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Study on Anti-inflammatory, Anticoagulant, Antiplatelet Activity of *Grewia serrulata* DC. and *Grewia heterotricha* Mast

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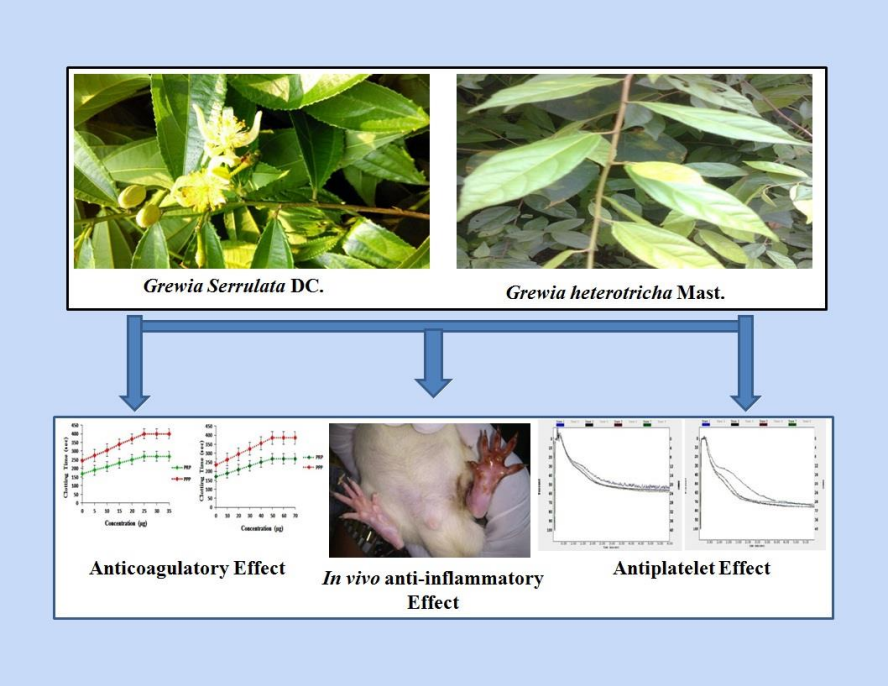
Abstract

Aim: To evaluate anti-inflammatory, anticoagulant, antiplatelet activity of aqueous leaf extract of *Grewia heterotricha* Mast.*and Grewia serrulata* DC. **Methods:** The aqueous leaf extract of these plants were assessed for their *in-vitro* anti-inflammatory activity by inhibition of albumin denaturation, Protein inhibitory action, heat- induced hemolytic activity methods and *in-vivo* anti-inflammatory activity by Carrageenan Induced Rat Paw Edema method. Primary phytoconstituents were screened by RP-HPLC, FTIR and GC-MS. **Results:** Aqueous extracts of both the plants showed promising anti-inflammatory activity by inhibiting protein denaturation with IC_{50} values 491.4µg/ml (AEGH) and 742.6µg/ml (AEGS). HRBC membrane stabilizing assay of AEGH and AEGS showed significant inhibition values of 56.26±0.37% and 55.03±0.37% respectively. Proteinase inhibitory activity of AEGH and AEGS was found to be 66.01±0.12% and 54.83±0.02%respectively. In addition, presence of different functional groups was adjudged by FTIR and primary phytoconstituents were screened by RP-HPLC and GC-MS. Furthermore, AEGH and AEGS prolonged the citrated human plasma in both PRP (Platelet Rich Plasma) and PPP (Platelet Poor Plasma) from control 190s to 290s, 270s to 420s, 150s to 250s and 260s to 410s respectively. This confirms the anticoagulant nature of AEGH and AEGS. Moreover, both AEGH and AEGS did not alter ADP induced platelet aggregation suggested that they have no role on platelets. **Conclusion:** The aqueous leaf extract of *Grewia serrulata* DC. (AEGS) and *Grewia heterotricha* Mast.(AEGH) have promising anti-inflammatory and anti-coagulant activity. The active components in the extracts may be responsible for these properties. Isolation of the active components might help in the field of anti-inflammatory drug research.

