

EFFECT OF ETHANOLIC EXTRACT OF LEAVES OF *Vitex negundo* L. ON ACETIC ACID INDUCED COLITIS IN ALBINO RATS.SWARNAMONI DAS^{1*}, LALIT KANODIA²

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ABSTRACT

Objective- As the leaves of *Vitex negundo* L. possess anti-inflammatory and antioxidant properties, this study has been undertaken to evaluate the effect of *Vitex negundo* L. in experimentally induced inflammatory bowel disease (IBD) and to find its probable mechanism of action including its antioxidant potential. **Methods-** The extract of *Vitex negundo* L. was prepared by percolation method. Acute toxicity test was done by using OECD guidelines. Albino rats were divided into four groups (n=5). Group A and B received 3% gum acacia. Group C and D received ethanolic extract of *Vitex negundo* L. (EEVN) 500 mg/kg BW and 5-aminosalicylic acid (5-ASA) 100 mg /kg BW respectively. Colitis was induced by transrectal administration of 4% acetic acid on 5th day. All animals were sacrificed after 48 hour of colitis induction and distal 10 cm of the colon was dissected. Colon was weighed for disease activity index (DAI) and scored macroscopically and microscopically. Biochemical assessment of tissue myeloperoxidase (MPO), catalase (CAT) and superoxide dismutase (SOD) was done in colonic tissue homogenate and malondialdehyde (MDA) was estimated in serum. **Results-** *Vitex negundo* L. showed significant (p<0.05) reduction in DAI, macroscopic and microscopic lesion score as well as significant (p<0.05) improvement in MPO, MDA, CAT, and SOD level as compared to Group B. **Conclusion-** The ethanolic extract of leaves of *Vitex negundo* L. showed significant amelioration of experimentally induced colitis, which may be attributed to its anti-inflammatory and antioxidant property.

Keywords: Colitis, *Vitex negundo* L., antioxidant.

INTRODUCTION

Inflammatory bowel disease (IBD) comprises Crohn's disease (CD) and ulcerative colitis (UC) which are defined as chronic and relapsing inflammations of the gastrointestinal tract caused by variable pathophysiological mechanisms characterized by clinical manifestations including diarrhoea, blood in the stool, abdominal pain, and weight loss [1]. Despite the fact that aetiology of IBD still remains poorly understood, complex interactions among genetic, environmental, immunological and reactive oxygen species (ROS) have been implicated in the pathogenesis of IBD [2,3]. In many studies, it has been reported that antioxidants show beneficial effects on experimental colitis [4, 5].

Vitex negundo L. (Assamese- Pachatiya, Hindi- Nirgund, English-Indian pivet) [6] belongs to the family Lamiaceae. It is an aromatic large shrub. It commonly bears tri- or penta-foliolate leaves on quadrangular branches, which give rise to bluish-purple coloured flowers in branched tomentose cymes [7]. It is almost found throughout India. Phytochemical analysis of plant showed that its leaves contains alkaloid (nishundine), flavonoids like flavones, luteolin-7-glucoside, casticin, iridoid glycosides, an essential oil and other constituent like vitamin C, carotene, gluco-nonital, benzoic acid, β -sitosterol and C-glycoside. Seeds contain hydrocarbons, β -sitosterol, benzoic acid and phthalic acid, diterpenoids, flavonoids, artemetin and triterpenoids. Bark contains fatty acids, valinic acid, p-hydroxybenzoic acid and luteolin [8]. Traditionally it is used as vermifuge, in headache, catarrh, acute rheumatism, expectorant, fever, sinusitis [9], to increase memory, as hypolipidaemic, in bodyache [6]. It has got antioxidant [10], anti-inflammatory and immunomodulatory [11] hypolipidaemic [6], and anticonvulsant activities [8].

As the leaves of *Vitex negundo* L. possess anti-inflammatory and antioxidant properties, this study has been undertaken to evaluate the effect of in experimentally induced IBD and to find its probable mechanism of action including its antioxidant potential.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Ethical Committee (Registration no.-634/02/a/CPCSEA). The whole experiment was conducted according to CPCSEA guidelines. Animals were dissected using ether by inhalation and proper aseptic conditions were maintained during whole procedure. After the experiment, the animals were euthanized by using proper dose of ether and then carcasses were sent to Central Incinerator, Assam Medical College and Hospital.

Collection, Identification and Extraction of Plant Materials

Approximately 1 kg of fresh tender leaves of *Vitex negundo* L. collected during April-May was used for the study. The plant was authenticated by Dr. M. Islam, Professor of Life Science, Dibrugarh University, Assam, India. The plant material was air-dried at room temperature. The dried leaves were grounded to a fine powder and stored in an air tight container.

