RESEARCH PAPER

Evaluation of Antioxidant Capacity (FRAP) and Antimicrobial Efficacy (ZOI and MIC) of Polyherbal Immunomodulatory Formulations

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ABSTRACT

Objective: The present study aimed to evaluate and compare the in vitro total antioxidant capacity and antimicrobial efficacy of individual natural extracts (Amla, Chyavanprash, Marking Nut tree, and Gorakhmundi) alongside their optimized polyherbal dosage forms—Tablet Formulation F05 and Granules Formulation F06.

Methods: Total antioxidant capacity was assessed via the Ferric Reducing Antioxidant Power (FRAP) assay using Trolox as a reference standard across 10–100 µM. Antimicrobial efficacy against pathogenic Gram-negative bacteria belonging to the family Enterobacteriaceae was evaluated using the Zone of Inhibition (ZOI) agar disc diffusion method and the Minimum Inhibitory Concentration (MIC) broth macrodilution assay. Statistical analysis was performed using One-Way ANOVA to verify concentration-dependent antibacterial effects.

Results: The FRAP assay demonstrated superior ferric reducing power for the combined dosage forms—Tablet Formulation F05 (60.67 µmol TE/g) and Granules Formulation F06 (60.00 µmol TE/g)—compared to individual extracts (25.33–54.00 µmol TE/g). In the ZOI assay, at 0.10 mL application volume, Granules Formulation F06 (27 mm) and Tablet Formulation F05 (25 mm) exhibited significantly larger inhibition zones than single Amla (20 mm) and Chyavanprash (18 mm) extracts. One-Way ANOVA confirmed a statistically significant concentration-dependent antibacterial response ($p = 0.011485$, $F = 12.8977$). In MIC testing, both polyherbal formulations achieved complete microbial growth suppression at 100 µg/mL, whereas single extracts required 199 µg/mL.

Conclusion: Co-formulating botanical extracts into tablet and granule dosage forms produces marked synergistic antioxidant and antimicrobial effects, confirming their potential as effective immunomodulatory therapeutics.

Keywords: Polyherbal Formulations, FRAP Assay, Zone of Inhibition, Minimum Inhibitory Concentration, Enterobacteriaceae, Antioxidant Capacity, Synergism.

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1. INTRODUCTION

Immunity is defined as a complex biological system endowed with the capacity to recognize and tolerate self-structures while recognizing and rejecting non-self entities [1-5]. In medicine, the immune system serves as the core defense network against infectious diseases [2]. Biological immunity encompasses three principal arms: innate immunity (constitutive physical barriers like skin and mucous membranes, as well as cellular components like natural killer cells), adaptive immunity (acquired in response to infection or vaccination through antigen-specific cellular memory that can last a lifetime), and passive immunity (rapid, short-term protection acquired via transfer of exogenous antibodies) [3-5].

Maintaining robust immunity is essential for preserving overall health and preventing infectious illnesses [6]. However, multiple physiological and environmental factors can severely depress immune function. Advancing age precipitates

immunosenescence, leading to thymus involution, diminished naive T and B lymphocyte output, accumulation of non-functional memory cells, reduced TLR expression on dendritic cells and macrophages, and impaired neutrophil oxidative burst [7]. Other debilitating factors include environmental toxins (tobacco smoke, air pollutants, excessive alcohol), obesity (low-grade chronic inflammation mediated by adipocytokines), poor diet, chronic psychological stress (cortisol-mediated leukocyte suppression), and rest deprivation [8,9].

To bolster immune defense in a natural way, traditional systems of medicine—such as Ayurveda ('The Science of Life')—utilize Rasayana remedies to nourish body tissues and strengthen biological resistance [10]. Notable medicinal botanicals with established immunomodulatory and anti-inflammatory properties include Phyllanthus emblica (Amla), Chyavanprash, Semecarpus anacardium (Marking Nut tree), and Sphaeranthus indicus (Gorakhmundi) [11-14]. P. emblica is exceptionally rich in ascorbic acid and hydrolyzable tannins, providing potent free-radical scavenging capabilities [11]. Chyavanprash is a traditional polyherbal rejuvenative formulation known to enhance respiratory immunity [12]. S. anacardium contains bioactive anacardic acids and flavonoids that possess immunomodulatory and antimicrobial activity [13], while S. indicus yields sesquiterpene lactones and alkaloidal fractions with marked antimicrobial and tissue-protective properties [14].

