Pharmacological Evaluation of Anti-hemorrhoidal activity in ethanolic leaf extract of *Ageratum conyzoides* L.

Nitisha Shah[1], Dr. Virender Kaur,[2] Dr. Tirath Kumar[3]

[1]Department of Pharmaceuticals Sciences, Sir J.C. Bose Technical Campus, Kumaun University, Bhimtal, Uttarakhand, India
Email id- nitishashah1002@gmail.com
[2]Associate Professor,
College of Pharmacy, Graphic Era Hill University, Bhimtal Campus, District -Nainital, Uttarakhand, India
Email: verumalwa@gmail.com
(Corresponding Author)
[3]Associate Professor,
Department of Pharmaceuticals Sciences, Sir J.C. Bose Technical Campus, Kumaun University, Bhimtal, Uttarakhand, India
Email id- tirath.kamboj@gmail.com

Corresponding Author:

Dr. Tirath Kumar
Associate Professor, Department of Pharmaceuticals Sciences, Sir J.C. Bose Technical Campus, Kumaun University, Bhimtal, Uttarakhand, India (tirath.kamboj@gmail.com)

KEYWORDS

Anti-hemorrhoid, *Ageratum conyzoides*, Croton oil, Inflammation, Antioxidant

ABSTRACT

Objective- Millions of people throughout the world suffer from hemorrhoids, a common medical illness characterized by enlarged and irritated blood vessels in the rectal and anal regions. Modern medicine is still in its early stages of finding a definitive cure for hemorrhoids, as there is no established and clear-cut solution for this particular ailment. The purpose of this study was to examine the ethanolic leaf extract of Ageratum conyzoides L. (ELEAC) has any anti-hemorrhoidal properties in experimental animal models.

Method- *A. conyzoides* ethanolic leaf extract was made, and its phytochemical screening was examined. Rats were separated into treatment groups after being given a standardized procedure to produce hemorrhoids. The effects of ELEAC on hemorrhoid size, rectal bleeding, and histological alterations in the anal area were assessed after it was given orally and topically. In addition, ano rectal coefficient, liver enzyme results were evaluated.

Results- Flavonoids, alkaloids, and tannins were found in the ELEAC, according to the phytochemical examination. Treatment with the test extract dose-dependently decreased inflammation, rectal bleeding and hemorrhoid size, when compared to the control group, histopathological analysis of the anal area of treated rats revealed a reduction in congestion, edema, and vascular dilatation. Additionally, the extract exhibited antioxidant activity by reducing oxidative stress markers and anti-inflammatory activity.

Conclusion- In experimental animal models, the ELEAC showed significant anti-hemorrhoidal action, presumably because of its antioxidant and anti-inflammatory characteristics. Additional research is required to understand the underlying processes and assess its therapeutic applicability in the treatment of hemorrhoids.

INTRODUCTION

Since ancient times, people have employed plants as a significant source of medicine. The scientific examination of traditional knowledge regarding the use of herbs in the treatment of various ailments is one of the research's main focal areas. Herbal medications are the most in-demand primary healthcare in both developed and developing countries due to their vast biological and therapeutic action, higher safety margins, and lower costs[1].

Hemorrhoids, often known as piles, are a very prevalent anorectal ailment that affects millions of individuals today. One of the most prevalent anorectal disorders, hemorrhoidal disease (HD) affects 13–36% of the general population. According to current estimates, 75% of people will at some point in their lives encounter bleeding hemorrhoids[2,3]. They are characterized by symptomatic swelling and distal displacement of the natural anal cushions. Over 50 percent of men and women over the age of 50 may experience hemorrhoid symptoms at some point in their lives, making it a highly prevalent condition among adults[4]**.** An expanded rectal venous plexus and anal canal are the hallmarks of hemorrhoids, a digestive problem that can result in discomfort, bleeding, itching, and inflammation[5]. Pain and rectal bleeding are the most common symptoms of the disease. Constipation is a risk factor that can aggravate the disease[6]**.**

Swollen veins at the anus or in the lower rectum are known as hemorrhoids. Internal and external hemorrhoids are two different varieties. **Internal hemorrhoids** refers to swollen veins within the rectum. Hemorrhoids inside the body i.e., internal hemorrhoids might occasionally protrude outside the anus. **External hemorrhoids** are the enlarged veins that are located outside of the anus. External hemorrhoids are the most common and difficult to treat[7].

Many patients with hemorrhoids or piles do not seek medical attention as soon as the symptoms appear. This tendency may be because the disease affects the anal region and they feel embarrassed to seek medical care. Other patients may choose not to go to the hospital for financial reasons[8].

Hemorrhoids can result in fecal leakage, painful bowel movements, blood on tissue after having a bowel movement, irritation, soreness and acute itching around the anus, as well as itchy or painful lumps or swelling close to the anus. The major goal is to deal with the underlying issue. Simple cases of hemorrhoids are best managed by eating a diet high in fiber, drinking a lot of water, and applying lotions containing local anesthetic to relieve pain and itch[7].

Internal hemorrhoids, not external, are graded based on their appearance and degree of prolapse, known as Goligher's classification:-
Grade I: The anal cushions bleed, but do not prolapse;
Grade II: The anal cushions prolapse through the anus on straining, but reduce spontaneously;
Grade III: Like grade II the anal cushions prolapse through the anus on straining or exertion but require manual replacement into the anal canal;
Grade IV: The anal cushion prolapse, stays out at all times and is irreducible;
Treatments of Grade I and Grade II type hemorrhoids are possible without surgical operation but in Grade III and Grade IV surgical procedure is required[9].

The sliding anal canal, or cushion theory, is the most widely accepted theory, and it proposes that the major pathologic event is the aberrant slipping of cushions through the anal canal. Anal cushion injury, a form of supporting tissue deterioration, and hard stool defecation are two factors that might lead to internal hemorrhoids. Due to this damage, the hemorrhoid plexus blood flow became stagnant, and further edema resulted in an expanded anal cushion. A problem could cause the cushion to prolapse permanently or show up as anal hemorrhage if the affected area is irritated[10].

Current research suggests that treatment options for hemorrhoids include medication, drastic surgery, dietary and lifestyle changes. Haemorrhoid remedies have venotonic, local anesthetic, anti-inflammatory, and analgesic effects. It can be treated with a variety of pharmaceutical treatments, including anesthetics, corticosteroids, and calcium dobesilate[6]. Plants containing phenolic compounds such as flavonoids, tannins, stilbenoids, catechins, lignin and phenolic acids are useful for a large number of inflammatory disorders such as allergies, asthma, autoimmune diseases, inflammatory bowel disease and hemorrhoids. The most popular oral plebotonic medications used for hemorrhoidal treatment are phenolic compounds. Phenolic substances have anti-inflammatory properties, can enhance lymphatic drainage, decrease capillary permeability and raise venous tone[5].

Hemorrhoid treatment in modern medicine is still in its early stages. There is now substantial study being done in the field of Ayurveda for using natural sources for treating hemorrhoids because there is no particular medication to cure hemorrhoids. Over the years, The Himalaya Drug Company (Bangalore, India) has been using Pilex, a polyherbal proprietary formulation, in clinical settings to treat hemorrhoids and other associated diseases[11].
Research into medicinal plants is expanding quickly. The use of plants as traditional medicines and alternative medicines is increasingly in demand because it is relatively safer compared to synthetic drugs. *Ageratum* is one of the genera included in the Asteraceae family. *Ageratum conyzoides* is called goatweed, Billygoat weed and Jangli pudina (in Hindi)[12]. It grows incredibly well during the rainy season. *A. conyzoides* often grow in damp areas like gardens, roadside ditches, and streams[13]. *Ageratum conyzoides* L. was traditionally used to treat hemorrhoids, also ethano-botanical survey results show that this plant was used to treat hemorrhoids[14,15].

