"Effect of hydro-alcoholic extract of *Terminalia arjuna* bark and Arjunarishta in Inflammatory bowel disease"

Thesis submitted to

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For the award of the degree of

Doctor of Philosophy

In the Faculty of Pharmacy

By

Ms. Cota Damita Lourdes M.Pharm

(Registration No: KLEU/Ph.D./15-16/DO1215007)

Under the Guidance of

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Asso. Prof. and Scientist, KAHER's Dr. P.K. BSRC

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Ms. Cota Damita Lourdes

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LIST OF ABBREVIATIONS

5 -FU:	5-Fluorouracil
AA:	Arjunarishta
ACN:	Acetonitrile
BHI:	Brain heart infusion
CAT:	Catalase
CD:	Crohns Disease
CRC:	Colorectal cancer
DAI:	Disease activity index
DMEM:	Dulbecco’s modified eagle’s medium
DMSO:	Dimethyl sulfoxide
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
FRAP:	Ferric Reducing Antioxidant Power
GAPDH:	Glycerol 3-phosphate dehydrogenase
GI:	Gastrointestinal
GSH:	Glutathione
HPLC-PDA:	High Performance Liquid Chromatography and Photo Diode Array
IBD:	Inflammatory bowel disease
IC50:	Half maximal inhibitory concentration
IHD:	Ischemic heart disease
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
MCP-1:	Monocyte chemoattractant protein-1
MDA:	Malondialdehyde
mg/dL:	Milligram per decilitre

mg/kg:	Milligram per kilogram
Min:	Minutes
MIC:	Minimum inhibitory concentration
MBC:	Minimum bactericidal concentration
mL/kg:	Milliliter per kilogram
MPO:	Myeloperoxidase
MTT:	3 - (4, 5-dimethylthiazol- 2 - yl)-2, 5-diphenyltetrazolium bromide
NF-kB:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
nm:	Nanometer
NO:	Nitric Oxide
R^2:	Coefficient of regression
ROS:	Reactive oxygen species
R_t:	Retention time
SOD:	Superoxide Dismutase
TA:	*Terminalia arjuna*
TAHA:	*Terminalia arjuna* Hydro-alcoholic extract
TBARS:	Thiobarbituric acid reactive substances
TFC:	Total flavonoid content
TNF-α:	Tumor Necrosis Factor- alpha
TPC:	Total phenolic content
UC:	Ulcerative colitis
v/v:	Volume/volume
Vs:	Versus
WHO:	World health organization
Zn:	Zinc

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ABSTRACT

Background:

Terminalia arjuna Roxb. (Combretaceae) a medicinal plant and its formulation Arjunarishta, are traditionally utilized in cardiovascular diseases. According to Ayurveda, it stabilizes the three doshas - Vata, Pitta and Kapha. This bark possesses anti-inflammatory, antibacterial, antidiarrheal and antioxidant properties which would be useful for the treatment of inflammatory bowel disease (IBD), which is associated with bacterial microflora dysbiosis, oxidative stress, inflammatory response activation and immune dysfunction. With the rising disease burden, research should also focus on developing low-cost therapies which would not only help the patients in India but also in entire Asia, which like India are experiencing a rise in IBD disease burden along with scarce resources to tackle this problem.

Phytochemicals like gallic acid, ellagic acid and quercetin, present in *Terminalia arjuna* bark have exhibited protective effects in rat colitis models. Thus, this study evaluates the effect of hydro-alcoholic extract of *Terminalia arjuna* bark (TAHA) and Arjunarishta against IBD.

Study Objectives

To study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta on IBD related microbes, colorectal adenocarcinoma cells and TNBS induced inflammatory bowel disease in rats.

Methodology

The phytochemical profile of test materials was confirmed via investigation of total phenolic and flavanoid content and standardized by HPLC-PDA method. In vitro antioxidant activity was carried out using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and

ferric reducing ability of plasma (FRAP) assay. Antimicrobial potential was tested against clinical isolates of IBD patients (HM95, HM233, HM251, HM615). Cytotoxicity was determined against human colorectal adenocarcinoma cells (Caco2, COLO.205), whereas, cytocompatibility against normal rat intestinal epithelial (IEC-6) and mouse fibroblast cells (L929). In vitro oxidative cell damage stress was estimated by the level of malondialdehyde. The phenolic compounds in TAHA and AA were identified and quantified using HPLC. In-vivo studies were performed in the TNBS induced colitis model. TNBS was administered intrarectally, thereafter TAHA (500, 250, 125 mg/kg), AA (1.8, 0.9 and 0.45 mL/kg) was administered orally for 28 days. Response to treatment was assessed by scoring Disease activity index (DAI), Macroscopic and Histological damage, determining Myeloperoxidase (MPO), Malondialdehyde (MDA), Nitric oxide (NO), Catalase (CAT), Superoxide dismutase (SOD), reduced Glutathione (GSH) levels, gene expression of pro-inflammatory cytokines such as Interleukin (IL)-6, IL-1β,TNF-α and chemokine-Monocyte Chemoattractant protein (MCP)-1. The role of TAHA and AA in altering the plasma zinc status and gut microbiota profile was also evaluated in this study

Results

TAHA demonstrated higher antioxidant capacity as compared to AA formulation. Different trend in results were observed against different study cell lines in dose dependent manner. Similarly, significant ($p<0.05$) enhanced MDA concentrations in test materials treated colorectal adenocarcinoma cells was detected as compared to control cells. TAHA and AA exhibited antimicrobial activity against IBD associated clinical isolates. These study findings may provide biological evidence for the application of TAHA and AA in IBD and colorectal cancer treatment. Treatment of TNBS colitic rats with Test materials resulted in decreased DAI scores,

macroscopic and histologic damage, it also decreased the colonic neutrophil infiltration indicated by a lower MPO level, lipid peroxidation indicated by MDA and NO level, prevented depletion of plasma CAT, SOD and GSH levels in colitic rats. In addition, test material treatment downregulated the expression of pro-inflammatory mediators in TNBS induced rats. Administration of test materials exhibited altered faecal microbiota in colitic rats. The treatment with test drugs also improved the plasma zinc status in colitic rats. The beneficial effect of test materials seen in colitic rats was found to be dose dependent, 500 mg/kg of TAHA and 1.8 mL/kg AA producing the most significant effects.

Conclusion

These study findings may provide biological evidence for the application of TAHA and AA in IBD, demonstrated through its antibacterial activity against IBD strains, cytotoxic effect and ameliorated symptoms in TNBS induced colitis by decreasing oxidative stress, reduced expression of proinflammatory cytokines/chemokine and improvement in structure of gut microbiota and plasma zinc status.

Keywords

Terminalia arjuna; Arjunarishta; Antibacterial; Cytotoxicity; Inflammatory bowel disease; Colorectal cancer; TNBS rat colitis; Gut microbiota.

1. INTRODUCTION

1.1. Background

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal (GI) tract, characterized into two main types: Ulcerative colitis (UC) and Crohn’s disease (CD). German surgeon Wilhelm Fabry first identified Crohn’s disease in 1623, However it was later named after the US physician Burril B Crohn. Ulcerative colitis was discovered in 1859 by the British Physician Sir Samuel Wilks [1].

IBD was traditionally considered as a disease of developed nations, however in recent years a change in epidemiology has been noted. High income countries with greater disease burden and prevalence rates are indicating stabilizing occurrence, simultaneously in the newly developing countries of South America, Eastern Europe, Asia and Africa a rapid rise in cases is noted. These rapid changes occurring worldwide suggests call for global estimates to provide insight into the burden and trends. Moreover, defining the varying incidence and diagnosis of IBD in different geographical regions might provide evidence to the cause of the disease which is not yet clearly known [2].

UC is a gastrointestinal disease that is confined to the colon, where inflammation is involved either in the entire colon or a part of it. However, CD can affect any part of the GI tract from mouth to the anus. Although CD is more severe than UC, the global prevalence is much lower. Approximately 1.86 billion UC patients have been diagnosed globally, with 1.54 billion patients currently receiving treatment and 1.3 million CD patients diagnosed and 0.8 million receiving treatment.

Traditional therapies have yielded $4.18 billion for UC and $3.17 billion for CD in annual sales worldwide, and expected to increase further to $6.85 billion and $4.20 billion respectively by 2022 with the approval of various pipeline drugs [3].

The pathological process of IBD is still not clear however it is known to involve a complex interrelationship between genetic or environmental factors, gut microflora and mucosal immune response [4]. The immune system imbalance and/or altered microbial interactions leads to progression of chronic intestinal inflammation in genetically susceptible hosts when certain environmental factors are triggered. Th1 cells were considered to play an important role in CD pathogenesis and Th2 cells were shown to be involved in pathogenesis of UC. However, newer developments suggest Th17 cell activation and imbalance of Th17/regulatory T (Treg) cells to be a prime component in the development of gut inflammation [5]. Mucosal inflammatory reactions to unidentified antigens or consequence of an abnormal immune system results in cellular infiltration of neutrophils, plasma cells, mast cells, lymphocytes and macrophages. Activation of these infiltrating cells cause release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1, IL-6 and IL-12 which contribute to epithelial intestinal damage and ultimate clinical disease [6,7].

Clinical features and complications of Ulcerative colitis are severe diarrhoea, blood loss and progressive loss of peristaltic function leading to rigid colonic tube. Depending on its severity it can lead to toxic megacolon and perforations and those of Crohn's disease include diarrhoea, pain, gut lumen narrowing leading to strictures and intestinal obstruction, abscess formation, skin and internal organ fistulation. Inflammatory expressions can occur in other organs like joints, eyes, skin, mouth and liver. It has been associated with prolonged risk of colon carcinoma, predominantly in UC [8].

The existing treatment regimen in IBD management includes amino-salicylates, corticosteroids, immunosuppressants, antibiotic and biological agents as choice of drugs, based on disease severity. However, these have been associated with serious side effects and develop resistance over long term use [9,10].

Trinitrobenzenesulfonic acid (TNBS) is a hapten and administered intrarectally in rats for chemical induction of IBD. TNBS (100 mg/kg) is co-administered with ethanol as ethanol aids in disruption of the intestinal mucosal barrier and leads to the interaction of TNBS with colonic proteins. TNBS when coupled with high molecular weight proteins results in significant immunologic reaction by making those proteins immunogenic to the host immune system. Even a single administration leads to development of an extreme cell mediated immune response reflected by acute Th1 inflammation [11].

Modern system of medicine has been in practice for the treatment of chronic inflammatory diseases. However, there is increasing interest in natural products as a source of Complementary and alternative medicine (CAM) for the treatment of IBD. CAM includes herbal therapy or phytotherapy, dietary supplementation, probiotics, Chinese medicinal practices mindfulness or other mind-body therapies, which are not considered as conventional treatment options [12]. The use of natural products is continuously increasing despite the fact that only a few research studies of controlled trials dealing with either their safety or efficacy exist. The prevalence of CAM use ranges from 21 to 60%, while herbal medicines are being used by 5 to 58% of IBD patients. Plants constitute of proteins, lipids, organic acids (intermediate metabolites) and secondary cellular constituents such as alkaloids, glycosides, flavonoids, saponins, tannins and essential oils. The secondary cellular constituents are generally assumed to be pharmacologically relevant and may have anti-inflammatory, antiphlogistic or astringent properties, as well as exerting mucosal protective effects or even influencing fecal microflora. Most herbal medicines, however, act via the combination and interaction of all of their active ingredients and further target multiple pathways involved in the inflammatory process [13].

Terminalia arjuna (T. arjuna), belonging to the family Combretaceae has been used traditionally in the treatment of cardiovascular diseases and certain forms of cancer. It has been utilized in balancing three doshas - Vata, Pitta and Kapha in Ayurveda [14]. The active constituents include triterpenoids, saponins, tannins, flavonoids, ellagic acid, gallic acid, oligomeric proanthocyanidins, phytosterols, magnesium, calcium, zinc, and copper [15]. *T. arjuna* is used as folk medicine by ‘Bhoxa’ community in Dehradun – India in cases of dysentery and diarrhoea [16] as well as documented to have anti-inflammatory, anti-nociceptive and immunomodulatory activities [17]. Furthermore, bark extract is reported to protect liver and kidney tissues against CCl_4-induced oxidative stress [18] and induce *in vitro* cytotoxicity in Hepatocellular carcinoma cells [19]. The stem bark, in particular, is made up of an appreciable number of secondary metabolites, which may act as a resource of pharmacologically active substances and antioxidants of natural origin [20].

Arishtas are Ayurvedic formulations, which are traditional decoction of certain herb combinations. These liquid dosage forms constitute of self-generated alcohol, which improves the extraction efficiency of molecules soluble in alcohol and water, resulting in improved drug delivery. Arjunarishta (AA), an arishta formulation supports the improvement of cardiac functions, appetite and balances immune response [21]. AA has been used traditionally as a cardiotonic and also reported for its action of cleansing intestines. This formulation contains T. arjuna, *Madhuca indica, Vitis vinifera* and *Woodfordia fruticosa* [22]. Phytochemicals such as gallic acid, ellagic acid, quercetin, which have been reported to be present in *T. arjuna* bark and AA hold varying degree of antioxidant, protective and intestinal anti-inflammatory effect in rat models of colitis [23-25].

Based on above evidences, the study was planned to evaluate the potential of *Terminalia arjuna* hydroalcoholic extract and Arjunarishta in inflammatory bowel disease.

1.2. Review of literature

1.2.1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) which are inflammatory bowel diseases (IBD) are chronic and relapsing inflammatory disorders of the GIT. IBD is an autoimmune disease wherein the patient's digestive system is targeted by its own immune mechanism It is believed to be an outcome from an altered and persistent inflammatory reaction to the host microorganisms in a genetically predisposed individual. UC originates in the rectum and spreads continuously mostly involving the peri appendiceal region. The inflammation of UC is limited to the colon. However, CD occurs in a non-continuous pattern. It can originate in any part of the GIT, most commonly the terminal ileum or the perianal section are involved [26,27].

1.2.2. Epidemiology of IBD

A study of 2011 showed that in the last 50 years the occurrence of IBD has increased in the Western countries. Incidence and prevalence of UC is up to 8–14/100,000 and 120–200/100,000 persons and for CD it was 6 –15/100,000 and 50 – 200/100,000 persons, respectively. However, populations of developing countries including migrant population exhibited slow rise in the incidence of UC compared to CD, but eventually the incidence levels of both were similar [28].

Another study of 2012 reported that in the West, the incidence and prevalence of IBD is higher than in Asia. Due to increased contact of Asians with the West, diet alteration influenced by the West, increased use of antibiotics and vaccines, hygiene improvement leading to changes in the gut microbiota could have led to rapidly increased IBD incidence and prevalence over the last forty years [29].

The disease burden of UC in India is based on the population of two states in the country. In 1984 Khosla et al. conducted the first study in Haryana, North India wherein 21,971 individuals participated in the study and UC prevalence of 42.8/100,000 persons was reported. A few years later Sood et al. conducted the second study in Punjab wherein 51,910 individuals participated and prevalence rate of UC was 44.3/100,000 persons. The incidence rate of UC was 6.02/100,000 persons. In 2010 India was ranked as the second highest with 1.4 million after USA with 1.64 million of IBD population. Therefore, presenting India's IBD population to be among the largest in the world [30].

A study suggested that children and adults (under 40 years) constitute 7-20% and 60- 85% of IBD patients respectively. Population in the age group of 20-30 and

60-70 years show higher incidence of CD. Whereas UC shows the incidence peaks between the age group of 20-30 [31]. Findings of a study in 2018 which included IBD incidence of 207,600/ 478 million people suggested that sex hormones might have a role in IBD pathogenesis in genetically susceptible patients and also concluded that age of IBD onset varied by gender [32].

1.2.3. Pathogenesis of IBD

IBD pathogenesis is a result of interplay between genomic susceptibility, environmental impact on the microbiome that through a deteriorated intestinal barrier, leads to inappropriate intestinal immune stimulation [33]. The various factors involved in IBD pathogenesis are displayed in figure 1.

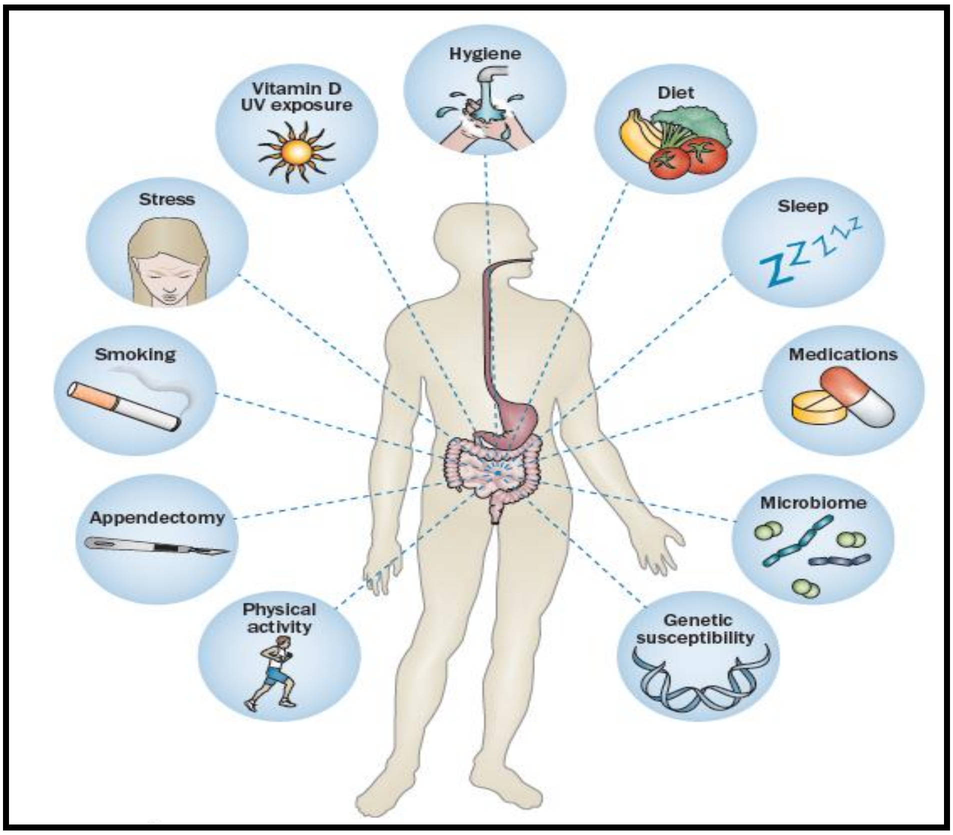

Figure 1. The interaction between genetics, immunology, environment and microbiome [34].

1.2.3.a. Role of Microbes in IBD pathogenesis

The gut bacteria perform vital metabolic and host defensive functions. On the contrary it is also involved in the pathology of the intestinal mucosal inflammation. Various screening experiments in animal models confirmed development of IBD which could be due to extreme passage of bacteria through the gut wall into the extra-intestinal sites or altered behaviour of microbes due to genetic predisposition [35]. Figure 2 illustrates the involvement of gut bacteria in the induction of intestinal inflammatory response

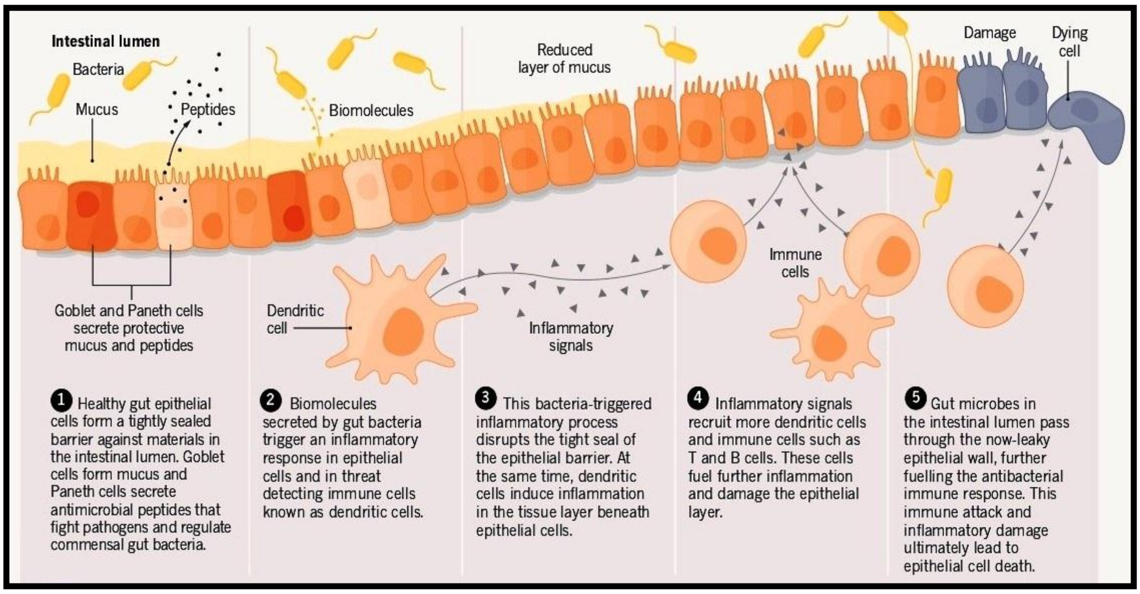

Figure 2. Role of gut bacteria in inflammation [36].

Dysbiosis, which is an imbalance in gut bacteria is considered a novel factor associated in IBD pathogenesis (Figure 3). The main factor in induction and inhibition of colonic inflammation particularly, balancing of T helper 17 (Th17)/Treg is due to the gut microbiota [37].

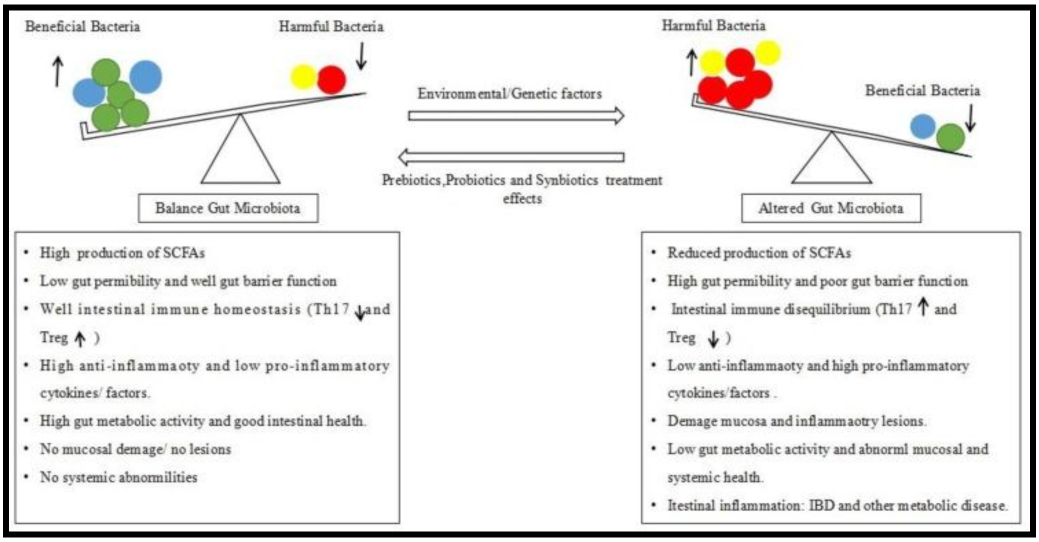

Figure 3. The gut microbiota of the healthy individual (left), the gut microbiota of the IBD patient [37].

The IBD patients demonstrate a rise in the pathogenic microorganisms such as Klebsiella Enterobacter, Proteus and fungi [38-41].

Lactobacilli and Bifidobacteria are reduced whereas Bacteroides and Prevotella spp. are increased in the gut mucosa of IBD patients [42,43]. Lactobacilli and Bifidobacteria bacteria play a role in the host defence mechanisms. Therefore, the decrease in their count could lead to dysregulated immune responses [44]. Microorganisms such as *Chlamydia*, *Mycoplasma*, *Mycobacteria*, *Clostridium difficile*, *Salmonella spp.*, *Listeria monocytogenes*, *Streptococcus spp.*, *Escherichia coli (E.coli*), *Desulfovibrio spp.*, *Fusobacterium nucleatum* present in IBD patients indicate their role in disease pathogenesis [45-52]

Intestinal microbiome dysbiosis characterised by an increase in the number of mucosa-associated studies have reported increased *E.coli* count in the gut of UC patients and several animal models [53]. Bacteria such as Adherant –invasive *E.coli (*AIEC) are reported to be involved in pathologic process of CD, working by deregulation of immunity. These variants of *E.coli* attach and invade the mucosal cells to produce extensive biofilms that protect the bacteria from the host immune system and treatment with antibiotics, causing diseases in living organisms. The invasion of macrophages by these AIEC strains stimulates expression of proinflammmatory cytokines such as TNF-α and interleukin 6 [54]. *Bdellovibrio bacteriovorus* a predator bacterium, invades Gram-negative bacteria. It is found to counteract the pathogenicity of AIEC strains [55].

Human commensal *Bacteroides thetaiotaomicron* induces colitis in Healthy germ-free HLA-B27 transgenic rats. This induction could be due to significant alteration of luminal bacterial transcription that affect metabolic, growth, and nutrient binding pathways. The study also suggests that commensal gut microbes are involved in chronic intestinal inflammation [56].

A study showed that UC patients have a higher risk of *Clostridium difficile* infection (CDI) and the incidence of CDI in IBD patients in USA has doubled from 2.66 to 5.12% over a period of 7 years, and the risk factor includes the use of broad-spectrum antimicrobial agents, old age, chemotherapy and hospitalisation [57]. Decreased clostridial counts were observed in active hexose correlated compound administered colitic rats and raised the decreased levels of lactobacilli and bifidobacterium along with reduced inflammation [58].

1.2.3.b. Role of inflammatory mediators in IBD

The immune response in IBD is regulated by T cells. Any changes in T cell proliferation cause excessive release of cytokines and chemokines promoting the mucosal inflammation. Th1 cells secrete proinflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8, IL-12, TNF-α, and IFN-γ. Th2 cells secrete IL-4, IL-5, IL-9, and IL-13. Imbalance of Th1/Th2 leads to inflammation in IBD [59].

Macrophages in the intestine maintain tissue homeostasis and induce resolution after inflammation. Therefore, macrophages play a major role in IBD remission [60]. Dendritic cells have a vital role in immune response and IBD pathogenesis. The mechanism of pathogenesis is due to its involvement in genetics and microbial interactions. IBD in humans and animal models show accumulation of activated dendritic cells [61].

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) is an inducible transcription factor. It activates transcription of various genes encoding cytokines and chemokines thus controlling the process of inflammation. It also plays an important role in modulating programmed cell death, morphogenesis, differentiation and proliferation (Figure 4). Dysregulated NF-κβ pathway and increased proinflammatory cytokine levels leads to altered immunity and uncontrolled inflammation in IBD patients [62,63]

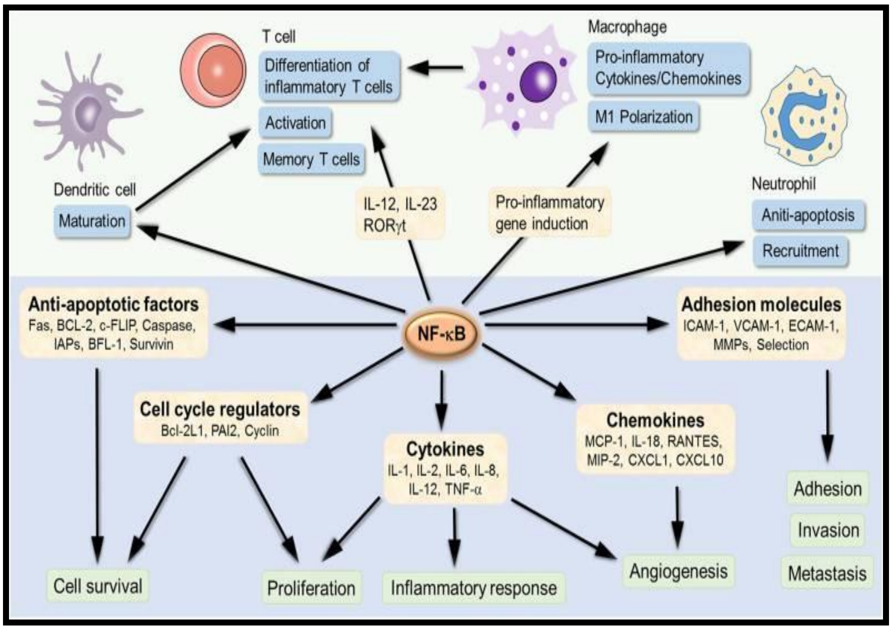

Figure 4. NF-κB targeting genes involved in inflammation development and progression [62].

Adhesion molecules (cell adhesion molecule – CAM) participate in leukocyte attachment to endothelial cells, which enables their penetration to the site of inflammation. The intercellular adhesion molecule (ICAM) enhances rolling of leukocytes. Vascular cell adhesion molecules (VCAM) and selectins, cause leukocytes adhesion to the endothelium. Integrins are activated by the release of proinflammatory cytokines, aiding communication between leukocytes and endothelial cells. Which further leads to leukocyte extravasation and migration contributing to the inflammatory process in IBD [64].

The driving force which regulates different aspects in intestinal inflammation is brought about by cytokines in the immunopathogenesis of IBD. The tissue damage in IBD and the disease progression is a result of the imbalance between the pro inflammatory and anti-inflammatory cytokines. The activated lamina propria cells in the tissues secrete a large number of soluble mediators including proinflammatory and anti-inflammatory cytokines. Currently several drugs targeting cytokines are being tested for their clinical efficacy against IBD. These cytokines have been intensely studied for potential therapeutic targets. Suppression of one cytokine may activate several other proinflammatory cytokine pathways in the patients as a result of imbalance. Therefore, using several cytokine inhibitors which could target multiple cytokines or their signal pathways simultaneously may prove beneficial in achieving a good clinical response in IBD patients [65]. Chemokines are implicated in IBD pathogenesis by regulating leukocyte trafficking. For IBD treatment, chemokine/chemokine-receptor antagonists serve as innovative therapeutic targets [66]. IBD patients have reported the increased levels of cytokines (TNF-α, IL-6, IL-1β) and Chemokine MCP-1[67].

1.2.3.c. Role of Zinc in IBD

Zinc (Zn) plays a fundamental role in immune response, wound repair and tissue regeneration. Zn deficiency in IBD occurs due to reduced food intake (anorexia), decreased absorption of food and increased loss of food [68,69]. Intestinal diseases such as IBD, that occur due to changes in the intestinal permeability can be modulated with Zn supplementation [70]. It controls cell turnover and repair systems. Restabilising Zn deficiency modulates tight junctions of the gut and restores intestinal permeability in CD patients [71].

Zn finger protein (A20) decreases progression of colitis by decreasing inflammation via inhibition of NF-κB, phosphorylated STAT3 and IL-17. Zn supplementation upregulated A20 inhibiting NF-κB activation and further decreased inflammatory cytokines generation [72,73]. Zn acts as a co-factor for endogenous enzymatic antioxidant defences. Zn supplementation raised the concentration of Zn-dependent enzymes (pSOD and eSOD) and plasma Zn [74]

In a cohort study with IBD patients, it was estimated that 8.5% CD and 2.9% UC patients had inadequate Zn intake [75]. Another study suggested that Zn deficiency and high-dose Zn supplementation reduced the rate of wound closure and mRNA levels of IL-1β and TNF-α and attenuated infiltration of neutrophils at the wound site compared with controls. Thus, adequate Zn supplementation is vital for inflammatory responses to hasten the wound healing [76].

1.2.3.d. Oxidative stress in IBD

Oxidative stress levels in IBD patients could be related to the severity of inflammation in the mucosa indicating its role in the pathogenesis of the disease.

Determining total antioxidant status, total oxidant status, oxidative stress index and paraoxonase1/arylesterase levels in IBD patients would help in assessing oxidative stress development in the host which arises due to disbalanced equilibrium between the antioxidant and oxidant mechanisms [77].

In IBD, the imbalance between ROS production and elimination occurs in the inflamed gut mucosa, deeper layers of intestinal wall and also systemic circulation. ROS and other species like reactive nitrogen species (RNS) and reactive sulphur species (RSS) participate in the inflammatory reactions of IBD. Nutritional malabsorption, gut motility disturbance and elevated intestinal permeability are a result of compromised gastrointestinal function due to long lasting exposure of inflamed intestinal mucosa to raised ROS levels [78].

Nitric oxide (NO) is involved in the inflammatory process development of IBD. Th1 and Th17 cytokines upregulate inducible nitric oxide synthase (iNOS) and leads to increased NO production. NO is associated with mucosal vasodilation, increased epithelial/vascular permeability and motility disorders which are observed in IBD. IBD patients with inflamed mucosa expressed increased concentration NO and iNOS, leading to the deterioration and exacerbation of immune responses [79,80]. ROS generation and uncontrolled lipid peroxidation in IBD is a result of severe oxidative stress. Polyunsaturated fatty acids on oxidation form a mixture of complex aldehyde products, such as alkanals, alk-2-enals, alka-2, 4-dienals, and malondialdehyde (MDA). Lipid peroxidation levels are estimated by MDA assessment [81]. Myeloperoxidase (MPO) is an enzyme that indicates neutrophil infiltration and it acts as a catalyst in ROS production. Mucosa of IBD patients display increased levels of both oxidative damage markers and MPO level [82].

In human cells antioxidant enzymes such as superoxide dismutase (SOD1), catalase (CAT) and glutathione peroxidase (GPx1) act as a first line of defence against damage caused due to ROS/RNS [83].

1.2.4. Colorectal cancer and IBD

Colorectal cancer (CRC) accounts for 10-15% mortality in IBD. The pro-neoplastic effects occurring in chronic intestinal inflammatory conditions in IBD patients provide a great risk of CRC. Risk factors of IBD related CRC includes disease period, extent/ severity of the disease, inflammatory pseudo polyps, coexistent primary sclerosing cholangitis and heredity. The development of IBD related CRC progresses through stages such as early/indefinite, low grade dysplasia (LGD), high grade dysplasia (HGD) and invasive adenocarcinoma [84].

IBD in CRC patients occurs at a younger age compared to sporadic CRC patients [85]. Rise in paediatric IBD cases suggests increasing disease burden, associated morbidity and rising healthcare costs. Its associated neoplastic transformation may significantly impact paediatric gastroenterology and adult CRC care [86].

1.2.5. Signs/symptoms and Scoring patterns

1.2.5.a. Ulcerative colitis –symptoms

Diarrhoea, passage of mucus, bloody mucus, bloody diarrhoea, cramps, rectal urgency, tenesmus, rectal bleeding and incomplete evacuation. The treatment and disease nature causes exacerbations and remissions in patients. The histologic examination demonstrates crypt distortion, basal plasma cell and lymphoid aggregates with focal haemorrhage in lamina propria [87].

Table 1. Disease activity in UC, adapted from Truelove and Witts [88].

	Mild	**Moderate**	**Severe**
Bloody stools/day	< 4	4-6	≥ 6 and
Pulse	< 90 bpm	≤ 90 bpm	>90 bpm or
Temperature	< 37.5ºC	≤ 37.8ºC	>37.8ºC or
Haemoglobin	> 11.5 g/Dl	≥10.5 g/dL	<10.5 g/dL or
Erythrocyte sedimentation rate	< 20 mm/h	≤30 mm/h	>30 mm/h or
C- reactive protein	Normal	≤ 30 mg/L	> 30 mg/L

Table 2. Mayo scores for UC [88].

Mayo score	**0**	**1**	**2**	**3**
Stool frequency	Normal	1-2/day > Normal	3-4/day > Normal	5/day > Normal
Rectal bleeding	None	Streaks	obvious	Mostly blood
Mucosa	Normal	Mild friability	Moderate friability	Spontaneous bleeding
Physicians global assessment	Normal	Mild	moderate	Severe

1.2.5.b. Crohn's Disease Symptoms

Chronic diarrhoea, (decrease in faecal consistency for more than four weeks.) which is the most common symptom, Abdominal pain (70 %), weight loss (60 %) and blood, mucus or both in stools (40-50 %) are also common features of CD. Most common extra intestine/ manifestation is primary peripheral arthritis (33%), aphthous stomatisis, uveitis, erythema nodosum and ankylosing spondylitis, pyoderma gangrenosum psoriasis and primary sclerosing cholangitis, fistulae [89].

A simple index for CD activity [90].

1. General wellbeing (0=very well, 1=slightly below par, 2=poor, 3= very poor, 4=terrible
2. Abdominal pain (0=none, 1=mild, 2= moderate, 3=severe)
3. No of liquid stools per day
4. Abdominal mass (0=none, 1=dubious, 2=definite, 3=definite and tender)
5. Complications: Arthralgia, uveitis, erythema, nodosum, aphthous ulcers, pyoderma, gangrenosum, abscess (score 1 per item)

1.2.6. Current treatment options of IBD

The backbone of therapy for induction and maintenance of IBD are the pharmacological agents with surgery as the option as when needed. The drug therapy choice is dependent on severity and location of the disease, the efficacy of the drug with a view to minimise the side effects of drugs. Owing to the recurrent nature of the disease, long term maintenance therapy is required [91].

Table 3. Established drug categories used in treatment of IBD [92-94].

