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Protective Effect By Herbal On Experimental Models For Inflammatory Bowel Disease

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Bark extract of H. indicus root bark Linn. is screened for inflammatory bowel disease. Aqueous and methanolic extract of H. indicus root bark Linn have been shown to progressively reduced rat paw edema induced by sub plantar injection of albumin, which suggest that this extract might have antiinflammatory principals. The bark extract of H. indicus root bark Linn. has antioxidant and antiproliferative effects on human cancer cells. In a study H. indicus root barkLinn showed significant in vivo analgesic and antiinflammatory activities. The models used for study of IBD are acetic acid induced ulcerative colitis and indomethacin induced enterocolitis. Intrarectal instillation of acetic acid in rats affected only the distal colon portion. The inflammation was not transmural. Massive necrosis of mucosal and submucosal layers was observed. This model shares many of the histologic features of ulcerative colitis in human beings including mucosal edema, neutrophil infiltration of the mucosa and submucosal ulceration. Bark extract of H. indicus root bark Linn. has potent protection against inflammatory bowel activity in both indomethacin induced enterocolitis and acetic acid induced ulcerative colitis. Collectively, these findings indicate that the anti-oxidant effect of fraction may be an important contributor to its anti-inflammatory activity in IBD. This present investigation has also opened avenues for treatment of IBD from the title plant.

Keywords: Inflammatory bowel disease (IBD), gastrointestinal tract (GI), Hemidesmus indicus, . Crohn's disease, COX Inhibition.

INTRODUCTION

The medicinal use of natural herbs and spices is deeply rooted in the history and folklore of human beings, and many herbs and spices have been incorporated into the traditional medicine of virtually all human cultures[1]. Throughout history, people have used a variety of herbs and plants to prevent or treat diseases. In a study done by Barrett and colleagues, it was found that as many as 50% of Americans use herbal remedies in a given year [2], indicating that the use of herbal medicine is both widespread and growing dramatically. It is the gentle, nourishing, and synergistic actions of herbal remedies that make them an excellent treatment choice [3]. Whether a treatment is approached using natural herbs or chemical drugs, whose synthesis is based on properties and actions of those herbs, medicinal herbs have played a major role in

the development of modern, conventional medicine, and they will remain, like a historical treasure, as a source of therapy.

Inflammatory bowel disease (IBD) is inflammation within the gastrointestinal (GI) tract characterized by chronic or relapsing immune system activation. There are two types of IBD: ulcerative colitis and Crohn's disease. Inflammatory bowel disease is characterized by chronic recurrent ulceration of the bowel and of unknown etiology[4]. The pathogenesis likely involves genetic, environmental, and immunologic factors. Crohn's disease is a chronic autoimmune inflammatory bowel disease affecting digestion. In this disease inflammations in the small intestine occur. It usually occurs in the lower part of the small intestine, called the ileum, but it can affect any part of the digestive tract, from the mouth to the anus. Ulcerative colitis is an idiopathic, chronic infl ammatory disorder of the colonic mucosa, which starts in the rectum and generally extends proximally in a continuous manner through part of, or the entire, colon; however, some patients with proctitis or left-sided colitis might have a caecal patch of inflammation. Bloody diarrhoea is the characteristic symptom of the disease[5].

Hemidesmus indicus R. Br (Asclepiadaceae) commonly known as Anantmul. It is a common herb found throughout India. It is widely used in traditional Indian medicine and has been extensively studied for its pharmacological effects [6].
It is a perennial slender lactiferous, prostrate, or semierect climber widely distributed throughout India, from upper Gangetic plains east-wards to Assam, throughout Central, Western and Southern India. The traditional name is Sariva, Ananta, Anantamula but it is commonly known as ‘‘Indian Sarsaparilla’’. The drug consists of root with a characteristic pleasant smell of vanilla and acrid taste [7].
In Ayurvedic tradition, this plant is considered effectual as an antiarthritic and antirheumatic remedy. Methanolic extract of root was found to be antihepatotoxic, in Ayurveda the root of H. indicus is considered to be demulcent, diaphoretic, diuretic and tonic. It is useful in synphilis, leucoderma, hemicrania, rheumatism and also in liver and kidney disorders [8].

MATERIAL AND METHOD

Collection and air drying of the root of H. indicus plant was taken from the Satpura region of Madhya Pradesh, India. It was verified by the botanologist safia college bhopal, under voucher specimen number of 147 Herbarium. A sample specimen of collected material was stored in the herbarium for future reference. The H. indicus root parts were dried under shade at 30 0C for 20 days.

Phytochemical profiling

Determination of the total phenol content (TPC)

Phenolic content in the different extracts of H. indicus was determined by adopting the method described by Singleton and Rossi. 23 A mixture of 10% Folin–Ciocalteau's reagent (2.5 mL) and 2% sodium carbonate solution (2 mL) was added to different extracts (0.5 mL) of H. indicus (1 mg/mL). After incubation for 15 min at 45 °C, absorbance was measured at 765 nm.

Based on the calibration curve prepared using gallic acid as standard, total phenol content was calculated[10].

Determination of total flavonoid contents (TFC)

Aluminium chloride ($AlCl_3$) colorimetric method was used in order to determine total flavonoid content. Different extracts of H. indicus (1mL) was added to the mixture of methanol (3 mL), 10% $AlCl_3$ (0.2 mL), potassium acetate (1 M) (0.2 mL), and distilled water (5.6 mL). Absorbance was read at 420 nm after 30 min. Based on a standard curve prepared using rutin as standard, total flavonoid content was measured[11].

Determination of total tannin contents (TTC)

By using the method developed by Sun (1998), total tannin content was measured where a mixture of extract (1 mL) and acidic methanol (3 mL) was allowed to stand for 10 min at room temperature followed by addition of Vanillin HCl reagent (6 mL). Absorbance was measured at 500 nm. Quantification of total tannin was done on the basis of standard curve of catechin as standard.25

Determination of total terpenoid contents (TTRC)

Total terpenoid content in the different extracts of H. indicus was quantified based on the method described by Narayan Ghorai et al.26 To the assay mixture of extract (160 µL) and chloroform (1.2 mL), conc. H_2SO_4 was slowly added and incubated for 2 h at room temperature. The supernatant of reaction mixture was decanted and 95% methanol (1.5 mL) was added to dissolve the resultant reddish brown precipitate and absorbance was recorded at 538 nm. Total terpenoid content was determined with the aid of standard curve of linalool as standard[12].