However, to the best of our knowledge, there is no recorded evidence-based animal study/ethnopharmacological data on the anti-hemorrhoidal activity of the *Ageratum conyzoides*. Scientific research indicates that the plant has significant anti-inflammatory and analgesic properties, although traditional usage of the plant's leaves to cure hemorrhoids is still unproven. Thus, utilizing a good animal model and taking into account this evidence, the current work was carried out to establish the anti-hemorrhoidal potential of leaves from the plant A. conyzoides.

MATERIAL AND METHODS

Chemical and Drugs

Croton oil (Sigma Aldrich, St. Louis, USA), Pilex (The Himalaya Drug Company, Bangalore, India), quercetin, gallic acid, and all the other chemicals used in the experiments were of analytical grade from reputed suppliers.

Plant material

The plant was collected from the Naukuchiyatal area of Bhimtal region in the Nainital district of Uttarakhand. The plant was authenticated by Dr. S.K. Singh from the Botanical Survey of India (BSI), Northern Regional Centre, Dehradun.

Preparation of plant extract

Leaves of the plant *Ageratum conyzoides Linn* were collected, cleaned with distilled water and was air and shade dried at room temperature for a period of 15 days. Dried leaves were blended to a coarse powder. Blended leaves powder was soaked in ethanol for 3 days in tightly sealed

vessels at room temperature. The suspension was first filtered through sterile muslin cloth, decanted and then filtered using sterile Whatman No. 1 filter paper inserted in a funnel. The filtrates were concentrated using a rotary evaporator at 40° C and it was solidified by means of water bath[16].

Preliminary phytochemical screening of leaf extracts

Ethanolic extract of Ageratum conyzoides was taken and various qualitative reagents were utilized to confirm the presence of various phytomolecules.

Quantitative Phytochemical estimation

Estimation of Total Phenolic Content-

The Folin-Ciocalteau reagent-based test was used to assess the total phenolic content, which has been previously described[17] with little modification. 5mL of the Folin-Ciocalteau reagent, which had been diluted ten times, and 4mL of 7% Na2CO3 were added to 1mL of each extract (100g/mL) in methanol. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed colour was recorded at 765nm using a UV-VIS spectrophotometer. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).nm was measured which showed blue complex. 3 repetitions were made. The results were expressed as mg gallic acid equivalent/g of extract.

Estimation of total Flavonoid Content-

The calorimetric aluminium chloride technique was used to determine the ELEAC total flavonoid content, which has been previously described[18] with little modification. 1 mL of 10% aluminium chloride, 1 mL of potassium acetate (1M), and 2.5 mL of distilled water were combined with 0.5 mL of the sample (1 mg/mL). The calibration curve was made using quercetin. The absorbance of the mixtures was measured at 415 nm by using a UV-spectrophotometer. The total flavonoid content was expressed in terms of quercetin equivalent (mg QE/g of sample). All the analyses were repeated three times and the mean value of absorbance was obtained.

DPPH (1-diphenyl-2-picryl-hydrazine) Free Radical Scavenging Activity

The free radical scavenging activity of Ageratum conyzoides ethanolic leaf extract was measured by the DPPH method. 0.1 mM DPPH solution in ethanol and different concentrations (10, 20, 30, 40, 50 µg/ml) of Ageratum conyzoides extract were prepared. One milliliter of DPPH solution was mixed with 3 ml of different concentrations of the extract. After 30 min, the absorbance of each solution was measured at 517 nm using UV-visible spectrophotometer. Ascorbic acid was used as a standard for comparison. Ethanol was taken as blank[19]. The percentage of free radical scavenging was determined as:

$$\%RSA = (A_{control} - A_{sample})/A_{control} \times 100$$

where,

A_{sample} is the absorbance of the test sample

$A_{control}$ is the absorbance of the control.

Nitric Oxide Scavenging Assay

The stock solution of the standard and the plant extract (1mg/ml) were prepared. Different concentration (10, 20, 30, 40, 50 ug/ml) of standard and plant extract was prepared by stock solution. Sodium nitroprusside (3.0ml of 5mM) in phosphate-buffered saline pH 7.4 was prepared. It was mixed with different concentrations of the compound prepared in solvent and incubated at 25 °C for 30 min. A control without the test compound, with an equivalent amount of solvent, was taken. After 30 min, 1.5 mL of the incubated solution was removed and diluted

with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard (Ascorbic acid)[20].

$$\textbf{Scavenging (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where,

A_{sample} is the absorbance of the test sample

$A_{control}$ is the absorbance of the control.

Inhibition of protein denaturation method

Initially phosphate buffer saline (pH 6.4) and different concentrations (10, 20, 30, 40, and 50 µg/ml) of Ageratum conyzoides ethanolic leaf extract were prepared. The 5 ml of reaction mixture consisted of 0.2 ml of egg albumin, 2.8 ml of phosphate buffer saline, and 2 ml of different concentrations of the extract were added. In the case of reference, ascorbic acid was used in place of the extract. The mixture was incubated at 37°C±2 for 15 min and then heated for 5 min at 70°C. It was then left for cooling, and absorbance was measured using ultraviolet (UV)-visible spectrophotometer (Shimadzu) at 660 nm. Ethanol was used for blank[21].

The % inhibition of protein denaturation was estimated using the procedure below:

$$\textbf{\% Inhibition} = 100 \times [V_t / V_c - 1]$$

where,

V_t = Absorbance of the test sample,

V_c = Absorbance of control

Formulation:

The next stage was to create an appropriate formulation after extraction and phytochemical research. Polyethylene Glycols (PEGs) were selected as a base for the preparation of ointment. A mixture of PEG bases (600 and 4000) in a ratio of 7:3 was found to have the desired consistency. The formulation was prepared by the Fusion method containing *Ageratum conyzoides* leaf extract (10% w/w) in the selected base. The created formulation was then assessed using a number of criteria, including consistency, stability, etc[22].

Experimental Animals.

The male Wistar rats (200– 250 g) that were housed in standard conditions of temperature (22 ± 3∘ C), relative humidity (55 ± 5%), and light (12 h light-dark cycle) before and during the study were included in the experiment. They were fed with a standard pellet diet and water ad libitum. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC). The animals received humane care as per the guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Experimental design

Half an hour after the injection of EB dye, hemorrhoids were induced in rats by application of croton oil preparation, prepared by mixing deionized water, pyridin, diethyl and 6% croton oil in diethyl ether in the ratio of 1: 4: 5: 10. Except normal group, the croton oil preparation was inserted in the rectoanal portion of the rats, Soft cotton swabs (0.4 cm diameter) absorbed in 200 µL of croton oil solution were inserted into the anus (ano-rectal section, 2 cm from anal slot) of each rat and kept it there for 30 sec. The development of edema can be observed after 7 to 8 hours after application of croton oil. Administration of croton oil is done once a day for 3 consecutive days. On the 4th day animals from each group were given treatment according to their group for 7 days.

Treatment of hemorrhoid-

A total number of 54 animals were randomly divided into following 9 groups comprising 6 animals per group.

For topical application-

- **Group 1:** Normal control
- **Group 2 (Disease-induced):** Croton oil.
- **Group 3 (Ointment Base):** Croton oil and ointment base (Polyethylene glycol- PEG)
- **Group 4 (Topical standard group):** Croton oil + Standard Pilex® cream was applied
- **Group 5 (Topical group):** Croton oil + 10% w/w of extract in PEG base

For oral administration-

- **Group 1 (1% Tween 80):** Croton oil solution + 1% Tween 80
- **Group 2 (Oral standard group):** Croton oil-induced + 200 mg/kg pilex tablet (Pilex® tablet with 1% Tween 80)
- **Group 3 (Oral test group):** Croton oil + *Ageratum Conyzoides* extract (ELEAC LOW DOSE) 300 mg/kg extract/body weight per day.
- **Group 4 (Oral test group):** Croton oil + *Ageratum Conyzoides* extract (ELEAC HIGH DOSE) 600 mg/kg extract/body weight per day.

