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RESEARCH ARTICLE

Ameliorative Effect of *Caesalpinia sappan* Heartwood Extract in Acetic Acid-induced Ulcerative Colitis in Rats

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Abstract

Background: Decoction prepared from slices of *Caesalpinia sappan* (CS) heartwood was consumed for various health benefits. This drink is considered to be anti-oxidant and anti-inflammatory. Furthermore, *C. sappan* is a rich source of bioactive flavonoids. **Aim:** The titled study was intended to explore the protective effect of Hydroalcoholic Extract of *C. sappan* Linn. (HAECS) against acetic acid-induced colitis in rats. **Methods:** The animals in groups were administered with HAECS (200 and 400 mg/kg, p.o.) and sulfasalazine (100 mg/kg, p.o.) for 5 successive days before induction of colitis, which was done by intra-rectal instillation of acetic acid (2 ml of 4% v/v) and the treatment was sustained up to 7 days. After 72 h of colitis induction, rats were sacrificed, and mucosal injury at the distal colon was assessed both macroscopically and microscopically. Also, the mucosal levels of lipid peroxidation (LPO), reduced glutathione (GSH), colonic myeloperoxidase (MPO) activity, and serum lactate dehydrogenase (LDH), were assessed. **Results:** HAECS treatment exhibited significantly reduced macroscopic score, clinical activity score, wet colon weight and improved histology scores when compared to the colitis control. Also, it showed significantly attenuated colonic levels of MPO, LPO, LDH and elevated levels of GSH. **Conclusion:** *C. sappan* heartwood possesses an ameliorative effect against acetic-acid-inflicted colitis owing to its antioxidant and anti-inflammatory potential.

Major Finding: The *C. sappan* extract effectively healed colitis in rats, drastically reducing visible injury, colon weight, oxidative stress and improving tissue histology. Thus, proposed study provided the scientific footage to the folkloric claim of titled plant.

Keywords: Anti-inflammatory, Anti-oxidant, Inflammatory Bowel Disease, *Sappan* Wood

1. Introduction

Inflammatory Bowel Disease (IBD) is a persistent inflammatory ailment of an idiopathic cause, mainly affecting the alimentary canal. It is represented as Ulcerative Colitis (UC) and Crohn's Disease (CD)[1]. The incidence of IBD, including UC is usually high, with an incidence of 250 cases per 100,000 people in the Western world, yet it is prevalent elsewhere in the globe owing to the inculcation of Western culture[2].

Ulcerative colitis generally affects the mucosa of the colon, including the rectum, while Crohn's disease is an inflammation that afflicts the entire alimentary tract. Generally, patients with UC present the symptoms like diarrhoea, hematochezia, abdominal cramping, fever, and reduction of the colon length, and weight loss can occur in severe cases[3]. The exact root cause of UC is unknown, but associated with multifaceted interaction amongst genetic, immune system, and environmental factors. Alteration in dietary and colonic bacteria

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contributes to a pathogenic aspect of the IBD genesis[4]. Though the etiology of UC is not fully understood, enlarged production of free radicals and diminished antioxidant competence are imperative features of IBD. Reactive Oxygen Species (ROS) following oxidative degradation of lipids, and a decline in activity of cellular antioxidants, could potentially ensue in inflammation of the colon[5].

The generation of oxygen-free radicals, neutrophil infiltration, and the activation of inflammatory cytokines are among the characteristics of the inflammatory response. It has been demonstrated that the recruitment of neutrophils to inflammatory areas is linked to the release of proinflammatory cytokines like Tumor Necrosis Factor-alpha (TNF-α), a key player in inflammation. Furthermore, ROS, when interacting with biological macromolecules, imposes damage to lipids, nucleic acids, and proteins. Malondialdehyde (MDA) is a byproduct of oxidative stress that is produced when lipid peroxyl radicals break down. On the other hand, the sulfhydryl (SH) groups of proteins in extravascular regions or intracellular glutathione (GSH) systems are thought to be one of the key mechanisms of lowering oxidative stress. Thus, acute inflammation and an alteration in the integrity of the mucosa may ensue from an imbalance between gastro-toxic and protective substances[6].

Currently, immunomodulators, aminosalicylates, and glucocorticoids are largely prescribed to treat IBD. However, these agents are sometimes ineffectual or associated with unbearable effects. Hence, using herbal medicines, complementary and alternative treatments are becoming more prominent in the effective management of UC[7]. Although several models are established on experimental colitis, due to the similarity in the pathological changes of acetic acid-prompted colitis and human inflammatory bowel disease, it is a commonly used model to evaluate new drugs for the treatment of IBD. Acetic acid induces massive intracellular acidification, leading to the destruction of epithelial cells and substantial inflammatory colonic response[8].

