known for antioxidant and anti-inflammatory effects. Gyrocyanin ( $C_{17}H_{12}O_5$ ; MW 296.07 Da, RT 12.92–13.29 min) exhibited vasoprotective and free radical scavenging properties. Gravelliferone ( $C_{19}H_{22}O_3$ ; MW 298.16 Da, RT 21.32 min) demonstrated smooth muscle relaxant and mild anticoagulant potential, while piceatannol 4'-galloylglucoside ( $C_{27}H_{26}O_{13}$ ; MW 560.15 Da, RT 10.45–11.09 min) showed strong anti-inflammatory and vascular protective activities. Together, these compounds suggest a synergistic mechanism involving antioxidant defense, anti-inflammatory response, vascular strengthening and smooth muscle modulation, contributing to the extract's anti-hemorrhoidal potential [40].

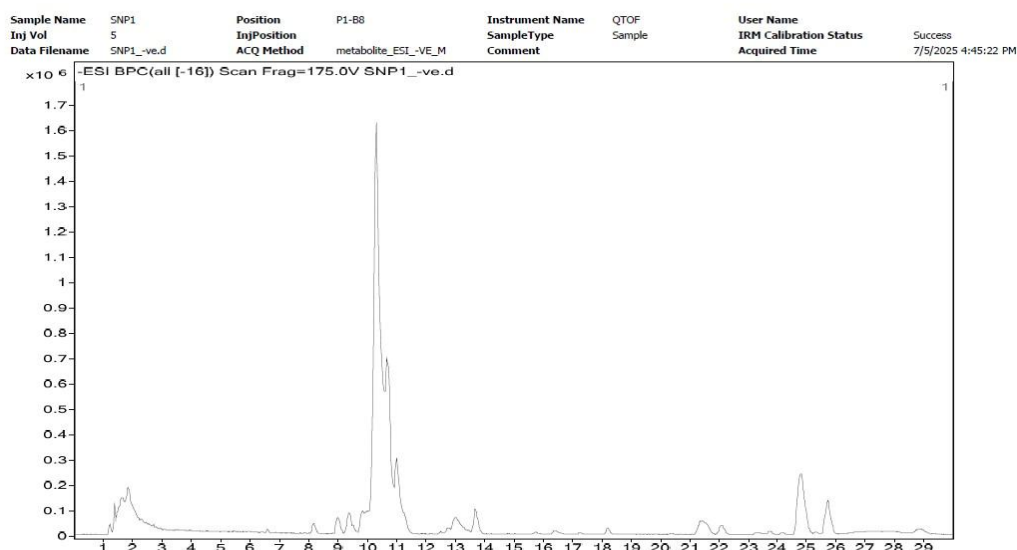


**Figure No-6 Analysis of capped phytoconstituents by LCMS analysis**

### 3.3.7 Anti-hemorrhoidal activity

#### a) Recto anal coefficient

The RAC evaluated the structural and functional integrity of the recto anal area. A higher RAC typically reflects a more inflammatory state and altered recto anal morphology, often associated with hemorrhoidal pathology. In this study, the anti-hemorrhoidal efficacy of various therapies (Figure No-7) was determined by its ability to reduce RAC values when compared with control group. Control group exhibited the highest RAC value  $1.21 \pm 0.039$ , indicating significant recto anal tissue disruption and inflammation typical of induced hemorrhoidal conditions. The standard treatment group, treated with standard ointment, showed a significantly lower RAC value of  $0.94 \pm 0.026$ , validating its effectiveness in restoring recto anal integrity. Treatment with AFHAE resulted in an RAC of  $1.08 \pm 0.010$ , indicating a moderate improvement compared to the control group. The group treated with SNP (0.02%) showed an RAC value of  $0.97 \pm 0.018$ , slightly better than the AFHAE group. This suggests that the SNP formulation at this concentration may have a stronger effect in reducing recto anal inflammation or tissue remodelling [41].