Oxidative stress, arising from an imbalance between reactive oxygen species (ROS) and endogenous antioxidant defenses, leads to cellular damage and immune suppression [15]. Plant extracts rich in polyphenols and flavonoids act as electron donors to neutralize free radicals. Evaluating total antioxidant capacity via the Ferric Reducing Antioxidant Power (FRAP) assay provides a simple, reproducible, and direct measure of electron-donating reducing capacity [16].

Concurrently, rising bacterial resistance—particularly among Gram-negative pathogens belonging to the family Enterobacteriaceae—underscores the need for effective natural antimicrobial formulations [17]. Gram-negative bacteria possess a complex cell envelope that restricts drug entry. Multi-component herbal remedies offer a distinct therapeutic advantage by targeting multiple cellular mechanisms simultaneously, minimizing resistance development [18]. Assessing antimicrobial efficacy through Zone of Inhibition (ZOI) agar disc diffusion and Minimum Inhibitory Concentration (MIC) broth macrodilution assays provides quantitative validation of antibacterial potency and reveals potential synergistic interactions in polyherbal dosage forms [19,20].

Therefore, the objective of the present study was to systematically evaluate and compare the in vitro antioxidant capacity (FRAP assay) and antimicrobial efficacy (ZOI and MIC studies) of individual natural extracts (Amla, Chyavanprash, Marking Nut tree, and Gorakhmundi) versus two optimized polyherbal dosage forms: Tablet Formulation F05 and Granules Formulation F06.

2. MATERIALS AND METHODS

2.1. Botanical Extracts and Dosage Form Preparation

Standardized herbal extracts of Amla (Phyllanthus emblica), Marking Nut tree extract (Semecarpus anacardium), and Gorakhmundi extract (Sphaeranthus indicus) were procured from NJP Healthcare, and Chyavanprash extract was procured from Konark Herbals. Two solid polyherbal formulations were evaluated:

- **Tablet Formulation F05:** Composed of Amla extract (58.82%), Chyavanprash extract (18.82%), and Marking Nut tree extract (11.76%) along with standard pharmaceutical excipients.
- **Granules Formulation F06:** Composed of Amla extract (58.82%), Chyavanprash extract (18.82%), and Gorakhmundi extract (11.76%) formulated into free-flowing granules.

2.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay measures the total antioxidant capacity based on the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to its ferrous (Fe^{2+}-TPTZ) form at acidic pH [16]. Trolox (a water-soluble vitamin E analog) served as the reference antioxidant standard across 10–100 µM. Fresh FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 (v/v/v) ratio. Test solutions of individual extracts (Amla 58.82%, Chyavanprash 18.82%, Gorakhmundi 11.76%, Marking Nut tree 11.76%) and finished formulations (Tablet F05 and Granules F06) were prepared at

appropriate dilutions. Sample aliquots were mixed with FRAP reagent, incubated at 37°C for 30 minutes, and absorbance was measured spectrophotometrically at 593 nm against a blank. Measurements were performed in triplicate, and antioxidant potential was expressed as µmol Trolox equivalents per gram (µmol TE/g).

2.3. Zone of Inhibition (ZOI) Assay

Antimicrobial activity was evaluated using the agar disc diffusion method against pathogenic Gram-negative bacteria belonging to the family Enterobacteriaceae [19]. Sterile agar plates were inoculated with standardized bacterial suspension. Tablet samples (F05) were prepared by dispersing one tablet in 28.90 mL of distilled water. Granule samples (F06) were prepared by dispersing 850 mg of granules in 28.90 mL of distilled water. Test solutions of single extracts and formulations were tested at application volumes of 0.05 mL and 0.10 mL, maintaining a standardized dose of 10 µg/disc (100 µg/mL dispersion). Sterile filter paper discs impregnated with sample solutions were placed on inoculated agar plates and incubated at 37°C for 24 hours. The diameter of clear inhibition zones was measured in millimeters using a digital caliper. Quantification of chemical marker content (% ascorbic acid and % gallic acid) was performed simultaneously using validated reversed-phase chromatographic techniques [21-27]. Data were analyzed using One-Way Analysis of Variance (ANOVA) at $p < 0.05$ level of significance.