Caesalpinia sappan Linn. (*Fabaceae/Caesalpiniceae*), commonly known as sappan wood or patang in Hindi, is a small-sized tree that develops to a height of ten meters. Species are being used traditionally as they possess a wide variety of medicinal properties. The heartwood contains diverse chemical constituents such as triterpenoids, phenolic compounds (coumarin, xanthone, chalcones, homoisoflavonoids, flavones), saponins, flavonoids (quercetin, rutin, gallic acid, ombuin), aromatic compounds, brazilin, sappan chalcone, Caesalpin J and P, protosappanin A and B, and steroids (β-sitosterol)[9]. The review of the literature revealed that the plant has been reported for numerous biological activities, including anti-inflammatory[10], antioxidant[10], anti-ulcer[11], hepatoprotective[12], and antibacterial[13]. Considering its beneficial actions owing to its antioxidant potential and anti-inflammatory action, we designed this research work to explore the effect of *C. sappan* Linn. against acetic-acid-inflicted UC in experimental models of IBD in rats anticipating its beneficial effects in conditions of colitis.

2. Materials and Methods

2.1 Chemicals

Acetic acid (Himedia, Bombay, India), Sulphasalazine tablets (Panacea Biotech Ltd, New Delhi, India), and Lactate dehydrogenase kits were obtained from ERBA Diagnostics, Mannheim, Germany.

2.2 Collection and Preparation of Plant Extract

The *C. sappan* Linn. heartwood was garnered in October 2018 from Kerala, India. Assistant Professor and Head G. Chidananda Murthy, Department of Botany, Sree Siddaganga College of Arts, Science, and Commerce for Boys, identified and authenticated the plant, and a specimen voucher (SSCP14PC012) was deposited in the herbarium. The shade-dried powdered heartwood was extracted by the Soxhlation process (80% v/v ethanol). Then the solvent residue was freed, dried, and stored for further use[11].

2.3 Dose Selection

From the previously published available reports, HAECS was found to be safe at the dose of 2000 mg/kg in the acute toxicity study. Therefore, 200 mg and 400 mg/kg doses were chosen for this study[14].

2.4 Preliminary Phytochemical Screening

To affirm, the HAECS was analysed using various preliminary phytochemical trials to affirm the presence

of flavonoids, alkaloids, tannins, glycosides, and saponins[15].

2.5 Total Phenolic Estimation

The total phenolic content in the hydroalcoholic extract of *C. sappan* Linn. heartwood was measured using a modified colorimetric Folin-Ciocalteu method. The polyphenols give a blue color chromogen in an alkaline medium with Folin-Ciocalteu reagent, having absorption maxima at 760 nm. The stock solution of HAECS (1 mg/ml) was prepared with methanol and filtered through Whatman No. 1 paper. This stock solution (0.3 ml) was then mixed with 2.5 ml of 0.2 N Folin–Ciocalteu reagent for 5 min and 2 ml of sodium carbonate (7.5 g/dl) was then added. After 2 h of incubation at room temperature, the absorbance of the reaction mixture was measured at 760 nm against methanol as blank. Gallic acid (0–10 µg /ml) was used as standard to produce the calibration curve. The mean of two readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE) /g of HAECS[16].

2.6 Determination of Total Flavonoid Content

Caesalpinia sappan contains abundant flavonoids, which contribute to its antioxidant activity. Therefore, estimating the total flavonoid content is essential to assess its phytochemical strength.

2.6.1 Preparation of Standard Quercetin for Calibration Curve

The total flavonoid concentration of the extracts was measured using an aluminium chloride colorimetric test. A stock solution of quercetin (4 mg/mL) was prepared. The standard solution was serially diluted to achieve concentrations of 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, and 1 mg/mL. In a test tube with 4 mL of distilled water, 1 mL of each quercetin concentration was introduced. The test tube was filled with 0.3mL of 5% $NaNO_2$ and 0.3mL of 10% $AlCl_3$ after 5 minutes. Then, 2 mL of 1 M NaOH was added to the mixture after 6 min. The volume of the mixture was made to 10 mL by immediately adding 4.4 mL of distilled water. The total flavonoid content was expressed as quercetin equivalents using the linear equation based on the calibration curve.

2.6.2 Preparation of Samples for Total Flavonoid Content

The extracts were diluted serially to yield solutions with varying concentrations starting from stock solutions that had a 4 mg/mL concentration in methanol. A spectrophotometer was used to test the extracts' absorbance at 510 nm, using a similar method that was previously described for quercetin. The absorbance value was taken as an average over three readings, and the total flavonoid content was determined using this value. Equating the flavonoid content using the linear equation based on the standard calibration curve yielded the quercetin equivalent (mg QE/g)[17].