Determination of total saponin contents (TSC)

For estimation of total saponin content in the extract, vanillinsulphuric acid assay was incorporated. Extracts (0.25mL) were mixed with 8% (w/v) vanillin in ethanol (0.25 mL) and 72% (v/v) sulphuric acid in water (2.5 mL) and incubated at 60 °C in water bath for 15 min. Absorbance was then measured at 560 nm after cooling at room temperature for 5 min.27

Antioxidant activity Nitric oxide (NO)

scavenging activity To sodium nitroprusside solution (1mL of 25 mM), 4 mL of different concentrations (6.25-100 µg/mL) of extracts was added and incubated for 3 h at 37° C. Griess reagent (0.3 mL) was added to the solution (0.5mL) and the absorbance of the chromophore formed was measured at 570 nm. Control was prepared in a similar way, but without the extract. Butylated hydroxytoluene (BHT) was used as a reference standard.28 1, 2-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity Effect of H. indicus extracts on DPPH radical has been carried out by employing the method described earlier by Mary et al.29 2mL of various concentrations (2 mL) of extracts (6.25-100 µg/mL) was mixed with DPPH (0.5 mL of 0.01 M) in methanol. Absorbance of the solution was analysed at 517 nm. Ascorbic acid was used as reference standard.

Neuro-protective potential Evaluation

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) inhibition assay Both AChE and BuChE inhibitory ability of the extracts was evaluated by a modified colorimetric method of Ellman et al. Som, et al.: Phytochemical Profiling of Hemidesmus indicus (L.) R. Br. ex Schult and its Antioxidant, Anti-Inflammatory and Neuroprotection Linked Enzyme Inhibitory Properties mixture consisting of 5,5-dithiobis-2-nitrobenzoate (125 µL of 3mM), acetylthiocholine iodide (25 µLof 15mM), different concentrations (6.25-100 µg/mL) of

extracts and phosphate buffer pH 8.0 (50 µL of 0.1 M) was incubated at 25 °C. After 20 min, AChE (25 µL of 0.22 U/ mL) or BuChE (25 µL of 0.22 U/mL) was added to initiate the reaction and the activity was determined by measuring absorbance at 412 nm[13].

Screning for Inflammatory Bowel Disease:

Indomethacin-induced Enterocolitis in Rats: The male Wistar albino rats (200 - 250 gm) were selected and randomized into five groups of six animals in each group. Group 1 served as Normal or untreated animals, group 2 was positive control (induced) animals receive only indomethacin (7.5 mg/kg) s.c. group 3 was treated group with lower dose received indomethacin (7.5 mg/kg) s.c along with TGE, group 4 was treated group with higher dose received indomethacin (7.5 mg/kg) s.c along with TGE and group 5 standard group received indomethacin (7.5 mg/kg) s.c along with prednisolone (2 mg/kg p.o). Animals pretreated with Tectona grandis Linn. bark extract for 7 days will be administered Indomethacin (7.5 mg/kg, s.c.) on 8th and 9th day of treatment. Extract will be administered till 11th day. On the 11th day the animals will be sacrificed by cervical dislocation and dissected[14]. Ileum and colon will be taken out to assess inflammation, based on physical parameters, macroscopy and microscopic features. Quantification of inflammation would be done using biochemical assay (MPO, lipid peroxides, GSH).

Acetic Acid-induced Colitis in Rats: The male Wistar albino rats (200 - 250 gm) were selected and randomized into five groups of six animals in each group. Group 1 served as normal or untreated animals, group 2 was positive control (induced) animals receive 2 ml of 4% (v/v) acetic acid. group 3 was treated group with lower dose received 2 ml of 4% (v/v) acetic acid along with TGE, group 4 was treated group with higher dose received 2ml of 4% (v/v) acetic acid along with Extract and group5 standard group received 2 ml of 4% (v/v) acetic acid along with Prednisolone (2 mg/kg p.o). Animals will be treated with Extract Linn. bark extract for 7 days. On the 8th day, overnight fasted animals will be anaesthetized using pentobarbitone sodium and 2 ml of 4% acetic acid solution will be instilled into rectum. After 48 h animals will be sacrificed by cervical dislocation and dissected to remove colon. Waste material will be removed from colon and it will be flushed with saline gently[15]. Inflammation will be assessed based on physical parameters, macroscopy and microscopic features. Quantification of inflammation would be done using biochemical assay (MPO and lipid peroxides)

Evaluation Based on Macroscopic Characters:

Scoring for Rat Colon: 13 for each animal, the distal 10 cm portion of the colon was removed and cut longitudinally, and slightly cleaned in physiological saline to remove faecal residues. Pieces of rat ileum and colon (10 cm long each) were scored for macroscopic[16].

Percentage Area Affected of Rat Colon: 13 Rat colon (5 cm long) was scored for macroscopic features. Score for an individual rat was calculated as the combined score of ileum, colon, and caecu.

Evaluation Based on Microscopic (Histologic) Characters: The colon from each animal was removed after sacrificing the animal and was collected and preserved in 10% formalin solution. The samples were submitted to Jeevan Regional diagnostic health care and research centre Pvt. Ltd., (Belgaum, India) for histological examination[17].