Graphical abstract



Keywords
Denaturation; hemolysis; GCMS; HPLC; FTIR

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1. INTRODUCTION

Inflammation is a complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants [1]. The signs of inflammation are heat, pain, redness, swelling, and loss of function. Inflammation can be classified as either acute or chronic [2]. Bennett. The initial response of the body to harmful stimuli is acute inflammation and is achieved by the increased movement
of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. Prolonged inflammation is also known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells, and is characterized by destruction and healing of the tissue simultaneously from the inflammatory process. Inflammatory diseases include number of disorders and conditions that are characterized by inflammation. Examples include allergy, asthma, autoimmune diseases, celiac disease, glomerulonephritis, hepatitis, inflammatory bowel disease, cancer and transplant rejection [3-5].In order to prevent blood loss during tissue injury, hemostasis plays a major role and it was highly regulated [6]. In physiological condition when tissue gets injured and exposed to surface collagen platelets get activated and form platelet plug at the site of injury, meanwhile blood coagulation cascade gets activated serially from factor-XII to factor-X and it finally leads to the formation of fibrin clot mesh to prevent the loss of blood [7]. But in pathological condition due to some genetic aberrations and few environmental factors leads to formation of unusual clots in arteries and veins which also called as thrombus and the disorder known as thrombotic disorder. In order to treat thrombotic disorder anticoagulant and antiplateletagents were essential [8]. During the past decade, the therapeutic use of herbal medicine is gaining considerable momentum in the world due to toxicity and side effects of allopathic medicines [9]. Many bioactive compounds from medicinal plants showed anti-inflammatory activities. It is evident that several plants have been used in traditional Ayurveda medicine for treatment and management of distinct inflammatory disorders and wound healing activities [10].

In the present study, attention will be focused on *Grewia serrulata* DC. and *Grewia heterotricha* Mast. It was reported that various parts of *G.serrulata* DC. and *G.heterotricha* Mast. are used in wound healing, fever, bronchitis and also to cure upset stomachs and some skin and intestinal infections and seem to have

anti-inflammatory and analgesic properties. Thus, in the current study, aqueous extracts of leaves of *G.serrulata* DC and *G.heterotricha* Mast were used to investigate the anticoagulant, antiplatelet and anti-inflammatory activity *in vitro* and *in vivo* in animal models.

2. Materials and methods

All the chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the preparation of RBCs suspension, Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP).

2.1. Preparation of extracts

The aqueous extract was also prepared by mixing leaf powder in distilled water and stirred continuously using magnetic stirrer for 48hr. The mixture was filtered, and the filtrate was then concentrated. The extract was stored in a refrigerator and was used for further study.

2.2. In-vitro anti-inflammatory activity

2.2.1. Inhibition of albumin denaturation

Method of Mizushima and Sakat et al was followed with minor modifications [11-12]. The reaction mixture was consisting of extract at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted with a small amount of 1N HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 20 min. After cooling, the turbidity of the samples was measured spectrophotometrically at 660 nm. Percent inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Abs control – Abs sample) X 100/ Abs control

2.2.2. Proteinase Inhibitory Activity

The test was performed according to the modified method of Oyedepo et al.,[13]. The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM TrisHCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. The turbid suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

2.2.3. Membrane stabilization

2.2.3.1. Preparation of Red Blood cells (RBCs) suspension [12-13]**:** The Blood was collected from a healthy human volunteer who has not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and regenerated as 10% v/v suspension with normal saline.

2.2.3.2 Heat-induced hemolysis

This assay was performed as per the method described by Shinde et al., [14]. The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min and cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was carried out in triplicates for all the test samples. The Percentage inhibition of hemolysis of RBCs was calculated as follows:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

2.3. *In-vivo* anti-inflammatory activity

2.3.1. Animals

Adult Wister albino rats (150 g -200 g) of either sex was used for the in vivo evaluation. They were housed under standard laboratory conditions and were fed with standard animal feed and water ad libitum. The experimental protocol was approved by the institutional animal ethical committee.