2.7 Experimental Animals

Male albino Wistar rats of weight 200-250 g, kept under optimum conditions of temperature (22 ± 2 °C), and 12 h light-dark cycles were procured from the animal facility of Sree Siddaganga College of Pharmacy, Tumakuru. The acclimatised animals were randomised into respective treatment groups and were housed individually in sanitised cages bedded with sterilised paddy husk, free access to pellet diet, and water ad libitum. The study was authorised by the Institutional Animal Ethical Committee (IAEC) of Sree Siddaganga College of Pharmacy, Tumakuru, Karnataka. Approval No. 183 SSCP/IAEC-.Clear//2018-19 dated 09/03/2018, as per the guidelines prescribed by the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India.

2.8 Experimental Design

The rats were randomized and allocated equally into six groups (n=8). Group I (control group) orally received 0.3 % w/v of sodium CMC, Group II was orally administered with HAECS 400 mg/kg, colitis control Group III received sodium CMC till day 5, and on the 5th day 2 ml of 4% v/v acetic acid intrarectally, while Group IV and V were orally administered with HAECS (200 and 400 mg/kg) respectively while group VI with sulphasalazine (100 mg/kg, p.o).

Pretreatments were carried out for seven days. On day 4, of the treatment, the animals were kept overnight fasting with free access to water ad libitum. On day 5, following 1 h of the foregoing treatments, rats in all the Groups except I and II were anaesthetized. After that, intrarectal instillation of acetic acid (2 ml of 4% v/v)

in 0.9% saline using a polyethylene catheter placed through the rectum of the colon around 8cm. Animals were held in the Trendelenburg position for 2–3 min to prevent the instant anal seepage of the intracolonic instillate. On day 8, the blood was collected retro-orbitally for serum LDH estimation. Furthermore, the experimental animals were sacrificed, and colonic tissues were collected for macroscopical, biochemical, and histopathological assessments[18-20].

2.9 Disease Assessment Parameters

2.9.1 Clinical Activity Score

The clinical activity scores of UC were assessed by summing up the scores of weight loss, bleeding, and stool consistency within 48 hours after colitis infliction, and the assessment criteria were 0 = No weight loss, weight loss of 1 to 5% = 1, 5 to 10% = 2, 10 to 20% = 3, and 20% = 4. Stool consistency was scored as 0 = well-formed pellets, 2 = Pasty and semi-formed stools that did not stick to the anus, 3 = Liquid stools that did stick to the anus, and bleeding was scored as 0 = No blood in hemoccult, 2 = Positive hemoccult, 4 = Gross bleeding. The scores were divided by 3, which gave a total clinical score that varied from 0.0 (healthy) to 4.0 (the highest l activity of colitis)[21].

2.9.2 Colon Weight - Length Ratio

This parameter is used to review the extent of edema in the colon, which reveals the severity of colitis. After scarification, the colon (distal end) was longitudinally cut, and fecal matter was gently cleaned using saline. For each specimen, the wet weight (mg) and the ratio of weight to length (mg/cm) were measured and noted[22].

2.9.3 Macroscopic Evaluation

The macroscopic changes were assessed by an independent observer using the grading scale from 0-8 and scored as 0 = no visible changes, 1= showing hyperemia at sites, 2 = lesions having diameter 1 mm or less, 3 = lesions having diameter 2 mm or less (no. < 5), 4 = lesions having diameter 2 mm or less (no. 5-10), 5 = Lesions having diameter 2 mm or less (no. > 10) 6 = lesions having diameter more than 2 mm (no.< 5), 7 = lesions having diameter more than 2 mm (no. 5-10), 8 = lesions having diameter more than 2 mm (no. > 10). The percentage protection for clinical activity and macroscopic scores was calculated as follows[8].

$$\%\,\mathrm{Protection} = \frac{(Disease\ control\ score - Treatment\ score)}{Disease\ control\ score} \times 100$$

2.9.4 Study of Biochemical Parameters

A portion of the inflamed tissue of the colon was homogenised in ice-cold potassium phosphate buffer (pH 7.4), and the homogenate was used for the assessment of oxidative stress markers parameters such as MDA, reduced GSH and MPO activity. Besides, the serum LDH activity was measured utilising an ERBA diagnostics kit.

2.9.5 Assessment of Myeloperoxidase Activity of the Colon

The myeloperoxidase activity of the colon was measured as an indicator of neutrophil infiltration. The colonic tissue homogenate (10%) was subjected to centrifugation for 30 minutes at a temperature of 4°C. The supernatant liquid was separated from the pellets, and the pellets were added to 0.5% w/v HETAB in the solution (prepared using 0.5g hexadecyltrimethylammonium bromide in 100 ml of ice-cold 50 mM potassium phosphate buffer (pH 6)). Further, the solution was subjected to centrifugation for 20 min, and supernatant liquid (0.1 ml) was added to the phosphate buffer (2.9 ml 50 mM, pH 6.0) containing O-dianisidine hydrochloride (0.167 mg/ml) and 0.0005% hydrogen peroxide. Change in the absorbance was recorded at 460 nm using a UV spectrophotometer for about 5 min. The activity of myeloperoxidase was calculated using the formula as follows[23].

$$MPO\ activity\ (U/g) = \frac{x}{Weight\ of\ the\ piece\ of\ tissue\ taken(mg)}$$

Where X = 10 × change in absorbance per min/ volume of supernatant taken in the final concentration.