ELEAC was administered for 7 days for the treatment after the development of croton oil-induced hemorrhoids. The samples of 5 mL blood were collected through retro-orbital route from all rats on 8th day under anesthesia for evaluation of biochemical parameters[23]. Subsequently, the animals were sacrificed and anorectal tissues were harvested. Ano-rectal tissues were weighted for anorectal coefficient (ARC) calculation, and then histomorphometric analyses were performed.

Estimation of rectoanal coefficient

For calculating rectoanal coefficient (RAC), the previously weighed rectoanal tissues were compared with the body weight of the individual rats and the obtained value represented the RAC, which helps in judgement of the severity of inflammation[9] using the formula:

Rectoanal coefficient = weight of rectoanal tissue (mg)/Body weight (g).

Biochemical parameters

Parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP). The serum was separated by centrifuging blood at 3000 rpm for 10 minutes and the levels of SGOT, SGPT and ALP were analyzed by using diagnostic kits ERBA[24].

Histopathological studies

Hemorrhoidal tissue samples obtained on the 5th day of treatment were also utilized for performing the histopathological examinations. The tissues were immediately blotted, dried and fixed in 10% formalin. Rectoanal tissue underwent histological examination to look for signs of inflammation, congestion, haemorrhage, vasodilatation, and moderate to severe necrosis.

Statistical analysis

All the results of the research were expressed as mean ± SEM. Results obtained were analysed by one way ANOVA with Dunnett's post hoc test with the help of Graph Pad Prism version 8 for Windows. Values are expressed as mean ± SEM #P < 0.001 compared to normal control; ** P< 0.01 compared to positive control and *** P < 0.001 compared to positive control.

Results

The percentage yield of ethanolic leaf extract of *Ageratum conyzoides* was found to be 10.75%.

Table 1: Phytochemical screening of ethanolic extract of A. conyzoides leaves

Plant phytochemicals	Observations
Alkaloids	+
Saponins	-
Flavonoids	+
Steroids	+
Carbohydrates	+
Tannins and Phenolic Compounds	+
Proteins	+

Where, + present and – negative

Estimation of Total Phenolic Content:

The total phenolic content of the crude extracts of *Ageratum conyzoides* leaf using Folin-Ciocalteu's reagent was expressed in gallic acid equivalent. The absorbance of serial concentrations of gallic acid was plotted against concentration to yield a linear calibration curve of gallic acid (y = 0.0004x + 0.017) with a correlation coefficient, $R^2 = 0.9944$ (Figure 1). The total phenolic content in the *Ageratum conyzoides* leaf extracts was found to be 37.5mg GAE/g extract.

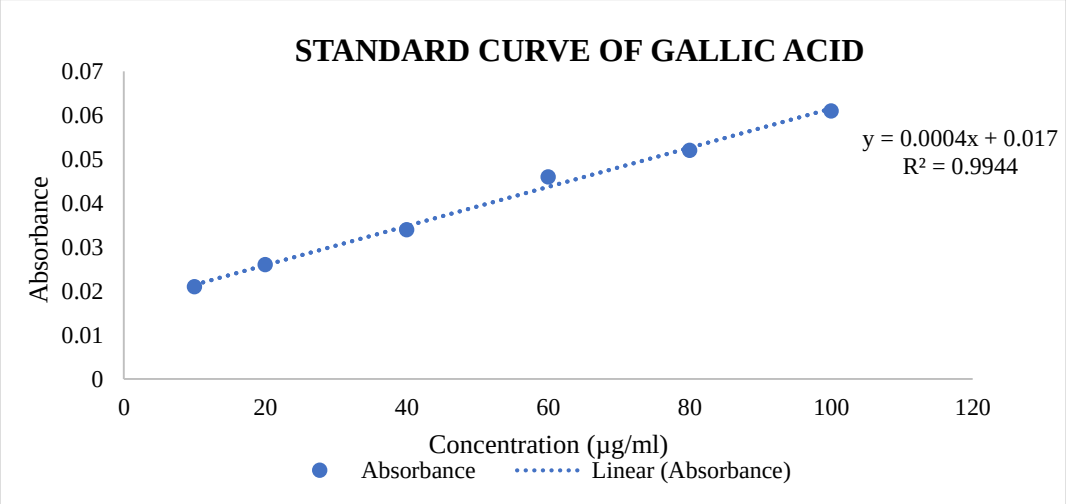


Fig 1. Standard Gallic acid calibration curve for total phenolic content at different concentrations

Estimation of Flavonoid Content-

The absorbance of serial concentration (10, 20, 40, 60, 80, 100) of quercetin standard was plotted against concentration to yield a linear curve of quercetin equivalent (y = 0.0034x and R2 = 0.9918) of QE/g of extract (Figure 2). The total flavonoid content in the *Ageratum conyzoides* leaf extracts was found to be 10.132mg QE/g extract.

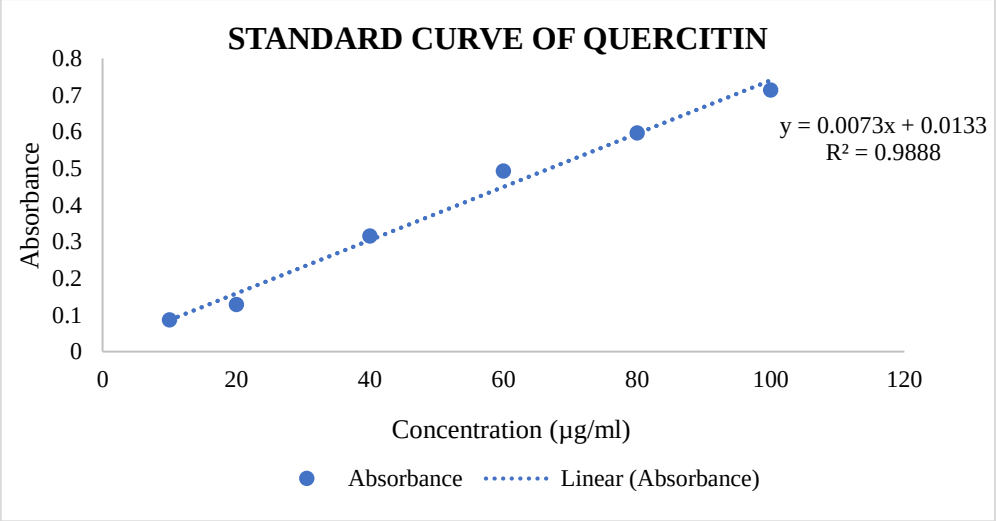


Fig 2. Standard Quercetin calibration curve for total flavonoid content at different concentrations

DPPH FREE RADICAL SCAVENGING METHOD:

The DPPH scavenging activity of ELEAC increased with increasing concentration DPPH scavenging potential of extract at various concentrations was measured and results are duplicated in Fig.3 and table 2. The extract shows a radical scavenging ability of 89.79 ± 0.159% whereas ascorbic acid showed 97.56 ± 0.032% at the highest concentration. The extract showed significant activity when compared to the standard. The IC50 value of the extract calculated was 15.27 µg/ml. With an increase in the concentration of the reaction mixture, the absorbance decreases, indicating higher free radical scavenging activity.

NITRIC OXIDE SCAVENGING ASSAY

The Nitric Oxide scavenging activity of ELEAC increased with increasing concentration. Nitric Oxide scavenging potential of extract at various concentrations was measured and results are shown in Fig.4 and Table 2. The extract shows a radical scavenging ability of 65.29 ± 0.577% whereas ascorbic acid showed 77.03 ± 0.439% at the highest concentration. The extract showed significant activity when compared to the standard. The IC50 value of the extract calculated was 36.44 µg/ml.