Drug	Use	Mechanism of action	Side effects
Amino salicylates	Maintenance of remission in UC Their use in remission of CD is controversial	Blocks the production of Arachidonic acid metabolites, cytokines and reactive oxygen species	Side effects of sulfasalazine occur due to dose in 10-45% of UC patients which includes headache, nausea, fatigue, serious allergic reactions (rash, fever, Stevens johnsons syndrome, pancreatitis, agranulocytosis or alveolitis, inhibits intestinal folate absorption)
Corticosteriods	Used to treat mild to moderate active forms of UC and CD	Act through inhibition of several inflammatory pathways-interleukin suppression, arachidonic acid metabolism suppression and stimulates apoptosis of lymphocytes	Occur at dose higher than 40 mg/day and includes moon face, acne, edema and sleep/mood disturbances in early stages, over long term use unusual defects are observed, osteoporosis, osteonecrosis and increased susceptibility to infection is noticed
Immunosuppres ants	Azathioprine and 6-mercaptopurine is used in patients unresponsive to steroids and aminosalicylates, or in cases of relapse when steroids are withdrawn	Their metabolites inhibit purine synthesis and inhibit T cell proliferation	Headache, myalgia, nausea, diarrhoea in 3-5% patients, drug induced pancreatitis, leukaemia is rarely reported
	Methotrexate effective in inducing remission or preventing relapse of CD, especially cases which are refractory or intolerant to	It inhibits dehydrofolate reductase which inturn blocks DNA synthesis which further	Nausea, vomiting, stomatitis, leukopenia, pneumonitis and hepatic fibrosis. MTX is contraindicated in pregnancy because of severe side effects on both the foetus and the

	azathioprine and 6-mercaptopurine	leads to cell death	course of the pregnancy.
	Cyclosporine- used in the treatment of UC who are refractory to corticosteroid therapy	It is a calcineurin inhibitor which inhibits the release of IL-2 and prevents formation of ctotoxic lymphocytes	Tremors, headache, gum hyperplasia and hirsutism
Biologicals	Infliximab- UC, CD with moderate and severe activity	Binds to TNF-α and neutralises activity	Urticaria, flushing, headache, contraindicated in patients with CHF, rarely develops lupus like syndrome.
	Adalimumab- UC and CD not responsive to conventional therapy	Binds to TNF-α receptor but does not bind to TNF-β receptors	Injection site pain, upper respiratory tract infection, increased creatine phosphokinase, headache, rash, sinus infection, nausea, urinary tract infection
Antibiotics	Metronidazole- most widely used antibiotic, especially in treating perianal CD	Active against parasites and anaerobic bacteria	short-term side effects in around 50% patients (gastrointestinal intolerance, metallic taste, reaction to alcohol), polyneuropathy secondary to metronidazole limits long-term use
	Ciprofloxacin	Selective suppressive effect on the intestinal micro flora.	Tendonitis and Achilles tendon rupture. The most frequent side effects reported are of gastrointestinal origin; skin reactions and an increase in transaminase level

1.2.7. Alternative medicine

The choice of treatment for chronic inflammatory diseases for many years has been modern system of medicine. Nowadays Western countries as well as many Asian countries like China and India use herbal therapy for IBD management. Natural products are being increasingly utilized as alternative or complimentary IBD therapy. Their use continues to rise even though there are no sufficient clinical trials to support the claims of their safety and efficacy. Research however suggests that these natural substances target various mechanisms such as antioxidant pathway and cell signaling, pro-inflammatory mediators, and alteration of bacterial flora thereby disrupting the proinflammatory cascade and maintaining the immune balance [95].

1.2.8. Some medicinal plants under investigation for the treatment of IBD

Table 4. Some Medicinal plants investigated for IBD management [96-99]

Plant	Observed parameters	Conclusion
Punica granatum L. (2019)	Fecal calprotectin, C-reactive protein, erythrocyte sedimentation rate, IL-1β, IL-6, IL-8, IL-10, and TNF-α, Trimethylamine *N*-oxide	The primary outcome is change in the faecal neutrophil-derived protein calprotectin, a surrogate marker of mucosal improvement. The secondary outcomes include transcriptomic changes in peripheral blood mononuclear cells and intestinal biopsies and changes in circulating inflammatory markers and trimethylamine-*N*-oxide levels.
Zingiber officinale (2019)	Malondialdehyde, serum total anti-oxidant capacity. Severity of disease activity was assessed	Data indicates that ginger supplementation improved treatment of patients with UC. Further clinical trials with different dosages and duration of ginger or its standard extract supplementation are needed to obtain firm conclusion.
Cannabis (marijuana) (2014)	Information regarding demographics (age, sex, educational level, income levels, and ethnic background) was gathered and the clinical characteristics of their disease including diagnosis (CD, UC, or indeterminate colitis), years with IBD, number of flares, cardinal symptoms, previous hospital stays and surgeries, medication use in the last 12 months, and severity of their disease was noted	Cannabis use is common in patients with IBD and subjectively improved pain and diarrheal symptoms. However, Cannabis use was associated with higher risk of surgery in patients with CD.

Achillea wilhelmsii C Koch. (2019)	The disease activity index, Partial Mayo Score, haemoglobin, platelet count, erythrocyte sedimentation rate and serum level of C-reactive protein were measured at the entry and the end of the treatment.	Oral administration of *A. wilhelmsii* powder for 4 weeks did not create a clinical response more than placebo. It seemed to be safe in UC patients.

1.2.9. Plant profile

Terminalia arjuna (Roxb) Wt. and Arn. is a member of the family of Combretaceae. Its conventional names include: Arjuna, Nadisarja, Indradru Kakubha, Dhavala, Veeravriksha and Partha. It is found in lower Himalayas, Bihar, Bengal, Chota Nagpur, Central and Southern India and Ceylon [100,101].

Table 5. Taxonomical classification of *Terminalia arjuna* [102]

PLANT PROFILE	
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	Combretaceae
Genus	*Terminalia*
Species	*arjuna*

Ethnomedical considerations

Arjuna bark was used in Kangra region of Punjab to cure sores. In Assam heart diseases were treated by Golaghat tribes. Leucorrhoea was treated by the tribes of Andhra Pradesh. Paste of Arjuna and *Nyctanthes arbortristis* L. barks was used to treat internal injuries and broken bones from falls [103].

It has been used to treat various ailments in Ayurveda, the traditional system of medicine of Indian subcontinent, by regulating a balance among the three humours *vata, pitta* and *kapha***,** and was widely known for its cardiotonic property [100].

It is used in gastrointestinal disorders such as antidysenteric, purgative and laxative and other ailments like urinary discharge, strangury, leucoderma, anaemia, ulcers, tumors, hyperhidrosis and asthma [104].

Newer Research Findings

Terminalia arjuna (TA) (Roxb.) aqueous extract showed protective effects on Isoproterenol-induced cardiac hypertrophy in rats. It modulates genes responsible for metabolism, cell signalling, receptor activation, cardiovascular functions and other diseases [105].

TA extract increased alkaline phosphatase and calcium release in bone substitutes compared with control. The extract helped in *in- vitro* biomineralization. It also influenced cell differentiation. Suggested to be used as low-cost alternative for growth factors for treatment of fractured bones [106].

TA effect was studied on total platelet count, lipid profile, clinical parameters in patients of coronary artery disease. Significantly reduced blood pressure and altered lipid profile was observed in this study population [107].

A study based on tribal claim was conducted for assessing analgesic property of TA in Swiss albino mice by tail flick method, acetic acid-induced writhing reflex and formaldehyde-induced paw licking method. Statistically significant analgesic activity was observed which provided a scientific validation of the tribal claim [108].

TA hydroalcoholic extract and its three fractions were assessed for wound healing property in rat model on topical application. This study exhibited maximum tensile strength of the incision wounds was exhibited by the fraction consisting of tannins. The percent epithelialization of excision wounds was also maximum in this case. This tannin fraction did not possess activity against *Candida albicans* but displayed antimicrobial activity against microorganisms such as *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* [109].

TA exhibited anti-inflammatory effects in formalin induced paw edema model. Its immunomodulatory effects were evaluated by the increase in anti- sheep red blood cell antibodies [110].

The aqueous bark extract is an antioxidant which also downregulated anaerobic metabolism in lymphoma bearing mice [111]. and cytotoxic in Hepatocellular carcinoma cells [19]. Casuarinin, a tannin from TA bark induced cell cycle arrest in G0/G1 phase and apoptosis in human lung cancer cell line [112].

TA bark also modulated the lipid profiles of hypercholesterolemic diet fed rabbits [113]

Arjunarishta

Arishtas are made with decoctions of herbs in water. These are unique liquid formulation that contains self-generated alcohol. Its therapeutic efficacy and stability makes it valuable in Ayurveda.

The dried plant materials, dried flowers of *Woodfordia fruticosa* (L.) Kurz (Lythraceae) and sugar source are allowed to ferment under anaerobic conditions in an airtight container. This fermentation process increases potency and absorption rate however decreases the toxicity of the plant material in the arishta preparation which is brought about by several transformation reactions during the process. Arjunarishta (AA) is a frequently used oral arishta having cardiotonic effect with *Terminalia arjuna* as an active constituent. The other ingredients used in preparing AA are *Madhuca indica, Woodfordia fruticosa Vitis vinifera,* and *Saccharum officinarum*. Its cardiotonic activity is through regulation of cholesterol and the blood pressure [114].

AA administration in High fat diet fed rats reduced fasting blood glucose, systolic blood pressure, total cholesterol and triglyceride levels. The anti-hyperglycemic and anti-hyperlipidemic effect of AA may be a result of decreased expression of TNF-α and insulin sensitizer genes (insulin receptor substrate-1 and peroxisome proliferators activated receptor γ coactivator 1-α) [115].

1.2.10. TNBS induced colitis model

TNBS induced colitis is a widely used animal model that shares significant characteristics with human CD with advantages of reproducibility simplicity and low cost.

Pathophysiology of TNBS induced colitis model

Ethanol and TNBS is co-administered intrarectally to rats at a dose of 125 mg/kg. Ethanol disrupts the intestinal barrier lining, which allows the TNBS to interact with the proteins in the colonic tissue. TNBS, serves as a hapten which when coupled with high molecular weight proteins exhibit immunologic reactions by rendering those proteins immunogenic to the host immune system. A single episode of co-administrating ethanol and TNBS, leads to the development of an excessive cell mediated immune response, demonstrated through acute Th1 mediated inflammation [11].

Clinical course of TNBS – Colitis

Intracolonic administration of TNBS/ethanol causes inconsistent stool formation, occult or bloody diarrhoea, and significant weight loss during the first week. After one week, body weight slowly increases, however the diarrhoea still persists longer. Other nonspecific signs include piloerection of fur and decreased animal movements. The onset and severity of these symptoms varies depending on the specific species or strain used and the dose of TNBS and ethanol utilised. The severity of the symptoms is reflected on the scores that quantify the level of inflammation in the gut. TNBS colitis histology shows mucosal and submucosal infiltration by, lymphocytes / macrophages polymorphonuclear leukocytes, mast cells and fibroblast cells, ulceration, mucosal edema, necrosis and bleeding [116].

1.3. Aim and Objectives

1. To study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta against IBD related microorganisms.

2. To study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta on colorectal cell lines.

3. To study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta on TNBS induced inflammatory bowel disease in rats.

2. MATERIAL AND METHODS

2.1. Materials

(Details are cited in 'Annexure I')

2.1.1. List of chemicals and materials used for experiments is given below:

Table 6. List of chemicals and materials

Sr. No.	Particulars	Make/Source
1	TA bark	KLE Society's Ayurved Pharmacy, (collected in February 2015 from the Western Ghats, Belagavi region, Karnataka – India) The authentication of T. arjuna was performed by AYUSH approved ASU drug testing laboratory at Shri BM Kankanwadi Ayurveda Mahavidyalaya, Belagavi, Karnataka–India and assigned the voucher number CRF/645/2015).
2	Arjunarishta (AA)	Sandu Pharmaceuticals Ltd., Mumbai – India (Batch no. 25)
3	HPLC grade methanol	Merck, Life Science Pvt. Ltd, Mumbai – India
4	Water	Milli-Q (Millipore)
5	HPLC grade acetonitrile	Merck, Life Science Pvt. Ltd, Mumbai – India

6	Oxiselect™. TBARS Assay Kit	Cell Biolabs, Inc, San Diego, CA, U.S.A. TNBS was purchased from Sigma-Aldrich, U.S.A.
7	Trizol ® reagent	Sigma-Aldrich
8	Hemospot kit.	Coral Clinical Systems, Tulip Diagnostics (P) Ltd. Verna, Goa – India
9	Culture media (for the bacteriological study)	HiMedia Laboratories Pvt. Ltd
10	Four clinical bacterial isolates - *E.coli* HM95 (AIEC), *E.coli* HM615 (colonic mucosa associated. *E.coli*), *E.coli* HM233 and *E.coli* HM251 (colonic mucus associated patient strains)	Received under Material Transfer Agreement with University of Liverpool, United Kingdom.
11	Human colorectal adenocarcinoma cells (Caco2, COLO.205) and normal rat intestinal cells (IEC-6) and mouse fibroblast cells (L929)	National Center for Cell Sciences, Pune-India.

2.1.2. List of equipment used for experiments

Table 7. List of equipment used in the study

Sr. No.	Particulars	Make/Source
1	Rotary evaporator	Buchi Rotavapor
2	HPLC system	Dionex P680
3	Step one real-time PCR system	Applied Biosystems
4	Compact-MG Anaerobic workstation	Kim microsystems
5	UV Spectrophotometer	UV-1800, Shimadzu, Japan
6	Atomic Absorption Spectrophotometer	AA-7000; Shimadzu, Japan
7	NanoDrop® Spectrophotometer	JH BIO, U.S.A.

2.2. Experimental Design

The plan of work was as follows-

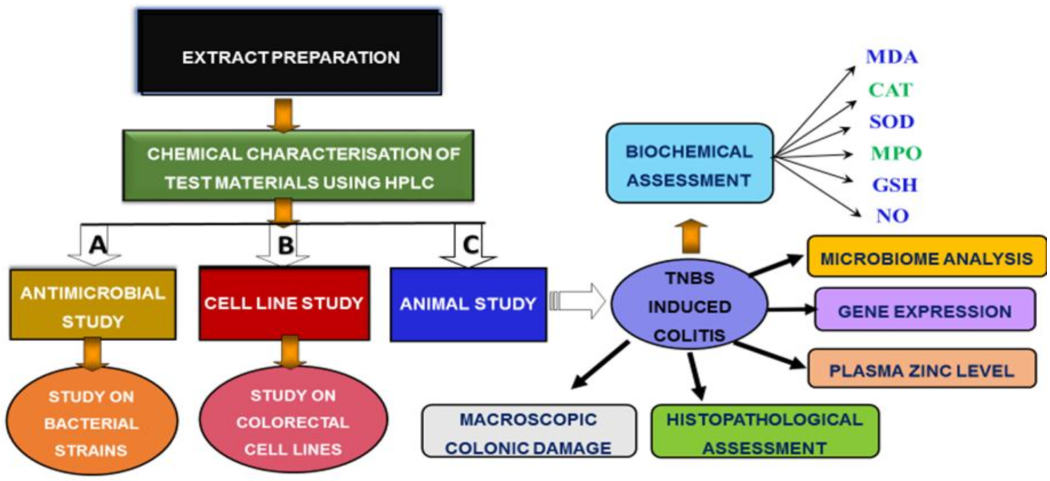

Figure 5. Study plan

2.3. Preparation of plant extract and preliminary phytochemical analysis

The dried bark of TA was powdered and extracted with ethanol: water (70:30 v/v) using cold maceration method in a conical flask.

For detailed methodology please refer 'Annexure I'

2.4. In vitro quantitative antioxidant assay of TAHA and AA

2.4.1. Total phenolic content (TPC)

For detailed methodology please refer 'Annexure I'

2.4.2. Total flavonoid content (TFC)

For detailed methodology please refer 'Annexure I'

2.4.3. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

For detailed methodology please refer 'Annexure I'

2.4.4. Ferric Reducing Antioxidant Power (FRAP) Assay

For detailed methodology please refer 'Annexure I'

2.5. HPLC-PDA Analysis: TAHA and AA

For detailed methodology please refer 'Annexure I'

2.6. Antibacterial activity

Four clinical bacterial isolates - *E.coli* HM95 (AIEC), *E.coli* HM615 (colonic mucosa associated. *E.coli*), *E.coli* HM233 and *E.coli* HM251 (colonic mucus associated patient strains) were used to test the antibacterial potency of the test materials.

For detailed methodology please refer 'Annexure I'

2.6.1. Agar well diffusion assay method

For detailed methodology please refer 'Annexure I'

2.6.2. Serial dilution method

For detailed methodology please refer 'Annexure I'

2.7. Cell line studies

For detailed methodology please refer 'Annexure I'

2.7.1. Determination of cytotoxicity and cytocompatibility

For detailed methodology please refer 'Annexure I'

2.7.2. Lipid peroxidation in cells

For detailed methodology please refer 'Annexure I'

2.8. Animal study

2.8.1. Ethical Approval

Female Wistar rats (180-200 g) obtained from Shri Venkateshwara Enterprises (Bangalore, India) after approval by Institutional Animal Ethics Committee of College of Pharmacy-KLE Academy of Higher Education and Research, Belagavi (resolution No. KLECOP / CPCSEA- Reg. No. 221/Res. 23-3 / 09 / 2016). The CPCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals) were followed throughout the experiment. The rats were housed in polypropylene cages and maintained at 22 ± 2 °C, under standard lighting conditions (12- h light/dark cycle).

2.8.2. Trinitrobenzene sulphonic acid (TNBS) model of rat colitis

After a period of acclimatization, the rats were randomly divided into nine groups consisting of eight animals in each group each described in Table 8.

For detailed methodology please refer 'Annexure I'

Table 8. Grouping for Animal study

Group 1	Normal saline administration
Group 2	TNBS control
Group 3	TNBS + 2 mg/kg Prednisolone
Group 4	TNBS + 500 mg/kg TAHA
Group 5	TNBS + 250 mg/kg TAHA
Group 6	TNBS + 125 mg/kg TAHA
Group 7	TNBS + 1.8 mL/kg AA
Group 8	TNBS + 0.9 mL/kg AA
Group 9	TNBS + 0.45 mL/kg AA

2.8.3. Macroscopic Scores in colitis model

Scoring pattern for inflammation based on earlier report is depicted in Table 9.

Table 9. Macroscopic damage scores

Score	Observations
0	No macroscopic change
1	Mucosal erythema alone
2	Mild mucosal edema, slight bleeding or small erosions
3	Moderate edema, bleeding ulcers or erosions
4	Severe ulceration / erosions, edema, and tissue necrosis

2.8.4. Histopathological scoring pattern of colitis

Previously reported histopathological scoring pattern was adopted for evaluation as depicted in Table 10.

For detailed methodology please refer ‘Annexure I’

Table 10. Histopathological scoring pattern in colon tissue

Histopathological conditions	**Scoring pattern**		
	'0'	**'1'**	**'2'**
Infiltration of acute inflammatory cells	No	Mild increasing	Severe increasing
Infiltration of chronic inflammatory cells	No	Mild increasing	Severe increasing
Deposition of fibrotin protein	Negative	Positive	-
Submucosa edema	No	Patchy – like	Fusion - like
Epithelium necrosis	No	Limiting	Widening
Epithelium ulcer	Negative	Positive	-

2.8.5. Biochemical assays

2.8.5.a. Myeloperoxidase (MPO) Assay

For detailed methodology please refer 'Annexure I'

2.8.5.b. Malondialdehyde (MDA) Assay

For detailed methodology please refer 'Annexure I'

2.8.5.c. Catalase Assay

For detailed methodology please refer 'Annexure I'

Table 11. Procedure for Catalase

Reagents	Sample (µL)	Blank 1 (µL)	Blank 2 (µL)	Blank 3 (µL)
Plasma	50	50	-	-
Substrate (H_2O_2)	1000	1000	1000	-
Phosphate Buffer	-	-	50	1050
Ammonium Molybdate	1000	1000	1000	1000

2.8.5.d. Superoxide Dismutase (SOD) Assay

For detailed methodology please refer 'Annexure I'

Table 12. Procedure for Superoxide Dismutase Assay

Reagents	Test (µL)	Control (µL)
Serum	50	-
Tris buffer	1000	1000
d.$H_2$0	-	50
Pyrogallol	1000	1000

2.8.5.e. Glutathione (GSH) (Reduced) Assay

For detailed methodology please refer 'Annexure I'

2.8.5.f. Nitric Oxide Estimation Assay

For detailed methodology please refer 'Annexure I'

2.8.6. Plasma zinc estimation

Zinc level was estimated using Atomic Absorption Spectrophotometer (AA-7000; Shimadzu, Japan). Plasma samples were allowed to thaw at room temperature. Working standards of zinc were prepared using standard zinc solution (S. D. Fine Chem. Ltd., Mumbai, India) and samples were diluted with de-ionized water. The instrument, gas-flow setting and aspiration rate was precisely established, to optimize signal and minimize background noise. The zinc concentration in the samples was calculated from absorbance readings, by interpolation from the working curve and the results were expressed in milligram/deciliter.

2.8.7. Analysis of gene expression by real-time polymerase chain reaction

Total RNA was isolated from colon samples. The primer sequences used in gene expression study are shown in Table 13.

Table 13. Primer sequences used in RT-PCR assays in colonic tissue

Primers	Sequences (5'-3')	Annealing temperature (°C)
IL-6	Forward AACTCCATCTGCCCTTCAGGAACA Reverse AAGGCAGTGGCTGTCAACAACATC	62.7
IL-1β	Forward AGCAGCTTTCGACAGTGAGGAGAA Reverse TCTCCACAGCCACAATGAGTGACA	62.7
MCP-1	Forward TGCTGTCTCAGCCAGATGCAGTTA Reverse TACAGCTTCTTTGGGACACCTGCT	62.7
TNF-α	Forward AGAACAGCAACTCCAGAACACCCT Reverse TGCCAGTTCCACATCTCGGATCAT	62.7

2.8.8. Bacteriological analysis of feces

For detailed methodology please refer 'Annexure I'

3. STATISTICAL ANALYSIS

All *in vitro* determinations were carried out in triplicate. The results are presented in the form of Mean ± standard deviation (SD). Calculation of IC50 value was carried out using GraphPad Prism 7 for Windows.

Animal study data obtained are expressed as the mean ± standard error of mean (SEM). Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) with Tukey post hoc test. Differences were considered statistical significant when $p \leq 0.05$ (GraphPad Prism version 5.01 software, San Diego, CA, U.S.A.)

4. RESULTS

4.1. Preliminary phytochemical screening

TAHA prepared by cold maceration process and AA were tested for its phytochemicals. The phytochemical evaluation of TAHA and AA displayed presence of proteins, steroids, flavonoids and tannins which are known to have several pharmacological effects. TAHA demonstrated presence of alkaloids, however were absent in AA. The Results of phytochemical tests are summarized in Table 14.

4.2. Antioxidant potential

Folin-Ciocalteu method was utilized to estimate the TPC in test materials and it was expressed as TAE calculated from the calibration curve (R^2= 0.991). TPC was seven TAHA (502.6 mg TAE/g) exhibited TPC value seven-fold higher as compared to AA (79.53 mg TAE/g). Standard quercetin calibration curve (R^2= 0.994) was used to evaluate TFC in test materials, where TAHA demonstrated a value of 488.25 mg QE/g and AA was 62 mg QE/g.

The DPPH and FRAP assay were used in this study to demonstrate the antioxidant potential of test materials (Table 15, Figure 6, 7). TAHA antioxidant activity was higher compared to AA. In DPPH assay, Gallic acid antioxidant potential was higher compared to TAHA. However, antioxidant potential of TAHA was greater than Gallic acid in the FRAP assay.

Table 14. Preliminary phytochemical screening of *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA)

TESTS	TAHA	AA
Carbohydrates	Positive	Positive
Pentose sugars	Negative	Positive
Hexose sugars	Positive	Positive
Proteins	Positive	Positive
Steroids	Positive	Positive
Flavonoids	Positive	Positive
Alkaloids	Positive	Negative
Tannins	Positive	Positive
Glycoside		
a. Saponin Glycoside	Positive	Positive
b. Coumarin Glycosides	Negative	Negative
c. Cardiac Glycosides	Positive	Positive

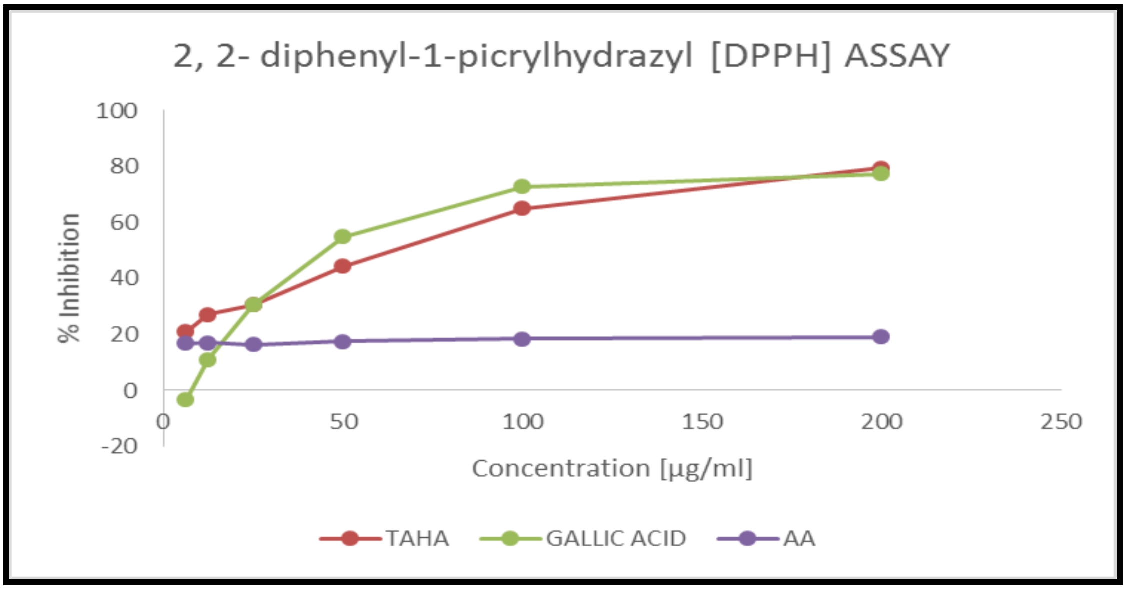

Figure 6. Comparative DPPH radical scavenging activity of *Terminalia arjuna* Hydro-alcoholic extract (TAHA), Arjunarishta (AA) and Gallic acid.

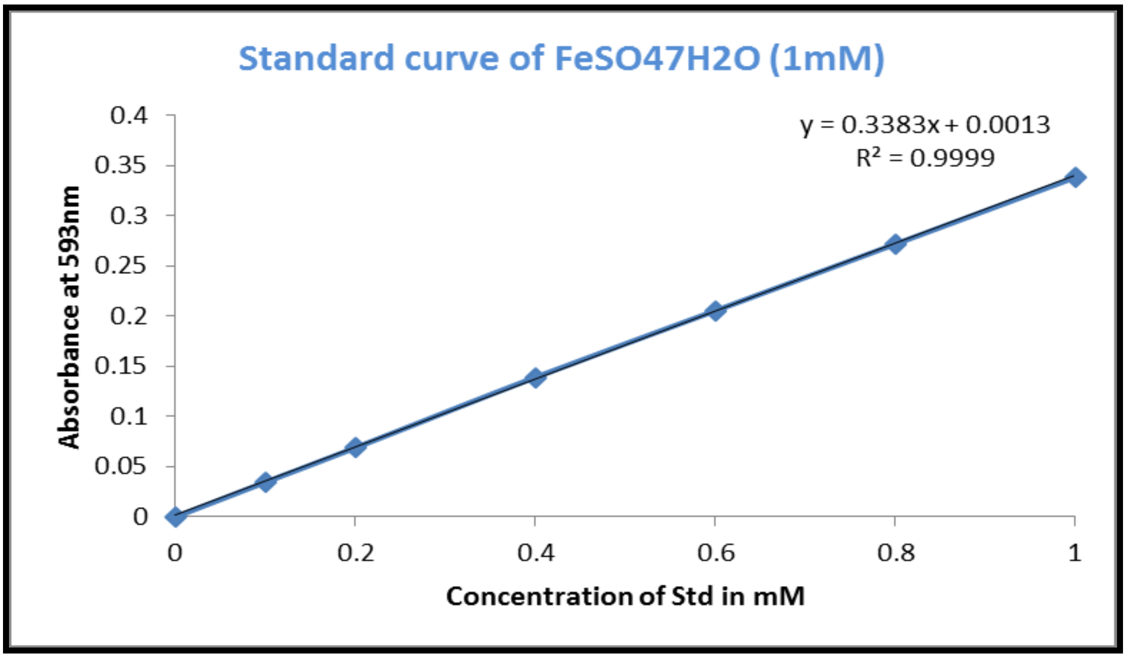

Figure 7. Calibration curve of $FeSO_4 7H_2 0$

Table 15. Antioxidant activity of *Terminalia arjuna* Hydro-alcoholic extract (TAHA), Arjunarishta (AA) and Gallic acid

Test Materials	**DPPH activity (IC_{50} µg/mL)**	**FRAP activity (mM $Fe2^{+}$ /g)**
TAHA	51.31 ± 1.20	1907.00 ± 0.83[a]
AA	1025.00 ± 1.12[a]	290.00 ± 1.19[a]
Gallic Acid	49.89 ± 1.05	1643.00 ± 1.34

Data are expressed as mean ± SD of three individual determinations. The data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Compared with standard-gallic acid, [a] <0.05; DDPH: 2, 2-diphenyl -1-picrylhydrazyl; FRAP: ferric reducing ability of plasma.

4.3. HPLC-PDA Analysis

Phytochemical standardization of TAHA and was carried out using selected marker-based approach. Polyphenolic compounds such as gallic acid, ellagic acid, and quercetin were used as chemical markers for standardization purpose. The marker contents were estimated using earlier reported HPLC-PDA method suitably modified with respect to column and mobile phase gradient. The optimized chromatographic conditions showed good resolution of the all the peaks. The presence of marker contents in TAHA and AA was identified using retention time (R_t) and UV spectra matching with corresponding reference standards (Figure 8-9). The R_t for gallic acid, ellagic acid, and quercetin were found to be 2.36, 24.71 and 31.98 min respectively. Quantitative estimation of these marker compounds was carried out using external standard calibration method.

The content of gallic acid, ellagic acid, and quercetin was 2.443 ± 0.090, 7.901 ± 0.786 and 3.20 ± 0.351 mg/g respectively in TAHA whereas 92.18 ± 1.184, 244.6 ± 8.676 and 15.82 ± 1.832 µg/mL respectively in AA (Table 16). These quantitative estimations were consistent with earlier reports on AA and TAHA.

Table 16. Retention time and relative concentration of polyphenols in *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA)

Sr. No.	Compounds	R_t (min)			(mg/g of TAHA)	(µg/mL of AA)
		Reference Standard	TAHA	AA		
1	Gallic Acid	2.362	2.394	2.248	2.443 ± 0.090	92.18 ± 1.184
2	Ellagic Acid	24.711	24.709	24.720	7.901 ± 0.786	244.6 ± 8.676
3	Quercetin	31.987	31.974	31.944	3.20 ± 0.351	15.82 ± 1.832

Data are expressed as mean ± SD of three individual determinations.

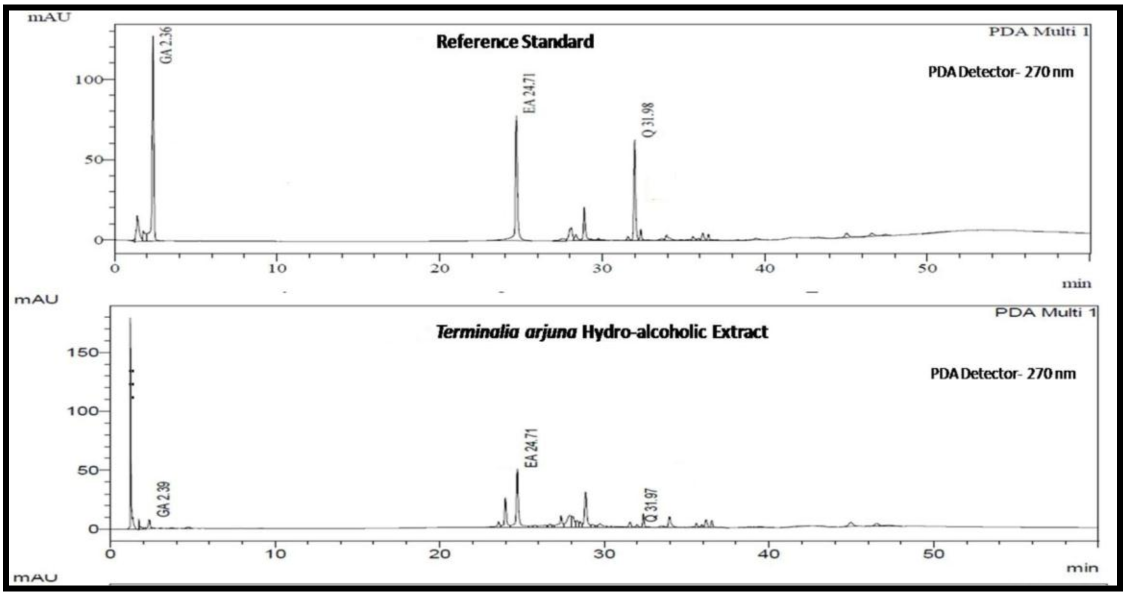

Figure 8. HPLC chromatogram of Reference standard polyphenols: Gallic acid, Ellagic acid, Quercetin and of *Terminalia arjuna* Hydro-alcoholic extract (TAHA)

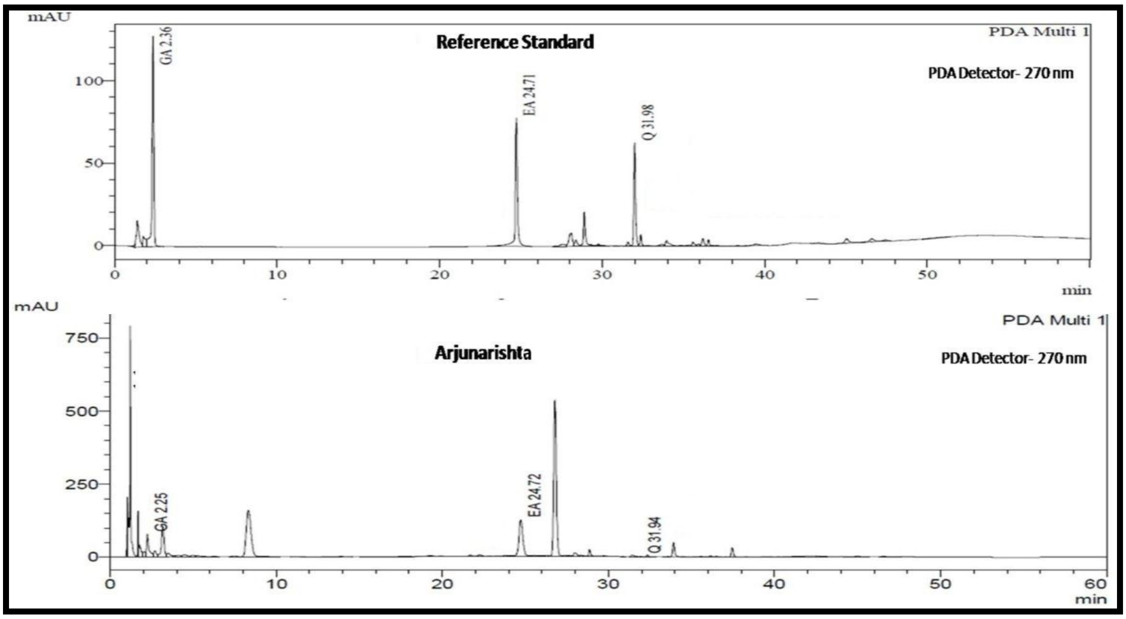

Figure 9. HPLC chromatogram of Reference standard polyphenols: Gallic acid, Ellagic acid, Quercetin and Arjunarishta (AA)

4.4. Antibacterial assay

The antibacterial activity results of TAHA and AA against the four IBD clinical isolates were depicted by their zone of inhibition and MIC values which are listed in Table 17 and Table 18 respectively. AA did not demonstrate an inhibitory zone. In the well diffusion assay, only the zone of inhibition by TAHA at both doses against HM95 showed no significant difference when compared to the standard ciprofloxacin inhibitory zone. The MIC and MBC values of TAHA, AA and ciprofloxacin remained constant against all the four study strains i.e. 6.25 mg/mL, 12.5%, 1.25 µg/mL as MIC and 25 mg/mL, 50%, 5 µg/mL as MBC respectively.

Table 17. Antimicrobial activity of *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) by agar well diffusion method

Microbial Strains	Zone of inhibition (mm)		
	TAHA (50 mg/mL)	**TAHA (25 mg/mL)**	**Ciprofloxacin**
HM95 (CD)	22.67 ± 0.58	21.33 ± 1.53	21.00 ± 1.00
HM233 (UC)	24.67 ± 0.58 [a]	21.00 ± 1.00 [a]	27.00 ± 1.00
HM251 (UC)	24.00 ± 1.00 [a]	22.67 ± 1.53 [a]	29.67 ± 0.53
HM615 (CD)	24.33 ± 0.58 [a]	22.33 ± 1.16 [a]	20.33 ± 0.58

Data are expressed as mean ± SD of three individual experiments. The data were analysed by one-way ANOVA followed by Dunnett's test. Compared with standard-ciprofloxacin for respective microbial strain, [a] $p<0.05$.