Preparation of the Extract

Two hundred and fifty grams of the dry powder obtained was soaked in 95% ethanol for 24 hours in a percolator. After 24 hours, it was allowed to percolate slowly and the extract was collected in petri dishes [12]. The extract was concentrated in vacuum using a rotary flash evaporator. There was a net yield of 22.6 g of the concentrated extract (9.12%).

Animals

The experiments were carried out in albino rats of the species *Rattus norvegicus* of either sex, weighing 150–200 g. The animals were acclimatized for 1 week under laboratory conditions. They were fed with standard diet, and water was provided *ad libitum*.

Acute Toxicity Studies

Acute oral toxicity test for the ethanolic extract of leaves of *Vitex negundo* L. was carried out as per Organization for Economic Cooperation and Development (OECD) Guidelines 425 [13]. One

arbitrary dose of 500 mg/kg was selected for the study, as the extract was found safe even at doses more than 2000 mg/kg without any sign of toxicity or mortality.

Experimental Design

Twenty healthy albino rats of the species *R. norvegicus*, weighing 150–200 g, were used in the study and were divided into four groups with five animals in each group (n=5) as follows:

- Group A (normal control) – received 3% gum acacia 10 ml/kg/day p.o.
- Group B (experimental control) – received 3% gum acacia 10 ml/kg/day p.o.
- Group C (test) – received ethanolic extract of *Vitex Negundo* L. (EEVN) 500 mg/ kg/day p.o.
- Group D (standard) – received 5-aminosalicylic acid (5-ASA) 100 mg/kg/day p.o.

Induction of colitis

The experiment was performed using acetic acid for inducing colitis [9]. All the animals were pre-treated with the respective drugs (volume of drugs was kept constant at 10 ml/kg) for 5 days, along with the normal diet. On the fifth day, animals were fasted for 12 hours (overnight) and IBD was induced the next morning in Groups B, C and D by administration of 1 ml of 4% acetic acid solution transrectally (TR). Group A (normal control) received 0.9% normal saline transrectally instead [14].

IBD induction was done using an 8-mm soft paediatric catheter which was advanced 6 cm from the anus under low-dose ether anaesthesia. Rats were in Trendelenburg position during this process and 1 ml of 4% acetic acid or 0.9% normal saline solution was slowly administered TR. The rats were maintained in head-down position for 30 seconds to prevent leakage. After this process, 2 ml of phosphate buffer solution of pH 7 was administered TR [14].

All the animals were sacrificed after 48 hours of IBD induction, by ether overdose. Abdomens were opened and colons were exposed. Distal 10 cm of colon was excised and opened by a longitudinal incision. After washing the mucosa with saline solution, mucosal injury was assessed macroscopically using the scale of Morris et al [15]. – no damage (score 0); localized hyperaemia but no ulceration (score 1); linear ulcer without significant inflammation (score 2); linear ulcer with significant inflammation at one site (score 3); two or more sites of ulceration and inflammation (score 4) and two or more sites of ulceration and inflammation or one major site of inflammation and ulcer extending >1 cm along the length of colon (score 5). Disease activity index (DAI) was also measured, and the ratio of colon weight to body weight, which was used as a parameter to assess the degree of tissue oedema and reflects the severity of colonic inflammation, was measured [16].

A 6–8 mm sample block of the inflamed colonic tissue with full thickness was excised from a region of grossly visible damage for histological analysis. Formalin-fixed tissue samples were embedded in paraffin and stained with haematoxylin–eosin (HE). Colonic tissues were scored for histological damage using the criteria of Wallace and Keenan [17]. 0=intact tissue with no apparent damage; 1=damage limited to surface epithelium; 2=focal ulceration limited to mucosa; 3=focal, transmural inflammation and ulceration; 4=extensive transmural ulceration and inflammation bordered by normal mucosa; 5=extensive transmural ulceration and inflammation involving entire section [17].

Biochemical Assessments

Preparation of the sample

The proximal 5 cm of the dissected colon specimen was used for biochemical analysis [14] of myeloperoxidase (MPO), tissue catalase

(CAT) and superoxide dismutase (SOD). The colonic samples were minced and homogenized using a polytron homogenizer. The supernatant was obtained by centrifuging at 3000 rpm for 20 minutes.