2.3.2. Acute toxicity study

Acute toxicity study was performed as per OECD guidelines 423[2]. (Acute toxicity class method).

2.3.3. Carrageenan-Induced Rat Paw Edema:

The carrageenan-induced rat paw edema was carried out to evaluate acute anti-inflammatory activity. Paw edema was induced by injecting 0.1 mL of 1% (w/v) carrageenan suspension in 0.9% (w/v) sterile saline into the plantar tissue of the left hind paw of all animals, one hour following oral administration of either control vehicle, Diclofenac sodium or plant extracts. The right paw will serve as a reference to measure the degree of inflammation in the left one. Increase in paw volume was measured in the interval of 0min, 30min, 60min 120min, 240min and 24hr following carrageenan injection, using a plethysmograph [15]. The percentage inhibition of inflammation was calculated as inhibition of edema

volume in extract treated groups and was compared with control.

2.4. Anti-coagulant activity

2.4.1. Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) preparation

PRP and PPP were generated as per the method described by Ardlie and Han [16]. Adjust the concentration of PRP to 3.1×10^8 platelets/ml with PPP and it was carried out using plastic wares or siliconized glasswares.

2.4.2. Plasma re-calcification time

The plasma re-calcification time was evaluated as per the method described by Quick et al., [17]. Briefly, PRP/PPP was incubated with AEGS(5-35µg) and AEGH (10-70µg) in the presence of 10mM TrisHCl (20µl) buffer (pH 7.4) for 1min at 37°C. Clotting time was recorded upon 20µl addition of 0.25M $CaCl_2$.

2.5. Anti-platelet activity

2.5.1. Platelet aggregation

Platelet aggregation was assayed as per the method described by Born [18] in lumi aggregation system (Model-700). Different concentrations of AEGS(0–30µg) and AEGH(0-30µg) were pre-incubated with PRP in a reaction volume of 0.25ml. Process of platelet aggregation was initiated by adding ADP as agonist and followed for 6min.

2.6. Statistical analysis

Statistical analysis was done using one-way ANOVA, followed by Tukey's post hoc multiple comparison tests. $p<0.01$ were considered as significant. Data are represented as mean ± S.E.M.

2.7. Reverse Phase High Performance Liquid Chromatography analysis

The RP-HPLC instrument endures manual injector, binary pumps, column and Photo Diode Array detector. Samples were eluted using mobile phase A (Water) and B (Acetonitrile) in C_{18} column with the flow rate of 1mL/min. The detector wavelength was 280nm and the volume of injection was 20µL. All solvents were HPLC-grade [19]

2.8. Fourier Transform Infrared spectrum analysis

FT-IR spectra were recorded on Agilent FT-IR-4100 spectrophotometer in the spectral range of 650-4000 cm^{-1} taking the sample in the ATR disc. The extracted powder was kept on the ATR disc and subjected to spectral analysis and the results are predicted [20].

2.9. Gas Chromatography Mass Spectroscopy analysis

GC-MS analysis of samples was analyzed on quadrupole mass spectrometers in the electron-capture negative-ion chemical ionization (ECNICI) mode with capillary column (30X0.25mm IDX1EM df, composed of 100% Dimethyl poly siloxane). Helium (99.9%) gas was used as carrier gas at the flow rate of 1ml/min and the injection volume of 0.5 El (split ratio of 10:1). The temperature program was set as follows, injector temperature 250°C; ion-illuminator temperature 280°C, oven temperature 110°C (isothermal for 3min) with an increase in temperature of 20°C/min to 220°C, thereafter 5°C/min to 300°C. Mass spectrum was taken at 80ev; a scan interval of 0.5s[21].