Table 2: % Inhibition obtained from DPPH model

S. No.	Concentration (µg/ml)	DPPH		NO	
		Standard (Ascorbic acid)	Test (ELEAC)	Standard (Ascorbic acid)	Test (ELEAC)
1	10	96.51 ± 0.039	72.00 ± 0.468	45.20 ± 0.317	22.33 ± 0.649
2	20	96.94 ± 0.029	79.99 ± 0.058	55.52 ± 0.382	30.20 ± 0.559

3	30	97.21 ± 0.018	85.22 ± 0.313	68.31 ± 0.401	44.92 ± 0.406
4	40	97.35 ± 0.023	87.34 ± 0.120	72.64 ± 0.275	55.21 ± 0.696
5	50	97.56 ± 0.032	89.79 ± 0.159	77.03 ± 0.439	65.29 ± 0.577
	IC50	**28.32**	**15.27**	**24.1844**	**36.44**

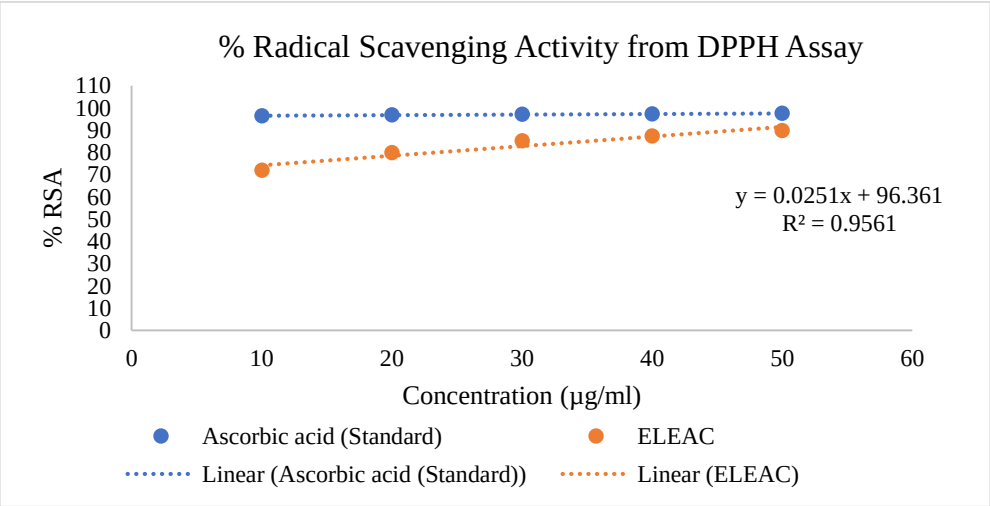


Fig.3. Percentage inhibition of DPPH radical scavenged by the ELEAC and outcome is in comparison with Ascorbic acid. The values are expressed as mean ± SEM. The standard (Ascorbic acid) exhibited significantly higher DPPH radical scavenging activities than the DPPH radical scavenging activity of ELEAC.

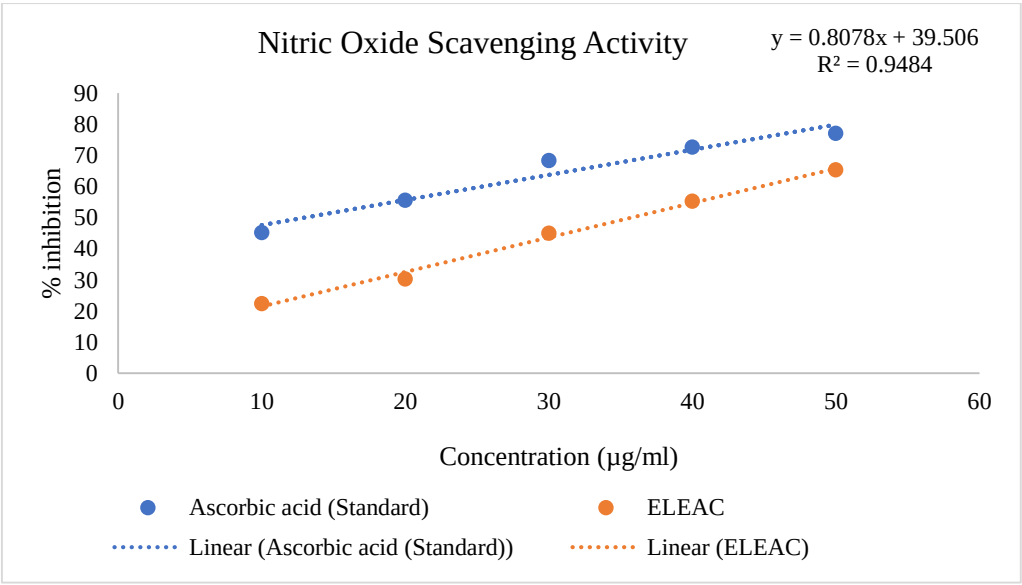


Fig.4. Percentage inhibition of NO scavenged by the ELEAC and outcome is in comparison with Ascorbic acid. The values are expressed as mean ± SEM. The standard (Ascorbic acid) exhibited significantly higher Total antioxidant activity than the ELEAC.

Anti-inflammatory activity (Protein denaturation) using Egg's albumin

In-vitro percentage inhibition of protein (egg albumin) denaturation activity of plant was determined. A dependent type of effect is noticed where an increase in response is obtained by raising the dose of ELEAC. On the other hand standard drug diclofenac sodium at similar concentrations was also observed to inhibit egg albumin protein denaturation as shown in (Fig.6). After the interpretation, of the obtained results, it became evident that ELEAC had an inhibitory effect on protein denaturation, which is a positive effect for reducing inflammatory mediators in hemorrhoids. The IC 50 value for diclofenac sodium was found to be 46.245 µg/ml. The IC50 value for extract was found to be 42.48 µg/ml using GraphPad Prism 8 (Table 4).

Table 4: % Inhibition obtained from Anti-inflammatory activity

S. No.	Concentration (µg/ml)	Standard (Diclofenac)	Test (ELEAC)
1	10	78.79 ± 0.289	66.13 ± 0.589
2	20	84.93 ± 0.364	72.37 ± 0.292
3	30	88.68 ± 0.307	75.94 ± 0.143
4	40	91.02 ± 0.113	79.80 ± 0.295
5	50	92.49 ± 0.610	84.96 ± 0.584
	IC50	**46.245**	**42.48**

Values are expressed in Mean ±SEM (n=3). The anti-inflammatory effect of plant extract was studied. The maximum inhibition shown by the test was 84.96 ± 0.584% at the concentration of 50µg/ml having very significant activity as compared to standard at the same concentration having anti-inflammatory activity at 92.49 ± 0.610%

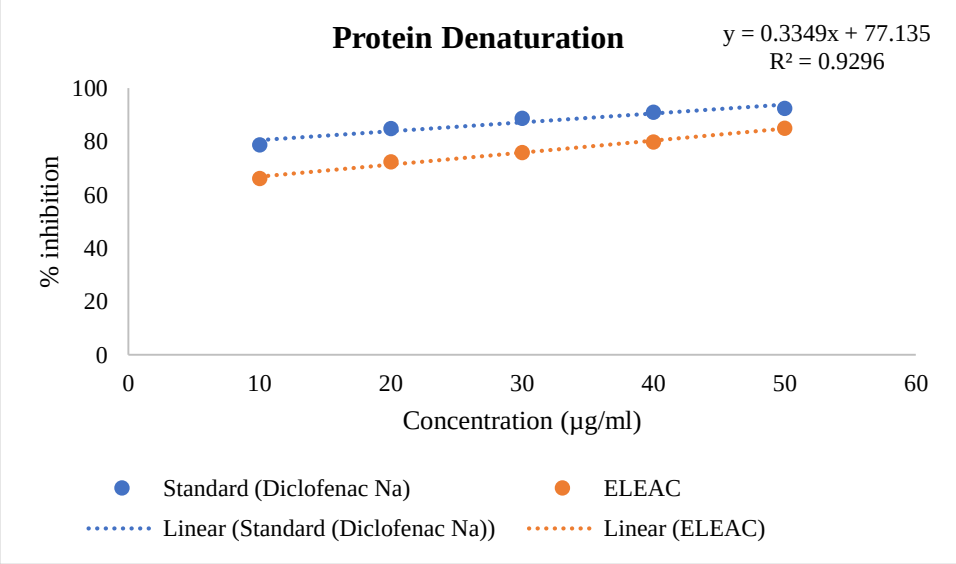


Fig. 5. In vitro anti-inflammatory activity of ELEAC using inhibition of egg albumin denaturation Diclofenac sodium was used as standard. Values are expressed in Mean ± SEM (n=3).