Myeloperoxidase activity

The minced colonic samples were homogenized in 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB). The homogenates were then sonicated and centrifuged for 20 minutes at 12,000 rpm. MPO activity was measured spectrophotometrically as follows. Exactly 0.1 ml of supernatant was combined with 2.9 ml of 50 mM phosphate buffer containing 0.0005% H₂O₂. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute at room temperature, in the final reaction.

MPO activity (U/g) = X/weight of the piece of tissue taken, where X=10×change in absorbance per minute/volume of supernatant taken in the final reaction [18].

Malondialdehyde (MDA) level

MDA was estimated by Satoh K method [19]. 75mg of Thiobarbituric acid (TBA) was dissolved in 15% TCA, to this 2.08ml of 0.2N HCL was added, the volume was made up to 100 ml using 15% TCA. 3.0 ml of this reagent was added to 0.75 ml of serum of the rats. The test tubes were kept in a boiling water bath for 15 minutes. They were cooled and centrifuged for 10 minutes at 10000 rpm. Absorbance of the supernatant was read against the blank at 535nm. The results were expressed in nmol/ml of serum.

Assessment of antioxidant status in colonic tissue

CAT was measured by the method of Beers and Sizer [20]. Phosphate buffer (2.5 ml, pH 7.8) was added to the supernatant and incubated at 25°C for 30 minutes. After transferring into the cuvette, the absorbance was measured at 240 nm spectrophotometrically. Hydrogen peroxide (650 µl) was added and change in absorbance was measured for 3 minutes. Values were expressed as µmol/min/mg of proteins.

SOD was assayed according to the method of Kakkar *et al.* [21]. The colonic samples were ground with 3.0ml of potassium phosphate buffer, centrifuged at 2000 rpm for 10 minutes and the supernatants were used for the assay. The reaction was initiated by the addition of 0.2ml of NADH to the mixture and incubated at 30°C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

Statistical Analysis

For all the above methods, the results were expressed as mean ± SEM. Statistical analysis was done using one-way analysis of variance (ANOVA), followed by Dunnett's. P<0.05 was considered significant.

RESULTS

Acute Toxicity Studies- There was no mortality among the animals. So the LD₅₀ was calculated more than 2000 mg/kg body weight.

As observed from this study, acetic acid administration to the experimental control group caused significant macroscopic ulcerations and inflammations (P<0.05) in rat colon along with significant mucosal injury microscopically (P<0.05), when compared to the normal control group [Table 1, Figures 1 and 2].

Table1: Effect of *Vitex negundo* L. leaves on induced colitis.

Groups	Macroscopic score	DAI score	Microscopic
A (Normal)	0 ± 0	0.53 ± 0.01	0 ± 0
B (Experimental control)	4.814 ± 0.08*	1.66 ± 0.01*	4.4 ± 0.04
C (Test drug)	2.72 ± 0.83**	1.16 ± 0.04**	3.5 ± 0.03**
D (Standard)	1.41 ± 0.05**	0.70 ± 0.01**	1.4 ± 0.03**

Values expressed as mean ± SEM (n=5). *P<0.05 when compared to normal control; **P<0.05 when compared to experimental control; ANOVA followed by Dunnet's test; DAI – Disease activity index.

Also, there was significant derangement of biochemical parameters including tissue levels of MPO, CAT and SOD (P<0.05) and serum

MDA (P<0.05) indicating oxidative stress due to colon damage and colonic inflammation [Table -2]

Table: 2Effect of *Vitex negundo* L. on acetic acid induced colitis.

Groups	Tissue MPO (U/g)	Serum MDA (nmol/ml)	Tissue CAT (µmol/min/mg)	Tissue SOD (U/mg of proteins)
A (Normal control)	0.35±0.01	3.53±0.13	396.2±0.86	7.3±0.08
B (Experimental Control)	4.1±0.04*	5.91±0.04*	143.8±0.37*	2.96±0.13*
C (Test drug)	2.05±0.04**	2.26±0.07**	293.4±1.43**	5.2±1.13**
D (Standard)	0.86±0.05**	3.03±0.20**	327 ±0.38**	5.12±0.03**

Values expressed as mean ± SEM (n=5). *P<0.05 when compared to normal control; **P<0.05 when compared to experimental control. ANOVA followed by Dunnet's test.

Ethanol extract of *Vitex Negundo* L. leaves has shown significant activity against experimentally induced IBD when compared to that of the experimental control (P<0.05) animals, with an improved picture of colon architecture both macroscopically as well as microscopically [Table 1, Figures 2,3]. There is reduction of oxidative stress with significant improvement in tissue levels of CAT, SOD (P<0.05), showing its antioxidant potential [Table-2]. There is also significant improvement in the levels of MPO and MDA showing its potential anti-inflammatory activity (P<0.05) [Table 2]. The 5-ASA showed near normalisation of DAI and macroscopic and microscopic score as compared to normal control [Table 1, Figure 4].