Myeloperoxidase Assay for Quantification of Inflammation: Pieces of inflamed tissues (colon-4 cm) were taken. The tissue was then rinsed with ice-cold saline, blotted dry, weighed and excised. Minced tissue was homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4), using Remi tissue homogenizer (RQ-127A). The homogenate was centrifuged at 3500 rpm for 30 min at 4 ºC (Remi centrifuge C23). The supernatant was discarded. 10 ml of ice- cold 50 mM potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB) and l0 mM EDTA was then added to the pellet. It was then subjected to one cycle of freezing and thawing and brief period (l5 s) of sonication.
After sonication solution was centrifuged at 15,000 rpm for 20 min. (Remi centrifuge, R24). Myelo-peroxidase (MPO) activity was measured spectro-photometrically as follows. 0.1 ml of supernatant was combined with 2.9 ml of 50 mM phosphate buffer containing 0.167 mg/ml O-dianisidine hydrochloride and 0.0005% H2O2. The change in absorbance was measured spectrophotometrically (Shimadzu UV 1800), at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute by 1.0 at room temperature, in the final reaction[18].

Calculation of MPO Activity:
MPO activity (U/g) = X / (Wt of piece of tissue taken)
Where X = (10x Changes in absorbance per minute)/(Volume of supernatant taken in the final reaction)
Measurement of Colonic Lipid Peroxides Concentration: Lipid peroxidation, an indicator of mucosal injury induced by reactive oxygen species was measured as thio barbituric acid reactive substance. The amount of colonic lipid peroxides was measured by the thio barbituric acid assay (TBA). Briefly, 0.5 ml of colonic tissue homogenates prepared were reacted with 2 ml of TBA reagent containing 0.375% TBA, 15% trichloroacetic acid and 0.25 N HCl. Samples were boiled for 15 min, cooled and centrifuged. Absorbance of the supernatants was spectro-photometrically measured at 532 nm. TBARS concentrations were calculated by the use of 1, 3, 3, 3 tetra-ethoxypropane as a standard. The results were expressed as µmol/g wet tissue weight[19,20].

COX Inhibition Assay: The assay was performed by using Colorimetric COX (human ovine) inhibitor Screening assay kit. A reaction mixture contains, 150 µl of assay buffer, 10 µl of heme, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of TGE (1 mg/ml). The percent COX inhibition was calculated by colorimetrically by monitoring the appearance of oxidized N, N, N, N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm[21]. Aspirin was used as a standard drug. The percent COX inhibition was calculated using following equation:
% Inhibition of COX = 1− (T/C) × 100
Where T = Absorbance of the inhibitor well at 590 nm. C = Absorbance of the 100% initial activity without inhibitor well at 590 nm.

Screening of Prostaglandins Inhibition: Screening of Prostaglandins inhibition was determined by inhibition of Castor oil induced diarrhea in rats. In the present study animals were divided into three groups of six rats each. Group 1 was administered vehicle orally and served as control. Group 2 served as standard and received aspirin (150 mg/kg), orally.
Group 3 was administered with TGE 300 mg/kg by orally. Before administration of test and standard drugs animals were fasted overnight but allowed free access to water. After 30 min

of administration of above dose all the rats were given with 1 ml of castor oil orally. The numbers of wet fecal dropping were measured for four hours[22].

Statistical Analysis: All data was expressed as mean ± standard error of the mean (S.E.M.) of 6 rats per experimental group[23]. Statistical analysis was performed using Graph pad prism 5.0 statistical software. Parametric one way analysis of variance (ANOVA) followed by Tukey's post test. The minimal level of significance was identified at $p < 0$.

RESULT AND DISCUSSION

Preliminary phytochemical analysis, TLC finger printing of methanolic extract of root bark of H. indicus

The methanolic extract was subjected to preliminary phytochemical testing for the detection of major chemical groups (Table 1). The details of the tests are as follows[24]:

Table 1: Preliminary phytochemical screening of methanolic extract of H. indicus root bark.

S.No.	Taste	Presence/Absence	Test Perform
1	Phenols	+++	Phosphomolbdic acid test
2	Tannins	+++	Braemaers Test
3	Steroids	++	Libermann Burchardt Test
4	Terpenoids	+	Burchardt test
5	Alkaloids	-	Dragendrorff's Test
6	Anthraquinones	-	Borntrager test
7	Flavonoids	++	Shinodas test

– absent; + Traces; +++ Abundant

Ash value

The proximate parameters of a plant material indicate the nature of the plant constituents. The total ash value of a plant material indicates the amount of minerals and earthy materials in the plant material. The amount of acid insoluble ash indicates the amount of silacious matter in the plant material. The alcohol soluble extractive value indicates the presence of constituents such as flavonoids, alkaloids, steroids and their glycosides; Water soluble extractive value indicates the presence of sugars, acids and inorganic components of a plant material. The proximate parameters as reported[25] for the tuberous roots of H. indicus indicate that the roots possess a higher percentage of water soluble constituents (18.6-18.8% w/w). Being a root material, the acid insoluble ash (15.5-18.8% w/w) was found to be higher.

Extractive value

The extractive values obtained by extraction of the roots with various solvents indicate a higher extractive value with of water.

Table 2: Extraction Yield of Different extracts of H. Indicus Roots.

Extract	% Extractive Value	
	Soxhlet Extraction	**Cold Extraction**
Hexane	4.2	0.2
Petroleum ether	3.25	-
Ether	0.46	-
Benzene	0.4	-
Choloroform	0.42	2.6
Ethanol (90%)	6.25	7.1
Methanol (90%)	9.12	-
Water	6.4	4.2

Elemental analysis

Trace elements present in the methanol extract of roots of H. indicus was analyzed by Atomic Absorption Spectroscopy as reported. The percentage of elements present in the hexane and hydro ethanol extract (90:10 ethanol -water mixture) of roots of H. indicus was analyzed by energy dispersive X-Ray analysis in our laboratory (Instrument make: MIRA3 TESCAN). The nonpolar hexane extract is found to contain almost equal elemental percentage of carbon and oxygen in addition to the elements Mg, Fe, and Ca. This is indicative of the presence of oxygenated low polar molecules in the roots to a greater extent. The respective energy dispersive X-Ray Spectra depict this[26].

Table 3: Percentage of Elements Detected in Root Extracts.