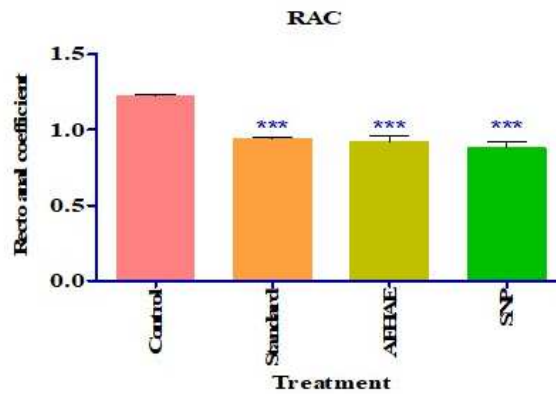


**Figure No: 7 Recto anal coefficients of experimental animal groups**

**All the values expressed as mean  $\pm$ SEM, n=24,  $p<0.05$ ,  $p<0.001$  (one way Analysis of Variance [ANOVA] followed by Dunnet test) as compared to control group**

#### **b) Histopathological assessment**

Histopathological evaluation of anorectal tissues revealed distinct variations among all experimental groups, reflecting the degree of tissue protection and healing given by each treatment. In the C1 (Figure No:8a), the mucosal and submucosal layers exhibited severe pathological alterations, including marked vascular congestion, oedema, intense inflammatory infiltration, and the presence of granulation tissue with superficial and deep ulceration. Degenerative and necrotic changes were also evident, indicating ongoing mucosal damage and impaired tissue integrity, which are characteristic of hemorrhoidal inflammation. These findings correlate with acute inflammatory reactions, dilated blood vessels, and focal haemorrhagic areas observed in untreated conditions. In contrast, the STD (Figure No:8b) and AFHAE (Figure No:8c) groups demonstrated notable recovery of normal architecture, with mild congestion and minimal inflammatory response. The AFHAE treated tissues showed moderate healing, evidenced by reduced inflammation, presence of mild granulation tissue, and early re-epithelialization. The most remarkable improvement was observed in the SNP (Figure No:8d) group, where the mucosal, submucosal, and muscular layers appeared nearly normal with an

absence of necrosis, oedema, and ulceration. Minimal inflammatory cell infiltration and lack of vascular congestion suggested effective resolution of the disease process. Overall, the SNP ointment exhibited superior anti-inflammatory and tissue-healing effects, promoting near-complete restoration of the anorectal tissue structure, followed by the standard ointment and AFHAE treatments, while the control group remained severely affected [42-43].