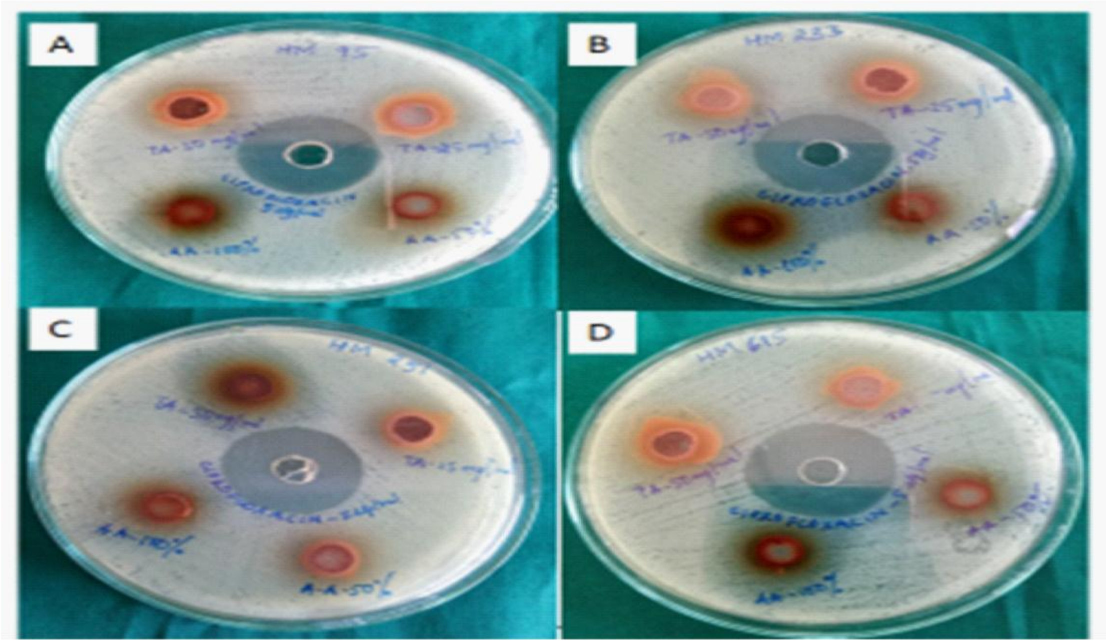

Figure 10. Zone of inhibition of *Terminalia arjuna* Hydro-alcoholic extract (TAHA), Arjunarishta (AA) and Ciprofloxacin against (A) HM95, (B) HM233, (C) HM251, (D) HM615

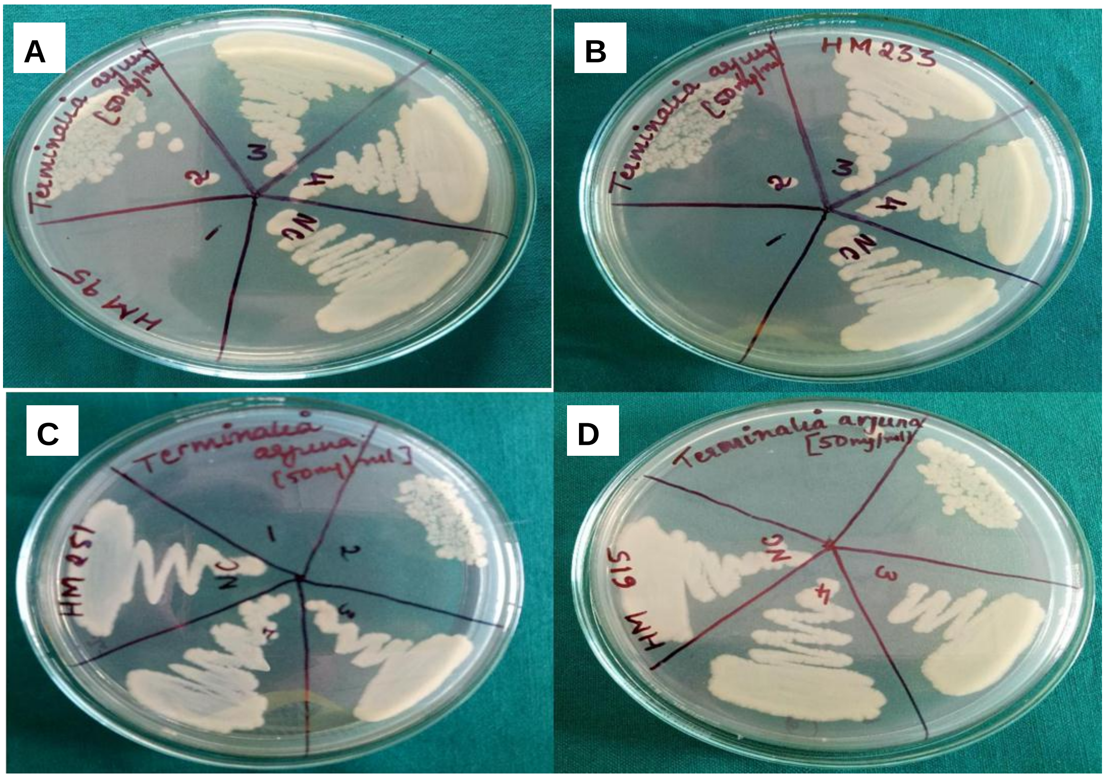

Figure 11. MBC of *Terminalia arjuna* hydro-alcoholic extract (TAHA) against (A) HM95, (B) HM233, (C) HM251, (D) HM615

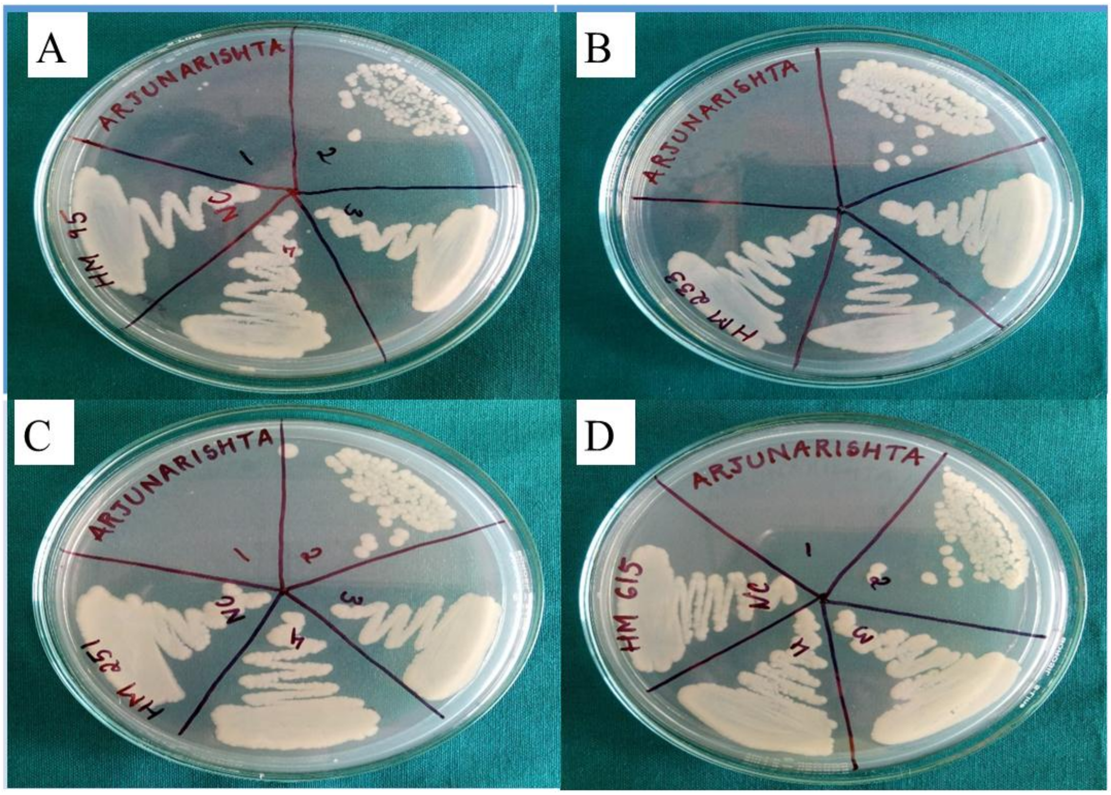

Figure 12. MBC of Arjunarishta (AA) against (A) HM95, (B) HM233, (C) HM251, (D) HM615

Table 18. Minimum Inhibitory Concentration (MIC) values of *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA)

Microbial Strains	TAHA (mg/mL)	AA (%)	Ciprofloxacin (μg/mL)
HM95 (CD)	6.25	12.5	1.25
HM233 (UC)	6.25	12.5	1.25
HM251 (UC)	6.25	12.5	1.25
HM615 (CD)	6.25	12.5	1.25

4.5. Cytotoxicity and Cytocompatibility

In this study both test materials tested at concentrations ranging from 400-12.5 µg/mL and 5-fluorouracil (5-FU) from 100-1.562 µg/mL demonstrated a decrease in percent cell viability in the cytotoxicity assay. MTT assay was utilized to assess the cytotoxicity potential of the test compounds. Two human colorectal adenocarcinoma cells, COLO.205 and Caco2 were used to determine the cytotoxicity potential. Whereas, L929 (Mouse fibroblast cells) and IEC-6 (Rat intestinal cells) using colorimetric MTT assay were utilized to conduct the cytocompatibility assay. On 48 h treatment on cells, TAHA, AA and 5-FU demonstrated a concentration-dependent deduction in percent cell viability (Table 19) and 50% cell viability of the cells noted as the IC_{50} value were estimated. Test materials displayed good cytocompatibility against the study cell lines (Figure 13, 14). Similarly, 5-FU was also analysed for cytocompatibility as per IC_{50} value (Figure 15).

Table 19. Effect of TAHA, AA, and 5-FU treatment on cell lines

Sample (µg/mL)		12.5	25	50	100	200	400	IC_{50} (µg/mL)
TAHA	COLO.205	98.82 ± 0.36	97.81 ± 0.30 [a]	94.42 ± 0.50 [a]	51.76 ± 1.06 [a]	33.39 ± 0.86 [a]	30.74 ± 1.72 [a]	**145.3 ± 0.53**
	Caco2	88.25 ± 0.44 [a]	73.03 ± 0.31 [a]	53.70 ± 1.11 [a]	48.77 ± 0.38 [a]	42.00 ± 0.41 [a]	37.00 ± 1.99 [a]	**90.4 ± 0.66**
AA	COLO.205	97.49 ± 2.61	97.13 ± 3.25	94.31 ± 0.06 [a]	69.50 ± 1.38 [a]	40.63 ± 1.01 [a]	30.03 ± 2.52 [a]	**183.6 ± 1.19**
	Caco2	87.67 ± 0.48 [a]	83.93 ± 0.95 [a]	70.11 ± 0.96 [a]	53.97 ± 0.67 [a]	47.79 ± 0.98 [a]	40.00 ± 1.00 [a]	**182.9 ± 0.21**
Sample (µg/mL)		**3.12**	**6.25**	**12.5**	**25**	**50**	**100**	**IC_{50} (µg/mL)**
5-FU	COLO.205	79.23 ± 1.07 [a]	68.00 ±1.27 [a]	58.18 ± 0.67 [a]	50.63 ± 0.88 [a]	38.07 ± 2.02 [a]	31.14 ± 1.03 [a]	**24.12 ± 1.29**
	Caco2	83.00 ± 2.11 [a]	76.00 ± 1.59 [a]	67.28 ± 1.91 [a]	55.18 ± 1.09 [a]	42.27 ± 2.17 [a]	30.34 ± 1.67 [a]	**32.42 ± 0.78**

Data are expressed as mean ± SD of three individual experiments. The data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Compared with control (considered as 100 %), [a] $p<0.05$.

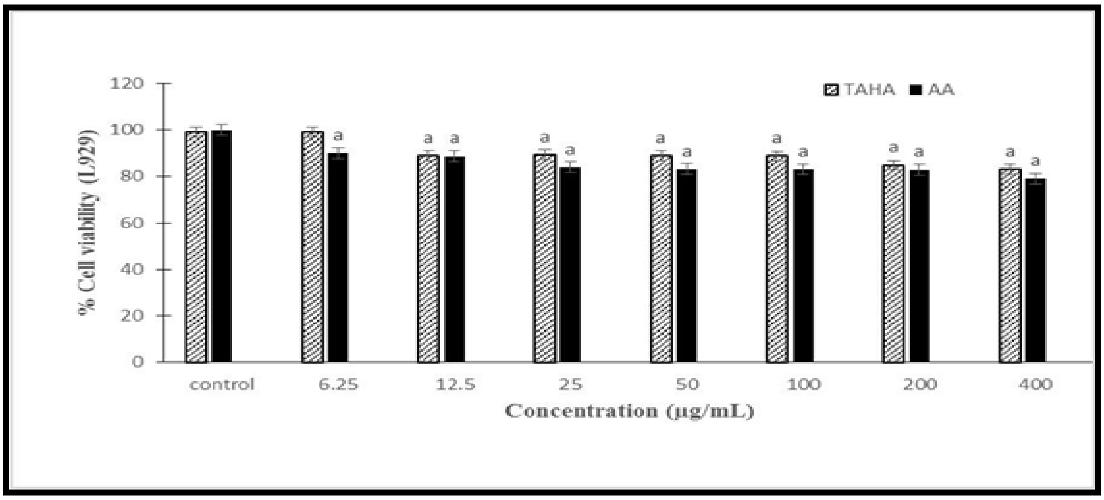

Figure 13. Cytocompatibility of *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) against L929 Cells derived from mouse fibroblast. Cytocompatibility evaluated by % cell viability considering viability of control as 100% (expressed as Mean ± SD of three experiments). Alphabet 'a' represents significant differences in mean ($p<0.05$) compared to control group.

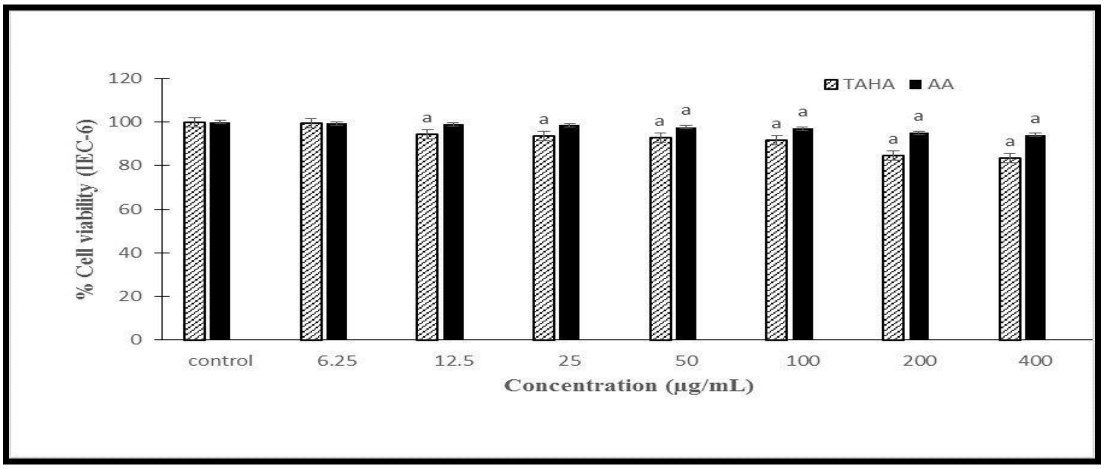

Figure 14. Cytocompatibility of *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) against IEC-6 Cells derived from rat intestinal epithelium. Cytocompatibility evaluated by % cell viability considering viability of control as 100% (expressed as Mean ± SD of three experiments). Alphabet 'a' represents significant differences in mean ($p<0.05$) compared to control group.

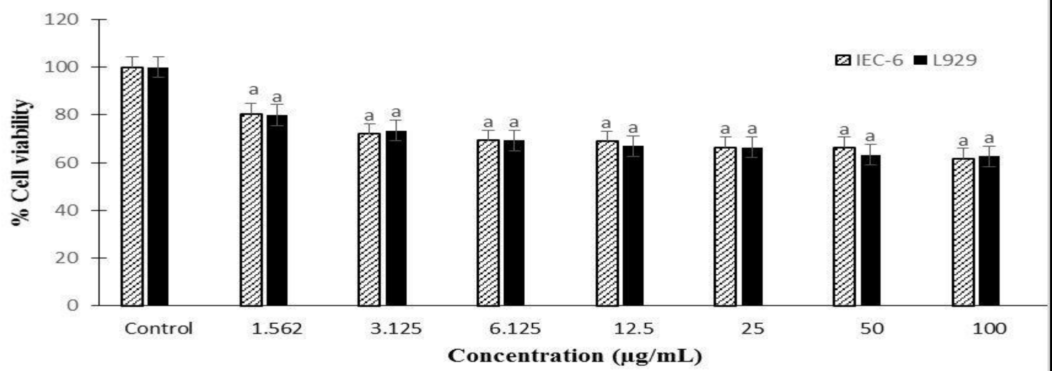

Figure 15. Cytocompatibility of 5-FU against IEC-6 and L929 Cell lines. Cytocompatibility evaluated by % cell viability considering viability of control as 100%, expressed as Mean ± SD of three experiments. Alphabet ‘a’ represents significant differences in mean ($p<0.05$**)** compared to control group.

4.6. Lipid peroxidation in Cell lines

The standard calibration curve of MDA with R^2 value 0.9985 is depicted in Figure 16. The MDA content in each group on exposure to TAHA, AA and 5-FU have been noted in Table 20. Normal cells (IEC-6 and L929 cells) treated with TAHA and AA did not exhibit a significant change in MDA level when compared to its control group. However, 5-FU exposure resulted in significantly ($p<0.05$) increased MDA concentration compared to the control, TAHA, AA and 5-FU treatment in COLO.205 and Caco2 cells indicated significant ($p<0.05$) elevated lipid peroxidation compared to its untreated control. The enhanced MDA level for each test compound was concentration dependent. This increase demonstrates that both test materials amplifies MDA production in carcinoma cells by 52% and 48% at 200 µg/mL of TAHA exposure and 25% and 26% at 200 µg/mL of AA exposure on COLO.205 and Caco2 cells respectively when compared to the control.

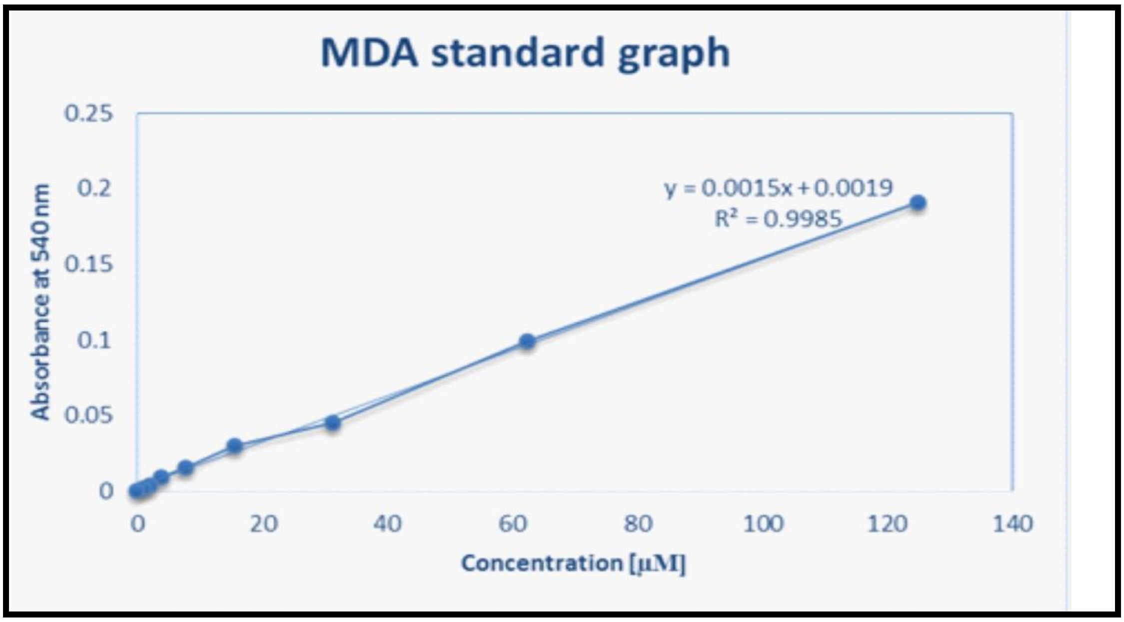

Figure 16. Calibration curve of Malondialdehyde (MDA)

Table 20. Lipid Peroxidation (MDA) concentration in the various treated cells.

Test materials (µg/mL)	Lipid Peroxidation (MDA conc./10^6) µM			
	L929 cells	IEC-6 cells	COLO.205 cells	Caco2 cells
Control	21.71 ± 0.67	24.84 ± 0.39	23.26 ± 0.93	22.82 ± 1.00
TAHA (200)	23.29 ± 0.17	25.82 ± 0.25	35.37 ± 0.91[a]	33.79 ± 0.58[a]
TAHA (100)	22.91 ± 0.09	25.64 ± 0.02	28.74 ± 0.97[a]	27.82 ± 0.41[a]
AA (200)	23.08 ± 0.04	25.59 ± 0.23	28.97 ± 0.79[a]	28.78 ± 0.47[a]
AA (100)	20.89 ± 0.03	24.34 ± 0.23	26.76 ± 0.99[a]	26.07 ±1.48[a]
5 - FU (35)	26.92 ± 0.88[a]	32.03 ± 1.23[a]	31.44 ± 1.00[a]	31.11 ± 1.40[a]
5 - FU (25)	24.62 ± 0.58[a]	29.53 ± 0.29[a]	29.35 ± 0.87[a]	28.67 ± 0.79[a]

Data are expressed as mean ± SD of three individual determinations. The data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Compared with control: [a] $p<0.05$.

4.7. Animal Study

4.7.1. Effects of TAHA and AA on gross appearance and DAI

The TNBS colitis induced experimental rats demonstrated prostration, piloerection, inability to move, appetite loss, faecal adhesion to the anus associated with blood, bloody diarrhea and weight loss. Test compound administration ameliorated the symptoms of colitis. It was observed that the colitic symptoms gradually decreased from Day 10 in TNBS control animals, which kept decreasing further during the study. While administration of test drugs and the standard Prednisolone hastened the disappearance of colitis symptoms. Figure 17 depicts the disease activity index for each group during the 28 days study period. The improvement in test groups treated rats was higher as compared to standard prednisolone. TAHA administration resulted with no DAI (500 mg/kg by day 14, 250 mg/kg by day 26 and 125 mg/kg by day 28). While AA administration resulted with no DAI (1.8 mL/kg by day 20 and 0.9, 0.45 mL/kg by day 24). Whereas, Prednisolone treated rats were observed with DAI till the end of the study.

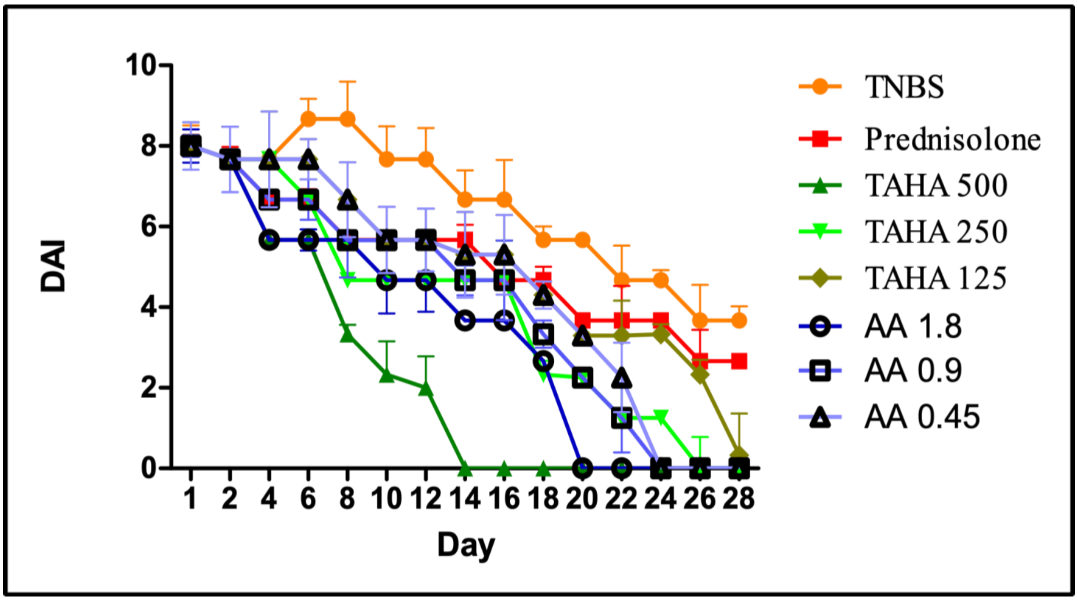

Figure 17. Disease activity index (DAI) scores (expressed as mean ± SEM, n = 8) reflecting the recovery process of TNBS induced treatment groups. DAI scores were calculated according to weight loss, stool consistency and blood in the stool

4.7.2. Effects of TAHA and AA on macroscopic and histological changes

Macroscopic signs of colitis were observed in rats administered with TNBS intrarectally. Macroscopic colonic mucosal oedema, necrosis and ulceration was compared with control group. The macroscopic score increased significantly as compared to the control group (0.60 ± 0.55 vs. 3.00 ± 0.71, $p < 0.05$). Treatment with test drugs at different doses and prednisolone decreased both hyperaemia and inflammation in colonic tissue (Figure 18). Significantly reduced macroscopic scores were observed at Prednisolone (1.4 ± 0.54), TAHA 500 mg/kg (0.8 ± 0.44), TAHA 250 mg/kg (1.6 ± 0.54), AA 18 mL/kg (1.2 ± 0.44), AA 0.9 mL/kg (1.4 ± 0.54), AA 0.45 mL/kg (1.6 ± 0.54) compared to TNBS group. However, TAHA 125 mg/kg (2.6 ± 0.54) did not show significant decrease compared to TNBS group.

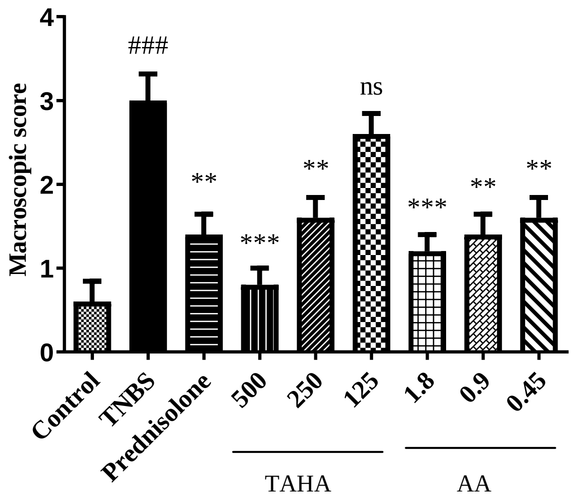

Figure 18. Effects of prednisolone (2 mg/kg**)** and different doses of *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on colonic macroscopic damage. Data are expressed as the mean ± SEM, (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. normal control; ns=Non significant, * $p < 0.05$, ** $p < 0.01$, $^{***}p < 0.001$ vs. TNBS control

Intrarectal TNBS induction in rats induced Histopathological changes in the colon displaying severe infiltration of acute and chronic inflammatory cells, deposition of fibrin protein, epithelial necrosis and ulceration. No histological changes were observed in normal control group. The histologic samples of TAHA 500 mg/kg (1.5 ± 0.70), TAHA 250 mg/kg (2.5 ± 0.70), TAHA 125 mg/kg (3.5 ± 0.70), AA 1.8 mL/kg (1.5 ± 0.70) and 0.9 mL/kg (2.5 ± 0.70) and prednisolone (2.5 ± 0.70) treated groups indicated significant progressive restoration, reduction in oedema and necrosis as compared to TNBS group (5.5 ± 0.70) as observed in Figure 19. AA 0.45 mL/kg (4.5 ± 0.70) displayed no significant changes compared to TNBS control group. Representative histological samples from each group are shown in Figure 20.

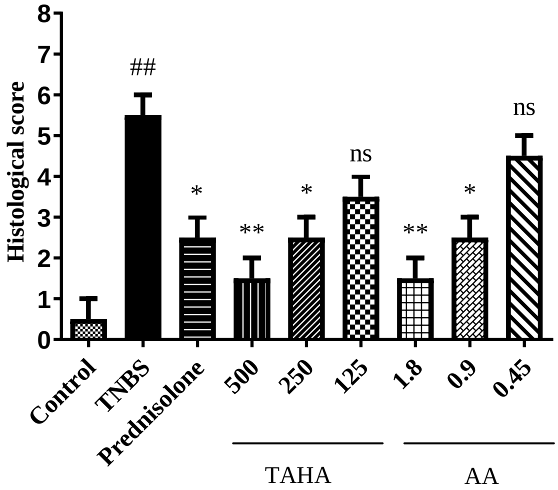

Figure 19. Effects of prednisolone (2 mg/kg) and different doses of *Terminalia arjuna* Hydro- alcoholic extract (TAHA) and Arjunarishta (AA) on Histological score. Data are expressed as the mean ± SEM, (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. normal control; ns=Non significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control

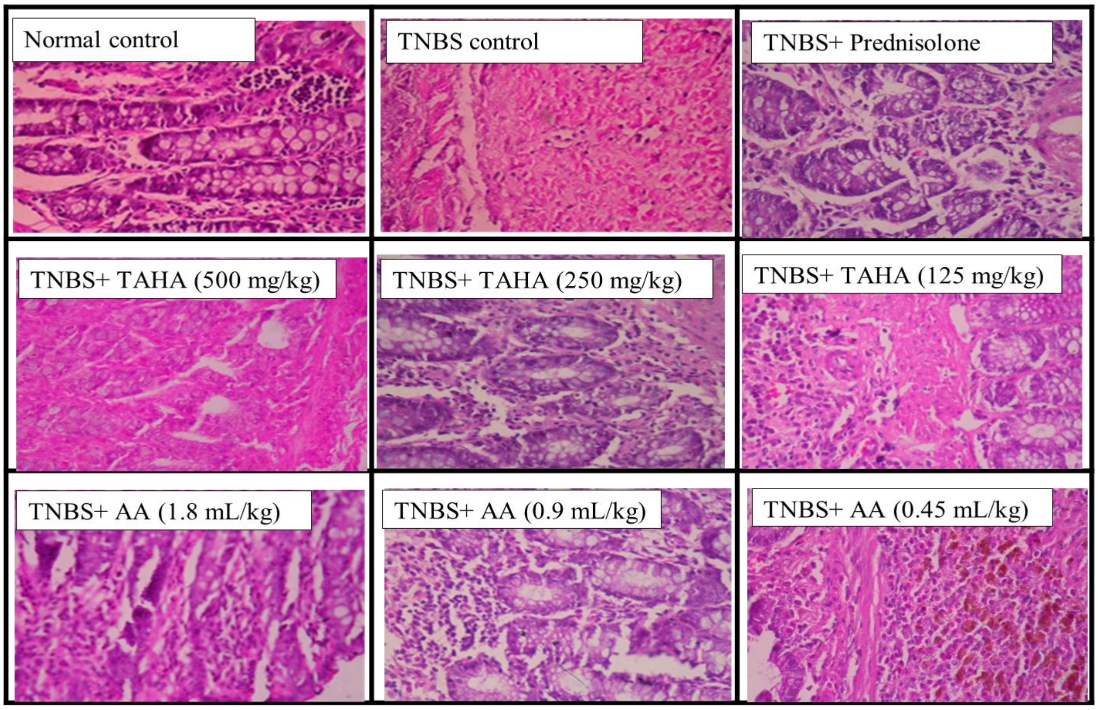

Figure 20. Histopathological changes. H and E staining, Magnification 40x

4.7.3. Effects of TAHA and AA on biochemical parameters

The colonic MDA levels in TNBS control group significantly increased compared to the Normal control group (4.20 ± 0.12 nmol/g vs. 0.37± 0.03 nmol/g) and it was significantly decreased by the treatment of prednisolone (2.45 ± 0.13 nmol/g), TAHA 500 mg/kg (0.76 ± 0.06 nmol/g), 250 mg/kg (1.59 ± 0.18 nmol/g), 125 mg/kg (3.38 ± 0.14 nmol/g), AA 1.8 mL/kg (1.76 ± 0.05 nmol/g) and 0.9 mL/kg (2.51 ± 0.20 nmol/g), compared to TNBS control. However, 0.45 mg/kg (3.62 ± 0.19 nmol/g) showed no significant difference in MDA level compared to control group as seen in (Figure 21).

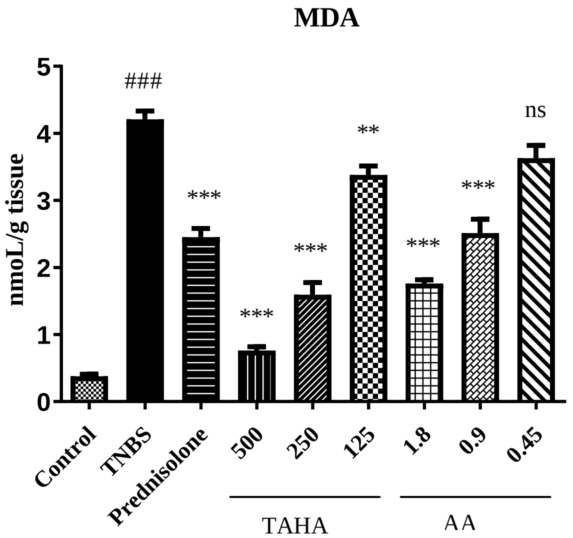

Figure 21. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Malondialdehyde (MDA**)**. Data are expressed as the mean ± SEM, (n=8). $^{\#}$p<0.05, $^{\#\#}$p< 0.01$^{\#\#\#}$p< 0.001 vs. normal control; ns=Non significant, *p< 0.05, ** p< 0.01, ***p< 0.001 vs. TNBS control.

The colonic MPO levels in TNBS control group significantly increased in comparison with normal control group (55.00 ± 1.93 U/mg vs. 12.79 ± 0.84 U/mg) and colonic MPO levels decreased significantly by the treatment of Prednisolone 27.37 ± 1.29, TAHA 500 mg/kg (19.70 ± 3.24 U/mg), TAHA 250 mg/kg (31.81 ± 3.42 U/mg), TAHA 125 mg/kg (38.89 ± 1.98 U/mg), AA 1.8 mL/kg as (14.34 ± 2.02 U/mg), AA 0.9 mL/kg (17.24 ± 0.48 U/mg) compared to TNBS control, whereas AA 0.45 mL/kg (33.90 ± 6.91 U/mg) showed no significant difference. (Figure 22).

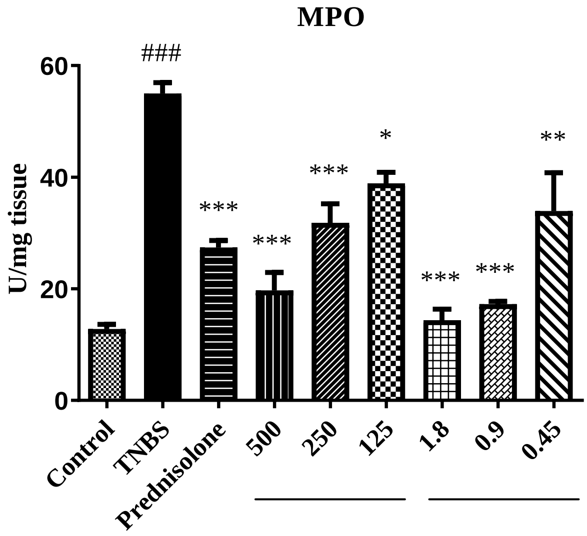

Figure 22. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Myeloperoxidase (MPO). Data are expressed as the mean ± SEM, (n=8). $^{\#}p<0.05$, $^{\#\#}p< 0.01$ $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

The calibration curve of nitric oxide with R^2 value of 0.999 was used for estimating plasma NO levels as depicted in Figure 23.The plasma NO levels in TNBS control group significantly increased compared to the Normal control group (1.25 ± 0.17 nmol/mL vs. 0.61 ± 0.004 nmol/mL) and it was decreased significantly by the treatment of prednisolone (0.65 ± 0.02 nmol/mL) TAHA 500 mg/kg (0.66 ± 0.02 nmol/mL), TAHA 250 mg/kg (0.73 ± 0.05 nmol/mL, TAHA 125 mg/kg (0.92 ± 0.01 nmol/mL), AA 1.8 mL/kg (0.73 ± 0.02 nmol/mL), AA 0.9 mL/kg (0.81 ± 0.03 nmol/mL). However, AA 0.45 mL/kg (0.97± 0.01 nmol/mL) exhibited no significant difference when compared with the TNBS control group (Figure 24).