Elements	Xexane Extract (Weights %)	Hydroethanol Extract (Weights %)	Methanol Extract (Weights %)
C	445.36	81.28	-
O	43.56	16.72	-
Mg	1.07	-	-
Si	1.58	0.36	-
Cu	2.56	-	0.85
K	2.36	0.0	-
Ca	0.42	-	-
Fe	2.36	-	10.09
Al	-	0.65	-
Zn	-	-	2.76
Mn	-	-	1.15

Free radical scavenging activity

• Assay for antiradical activity: Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of coloured DPPH brought about by the sample. A stock solution of DPPH (1.3 mg/ml methanol) was prepared such that 75 µl of it in 3 ml methanol gave an initial absorbance of 0.9. This stock solution was used to measure the antiradical activity[27]. Decrease in the absorbance in the presence of methanolic extract of H. indicus

root bark at different concentrations was noted after 15 min. EC50 was calculated from % inhibition. Pyrogallol was used as positive control. Suitably diluted stock solution of methanolic extract was spotted on TLC plates and they were developed in different solvent systems for resolving compounds of different polarities as described above. Then they were sprayed with 0.2% DPPH in methanol. Bleaching of DPPH by the resolved bands was observed for 10 minutes and the details recorded[28].

• Assay for superoxide radical scavenging activity:

The assay was based on the capacity of the methanolic extract of H. indicus root bark to inhibit formazon for mation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. The reaction mixture contains 50 mM phosphate buffer pH 7.6, 20 µg riboflavin, 12 mM EDTA, NBT 0.1 mg/3ml, added in that sequence. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. Ascorbic acid was used as positive control[29].

Table 4: TLC figure printing profile of methonolic extract of H. indicus root bark

Scanned at	**Solvent system 1**			**Solvent system 2**			**Solvent system 2**		
	Rf	Lamda max	Relative %	Rf	Lamda max	Relative %	Rf	Lamda max	Relative %
254 nm	0.11	212	13.12	0.15	212	9.01*	0.05	229	3.75*
	0.20	254	2.58	0.25	282	9.02*	0.40	282	28.62/
	0.30	242	15.63	0.42	211	52.28*	0.64	284	29.39*
	0.54	233	3.08	0.55	266	5.72	0.95	280	38.25
	0.62	295	17.73	0.72	300	6.90			
	0.69	293	8.52	0.96	285	11.04			
	0.77	284	3.52						
	0.93	282	35.25						
366 nm**	0.32	317	2.52	57.25	244	9.52			
	0.50	315	57.25	2.74	364	5.72			
	0.64	295	20.95	9.42	265	14.52			

	0.77	288	9.40	2.43	399	22.23			
	0.83	303	2.43	9.92	315	48.56			

*bands which showed positive for Phenolic compounds with methanolic ferric chloride;
**all band are fluorescent solvent system 1. Toluene/ethyl acetate (1:1v/v): Solvent system 2. N-butanol/glacial acetic acid/water (4:1:2v/v) Solvent system 3.n-butonal/glacial acetic acid/water/methanol/ethyl acetate (5:1:4:2:4v/v)

• Assay for nitric oxide scavenging activity:

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of methanolic extract of root bark of H. indicus dissolved in methanol and incubated at room temperature for 150 min. The same reaction mixture without the methanolic extract of sample but with equivalent amount of methanol served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm. Curcumin was used as positive control[30].

Measurement of effect on lipid peroxidation in rat liver homogenate

Rat liver homogenate (10% w/v) was prepared according to the procedure described by Tripathi et al. (1996). Peroxidation was induced in liver tissue by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted 0.5 ml of liver homogenate (10% w/v), 100 µM FeCl3, 1.7 µM ADP, 500 µM of ascorbate and different concentrations of extract in 2 ml of total incubation medium. The medium was incubated for 20 min at 37 °C. Extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) content.

• Assay for phenylhydrazine induced haemolysis of erythrocytes (membrane stabilization study):

The incubation mixture comprises of 1 ml of phenylhydrazine hydrochloride (0.5 mM), different concentrations of sample extract and 0.1 ml of 20% erythrocyte suspension made to a total volume of 3 ml with phosphate buffered saline (PBS) solution. The mixture was incubated at 37 °C for 1 hour and centrifuged at 1000 g for 10 min. The extent of haemolysis was measured by recording the absorbance of the supernatant at 540 nm. Suitable controls were kept to nullify the effect of solvents and inherent haemolysis. α-tocopherol acetate was used as a positive control for the inhibition of phenylhydrazine induced haemolysis of erythrocytes[31].

Neuroprotective potential Evaluation:

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) inhibition assays Based on our exploratory study, we can convey that, methanol extract of H. indicus demonstrated the highest AChE and BuChE inhibitory activity with IC50 of (17.46±0.49 µg/mL) and (31.05±0.39 µg/mL) respectively. Likewise, aqueous extracts too showed equally satisfactory results against AChE (IC50 =21.19±0.4 µg/mL) and BuChE (IC50 =39.23±0.11 µg/mL),

while, pet ether extract displayed marginal inhibition potential (Table 4). Interestingly, a significant correlation was observed between AChE (R=-0.888) and BuChE (R=-0.915) inhibitory activities and concentration of TTRC (Table 7).

Table 7: Correlation Coefficient between phytochemical compounds and antioxidant, anti-inflammatory, cholinesterase and MAO-B inhibitory activities of H. indics roots

Assays	**TPC**		**TTC**		**TFC**	
	r	**p**	**r**	**p**	**r**	**p**
NO	-0.975	0.032*	-0.969	0.289	-0.979	0.042
DPPH	-0.972	0.676	-0.998	0.0323*	-0.987	0.344
Anti-inflammatory	-0.973	0.042*	-0.998	0.038*	-0.986	0.29*
AChE	-0.972	0.666	-0.888	0.0027**	-0.839	0.989
BuChE	-0.925	0.568	-0.915	0.0442*	-0.890	0.879
MAO-B	-0.882	0.035*	-0.796	0.698	-0.850	0.49*

** indivates P 0.01 and P 0.01. TPC-Total Phenolic Content, TTC-Total Terpenoid content, TFC- Total Flavonoid Content,

Antioxidant activity

Plant extract shows inhibition of Fe^{3+} ADP induced lipid peroxidation and ascorbate system in liver. It appears that phenylhydrazine can generate various reactive species, including superoxide radical, hydrogen peroxide, hydroxyl radical, and phenyl radical, under aerobic conditions. These reactive species can initiate the peroxidation of unsaturated fatty acids in endogenous membrane phospholipids, potentially affecting red cell membrane structure and function. This is significant because erythrocyte hemolysis in this modelis caused by the generation of different free radicals[32]. One of the most accepted methods of antioxidant activity is DPPH radical scavenging activity assay.