3. RESULTS AND DISCUSSION

3.1. Inhibition of albumin denaturation

Denaturation of proteins is a widely known to cause inflammation. On denaturation, most of the proteins in the biological system lose their activity [11,22]. The present investigated the ability of plant extract to inhibit protein denaturation. The aqueous leaf extracts of selected plants showed significant inhibition of egg albumin denaturation in concentration dependent manner. IC50 of AEGH and AEGS was observed as 491.4µg/ml and 742.6µg/ml respectively. Diclofenac sodium was used as astandard anti-inflammatory drug showed IC50 value 298µg/ml. Results are presented in Fig. 1.

3.2. Proteinase Inhibitory Activity

Neutrophils are major leukocytes present during the inflammatory response. Neutrophils carry lysosomes which are rich in proteinases and when released during inflammatory reactions can cause tissue damage. Proteinase inhibitors can provide a significant level of protection against proteinases during inflammatory reactions.[23-24]AEGH and AEGS exhibited significant antiproteinase activity 66.01±0.12% (IC50 value 543.6) and 54.83±0.02% (IC50 value 754.2) respectively at 1mg/ml. Standard Diclofenac sodium (100 µg/ml) showed maximum inhibition of 91.27±0.09% (IC50 value 88.4) (Fig. 2).

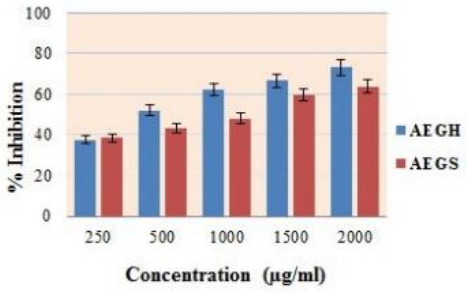


Fig 1. Inhibition of Protein denaturation activity of AEGH and AEGS
AEGH and AEGS were incubated with 1% aqueous solution of bovine albumin fraction for 20 min at 37°C. The turbidity was measured at 660nm.

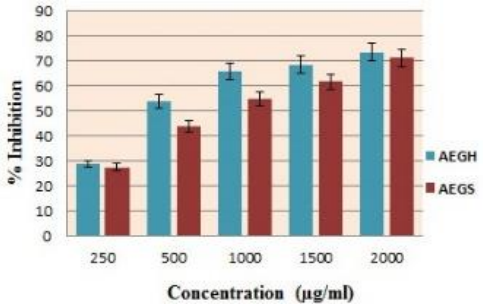


Fig. 2. Proteinase inhibitory activity of AEGH and AEGS.
AEGH and AEGS were incubated with trypsin in 1 ml of Tris HCLbuffer (pH 7.4) for 5 min at 37°C. Cloudy suspension was centrifuged & the absorbance was read at 210 nm against buffer as blank.

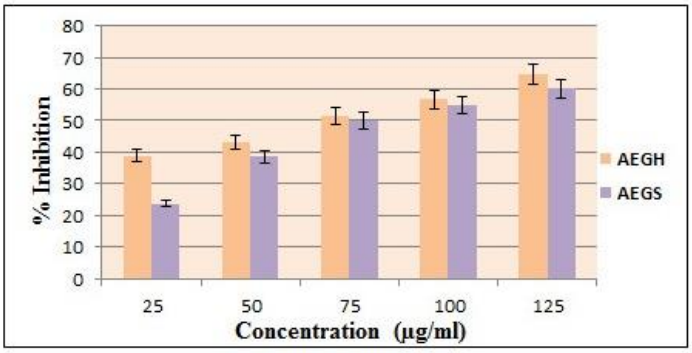


Fig. 3. Inhibition of heat induced haemolytic activity of AEGH and AEGS.
Different concentration of AEGH and AEGS were incubated with 10% RBC suspension for 30 min at 56°C in water bath. The mixture was centrifuged at 2500 RPM for 5 min and the absorbance was read at 560 nm. Aspirin was used as standard drug and saline serves as negative control.

3.3. Membrane stabilization

At the site of inflammation, activated neutrophils release lysosomal proteases which can cause further inflammation and tissue damage. Stabilization of lysosomal membrane can prevent the release of these lysosomal contents and may inhibit tissue inflammation. As erythrocyte membrane is analogous to lysosomal membrane, stabilization of erythrocyte membrane implies to the stabilization of lysosomal membrane [25-26].