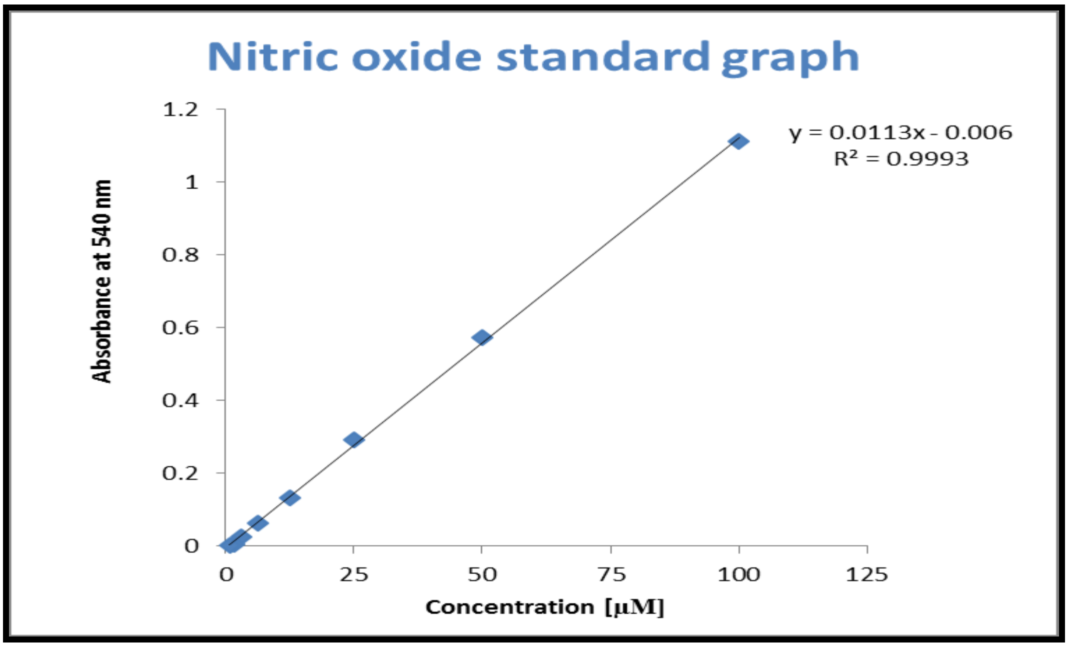

Figure 23. Calibration curve of Nitric oxide

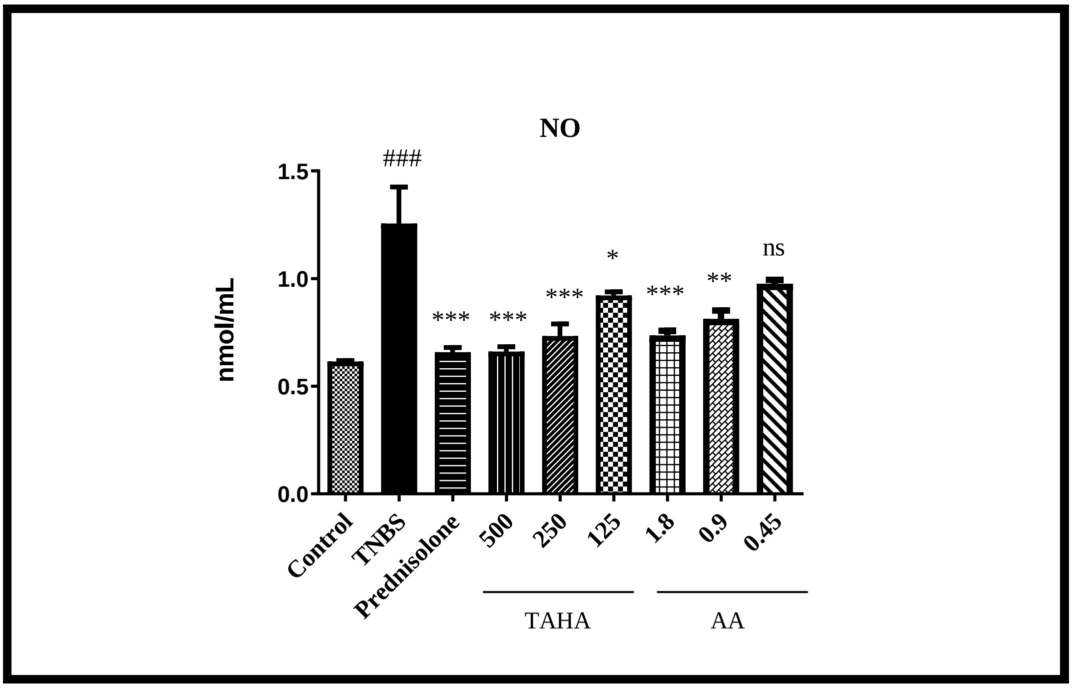

Figure 24. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Nitric Oxide (NO**)**. Data are expressed as the mean ± SEM, (n=8). $^{\#}$p<0.05, $^{\#\#}$p< 0.01$^{\#\#\#}$p< 0.001 vs. normal control; ns=Non significant, *p< 0.05, ** p< 0.01, ***p< 0.001 vs. TNBS control.

TNBS administration significantly decreased SOD level in TNBS control (2.97 ± 0.25 U/mL) compared to Normal Control (5.82 ± 0.18 U/mL). The treatment groups Prednisolone (4.52 ± 0.08), TAHA 500 mg/kg (4.41 ± 0.18 U/mL) AA 1.8 mL/kg (4.61 ± 0.15 U/mL) and AA 0.9 mL/kg (4.49 ± 0.15 U/mL), AA 0.45 mL/kg (4.17 ± 0.25 U/mL) significantly increased SOD level compared to TNBS control. However, 250 mg/kg (3.73 ± 0.22 U/mL), 125 mg/kg (3.65 ± 0.20 U/mL) displayed non-significant increase compared to TNBS control (Figure 25).

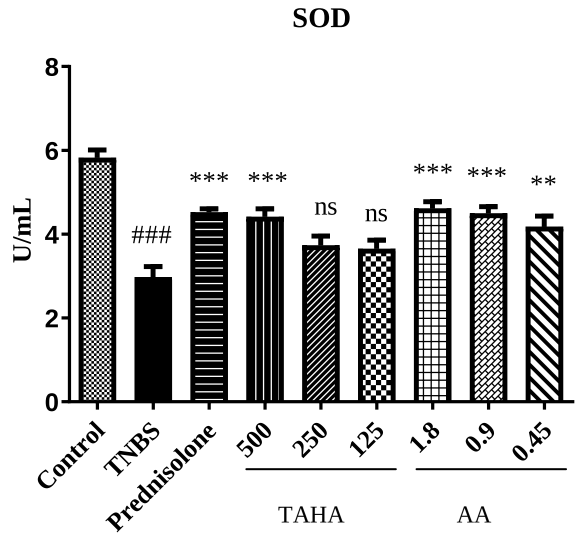

Figure 25. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Superoxide dismutase (SOD). Data are expressed as the mean ± SEM, (n=8**).** $^{\#}p<0.05$, $^{\#\#}p< 0.01$ $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

The calibration curve of GSH with R^2 value of 0.998 was used for estimating plasma GSH levels as depicted in Figure 26. TNBS administration significantly decreased GSH level in TNBS control (3.36 ± 0.75nmol/mL) compared to Normal

Control (13.57 ± 1.26 nmol/mL). Among the treatment groups only TAHA 500 mg/kg (10.10 ± 0.58 nmol/mL) significantly increased GSH level compared to the TNBS control group. The increase in other treatment groups was not significant. Prednisolone and other treatment groups showed a non-significant increase (3.52 ± 0.91nmol/mL), 250 mg/kg (5.61 ± 1.24 nmol/mL), 125 mg/kg (3.95 ± 1.32 nmol/mL), AA 1.8 mL/kg (9.43 ± 1.48 nmol/mL) and AA 0.9 mL/kg (3.43 ± 0.45 nmol/mL), AA 0.45 mL/kg (3.16 ± 0.56 nmol/mL) compared to TNBS control (Figure 27).

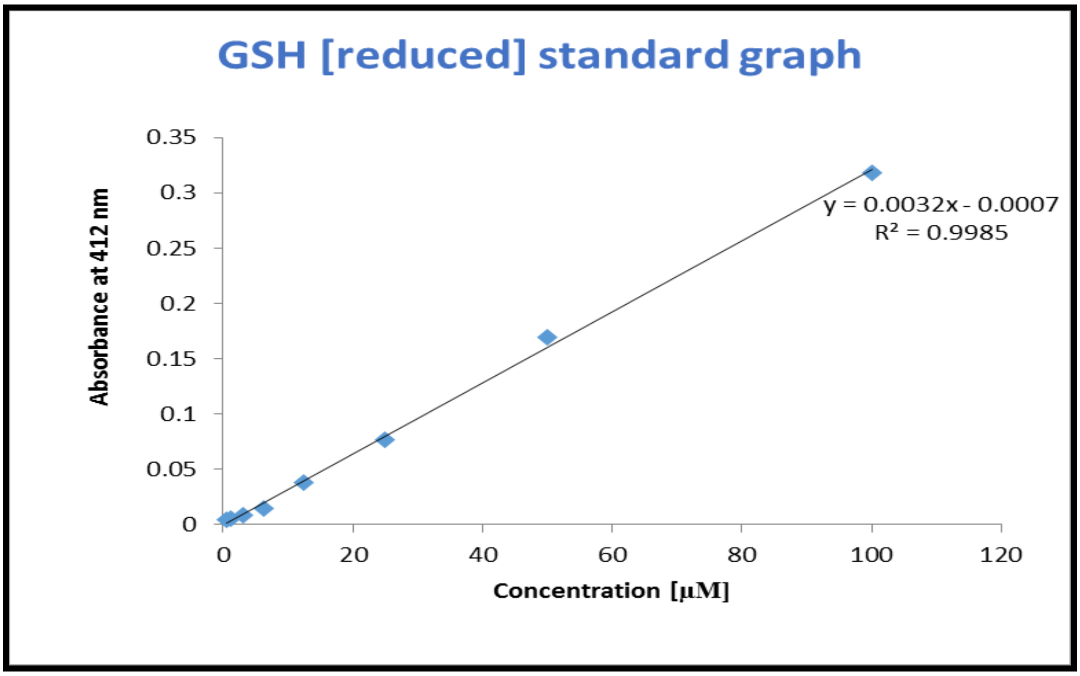

Figure 26. Calibration curve of Glutathione

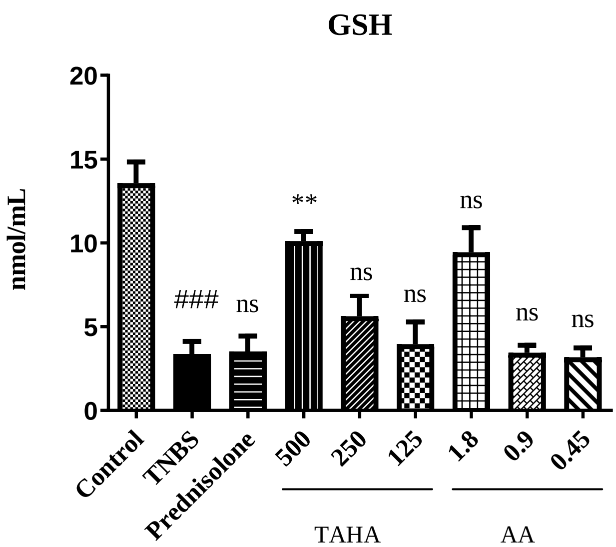

Figure 27. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Glutathione (GSH). Data are expressed as the mean ± SEM, (n=8). $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

The plasma CAT levels in TNBS control group significantly decreased compared to the Normal control group (10.96 ± 0.44 kU/I vs. 31.48 ± 2.17 kU/I) and it was increased significantly by the treatment of prednisolone (20.17 ± 0.79 kU/I) TAHA 500 mg/kg (25.20 ± 2.14 kU/I), TAHA 250 mg/kg (20.56 ± 0.80 kU/I, AA 1.8 mL/kg (23.45 ± 0.33 kU/I), AA 0.9 mL/kg (18.31 ± 0.79 kU/I). However, TAHA 125 mg/kg (15.64 ± 0.92 kU/I) and AA 0.45 mL/kg (13.70± 0.58 kU/I) exhibited no significant difference when compared with the TNBS control group (Figure 28).

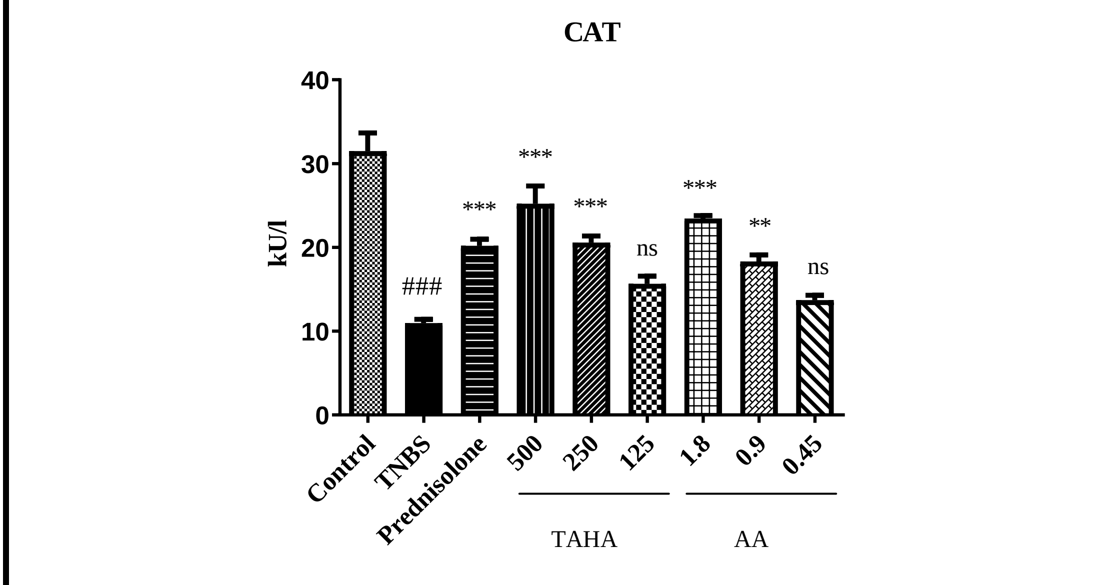

Figure 28. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Catalase **(**CAT**)**. Data are expressed as the mean ± SEM, (n=8**)**. #p<0.05, ##p< 0.01 ###p< 0.001 vs. normal control; ns=Non significant, *p< 0.05, **p< 0.01, ***p< 0.001 vs. TNBS control.

4.7.4. Effect of TAHA and AA on the expression of cytokines in rat colon samples

The intestinal inflammatory process by TNBS induction resulted in significant up-regulation of the colonic expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and chemokine MCP- 1, when compared to the normal control group (Figure 29-32) (2.60 ± 0.33, 2.38 ± 0.40, 2.60 ± 0.29, 2.60 ± 0.23) respectively. The administration of test substances and prednisolone decreased the expression of these inflammatory cytokines in tissue, showing a dose-dependent response.

The IL-1β level was significantly reduced on administration of Prednisolone (1.22 ± 0.21), TAHA 500 mg/kg (1.13 ± 0.29) and AA 1.8 mL/kg (1.16 ± 0.38) in colitic rats compared to TNBS control group. However, TAHA 250 mg/kg (1.26 ± 0.14), TAHA 125 mg/kg (2.04 ± 0.31), AA 0.9 mL/kg (1.36 ± 0.31) and AA 0.45 mL/kg (1.61 ± 0.26) treatment showed a non-significant decrease.

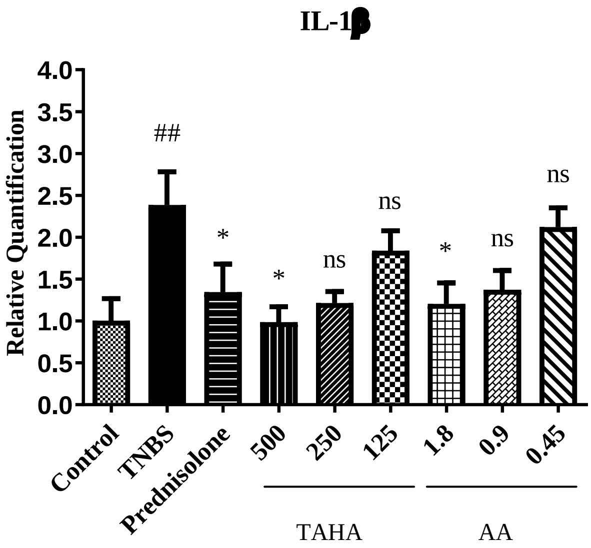

Figure 29. Effect of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on IL-1β. Data are expressed as the mean ± SEM, (n=8). $^{\#}$p<0.05, $^{\#\#}$p< 0.01, $^{\#\#\#}$p< 0.001 vs. normal control; ns=Non significant, *p< 0.05, ** p< 0.01, ***p< 0.001 vs. TNBS control.

IL-6 level was significantly reduced on administration of TAHA 500 mg/kg (0.98 ± 0.18) in colitic rats compared to TNBS control group. However, Prednisolone (1.34 ± 0.33), TAHA 250 mg/kg (1.21 ± 0.13), TAHA 125 mg/kg (1.83 ± 0.23), AA 1.8 mL/kg (1.20 ± 0.25), AA 0.9 mL/kg (1.37 ± 0.23) and AA 0.45 mL/kg (2.12 ± 0.22) treatment showed a non-significant decrease.

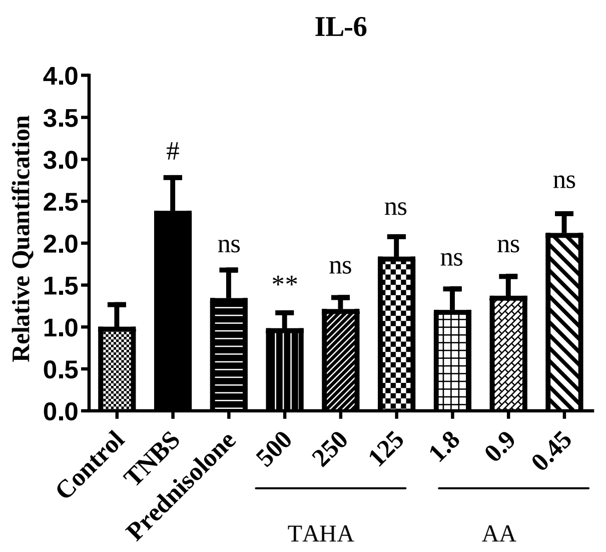

Figure 30. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on IL-6. Data are expressed as the mean ± SEM, (n=8**)** $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

The TNF-α level was significantly reduced in colitic rats on administration of Prednisolone (1.01 ± 0.20), TAHA 500 mg/kg (0.87 ± 0.14), TAHA 250 mg/kg (1.37 ± 0.14) and AA 1.8 mL/kg (1.10 ± 0.21) compared to TNBS control group. However, TAHA 125 mg/kg (1.95 ± 0.25), AA 0.9 mL/kg (1.64 ± 0.19) and AA 0.45 mL/kg (2.41 ± 0.29) treatment showed a non-significant decrease.

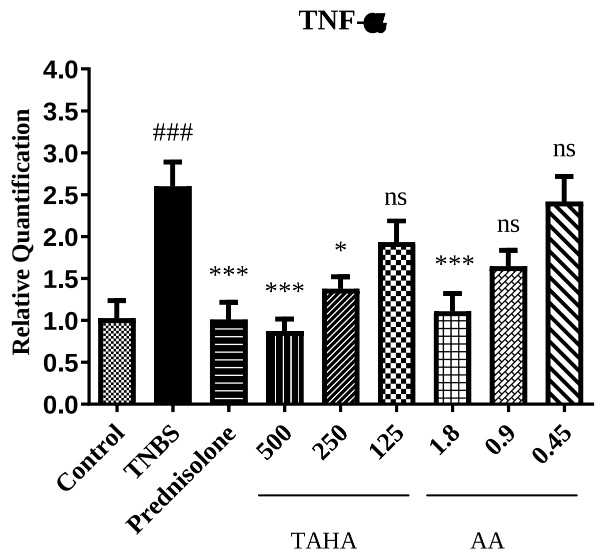

Figure 31. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on TNF-α. Data are expressed as the mean ± SEM, (n=8). $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

The MCP-1 level was significantly reduced in colitic rats on administration of Prednisolone (0.95 ± 0.12), TAHA 500 mg/kg (0.85 ± 0.74), TAHA 250 mg/kg (1.33 ± 0.49) and AA 1.8 mL/kg (1.10 ± 0.28) compared to TNBS control group. However, TAHA 125 mg/kg (1.70 ± 0.42), AA 0.9 mL/kg (1.25 ± 0.39) and AA 0.45 mL/kg (1.38 ± 0.24) treatment showed a non-significant decrease.

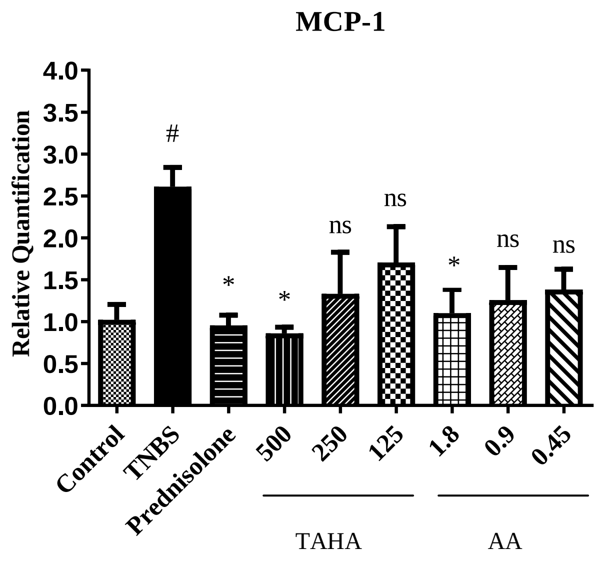

Figure 32. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on MCP-1. Data are expressed as the mean ± SEM, (n=8). $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, ** $p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

4.7.5. Effects of TAHA and AA on plasma zinc level

The plasma Zn levels in TNBS control group significantly decreased compared to the Normal control group (66.19 ± 2.99 mg/dL vs. 140.6 ± 4.04 mg/dL) and it was increased significantly by the treatment of prednisolone (131.5 ± 5.01 mg/dL) TAHA 500 mg/kg (124.6 ± 11.29 mg/dL), TAHA 250 mg/kg (112.0 ± 8.63 mg/dL, TAHA 125 mg/kg (101.3 ± 2.57 mg/dL), AA 1.8 mL/kg (104.0 ± 8.65 mg/dL), AA 0.9 mL/kg (90.16 ± 4.92 mg/dL). However, AA 0.45 mL/kg (84.66 ± 3.99 mg/dL) exhibited no significant difference when compared with the TNBS control group (Figure 33).

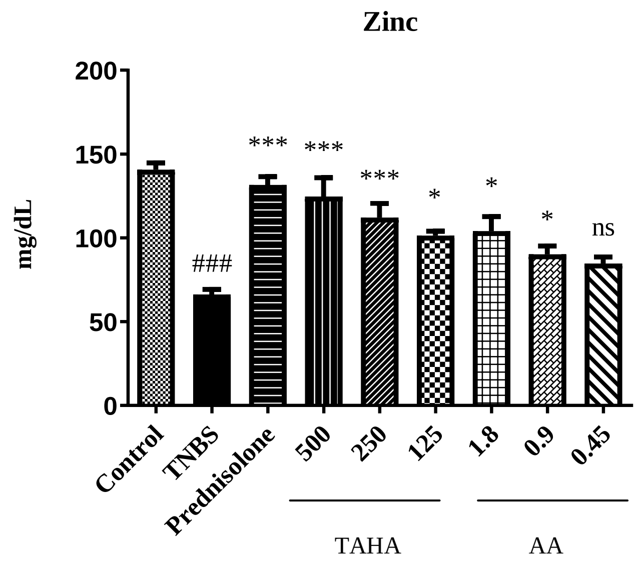

Figure 33. Effects of prednisolone (2 mg/kg), *Terminalia arjuna* Hydro-alcoholic extract (TAHA) and Arjunarishta (AA) on Plasma zinc level. Data are expressed as the mean ± SEM, (n=8**)**. $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. normal control; ns=Non significant, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$ vs. TNBS control.

4.7.6. Effects of TAHA and AA on the structure of gut microbiota

The faecal matter evaluation revealed the effect of prednisolone and test drugs on gut microbiota *in vivo* (Table 21). The normal microbial flora was altered after TNBS administration. TNBS administration significantly increased Clostridial counts compared to normal group. Prednisolone, TAHA 500 mg/kg, AA 1.8 mL/kg, AA 0.9 mL/kg significantly lowered the Clostridial count compared to the normal group. While TAHA 250 mg/kg, TAHA 125 mg/kg significantly increased and AA 0.45 had non- significant increase in Clostridial count compared to Normal group. Prednisolone and all the test groups showed significant decrease in Clostridial count except TAHA 250 mg/kg and 125 mg/kg had a non-significant decrease compared to TNBS control.

TNBS control showed non-significant decrease in Bifidobacterial count compared to Normal group, However, prednisolone administration showed significant decrease in Bifidobacteria compared to normal control. TAHA 500mg/kg, AA 1.8 mL/kg and AA 0.9 mL/kg significantly increased Bifidobacterial count compared to Normal group. prednisolone group showed non-significant alteration in Bifidobacterial count compared to TNBS control. All other test groups significantly increased Bifidobacterial count compared with TNBS control. Except AA 0.45 mL/kg which showed non-significant alteration in Bifidobacterial count.

TNBS control significantly decreased Lactobacilli count compared to Normal group, Lactobacilli count was zero in faecal samples of rats treated with prednisolone, TAHA 500 mg/kg and TAHA 250 mg/kg which is significantly lower than the Normal control and TNBS control rats. However, TAHA 125 mg/kg significantly increased Lactobacilli count compared to Normal and TNBS control group. No significant difference was observed at AA 1.8 mL/kg compared to the normal control group. AA 0.9 mL/kg and AA 0.45 mL/kg exhibited significant decrease in Lactobacilli compared to Normal group. AA at all test doses showed significant increase in the Lactobacilli count compared to TNBS control rats.

Table 21. Bacterial faecal level in the different experimental groups

Groups	Clostridium	Bifidobacteria	Lactobacilli
Normal	3.531 ± 0.002	3.428 ± 0.023	3.536 ± 0.004
TNBS	3.614 ± 0.008 $^{###}$	3.394 ± 0.003 ^{ns}	3.375 ± 0.003$^{###}$
Prednisolone	2.505 ± 0.002 $^{###,***}$	3.365 ± 0.002$^{##, ns}$	0.000 ± 0.000$^{###, ***}$
TAHA 500	2.505 ± 0.004 $^{###, ***}$	3.570 ± 0.003$^{###, ***}$	0.000 ± 0.000$^{###, ***}$
TAHA 250	3.579 ± 0.012 $^{##, ns}$	3.473 ± 0.003 $^{ns, **}$	0.000 ± 0.000$^{###, ***}$
TAHA 125	3.584 ± 0.009 $^{##, ns}$	3.457 ± 0.005 $^{ns, **}$	3.647 ±0.001$^{###, ***}$
AA 1.8	3.476 ± 0.001$^{##, ***}$	3.491 ± 0.004$^{##, ***}$	3.525 ± 0.003$^{ns, ***}$
AA 0.9	2.908 ± 0.002$^{###, ***}$	3.552 ± 0.001$^{###, ***}$	3.410 ± 0.002$^{###, **}$
AA 0.45	3.533 ± 0.017$^{ns, ***}$	3.435 ± 0.002$^{ns, ns}$	3.473 ± 0.004$^{###, ***}$

Data are expressed as mean ± SEM, (n=6), ns=Non significant, $^{#}$p<0.05, $^{##}$p< 0.01, $^{###}$p< 0.001 vs. Normal control; ns=Non significant, *p< 0.05, ** p< 0.01, ***p< 0.001 vs. TNBS control

5. DISCUSSION

Inflammatory bowel disease (IBD) is a cluster of gastrointestinal (GI) inflammatory disorders that are categorized into two major types – UC and CD. The most common exhibited symptoms in UC are bloody bowel movements and diarrhoea. however, in CD it is fatigue and abdominal pain [139].

UC affects superficial mucosal layer causing infiltration of neutrophils, lymphocytes, granulocytes and goblets cells. Conversely, CD affects the entire bowel wall and dense infiltration of lymphocytes and macrophages is observed [8]. The aetiology and pathogenesis of IBD is still uncertain, while it is known to involve a complex interaction between genetic risk factors, exposure to environmental risk factors, the host immune system dysfunction and the commensal microbiota [140].

The current pharmacotherapy for IBD focuses on maintaining remission in patients and decreasing the secondary effects of the disease, rather than targeting the basic pathogenic mechanism [141]. Amino salicylates, corticosteroids, immunosuppressant's, antibiotics and biologicals are the drugs of choice for IBD. However, the current drug therapy is associated with several side effects and develop resistance upon long term use, especially when the disease is chronic and prolonged drug treatment is required in certain cases [10,142].

In certain cases, patient non-compliance to conventional drug therapy is also noted. This has encouraged the use of complementary and alternative medicine to achieve the desired efficacy, safety and higher compliance in IBD patients [94,143].

Several medicinal plants as part of complementary and alternative medicine are being used to treat various intestinal inflammatory conditions. A number of active phytoconstituents occurring in medicinal plants simultaneously work at different targets in the inflammatory pathway [144]. Medicinal plants with their multiple mechanism of actions may synergize to produce their overall efficacy in IBD exhibited through results of preclinical evaluation, improving symptoms in patients and reduced medical cost of treatment [14].

This may be helpful in treating the disease as a whole and not just supress the symptoms like the conventional drug therapy. Through this study we plan to evaluate new safer complementary treatment options for IBD management.

The present study was carried out using TAHA and AA along with standard drugs. The parameters evaluated are Phytochemical screening and quantification using marker components, *in-vitro* antioxidant evaluation, antimicrobial assessment against clinical isolates of IBD, colorectal cancer cytotoxicity along with MDA estimation and also the assessment of the effect of TAHA and AA against inflammatory bowel disease in chemically induced rat models.

The results of phytochemical evaluation of TAHA are as per previous studies using methanolic extract of TA bark [145,146]. Similarly, the phytochemical screening findings in our study showing presence of certain constituents but absence of alkaloids in AA is also supported by previous reports [147]. These reported phytoconstituents in the test materials are well reported to have antioxidant, antimicrobial, anticancer, and anti-inflammatory potential [148], which may also be effective in this study involving its antimicrobial, cytotoxic and anti-inflammatory properties.

The free radical scavenging ability of an antioxidant is seen in phenolic compounds. Therefore, the antioxidant potential of a compound can be established through its total phenolic concentration [149]. DPPH and FRAP assay confirmed the antioxidant potential of the test materials. In this study gallic acid and TAHA displayed comparable antioxidant potential result.

The dietary nutrients are absorbed and digested in the GI tract. These dietary nutrients offer immunity against foreign antigens, digests and absorbs dietary nutrients and defends the body against physical and chemical injury. The GI tract also serves an ideal environment for the gut microbiome. These regulatory functions ideally performed by the GI tract are not seen in case of IBD. Antioxidant supplements are administered to IBD patients as this disease is linked with oxidative stress. These supplements help to maintain the gut function and also recover the damage. Their mechanism of action is not by simply acting as scavengers but by inhibiting stress pathways in a specific manner in tissue and environment [150].

Growing research indicates that pathogenesis of IBD could be linked to the role of mucosa-associated microbiota [151]. Virulent strains of *E. coli* and similarly other enteric gram-negative bacteria associated with IBD pathogenesis can be targeted by antibiotics like Ciprofloxacin. However, the negative outcomes such as tendonitis, tendon rupture, photosensitivity, foetal/ child cartilage growth inhibition and QT prolongation on an electrocardiogram is linked to the use of antibiotics [152]. There is no study as yet claiming the antibacterial potency of TA and AA against clinical isolates of IBD. Although reports suggest the antimicrobial activity of TA against Gram positive/negative ear pathogens [153]and also broad-spectrum activity against bacteria that causes diarrhoea [154]. TA use in treatment of GI disorders is also supported by data that 'Bhoxa community' of Dehradun district, Uttarakhand, India use this medicinal plant for treatment of dysentery and diarrhoea [16]. TA showed a zone of inhibition against the test bacteria. Our study results revealed that TA exhibited an inhibitory zone against the clinical isolates of IBD. Whereas, AA displayed no inhibitory zone which could possibly be due to its inability to diffuse from the well. However, in the broth dilution method both TA and AA displayed antibacterial potential against IBD isolates. Thus, this study results support the earlier claims concerning the antimicrobial properties of TA.

The validation of medicinal plant anticancer potential is conducted through the assessment of cytotoxicity. Hence, we studied the cytotoxic potential of TA and AA on two human colorectal adenocarcinoma cell lines. Earlier reports suggest activity of TA bark phytoconstituent i.e. Arjunic acid against human oral, ovarian and liver cancer cell lines [155].

Additionally, the carbohydrate metabolizing enzymes were targeted by TA extracts in N-nitrosodiethylamine induced hepatocellular rat carcinoma [19]. Similarly, this study against colorectal adenocarcinoma cells exhibited dose-dependent cytotoxicity of test materials. This supports the traditional use of TA in cancer treatment [156].

Bioactive substances through oxidative stress molecular mechanisms can cause apoptosis and toxicity in cells [19]. To investigate the degree of oxidative cell damage in colorectal adenocarcinoma cells exposed to test materials and 5-FU, we carried out lipid peroxidation study. Lipid peroxidation study was performed in colorectal adenocarcinoma cells to investigate the extent of oxidative cell damage on exposure to test materials and 5-FU. In our study TAHA and AA - treated carcinoma cells showed significantly elevated MDA (a by-product of lipid peroxidation and biomarker of oxidative stress) levels as compared to control cells. Similarly, HepG2 cells which is a human liver cancer cell lines induced ROS production on exposure to TA extract causing cell apoptosis.

In addition, human prostate cancer cells on exposure to Gallic acid which is present in TA produced ROS-induced cell death through the mechanism of autoxidation [157]. Similarly, in this study colorectal cancer cell lines on exposure to standard drug and both the test materials demonstrated a rise in lipid peroxidation levels and decreased the cell viability. This effect could be a result of generating oxidative stress mediated apoptosis in the cells [158,159].

The effect of TAHA and AA was also evaluated in TNBS induced rat colitis model. It is a well recognized animal model which resembles biochemical and histological features of the human disease and has manifestations such as inconsistent stool formation and occult/bloody diarrhea . The anti-inflammatory and anti-oxidative potentials of various natural and chemical moieties have been preclinically tested using this animal model. Progression of excessive cell mediated immune reaction is obtained by a single administration of TNBS and ethanol. This inflammatory response is a result of an acute Th1 inflammation through secretion of various pro-inflammatory cytokines. Colitis induction causes manifestations like inconsistent stool formation and occult or even bloody diarrhea. [11, 160] .

Previous reports indicated TNBS administration in rats showed significantly increased disease activity score owing to body weight loss scores, diarrhoea score and bleeding scores [161]. This study findings suggests beneficial effect of test drugs, observed during experimental schedule in terms of reduced DAI and faster recovery from TNBS induced damage as compared to colitic rats. Thus, revealing an improvement in the health status of experimental rats treated with TAHA and AA, which was further confirmed through improved histological damage scores.

TNBS model showed an anti-inflammatory effect on test compound administration. On treatment with TAHA and AA, histological damage in rat colon was recovered in a dose-dependent manner. The extent of inflammation, ulceration and hyperemia was found to be reduced in treated animals. Our study results are in agreement with previous reports suggesting colonic damage even after 28days on TNBS induction in TNBS control group [162].

The inflammatory and biochemical marker investigations supported the protective effects of the treatment in TNBS model. IBD development is a result of oxidative stress and increased ROS production has been associated with chronic intestinal inflammation. These enhanced levels could result in relapse of active infection in IBD patients in spite of being on maintenance therapy and may cause requirement of additional antioxidant treatment [163, 164]. The disbalance between protective free radical scavengers and ROS production in the TNBS induced colitis model may lead to tissue injury [165]. Although uncontrolled oxidative stress is destructive to GI tract, body's defenses can counteract the effects triggered by excess reactive oxygen species (ROS) production. The reactive oxygen species and reactive nitrogen species modulate the gene expression associated with the immune response in the GIT. Radiation, chemotherapy, smoking, luminal antigens, alcohol, drugs, and the use of xenobiotics can activate the oxidative stress which can contribute to IBD pathogenesis. Intracellular enzymatic antioxidants such as Superoxide dismutase (SOD)s present in mammalian cells exist in three isoforms SOD1(major isoform,70%), SOD2 and SOD3. SODs catalyse the dismutation of $O_2^{\cdot -}$ into H_2O_2.

Another intracellular enzymatic antioxidant, Catalase (CAT) which occurs in peroxisomes and catalyses the reduction of H_2O_2 into H_2O and O_2 molecules. Glutathione (GSH) is an intracellular nonenzymatic antioxidant and highly expressed in the nucleus, cytoplasm and the mitochondria. It participates in the membrane barrier function in the gut [166].

TAHA and AA administration displayed significantly enhanced (SOD, CAT, GSH) level showing the anti-inflammatory potential in comparison to TNBS control animals confirming favourable antioxidant effects of treatment.

Nitric oxide (NO) and malondialdehyde (MDA) are the primary ROS/NOS products in IBD. Inducible nitric oxide synthase (iNOS) and NO are actively involved in IBD progression [167]. As per previous reports, decrease in overproduction of NO by inhibition of iNOS may ameliorate the intestinal inflammation in IBD conditions [168,169]. MDA indicates lipid peroxidation and gives a measure of oxidative stress and increased levels are depicted in experimental colitis models [170,171]. Further, another marker, MPO level was estimated in rat colon samples. MPO indicates the extent of neutrophil influx into inflamed gastrointestinal tissue. Inhibiting MPO activity might help in preventing the mucosal inflammation [172].

The advancement of the disease resulted in elevated levels of NO and MPO in colonic biopsies of TNBS induced rats. Similar results have been obtained in our study [173]. While TAHA and AA administration displayed the anti-inflammatory potential reducing NO and MPO level significantly as compared to TNBS control animals.