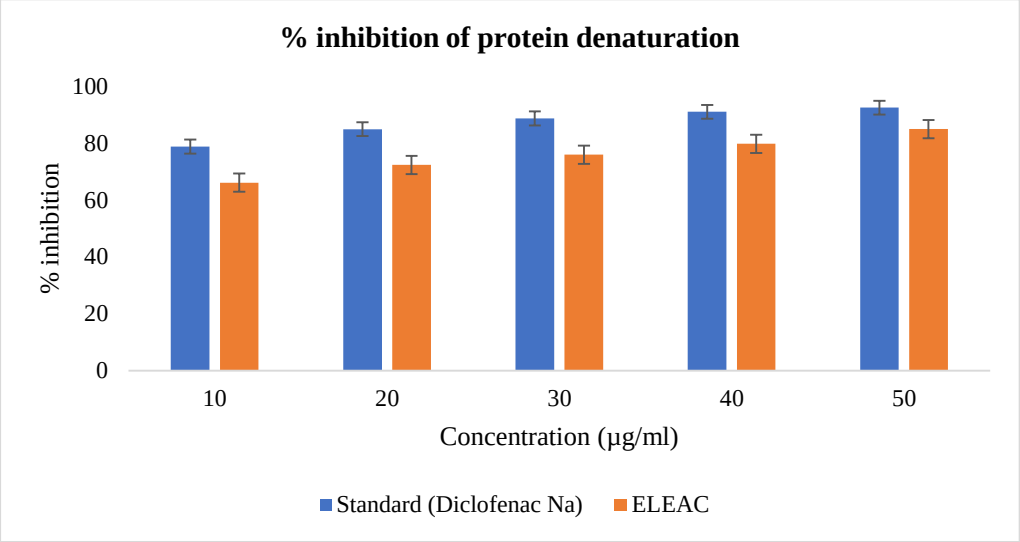
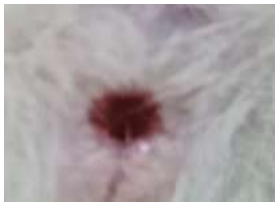
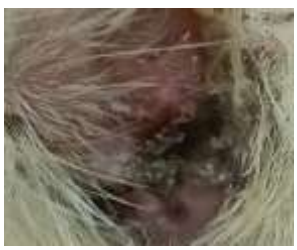


Fig. 6. % Inhibition Vs. concentration protein denaturation (Egg's albumin) between Diclofenac sodium and ELEAC which shows an increase in % inhibition with an increase in concentration.

Table 5: Images of topical application on rectal portion of rats for the treatment of hemorrhoids

Topical Group	Day 3 (Croton oil Induced)	Day 3 (Treatment)	Day 7 (Treatment)
Normal Control Group			
Disease Induced			

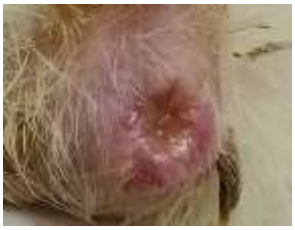
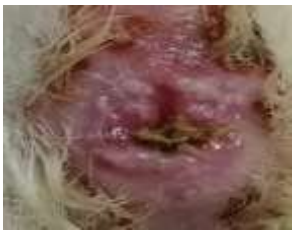
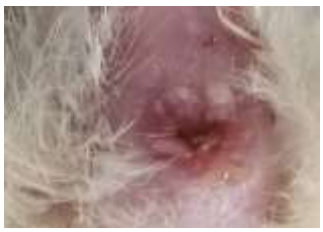
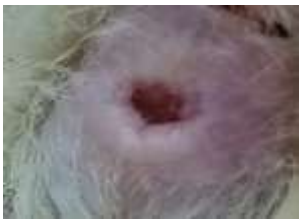
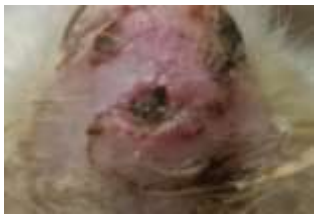
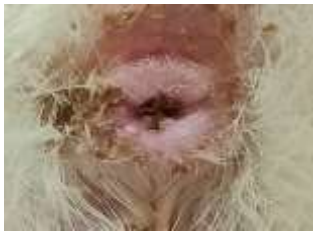
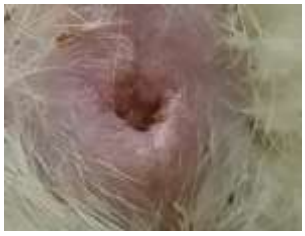
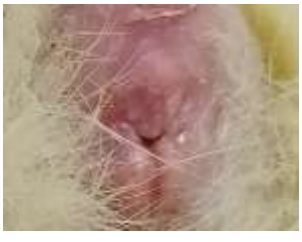
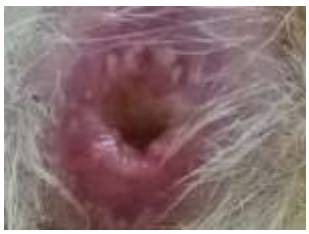
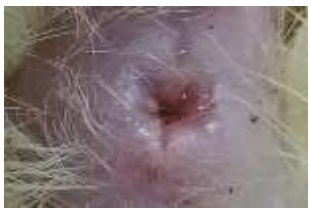
Ointment Base			
Ointment (Standard)			
Ointment (Test)			

Table 6: Images of oral administration on rectal portion of rats for the treatment of hemorrhoids

Topical application Group	Day 3 (Croton oil Induced)	Day 3 (Treatment)	Day 7 (Treatment)
Tween 80			
Oral Standard			

Test Low dose 300mg/kg			
Test High dose (600mg/kg)			

Effect on recto-anal weight

Croton oil preparation, in experimental animals found to induce extravasation in study animals can cause rectal inflammation. Hence, in the current research, ARC was determined to measure the intensity of rectal inflammation developed in study animals. Significant, larger ARC values, were reported in disease-induced group animals 3.380 ± 0.053 when compared to normal group animals at 0.988 ± 0.046 ($P<0.0001$) indicating the development of rectal inflammation by croton oil in these group animals. Whereas, lowered ARC values 1.093 ± 0.004 reported in the Pilex granules treated group and 1.019 ± 0.003 reported in the Pilex ointment treated group indicate the protective role of standard against croton oil-induced inflammation. The result of ELEAC was found to be (low dose 300 mg/kg) 1.980 ± 0.005 and (high dose 600mg/kg) 1.143 ± 0.004 and also in plant extract ointment 1.117 ± 0.002. The plant has shown a significant effect in comparison to disease disease-induced group.