2.9.6 Assessment of Colonic MDA Level

0.1 ml of homogenate was added to the 2ml of TBA-TCA-HCl reagent (containing equal proportions of Trichloroacetic acid 15%, Thiobarbituric acid 0.375%, and HCL 5N) and boiled for 15 min, cooled for 10 minutes, centrifuged, and the absorbance of the clear liquid (supernatant) was recorded at 532 nm against a blank reagent. The amount of lipid peroxides MDA was assessed and determined stated as µmol/g of wet tissue weight[19].

Concentration of MDA = absorbance of test x total volume

ε sample volume

ε (molar extinction co-efficient) = 1.56 x 10^5 M^{-1} cm^{-1}

2.9.7 Determination of GSH Content

The assessment of GSH is based on the reaction between sulphydryl groups and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), resulting in a relatively stable yellow product. Proteins were precipitated by the method of centrifugation using TCA (10%). Then the supernatant liquid (0.5 ml) was added to the phosphate buffer (0.2 M, pH 8.0) and DTNB (10 mM). This mixture was allowed for incubation (10 min), and the absorbance of the sample was measured at 412 nm against the appropriate blank. The results were reported as nmol/g of wet tissue weight[24].

2.9.8 Determination of LDH Levels

Serum LDH levels were assessed using a commercially available ERBA diagnostics kit[25].

2.9.9 Histopathological Evaluation

The histopathological evaluation was done by an examiner who was blind to the study protocol. To determine the induction of UC, from each rat, a portion (2 cm) of the inflamed colonic tissue was collected and stored in 10% formalin, sectioned into 5µm thickness, stained using haematoxylin and eosin (H and E), and studied for histopathological alterations and changes due to inflammation like submucosal edema, goblet cell loss and to cellular infiltration. In addition, Periodic Acid and Schiff's (PAS) stain of histological slides was also performed to know the extent of mucosal damage and the number of goblet cells[7].

2.9.10 Statistical Analysis

The statistical results were stated as mean ± SEM. Variances between groups were carried out by one-way analysis of variance (ANOVA) followed by multiple comparisons, Tukey's test using GraphPad Prism 5.0 software. The statistical significances were identified at $p< 0.05$, $p< 0.01$, and $p< 0.001$.

3. Results

3.1 Percentage Yield and Preliminary Phytochemical Screening of HAECS

The percentage yield of HAECS was 7.80 % w/w, and qualitative phytochemical analysis revealed that contains flavonoids, alkaloids, glycosides, tannins, and saponins.

3.2 Total Phenolic and Flavonoid Content of HAECS

A standard calibration curve of gallic acid and quercetin was used to quantify total phenolic and total flavonoid content in HAECS. The amount of total phenolic content and total flavonoid content of HAECS was found to be 377.62 ± 7.86 mg (gallic acid equivalent / g dry material) and 343.56 ± 0.734 mg (quercetin equivalent / g dry material).

3.3 Effects of HAECS on Clinical Activity Score (CAS)

After induction of colitis in rats, CAS was evaluated by alterations in the total mean weight loss, bleeding, and stool consistency. A significant ($p< 0.001$) augment in the clinical activity score of rats in colitis control

Table 1. Effect of HAECS on the clinical score, macroscopic score, and colon weight-to-length ratio in acetic acid-induced colitis rats

Sl. No.	Groups	Clinical activity score	Macroscopic score (% protection)	Colon weight/length ratio (mg/cm)
1	Normal control 10 ml/kg	0.0±0.0	0.0±0.0	86.81 ± 3.01
2	HAECS alone (400 mg/kg, p.o.)	0.18±0.16	0.0±0.0	118.07 ± 6.65
3	Colitis control (2 ml of 4% v/v AA, intra rectal)	3.56±0.57###	6.57 ±0.22###	137.0 ±1.15###
4	AA+HAECS (200 mg/kg, p.o.)	1.27 ± 0.23***	2.59±0.42***(60.57)	121.2 ± 9.74**
5	AA+HAECS (400 mg/kg, p.o.)	0.98 ± 0.03***	1.83±0.47***(72.14)	102.2 ± 1.83***
6	AA+SLZ (400mg/kg, p.o.)	0.76 ± 0.03***	1.33±0.55***(79.75)	98.51 ± 2.77***

Data are expressed as mean ± standard error of the mean (SEM); n = 6. One-way ANOVA followed by Tukey's post hoc test. ###$p < 0.001$ significantly different from normal control. *$p < 0.05$, **$p<0.01$and***$p<0.001$ significantly different from Colitis control.

in contrast to the normal control. Pretreatment with HAECS at both dose levels (200 and 400 mg/kg, p.o.) exhibited significant ($p< 0.001$ and $p< 0.001$) attenuation in the clinical scores. The SLZ pre-treatment significantly inhibited the AA-induced alterations and showed amelioration of all the parameters mentioned above, indicating its potent action at the dose tested, as shown in Table 1.