Cytokines plays a major role involving the development, relapse and exacerbation of the inflammatory process in IBD [174]. Prolonged activation of the intestinal immune system plays an essential role in pathophysiology of chronic mucosal inflammation as observed in various animal models and clinical studies. Cytokines are synthesized and secreted by specific cells of the immune system, which further induce the production of adhesion molecules and other inflammatory mediators such as ROS, nitric oxide and lipid mediators [175]. Th1 and Th17 $CD4^+$ T cell differentiation plays a major role in CD and comprises of interferon (IFN)-γ and IL-17/IL-22. However, Th2-like differentiation process causing expansion of Natural killer T (NKT) cells which produce IL-13 has a role in UC [176]. UC patients show increased expression of IL-6 mRNA. Similarly, in CD IL-6 levels could be an indicator to stratify patients with high risk of relapse and chronic active disease progression [177]. IL-1β is reported to facilitate the accumulation of IL-17A secreting cells. IL-17A causes chronic inflammation of the gut. Therefore, targeting IL-1β may reduce this accumulation which could be beneficial in IBD treatment [178]. The proinflammatory cytokine, Tumor necrosis factor (TNF)-α contributes to the pathogenesis of IBD by various mechanisms such as neutrophil migration, initiation of edema and granuloma formation, stimulation of coagulation at the site of inflammation in the tissue [179,180]. Clinical and experimental model CD and UC have revealed raised level of chemokine Monocyte chemoattractant protein (MCP)-1. Exposure to cytokines (IL-1 and TNF-α) upregulates MCP-1 expression in colonic mucosa [181].

Therefore, we studied the colonic expression of the pro-inflammatory cytokines: IL-6, IL-1β, TNF-α and Chemokine: MCP-1 in colitic rats to evaluate the altered immune response. Earlier studies have indicated elevated levels of IL-6, IL-1β, TNF-α in mucosa samples of IBD patients as compared to normal mucosa [182,183].

A number of biological therapies targeting cytokine networks have been developed for the treatment of IBD. Anti-TNF-α biologicals have been known to improve clinical score, mucosal healing and also extend relapse-free periods in IBD cases. Similarly, anti-interleukin (IL)-12p40 therapy is effectively beneficial in CD patients [184]. In agreement to previous findings, we observed increased MDA, MPO and NO levels in TNBS control group along with enhanced cytokine levels which were significantly reduced on test drug administration. Pre-treatment with hydroalcoholic extract of TA at a dose of 100 mg/kg significantly reduced IL-6 and TNF levels in myocardial infarcted rats [185]. AA is previously reported to have down regulated the gene expression of TNF-α in High fat fed animals [114].

Zinc performs functions such as maintaining intercellular junctions in the membrane barrier and aids to the defence mechanisms of the host thereby contributing to the anti-inflammatory response. Zn deficiency affects the membrane barrier permeability. Consequently, low Zn concentrations have frequently been associated with IBD. Whereas Zn supplementation reduces the severity of diarrhoea [186]. Zn is also involved in pathways that neutralise the Reactive oxygen species (ROS) and modulates the expression of inflammatory cytokines. Zn supplementation reduces gene expression of metalloproteins and maintains the zinc homeostasis by regulating intracellular levels thereby improving the immunity [187].

IBD patients have represented lowered levels of Zn in the urine, plasma and hair samples [188]. It is found that the risk of CD reduces with higher intake of Zn but it does not apply in case of UC. It is also supported by the claim that Zn intake from diet is more effective than Zn supplementation [189]. In our study, we observed increased plasma Zn status in prednisolone, TAHA and AA treated rats as compared to TNBS control group which could be associated with increase in the antioxidant status along with improvement in the immune response which may be effective in tissue restoration and healing of colon tissue.

IBD is considered to be an immune-mediated inflammatory disorder of the intestine resulting from the abnormal interaction between intestinal microbes and the local immune system [190]. Bifidobacteria offer positive health benefits to the host [191] and intestinal bifidobacterial count alteration has been previously noticed in IBD and colorectal cancer case [192]. In the same way, Lactobacilli population is also decreased in UC patients and a study suggested that administration of Lactobacillus spp. prevented colitis in IL-10 gene deficient mice [193]. Treatment with traditional Japanese medicine 'Strong Wakamto®' in TNBS induced colitic rats demonstrated beneficial effect through mechanism of balancing the fecal lactobacillus spp. [194]. On the other hand, the Clostridial bacteria count in rat feces increased on TNBS administration [195] and decreased in prebiotic treated colitic rat [57]. Therefore, we have explored the TAHA and AA treatment effect on gut microflora using a bacteriological analysis of feces from experimental animals. Our study results directed that the administration of TNBS decreased fecal Bifidobacteria, Lactobacilli and increased the counts of Clostridium as compared to control. The mechanism whereby TNBS modulates colonic flora is unknown and needs further investigation. TAHA treatment normalized the microflora profile by increasing the count of Bifidobacteria, only at dose 125 mg/kg TAHA showed raised lactobacilli while decreasing clostridium counts as compared to TNBS control group. AA administration raised Bifidobacterial and Lactobacilli counts and decreased Clostridial counts which were altered on TNBS administration. In contrast, prednisolone treatment further decreased the Bifidobacteria and Lactobacilli compared to TNBS alone. However, its effect on Clostridium was comparable to TAHA. This fecal microbiota alteration by test drug administration could play a major role in restoring the intestinal damage seen in colitis. This finding needs further evaluation to claim its efficacy.

6. SUMMARY

The present thesis **"Effect of hydro-alcoholic extract of *Terminalia arjuna* bark and Arjunarishta in Inflammatory bowel disease"** aimed to study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta against IBD related microorganisms. To study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta on colorectal cell lines and to study the effect of hydro-alcoholic extract of *Terminalia arjuna* and Arjunarishta on TNBS induced inflammatory bowel disease in rats.

The current drugs used in IBD management are associated with a series of side effects upon long term use. Also considering the fact that the pathogenesis of the disease has not been clearly determined, disease management is difficult. Review suggests that increased DNA damage may be a risk factor for developing colon cancer in IBD patients. Administration of external antioxidants may be recommended as a supportive therapy to reduce the oxidative DNA damage. Inflammatory cytokines have been studied in experimentally induced colitis and colon carcinogenesis. The objective of modulating the intestinal flora to promote the growth of host friendly bacteria and inhibit the proliferation of potentially harmful microorganisms is also considered and studied in IBD treatment.

TA bark has been reported for a vast number of pharmacological activities such as antioxidant, immunomodulatory, antibacterial, anticancer. TA bark and AA have been traditionally used in treating various gastrointestinal ailments. Considering this traditional knowledge and its pharmacological claims, we planned to evaluate its efficacy in IBD treatment since its potential in IBD management is not yet established.

In the current study the antibacterial potential of TAHA and AA was estimated against the clinical isolates of IBD. The colorectal cancer cells treated with test materials demonstrated increased malondialdehyde levels which indicate increased lipid peroxidation along with cytotoxicity.

TAHA and AA treatment in TNBS induced animal model decreased oxidative stress and proinflammatory cytokines/chemokine levels. The faecal microbiota composition was also altered upon treatment in colitic rats. It also increased the plasma zinc levels which is essential in maintaining and restoring intestinal barrier functions.

The traditional use of TA and Arjunarishta by certain communities in the treatment of gastrointestinal disorders may be related to results observed in our study. This study suggests the beneficial role of test materials; TAHA and AA in IBD management. However, further clinical studies are necessary to justify its possible use in IBD treatment and to establish the exact mechanism

7. CONCLUSION

In the current study the antibacterial potential of TAHA and AA was found against the bacterial isolates from CD and UC patients used in the study. It also increased the lipid peroxidation with a simultaneous decline in cell viability in colorectal cancer cell line with its mechanism of generation of oxidative stress-mediated apoptosis.

TAHA and AA treatment relieved the symptoms of TNBS induced colitis by decreasing oxidative stress and proinflammatory cytokines/chemokine. It also altered the composition of gut microbiota and plasma zinc levels which could be the mechanism of maintaining the gut barrier function and inhibiting inflammatory pathways.

The traditional use of TA and AA by certain communities in the treatment of gastrointestinal disorders may be related to results observed in our study. This study suggests the beneficial role of test materials; TAHA and AA in IBD management. However, further clinical studies are necessary to justify its possible use in IBD treatment and to establish the exact mechanism.

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2. MATERIAL AND METHODS

2.1. Materials

2.1.1. List of chemicals and materials used for experiments is given below:

Table 6. List of chemicals and materials

Sr. No.	Particulars	Make/Source
1	TA bark	KLE Society's Ayurved Pharmacy, (collected in February 2015 from the Western Ghats, Belagavi region, Karnataka – India) The authentication of T. arjuna was performed by AYUSH approved ASU drug testing laboratory at Shri BM Kankanwadi Ayurveda Mahavidyalaya, Belagavi, Karnataka–India and assigned the voucher number CRF/645/2015).
2	Arjunarishta (AA)	Sandu Pharmaceuticals Ltd., Mumbai – India (Batch no. 25)
3	HPLC grade methanol	Merck, Life Science Pvt. Ltd, Mumbai – India
4	Water	Milli-Q (Millipore)
5	HPLC grade acetonitrile	Merck, Life Science Pvt. Ltd, Mumbai – India
6	Oxiselect™. TBARS Assay Kit	Cell Biolabs, Inc, San Diego, CA, USA. TNBS was purchased from Sigma-Aldrich, U.S.A.

7	Trizol ® reagent	Sigma-Aldrich
8	Hemospot kit.	Coral Clinical Systems, Tulip Diagnostics (P) Ltd. Verna, Goa - India
9	Culture media (for the bacteriological study)	HiMedia Laboratories Pvt. Ltd
10	Four clinical bacterial isolates - *E.coli* HM95 (AIEC), *E.coli* HM615 (colonic mucosa associated. *E.coli*), *E.coli* HM233 and *E.coli* HM251 (colonic mucus associated patient strains)	Received under Material Transfer Agreement with University of Liverpool, United Kingdom.
11	Human colorectal adenocarcinoma cells (Caco2, COLO.205) and normal rat intestinal cells (IEC-6) and mouse fibroblast cells (L929)	National Center for Cell Sciences, Pune-India.

2.1.2. List of equipment used for experiments

Table 7. List of equipment used in the study

Sr.No.	Particulars	Make/Source
1	Rotary evaporator	Buchi Rotavapor
2	HPLC system	Dionex P680
3	Step one real-time PCR system	Applied Biosystems
4	Compact-MG Anaerobic workstation	Kim microsystems
5	UV Spectrophotometer	UV-1800, Shimadzu, Japan
6	Atomic Absorption Spectrophotometer	AA-7000; Shimadzu, Japan
7	NanoDrop® Spectrophotometer	JH BIO, U.S.A.

2.2. Experimental Design

The plan of work was as follows-

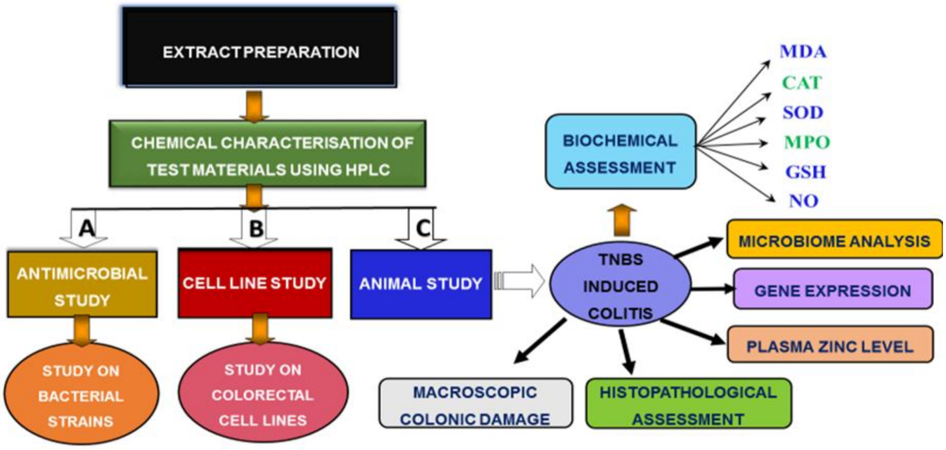

Figure 5. Study plan

2.3. Preparation of plant extract and preliminary phytochemical analysis

The dried bark of TA was powdered and extracted with ethanol: water (70:30 v/v) using cold maceration method in a conical flask. The extract was manually shaken every hour for initial six hours. Later, it was kept in a shaker at 200 rpm. The extract was filtered and concentrated in a rotary evaporator at 40ºC followed by complete drying using a water bath. The yield of hydroalcoholic extract (expressed as percentage w/w) was 22.2 %. The extract was stored in an air tight container at -20ºC until further analysis. The test materials were subjected to preliminary phytochemical screening following the standard methods [117].

2.4. In vitro quantitative antioxidant assay of TAHA and AA

2.4.1. Total phenolic content (TPC)

TPC was determined by the Folin-Ciocalteu reagent method. Test materials/standard (0.5 mL) of different concentrations were mixed with 1N Folin-Ciocalteu reagent and 20% sodium carbonate. The tubes were vortexed and allowed to stand for 40 min at 20°C for color development. The absorbance was read at 725 nm using spectrophotometer (UV-1800, Shimadzu, Japan) against blank. The total content of phenolic compounds was expressed in Tannic acid equivalents (TAE)/g of dry extract [118].

2.4.2. Total flavonoid content (TFC)

TFC was analyzed using previously reported method with suitable minor modifications. Briefly, 240 μL sample, Sodium nitrite (50 mg/mL), Aluminium chloride (100 mg/mL in methanol) were added and mixed. After five Min, 1M Sodium hydroxide was added. The TFC was calculated from a calibration curve using

Quercetin as standard (12.5–800 µg/mL) and expressed as (Quercetin equivalent) QE/g of dry extract [119].

2.4.3. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging capability of each extract solution on DPPH radicals was investigated as reported previously. Briefly, 4 mL of 0.1mM DPPH in methanol was mixed with one mL of each of extract (solution at different concentrations, 200 - 6.25 µg/mL). These mixtures were incubated in a dark room for 30 minutes, and the free radical scavenging ability was estimated by measuring the absorbance at 517 nm using a spectrophotometer [120].

2.4.4. Ferric Reducing Antioxidant Power (FRAP) Assay

The capability to reduce ferric ions was estimated using the standard method described by Benzie and Strain. The working FRAP reagent was freshly prepared by adding 300 mM sodium acetate buffer (pH 3.6), 10.0 mM tripyridyl triazine (TPTZ) solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 (v/v/v). Test materials (1 mg/mL) and standard $FeSO_4$ (0.1-1 mM) were then mixed with 3 mL of FRAP reagent, and the reaction mixture was incubated at 37°C for 30 Min followed by absorbance measurement at 593 nm. Calibration was carried out with a fresh working solution of $FeSO_4$. The antioxidant capacity based on ability to reduce ferric ions of the sample was calculated from the linear calibration curve [121].

2.5. HPLC-PDA Analysis: TAHA and AA

Chemical characterization was carried on the basis of polyphenolics such as gallic acid, ellagic acid, and quercetin using HPLC method [122].

The method was modified as per laboratory conditions. Briefly, Prominence HPLC system (Schimadzu, Japan) was equipped with the binary pump, autosampler, a column oven and a photodiode array detector. Chromatographic separations were carried out using C-18 analytical column (150 x 4.6 mm, 5 µm particle size; Syncronis, Thermo Scientific, U.S.A.). Following gradient elution with water containing 0.5% acetic acid (component A) and acetonitrile: water containing 0.5% of acetic acid (80:20 v/v) as a component B was used.

The nonlinear gradient elution program: 0-10 min 10% of B; 10-20 min 20% of B; 20-30 min 40% of B; 30-40 min 60% of B; 40-45 min 70% of B; 45-55 min 10 % of B and equilibrated with initial conditions for another 5 min. The flow rate and oven temperature were used at 1.0 ml/min and 25 °C respectively. All chromatograms were monitored at 270 nm. The method was validated for linearity, accuracy and precision.

Reference compound preparation

Each reference compound (10 mg) was dissolved in 10 mL of methanol. Serial dilutions were carried out from the working stock solution in methanol (600 µg/mL). Calibration curves were plotted from concentration range of 3.125-100 µg/mL in triplicate.

Sample preparation

Sample preparation was done as per previously reported method. Briefly, 10 mg of TAHA extract was dissolved in 10 mL methanol and the solution was filtered through 0.45 µm membrane filter. AA (1 mL) was dried on a rotary evaporator for 0.5 h and 5 mL of methanol was added. It was sonicated for 10 Min and then centrifuged at 3000 rpm for 10 min. Supernatant (1 mL) was passed through 0.45 µm membrane filter. Sample solutions of 20 µL were used for HPLC analysis. The peak of ellagic acid, gallic acid, and quercetin were identified by comparing their retention time values and UV spectra with those of standards. The marker contents for TAHA and AA were expressed as mg/g and µg/mL respectively.

2.6. Antibacterial activity

Four clinical bacterial isolates - *E.coli* HM95 (AIEC), *E.coli* HM615 (colonic mucosa associated. *E.coli*), *E.coli* HM233 and *E.coli* HM251 (colonic mucus associated patient strains) were sub cultured on MacConkey agar plates and incubated aerobically at 37ºC. The antimicrobial activity of TAHA and AA was evaluated by agar well diffusion method and MIC/MBC was detected by broth dilution method.

2.6.1. Agar well diffusion assay method

TAHA and AA were analysed for its antibacterial activity by agar well diffusion assay method. Antibacterial activity was performed against 4 clinical isolates of *E. coli* from IBD patients. Brain heart infusion (BHI) agar media was prepared, sterilized followed by aseptically transfer to petri plates. These petri plates were solidified and later kept in incubator overnight to validate sterility of the prepared plates. The bacterial culture of 10^6 cfu/mL (0.5 McFarland Standard) was uniformly spread on the surface of nutrient agar plates using sterile cotton swabs. The wells were punched with the cork borer (6 mm) in the agar. Dried TAHA extract was dissolved in dimethyl sulfoxide (20 % DMSO) to obtain a concentration of 50 mg/mL and 25 mg/mL. AA formulation was used at a concentration of 100% and 50%. Ciprofloxacin a standard drug was used as positive control. 100 µl volume of each test drug was added into the wells of the inoculated agar plates for each test organism. The plates were allowed to stand at room temperature for about 2 h and incubated at 37ºC. After 24 h of incubation, the zone of inhibition was measured using a HiAntibiotic Zone scale-C (Himedia Biosciences, Mumbai, India). All the experiments were performed thrice and results were recorded as mean ± standard deviation [123].

2.6.2. Serial dilution method

Serial dilution technique was employed to determine MIC and MBC of TAHA and AA. The least concentration of TAHA and AA displaying growth inhibition was considered as the MIC (determined by visual observation), whereas complete inhibition on inoculation on BHI agar plates and incubation for 24 hours was considered as MBC. Assay was performed thrice with appropriate Negative control (containing only media and bacterial culture) [123].

2.7. Cell line studies

Human colorectal adenocarcinoma cells (Caco2, COLO.205) and normal rat intestinal epithelial and mouse fibroblast cells (IEC-6 and L929) were obtained from National Center for Cell Sciences, Pune-India**.** The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with two mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% FBS procured from Gibco Life Technologies, Bangalore-India.

2.7.1. Determination of cytotoxicity and cytocompatibility

Viable cell suspension 50 µL with a density of 1×10^5 cells/mL (determined by Trypan blue exclusion method) was seeded into each well in a 96 well micro titre plate and final volume made upto 150 µL with DMEM media. Test materials were diluted in DMEM media to obtain different concentrations. 100 µL of TAHA and AA (400 - 6.25 µg/mL) and Standard drug 5-FU (100 - 1.562 µg/mL) was added to the wells followed by incubation for 48 h in the presence of 5% CO_2 at 37°C into CO_2 incubator (Eppendorf Brunswich, Germany). After the incubation period, 20 µL of MTT reagent (3 - (4, 5-dimethylthiazol- 2 - yl)-2, 5-diphenyltetrazolium bromide, 5 mg/mL in PBS) procured from HiMedia laboratories, was added to each well following 4 h incubation in dark. The supernatant was removed without disturbing the precipitated Formazan crystals. Formed crystals were dissolved by addition of 100 µL of DMSO and optical density (OD) was calculated at a wavelength of 492 nm using ELISA plate reader plus, India [124].

The study was performed in triplicates, and percent cell viability was calculated using equation –

$$\text{Percent cell viability} = \frac{OD\ of\ Test\ material}{OD\ of\ Control} \text{ X } 100$$

2.7.2. Lipid peroxidation in cells

IEC-6, L929, COLO.205, and Caco2 cells were plated into 12-well plates at a density of 1 x 10^7cells/mL in complete medium.

Sample preparation

Cells were re-suspended at 1-2 x 10^7 cells/mL in PBS containing 1X BHT and cells were sonicated on ice. The whole homogenate was used in the assay.

Procedure

- Series of dilution were prepared of MDA standards in the concentration range of 125 µM – 0 µM by diluting the MDA Standard in distilled or deionized water. It is recommended that standards be performed in duplicate. Absorbance was read at 532nm.
- Samples or MDA standards (100 µL) was added to separate microcentrifuge tubes.
- SDS Lysis Solution (100 µL) was added to the tubes. Thoroughly mixed. Incubated samples for 5 Min at room temperature.
- TBA Reagent (250 µL) was added to the tubes.
- Each tube was closed and incubated at 95°C for 45-60 min.
- Tubes were cooled to room temperature in an ice bath for 5 Min.
- Tubes were centrifuged at 3000 rpm for 15 Min and supernatant separated for further analysis.
- The supernatant (300 µL) was added to another tube containing 300 µL of n-Butanol. Vigorously vortexed for 1-2 Min and centrifuged for 5 min at 10,000 g. b. The butanol fraction was further assessed. The concentration of MDA in samples was calculated using MDA standards as reference [125].

2.8. Animal study

2.8.1. Ethical Approval

The experimental protocol used in the study was carried out in compliance with the CPCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals) Government of India. Female Wistar rats (180-200 g) obtained from Shri Venkateshwara Enterprises (Bangalore, India) were housed in polypropylene cages and maintained at 22 ± 2 °C, under standard lighting conditions (12- h light/dark cycle). The study was approved by Institutional Animal Ethics Committee of College of Pharmacy-KLE Academy of Higher Education and Research, Belagavi (resolution No. KLECOP / CPCSEA- Reg. No. 221/Res. 23-3 / 09 / 2016).

2.8.2. Trinitrobenzene sulphonic acid (TNBS) model of rat colitis

The animals were randomly distributed into nine groups of eight animals each. Two of the groups served as control (Noncolitic and Untreated colitic). The remaining four groups were colitic groups treated with standard (Prednisolone 2 mg/kg) and three different doses of TAHA (500, 250 and 125 mg/kg) and three doses of AA (1.8, 0.9, 0.45 mL/kg), each orally for 28 days after induction of colitis. Briefly, rats were anesthetized with thiopentone sodium, and then a flexible rubber plastic tube with an external diameter of 2 mm was inserted rectally into the colon. The tip was 8 cm proximal to the anus verge. TNBS (120 mg/kg) dissolved in ethanol (50%, v/v) was instilled into the colon via the cannula to induce colitis. To distribute the agents within the entire colon, rats were held in the head-down position for 2-3 min after the instillation of TNBS and then returned to their cage. The non colitic group received 50% ethanol rectally. Treatment with TAHA and standard continued for 28 days after which blood was withdrawn by cardiac puncture, and the rats were sacrificed with thiopentone sodium overdose. Rat body weight, stool consistency, and presence or absence of blood in stool was recorded daily throughout the experiment to calculate disease activity index (DAI)After the sacrifice, the colon was removed aseptically and placed on a cold plate. The colon was freed from surrounding tissues or fat and cleaned from luminal contents with cold saline. Colon specimens and rat plasma from different groups were kept frozen at -80 ºC until further analysis [126, 127].

Table 8. Grouping for Animal study

Group 1	Normal saline administration
Group 2	TNBS control
Group 3	TNBS + 2 mg/kg Prednisolone
Group 4	TNBS + 500 mg/kg TAHA
Group 5	TNBS + 250 mg/kg TAHA
Group 6	TNBS + 125 mg/kg TAHA
Group 7	TNBS + 1.8 mL/kg AA
Group 8	TNBS + 0.9 mL/kg AA
Group 9	TNBS + 0.45 mL/kg AA

2.8.3. Macroscopic Scoring

Macroscopic scores for inflammation were assigned based on earlier reported scoring pattern [128].

Table 9. Macroscopic damage scores

Score	Observations
0	No macroscopic change
1	Mucosal erythema alone
2	Mild mucosal oedema, slight bleeding or small erosions
3	Moderate oedema, bleeding ulcers or erosions
4	Severe ulceration / erosions, oedema, and tissue necrosis

2.8.4. Histopathological assessment of colitis

The colon tissue samples for histological examination were fixed overnight in 4% neutral buffered formalin, processed, sectioned (4 μm thick) and stained with hematoxylin and eosin (H & E). Previously reported histopathological scoring pattern was adopted for evaluation as depicted in Table 10 [129, 130].

Table 10. Histopathological scoring pattern in colon tissue

Histopathological conditions	Scoring pattern		
	'0'	**'1'**	**'2'**
Infiltration of acute inflammatory cells	No	Mild increasing	Severe increasing
Infiltration of chronic inflammatory cells	No	Mild increasing	Severe increasing
Deposition of fibrotin protein	Negative	Positive	-
Submucosa oedema	No	Patchy - like	Fusion - like
Epithelium necrosis	No	Limiting	Widening
Epithelium ulcer	Negative	Positive	-

2.8.5. Biochemical assays

2.8.5.a. Myeloperoxidase (MPO) Assay [131]

50 mM solution of Potassium Phosphate buffer

Solution B (K_2HPO_4, 8.7 g of dibasic potassium phosphate in 1 L of dH_2O) was added to solution A (KH_2PO_4, 6.8 g of monobasic potassium phosphate in 1L of dH_2O) until a pH of 6.0 was achieved.

Hexadecyltrimethylammonium bromide (HTAB) buffer

5g HTAB was added to 1 L of Potassium Phosphate buffer (50 mM, pH=6.0). Gently heated to dissolve and stored at 2-8°C until use.

o-dianisidinedihydrochloride (o-dianisidine)

16.7 mg of o-dianisidinedihydrochloride, 90 mL of dH_2O, and 10 mL of potassium phosphate buffer were mixed together.

Procedure

- Colon samples were homogenized in 10 mM hexadecyltrimethylammonium bromide buffer (25 mg tissue/mL) and the supernatant was used to determine MPO activity
- 200 μL of supernatant was added in triplicate into a 96-well plate.
- 200 μL of the o-dianisidine mixture containing H_2O_2 (4 μL of 30% H_2O_2 diluted in 96 μL of dH_2O) was added to each of the wells.
- Absorbance was measured at 450 nm using a spectrophotometer.
- MPO activity was measured in units (U) of MPO/mg tissue.

2.8.5.b. Malondialdehyde (MDA) Assay [132]

- Tissue malondialdehyde (MDA) levels as a marker of lipid peroxidation, which is related to colitis disease severity, were analysed using the thiobarbituric acid reactive substances (TBARS) assay and the novel radical formation analysis method of Mihara and Uchiyama .
- Colon tissues were homogenized with cold 1.5% potassium chloride to make a 10% homogenate for MDA analyses.
- Three mL of 1% phosphoric acid and 1 ml of 0.6% thio- barbituric acid (TBA) aqueous solution were added to 0.5 mL of 10% homogenate.
- The mixture was heated for 45 Min and, after cooling, 4 mL of n-butanol was added and mixed. Absorbance was measured at 535 and 520 nm.
- The difference of the two measurements defined the level of MDA (nmoL/g tissue).

2.8.5.c. Catalase Assay [133]

(0.065 M) Hydrogen Peroxide (H_2O_2)

A volume of 6.639 ml of H_2O_2 (30%) was diluted to a final volume of 1 liter of Na-K-phosphate buffer.

60 mmol/l Sodium-Potassium Phosphate Buffer, pH 7.4

A weight of 11 g of disodium monohydrogen phosphate (Na_2HPO_4) and 2 g of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 1 liter of dH_2O. The pH was adjusted to 7.4 by the addition of 1M of HCl.

32.4 mmol/l Ammonium Molybdate ($(NH_4)_6 Mo_7O_{24} .4 H_2O$)

A weight of 40.04 g of ammonium molybdate was dissolved in 1 liter of dH_2O.

Procedure

All reagents and plasma were brought to 37°C. Sample, blank 1, blank 2, and blank 3 test tubes were prepared then pipetted into test tubes as shown in Table 11.

Table 11. Procedure for Catalase

Reagents	Sample (µL)	Blank 1 (µL)	Blank 2 (µL)	Blank 3 (µL)
Plasma	50	50	-	-
Substrate (H_2O_2)	1000	1000	1000	-
Phosphate Buffer	-	-	50	1050
Ammonium Molybdate	1000	1000	1000	1000

Tubes were incubated for 60 sec.at 37°C. Absorption was read at 405 nm against blank 3.

$$\text{CAT activity (kU/l)} = \frac{A\,(Blank\ 1) - A(Sample)}{A\,(Blank\ 2) - A(Blank\ 3)} \text{ X } 271$$

2.8.5.d. Superoxide Dismutase (SOD) Assay [134]

Tris- EDTA buffer pH 8.2

A weight of 2.85 g of Tris and 1.11 g of EDTA-Na_2 were dissolved in 1 liter of dH_2O

Pyrogallol Solution (0.2 mM)

A weight of 0.252 g of pyrogallol was dissolved in a solution of 0.6 mL of concentrated hydrochloric acid diluted in 1 liter of dH_2O.

Table 12. Procedure for Superoxide Dismutase Assay

Reagents	**Test (µL)**	**Control (µL)**
Serum	50	-
Tris buffer	1000	1000
$d.H_20$	-	50
Pyrogallol	1000	1000

Absorption was read at the wavelength of 420 nm against Tris-EDTA buffer at zero time and after 1 Min of the addition of pyrogallol.

Calculation of SOD Activity

$$\%\ \text{Inhibition of pyrogallol autoxidation} = \frac{\Delta A\ Test}{\Delta A\ Control} \text{ X } 100$$

$$\text{(Cu-Zn) SOD Activity (U/mL)} = \frac{\%\ \text{inhibition of pyrogallol autoxidation}}{50}$$

2.8.5.e. GSH [Reduced] Assay [135]

Assay buffer solution (0.1M sodium phosphate with 5 mM EDTA, pH 7.4)

Solution 1: Weigh 14.196 g Na-Phosphate dibasic and dissolve in approximately 800 ml dH_2O. Add 1.86 g EDTA and dissolve completely.

Solution 2: Weigh 3.45 g Na-Phosphate monobasic and dissolve in 250 mL dH_2O. Add 0.47 g EDTA and dissolve completely

Add monobasic solution to dibasic solution (#2 to #1) to achieve pH of 7.4. Bring final volume to 1000 mL by adding dH_2O after pH of 7.4 has been reached.

5,5- dithiobis 2-nitrobenzoic acid (DTNB) stock solution (10 mM)

Weigh 99.1 mg DTNB and dissolve in approximately 20 ml assay buffer. Transfer to volumetric flask and bring volume up to 25 ml by adding assay buffer.

Procedure

- 0.6% sulfosalicylic acid was added to plasma.
- The tubes were centrifuged at 8000 g for 10 Min. at 4°C.
- Supernatant obtained was mixed with 2 mL of buffer.
- 0.1 mL of freshly prepared DTNB was added, and absorbance was measured spectrophotometrically at 412 nm.
- A standard curve was plotted using 10-100 μM of reduced form of glutathione and results were expressed as nanomoles of reduced glutathione per milligram of protein.

2.8.5.f. Nitric Oxide Estimation Assay [136]

NED Solution

(0.1% N-1-napthylethylenediamine dihydrochloride in water)

Nitrite Standard

(0.1M sodium nitrite in water)

Sulfanilamide Solution

(1% sulfanilamide in 5% phosphoricacid)

Procedure

The NO production was assessed indirectly by measuring the nitrite levels in plasma determined by a calorimetric method based on the Griess reaction

Standard nitrite concentration in the range 0-100 µM were prepared

Plasma samples were deproteinized by zinc sulphate and NaOH, centrifuged and supernatant was used in the assay.

Sulfanilamide Solution and NED Solution was allowed to equilibrate to room temperature (15–30 Min).

- 50 µL of each experimental sample was added to wells in duplicate or triplicate.
- 50 µL of the Sulfanilamide Solution was added to the wells.
- Incubated for 5–10 Min at room temperature, protected from light.

- 50 μL of the NED Solution was added to all wells.
- Incubated at room temperature for 5–10 Min, protected from light. A purple/magenta color soon developed.
- Absorbance was measured within 30 Min in a plate reader at 540 nm.

2.8.6. Plasma zinc estimation

Zinc level was estimated using Atomic Absorption Spectrophotometer (AA-7000; Shimadzu, Japan). Plasma samples were allowed to thaw at room temperature. Working standards of zinc were prepared using standard zinc solution (S. D. Fine Chem. Ltd., Mumbai, India) and samples were diluted with de-ionized water. The instrument, gas-flow setting and aspiration rate was precisely established, to optimize signal and minimize background noise. The zinc concentration in the samples was calculated from absorbance readings, by interpolation from the working curve and the results were expressed in milligram/deciliter [137, 138].

2.8.7. Analysis of gene expression by real-time polymerase chain reaction

The analysis of pro-inflammatory mediators in colonic samples were performed by real-time PCR (RT-PCR). Total RNA was isolated from colon tissues using the Trizol ® reagent (Sigma-Aldrich), according to the manufacturer's instructions. Isolated RNA samples were quantified by using NanoDrop® Spectrophotometer (JH BIO, USA) and 2 µg of total RNA was reverse transcribed into first-strand cDNA (complementary Deoxyribonucleic acid) following the manufacturer's procedure. The synthesized cDNA was then used as a template for polymerase chain reaction (PCR) amplification. RT-PCR was performed using step one real-time PCR system (Applied Biosystems). IL-6, IL-1β, MCP-1 and TNF-α SYBR (synergy brands.inc) green primers were used for RT-PCR analysis. The primer sequences used are shown in Table 13. The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene GAPDH [115].

Table 13. Primer sequences used in RT-PCR assays in colonic tissue

Primers	**Sequences (5'‾3')**	**Annealing temperature (°C)**
IL-6	Forward AACTCCATCTGCCCTTCAGGAACA Reverse AAGGCAGTGGCTGTCAACAACATC	62.7
IL-1β	Forward AGCAGCTTTCGACAGTGAGGAGAA Reverse TCTCCACAGCCACAATGAGTGACA	62.7
MCP-1	Forward TGCTGTCTCAGCCAGATGCAGTTA Reverse TACAGCTTCTTTGGGACACCTGCT	62.7
TNF-α	Forward AGAACAGCAACTCCAGAACACCCT Reverse TGCCAGTTCCACATCTCGGATCAT	62.7

2.8.8. Bacteriological analysis of faeces

The bacteriological analysis of faeces was carried out as per previously described method. The feces sample was collected the day rats were sacrificed and stored at -80 ºC until further analysis. Faecal matter (0.1 g) was homogenized with PBS buffer (0.9 mL) and 10^{-5} dilution was made followed by plating 100 µL on the different culture media: reinforced clostridium agar medium (Clostridia) and Beerens (Bifidobacteria). The plates were subsequently incubated anaerobically at 37 ºC for 72 hours and colonies appearing were counted. The results were expressed as Logarithm of the number of colony forming units [58].

ANNEXURE II

A. Authentication letter of *Terminalia arjuna* bark

K.L.E.U'S
Shri B.M.K.Ayurveda Mahavidyalaya
Post Graduate Studies and Research Centre
Shahapur,Belagavi – 03.

Central Research Facility

ANALYTICAL LABORATORY

(AYUSH Approved ASU Drug testing Laboratory)

KLE UNIVERSITY

CRF/52/2015

Name: Damita Cota — Date: 14/10/2015

MD / **PhD Scholar** / Others — Sample code: CRF/645/2015

Name of specimen: Sl No: 1 — **Dry** / Wet / Others

AUTHENTICATION CERTIFICATE

This is to certify that the below mentioned specimen is identified and authenticated by concerned expert as.

	Name	Code	Scientific name	Part Used	Varg/Family
1.	Arjuna	645	*Terminalia arjuna* Roxb	Stem bark	Combrataceae

CRF SHAHAPUR, BELGAUM

CO-ORDINATOR
Central Research Laboratory
KLE'S Shri B. M. Kankanawadi Ayurved Mahavidhyalaya, Shahapur - BELGAUM.

B. Institutional Animal Ethics Committee Approval certificate

KLE UNIVERSITY EMPOWERING PROFESSIONALS

K.L.E. University's

COLLEGE OF PHARMACY

J.N.M.C Campus, Nehru Nagar Belgaum. Karnataka, India.

(Recognised by PCI, AICTE & Accredited 'A' Grade by NBA, AICTE, New Delhi

Phone No. 0831-2471399, Fax 0831-2472387

ಕೆ.ಎಲ್.ಇ. ವಿಶ್ವವಿದ್ಯಾಲಯದ ಔಷಧೀಯ ಮಹಾವಿದ್ಯಾಲಯ ಬೆಳಗಾವಿ – 590010

KLE SOCIETY'S COLLEGE OF PHARMACY BELGAUM

Ref No. KLEU/COP ____________ Date : 03/09/2016

CERTIFICATE

This is to certify that the research project," **Effect of hydro-alcoholic extract of *Terminalia arjuna* bark and *Arjunarishta* in inflammatory bowel disease.** ", Submitted by **Miss. Damita Cota** has been approved in the Institutional Animal Ethics Committee meeting held on 3rd September 2016, resolution No. KLECOP/CPCSEA-Reg.No.221/Res.23 - 3/09/2016 and was permitted to use 72, sex Either Rats/ Mice/ Rabbits/Guinea pig

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, **Form D.**

MEMBER SECRETARY
Institutional Animal Ethical Committee,
KLES's College of Pharmacy,
BELGAUM - 590010

(M.S. Kolhar) 03/9/2016
CPCSEA Nominee
Institutional Animal Ethics Committee
KLES's College of Pharmacy,
BELGAUM.