3.3.1. Heat induced hemolysis

The extracts inhibited the heat induced hemolysis of RBCs at different concentrations. AEGH and AEGS showed maximum inhibition of 56.26±0.37% (IC50 value 62.26) and 55.03±0.37% (IC50 value 80.29) respectively at 100 µg/ml. Standard Dichlofenac sodium (100 µg/ml) showed maximum inhibition of 91.56±0.25% (IC50 value 8.66) (Fig 3).

3.4 Carrageenan Induced Rat Paw Edema

The AEGH and AEGS showed significant reduction in the paw volume of rats at a concentration of

100mg/ml and showed potent activity compared with the reference standard Diclofenac sodium. At 100 mg/kg, the paw edema inhibition following treatment with AEGH and AEGS was 70.42% and 67.18% respectively. The anti-inflammatory activity of both extracts is comparable to Diclofenac (10mg/kg) with the paw edema inhibition of 81.69% at 2h. AEGH appears to be more effective. The results are tabulated in Table 1.

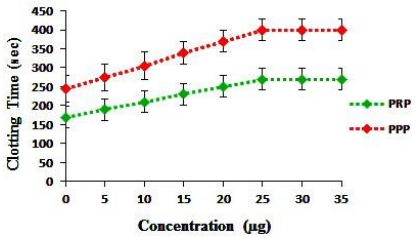
Table 1: Effect of AEGH and AEGS in carrageenan induced rat paw edema.

Drug	Dose (mg/kg)	Carrageenan induced edema (Volume in ml)					% Inhibition	
		30min	60min	120min	240min	24hr	2hr	4hr
Control		0.39±0.02	0.86±0.05	0.50±0.03	0.28±0.03	0.16±0.03	-	-
Dichlofenac	10	0.11±0.16*	0.25±0.02*	0.13±0.02*	0.05±0.02*	0.01±0.01*	81.69	84.84
AEGS	100	0.24±0.02*	0.41±0.03*	0.23±0.02*	0.09±0.02*	0.03±0.02*	67.18	72.24
AEGH	100	0.22±0.02*	0.33±0.02*	0.21±0.03*	0.08±0.02*	0.01±0.01*	70.42	74.84

Note: All the result are expressed in terms of Mean ± S.E.M., n=6 animals in each group;
*p<0.01 vs control, statistically significant

3.5. Plasma re-calcification time

To identify the role of AEGS and AEGH on plasma coagulation cascade, plasma re-calcification time was analyzed. Interestingly, both AEGS and AEGH exhibited anticoagulant property by enhancing the clotting time of citrated human plasma in both PRP and PPP from control 190s to 290s, 270s to 420s, 150s to 250s and 260s to 410s respectively (Fig. 4a & Fig.4b). Plasma coagulation cascade is a physiological phenomenon; it plays a major role in arrest the bleeding at the site of injury [27]. But some environmental factors and genetic aberrations alter the normal operating system of coagulation cascade which leads to formation of unusual clot (thrombus) in arteries and veins which termed as thrombosis. It is a pathological phenomenon which leads to cardio and cerebrovascular disorders [28]. Several synthetic anticoagulants were discovered in order to treat cardiovascular disorders, but it has some side effects such as nausea, vomiting and headache. To overcome such problems identifying the anticoagulants from natural sources was sounds good. Flax seeds, jackfruit seed, pea pod, bitter gourd and facile capped silver nanoparticles exhibited anticoagulant property [29-30]. AEGS and AEGH exhibited anticoagulant property, thus it could be better agent to treat thrombotic disorders.