Figure 1 Group A (Normal control)-Normal mucosal architecture of colon (Hematoxylin & Eosin Sections).

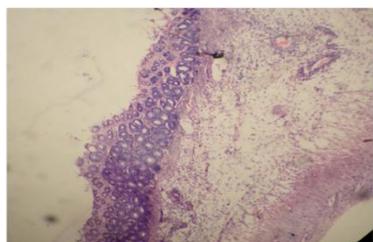


Figure 2: Group B (Experimental control)-Extensive necrosis with transmural infiltration in Hematoxylin & Eosin colonic Sections.

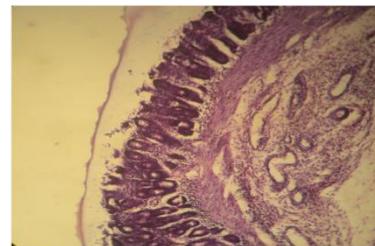


Figure3: Group C (Test group) - Focal ulceration limited to mucosa in Hematoxylin & Eosin sections of colon.

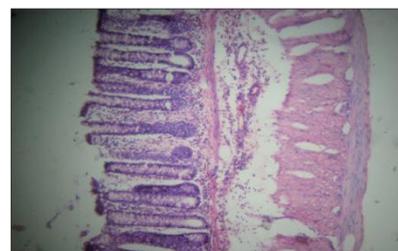


Figure 4: Group D (Standard) - Near normalisation of architecture of colonic section with mucosal infiltration only.

DISCUSSION

Acetic acid induced colitis model is similar to human ulcerative colitis in terms of histological features. It affects the distal colon portion and induces non-transmural inflammation, massive necrosis of mucosal and submucosal layers, mucosal oedema, neutrophil infiltration of the mucosa and submucosal ulceration. The protonated form of the acid liberates protons within the intracellular space and causes massive intracellular acidification resulting in massive epithelial damage. Inflammation is the pathogenesis of IBD, and several pathways are associated with inflammatory response in IBD due to mucosal intestinal flora [22]. The inflammatory response initiated by acetic acid includes activation of Cyclooxygenase and lipoxygenase pathways [23,24].

The results showed that ethanol extract of fruit extract of *Vitex negundo* L. has got a significant protective activity against experimental colitis in rats, as indicated by DAI, macroscopic, microscopic and biochemical evaluations.

Myeloperoxidase (MPO) is an enzyme mainly found in azurophilic granules of neutrophils. It can serve as a good marker of inflammation, tissue injury and neutrophil infiltration in gastrointestinal tissues. Pre-treatment with *Vitex negundo* L. exhibits decrease in polymorphonuclear infiltration demonstrated by significant reduction in MPO activity [1].

In the present study, there is decrease activity of catalase (CAT) and superoxide dismutase (SOD) with the concomitant increase in malondialdehyde (MDA) concentration in the homogenized colonic tissue samples and serum MDA after acetic acid administration.

Present study shows that treatment with *Vitex negundo* L. inhibits this decrease of CAT and SOD level and increase of MPO and MDA in rat treated with acetic acid which may be because of protection against the progression of the disease. Oxidative stress is believed to play a key role in the pathogenesis of IBD-related intestinal damage [25]. As a matter of fact, intestinal mucosal damage in the IBD, including Crohn's disease and ulcerative colitis, is related to both increased free radical production and a low concentration of endogenous antioxidant defense [26].

The leaves of *Vitex negundo* L. are rich in flavonoids and triterpenoids. Flavonoids are phenolic substances which have antioxidant property [27]. Further, previous studies have reported the protective action of flavonoids against oxidative stress induced cellular damage. Flavonoids can exert their antioxidant activity by various mechanisms, e.g., by scavenging or quenching free radicals, by chelating metal ions, or by inhibiting enzymatic systems responsible for free radical generation [28].

Triterpenoids (lupeol), present in *Vitex negundo* L. have the ability to protect cells and tissues from oxidative stress by increasing the transcriptional activity of NRF2, which induces the formation of cytoprotective enzymes like catalase, superoxide dismutase, etc [29].

CONCLUSION

As proved by the above study and as also described in literature, leaf extract of *Vitex negundo* L. possesses significant antioxidant property, proving its role in the management of experimentally induced IBD.

Conflict of interest – Nil

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