ANNEXURE III: RESEARCH PUBLICATIONS

1. Cota D, Mishra S, Shengule S. Arjunarishta alleviates experimental colitis via suppressing proinflammatory cytokine expression, modulating gut microbiota and enhancing antioxidant effect. Molecular Biology Reports. https://doi.org/10.1007/s11033-020-05766-z
2. Cota DL, Mishra S, Shengule SA, Patil D. Assessment of in vitro biological activities of *Terminalia arjuna* Roxb. bark extract and Arjunarishta in inflammatory bowel disease and colorectal cancer. Indian J Exp Biol. 2020; 58: 306-313.
3. Cota D, Mishra S, Shengule S. Beneficial role of *Terminalia arjuna* hydro-alcoholic extract in colitis and its possible mechanism. J Ethnopharmacol. 2019; 230: 117-125.

Oral/ Poster presentations (National/ International)

- Presented at 3rd International conference on healthcare and Allied Sciences nosy on Healthcare Transformation and New Age Technologies organised by Lincoln University College, Malaysia, on 25th and 26th September 2019.
- Awarded best poster at Two Days National Conference on 'Advances in Drug Discovery and Development', KLE College of Pharmacy, Belagavi on 26-27th October 2018.
- Presented at 'Current Trends in Pharmacological Research' organised by Indian Pharmacological Society, Local branch, Belagavi and Department of Pharmacology, J. N Medical College, Belagavi on 6th April 2018.
- Awarded best poster at Pharmacology CME 2016 on 'Current trends in the management of hypertension' organised by Department of Pharmacology, J. N. Medical College, Belagavi and Indian Pharmacological Society, Belagavi Branch on 22nd April 2016.
- Presented at 20th National Convention of Society of Pharmacognosy on 'Newer approaches for development and promotion of natural products' organised by KLE University's College of Pharmacy, Belagavi, Karnataka on 26th and 27th March 2016.

Molecular Biology Reports
https://doi.org/10.1007/s11033-020-05766-z

ORIGINAL ARTICLE

Arjunarishta alleviates experimental colitis via suppressing proinflammatory cytokine expression, modulating gut microbiota and enhancing antioxidant effect

Damita Cota[1] · Sanjay Mishra[1,2] · Sushant Shengule[1]

Received: 7 June 2020 / Accepted: 28 August 2020

Abstract

Traditional ayurvedic medicine, Arjunarishta (AA) is used to treat several inflammatory conditions including dysentery associated with blood. The formulation is a decoction of *Terminalia arjuna* (Roxb.) Wight and Arn. (TA), *Madhuca indica* J.F.Gmel., *Vitis vinifera* L., *Woodfordia fruticosa* (L.) Kurz., and *Saccharum officinarum* L. *Terminalia arjuna*, a major constituent of this formulation has been recognized for anti-inflammatory effects. This study aimed at evaluating beneficial effects of AA and probable mechanism of action in Trinitrobenzenesulphonicacid (TNBS) induced colitis model. Response to AA treatment was explored through determination of disease activity index (DAI), histological assessment and damage scores, colonic pro-inflammatory cytokine/chemokine expression and estimation of oxidative stress biomarkers. Improvement in gut microbiome and plasma zinc level was also assessed. Study findings directed therapeutic effects of AA treatment in colitis model by attenuating the colitis symptoms such as weight loss, diarrhoea, blood in stool; histological damage; and downregulated expression of pro-inflammatory cytokines/chemokine (TNF-α, IL-1β, IL-6) and MCP-1). Similarly reduced oxidative stress by decreased level of Nitric Oxide (NO), Myeloperoxidase (MPO), Malondialdehyde (MDA) and enhanced level of Catalase (CAT), Superoxide dismutase (SOD) and Reduced Glutathione (GSH) was also witnessed. In addition, an improved beneficial fecal microbiome profile and restored plasma zinc status was revealed compared to the TNBS control group. The present study directs that downregulated pro-inflammatory cytokines/chemokine expression, enhancement of antioxidant effect, increased plasma zinc status and promising role in modulating fecal microbiome might be potential mechanisms for the therapeutic effect of AA treatment against colitis.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-020-05766-z) contains supplementary material, which is available to authorized users.

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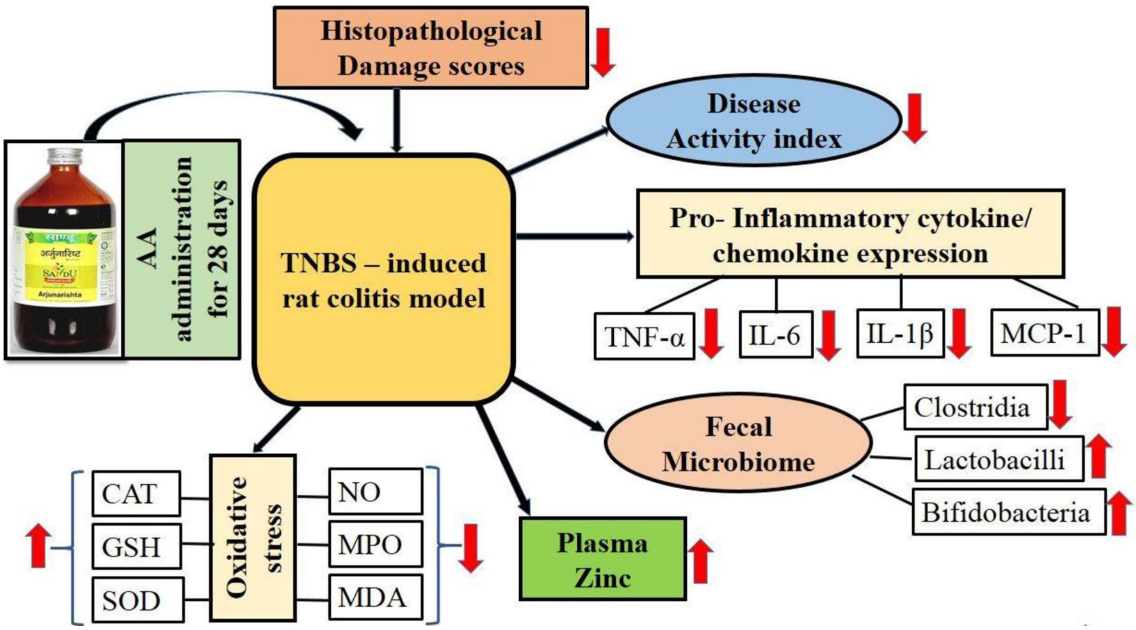
Published online: 03 September 2020

Graphic abstract



Keywords Arjunarishta · Inflammatory bowel disease · TNBS rat colitis · Cytokines · Chemokine · Gut microbiota

Abbreviations

AA	Arjunarishta
CAT	Catalase
DAI	Disease activity index
GSH	Glutathione
IBD	Inflammatory bowel disease
IL-6	Interleukin-6
IL-1β	Interleukin-1β
MCP-1	Monocyte chemotactic protein
MDA	Malondialdehyde
MPO	Myeloperoxidase
NO	Nitric oxide
SOD	Superoxide dismutase
TNF-α	Tumour necrosis factor-α
TNBS	Trinitrobenzenesulfonic acid
UC	Ulcerative colitis

Introduction

Ulcerative colitis (UC), one major form of inflammatory bowel disease is a chronic, relapsing inflammation that starts in rectum and extends proximally covering the entire colon in a continuous manner [1]. It affects superficial mucosal layer causing infiltration of neutrophils, lymphocytes, granulocytes and goblets cells along with excessive production of free radicals, initiating mucosal damage and ulceration. These infiltrating cells regulate release of pro-inflammatory cytokines and add to intestinal damage leading to clinical complications. The clinical features and associated complications such as severe diarrhoea, blood loss and progressive loss of peristaltic function leads to rigid colonic tube [2]. The disease pathogenesis is unclear; however interaction between genetic, environmental factors and intestinal microbiome leading to dysregulated immune responses may be accountable for the disease onset [3]. Based on the disease severity, existing treatment includes corticosteroids, antibiotics, amino-salicylates, immunosuppressants and biological agents as choice of drugs. However, are associated with several side effects and resistance upon long term use [4, 5]. Therefore, in recent years practice of novel approaches based on natural products/herbal therapies has been continuously increasing worldwide [6, 7].

Arjunarishta (AA) is a polyherbal hydro-alcoholic formulation. It is advised as cardiotonic and choice of medication in chronic respiratory ailments, bilious affection, blood dysentery and other inflammatory conditions. AA treatment also improves strength and aids in cleansing of intestines [8, 9]. The TA stem bark, major component of this formulation is traditionally known to treat various inflammatory conditions and gastrointestinal disorders [10, 11]. Likewise, we have previously reported the beneficial effects of TA bark extract in Trinitrobenzenesulphonicacid (TNBS) induced colitis model [12].

The present study aimed at evaluating the biological activities of AA in TNBS model of rat colitis. The efficacy of AA at three different doses was compared against standard drug prednisolone (glucocorticosteroid—used clinically to treat UC). The histological evaluation of rat colon, investigation of AA in modulating antioxidant enzyme activities, proinflammatory cytokines/chemokine levels, plasma zinc level and fecal microbiota was assessed.

Materials and methods

Drugs and chemicals

TNBS for colitis induction and Trizol ® reagent for RNA isolation was purchased from Sigma-Aldrich, USA. While, standard drug: Prednisolone (Medrol) was procured from local medical store. Analytical grade solvents were used for analysis (Fisher Chemicals). An ayurvedic formulation of *Terminalia Arjuna*—Arjunarishta was purchased from the local market (Batch no. 25, Sandu Pharmaceuticals Ltd., Mumbai—India). Hemospot kit (fecal occult blood detection) was procured from Coral Clinical Systems, Tulip Diagnostics (P) Ltd. Verna, Goa—India. IL-6, IL-1β, MCP-1 and TNF-α SYBR (synergy brands.inc) green primers were used for RT-PCR analysis. Media for fecal microbiome assessment was procured from HiMedia Laboratories Pvt. Ltd.

The phytochemical standardization of the Arjunarishta was analyzed according to previously reported method with suitable modification in column and mobile phase gradient [13] using three polyphenolic markers-gallic acid, ellagic acid and quercetin by High Performance Liquid Chromatography. In our previous published reports, we had described the presence of above mentioned three polyphenolic markers in Arjunarishta [14, 15]. (Data not presented here)

Animals

Female Wistar rats (180–200 g) obtained from Shri Venkateshwara Enterprises (Bangalore, India). Animals were housed in polypropylene cages at constant temperature with controlled 12–12 h light/dark cycle and access to food and water *ad libitum*. The experimental procedures were performed in compliance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines, Government of India and approved by Institutional Animal Ethics Committee of College of Pharmacy-KLE Academy of Higher Education and Research, Belagavi [resolution No. KLECOP/CPCSEA-Reg. No. 221/Res. 23-3/09/2016].

Colitis induction and experimental design

After 1 week of acclimatization, 48 Wistar rats were randomly distributed into six groups (n = 08): Normal control, TNBS control, TNBS + standard (Prednisolone 2 mg/kg), TNBS + AA (1.8, 0.9, 0.45 mL/kg). Before the intrarectal inoculation of TNBS solution (Sigma-Aldrich, USA), the rats were fasted for 24 h and lightly anesthetized with thiopentone sodium (40 mg/kg). Experimental colitis was induced using TNBS enema (120 mg/kg) dissolved in ethanol (50%, v/v) [16]. AA and prednisolone was administered orally for 28 days using an oral feeding needle. Body weight (BW) and health status of animals was monitored daily throughout the study period.

The AA treatment dose was converted using USFDA guidance considering the weight of experimental rat as 200 g. The dose conversion table based on surface area, the adult human dose multiplied by 0.018 provided the dose [17]. The dose was selected based on adult human dose (20 mL per day).

Sample processing and analysis

The rats were euthanized with thiopentone sodium overdose. Blood was withdrawn by cardiac puncture and plasma was separated. Laparotomy was performed, colon tissue was excised and distal colon was separated for analysis. Distal colon was opened longitudinally, the luminal content was removed and immediately washed with saline solution. The colon was freed from surrounding tissues or fat. Colon specimens and rat plasma from different groups were kept frozen at − 80 °C until for biochemical and Gene expression investigations.

Microscopic examination of colitis severity

The colon tissue samples for histological examination were fixed overnight in 4% neutral buffered formalin, processed, sectioned (4 µm thick) and stained with hematoxylin and eosin (H & E) [17]. Previously reported histopathological scoring pattern [18] was adopted for assessment: Infiltration of acute inflammatory cells (0 = No, 1 = Mild increasing, 2 = Severe increasing), Infiltration of chronic inflammatory cells (0 = No, 1 = Mild increasing, 2 = Severe increasing), Deposition of fibrotin protein (0 = Negative, 1 = Positive), Submucosa edema (0 = No, 1 = Patchy like, 2 = fusion like), Epithelial necrosis (0 = No, 1 = Limiting, 2 = Widening), and Epithelial ulcer (0 = Negative, 1 = positive).

Biochemical estimations and plasma zinc analysis

MPO and MDA activity in tissue homogenate was estimated using previously described methods with minor modification

[19, 20]. CAT activity [21], SOD activity [22], reduced GSH activity [23] and NO production [24] were performed in plasma samples of different study groups. All the assays were determined by spectrophotometer (UV-1800, Shimadzu, Japan). Whereas, Atomic Absorption Spectrophotometer (AA-7000; Shimadzu, Japan) was involved for the zinc level as previously stated protocol [12].

Gene expression analysis by RT-PCR

Total RNA was isolated from colon tissues using TRIZOL (Invitrogen, Carlsbad, CA, USA). All isolated RNA samples were quantified by NanoDrop® Spectrophotometer (JH BIO, USA) and 2 µg of total RNA was reverse transcribed into first-strand cDNA (complementary Deoxyribonucleic acid). The synthesized cDNA was then used as a template for (PCR) amplification. RT-PCR was performed using step one real-time PCR system (Applied Biosystems). The primer sequences used in RT-PCR are shown in Table 1. The relative expression levels of target genes were calculated as a ratio to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [17, 25].

Fecal microbiome assessment

The stored rat fecal samples at − 80 °C was attained for microbiome assessment according to previously described protocol with minor modifications [26]. The fecal specimen dilution was cultured on Clostridial agar (Clostridium spp.), Rogosa SL agar (lactobacilli spp.), and Bifidobacterium agar (Bifidobacterium spp.). The experiment was conducted in Compact-MG Anaerobic workstation (Kim microsystems).

Statistical analysis

The results were expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way ANOVA with Tukey post hoc test. Differences were considered statistical significant when $p \leq 0.05$ (GraphPad Prism version 5.01 software, San Diego, CA, USA).

Results

AA administration improved gross appearance and disease activity index in colitis rats

The TNBS induction in rats displayed prostration, piloerection, inability to move, appetite loss, fecal adhesion to anus associated with blood and weight loss. Test samples ameliorated the symptoms of colitis. These symptoms gradually decreased in TNBS control animals by Day 10 which kept on declining further, while AA and Prednisolone treatment enhanced the disappearance of the colitis symptoms. Figure 1a revealed the disease activity index throughout 28 days of study period. The improvement in AA treatment groups was higher as compared to prednisolone. AA treatment groups showed with no DAI (1.8 mL/kg by day 20; 0.9 mL/kg and 0.45 mL/kg by day 24). Whereas, Prednisolone group was observed with DAI till the end of the study.

Administration of AA displayed recovery of histological investigation

TNBS induction in rats induced pathological changes displaying severe infiltration of acute and chronic inflammatory cells, deposition of fibrin protein, epithelial necrosis and ulceration. These histological changes were not detected in normal control group. Similarly, the treatment groups of AA (1.8 mL/kg and 0.9 mL/kg) and prednisolone markedly ameliorated the IBD-induced histological alterations like the tissue restoration, edema and necrosis relative to TNBS control group (depicted as devised histological scores in Fig. 1b). Whereas, AA treatment group (0.45 mL/kg) displayed no significant changes when compared to TNBS control group. Representative histopathological specimens from each group are displayed in Fig. 2.

AA administration alters the biochemical parameters: oxidative stress biomarkers, enzymatic antioxidants and plasma zinc level

The levels of colonic MPO, MDA and plasma NO in TNBS control group were significantly enhanced compared to

Table 1 Primers used in gene expression study

Target gene	Forward primer	Reverse primer	Annealing temp. (°C)
IL-6	AACTCCATCTGCCCTTCAGGAACA	AAGGCAGTGGCTGTCAACAACATC	62.7
IL-1β	AGCAGCTTTCGACAGTGAGGAGAA	TCTCCACAGCCACAATGAGTGACA	62.7
MCP-1	TGCTGTCTCAGCCAGATGCAGTTA	TACAGCTTCTTTGGGACACCTGCT	62.7
TNF-α	AGAACAGCAACTCCAGAACACCCT	TGCCAGTTCCACATCTCGGATCAT	62.7

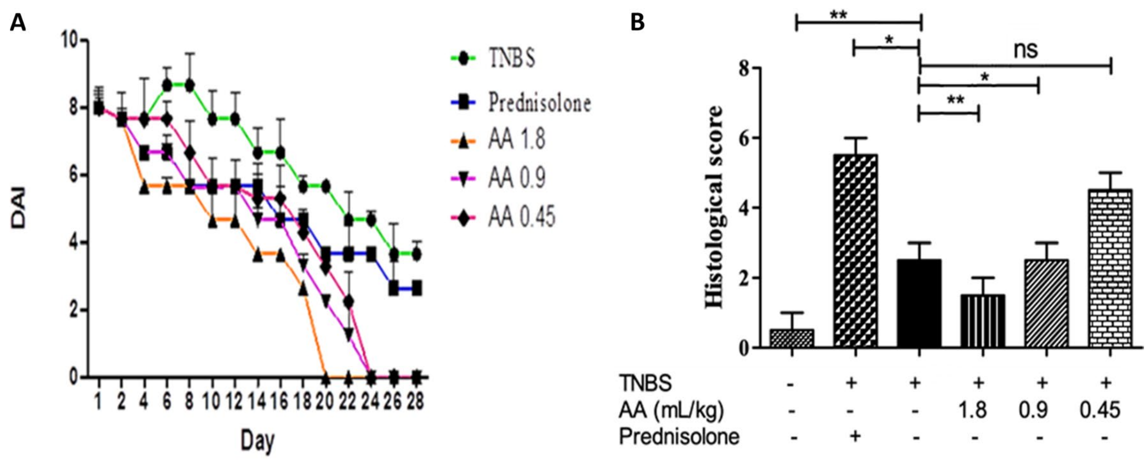


Fig. 1 Recovery effects of Arjunarishta treatment on TNBS-induced rat colitis (n = 08). **a** Disease activity index. **b** Histological scores. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Normal control and TNBS control respectively

normal control group. However, TNBS administration reduced significantly ($p < 0.001$) CAT, GSH and SOD activity as compared to normal control group (Fig. 3).

The enhanced MPO level was reduced in AA treatment groups (1.8 mL/kg) as 14.34 ± 2.02 U/mg, (0.9 mL/kg) as 17.24 ± 0.48 U/mg, and (0.45 mL/kg) as 33.90 ± 6.91 U/mg. While, TNBS mediated decrease in CAT, SOD and GSH was inhibited in the rats supplemented with AA and prednisolone. Raised MDA level by TNBS induction in rats was decreased by the treatments groups (AA 1.8 mL/kg) as 1.76 ± 0.05 nmol/g, (AA 0.9 mL/kg) as 2.51 ± 0.20 nmol/g, (AA 0.45 mg/kg) as 3.62 ± 0.19 nmol/g, and prednisolone as 2.45 ± 0.13 nmol/g. Similarly, the elevated plasma NO level was decreased by the treatment of AA (1.8 mL/kg) as 0.73 ± 0.02 nmol/ml,(0.9 mL/kg) as 0.81 ± 0.03 nmol/ml, (0.45 mL/kg) as 0.97 ± 0.01 nmol/ml. Whereas, prednisolone treatment revealed 0.65 ± 0.02 nmol/ml.

The plasma zinc level in TNBS control group was significantly downregulated compared to the Normal control group (66.19 ± 2.99 mg/dl vs. 140.6 ± 4.04 mg/dl) and was significantly upregulated by the AA treatment groups (1.8 mL/kg) as 104.0 ± 8.65 mg/dl, (0.9 mL/kg) as 90.16 ± 4.92 mg/ml. While, prednisolone treatment revealed 131.5 ± 5.01 mg/dl. The AA dose of 0.45 mL/kg did not affect significantly (Fig. 4).

AA administration downregulates proinflammatory cytokines and chemokine expression in colon

The intestinal inflammatory process induced by TNBS resulted in significant up-regulation of pro-inflammatory cytokines: IL-1β, IL-6, TNF-α and chemokine MCP- 1 expression in colon compared to the non-induced group (Fig. 5) (2.60 ± 0.33, 2.38 ± 0.39, 2.60 ± 0.28, 2.60 ± 0.23) respectively. The AA treatments downregulated the expression of these inflammatory cytokines in tissue. AA 1.8 mL/kg significantly reduced the level of IL-1β, TNF-α and MCP- 1 compared to TNBS control group, while reduction in IL-6 expression was not statistically significant. The AA treatment at 0.9 mL/kg displayed a significant reduction in MCP-1and TNF-α. AA 0.45 mL/kg displayed significance only in case of MCP-1 expression. Prednisolone treatment exhibited significant reduction except in IL-6.

Administration of AA beneficially improves the fecal microbiota

The fecal matter assessment revealed the AA treatment effect on gut microbiota (Table 2). The normal microbial flora was altered after TNBS administration, where it reduced Lactobacilli (significant) and Bifidobacteria (not significant) and significantly enhanced Clostridial counts compared to normal group. The AA treatments at 1.8 mL/kg and 0.9 mL/kg significantly elevated the Bifidobacteria count compared to TNBS control group. Whereas, prednisolone did not affect significantly Bifidobacterial count. Both AA and prednisolone treatments significantly reduced the clostridium count compared to TNBS group. No count was detected for Lactobacilli in fecal specimens of rats treated with prednisolone, which is lower than the TNBS control group. Interestingly, almost similar Lactobacilli count was detected in AA 1.8 mL/kg treatment group when compared with normal control group. Although, AA treatments at all doses resulted significant enhanced Lactobacilli count compared to TNBS control group.

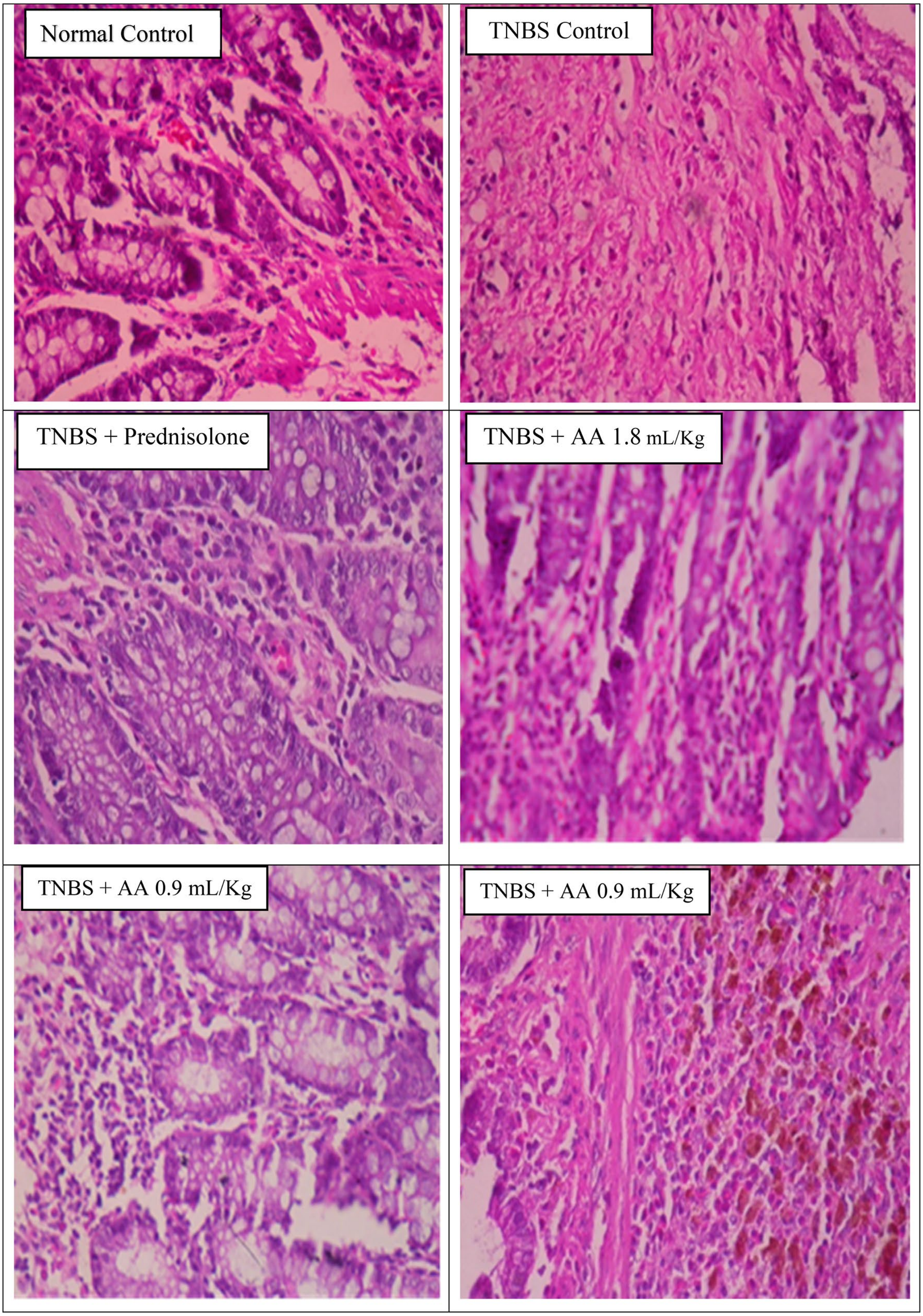


Fig. 2 Histopathological examination (Magnification ×40). Representative images of each study group

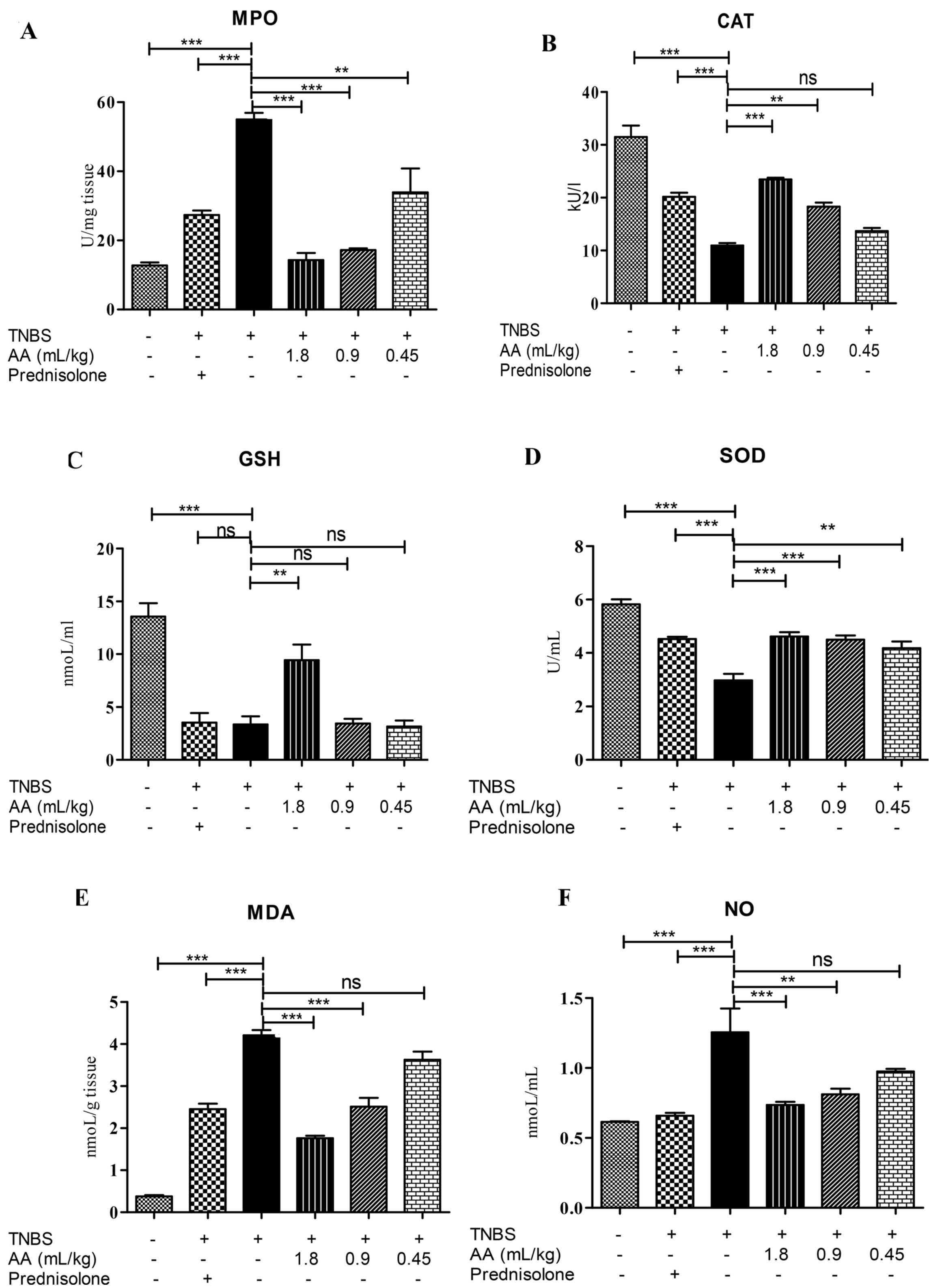


Fig. 3 Effects of prednisolone (2 mg/kg) and AA administration (1.8, 0.9,0.45 mL/kg) on Myeloperoxidase (MPO) (**a**), Catalase (CAT) (**b**), Glutathione (GSH) (**c**), Superoxide dismutase (SOD) (**d**), Malondialdehyde (MDA) (**e**), Nitric oxide (NO) (**f**). Treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 08). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ vs. normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control

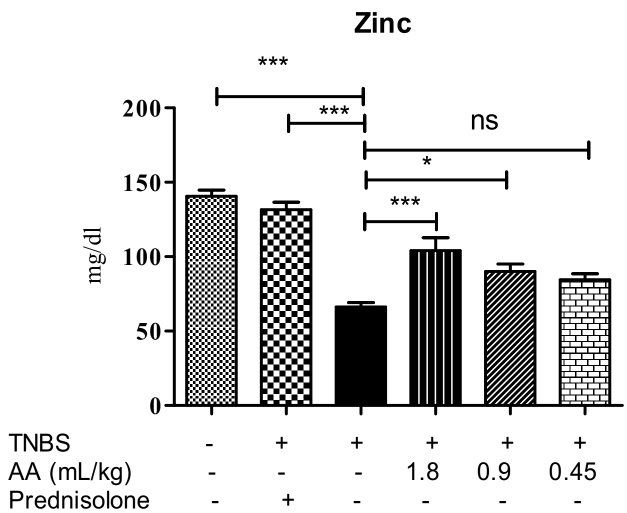


Fig. 4 Plasma Zinc levels in prednisolone (2 mg/kg) and AA treatment groups (1.8, 0.9, 0.45 mL/kg). Treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 08)

Discussion

The existing therapy in IBD includes anti-inflammatory agents, immunosuppressants and biologicals which are efficacious in most cases; however all these drugs are associated with certain serious side effects that limit their long term application. This has encouraged the need to identify and use naturally derived alternative medicines in order to attain efficacy, safety and compliance among IBD patients [27, 28].

The complex components in natural products and herbs with their multiple mechanism of action may synergize to produce their overall efficacy in ulcerative colitis with decreased medical cost [29].

In the present study we used the TNBS induced rat colitis model, mimicking human disease in its histological and biochemical features, which is established for inflammatory mechanism due to the generation of transmural oxidative stress and release of proinflammatory mediators [30, 31]. The study finding suggests beneficial treatment effect of AA, observed during experimental schedule in terms of reduced DAI and faster recovery from TNBS induced damage as compared to colitic rats. Thus revealing an improvement in the health status of experimental rats treated with Ayurvedic formulation, which was further confirmed by histological damage scores.

Oxidative stress is one of the major factor involved in the IBD progression and increased ROS production has been associated with chronic intestinal inflammation. These enhanced levels could result in relapse of active infection in ulcerative colitis patients despite being on maintenance therapy and may require additional antioxidant treatment [32, 33]. In TNBS induced colitis model, the natural balance between ROS production and protective free radical scavengers may be disturbed leading to tissue injury [34]. Enzymatic antioxidants such as GSH, SOD and CAT constitute the antioxidant system and these defense systems protect the cells against oxidative stress. SOD prevents oxidation in vascular endothelial cells exposed to ROS and the first enzyme to detoxify superoxide anion to hydrogen peroxide which is further converted to water by GSH and CAT. Substances with known antioxidant properties have previously been shown to be beneficial in patients with IBD [35, 36]. AA administration (1.8 mL/kg) displayed significantly elevated enzymatic antioxidant (GSH, CAT and SOD) levels in TNBS colitis model and stating its protective role which could be the mechanism for restoration of tissue damage.

NO is a main oxidant which maintains GI mucosal integrity by regulating gastric mucosal blood flow, epithelial secretion and barrier function. Up-regulated expression of inducible nitric oxide synthase and increased NO level has been observed in chronic UC patients. Which are established for harmful effects on the gut [37]. Similarly, increased MDA levels indicating lipid peroxidation in TNBS induced colitis are previously reported [38]. MPO concentration is a measure of neutrophil infiltration in tissues and also significant histological indicator of mucosal inflammation in IBD [39]. AA at 1.9 and 0.9 mL/kg showed significant inhibition of NO, MDA and MPO activity. Hence, we conclude that AA has a beneficial effect on TNBS –induced rat colitis.

Cytokines have a significant role in development, relapse and exacerbation of the inflammatory process in IBD [40]. Prolonged activation of the intestinal immune system plays an essential role in pathophysiology of chronic mucosal inflammation as observed in various animal models and clinical studies. Cytokines are synthesized and secreted by inflammatory cells, which induce the production of adhesion molecules and other inflammatory mediators such as ROS, nitric oxide and lipid mediators [41]. Earlier studies has indicated increased levels of TNF-α, IL-1β and IL-6 in mucosa samples of IBD patients as compared to normal mucosa [42, 43]. In agreement to previous findings, we observed increased MDA, MPO and NO levels in TNBS control group along with enhanced cytokine levels which were significantly reduced on AA administration. AA is previously reported to have down regulated the gene expression of TNF-α in High fat fed animals [17].

