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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 1×10^5 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 1×10^7 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 Fe^{2+} /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]

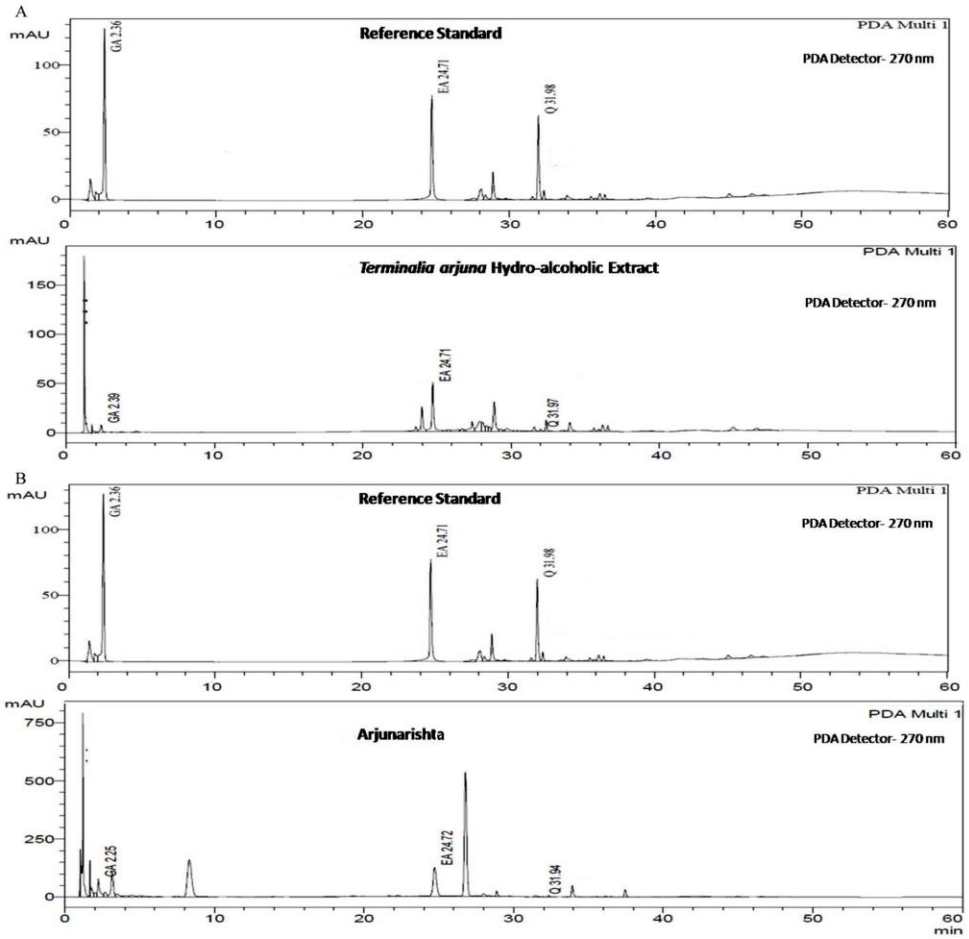

Fig. 1 — HPLC chromatogram of (A) TAHA; and (B) AA with reference standards

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]

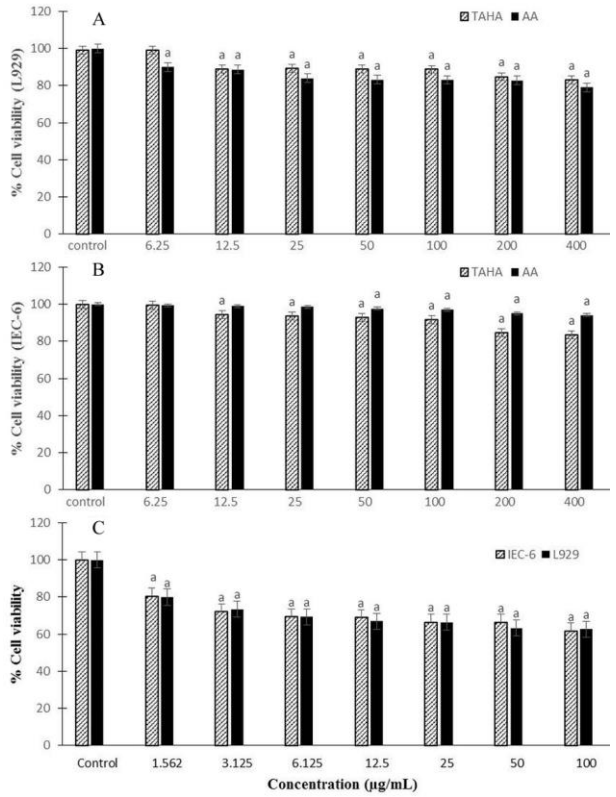

Fig. 2 — Cytocompatibility of TAHA and AA against (A) L929 cells derived from mouse fibroblast; (B) IEC-6 cells derived from rat intestinal epithelium; and (C) 5-FU against IEC-6 and L929 cell lines. [Cytocompatibility evaluated by % cell viability considering viability of control as 100% (expressed 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

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]

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].

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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