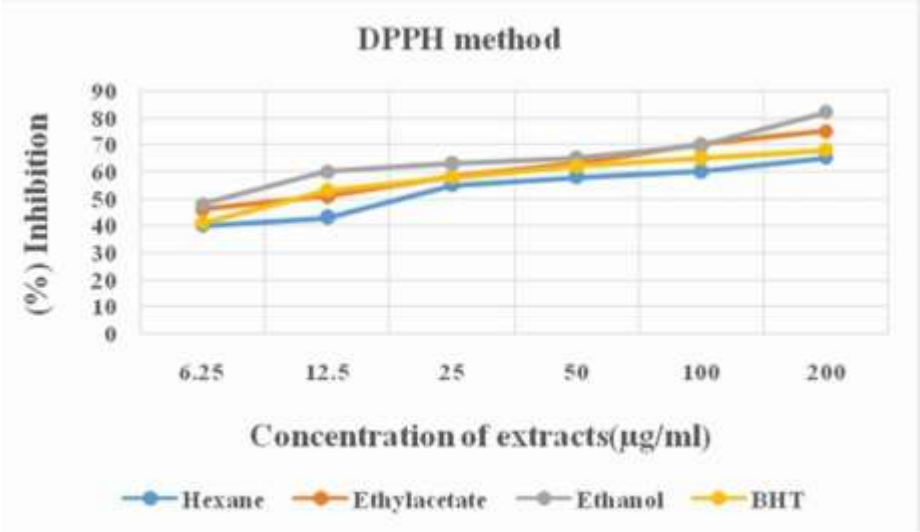

Fig. 3: DPPH method of Antioxidant property of Hemidesmus indicus root extracts [11].

Apoptosis activity

H. indicus was found to contain 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehydeand 2-hydroxy-4-methoxy benzoic acid which induces apoptosis.

Antiresorptive activity

Hemidesmus indicus appears to be a promising candidate due to its antiosteoclastic activity, which is like that of bisphosphonates but without toxic effects. Its high effectiveness at low concentrations suggests its potential as a therapeutic agent. This makes it worth exploring Hemidesmus indicus for its therapeutic potential, either as a standalone treatment or in combination with conventional drugs. Such an approach could potentially improve therapy adherence and have a positive impact on clinical outcomes [33].

Indomethacin-Induced Enterocolitis in Rats:

Two days treatment with indomethacin (7.5 mg/kg, s.c.), produced severe inflammation in rat intestine. The middle portion of the small intestine i.e. jejunum and proximal ileum showed more inflammation compared to proximal portion of the small intestine. Caecum was the most severely affected part, showing hemorrhagic spots. The ileum showed many lesions, which were transmural. In between there were skip areas of normal tissue. In some animals the large intestine was found to be affected with hemorrhagic lesions. Evaluation based on macroscopic features showed significantly.

Effect of H. Indicus Root Bark extract on macroscopic features in indomethacin induced entero colitis in rats

Treatment groups Mean of macroscopic scores ± S.E.M 1 Normal or untreated animals. 0 2 Control animals receive only indomethacin (7.5mg/kg) s.c. 8.35 ± 0.57 3 Animals treated with indomethacin (7.5 mg/kg) s.c + lower dose. (TGE) 5.34 ± 0.87* 4 Animals treated with indomethacin (7.5 mg/kg) subcutaneous + higher dose. (TGE) 3.33 ± 0.56x 5 Animals, which will receive Prednisolone (2 mg/kg p.o) and indomethacin (7.5 mg/kg) 1.6 ± 0.22x

Table 8: Effect of H. Indicus Root Bark extract on macroscopic features in indomethacin induced enterocolitis in rats

S.No.	Treatment Groups	Mean of Macorscopic Scores ± SEM
1	Normal or untreated animals	0
2	Control animals receive only indomethacin (7.5mg/kg) s.c.	8.35 ± 0.57
3	Animals treated with indomethacin (7.5 mg/kg) s.c + lower dose. (TGE)	5.34 ± 0.87*
4	Animals treated with indomethacin (7.5 mg/kg) subcutaneous + higher dose. (TGE)	3.33 ± 0.58x
5	Animals, which will receive Prednisolone (2 mg/kg p.o) and indomethacin (7.5 mg/kg)	1.6 ± 0.22x

Table represents significant reduction in macroscopic score value compare to Indomethacin alone. Each value represents mean of macroscopic scores ± S.E.M. (n = 6). Significant in macroscopic score values according to one-way ANOVA followed by Tukey's post-test, P values of *< 0.05 were considered statistically significant. X P < 0.001 compare to indomethacin alone.

The myeloperoxidase assay showed significant increase in MPO activity of positive control group compared to normal untreated group. The drug treated and standard treated group showed significant ($P < 0.01$, $P < 0.001$) reduction in MPO activity compared to the positive control group. MPO activity of the drug treated group was comparable with the standard treated group . The lactate dehydrogenase (LDH) assay showed significant ($P < 0.001$) increase in LDH activity of positive control group compared to normal untreated group. The drug treated and standard treated group showed significant ($P < 0.01$, $P < 0.001$) reduction in LDH activity compared to the positive control group. LDH activity of the drug treated group was comparable with the standard treated group.

The Lipid peroxidase (LPO) assay showed significant ($P < 0.001$) increase in LPO activity of positive control group compared to normal untreated group. The drug treated and standard treated group showed significant ($P < 0.01$, $P < 0.001$) reduction in LPO activity compared to the positive control group. LPO activity of the drug treated group was comparable with the standard treated group Table 5 and Fig. 5. Histological examination of positive control group showed advanced lesions as necrosis of even payers patches and fragmentation of nuclei. The drug treated group showed reduced intensity of lesions without any evidence of necrosis, regeneration or inflammatory reaction. Standard treatment showed suppressed inflammatory reaction[34].

