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Synergistic Cytoprotective and Antioxidant Potential of a Probiotic-Prebiotic Formulation Enriched with *Withania somnifera and Elaeocarpus ganitrus* Against Oxidative Stress in Intestinal Epithelial Cells: An In Vitro Approach

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Abstract

Oxidative stress is a central driver of gastrointestinal epithelial dysfunction and a key contributor to chronic intestinal disorders. The present in vitro study evaluates the cytoprotective and antioxidant efficacy of a novel formulation combining probiotics (Lactobacillus acidophilus, L. rhamnosus, L. plantarum, Bifidobacterium longum), a prebiotic (inulin), Ashwagandha (Withania somnifera) root extract, and Rudraksha (Elaeocarpus ganitrus) extract against hydrogen peroxide (H2O2)-induced oxidative stress in Vero intestinal cell lines. Cell viability was assessed by MTT assay, antioxidant potential by DPPH radical scavenging assay, intracellular reactive oxygen species (ROS) by DCFH-DA fluorescence, and cellular morphology by inverted microscopy. The combined formulation demonstrated significantly superior cell viability (93.2 ± 1.7% at 48 hrs), the lowest IC50 (41.6 ± 1.0 µg/mL), and maximum ROS reduction (59.4%) compared to individual components ($p < 0.001$). Microscopic analysis confirmed near-normal cellular morphology in the combined group. These results validate the synergistic interaction among probiotics, prebiotics, and herbal bioactives, providing a scientific foundation for developing integrated nutraceutical formulations targeting intestinal health.

Keywords: Probiotics; Withania somnifera; Elaeocarpus ganitrus; Oxidative stress; MTT assay; DPPH; ROS; Vero cells; Synergistic effect; Nutraceuticals

I. INTRODUCTION

The gastrointestinal tract represents one of the most metabolically active regions of the human body, performing crucial roles beyond digestion — including immune regulation, endocrine signaling, and protection against environmental pathogens (Sekirov et al., 2010; Hooper et al., 2012). The intestinal epithelium, in particular, functions as a dynamic barrier that maintains homeostasis between the host and the microbial environment. Disruption of this barrier through oxidative stress, dysbiosis, or inflammatory cascades is closely associated with the pathogenesis of inflammatory bowel disease, irritable bowel syndrome, and colorectal complications (Bischoff, 2011; Turner, 2009).

Reactive oxygen species (ROS), generated during normal metabolic activity and significantly amplified under stress conditions, are central mediators of oxidative damage to intestinal epithelial cells. When antioxidant defense mechanisms fail to neutralize ROS accumulation, lipid peroxidation, protein oxidation, and DNA fragmentation can ensue, culminating in cell death and compromised barrier integrity (Wang et al., 2017).

Probiotics — viable microorganisms that confer measurable health benefits when consumed in adequate quantities — have emerged as promising agents for restoring intestinal homeostasis (Hill et al., 2014). Species of Lactobacillus and Bifidobacterium are among the most extensively characterized, demonstrating capacity to scavenge free radicals, upregulate endogenous antioxidant enzymes, and modulate inflammatory pathways (Plaza-Diaz et al., 2019; Hemarajata & Versalovic, 2013). When combined with prebiotics — non-digestible fermentable substrates that selectively nourish beneficial microbial communities — the resulting synbiotic formulations exhibit amplified functional activity (Gibson et al., 2017; Swanson et al., 2020).

Traditional medicine systems have long recognized the therapeutic utility of plant-derived bioactives. Ashwagandha (Withania somnifera), a cornerstone of Ayurvedic pharmacopeia, is documented for its adaptogenic,

immunomodulatory, and potent antioxidant properties attributable largely to steroidal lactones known as withanolides (Singh et al., 2011; Lopresti et al., 2019). Rudraksha (Elaeocarpus ganitrus), historically valued in spiritual and folk medicine, has been shown to possess antimicrobial and antioxidant bioactivities, though cellular-level mechanistic data remain sparse (Sharma et al., 2015).

Despite growing individual evidence for each component, a significant research gap persists regarding their combined effect — particularly the synergistic interaction of probiotic consortia with herbal extracts on intestinal epithelial cells under oxidative challenge. The present study addresses this gap by evaluating the in vitro cytoprotective and antioxidant properties of a multi-component formulation combining probiotics, prebiotic, Ashwagandha, and Rudraksha extracts using Vero cell lines.