3.4 Effects of HAECS on Macroscopical Evaluation

Intra-rectal instillation of acetic acid into the colon caused grievous macroscopic edematous inflammation. There was a significant ($p < 0.001$) rise in the macroscopic score of colitis control as compared to the normal group. Pretreatment of HAECS at doses 200, 400 mg/kg, and SLZ 100 mg/kg to AA-administered rats showed significant ($p < 0.001$, $p < 0.001$) attenuation in macroscopic scores, showing 60.57 %, 72.14 %, and 79.75 % protection, respectively, compared to disease control, as shown in Figure 1 and Table 1.

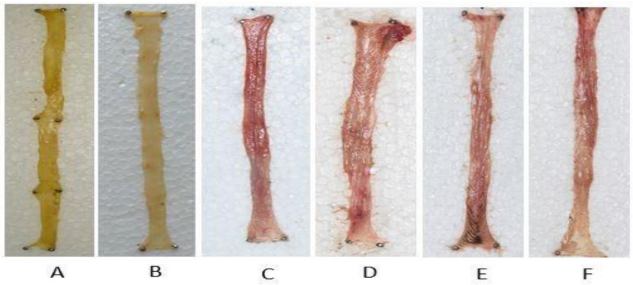


Figure 1. Macroscopic representation of colonic mucosa in acetic acid-induced colitis rats. A. Normal control. B. HAECS alone (400 mg/kg, p.o.). C. Colitis control (2 ml of 4% v/v acetic acid, intra-rectal). D. Acetic acid + HAECS (200 mg/kg, p.o.). E. Acetic acid + HAECS (400 mg/kg, p.o.). F. Acetic acid + Sulfasalazine (100 mg/kg, p.o.).

3.5 Effect of HAECS on Colon Weight / Length Ratio

The rats that received intra-rectal instillation of acetic acid alone showed a significant ($p< 0.001$) increase (137.0 ± 1.15 mg/cm) in colon weight/length ratio, in contrast to the normal control (86.81 ± 3.01 mg/cm). Rats orally pretreated with HAECS at 200 mg/kg, 400 mg/kg, and SLZ at 100 mg/kg exhibited significant ($p<0.01$, $p<0.001$, and $p<0.001$) decreases (121.2 ± 9.74, 102.2 ± 1.83, and 98.51 ± 2.77 mg/cm) in colon weight/length ratio respectively as shown in Table 1.

3.6 Effect of HAECS on Biochemical Studies

There was a significant increase in the levels of LDH (1379±22.08 U/L) in serum ($p<0.001$), in the colonic contents of MDA (141.99±1.6 nmol/g tissue), MPO (6.12±0.39 U/g), and attenuation in the levels of GSH (1.35±0.54 nmol/mg tissue) ($p<0.001$) in the colitis control, in contrast to the normal control. While, pre-treatment with HAECS at (200 mg/kg and 400 mg/kg) showed a significant and dose-dependent decline in MDA (80.16±5.7 and 81.18 ±2.4 nmol/g tissue) ($p < 0.01$), MPO (4.12 ± 0.34 and 3.23 ± 0.51 U/g) ($p < 0.05$), and LDH (909.7 ± 13.06 and 816.8± 86.79 U/ L) ($p < 0.05$) levels while HAECS at a dose of 400 mg/kg, resulted in a significant

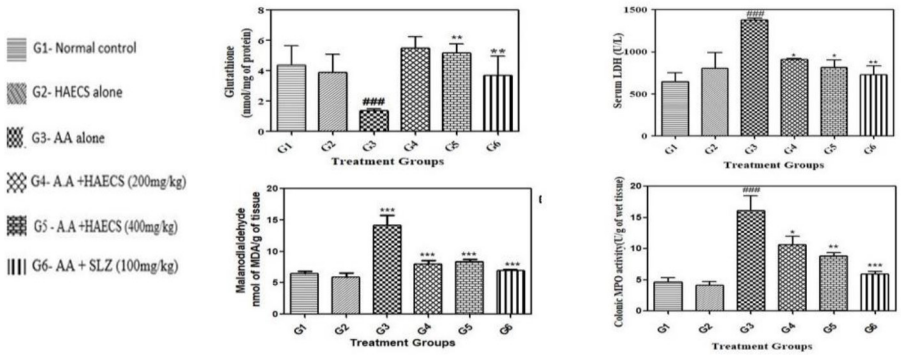


Figure 2. Effect of HAECS on colonic activities of MDA, GSH, MPO, and LDH of rats in acetic acid-induced ulcerative colitis. G1- Normal control, G2- HAECS alone, G3- Colitis control, G4- Acetic acid +HAECS (200 mg/kg, p.o.), G5-Acetic acid +HAECS (400 mg/kg, p.o.) , G6-Acetic acid +Sulfasalazine (100 mg/kg, p.o.).