Table 9: Effect of H. indicus root bark extract on MPO, LDH and LPO activity in indomethacin-induced enterocolitis

Groups	Treatment Groups	MPO activity (U/g) ± S.E.M	LDH activity (U/L) ± S.E.M	LPO Activity (µmol/g) S.E.M
1	Normal or untreated animals	2.16 ±0.34	514.67 ± 6.37	0.16 ±0.03
2	Control animals receive only Indomethacin (7.5mg/kg) s.c.	12.66 ±0.72	1153.67±60.68	0.70 ±0.05
3	Animals treated with Indomethacin (7.5mg/kg) s.c. + lower dose (TGE)	8.34 ±0.84B	908.17 ±75b	0.47 ±0.07c
4	Animals treated with Indomethacin (7.5mg/kg) s.c.+ higher dose(TGE)	4.00 ±1.13C	838.0 ±61a	0.3 ±0.07a
5	Animal treated group, which will receive Prednisolone (2mg/kg p.o) and indomethacin 7.5mg/kg) s.c.	1.68 ±0.21C	555.68 ±100b	100b 0.14 ±0.02b

Each value represents mean of MPO activity (U/g), LDH (U/L), LPO (µmol/g) ± S.E.M. (n = 6). (One-way ANOVA followed by Tukey's post-test)
c. Significant increase in MPO, LDH, LPO activity P
a. Significant decrease in MPO, LDH, LPO activity P
b. Significant decrease in MPO, LDH, LPO activity P

Acetic Acid-Induced Colitis in Rats: Intra-rectal instillation of acetic acid caused inflammatory reaction in the colon. The inflammation covered rectum and distal colon portion. The visible changes included severe epithelial necrosis and ulcerated mucosa. Drug treated and standard treated group showed significantly ($P < 0.001$) lower score values of macroscopic evaluation as compared to positive control group and values obtained for the drug treated group were comparable with the standard treated group

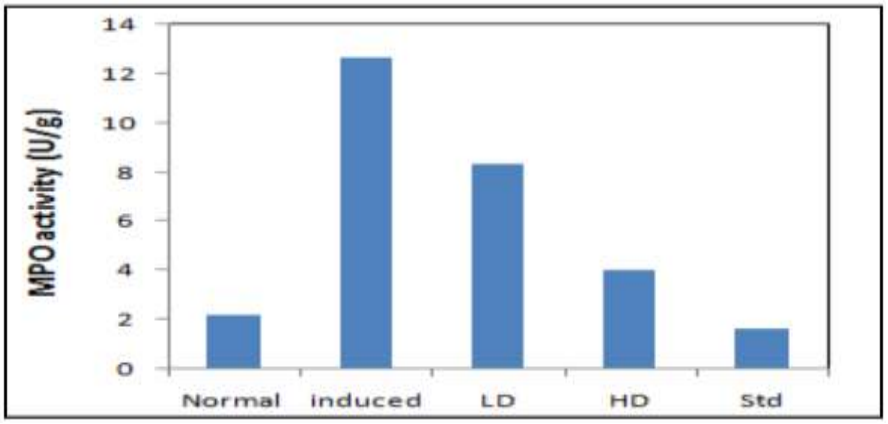

Figure 4: Effect of H. indicus root bark extract on MPO activity in Indomethacin-Induced Enterocolitis in rats

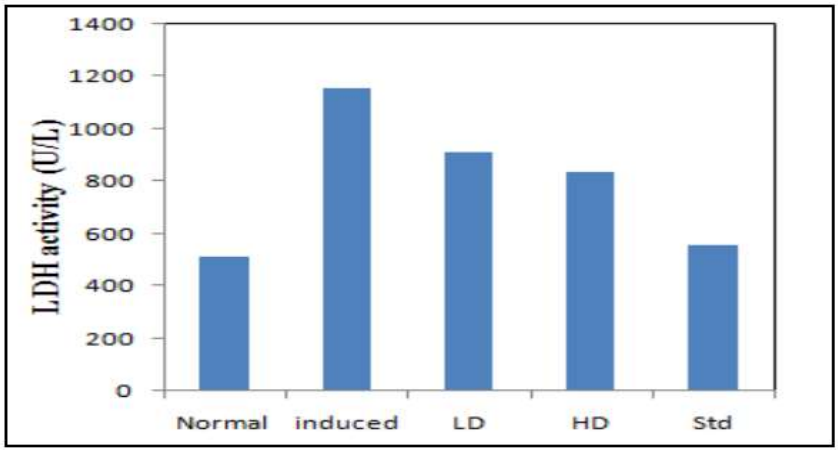

Figure 5: Effect of H. indicus root bark extract on LDH activity in Indomethacin-Induced Enterocolitis in rats

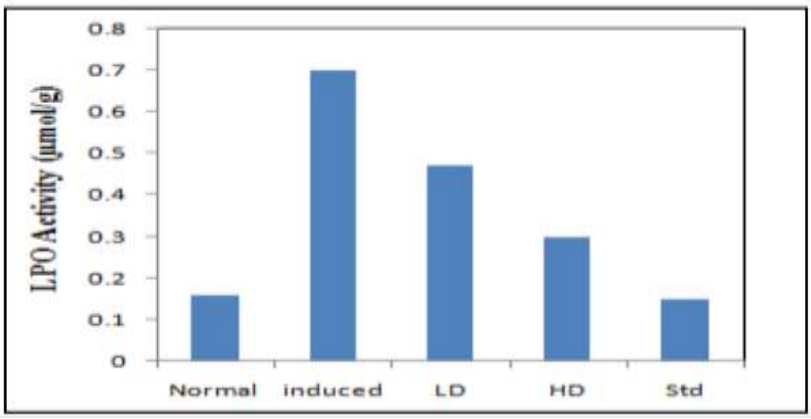

Figure 6: Effect of H. indicus root bark extract on LPO activity in Indomethacin-Induced enterocolitis in rats.