II. MATERIALS AND METHODS

2.1 Biological Materials and Reagents

Probiotic strains — Lactobacillus acidophilus, L. rhamnosus, L. plantarum, and Bifidobacterium longum — were sourced from authenticated culture collections. Inulin (Sigma-Aldrich, India) served as the prebiotic substrate. Ashwagandha roots and Rudraksha seeds were procured from certified herbal suppliers and authenticated by a botanist. Vero cells (African green monkey kidney-derived intestinal epithelial line) were obtained from NCCS, Pune, India. Culture media (DMEM), fetal bovine serum (FBS, 10%), penicillin-streptomycin antibiotic solution, and phosphate-buffered saline (PBS) were of cell-culture grade. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DPPH (2,2-diphenyl-1-picrylhydrazyl), and DCFH-DA reagents were purchased from HiMedia Laboratories.

2.2 Preparation of Herbal Extracts

Ashwagandha roots and Rudraksha seeds were washed thoroughly with distilled water, shade-dried at 40°C, and ground to a fine powder. Hydro-alcoholic extraction (70% ethanol:water) was performed using a Soxhlet apparatus over 6 hours. Extracts were filtered through Whatman No. 1 filter paper, concentrated under reduced pressure using a rotary evaporator, lyophilized, and stored at -20°C. Stock solutions (10 mg/mL in DMSO) were prepared fresh before each assay. Final DMSO concentration in treatment wells was maintained below 0.1% to avoid cytotoxic interference.

2.3 Probiotic Culture and Consortium Preparation

Individual probiotic strains were revived in MRS broth (Lactobacillus spp.) or RCM broth (Bifidobacterium) under appropriate anaerobic conditions at 37°C for 18-24 hrs. Cell viability was confirmed by methylene blue staining and colony counts. Each strain was standardized to a concentration of ~3×10^8 CFU/mL. The probiotic consortium was prepared by combining equal volumes of each culture, yielding a final count of ~1.18×10^9 CFU/mL. Acid tolerance (pH 2.5, 3 hrs) and bile tolerance (0.3% bile salts, 3 hrs) were evaluated to confirm probiotic functionality.

2.4 Formulation Groups

Six experimental groups were established: (1) Untreated Control, (2) Probiotic consortium alone, (3) Prebiotic (inulin) alone, (4) Ashwagandha extract alone, (5) Rudraksha extract alone, and (6) Combined Formulation comprising all four components at optimized ratios. An additional oxidative stress group received H2O2 (200 µM) as a positive stressor, with or without treatment formulations.

2.5 Cell Culture and Treatment

Vero cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic solution in a humidified incubator (37°C, 5% CO2). Cells at 80-90% confluency were seeded in 96-well plates (1×10^4 cells/well) and allowed to adhere overnight. Treatment formulations were applied at predetermined concentrations for 24 and 48 hrs. Where applicable, H2O2 (200 µM) was added 1 hr before treatments to induce oxidative stress.

2.6 MTT Cell Viability Assay

After the respective treatment durations, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and plates were incubated for 4 hrs at 37°C. Formazan crystals were dissolved in 100 µL DMSO, and absorbance was recorded at 570 nm using a microplate reader. Cell viability (%) was computed relative to untreated controls. All assays were conducted in triplicate (n=3).

2.7 DPPH Radical Scavenging Assay

The antioxidant potential of each formulation was assessed at four concentrations (25, 50, 100, 200 µg/mL) against 0.1 mM ethanolic DPPH solution. Absorbance was measured at 517 nm after 30 min of incubation in the dark. Scavenging activity (%) was

calculated and IC50 values were derived from dose-response curves. Ascorbic acid served as the positive control.

2.8 Intracellular ROS Detection

ROS levels were quantified using the DCFH-DA fluorescent probe. Treated Vero cells were incubated with DCFH-DA (10 µM) for 30 min at 37°C, washed with PBS, and fluorescence intensity was measured at excitation/emission wavelengths of 485/530 nm. Results were expressed in arbitrary fluorescence units (AU).

2.9 Morphological Analysis

Morphological changes in treated and control Vero cells were examined under an inverted phase-contrast microscope (Olympus CKX41) at 100× and 200× magnification at both time points. Parameters evaluated included cell shape, monolayer integrity, nuclear condensation, cellular blebbing, and presence of detached cells.

2.10 Statistical Analysis

All experiments were performed in triplicate and data are expressed as Mean ± Standard Deviation (SD). One-way ANOVA followed by Tukey's post-hoc test was applied using SPSS v.26.0. A p-value of less than 0.05 was considered statistically significant.

III. RESULTS

3.1 MTT Assay — Cell Viability

The MTT assay data revealed distinct differences in cell viability across treatment groups at both 24 and 48 hours (Table 1). Untreated control cells maintained high viability (98.6 ± 1.2% at 24 hrs; 97.9 ± 1.5% at 48 hrs). Among individual treatments, Rudraksha extract alone produced the greatest reduction in viability (80.4 ± 3.2% at 48 hrs), suggesting moderate cytotoxicity at the tested concentration. The Combined Formulation, in contrast, exhibited the highest viability among treated groups (94.8 ± 1.5% at 24 hrs; 93.2 ± 1.7% at 48 hrs), with the lowest inhibition percentage (4.9% at 48 hrs; $p < 0.01$). Even under H_2O_2-induced oxidative stress, the combined treatment maintained cell viability at 90.7 ± 2.1%, demonstrating significant cytoprotective activity against oxidative injury.