Data are expressed as mean ± standard error of the mean (SEM); n = 6. One-way ANOVA followed by Tukey's post hoc test. ###$p<0.001$ significantly different from normal control, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ significantly different from Colitis control.

decrease in the levels of MDA (70.91±1.0 nmol/g tissue) ($p<0.01$) and MPO (2.28 ± 0.53 U/g) ($p < 0.01$) in tissues of the colon and LDH (768.7±17.42 U/L) in serum ($p<0.05$), while the levels of GSH were significantly increased ($p<0.01$) in contrast to colitis group. Whereas, SLZ significantly ($p<0.001$) showed marked changes in MPO, MDA, GSH, and LDH parameters as shown in Figure 2.

3.7 Effect of HAECS on Histopathological Examination

H and E staining was used to evaluate changes in the cellular architecture of colonic tissues in rats. The colonic tissue of the normal group showed intact mucosal and submucosal architecture. Acetic acid-induced colitis revealed submucosal edema, neutrophil influx, total loss of goblet cells, necrosis, ulceration

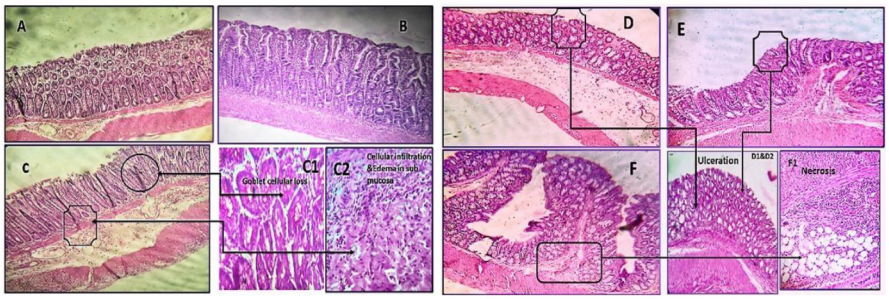


Figure 3. Photomicrograph of the hematoxylin and eosin-stained colonic sections of rats in the different groups. As it is shown, A) Normal control; B) HAECS alone C) Colitis control D) AA + HAECS (200mg/kg) E) AA + HAECS (400 mg/kg) F) AA + SLZ (100 mg/kg).

Table 2. Hematoxylin and eosin (HE)-stained colonic sections were scored as Nil: no abnormality detected; *mild, **moderate, ***/**** Severe damage

Sl. No.	Groups	Ulceration	Hyperemia	Necrosis	Edema	Cellular infiltration
1	Normal control 10 ml/kg	Nil	Nil	Nil	Nil	Nil
2	HAECS alone (400 mg/kg, p.o.)	Nil	*	Nil	Nil	**
3	Colitis control (2 ml of 4% v/v AA, intra rectal)	****	**	****	****	****
4	AA+HAECS (200 mg/kg, p.o.)	*	***	*	***	***
5	AA+HAECS (400 mg/kg, p.o.)	**	*	*	**	**
6	AA+SLZ (400mg/kg, p.o.)	Nil	Nil	Nil	Nil	**

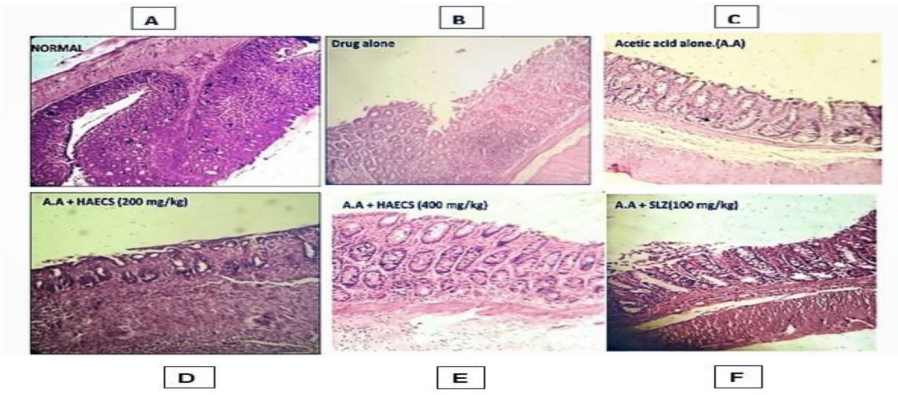


Figure 4. Photomicrographs of sections of colons from rats stained with PAS stain. A-Normal control, B- HAECS alone, C- Colitis control, D- A.A + HAECS (200 mg/kg), E- A.A + HAECS (400mg/kg), F- AA + SLZ (100mg/kg).

in the crypt, and distorted glands. Pretreatment with HAECS at 200mg/kg exhibited lessened damage in the mucosa, mild cellular infiltration, and submucosal edema, pretreatment with HAECS (400 mg/kg) and SLZ displayed minimal damage, showing improved colonic histoarchitecture, as shown in Figure 3 and Table 2.