Hemorrhoidal parameters

Table 7: Ano-rectal coefficient of Topical application and oral administration

Topical Application	RAW	ARC
Normal Control	209.5 ± 0.43	0.988 ± 0.046
Disease Induced	663.3 ± 1.94	3.380 ± 0.053****
Ointment Base	516.7 ± 0.42	2.286 ± 0.03****
Pilex Ointment (Std.)	202.5 ± 0.43	1.019 ± 0.003*
ELEAC Ointment	223.7 ± 0.71	1.117 ± 0.002**

Oral Administration	RAW	ARC
Normal Control	209.5 ± 0.43	0.988 ± 0.046
Disease Induced	663.3 ± 1.94	3.380 ± 0.053****
1% Tween 80	561.5 ± 0.43	2.547 ± 0.004****
Pilex granule (Std.)	218.8 ± 0.48	1.093 ± 0.004
ELEAC (300mg/kg)	435.3 ± 0.33	1.980 ± 0.005****
ELEAC (600mg/kg)	229.2 ± 0.60	1.143 ± 0.004**

Effect of extract on recto anal weight (RAW) and ano rectal coefficient (ARC) in rat model of croton oil-induced hemorrhoids. All the values are expressed as mean ± SEM. (Number of animals, n= 6), ** Significant at p<0.01, *** Significant at p<0.001, **** Significant at p<0.0001. The mean values of all the groups were compared to the normal control group by one-way ANOVA followed by Tukey's multiple comparison test)

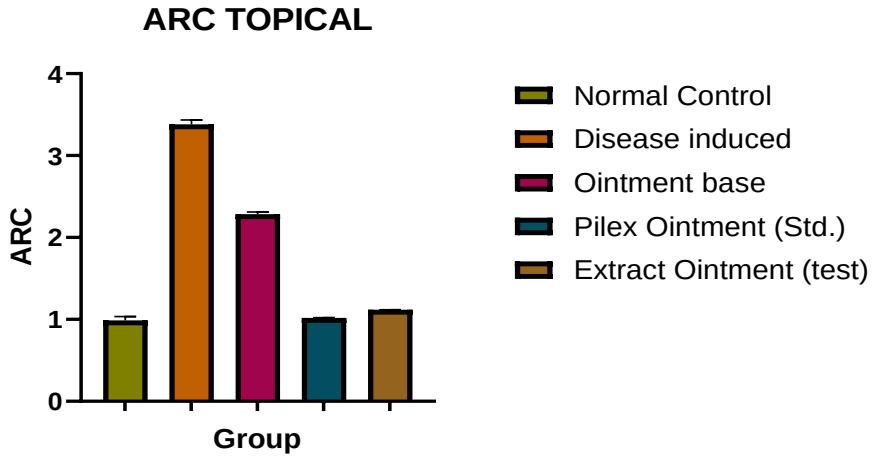


Fig 8. Effect on anorectal coefficient of Topical application

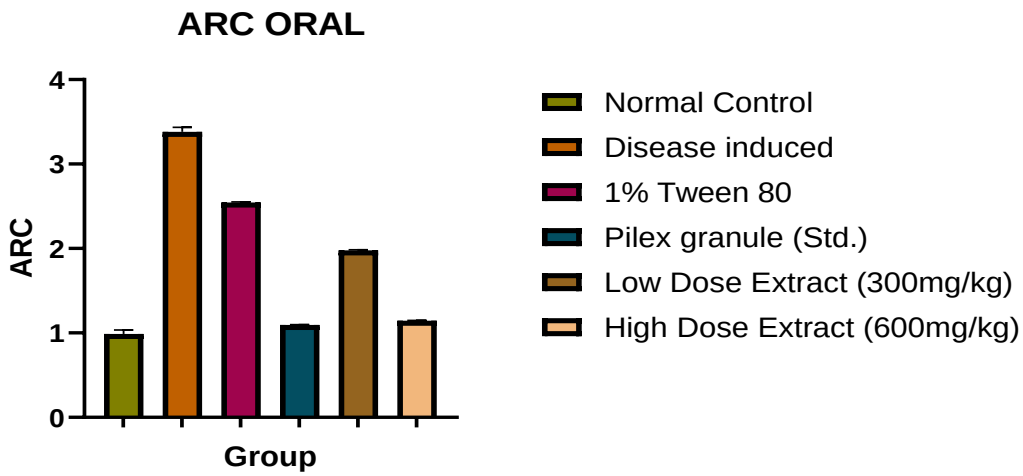


Fig 9. Effect on anorectal coefficient of Oral administration

The anti-hemorrhoidal effect of ELEAC after oral and topical application was studied by the Croton oil-induced method. In this method (fig.8 and 9), Wistar rats treated with two oral doses of plant extract (300, 600 mg/kg) and also treated topically showed a reduction in hemorrhoid, which was significant when compared with the control group in a dose-dependent manner.

Biochemical Parameters-

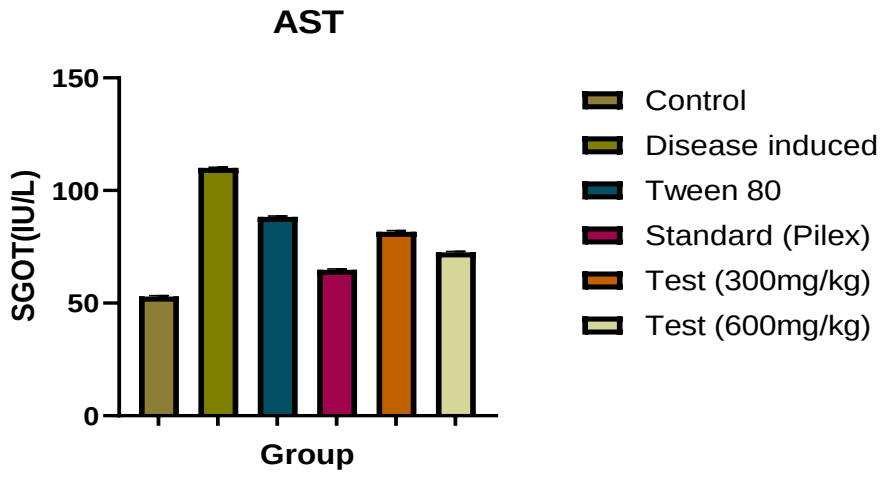
Estimation of AST (SGOT)-

Croton oil-induced hemorrhoid rats showed an increased level of SGOT, SGPT and ALP in their blood serum when compared to the normal control group. Treatment with ELEAC at a dose of 300 and 600 mg/kg caused a significant decrease in SGOT, SGPT and ALP levels when compared to the serum of croton oil administered rats (****P<0.0001 when compared to the normal control group).

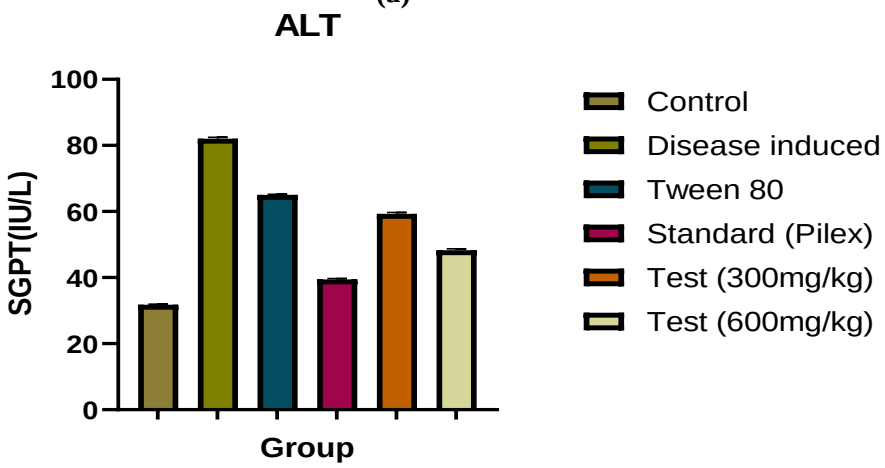
Table 8: Effect of *Ageratum conyzoides* extract on SGOT levels

S. No.	Groups	SGOT (U/L)	SGPT (U/L)	ALP (U/L)
1	Normal Control	52.92 ± 0.336	31.79 ± 0.490	60.42 ± 0.323
2	Disease Induced	110.0 ± 0.350****	82.07 ± 0.981****	117.6 ± 0.299****
3	Tween 80	88.27 ± 0.464****	65.01 ± 0.498****	113.1 ± 0.500****
4	Standard (Pilex granule)	64.78 ± 0.379**	39.44 ± 0.757***	71.56 ± 0.334**
5	ELEAC (300mg/kg)	81.70 ± 0.452***	59.31 ± 0.900****	94.69 ± 0.301***
6	ELEAC (600mg/kg)	72.60 ± 0.393***	48.31 ± 0.885****	80.29 ± 0.349**