Table 10: Effect of H. indicus root bark methanolic extract macroscopic features in acetic acid induced colitis in rats

S. no.	Treatment groups	Mean of macroscopic
1	Normal	0
2	Positive control – Acetic acid in Saline alone 2ml (4%,v/v) (once, intra-rectally)	10.16± 0.41

3	Methanolic extract of H. indicus root bark+ Aceticacidinsaline2ml (4%, v/v) (once, intra-rectally)[TGE]	6.68± 0.82*
4	Methanolic extract of H. indicus root bark+ Aceticacidinsaline2ml (4%, v/v) (once, intra-rectally)[TGE]	2.84 ± 0.47x
5	Standard (500mg/kg,p.o.) + Acetic acid in saline 2 ml (4%,v/v) (once, intra-rectally)	2.16± 0.18x

Table represents significant reduction in macroscopic score value compare to acetic acid alone. Each value represents mean of macroscopic scores ± S.E.M. (n = 6). Significant in macroscopic score values according to one-way ANOVA followed by Tukey's post-test, P values of < 0.05 were considered statistically significant. cP < 0.001 compare to acetic acid alone

The myeloperoxidase assay showed significant ($P < 0.001$) increase in MPO activity of positive control group compared to normal group. The drug treated and standard treated groups showed significant ($P < 0.01$, $P < 0.001$) decrease in MPO activity compared to positive control group Table 6 and Fig. 6. The lactate dehydrogenase (LDH) assay showed significant ($P < 0.001$) increase in LDH activity of positive control group compared to normal group. The drug treated and standard treated groups showed significant ($P < 0.01$, $P < 0.001$) decrease in LDH activity compared to positive control group Table 6 and Fig. 7**.**

The lipid peroxidase (LPO) assay showed significant ($P < 0.001$) increase in LPO activity of control group compared to normal group. The drug treated and standard treated groups showed significant ($P < 0.01$, $P < 0.001$) decrease in LPO activity compared to positive control group Table 6 and Fig. 8.

Histological examination of control group showed massive necrosis of the mucosa and submucosa. Payers patches appeared distorted with karyohexis and karyolysis. Drug treated group showed mild lesions, regeneration and inflammatory reaction. The standard treated group showed suppressed inflammatory reaction.

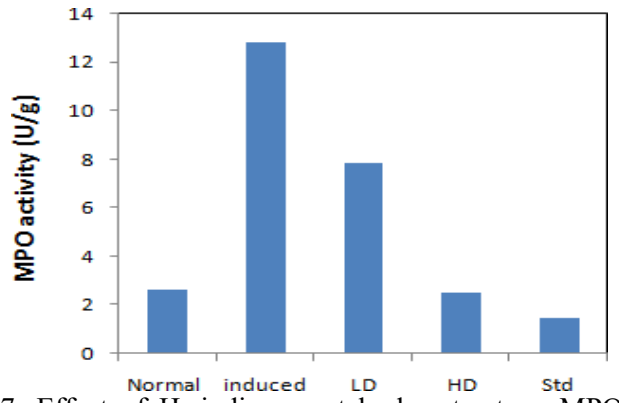

Figure 7: Effect of H. indicus root bark extract on MPO activity in acetic acid-induced enterocolitis in rats

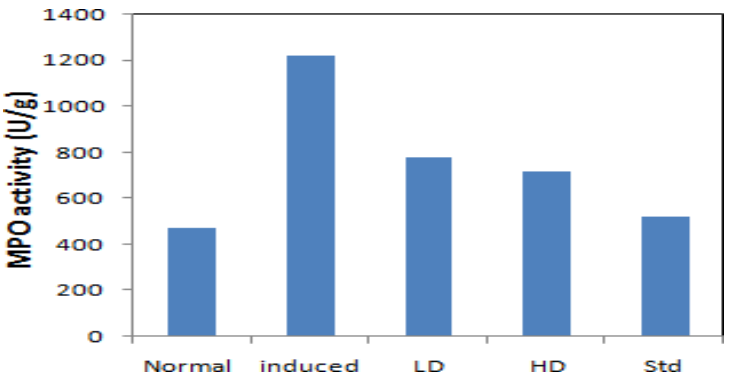

Figure 8: Effect of H. indicus root bark extract on LDH activity in acetic acid-induced entero colitis in rats

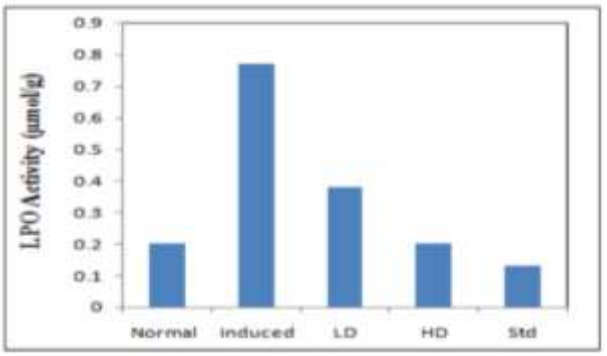

Figure 9: Effect of H. Indicus root bark extract on LPO activity in acetic-induced enterocolitis in rats

Table 10: Effect of Methanolic extract of H. Indicus root barkon MPO, LDH AND LPO activity in acetic acid-induced colitis in rats

Groups	Treatment groups	MPO activity (U/g) ±S.E.M	LDH activity (U/L) ± S.E.M	LPO Activity (µmol/g)S.E.M
I	Normal	2.67±0.21	472.33±46.22	0.20±0.04
II	Positive control- Acetic acid in salinealone 2 ml (4%,v/v) (once, intra-rectally)	12.83±0.87	1221.83±55.88	0.77±0.05
III	Methanolic extract of Tectonagrandis + Acetic acid in saline 2ml (4%,v/v) (once,intra-rectally [TGE]	7.83±0.87	778.50±96.20	0.38±0.09
IV	Methanolic extract of Tectonagrandis + Aceticacidin saline 2ml (4%,v/v) (once, intra-rectally) [TGE]	2.50±0.56	721.50±95.91	0.20±0.03
V	Standard (500 mg/kg,p.o.) + Acetic acid in saline 2ml (4%,v/v) (once ,intra- rectally)	1.50±0.22	523.33±83.62	0.13±0.02