Zn supplementation downregulates the expression of metalloproteins that transport Zn in normal pathway. Zn accumulation supports association between Zn homeostasis and immune response in UC patients, which could be due to its anti-inflammatory and antioxidant properties [44]. Higher intake of Zn has been associated with a reduced risk of Crohn's disease but not ulcerative colitis

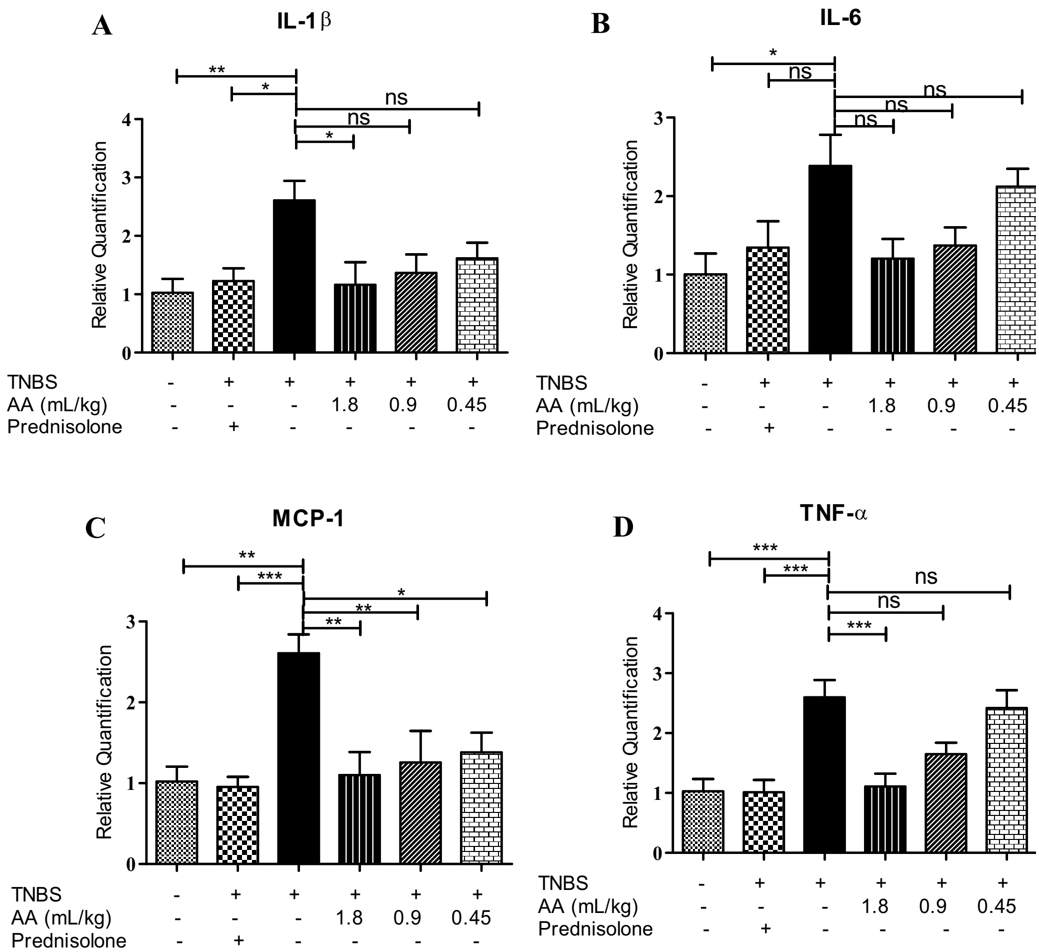


Fig. 5 Treatment effects of prednisolone (2 mg/kg) and AA (1.8, 0.9, 0.45 mL/kg) on IL-1β (**a**), IL-6 (**b**), MCP-1 (**c**), TNF-α (**d**). The treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 08). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control

Table 2 Fecal bacterial quantification in the different experimental groups

	Clostridium	Bifidobacteria	Lactobacilli
Normal	3.531 ± 0.002	3.428 ± 0.023	3.536 ± 0.004
TNBS	$3.614 \pm 0.008^{\#\#\#}$	3.394 ± 0.003^{ns}	$3.375 \pm 0.003^{\#\#\#}$
Prednisolone	$2.505 \pm 0.002^{\#\#\#,***}$	$3.365 \pm 0.002^{\#\#, ns}$	$0.000 \pm 0.000^{\#\#\#,***}$
AA 1.8	$3.476 \pm 0.001^{\#\#,***}$	$3.491 \pm 0.004^{\#\#,***}$	$3.525 \pm 0.003^{NS,***}$
AA 0.9	$2.908 \pm 0.002^{\#\#\#,***}$	$3.552 \pm 0.001^{\#\#\#,***}$	$3.410 \pm 0.002^{\#\#\#,**}$
AA 0.45	$3.533 \pm 0.017^{ns,***}$	$3.435 \pm 0.002^{ns, ns}$	$3.473 \pm 0.004^{\#\#\#,***}$

Data are expressed as mean ± SEM, (n = 06).

ns not significant,

$^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. normal control; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control

[45]. In our study, we observed increased plasma zinc status in prednisolone and AA treated rats as compared to TNBS control group which could be associated with increase in the antioxidant status along with improvement in the immune response.

Bifidobacteria are known to have positive health benefits to the host [46]. Alterations in intestinal bifidobacteria count have been previously detected in IBD and colorectal cancer case [47]. Similarly, Lactobacilli population is also known to be decreased in UC patients and administration of Lactobacillus spp. prevents colitis in interlukine-10 gene deficient mice [48]. Supporting these findings, a traditional Japanese medicine 'Strong Wakamto®' suggested beneficial effect in TNBS induced colitis by balancing the fecal lactobacillus spp. [49]. On the other hand, Clostridial spp. count in rat feces increased on TNBS administration [50] and decreased in prebiotic treated colitic rat [26]. In this study AA administration raised Bifidobacterial and Lactobacilli counts and decreased Clostridial counts which were altered on TNBS administration. This fecal microbiota alteration by AA administration could play a major role in restoring the intestinal damage seen in colitis. This finding needs further evaluation to claim its efficacy.

Conclusions

In conclusion, AA exhibited anti-inflammatory effects by decreasing oxidative stress, pro-inflammatory cytokines and chemokine. It also ameliorated colitis in rats by altering the gut microbiota composition. However, further clinical studies are warranted to justify its possible use in IBD treatment and to establish the precise mechanism.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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Assessment of *in vitro* biological activities of *Terminalia arjuna* Roxb. bark extract and Arjunarishta in inflammatory bowel disease and colorectal cancer

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Alternative or complementary therapies for several inflammatory disorders have gained considerable acceptability and popularity in recent years. The Arjuna tree, *Terminalia arjuna* Roxb. (Combretaceae) holds antidiarrheal and antioxidant potential useful in management of inflammatory gastro intestinal ailments. Here, we evaluated the possible effect of *T. arjuna* hydroalcoholic extract (TAHA) and traditional Ayurvedic formulation Arjunarishta (AA) for the treatment of inflammatory bowel disease (IBD) and colorectal cancer. The phytochemical profile of test materials was confirmed via investigation of total phenolic and flavanoid content and standardized by HPLC-PDA method. *In vitro* antioxidant activity was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) assay. Antimicrobial potential was tested against clinical isolates of IBD patients (HM95, HM233, HM251, HM615). Cytotoxicity was determined against human colorectal adenocarcinoma cells (Caco2, COLO.205), whereas, cytocompatibility against normal rat intestinal epithelial (IEC-6) and mouse fibroblast cells (L929). Additionally, *in vitro* oxidative cell damage stress was estimated by lipid peroxidation biomarker. TAHA displayed higher antioxidant capacity as compared to AA formulation. Different sensitivities were observed against different study cell lines in dose dependant manner. Similarly, significant (P <0.05) enhanced malondialdehyde (MDA) concentrations in test materials and 5-FU treated colorectal adenocarcinoma cells was detected as compared to control cells. TAHA and AA exhibited antimicrobial activity against IBD associated clinical isolates. These findings provide biological evidence for therapeutic application of TAHA and AA in IBD and colorectal cancer treatment.

Keywords: Antibacterial, Arjuna tree, Ayurveda, Cytotoxicity, IBD, Traditional medicine

Terminalia arjuna Roxb., (Fam. Combretaceae) has been used traditionally in cardiovascular diseases and cancer treatment. In ayurvedic concept, it helps in metabolic homeostasis[1]. The *T. arjuna* bark has been used in traditional system of medicine for various health benefits[2]. It has pharmacological activities, such as hypolipidemic, hypercholesterolemic, antimutagenic, antibacterial and antioxidant[3,4]. The active constituents include triterpenoids, saponins, tannins, flavonoids, ellagic acid, gallic acid, oligomeric proanthocyanidins, phytosterols, magnesium, calcium, zinc and copper[5].

Arishtas are conventional Ayurvedic formulations with decoction of herbs. These liquid dosage forms have self-generated alcohol, which improves extraction efficacy of molecules soluble in alcohol and water, resulting in improved drug delivery. Arjunarishta (AA), an arishta formulation supports improvement of cardiac functions, appetite and balances immune response[6]. This formulation contains *T. arjuna*, *Madhuca indica*, *Vitis vinifera* and *Woodfordia fruticosa*[7].

Inflammatory Bowel Disease (IBD) comprises of chronic, relapsing, inflammatory disorders of gastrointestinal tract that includes ulcerative colitis (UC) and Crohn's disease (CD)[8]. It is characterized by diarrhoea, rectal bleeding, the urgency to have bowel movements, stomach cramps, fever and weight loss[9]. Several people have been affected worldwide with rising incidence in developing countries. The overall IBD burden is growing in India, in view of latest report, India has a very high disease load globally[10]. It is known to be associated with a substantial increase in the threat of colorectal cancer (CRC), especially after 8-10 years of active disease. UC is one of the best

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Abbreviations: AA, Arjunarishta; AIEC, Adherent-invasive *Escherichia coli*; IBD, Inflammatory bowel disease; TAHA, *Terminalia arjuna* hydroalcoholic extract

clinically characterized examples of such correlation between inflammation and carcinogenesis[11].

A pathogenic variant of *Escherichia coli* termed as Adherent Invasive *E. coli* (AIEC) has been involved in IBD pathogenesis. It adheres or invades the intestinal cells and further replicates within epithelial cells and underlying mucosal macrophages[12]. Earlier *In vitro* studies on the activity of antibiotics and bovine lactoferrin against Crohn's disease associated with AIEC have established their potential for termination of *E. coli* from the gastrointestinal tract of patients with Crohn's disease[13,14].

Medicinal plants used in traditional system of medicine comprise of numerous constituents that can be used to treat various illnesses, infections and even chronic diseases including IBD, cancer, etc.[15-19]. We have reported earlier that *T. arjuna* hydroalcoholic extract (TAHA) administration relieved the disease activity in trinitrobenzenesulfonic acid (TNBS) induced colitis in rat model[20]. In the present study, we assessed the efficacy of standardized TAHA and AA for cytotoxicity and malondialdehyde (MDA) level. Furthermore, we studied their antibacterial potential against AIEC strain and other IBD associated bacterial isolates along with the antioxidant activity.

Material and Methods

Test materials

Dried stem bark of *T. arjuna* was procured from KLE Society's Ayurved Pharmacy, (collected in February 2015 from the Western Ghats, Belagavi region, Karnataka – India), and authenticatedfrom AYUSH approved ASU drug testing laboratory at Shri BM Kankanwadi Ayurveda Mahavidyalaya, Belagavi, Karnataka – India and assigned the voucher number CRF/645/2015). *T. arjuna* containing traditional ayurvedic formulation Arjunarishta (AA) was purchased from the local market.

Preparation of plant extract and preliminary phytochemical analysis

The dried bark of *T. arjuna* was powdered and extracted with ethanol: water (70:30 v/v) using cold maceration method in a conical flask. The extract was manually shaken every hour for initial six hours. Afterwards, it was kept in a shaker at 200 rpm. The extract was filtered and concentrated in a rotary evaporator at 40ºC followed by complete drying using a water bath. The yield of hydroalcoholic extract (expressed as percentage w/w) was 22.2%. The extract was stored in an air tight container at –20ºC until further analysis. The test materials were subjected to preliminary phytochemical screening following the standard methods[21].

Quantification of total phenolic content (TPC) and total flavonoid content (TFC)

TPC was determined by the Folin-Ciocalteu reagent method[22]. Test materials/standard (0.5 mL) of different concentrations were mixed with 1N Folin-Ciocalteu reagent and 20% sodium carbonate. The tubes were vortexed and allowed to stand for 40 min at 20°C for colour development. The absorbance was read at 725 nm using spectrophotometer (UV-1800, Shimadzu, Japan) against blank. The total content of phenolic compounds was expressed in Tannic acid equivalents (TAE)/g of dry extract.

TFC was analyzed using previously reported method[23] with suitable minor modifications. Briefly, 240 µL sample, sodium nitrite (50 mg/mL), aluminium chloride (100 mg/mL in methanol) were added and mixed. After 5 min, 1M sodium hydroxide was added. The TFC was calculated from a calibration curve using Quercetin as standard (12.5-800 µg/mL) and expressed as (Quercetin equivalent) QE/g of dry extract.

HPLC-PDA analysis: TAHA and AA

The phytochemical profile of TAHA and AA was performed as per previously reported method with suitable modification in column and mobile phase gradient using polyphenolic standards — gallic acid, ellagic acid and quercetin by HPLC[24-26]. Concisely, prominence HPLC system (Shimadzu, Japan) equipped with the binary pump, autosampler, a column oven and a photodiode array detector (PDA) was used. Chromatographic separations were carried out using C-18 analytical column (150X 4.6 mm, 5 mm particle size; Syncronis, Thermo Scientific, USA).

Assessment of *in vitro* antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging capability of each extract solution on DPPH radicals was investigated as reported previously[27]. Briefly, 4 mL of 0.1 mM DPPH in methanol was mixed with one mL of each of extract (solution at different concentrations, 200-6.25 µg/mL). These mixtures were incubated in a dark room for 30 min, and the free radical scavenging ability was estimated by measuring the absorbance at 517 nm using a spectrophotometer.

Ferric Reducing Antioxidant Power (*FRAP*) *assay*

The capability to reduce ferric ions was estimated using the standard method described by Benzie and

Strain[28]. The working FRAP reagent was freshly prepared by adding 300 mM sodium acetate buffer (pH 3.6), 10.0 mM tripyridyl triazine (TPTZ) solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 (v/v/v). Test materials (1.0 mg/mL) and standard $FeSO_4$ (0.1-1.0 mM) were then mixed with 3 mL of FRAP reagent, and the reaction mixture was incubated at 37°C for 30 min followed by absorbance measurement at 593 nm. Calibration was carried out with a fresh working solution of $FeSO_4$. The antioxidant capacity based on ability to reduce ferric ions of the sample was calculated from the linear calibration curve.

Antibacterial activity

Four clinical bacterial isolates, *E. coli* HM95 (AIEC), *E. coli* HM615 (colonic mucosa associated. *E.coli*), *E. coli* HM233 and *E. coli* HM251 (colonic mucus associated patient strains) were received under Material Transfer Agreement with University of Liverpool, United Kingdom. The bacterial isolates were subcultured on MacConkey agar plates and incubated aerobically at 37ºC. The media were procured from HiMedia Laboratories, Mumbai, India. The antimicrobial activity of TAHA and AA was evaluated by agar well diffusion method and MIC was detected by broth dilution method as previously reported with minor modifications[29]. Ciprofloxacin was used as positive control.

Determination of cytotoxicity and cytocompatibility

Human colorectal adenocarcinoma cells (Caco2, COLO.205) and normal rat intestinal epithelial and mouse fibroblast cells (IEC-6 and L929) were obtained from National Centre for Cell Sciences, Pune-India**.** The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% FBS procured from Gibco Life Technologies, Bangalore-India. Viable cell suspension 50 µL with a density of $1{\times}10^5$ cells/mL (determined by Trypan blue exclusion method) was seeded into each well in a 96-well micro titre plate and final volume made up to 150 µL with DMEM media. Test materials were diluted in DMEM media to obtain different concentrations. 100 µL of TAHA and AA (400-6.25 µg/mL) and Standard drug 5-FU (100-1.562 µg/mL) was added to the wells followed by incubation for 48 h in the presence of 5% CO_2 at 37ºC into CO_2 incubator. After the incubation period, 20 µL of MTT reagent (3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide, 5 mg/mL in PBS) procured from HiMedia laboratories, was added to each well following 4 h incubation in dark. The supernatant was removed without disturbing the precipitated Formazan crystals. Formed crystals were dissolved by addition of 100 µL of DMSO and optical density (OD) was calculated at a wavelength of 492 nm. Cell-viability assays were conducted as per previously reported standard procedure[30]. The study was performed in triplicates, and percent cell viability was calculated using the equation

$$\text{Percent cell viability} = \frac{OD\ of\ test\ material}{OD\ of\ control} \times 100$$

Lipid peroxidation in cells

IEC-6, L929, COLO.205, and Caco2 cells were plated into 12-well plates at a density of $1{\times}10^7$ cells/mL in complete medium. Pre-confluent cells were treated with test materials for 48 h. MDA, a marker of lipid peroxidation, was measured using an Oxiselect™ TBARS Assay Kit (Cell Biolabs, Inc, San Diego, CA, USA) following the manufacturer's protocol. Spectrophotometric measurements were recorded on the microplate reader at 532 nm. The concentration of MDA in samples was calculated using MDA standards as reference.

Data analysis

All determinations were carried out in triplicate. The results have been presented in the form of Mean ± SD. Calculation of IC_{50} value was carried out using GraphPad Prism 7 for Windows.

Results and Discussion

Preliminary phytochemical screening

The phytochemical evaluation of TAHA and AA directed the presence of therapeutically active phytoconstituents: proteins, steroids, flavonoids and tannins. Whereas, alkaloids were present in TAHA and found absent in AA.

In agreement with the present study, directed phytochemicals in *T. arjuna* bark extract has been reported previously[31]. On the other hand, the absence of alkaloids in Arjunarishta formulation along with the presence of other phytochemicals are as per previous report[32] supporting our finding. These phyto-constituents are well reported to have antioxidant, antimicrobial, anticancer, and anti-inflammatory potential[33]. Phenolic compounds are established for redox properties, and it allows them to act as an antioxidant through their free radical scavenging ability. Therefore, total phenolic concentration could be used for quick screening of antioxidant potential[16,19]. The antioxidant potential of the test materials was confirmed through DPPH and FRAP assay and TAHA expressed comparable results with standard gallic acid.

Total phenolic/flavonoid content

The TPC in test materials was estimated according to Folin-Ciocalteu method and expressed as TAE calculated from the calibration curve ($R^2 = 0.991$). TPC was seven folds higher in TAHA (502.6 mg TAE/g) as compared to AA (79.53 mg TAE/g). TFC was calculated from the standard quercetin calibration curve ($R^2 = 0.994$) was 488.25 and 62 mg QE/g in TAHA and AA, respectively.

Phytochemical standardization – HPLC analysis

The phytochemical standardization of TAHA and AA was performed using marker based approach. Gallic acid, ellagic acid and quercetin polyphenolic standards were utilized for standardization of TAHA and AA. The HPLC analysis findings depicted good resolution of peaks and the presence of polyphenolic markers in both test materials was recognised with the help of retention time (R_t) matching with equivalent to reference standards (Fig. 1 A and B).

Antioxidant properties of TAHA and AA

The antioxidant potential of the test materials was explored by DPPH and FRAP assay (Table 1). It was observed that TAHA showed better antioxidant activity compared to AA. The standard: Gallic acid indicated higher antioxidant potential as compared to TAHA in DPPH assay. However, TAHA displayed better antioxidant activity in comparison with Gallic acid using FRAP assay.

Table 1 — Antioxidant activity of TAHA and AA

Test materials	DPPH activity (IC_{50} µg/mL)	FRAP activity (mM $Fe2^+$ /g)
TAHA	51.31±1.23	1907.00±0.88[a]
AA	1025.00±1.15[a]	290.00±1.22[a]
Gallic acid	49.89±1.08	1643.00±1.37

[Data are expressed as mean±SD of three individual determinations. The data were analysed using one-way ANOVA followed by Dunnett's multiple comparison test. Compared with standard-gallic acid, [a] P <0.05; DDPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing ability of plasma]

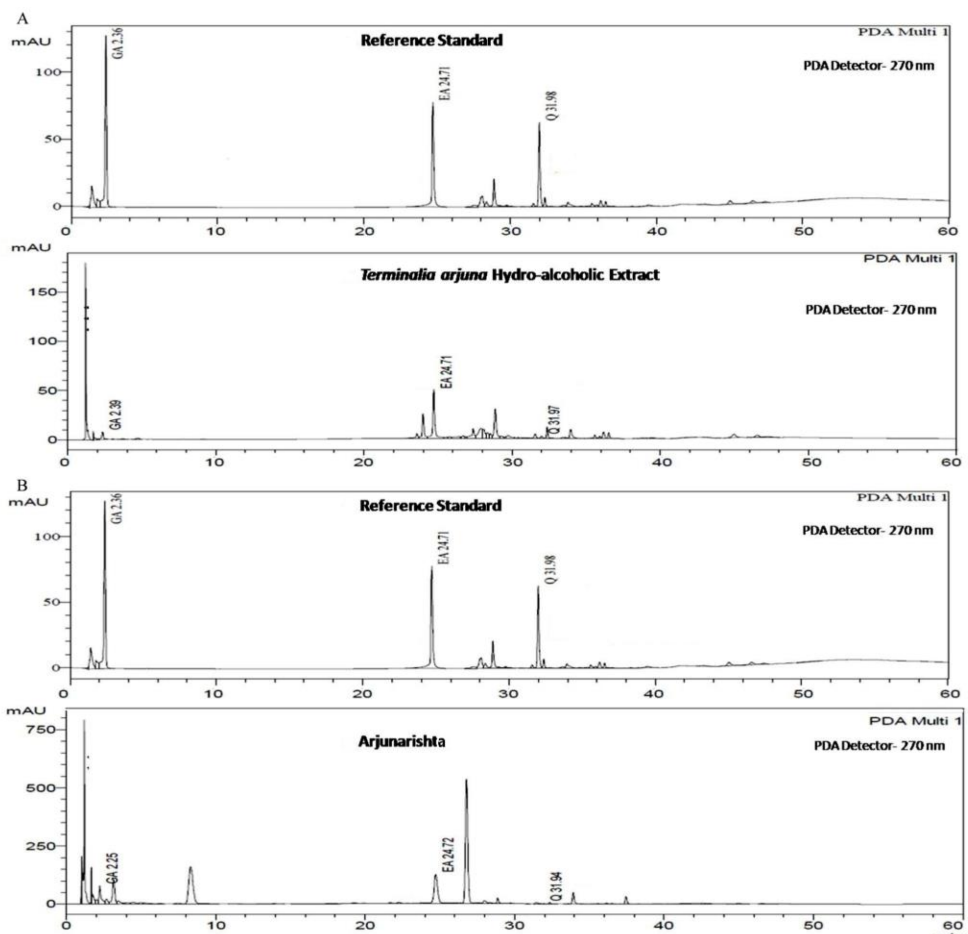

Fig. 1 — HPLC chromatogram of (A) TAHA; and (B) AA with reference standards

Antibacterial activity

The results of well diffusion assay and MIC values of TAHA and AA against the four IBD clinical isolates are listed in Tables 2 and 3, respectively. No inhibitory zone was detected for AA. Intestinal inflammation is a chronic condition that needs the administration of presently available drugs for extended duration and in several cases this can be linked with the onset of severe effects or non-compliance. This has stimulated the need for discovery of newer substances from natural origin, including medicinal plants as antimicrobial substances to attain efficacy and better tolerability. There is increasing evidence that the mucosa-associated microbiota, may be essential in the pathogenesis of the inflammatory bowel diseases: ulcerative colitis, and Crohn's Disease[34]. However, *T. arjuna* bark and Arjunarishta, antimicrobial activity against IBD isolates has not been reported till date. Although *T. arjuna* bark and leaves have been reported for its antimicrobial potential against Gram positive/negative ear pathogens[35] and broad-spectrum activity against diarrhea causing bacteria[36]. In addition, it is documented that 'Bhoxa community' of Dehradun district, Uttarakhand, India use this medicinal plant for treatment of dysentery and diarrhea[37]. *T. arjuna* showed a zone of inhibition against the test bacteria. Whereas, AA did not exhibit inhibition zone possibly could be owing to its inability to diffuse through media. These results support our earlier findings suggesting a beneficial role of TAHA in TNBS induced colitis[20].

Table 2 — Antimicrobial activity by agar well diffusion method

Microbial strains	Zone of inhibition (mm)		
	TAHA (50 mg/mL)	TAHA (25 mg/mL)	Ciprofloxacin
HM95 (CD)	22.67±0.58	21.33±1.53	21.00±1.00
HM233 (UC)	24.67±0.58 [a]	21.00±1.00[a]	27.00±1.00
HM251 (UC)	24.00±1.00[a]	22.67±1.53[a]	29.67±0.53
HM615 (CD)	24.33±0.58[a]	22.33 ±1.16[a]	20.33±0.58

[Data are expressed as mean±SD of three individual determinations. The data were analysed using one-way ANOVA followed by Dunnett's multiple comparison test. Compared with standard-ciprofloxacin for respective microbial strain, [a] P <0.05]

Table 3 — Minimum Inhibitory Concentration (MIC) values of TAHA and AA

Microbial strains	TAHA (mg/mL)	AA (%)	Ciprofloxacin (μg/mL)
HM95 (CD)	6.25	12.5	1.25
HM233 (UC)	6.25	12.5	1.25
HM251 (UC)	6.25	12.5	1.25
HM615 (CD)	6.25	12.5	1.25

***In vitro* anticancer and cytocompatibility assay**

Cytotoxicity assay resulted in reduction of percent cell viability when tested at concentrations ranging from 400-12.5 μg/mL for the test materials and 5-fluorouracil (5-FU) from 100-1.562 μg/mL. The cytotoxicity was assessed by MTT assay on two human colorectal adenocarcinoma cells, COLO.205 and Caco2. The IC_{50} value was obtained to assess its inhibitory concentration that causes 50% cell viability. The test materials and 5-FU presented a concentration-dependent deduction in percent cell viability after 48 h exposure (Table 4).

Test materials and 5-FU were examined for cytocompatibility assay at similar concentrations against L929 (Mouse fibroblast cells) and IEC-6 (Rat intestinal cells) using colorimetric MTT assay. Test materials displayed good cytocompatibility against the study cell lines (Fig. 2 A and B). Similarly, 5-FU was also analyzed for cytocompatibility as per IC_{50} value (Fig. 2C). Cytotoxicity assessment is important to validate the anticancer potential of medicinal plants. Therefore, we evaluated the cytotoxic potential of *T. arjuna* on human colorectal adenocarcinoma cells. Its phytoconstituent: Arjunic acid has been found active against human oral, ovarian and liver cancer cell lines[38]. In addition, *T. arjuna* extracts are reported to be effective against N-nitrosodiethylamine induced hepatocellular carcinoma in rats acting through carbohydrate metabolizing enzymes[39]. Similar dose

Table 4 — Effect of TAHA, AA, and 5-FU treatment on COLO.205 and Caco2 cells. % cell viability of treated cells and IC_{50} values of test materials and standard drug

Sample (μg/mL)		12.5	25	50	100	200	400	IC_{50} (μg/mL)
TAHA	COLO.205	98.82±0.36	97.81±0.30[a]	94.42±0.50[a]	51.76±1.06[a]	33.39±0.86[a]	30.74±1.72[a]	145.3±0.53
	Caco2	88.25±0.44[a]	73.03±0.31[a]	53.70±1.11[a]	48.77±0.38[a]	42.00±0.41[a]	37.00±1.99[a]	90.4±0.66
AA	COLO.205	97.49±2.61	97.13±3.25	94.31±0.06[a]	69.50±1.38[a]	40.63±1.01[a]	30.03±2.52[a]	183.6±1.19
	Caco2	87.67±0.48[a]	83.93±0.95[a]	70.11±0.96[a]	53.97±0.67[a]	47.79±0.98[a]	40.00±1.00[a]	182.9±0.21
Sample (μg/mL)		3.12	6.25	12.5	25	50	100	IC_{50} (μg/mL)
5-FU	COLO.205	79.23±1.07[a]	68.00 ±1.27[a]	58.18±0.67[a]	50.63±0.88[a]	38.07±2.02[a]	31.14±1.03[a]	24.12±1.29
	Caco2	83.00±2.11[a]	76.00±1.59[a]	67.28±1.91[a]	55.18±1.09[a]	42.27±2.17[a]	30.34±1.67[a]	32.42±0.78

[Data are expressed as mean ± SD of three individual experiments. The data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Compared with control (considered as 100 %), [a] P <0.05]

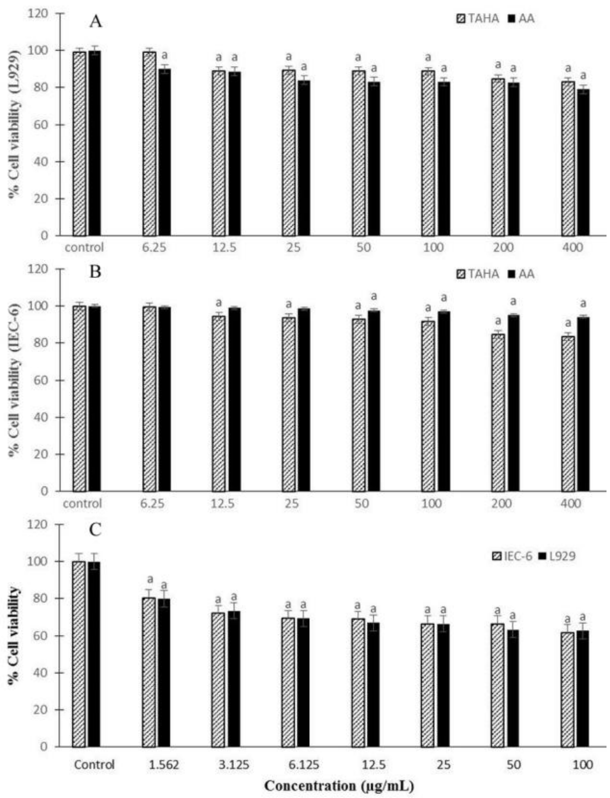

Fig. 2 — Cytocompatibility of TAHA and AA against (A) L929 cells derived from mouse fibroblast; (B) IEC-6 cells derived from rat intestinal epithelium; and (C) 5-FU against IEC-6 and L929 cell lines. [Cytocompatibility evaluated by % cell viability considering viability of control as 100% (expressed as Mean ± SD of three experiments. Alphabet a represents significant differences in mean (*P* <0.05) compared to control group]

dependent cytotoxicity has been observed in our study against colorectal adenocarcinoma cells supporting its traditional use in cancer treatment[40].

Lipid peroxidation

The levels of MDA content are shown in Table 5. TAHA and AA exposure indicated significant (*P* <0.05) enhanced lipid peroxidation in treated COLO.205 and Caco2 cells as compared to untreated control, which was concentration dependant. This increase demonstrates that both test materials amplified MDA production in carcinoma cells by 52 and 48% at 200 µg/mL of TAHA exposure on COLO.205 and Caco2 cells, respectively when compared to the control while AA exposure produced 25 and 26% at 200 µg/mL. Additionally, normal cells treated with 5-FU resulted in a significant (*P* <0.05) increase in MDA concentration as compared to its control.

Oxidative stress creates one of the molecular mechanisms by which bioactive substances induce cytotoxicity and apoptosis. To investigate the degree of oxidative cell damage in colorectal adenocarcinoma cells exposed to test materials and 5-FU, we carried out lipid peroxidation study. Our study findings indicated a significant rise in malondialdehyde (a by-product of lipid peroxidation and biomarker of oxidative stress) levels in TAHA and AA treated carcinoma cells as compared to control cells. Similarly, HepG2 cells exposed to *T. arjuna* extract directed induction of reactive oxygen species production and consequently causing apoptosis[41]. In addition, gallic acid present in *T. arjuna* is known to induce ROS induced cell death in human prostate cancer cells through its autoxidation[42]. Therefore, the present study demonstrated that both the test materials and standard drug increased the lipid peroxidation with a simultaneous decline in cell viability in colorectal cancer cell line with its mechanism of generation of oxidative stress-mediated apoptosis[43].

Table 5 — Lipid peroxidation (MDA) concentration in various treated cells

Test materials (µg/mL)	Lipid peroxidation (MDA conc./10^6) µM			
	L929 cells	IEC- 6 cells	COLO.205 cells	Caco2 cells
Control	21.71±0.67	24.84±0.39	23.26±0.93	22.82±1.00
TAHA (200)	23.29±0.17	25.82±0.25	35.37±0.91^a	33.79±0.58^a
TAHA (100)	22.91±0.09	25.64±0.02	28.74±0.97^a	27.82±0.41^a
AA (200)	23.08±0.04	25.59±0.23	28.97±0.79^a	28.78±0.47^a
AA (100)	20.89±0.03	24.34±0.23	26.76±0.99^a	26.07±1.48^a
5 - FU (35)	26.92±0.88^a	32.03±1.23^a	31.44±1.00^a	31.11±1.40^a
5 - FU (25)	24.62±0.58^a	29.53±0.29^a	29.35±0.87^a	28.67±0.79^a

[Data are expressed as mean ± SD of three individual determinations. The data were analysed using one-way ANOVA followed by Dunnett multiple comparison test. Compared with control: ^{a} *P* <0.05]

Conclusion

Results of the present study suggest that the *Terminalia arjuna* hydroalcoholic extract (TAHA) exhibited comparable *in vitro* antioxidant activity with gallic acid. Whereas, Antibacterial potential of both the test materials was observed against the bacterial isolates from CD and UC patients used in the study. TAHA and AA exhibited cytotoxicity in the cell lines where the lipid peroxidation was enhanced after test material exposure, which could be due to malon-dialdehyde formation associated cell death. However, further *in vitro* and *in vivo* investigations are required to understand precise mechanism.

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Conflicts of interest

Authors have declared no conflict of interests.

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Beneficial role of *Terminalia arjuna* hydro-alcoholic extract in colitis and its possible mechanism

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ABSTRACT

Ethnopharmacological relevance: Terminalia arjuna Roxb. (Combretaceae) is traditionally used in Ayurveda medicine and holds ethnomedicinal importance for treatment of gastrointestinal disorders. In view of its anti-inflammatory, antidiarrheal and antioxidant potential, it could be beneficial for the treatment of inflammatory bowel disease (IBD), which is associated with interaction between genetic, environmental factors and intestinal microbiome leading to dysregulated immune responses. This study evaluates the effect of hydroalcoholic extract of *Terminalia arjuna* bark (TAHA) in trinitrobenzenesulfonic acid (TNBS) model of rat colitis which resembles human IBD.

Materials and methods: TAHA (500, 250, 125 mg/kg) was administered orally for 28 days in TNBS induced rats. Response to treatment was assessed by comparing observations in diseased and treated groups using disease activity index (DAI); macroscopic/histological damage; determining oxidative stress indicators: myeloperoxidase, malondialdehyde, nitric oxide, catalase, superoxide dismutase, and reduced glutathione; gene expression of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α and chemokine: MCP-1. Furthermore, the role of TAHA in altering the gut microbiota profile in rat feces and plasma zinc was also studied.

Results: TAHA treatment in colitic rats directed decreased DAI scores, macroscopic and histologic damage. It also reduced myeloperoxidase, malondialdehyde and nitric oxide level. Whereas, prevented depletion of plasma catalase, superoxide dismutase and glutathione level. In addition, TAHA treatment down-regulated the gene expression of pro-inflammatory mediators and displayed altered beneficial effect on fecal microbiota. Furthermore, enhanced plasma zinc level supported the beneficial effect of TAHA in colitic rats. The dose of TAHA that produced most significant beneficial effect was 500 mg/kg.

Conclusion: TAHA administration relieved the disease activity in TNBS induced colitis by reducing expression of pro-inflammatory cytokines and chemokine, decreasing oxidative stress, and improving plasma zinc level and structure of gut microbiota.

1. Introduction

Inflammatory bowel disease (IBD) has been a global healthcare problem with a continuous growing incidence. The disease has two major forms: Crohn's disease (CD) and ulcerative colitis (UC) with chronic and relapsing gastro-intestinal inflammation, characterized by chronic diarrhoea, stomach pain, blood in stool and in certain circumstances associated with cancer. (Abiodun et al., 2016; Zhang and Li, 2014). The pathogenesis of IBD remains uncertain and involves a complex interplay between genetic threat, environmental aspects, gut microbiota and mucosal immune response (Hanauer, 2006). Mucosal inflammatory reactions to unidentified antigens or consequence of an abnormal immune system results in cellular infiltration of neutrophils, plasma cells, mast cells, lymphocytes and macrophages. Activation of these infiltrating cells cause release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1, IL-6 and IL-12 which contribute to epithelial intestinal damage and ultimate clinical disease (Shepherd et al., 2018; Stevens et al., 1992). The existing treatment regimen including amino-salicylates, corticosteroids, immunosuppressants, antibiotics, and biologic agents are effective, but

Abbreviations: CD, Crohn's Disease; CAT, Catalase; DAI, Disease Activity Index; GSH, Glutathione-; IBD, Inflammatory Bowel Disease; IL-6, Interleukin -6; IL-1β, Interleukin -1β; MCP-1, Monocyte Chemotactic Protein; MDA, Malondialdehyde; MPO, Myeloperoxidase; NO, Nitric Oxide; SOD, Superoxide dismutase; TAHA, *Terminalia arjuna* hydroalcoholic extract; TNF-α, Tumour Necrosis Factor – α; TNBS, Trinitrobenzenesulfonic acid; UC, Ulcerative Colitis

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associated with notable adverse effects with some being relatively severe (Triantafyllidi et al., 2015). Therefore, the search for new treatment options with a low incidence of adverse effects is much warranted.

Modern medicine has been in practice for the treatment of chronic inflammatory diseases. However, there is increasing interest in natural products which would be a source of alternative or complimentary medicine for IBD treatment. The usage of traditional and complementary medicine among patients with IBD, particularly in the form of herbal therapies is widespread in the Western as well as in many Asian countries including China and India. It seems that their use is continuously increasing despite the fact that only a small number of controlled trials dealing with either safety or efficacy of these natural products exist. Despite this, emerging evidence suggests that many of these substances can modulate the immune system and disrupt the pro-inflammatory cascade through a various mechanisms including antioxidant effects, variations in cell signaling, pro-inflammatory cytokines / mediators, and disruption of bacterial flora (Triantafillidis, 2008)

The present study aimed at assessing possible benefits of *Terminalia arjuna* Roxb. (Combretaceae) in IBD management based on its ethnomedical considerations of being used in several gastrointestinal disorders (Charak Samhita, 1941). *Terminalia arjuna* (*T.arjuna*) is widely known as 'Arjuna' and its bark, leaves, fruits have been used to treat various ailments in Ayurveda, the traditional system of medicine of Indian subcontinent (Warrier et al., 1996). The stem bark in particular has been attributed to possess antidysentric, purgative and laxative properties. It is advocated to treat inflammation, ulcers, tumors and diarrhoea associated with blood (Gogte, 2000; Vaidya, 1998; Gairola et al., 2013) as well as documented to have antinociceptive and immunomodulatory activities (Halder et al., 2009). Furthermore, bark extract is reported as antioxidant (Manna et al., 2006) and cytotoxic in Hepatocellular carcinoma cells (Sivalokanathan et al., 2006). The stem bark contains an appreciable amount of secondary metabolites such as phenolics and terpenoids, which may act as a resource of pharmacologically active agents and natural antioxidants (Mittal et al., 2015). Phytochemicals such as gallic acid, ellagic acid, quercetin, which have been reported to be present in *T. arjuna* bark hold varying degree of antioxidant, protective and intestinal anti-inflammatory effect in rat models of colitis (Dodda et al., 2014; Rosillo et al., 2011; Pandurangan, 2015).