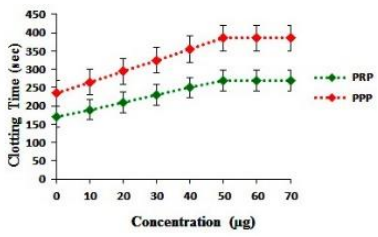
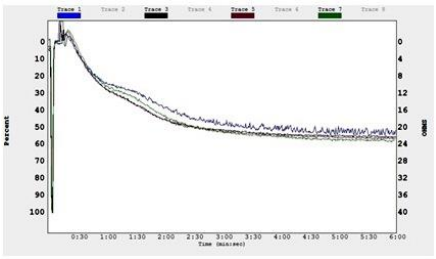


Fig. 4a and 4b. Plasma Recalcification time of AEGH and AEGS

AEGH (0–35 µg) and AEGS (0-70 µg) were pre-incubated with 0.2ml of citrated human plasma PRP/PPP in the presence of 20µl 10mM Tris–HCl buffer (pH 7.4) for 1min at 37°C. 20µl of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.



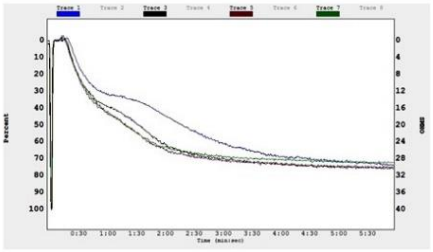


Fig 5a and 5b. ADP induced Platelet aggregation of AEGH and AEGS

Trace 1 (ADP 10µM); Trace 2 (ADP 10 µM+10 µg of AEGH); Trace 3 (ADP 10 µM+20 µg of AEGH); Trace 4 (ADP 10 µM+30 µg of AEGH and AEGS separately).

3.6. Platelet aggregation

To identify the role of AEGH and AEGS on platelet function, platelet aggregation assay was performed. Interestingly, both AEGH and AEGS did not inhibit or induce the ADP induced platelet aggregation, suggesting that there is no role of AEGH and AEGS on platelets (Fig. 5a & 5b). Together with blood coagulation factors, platelets do paly major role in order to treat thrombotic disorders. Hyperactivation of platelets leads to cause inflammation, tumour metastasis and atherosclerosis [31]. Thus, antiplatelet agents play a key role in order to inhibit the hyper activated platelets. Many antiplatelet agents were characterized from plants and animal sources [32]. Interestingly, AEGH and AEGS did not show any effect on platelets.

3.7. RP-HPLC

Phytochemical composition of leaf extract was determined by HPLC analysis. The RP-HPLC analysis was carried out using Schimadzu#LC prominence. Assessment of compounds was based on the combination of retention time and spectra. Since benzene ring, phenol compounds and polyphenols observed in the UV-range chromatogram was obtained at 280°C. Based on the obtained chromatogram due to the isomeric form of compounds are in the extract only 3 peaks were evolved that to only one major peak was evolved **(Supplementary Fig.1a & 1b)**. This suggests that AEGH with holds isomeric form of polyphenols in the extract. Although, AEGS extract possess similar kind of chromatogram in RP-HPLC analysis [33-34].

3.8. IR Spectrum

The IR spectrum of AEGH at ((ν, cm^{-1}) 3219; 80.146 was stretching vibrational band for OH and aromatic C-H group. Although new vibrational bands appeared at 1566; 63.925, 1406; 67.122 and 1253; 75.074 were characteristic of coordinated carbonyl (C=O) stretching of CHO group. Simultaneously bands appeared at 881; 72.640 and 1019; 53.924 indicates the bending form C-H aliphatic groups [35]. But in AEGS bands appeared at ((ν, cm^{-1}) 3566; 98.942, 3279; 94.615 and 2501; 98.977 were vibrational bands for aromatic C-H, C=O and OH groups. Bands appeared at 1711; 96.615, 1562; 88.306 and 1387; 89.476 were stretching of C=O and CHO groups. Bands appeared at 1268; 90.516, 1201; 92.822 and 1026; 87.317 indicates the bending form of C-H and C-C groups [36] **(Supplementary Fig. 2a & 2b)**.