Each value represents mean of MPO activity (U/g), LDH (U/L), LPO (µmol/g) ± S.E.M. (n = 6). (One-way ANOVA followed by Tukey's post-test) c. significant increase in MPO, LDH, LPO activity P

TGE was evaluated for its in vitro COX-1 and COX-2 inhibitory activities by using colorimetric COX (human ovine) inhibitor screening assay kit. The results showed that TGE inhibits COX-1 about 42.35% and that of COX-2 is 45.8% see Table 11 . Prostaglandins inhibition was studies by inhibition of Castor oil induced diarrhea in rats. Administration of Castrol induced diarrhea around 9 fecal drops with total weight of 5.5 grams in 4 h. This was reduced in both test and standard groups. Indicating TGE produces action by inhibiting the synthesis of prostaglandins see Table 12.

Table 11: Effect of KEE on Cox Inhibitory Activity

Extract	Inhibition of COX-1 (%)	Inhibition of COX-2 (%)
TGE	43.17	48.67

Table 12: Determination of Prostaglandins Inhibition

Groups	Treatment and dose	Mean frequency of diarrhea ± SEM	Mean no. of fecal drops ± SEM	Mean wt. of feaces ± after 4 hrs (gm)
1	Control (0.5 ml of DI water)	7.17±1.47	9.67±1.51	5.17±1.17
2	Aspirin150mg/kg	1.67 ±0.82	3.67±1.21	3.33±1.86
3	TGE	1.33±0.52	4.67±3.08	3.17±1.33

Bark extract of H. indicus root bark Linn. is screened for inflammatory bowel disease. Various parts of the plant have showed activities like astringent, depurative, diaphoretic, diuretic expectorant, febrifuge, odontalgic and ophthalmic. The aerial part of this plant has been reported to contain pyrrolizidine alkaloids and flavonoids 2 - 3. The plant is astringent, sweet, thermogeic, ntipyretic and antiasthmatic. Aqueous and methanolic extract of H. indicus root bark Linn have been shown to progressively reduced rat paw edema induced by sub plantar injection of albumin, which suggest that this extract might have antiinflammatory principals. The bark extract of H. indicus root bark Linn. has antioxidant and antiproliferative effects on human cancer cells. In a study H. indicus root barkLinn showed significant in vivo analgesic and antiinflammatory activities. The models used for study of IBD are acetic acid induced ulcerative colitis and indomethacin induced enterocolitis. Intrarectal instillation of acetic acid in rats affected only the distal colon portion. The inflammation was not transmural. Massive necrosis of mucosal and submucosal layers was observed. This model shares many of the histologic features of ulcerative colitis in human beings including mucosal edema, neutrophil infiltration of the mucosa and submucosal ulceration. The mechanism by which acetic acid produces inflammation appears to involve the entry of the protonated form of the acid into epithelium, where it dissociates to liberate protons causing intracellular acidification that most likely accounts for the epithelial injury observed. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipooxygenase pathways and generation of inflammatory mediators like prostaglandins and leukotrienes. Excess production of reactive oxygen metabolites e.g. superoxide, hydroxyl radical, hydrogen peroxide, hypochlorous acid and oxidant derivatives such as N-chloramines are detected in inflamed mucosa and may be pathogenic in IBD. Also, there is an increase in proinflammatory cytokine TNF -α production in colonic mucosa after acetic acid instillation. Indomethacin, a nonselective COX inhibitor produces enterocolitis in rats on sub cuteneous administration which is characterized by linear ulceration, thickening and transmural inflammation. The mechanism of indomethacin induced enterocolitis have not been fully illustrated, but previous reports suggests that, inhibition of protective prostaglandins PGE1, PGE2 and prostacyclin (PG12) may be one of the mechanism by which indomethacin induces injury. In addition, bacteria and bacterial products, biliary secretion and food intake have been demonstrated to be important for the development of the intestinal lesions. The treatment with bark extract H. indicus root barkLinn. has shown a decrease in the macroscopic scores for the inflammation. Since the intestine is in a constant

state of controlled inflammation, thus amplification of the inflammatory response activates infiltration of inflammatory cells that triggers pathological responses and symptoms of IBD. Our study showed that acetic acid raised the levels of colonic MPO, indicating infiltration of neutrophils and perturbation of the inflammatory system. This fact is documented in both animal models, and patients with IBD . H. indicus root barkLinn. bark extract ameliorated neutrophil infiltration as evidenced by suppression of colon MPO and improvement of histological features. Histopathology examination of drug treated group revealed less damage compared to control group. A significant decrease in MPO activity was also observed. All these observations support the findings that the barks extract of H. indicus root barkLinn. was able to offer significant protection in both the models studied. Acetic acid-induced colitis and indomethacin-induced enterocolitis simulate two different disease conditions, which are ulcerative colitis and Crohn's disease respectively. On this basis we can say that the constituents extract under study may be useful in treating UC as well as CD in humans. The prednisolone treatment has shown significant protection in both the animal models under our study. The bark extract H. indicus root barkLinn. was found comparable with standard drug. The role of oxygen-derived free radicals, such as hydroxyl radical and superoxide radical, in the inflammatory process is well known. It is also generally assumed that most of the antioxidants possess anti-inflammatory effect. Bark extract of H. indicus root barkLinn. may account for the observed anti-inflammatory properties.

CONCLUSION: Bark extract of H. indicus root bark Linn. has potent protection against inflammatory bowel activity in both indomethacin induced enterocolitis and acetic acid induced ulcerative colitis. Collectively, these findings indicate that the anti-oxidant effect of fraction may be an important contributor to its anti-inflammatory activity in IBD. This present investigation has also opened avenues for treatment of IBD from the title plant.

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