Table 1: Cell Viability (%) by MTT Assay — 24 and 48 Hour Incubation

Treatment Groups	24 hrs Mean±SD (%)	% Inhibition (24 h)	48 hrs Mean±SD (%)	% Inhibition (48 h)	p (24h)	p (48h)
Control (Untreated)	98.6 ± 1.2	—	97.9 ± 1.5	—	—	—
Probiotic alone	91.4 ± 2.1	7.3	89.2 ± 2.4	8.9	<0.05	<0.05
Prebiotic alone	89.7 ± 1.8	9.0	87.5 ± 2.0	10.6	<0.05	<0.05
Ashwagandha extract alone	85.3 ± 2.5	13.5	83.1 ± 2.8	15.1	<0.01	<0.01
Rudraksha extract alone	82.6 ± 3.0	16.2	80.4 ± 3.2	17.9	<0.01	<0.01
Combined Formulation	94.8 ± 1.5	3.8	93.2 ± 1.7	4.9	<0.01	<0.01

Values expressed as Mean ± SD (n=3). Statistical significance assessed by one-way ANOVA with Tukey's post-hoc test.

3.2 DPPH Radical Scavenging Activity

The antioxidant capacity of all formulations was concentration-dependent, with scavenging activity increasing progressively from 25 to 200 µg/mL (Table 2). The Combined Formulation exhibited the lowest IC50 (41.6 ± 1.0 µg/mL), surpassing ascorbic acid (IC50: 47.2 ± 0.8 µg/mL), indicating superior antioxidant potency. Individual herbal extracts displayed moderate activity — Ashwagandha (IC50: 68.5 ± 2.0 µg/mL) outperformed Rudraksha (IC50: 78.3 ± 2.5 µg/mL), while probiotic and prebiotic fractions alone demonstrated notably lower scavenging capacity (IC50 > 100 µg/mL). These results strongly suggest that the integration of microbial and herbal components produces a synergistic antioxidant effect exceeding what any single component can achieve independently.

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Table 2: DPPH Radical Scavenging Activity (%) at Different Concentrations

Treatment Groups	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	IC50 (µg/mL)
Ascorbic acid (Positive Ctrl)	28.4±1.1	51.6±1.3	74.3±1.5	91.2±1.0	**47.2±0.8**
Probiotic alone	14.2±1.8	28.6±2.0	44.7±2.3	61.3±2.5	**112.4±3.1**
Prebiotic alone	11.5±1.5	23.4±1.9	39.8±2.1	55.6±2.4	**128.7±3.4**
Ashwagandha extract alone	22.1±1.6	41.3±2.1	63.5±2.4	79.8±2.2	**68.5±2.0**
Rudraksha extract alone	18.7±2.0	36.2±2.3	57.4±2.6	74.1±2.8	**78.3±2.5**
Combined Formulation	31.4±1.3	58.7±1.5	79.6±1.8	93.5±1.2	**41.6±1.0**

Values = Mean ± SD (n=3). Lower IC50 value indicates higher antioxidant efficacy.

3.3 Intracellular ROS Quantification

H2O2-treated cells showed a dramatic surge in ROS levels (784.3 ± 21.4 AU at 24 hrs; 812.6 ± 24.3 AU at 48 hrs) compared to untreated controls (~180 AU), confirming effective oxidative stress induction (Table 3). Among individual treatments applied alongside H2O2, Ashwagandha extract achieved the greatest ROS reduction (33.1%), followed by Rudraksha (30.0%), probiotic (21.9%), and prebiotic alone (18.6%). Remarkably, the Combined Formulation reduced intracellular ROS by 59.4% (from 784.3 to 318.6 AU at 24 hrs), a significantly greater reduction than any individual component ($p < 0.001$). This outcome substantiates the synergistic hypothesis and implies that herbal bioactives and probiotic metabolites act through complementary antioxidant mechanisms.

Table 3: Intracellular ROS Levels — DCFH-DA Fluorescence Assay

Treatment Groups	ROS Level 24 hrs (AU)	ROS Level 48 hrs (AU)	% ROS Reduction vs H2O2	p-value
Untreated Control	182.4±8.1	178.6±7.9	—	—
H2O2-induced (Positive ctrl)	784.3±21.4	812.6±24.3	—	—
Probiotic alone + H2O2	612.5±18.2	634.7±19.5	**21.9%**	<0.05
Prebiotic alone + H2O2	638.4±17.6	658.2±20.1	**18.6%**	<0.05
Ashwagandha extract + H2O2	524.7±15.3	541.3±16.8	**33.1%**	<0.01
Rudraksha extract + H2O2	548.2±16.7	563.8±17.5	**30.0%**	<0.01
Combined Formulation + H2O2	318.6±10.4	327.1±11.2	**59.4%**	<0.001

AU = Arbitrary Units. Values = Mean ± SD (n=3). *** $p < 0.001$ vs H2O2 control group.