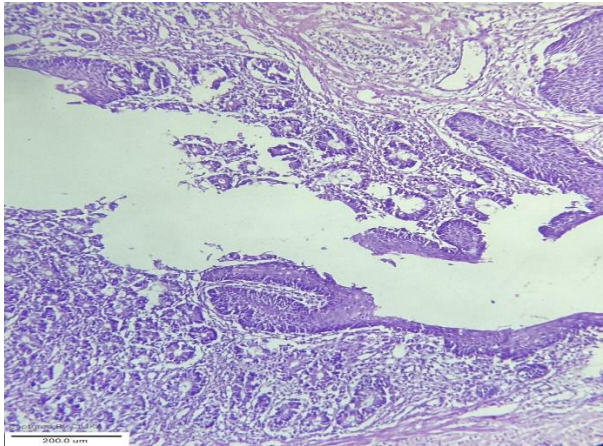


**Figure No-8a**

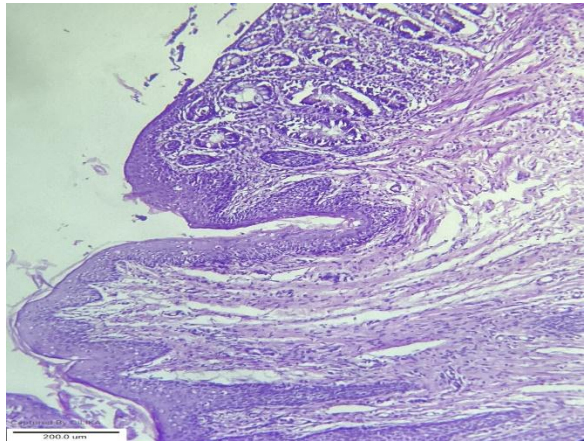


**Figure No-8b**

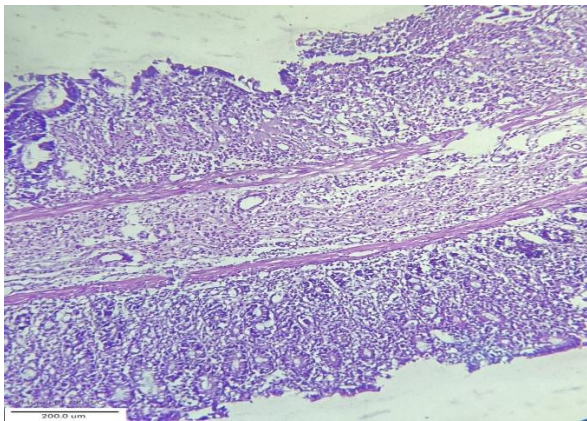


**Figure No-8c**

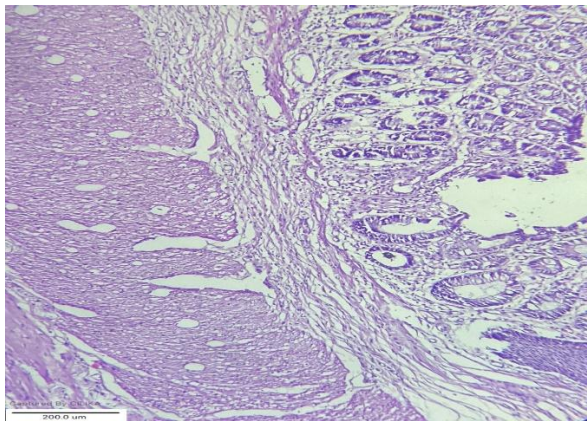


**Figure No-8d**

**Figure No- 8 Histology of Anorectal tissue A. Control, B. Standard, C. AFHAE and D.SNP**

**0.02%**

### 3.3.8 Network Pharmacology

Network pharmacology is rapidly developing as a key field in drug discovery and development by integrating systems medicine with computational and information sciences. Initially selected Plant is *Acacia ferruginea* based on the traditional claim to treat the haemorrhoids. Secondary metabolites were active components of the plants which shows pharmacological action. By studying LCMS reports and literature the phytoconstituents retrieved from PCIDB data bases. Among thirty compounds all thirty compounds were subjected to molsoft public data base for drug likeness score (DLS) prediction. In that fourteen compounds were selected based on Lipinski's Rule of Five model. The score range (-2 to 2) and also ADMET profile for biological destitution of drug. And finally, six compounds were shortlisted. Target prediction for the two compounds by using Superpred data base. All genes/proteins were exposed to string data base for the gene set enrichment analysis. Plant phytoconstituent gene which were extracted previously matched and KEGG pathway genes. Common proteins of both plant protein and KEGG path way proteins were exported to CYTOSCAPE 3.6.1. A network was built in Cytoscape version 3.6.1 to display the interaction between phytoconstituents, targets, and regulated pathways. The network analyser tool was used for network analysis, with the "edge count" parameter set. The size of each node reflected the level of interaction between phytoconstituents, proteins and pathways (Figure No-9). Most modulated pathway (AMPK and TNF- $\alpha$ ) were selected as target for molecular docking [44].



Gyrocyenin -6.0 Kcal/mol (Figure No-11c) and Gravelliferone -5.3 Kcal/mol (Figure No-11d).

Hemorrhoids is a condition which is associated with inflammation of anal cushion this inflammation can be reduced by inhibiting the TNF- $\alpha$  and by stimulating the AMPK receptor.

So, these compounds Gyrocyenin and Gravelliferone can reduce the inflammation in hemorrhoids by inhibiting the TNF- $\alpha$  and by stimulating the AMPK receptor [45].

## Molecular docking studies (TNF- $\alpha$ )

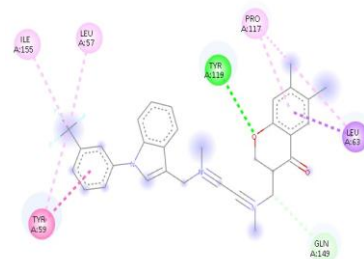
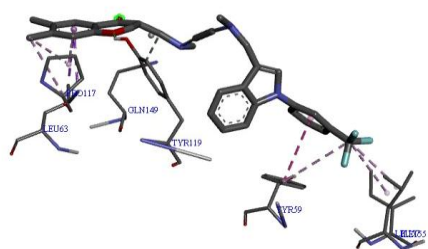


Figure No-10a 3D and 2D Interaction of cocystal ligand

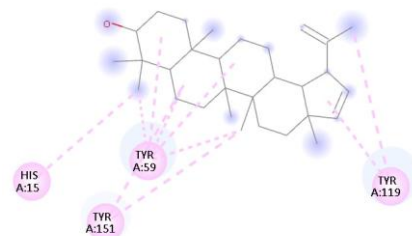
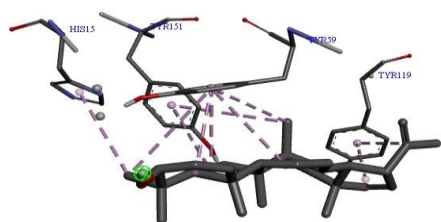