Supplementary Data

Fig 1a and 1b. HPLC chromatogram of AEGH and AEGS

AEGH and AEGS (50 µg) were eluted in C_{18}-Column by Acetonitrile and water as eluents in gradient mode with PDA as detector.

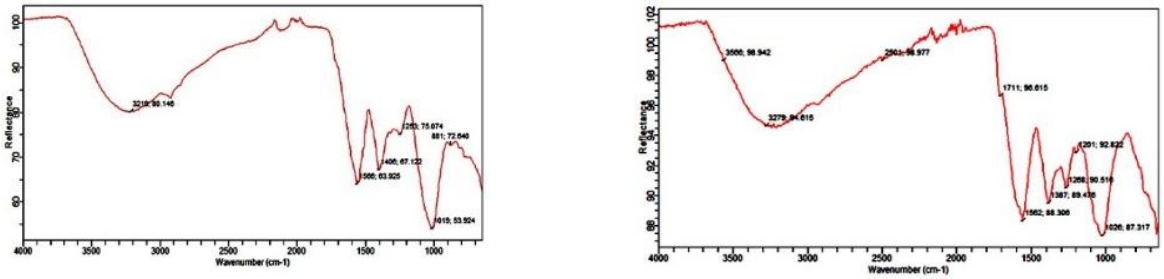


Fig 2a and 2b. Infrared chromatogram of AEGH and AEGS

IR-spectrum of AEGH and AEGS were obtained in Agilent FT-IR-4100 spectrophotometer at the spectral range of 650-4000 cm-1 in ATR disc.

3.9. GC-MS

GC-MS chromatography of AEGH (5µg) obtained by Thermo-fisher GC–MS in the SIM Mode. GC-MS study confirms the presence of several phytoconstituents which plays a major role in the medicinal field. As per the X-Caliber software prediction, the major peaks obtained in the extract AEGH and AEGS at 13.62min, 15.66min and 13.92, 15.67 were may be alkaloid and flavonoid respectively. The remaining minor peaks were may be isomeric forms of polyphenols [37-38] (Fig. 6a & 6b).

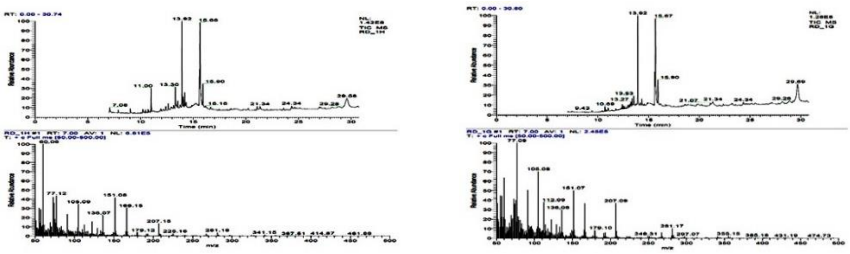


Fig 6a and 6b. GC-MS chromatogram of AEGH and AEGS

AEGH and AEGS (50 µg) was analyzed in GC-MS with quadrupole mass spectrometers in the electron-capture negative-ion chemical ionization (ECNICI) mode with capillary column (30X0.25mm). Helium was used as carrier gas at the flow rate of 1ml/min with gradient temperature system and the injection volume of 0.5 El (split ratio of 10:1).

4. CONCLUSION

Based on the results of *invitro* and *invivo* anti-inflammatory studies it can be concluded that the aqueous leaf extract of *Grewia serrulata* DC. (AEGS) and *Grewia heterotricha* Mast. (AEGH) have anti-inflammatory activity showing suppression of inflammation. Furthermore, plasma re-calcification time analysis revealed that AEGH and AEGS have anti-coagulant property. The active components in the extracts such as flavonoids, alkaloids, terpenoids and related polyphenols may be responsible for these properties and further RP-HPLC, FTIR and GC-MS results support their presence. Further studies are needed in order to purify bioactive compounds responsible for the anti-inflammatory property.

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Declaration of Conflict of Interest

The authors declared no potential conflict of interest with respect to the authorship and publication.

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