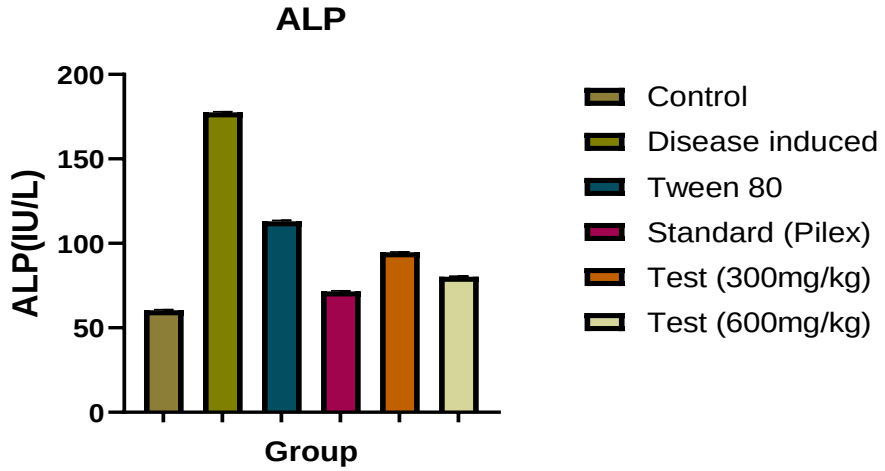
Results are represented as Mean ± SEM (Number of animals, n= 6), ** Significant at p<0.01, *** Significant at p<0.001, **** Significant at p<0.0001 when normal control group compared to the different treated group (One-way ANOVA followed by Tukey's multiple comparison test)



(a)



(b)



(c)

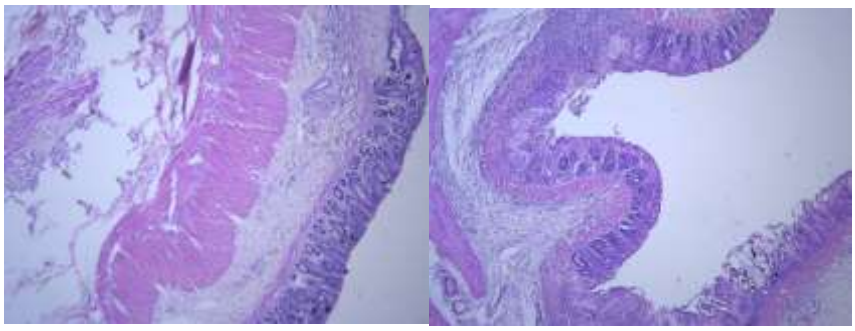
Fig. 10. Effect of ELEAC on (a) SGOT, (b) SGPT and (c) ALP values in Croton oil-induced hemorrhoids in rats

Histopathological studies

This section of the study contains histopathological studies carried out on recto-anal tissues of 54 Wistar rats. This study was done to know the effect of both oral and rectal application of

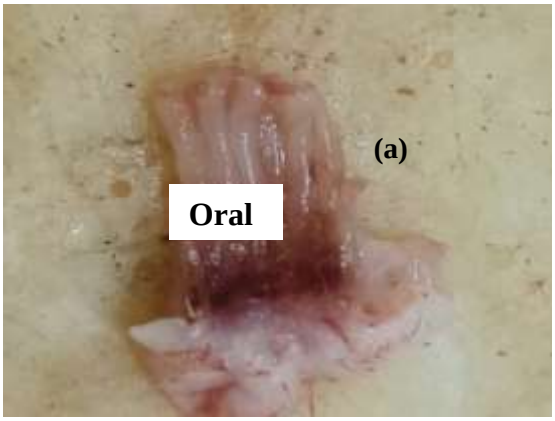
ELEAC on histological changes induced by croton oil preparation in recto-anal tissues. Histological study reports of normal control group I in section (a) showed normal histology with proper cell alignment and architecture as represented in (Fig.12). Whereas, croton oil preparation in group II positive control (disease induced) animals (b) was found to induce significant changes like hemorrhage, mucosal tissue damage, blood vessel dilation and necrosis with poor healing. Treatment with the standard drug in section (d) was found to exhibit nearly a normal recto-anal histology. Rectal tissues of animals treated with ELEAC at a dose of 300 mg/kg in section (e) showed mild changes, tissue damage followed by healing is seen, no hemorrhage is observed, intact mucosal linings are found and deposition of inflammatory cells noticed. Inflammation, hemorrhage, necrosis, blood vessel dilation is minimal in *Ageratum conyzoides* at a dose of 600mg/kg in section (f). It was found to exert a better protective role compared to Group 3 and Group 5 animals.

Section (g) was treated with an Ointment Base and showed mild inflammation, minimal congestion, dilatation of blood vessels, degeneration and necrosis. After treatment with pilex ointment in the treated group, section (h) showed that the inflammatory cells were seen less and tissue of the rectum lined found by mucosa forming luminal folds. Sections (i) treated with 10% w/w extract in Ointment Base showed minimal inflammation, congestion, dilatation of blood vessels, degeneration, and necrosis.



Control **Disease Induced**

Fig. 11. Tissue of rectal portion of Croton oil induced hemorrhoid in rats



(b)