3.8 Histopathology of the Colon (PAS stain)

PAS stain is specifically used to visualize and evaluate the structural integrity of goblet cells and basement membranes. PAS staining of normal control colon showed regular architecture with normal goblet cells. Whereas, the damaged colonic crypt structure, altered mucosal thickness, and reduced goblet cells were noticed in the colitis control group. Pre-treatment of HAECS showed a marked reduction in goblet cell depletion compared to the colitis control group Figure 4.

4. Discussion

Indigenous products of folklore medicine have been employed in every tradition all over the globe for centuries. In their quest for food, the ancient people recognised their specific properties of relieving or eliminating certain ailments and sustaining good health. Currently, prescribed drugs in modern medicine are obtained from herbs. In addition to their active constituents, these herbs also contain alkaloids, glycosides, vitamins, volatile oils, minerals, etc. In developing nations, relying on plant-derived sources is prevalent, where traditional medicine holds a key role in the health care system[26].

Caesalpinia sappan Linn, belonging to the family *Leguminosae* / *Caesalpiniaceae*, widely known as *C. sappan* (CS) or sappan wood, is dispersed in Southeast Asia. The heartwood is traditionally utilised in beverages/food. In folklore medicine, CS heartwood has been employed in treating vitiated conditions of burning sensation, wounds, and ulcers, and is useful in rheumatism. It has also been used as an analgesic and anti-inflammatory agent in traditional oriental medicine. Chemical constituent investigation of sappan wood resulted in the identification of various structural types of phenolic components, including brazilin, chalcones, flavones, flavonoids and homo isoflavonoids, etc[27].

Induction of UC in rats using AA through intrarectal instillation is a well-recognised experimental model that bears a close likeness to human colitis. Both diseases show common pathophysiological features as well as sensitivity to drug treatment[28]. Acetic acid exerts its damaging effect by an acute inflammatory response following colonic injury, accompanied by widespread hemorrhage, the release of mediators, formation of lesions and biochemical alterations are mainly due to exaggerated generation of reactive nitrogen and oxygen metabolites, the neutrophil influx, and enhanced oxidative stress, which provoke tissue injury in inflammatory ailments, diarrhoea, and weight reduction[3].

In the AA-inflicted UC model, necrosis of epithelial or mucosal tissue, transient inflammation, and edema, which occasionally extends into the lining of mucosa, submucosa or muscularis propria, subject to the concentration and duration of AA exposure, mucosal and sub-mucosal inflammation ensued by epithelial damage, which is generally correlated to the offset of arachidonic acid pathways[29].

The current research outlines the anti-inflammatory and antioxidant potential of HAECS against UC imposed by AA. Colitis induction using intrarectal instillation of 4% AA (2 ml) was confirmed by clinical activity score, macroscopic assessment, colon weight, and severity of diarrhoea in colitis control as compared with the normal control. In the present study, the groups that received 200 and 400 mg/kg of HAECS showed a significant attenuation in the macroscopic score, colon weight/length ratios, diarrhoea, and marked improvement in the colonic.

Increased lactic acid generation due to a shift towards anaerobiosis is indicated by higher serum levels of LDH in ulcerative colitis[30]. In our study, there was a marked increase in the levels of LDH in colitis control in contrast to the normal control. While the groups treated with HAECS showed significantly diminished LDH contents in contrast to the colitis control, the effect was also compared to the standard drug SLZ.

Myeloperoxidase (MPO) is an enzyme located in neutrophils and at much lower levels in monocytes and macrophages. The MPO activity is directly related to the neutrophil level in the damaged tissue, and enhanced MPO activity has been reported as an indicator of inflammation and neutrophil infiltration[31]. There was

a marked elevation in the MPO activities in colitis control as compared to the normal control. However, a significant attenuation in the MPO activity was noticed in rats treated with HAECS at both doses.

MDA is a principle final product of oxidative degradation of lipids. Enhanced contents of MDA might contribute to the augmented formation of free radicals and decrease the activity of the antioxidative shield. In the current research, acetic acid resulted in pronounced augmentation of lipid peroxidation, assessed as MDA level, which is consistent with the earlier literature. Pretreatment with HAECS significantly reduced the MDA levels as compared to colitis control, and this decrease can be ascribed to the HAECS potential in scavenging free radicals[8].