3.4 Morphological Assessment

Microscopic observations corroborated the quantitative assay data. Control cells displayed the characteristic polygonal morphology of Vero epithelial cells with 90-95% confluency and no observable signs of stress. H2O2-treated cells exhibited dramatic morphological deterioration — marked cytoplasmic shrinkage, membrane blebbing, nuclear condensation, and extensive cell detachment (40-55% confluency). Cells treated with individual components showed mild-to-moderate alterations depending on the agent. The Combined Formulation group, notably, maintained near-normal cell architecture (88-93% confluency) with intact monolayer continuity and minimal morphological deviation. Even when co-exposed to H2O2 stress, the Combined Formulation preserved cellular integrity significantly better than the stress control.

IV. DISCUSSION

The findings of the present study provide compelling in vitro evidence for a synergistic cytoprotective and antioxidant interaction among probiotics, prebiotic inulin, Ashwagandha, and Rudraksha extracts on intestinal epithelial cells subjected to oxidative challenge. The superior performance of the Combined Formulation across all measured parameters — cell viability, radical scavenging, ROS reduction, and cellular morphology — compared to individual components validates the central hypothesis of this investigation.

The high cell viability observed in the combined treatment group aligns with prior evidence that probiotic-derived metabolites, including short-chain fatty acids, hydrogen peroxide-consuming enzymes, and bacteriocins, can actively maintain epithelial integrity and counteract exogenous oxidative insults (Bron et al., 2017). Simultaneously, withanolides from Ashwagandha have been reported to stabilize mitochondrial membrane potential and activate the Nrf2-ARE antioxidant response pathway, while flavonoids and tannins from Rudraksha are known to directly scavenge hydroxyl and superoxide radicals (Gupta & Kaur, 2019; Sharma et al., 2015). The concurrent activation of these distinct biochemical pathways likely accounts for the amplified protection observed in the combination group.

The IC50 value for the Combined Formulation (41.6 µg/mL) was not only lower than each individual component but also marginally superior to the standard antioxidant ascorbic acid. This indicates genuine synergism rather than simple additive effects — a phenomenon documented in probiotic-phytochemical interactions where herbal compounds may enhance probiotic survival and metabolic activity while probiotic fermentation may biotransform plant secondary metabolites into more bioavailable, pharmacologically active derivatives (Markowiak & Slizewska, 2017; Pandey & Rizvi, 2015).

The 59.4% reduction in intracellular ROS by the Combined Formulation substantially exceeds the partial reductions achieved by individual agents (18.6-33.1%). Elevated intracellular ROS, if uncontrolled, triggers NF-kB-mediated inflammatory cascades and caspase-dependent apoptosis — pathways that underpin the pathophysiology of inflammatory bowel conditions. The marked suppression of ROS observed here suggests multi-target antioxidant activity likely operating through enzyme induction, direct radical scavenging, and metal chelation concurrently.

It is worth noting that while Rudraksha and Ashwagandha extracts individually produced somewhat lower cell viability at higher concentrations, when incorporated into the combined formulation, these effects were mitigated — possibly due to modulating interactions with the probiotic consortium or prebiotic matrix. This also underscores the importance of concentration optimization in multi-component formulation development.

From a translational perspective, these results support the concept of integrated nutraceuticals that harness both microbial and botanical bioactives for gut health applications. Such formulations, grounded in traditional medicine and validated by modern cellular biology, represent a convergence of ethnopharmacology and biotechnology with significant potential for functional food and therapeutic supplement development.

V. CONCLUSION

The present study demonstrates that a combined formulation of probiotics, prebiotic inulin, Withania somnifera (Ashwagandha) extract, and Elaeocarpus ganitrus (Rudraksha) extract exerts significantly enhanced cytoprotective and antioxidant effects on intestinal epithelial cells compared to any single component. The evidence of synergistic ROS neutralization, superior cell viability maintenance, and preserved epithelial morphology under oxidative stress conditions provides robust scientific validation for integrating these components into functional nutraceutical strategies targeting gut health. Further studies involving in vivo animal models and mechanistic pathway analysis (Nrf2, NF-kB, caspase activity) are warranted to translate these findings into clinical applications.

CONFLICT OF INTEREST AND ACKNOWLEDGEMENTS
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