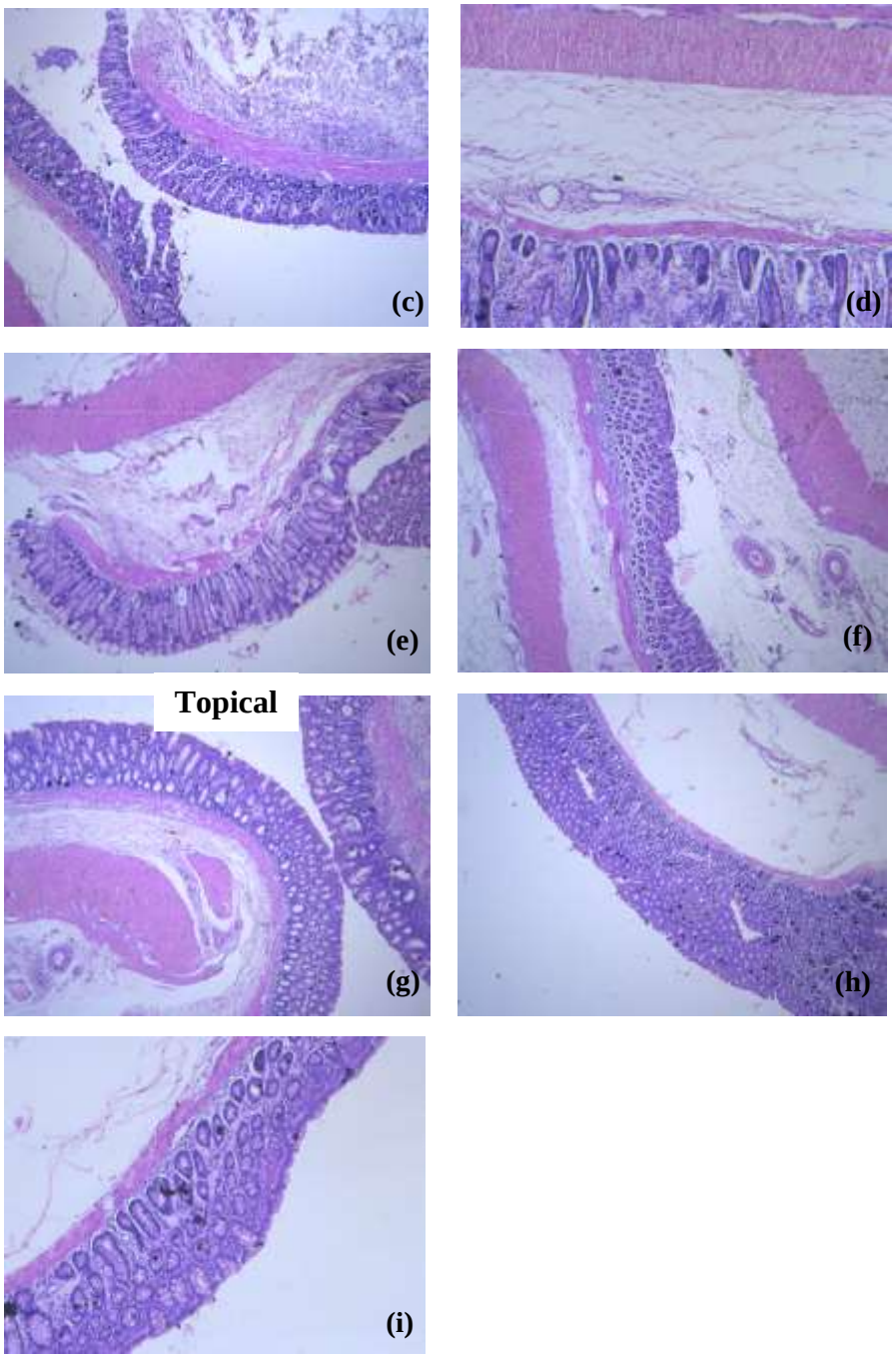


Fig. 12. Histopathology of Rectal portion. Effect of Leaf extract, Pilex ointment and Pilex granules on the histology of rectoanal tissue in croton-oil induced hemorrhoids. Rectoanal sections of rats:

(a) Group 1- Normal control,

(b) Group 2- Disease induced,

(c) Group 3- Tween 1%,

(d) Group 4- Oral Standard (Pilex granule) 200mg/kg,

(e) Group 5- ELEAC 300mg/kg,

(f) Group 6- ELEAC 600mg/kg,

(g) Group 7- PEG base,

(h) Group 8- Topical Standard (Pilex Ointment)

(i) Group 9- 10%w/w ELEAC in ointment base

DISCUSSION-

Phytochemical screening is a qualitative analysis carried out by observing the colour changes. Table 1. showed that the results of phytochemical tests on ELEAC positively contained flavonoids, alkaloids, tannins and phenolic compounds, steroids, and proteins, while the saponin content in ELEAC was negative.

There are several flavonoid compounds in *Ageratum conyzoides*. The phenolic compounds known as flavonoids have the ability to act as antioxidants because they can repair damaged body cells and stop the emergence of free radicals in the body[25].

Quantitative phytochemical estimation was also performed and total tannin content was found out to be 37.5mg GAE/g extract whereas total flavonoids was about 10.132mg QE/g extract.

the phenol content of A. conyzoides had antioxidant potential in diverse models of studies. From the antioxidant studies of DPPH scavenging activity, the ELEAC had the best antioxidant activity (IC50 of 15.27 µg/mL) even in comparison with the standard antioxidant (ascorbic acid) (with IC50 of 28.32 µg/mL).

In-vitro protein denaturation (Egg's albumin) of ELEAC exhibited significant anti-inflammatory activity by protein denaturation. The percentage inhibition of protein denaturation of plant extract increases with the increase in concentration. The IC50 diclofenac 46.245 while that of extract is 42.48 µg/mL.

Phase separation was not seen throughout the duration of the investigation in the plant extract ointment, nor were there any notable changes to characteristics like colour, odour, or consistency. Additionally, during the skin irritant test, no spots on the rat's skin were seen.

Hemorrhoids are a pathological condition characterized by a strong vasodilatation at the recto-anal area that causes inflammation of the surrounding tissues, further causing the hemorrhoids to result in additional issues including the extravasation of fluid into the fluid interstitial region primarily as a result of vascular permeability and the large-scale movement of Pro-inflammatory cells. In this study, croton oil was used as an inducer for the animal hemorrhoid model. Croton oil causes inflammation because it produces soluble inflammatory lipid metabolites such as nitric oxide (NO), leukotrienes, prostaglandins, TNF, IL-6, IL-17, and various cytokines, among others[11].

Numerous inflammatory conditions, including allergies, asthma, autoimmune illnesses, inflammatory bowel disease and hemorrhoids, can be treated using plants that contain phenolic chemicals such as flavonoids, tannins, stilbenoids, catechins, lignin, and phenolic acids, since the use of glucocorticoids in the treatment of hemorrhoids have many side effects. The most popular oral plebotonic medications used for hemorrhoidal treatment are phenolic compounds. Phenolic substances can have an anti-inflammatory impact, promote lymphatic drainage, decrease capillary permeability, and raise venous tone. The pathophysiology of hemorrhoids is developed by increased capillary permeability, which is largely a result of inflammation[3].

A recent study has reported that *Ageratum conyzoides* L. leaves extract containing quercetin actively inhibited TNF-α during the inflammation process by degrading the collagenase of cartilage as well as the matrix metalloproteinase-9 (MMP-9)[26]. Quercetin reduces capillary permeability by increasing vascular walls and inhibits inflammation[27]. Kaempferol is responsible for antioxidant, anti-inflammatory, antimicrobial[28]. Squalene is a bactericide and penetrant preferred for the substantial absence of sensitization reactions in patients having inflamed anorectal tissues[29].

To better understand the series of inflammatory reactions that occur during the development of hemorrhoids after the application of croton oil, researchers used ARC. The ARC value represents the edema of the recto-anal region. This implies that when the value of this rate rises, the severity of the edoema and haemorrhoids also rises[23]. ARC values of the disease induced, tween 80 and the base cream groups were observed at higher levels than the standard and the plant extract groups. Among the treatment groups, the lowest ARC value was observed in the ELEAC-administrated animal group showing that the treatment with the plant extract ointment is more effective than the other groups.
Croton oil increases the formation of ROS, leukocyte infiltration, and free radical scavengers, which reduces the antioxidant state by changing the levels of liver enzymes (ALP, AST, and

ALT). Similarly, our study also showed that croton oil application hampered the antioxidant status which shows an increase in the levels of liver enzymes like (ALT, AST, ALP)[30]. A. conyzoides extract at a dose of 300 and 600 mg/kg showed a significant decrease in ALT, AST and ALP levels when compared to disease-induced administered rats. So when compared to conventional pilex tablets, they result in a decrease in the biochemical parameters when compared to the ELEAC[31].

In this study, the effectiveness of *Ageratum conyzoides* in treating hemorrhoids in a rat model was evaluated. The oral and topically applied extracts of this plant decreased the total histomorphometric scores. Compared to the hemorrhoid-induced control group, experimental groups showed a significant improvement in inflammation, edema, congestion and constrained areas of necrosis and fibrosis in their histological sections. Histomorphometric scores of lesions excised from the recto-anal region of rats given *A. conyzoides* ointment was close to those of the control group. The same outcomes were seen with oral administration, the histomorphometric scores of the standard medicine Pilex® tablet, the *A. conyzoides* extract administration dosages, and the control all had comparable results.

The histomorphological scores and serum indicators of disease activity on the rat hemorrhoid model were successfully improved by topical and oral *A. conyzoides* therapies. ELEAC was discovered to be efficient in the anti-inflammatory pathway as well as in lowering capillary permeability. The typical medication employed in this study, a herbal formulation aids in treating piles by regenerating tissue.

CONCLUSION

We have justified the traditional claims of the plant *Ageratum conyzoides* L. in the treatment of hemorrhoids for the first time in Wistar rats. The observed anti-hemorrhoidal potential of the leaves from the plant was attributed to its anti-inflammatory and antioxidant potential, where Quercitin could play a major contributing factor in conjugation with other phytoconstituents. Based on the research conducted, it can be concluded that the ELEAC may be beneficial to cure hemorrhoids in humans. *Ageratum conyzoides* leaf extract that is rich in Quercitin, Kaempferol, Squalene may improve the health and quality of life of people who suffer from hemorrhoidal disease for which more advanced studies are required.

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