Oxidative stress is one of the key intracellular triggers that GSH defends against, and it also shields organs from oxidative damage[32]. In the colitis control, there was a decrease in the GSH level as compared to the normal control. A significant enhancement of protective GSH level was noticed in the test groups treated with HAECS at both doses when compared to the colitis control. The attenuation of neutrophil infiltration into the colon mucosa by HAECS is further apparent from the histopathological findings. The anti-inflammatory action was affirmed by the reversal of neutrophil infiltration.

Histopathological findings are the bench markers for the assessment of inflammatory colonic injury. The current investigation shows that HAECS action in the colon was substantially enhanced when compared to AA control, thus confirming that HAECS is very effective against AA-induced UC. AA administration to rats shows extensive ulceration in the mucosa, comprising degenerated epithelium, neutrophils, and necrotic cells. The goblet cells are decreased (>90%). The submucosa shows edema with mixed inflammatory infiltration consisting of lymphocytes and some neutrophils[33].

Pretreatment of HAECS (200 mg/kg) makes changes, i.e. scattered neutrophils, lymphocytes, and degenerated epithelial cells with occasional intact lining epithelium, and the goblet cells are decreased (<50%). Whereas HAECS (400 mg/kg) pretreatment shows intact colonic Mucosa with regenerative epithelial cells, scattered lymphocytes, and normal vascular spaces. The goblet cells appear adequate. The submucosa shows congested vascular spaces and scattered chronic inflammatory infiltration.

The preliminary phytochemical analysis of HAECS reveals the presence of phenolic components, glycosides, flavonoids, tannins, saponins, and proteins. The presence of such constituents is found to possess a wide range of pharmacological activities[15]. Due to the reactivity of the phenol moiety and presence of hydroxyl groups in their structure, phenolic compounds can scavenge and are recognised as antioxidants[34]. Therefore, determining the phenolic content of plants may yield more useful information on their capacity as antioxidants. The total phenolic content in the hydroalcoholic extract of *C. sappan* was found to be 49.71 ± 0.49 µg/mL equivalents to gallic acid. Similarly, the total tannin and flavonoid contents were found to be 55.76 ± 0.53 and 27.13 ±0.88 µg/mL equivalents to tannic acid and quercetin, respectively[35].

As reported in many publications, the free radical scavenging activity and reducing power of botanical extracts are well correlated with their total phenolics and total flavonoids. The phytoconstituents of HAECS, such as flavonoids, phenolics, and tannins ascribed to their anti-oxidant activity, which holds a key role in neutralizing free radicals and their actions[27]. Also, the plant under study has been documented for its antimicrobial activity, good inhibitory lipase activity, and good antioxidant activity. From the aforementioned details, the protective activity of HAECS is in part owing to its anti-inflammatory and antioxidative potential. Further study has to be done to unravel the exact mechanism of action[36].

Flavonoids and phenolic constituents are acknowledged as radical scavengers, metal chelators, and anti-oxidants[23] are documented to have significant anti-inflammatory activity[37]. Earlier experimental evaluations reported the benefits of flavonoids in colonic inflammation[38]. The heartwood of the hydroalcoholic extract of *C. sappan* has been recorded to contain substantial quantities of phenolic components and flavonoids, thus well correlating to the potential anti-inflammatory and antioxidative action. The heartwood is a rich source of bioactive flavonoids[37] and phenolics such as brazilin, protosappanin A and B, brazilein, sappan chalcone, Caesalpin, etc[38]. Several pieces of evidence have previously demonstrated the anti-inflammatory potential of *C. sappan* extract and isolated compounds. The anti-inflammatory activity of brazilin was reported due to its capacity to impede

the generation of NO. Brazilin has been suggested for the possible use of *Caesalpinia sappan* in treating inflammation and allied ailments[39]. Brazilin was also reported to suppress the release of IL-1β, TNF-α, NO, and PGE2, and these effects are mediated by heme oxygenase-1[40].

5. Conclusion

The observed beneficial effects of *C. sappan* could be ascribed to the occurrence of bioactive anti-inflammatory homoflavanoids like brazilin, phenolic components and their symbiotic attributes. In general, the results of this study manifest the effect of HAECS to avert acetic acid-inflicted colitis. Moreover, our results provided strong scientific evidence for the folklore and ethno-medical usage of *C. sappan* in the treatment of inflammatory ailments. Further experiments are required to elucidate the molecular mechanism of action and enrichment of exact bioactive compounds of the title plant.

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7. Author Contribution

S. Babitha and V.P. Veerapur contributed to the conceptualisation and study design. A.M. Thimmaraju conducted the experimental work and performed the data analysis. The manuscript was written by S. Babitha, B. Swetha, and M.B. Lavanya. S. Babitha and V.P. Veerapur reviewed and revised the draft. All authors have read and agreed to the published version of the manuscript.

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