Therefore, the purpose of current study is to investigate the anti-inflammatory potential of hydroalcohalic extract of *T. arjuna* (TAHA) in trinitrobenzenesulphonic acid (TNBS) induced experimental rat colitis model and associated mechanisms. It is a well established animal model of intestinal inflammation that has some biochemical and histological features resembling human disease. We analysed intestinal damage through disease activity index (DAI), macroscopic and histologic scoring, colonic mediators involved in inflammatory response: TNF-α, IL-1β, IL-6, MCP-1 (monocyte chemotactic protein) and oxidative stress parameters such as GSH (glutathione), CAT (catalase), SOD (superoxide dismutase), MDA (malondialdehyde), MPO (myeloperoxidase), NO (nitric oxide). In addition, we also investigated fecal microbiota profile alteration and plasma zinc level which supported anti-inflammatory potential of *T. arjuna* in gastrointestinal complaints.

2. Materials and methods

2.1. Chemicals and drugs

TNBS was purchased from Sigma-Aldrich, U.S.A. and standard drug: Prednisolone (Medrol) was purchased from Pfizer Products India Pvt. Ltd. Solvents (Fisher Chemicals) used for extraction and analysis were of analytical and HPLC grades, respectively. Hemospot kit for detection of fecal occult blood was procured from Coral Clinical Systems, Tulip Diagnostics (P) Ltd. Verna, Goa - India. Culture media for the bacteriological study was procured from HiMedia Laboratories Pvt. Ltd.

2.2. Plant material and extract preparation

The dried stem bark of *T. arjuna* was purchased from KLE society's Ayurved Pharmacy, Belagavi. The test material was identified and authenticated (sample code - CRF/645/2015) by AYUSH approved ASU drug testing laboratory: 'Central Research Facility - Analytical Laboratory' at Shri B M Kankanwadi Ayurveda Mahavidyalaya, Belagavi, Karnataka - India.

Briefly, the plant material was powdered and 100 g was extracted with 1000 ml of ethanol: water (70:30 v/v) using cold maceration method. The liquid extract was filtered and concentrated under vacuum at 45ºC using rotary evaporator. The yield of hydroalcoholic extract (expressed as percentage w/w) was 22.2 %. The extract was stored at -20 °C until further study.

2.3. Chemical characterization: HPLC-PDA analysis

The phytochemical profile of the TAHA extract was performed as per earlier reported method suitable modification in column and mobile phase gradient (Lal et al., 2009) using three polyphenolic markers namely gallic acid, ellagic acid and quercetin by High Performance Liquid Chromatography (HPLC). Our previous findings have shown the presence of polyphenolic markers in TAHA extract (Shengule et al., 2018a; data not shown). Concisely Prominence HPLC system (Shimadzu, Japan) equipped with the binary pump, autosampler, column oven and a photodiode array detector (PDA) was used. Chromatographic separations were carried out using C-18 analytical column (150 × 4.6 mm, 5 mm particle size; Syncronis, Thermo Scientific, USA). The standardized analytical method was validated for linearity, accuracy, and precision.

2.4. Ethical consideration

The experimental protocol used in the study was carried out in compliance with the CPCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals) Government of India. Female Wistar rats (180–200 g) obtained from Shri Venkateshwara Enterprises (Bangalore, India) were housed in polypropylene cages and maintained at 22 ± 2 °C, under standard lighting conditions (12- h light/dark cycle). The study was approved by Institutional Animal Ethics Committee of College of Pharmacy-KLE Academy of Higher Education and Research, Belagavi [resolution No. KLECOP / CPCSEA- Reg. No. 221/Res. 23-3 / 09 / 2016].

2.5. Trinitrobenzene sulphonic acid (TNBS) model of rat colitis

Experimental colitis was induced using TNBS enema following the previously reported method with minor modifications (Morris et al., 1989; Zhou et al., 2006). The animals were randomly distributed into six groups of eight animals each. Two of the groups served as control (Noncolitic and Untreated colitic). The remaining four groups were colitic groups treated with standard (Prednisolone 2 mg/kg) and Three different doses of TAHA (500, 250 and 125 mg/kg) each orally for 28 days after induction of colitis. Briefly, rats were anesthetized with thiopentone sodium, and then a flexible rubber plastic tube with an external diameter of 2 mm was inserted rectally into the colon. The tip was 8 cm proximal to the anus verge. TNBS (120 mg/kg) dissolved in ethanol (50%, v/v) was instilled into the colon via the cannula to induce colitis. To distribute the agents within the entire colon, rats were held in the head-down position for 2–3 min after the instillation of TNBS and then returned to their cage. The non colitic group received 50% ethanol rectally. Treatment with TAHA and standard continued for 28 days after which blood was withdrawn by cardiac puncture, and the rats were sacrificed with thiopentone sodium overdose. Rat body weight, stool consistency, and presence or absence of blood in stool was recorded daily throughout the experiment to calculate disease activity index (DAI) (Maheshwari et al., 2015). After the sacrifice, the colon was removed aseptically and placed on a cold plate. The colon was freed from surrounding tissues or fat and cleaned from luminal contents with cold saline. Colon specimens and rat plasma from different groups were kept frozen at -80 °C until further analysis.

2.6. Macroscopic scoring

Macroscopic scores for inflammation were assigned based on earlier reported scoring pattern (Millar et al., 1996). Briefly, the scores were assigned from 0 to 4: '0' - No macroscopic change; '1' - Mucosal erythema alone; '2'- Mild mucosal oedema, slight bleeding or small erosions; '3'- Moderate oedema, bleeding ulcers or erosions; '4'- Severe ulceration / erosions, oedema, and tissue necrosis.

2.7. Histopathological assessment of colitis

The colon tissue samples for histological examination were fixed overnight in 4% neutral buffered formalin, processed, sectioned (4 µm thick) and stained with hematoxylin and eosin (H & E) (Shengule et al., 2018b). Previously reported histopathological scoring pattern (Wei et al., 2003) was adopted for evaluation (Table 1).

2.8. Biochemical assays

Colon samples were homogenized in 10 mM hexadecyltrimethylammonium bromide buffer (25 mg tissue/ml) and the supernatant was used to determine MPO activity (Kim et al., 2012). Tissue MDA level (marker of lipid peroxidation) related to colitis disease severity was analyzed using the thiobarbituric acid reactive substances (TBARS) assay (Uchiyama and Mihara, 1978). Plasma samples were used to estimate CAT activity (Goth, 1991), SOD activity (Marklund and Marklund, 1974), reduced GSH activity (Rahman et al., 2006) and NO production (Green et al., 1982) using spectrophotometer (UV-1800, Shimadzu, Japan)

2.9. Plasma zinc estimation

Zinc level was estimated using Atomic Absorption Spectrophotometer (AA-7000; Shimadzu, Japan) (Smith et al., 1979; Fatmagul et al., 2002). Plasma samples were allowed to thaw at room temperature followed by gentle mixing by inverting sample tubes six times. Working standards of zinc were prepared using zinc standard solution: 1000 mg/l in nitric acid (S. D. Fine Chem. Ltd., Mumbai, India). Plasma samples (0.5 ml) were diluted with de-ionized water (2.0 ml) in tubes followed by immediate mixing the solution for 30 s. The instrument, gas-flow setting and aspiration rate was precisely established, to optimize signal and minimize background noise. The zinc concentration in the samples was calculated from absorbance readings, by interpolation from the working curve and the results were expressed in milligram/deciliter.

Table 1
Histopathological scoring pattern in colon tissue.

Histopathological conditions	Scoring pattern		
	'0'	'1'	'2'
Infiltration of acute inflammatory cells	No	Mild increasing	Severe increasing
Infiltration of chronic inflammatory cells	No	Mild increasing	Severe increasing
Deposition of fibrotin protein	Negative	Positive	–
Submucosa edema	No	Patchy - like	Fusion – like
Epithelium necrosis	No	Limiting	Widening
Epithelium ulcer	Negative	Positive	–

Table 2
Primer sequences used in RT-PCR assays in colonic tissue.

Primers	Sequences (5′ 3′)	Annealing temperature (°C)
IL-6	Forward AACTCCATCTGCCCTTCAGGAACA Reverse AAGGCAGTGGCTGTCAACAACATC	62.7
IL-1β	Forward AGCAGCTTTCGACAGTGAGGAGAA Reverse TCTCCACAGCCACAATGAGTGACA	62.7
MCP-1	Forward TGCTGTCTCAGCCAGATGCAGTTA Reverse TACAGCTTCTTTGGGACACCTGCT	62.7
TNF-α	Forward AGAACAGCAACTCCAGAACACCCT Reverse TGCCAGTTCCACATCTCGGATCAT	62.7

2.10. Analysis of gene expression in colonic samples by RT-PCR

The analyses of pro-inflammatory mediators in colonic samples were performed by real-time PCR (RT-PCR). Total RNA was isolated from colon tissues using the Trizol ® reagent (Sigma-Aldrich), according to the manufacturer's instructions. Isolated RNA samples were quantified by using NanoDrop® Spectrophotometer (JH BIO, USA) and 2 µg of total RNA was reverse transcribed into first-strand cDNA (complementary Deoxyribonucleic acid) following the manufacturer's procedure. The synthesized cDNA was then used as a template for polymerase chain reaction (PCR) amplification. RT-PCR was performed using step one real-time PCR system (Applied Biosystems). IL-6, IL-1β, MCP-1 and TNF-α SYBR (synergy brands.inc) green primers were used for RT-PCR analysis. The primer sequences used are shown in Table 2. The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene GAPDH (Shengule et al., 2018c).

2.11. Bacteriological analysis of feces

The bacteriological analysis of feces was carried out as per previously described method (Daddaoua et al., 2007). The feces sample was collected the day rats were sacrificed and stored at -80 °C until further analysis. Fecal matter (0.1 g) was homogenized with PBS buffer (0.9 ml) and 10^{-5} dilution was made followed by plating 100 µl on the different culture media: reinforced clostridium agar medium (Clostridia) and Beerens (Bifidobacteria). The plates were subsequently incubated anaerobically at 37 °C for 72 h and colonies appearing were counted. The results were expressed as Logarithm of the number of colony forming units.

2.12. Statistical analysis

Data obtained are expressed as the mean ± SEM (standard error of mean). Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) with Tukey post hoc test. All Statistical analysis were carried out using GraphPad Prism version 5.0 with statistical significance set at $p < 0.05$.

3. Results

3.1. Effects of TAHA extract on gross appearance and DAI

The TNBS treatment groups displayed prostration, piloerection, and hypomotility after colitis induction. Additionally, Inability to move, loss of appetite and gross blood adhesion to the anus and weight loss was also observed. These symptoms decreased without any medical treatment in TNBS control animals by Day 10 which kept on decreasing gradually, while treatment with TAHA and Prednisolone accelerated the process of disappearance of the symptoms of colitis (Fig. 1). The improvement in TAHA treated groups was higher compared to standard prednisolone groups. DAI reached normal level by Day 14 in 500 mg/kg TAHA treatment group.

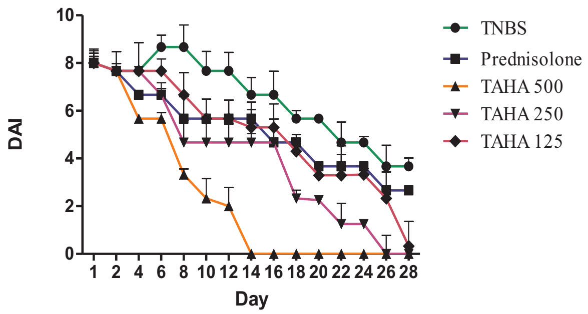

Fig. 1. DAI scores (expressed as mean ± SEM, n = 8) reflecting the recovery process of TNBS induced treatment groups. DAI scores were calculated according to weight loss, stool consistency and blood in the stool.

3.2. Effects of TAHA on macroscopic and histological changes

Macroscopic and histological signs of colitis were observed in rats administered with TNBS intrarectally. Macroscopic colonic mucosal edema, necrosis and ulceration was compared with control group. The macroscopic score increased significantly as compared to the control group (0.60 ± 0.55 vs. 3.00 ± 0.71, $p < 0.05$). Treatment with TAHA at different doses and prednisolone decreased both hyperaemia and inflammation in colonic tissue (Fig. 2 A). Histopathological examination of colonic tissue specimens from the TNBS control group revealed severe infiltration of acute and chronic inflammatory cells, deposition of fibrin protein, epithelial necrosis and ulcer. The histologic samples of TAHA (500, 250, 125 mg/kg) or prednisolone treated groups indicated progressive restoration, reduction in edema and necrosis as compared to TNBS control group (Fig. 2 B, C).

3.3. Effects of TAHA on biochemical parameters

The colonic MPO levels in TNBS control group significantly increased in comparision with normal control group (55.00 ± 1.93 U/mg vs. 12.79 ± 0.84 U/mg) and was decreased by the treatment of TAHA (500 mg/kg) as 19.70 ± 3.24 U/mg, (250 mg/kg) as 31.81 ± 3.42 U/mg, (125 mg/kg) as 38.89 ± 1.98 U/mg. TNBS administration decreased significantly ($p < 0.001$) CAT, GSH, and SOD activity as compared to normal control group (Fig. 3 B-D). TNBS mediated decrease in these parameters were inhibited in the rats supplemented with prednisolone and TAHA. The colonic MDA levels in TNBS control group significantly increased compared to the Normal control group (4.20 ± 0.12 nmol/g vs. 0.37 ± 0.03 nmol/g) and it was decreased by the treatment of TAHA (500 mg/kg) as 0.76 ± 0.06 nmol/g, (250 mg/kg) as 1.59 ± 0.18 nmol/g, (125 mg/kg) as 3.38 ± 0.14 nmol/g, prednisolone as 2.45 ± 0.13 nmol/g (Fig. 3 E). The plasma NO levels in TNBS control group significantly increased compared to the Normal control group (1.25 ± 0.17 nmol/ml vs. 0.61 ± 0.004 nmol/ml) and it was decreased by the treatment of TAHA (500 mg/kg) as 0.66 ± 0.02 nmol/ml, (250 mg/kg) as 0.73 ± 0.05 nmol/ml, (125 mg/kg) as 0.92 ± 0.01 nmol/ml, prednisolone as 0.65 ± 0.02 nmol/ml (Fig. 3F).

3.4. Effects of TAHA on plasma zinc level

The plasma zinc levels in TNBS control group significantly decreased compared to the Normal control group (66.19 ± 2.99 mg/dl vs. 140.6 ± 4.04 mg/dl) and it was increased by the treatment of TAHA (500 mg/kg) as124.6 ± 11.29 mg/dl, (250 mg/kg) as 112.0 ± 8.63 mg/ml, (125 mg/kg) as 101.3 ± 2.57 mg/dl, prednisolone as 131.5 ± 5.01 mg/dl (Fig. 4).

3.5. Effect of TAHA extract on the expression of cytokines in rat colon samples

The intestinal inflammatory process induced by TNBS resulted in significant up-regulation of the colonic expression of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and chemokine MCP- 1, when compared to the non colitic group (Fig. 5) (2.60 ± 0.33, 2.38 ± 0.40, 2.60 ± 0.29, 2.60 ± 0.23) respectively. The administration of TAHA decreased the expression of these inflammatory cytokines in tissue. TAHA 500 mg/kg significantly reduced the level of IL-1β, IL-6, and TNF-α, MCP- 1 compared to TNBS control group. TAHA 250 mg/kg showed a significant reduction in IL-1β, IL-6, and TNF-α. Prednisolone showed significant reduction except in IL-6.

3.6. Effect of TAHA on the structure of gut microbiota

The fecal matter cultivation revealed the effect of TAHA on gut microbiota *in vivo* (Table 3). The normal microbial flora was altered after TNBS administration, where it decreased Bifidobacteria (not significant) and significantly increased Clostridial counts compared to normal. TAHA administration significantly increased the Bifidobacterial count compared to TNBS control group. In contrast, prednisolone showed no significant difference in Bifidobacterial count compared to TNBS control. Both TAHA at 500 mg/kg and prednisolone significantly decreased the level of clostridium compared to TNBS group.

4. Discussion

The current pharmacological treatment of IBD mainly includes amino salicylates, corticosteroids, immunosuppressants and biologicals which have many side effects, especially when the prolonged administration is required to patients. In certain cases, patient non-compliance to conventional treatment is also seen. This has encouraged the use of complementary and alternative medicine to achieve efficacy, safety and compliance among IBD patients (Singh et al., 2012; Kondamudi et al., 2013). Natural products particular to medicinal plants are in practice to treat intestinal inflammatory conditions as complementary and alternative treatments. The presence of different active phytoconstituents in medicinal plants can act simultaneously on different targets in inflammatory pathway (Joshi et al., 2017).

In the present study, we performed phytochemical charecterization of TAHA extract using previously reported HPLC-PDA method for standardization purpose (Shengule et al., 2018a). The effect of hydroalcoholic extract of *T. arjuna* was evaluated in TNBS induced colitis rat model. It is established model for preclinical testing of various chemical or natural compounds towards their anti-inflammatory and anti-oxidative potentials, resembling human disease in histological and biochemical features (Antoniou et al., 2016; Strober et al., 2002). *In Vivo* study findings revealed an anti-inflammatory effect in TNBS model of experimental colitis. In particular, histological examination of rat colon displayed recovery in TAHA treated animals by reducing extension of ulceration, hyperemia and inflammation. This effect was further supported by biochemical and inflammatory markers investigations.

Oxidative stress is one of the most common pathogenic factors leading to inflammatory diseases and known to be associated with the pathogenesis of IBD (Yuksel et al., 2017; Jaiswal et al., 2018). Although uncontrolled oxidative stress is destructive to GI tract, body's defenses can counteract the effects triggered by excess reactive oxygen species (ROS) production. Intracellular enzymatic antioxidants such as SOD catalyzes the reduction of O_2^- into O_2 followed by conversion into H_2O_2 and CAT (located in peroxisomes) catalyses the reduction of H_2O_2 in forms of H_2O and O_2 molecules. Intracellular Nonenzymatic antioxidant i.e. GSH has been established as a biomarker for inflammation as well as oxidative stress (Tian et al., 2017). TAHA administration (500, 250 and 125 mg/kg) displayed significantly enhanced (SOD, CAT, GSH)

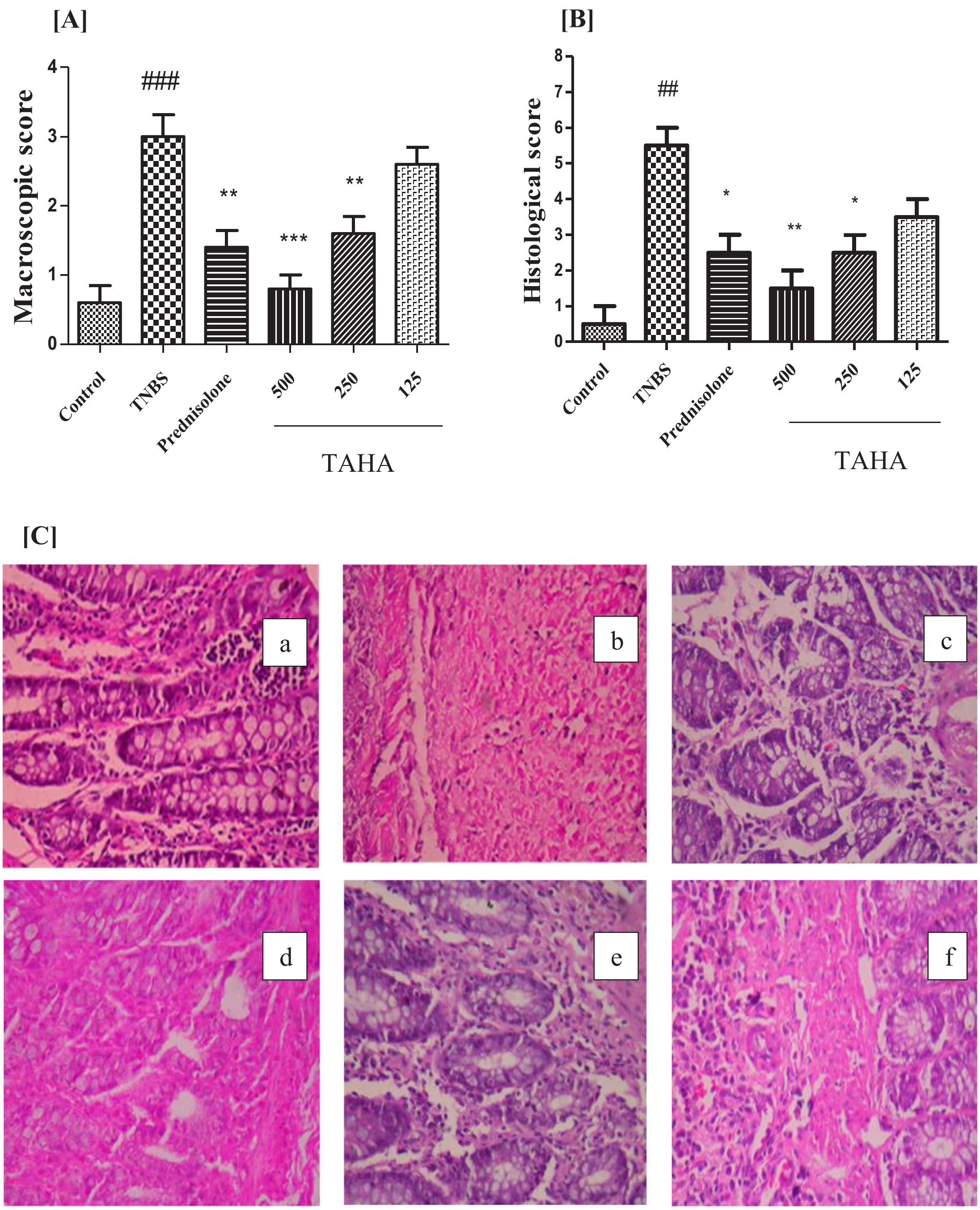

Fig. 2. Effects of prednisolone (2 mg/kg) and TAHA extract (500, 250,125 mg/kg) on colonic macroscopic damage [A], Histological score [B] and Histopathological changes [C]: a, normal control; b, TNBS control; c, prednisolone treatment; d-f, TAHA treatment (500, 250 and 125 mg/kg). Treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$$^{\#\#\#}p < 0.001$ vs. normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control. H&E staining, Magnification 40x.

level showing the anti-inflammatory potential in comparison to TNBS control animals confirming favourable effects of TAHA treatment.

The increased colonic mucosal iNOS (inducible nitric oxide synthase) and NO levels are seen in active period of IBD (Fei and Xu., 2016). As per previous reports, decrease in overproduction of NO by inhibition of iNOS may ameliorate the intestinal inflammation in IBD conditions (Xu et al., 2015; Gupta et al., 2018). Further, another marker, MPO level was estimated in rat colon samples as MPO (heme protein) signifies neutrophil infiltration which is the most prominent histological feature of mucosal inflammation in IBD. This indicates that decreasing MPO activity might play role for inhibition of gut mucosal inflammation (Choi et al., 2016). Our study finding revealed that TNBS control rats resulted in enhanced NO and MPO level in colonic biopsies as per previous findings is linked to disease advancement (Moura et al., 2015). While, TAHA administrations at all study doses displayed significantly reduced (NO and MPO) level indicating the anti-inflammatory potential as compared to TNBS control animals validating beneficial effects of TAHA treatment.

MDA is a by-product produced during oxidation of polyunsaturated fatty acids and is established indicator of lipid peroxidation as well as

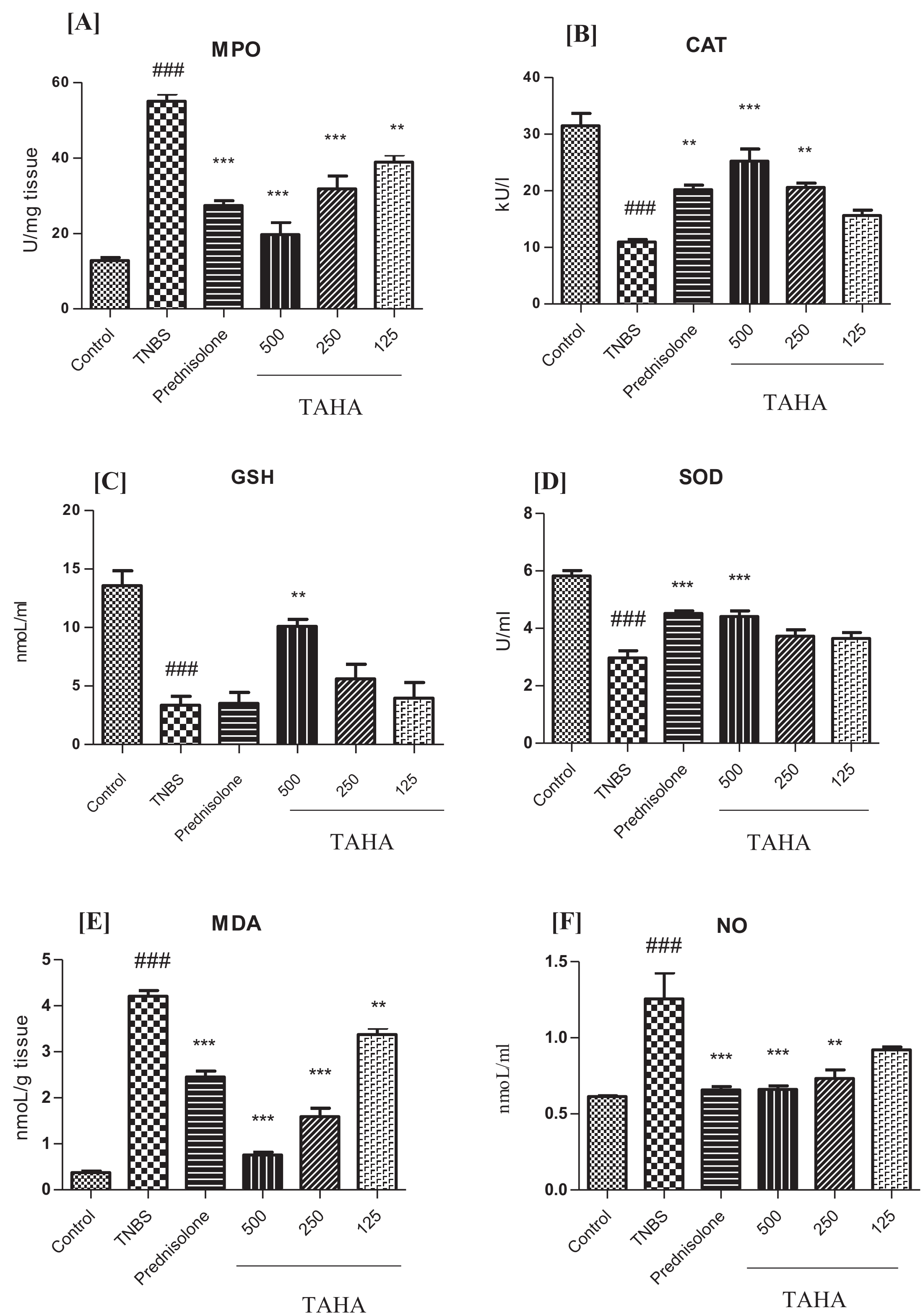

Fig. 3. Effects of prednisolone (2 mg/kg) and TAHA extract (500, 250, 125 mg/kg) on Myeloperoxidase (MPO) [A], Catalase (CAT) [B], Glutathione (GSH) [C], Superoxide dismutase (SOD) [D], Malondialdehyde (MDA) [E], Nitric oxide (NO) [F]. Treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$$^{\#\#\#}p < 0.001$ vs. normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control.

oxidative stress (Murad et al., 2016). It is also documented for enhanced level in colonic tissues of TNBS - induced colitic rats (Liu et al., 2012). This study result supports for effect of TAHA treatment on antioxidant mechanism by interfering in production of free radicals through interruption of lipid peroxidation process which is the main trigger for activation of inflammatory pathway. Hence, study findings indicate that TAHA treatment interferes with the inflammatory signalling cascade in TNBS - induced colitis.

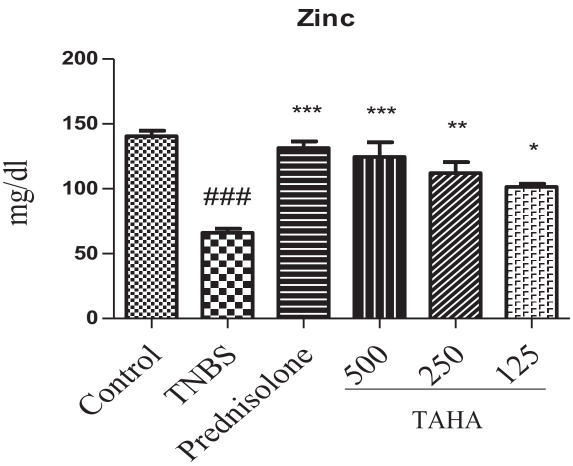

Fig. 4. Effects of prednisolone (2 mg/kg) and TAHA extract (500, 250, 125 mg/kg) on plasma Zinc levels. Treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control.

Table 3
Effect of TAHA on bacterial fecal level in the different experimental groups of rats with TNBS colitis.

	Bifidobacteria	Clostridium
Normal	3.428 ± 0.023	3.531 ± 0.002
TNBS	3.394 ± 0.003 ^{NS}	3.614 ± 0.008 $^{\#\#\#}$
Prednisolone	3.365 ± 0.002$^{\#\#, NS}$	2.505 ± 0.002 $^{\#\#\#, ***}$
TAHA 500	3.570 ± 0.003$^{\#\#\#, ***}$	2.505 ± 0.004 $^{\#\#\#, ***}$
TAHA 250	3.473 ± 0.003 $^{NS, **}$	3.579 ± 0.012 $^{\#\#, NS}$
TAHA 125	3.457 ± 0.005 $^{NS, **}$	3.584 ± 0.009 $^{\#\#, NS}$

Data are expressed as mean ± SEM, (n = 6). NS = not significant, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control.

Zinc an essential trace element is necessary in maintaining membrane barrier function and controlling inflammatory reaction, whereas depletion of zinc causes migration of neutrophils resulting in mucosal inflammation (Finamore et al., 2008). Low zinc levels have been earlier reported in the plasma, hair, and urine samples of IBD patients (Hendricks and Walker, 1988). Present study results directed similar findings in TNBS control rat plasma. Whereas, significant improved Zinc level was observed in TAHA treated experimental rats which might be beneficial in tissue restoration and healing of colon mucosa.

Intestinal microflora plays an essential role in the pathogenesis of

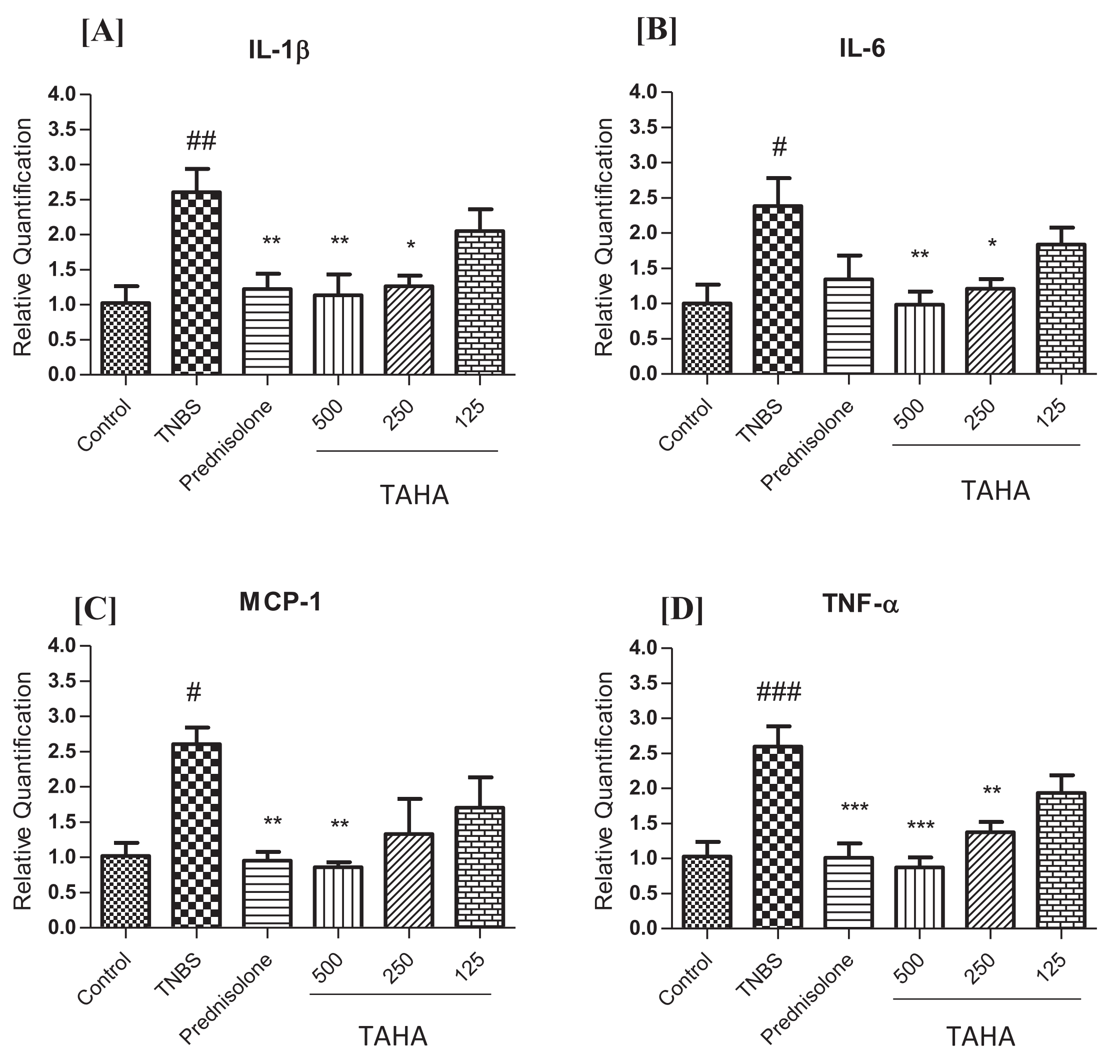

Fig. 5. Effects of prednisolone (2 mg/kg) and TAHA extract (500, 250, 125 mg/kg) on IL-1β [A], IL-6 [B], MCP-1 [C], TNF-α [D]. Treatment was administered 12 h after TNBS instillation and daily thereafter for consecutive 28 days. Data are expressed as the mean ± SEM, (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. normal control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNBS control.

intestinal inflammatory disorders. IBD is considered to be an immune-mediated disorder resulting from the abnormal interaction between intestinal microbes and the local immune system (Quing et al., 2015). Therefore, we have explored the TAHA treatment effect on intestinal microflora using a bacteriological analysis of feces from experimental animals. Our study results directed that the administration of TNBS decreased fecal Bifidobacteria and increased the counts of Clostridium as compared to control. The mechanism whereby TNBS modulates colonic flora is unknown and needs further investigation. TAHA treatment normalized the microflora profile by increasing the count of Bifidobacteria while decreasing clostridium counts as compared to TNBS control group. In contrast, prednisolone treatment further decreased the Bifidobacteria compared to TNBS alone. However its effect on Clostridium was comparable to TAHA.

Pro-inflammatory cytokines and chemokines have significant role in pathogenesis of IBD (Strober and Fuss, 2011). Therefore, experimental colitic rats in present study were evaluated for determining colonic expression of pro-inflammatory cytokines: IL-6, IL-1β, TNF-α and Chemokine: MCP-1. Previous study has reported an increased IL-6 mRNA expression in intestinal mucosa of ulcerative colitis (Atreya and Neurath, 2005). Since IL-1β facilitates chronic intestinal inflammation by promoting the accumulation of IL-17A. Therefore, targeting IL-1β may represent a useful therapeutic approach in IBD (Coccia et al., 2012). TNF-α contributes to the pathogenesis of IBD (Murch et al., 1993) and mediates multiple biologic effects including recruitment of neutrophils to local sites of inflammation, induction of edema, activation of coagulation, and induction of granuloma formation (Sandborn and Hanauer, 2007). Monocyte chemoattractant protein (MCP)-1 is a chemokine, and its expression is upregulated on exposure to inflammatory stimuli such as IL-1 and TNF-α. Elevation of MCP-1 is observed in colonic mucosa of clinical and experimental model CD and UC (Khan et al., 2006). In present study, we observed role of TAHA treatment (500 mg/kg) in the management of IBD through their significant down regulated gene expression of IL-1β (2 fold), IL-6 (2 fold), TNF-α (2.5 fold) and MCP-1 (2.5 fold) as compared to TNBS control rats. Whereas no significant expression was seen at lowest dose of TAHA treatment. On the other hand, prednisolone resulted significant reduction in IL-1β (2 fold), TNF-α (2.5 fold) and MCP-1 (2.5 fold). However, no significant reduction was seen for IL-6 expression.

5. Conclusion

TAHA treatment relieved the disease activity of TNBS induced colitis by decreasing oxidative stress and proinflammatory cytokines and chemokine. It also improved the composition of gut microbiota. The traditional use of *T.arjuna* by certain communities in the treatment of gastrointestinal disorders may be related to these effects. However, further clinical studies are necessary to validate its possible use in IBD treatment and to establish the exact mechanism.

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Conflicts of interest

The authors declare no conflict of interest.

Authors contribution

Damita Cota, has role for experimental protocol, conducting the experiments along with study parameters.

Sanjay Mishra, has provided the experimental concept, data analysis, and sincerely authored the article.

Sushant Shengule, has majorly performed the gene expression studies in the laboratory.

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