Figure No-10b 3D and 2D Interaction of reference drug lupeol

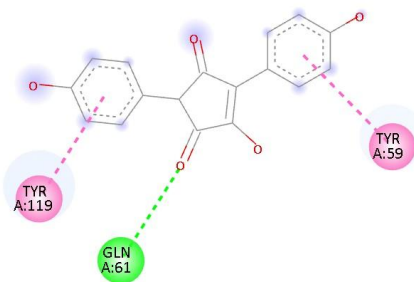
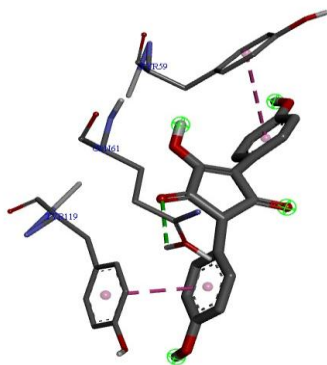


Figure No-10c 3D and 2D Interaction of Gyrocyanin (test drug)

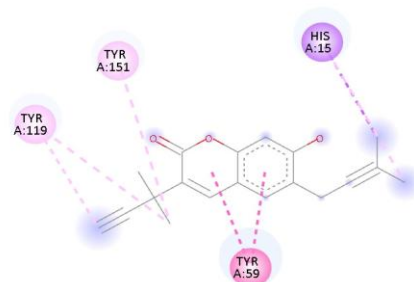
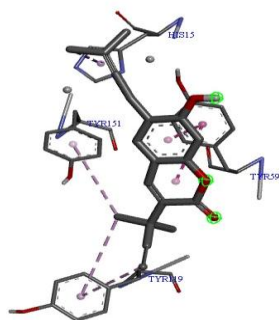


Figure No-10d 3D and 2D Interaction of Gravelliferone (test drug)

## Molecular docking studies (AMPK)



Figure No-11a 3D and 2D Interaction of co-crystal ligand



Figure No-11b 3D and 2D Interaction of reference drug lupeol



Figure No-11c 3D and 2D Interaction of Gyrocyanin (test drug)



Figure No-11d 3D and 2D Interaction of Gravelleferone (test drug)

## Conclusion:

By observing the above results, this study successfully demonstrated the green synthesis of SNPs using the AFHAE under dark conditions through a simple, cost-effective, and eco-friendly one-step method without the use of any chemical reagents or surfactants. Phytochemical investigation revealed the presence flavonoids, alkaloids, saponins, steroids, tannins and phenolic compounds, which played a key role as reducing and stabilizing agents in SNP production. Characterization studies, including UV-Visible spectroscopy, FTIR, DLS, TEM, and EDAX, verified the successful synthesis of predominantly spherical, well-dispersed nanoparticles. LCMS and network pharmacology analyses identified bioactive constituents particularly gyrocyanin and gravelliferone with strong anti-inflammatory and antioxidant potential, showing effective interaction with TNF- $\alpha$  and AMPK receptors, contributing to anti-hemorrhoidal activity. In vivo evaluation confirmed that SNP ointment (0.02%) significantly reduced recto-anal damage, restored tissue integrity, and enhanced healing compared to AFHAE and standard treatments. Histopathological findings further supported that the SNP ointment exhibited superior anti-inflammatory and tissue repairing effects. Therefore, it can be concluded that the green synthesized SNPs from *Acacia ferruginea* possess potent therapeutic efficacy and represent a promising, natural, and economical approach for the management of haemorrhoids.

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**Conflict of Interest.**

The author declares no conflicts of interest, financial or otherwise.

**Funding.**

The work received no funding from any place

**Ethical Considerations.**

All animal experiments were performed in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC) of BVVS Hanagal Shri Kumareshwar College of Pharmacy before the study commenced (Protocol No. IAEC/HSKCOP/July 2024/PG19).

**Statement of Contribution by Researchers**

KC : Investigation, Data Curation & Writing - Original Draft, MS: Methodology, Validation, Data Curation, Supervision, BK: Formal analysis and Editing, SA: Data Curation, BK: Visualization, SM: Validation, LV: Conceptualization, Methodology, Visualization, Validation, Data Curation and Supervision.

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