2.4. Minimum Inhibitory Concentration (MIC) Study

The MIC was determined via the broth macrodilution method against Enterobacteriaceae pathogens [19]. Test sample dilutions (individual extracts AE1–AE3 and CE1–CE3; Tablet F05 TF1–TF3; Granules F06 GF1–GF3) were prepared at application volumes of 0.05 mL, 0.10 mL, and 0.20 mL. Each tube was inoculated with a standardized bacterial culture and incubated at 37°C for 24 hours. Following incubation, tubes were visually evaluated for turbidity: '++++' indicated heavy bacterial growth, '++' indicated moderate growth, and '-' indicated complete absence of growth. The MIC was defined as the lowest sample concentration resulting in no visible turbidity.

3. RESULTS AND DISCUSSION

3.1. Ferric Reducing Antioxidant Power (FRAP) Assay Results

The FRAP assay demonstrated excellent linearity for the Trolox standard curve across 10–100 µM (Table 1), yielding a linear regression equation of $y = 0.015x$ ($r^2 = 1.000$). This confirms the sensitivity and analytical validity of the assay for quantitative evaluation of antioxidant potential.

Table 1: Standard Calibration Data for Reference Antioxidant (Trolox)

Trolox Concentration (µM)	Absorbance (593 nm)
0	0.000
10	0.150
20	0.300
30	0.450
40	0.600
50	0.750
60	0.900
70	1.050
80	1.200
90	1.350
100	1.500

Individual botanical extracts exhibited moderate ferric reducing ability, with FRAP values ranging from 25.33 to 54.00 µmol TE/g (Table 2). Amla extract (58.82%) produced the highest absorbance among individual components (0.81, corresponding to 54.00 µmol TE/g), attributed to its rich polyphenolic and ascorbic acid content. Chyavanprash extract (18.82%) produced an absorbance of 0.60 (40.00 µmol TE/g), while Marking Nut tree extract (11.76%) and Gorakhmundi extract (11.76%) recorded absorbances of 0.45 (30.00 µmol TE/g) and 0.38 (25.33 µmol TE/g), respectively.

Importantly, the finished polyherbal dosage forms displayed markedly superior antioxidant capacity. Granules Formulation F06 achieved a FRAP value of 60.00 µmol TE/g (absorbance 0.90), while Tablet Formulation F05 exhibited the highest antioxidant power at 60.67 µmol TE/g (absorbance 0.91). This notable increase in reducing capability demonstrates a synergistic antioxidant interaction among active constituents when combined into solid oral dosage forms.

Table 2: Comparative FRAP Absorbance and Total Antioxidant Capacity

Sample Description	Absorbance (593 nm)	FRAP Value (µmol TE/g)
Amla Extract (58.82%)	0.81	54.00
Chyavanprash Extract (18.82%)	0.60	40.00
Gorakhmundi Extract (11.76%)	0.38	25.33
Marking Nut Tree Extract (11.76%)	0.45	30.00
Combined Granules Formulation F06	0.90	60.00
Combined Tablet Formulation F05	0.91	60.67

3.2. Antimicrobial Zone of Inhibition (ZOI) Results

Chemical marker content determination confirmed that ascorbic acid and gallic acid levels in Tablet F05 (0.080% AA, 0.260% GA) and Granules F06 (0.079% AA, 0.261% GA) aligned precisely with expected theoretical target ranges (Table 3), verified via standardized chromatographic marker quantification [21-26].

Table 3: Active Marker Content (% Ascorbic Acid and % Gallic Acid) in Extracts and Formulations

Sample Description	% Ascorbic Acid	% Expected AA Range	% Gallic Acid	% Expected GA Range
Amla Extract (58.82%)	0.085%	—	0.140 %	—
Chyavanprash Extract (18.82%)	0.162%	—	1.030 %	—
Tablet Formulation F05	0.080%	0.072% – 0.088%	0.260 %	0.248% – 0.303%
Granules Formulation F06	0.079%	0.072% – 0.088%	0.261 %	0.248% – 0.303%

The Zone of Inhibition assay against Enterobacteriaceae demonstrated clear dosage-dependent antibacterial responses (Table 4). At 0.05 mL concentration, Amla and Chyavanprash produced zones of 10 mm and 8 mm, whereas Tablet F05 (15 mm) and Granules F06 (16 mm) exhibited significantly higher antibacterial activity. Increasing the application volume to 0.10 mL produced substantial zone enlargement: Amla (20 mm), Chyavanprash (18 mm), Tablet F05 (25 mm), and Granules F06 (27 mm).

Table 4: Dose-Dependent Zone of Inhibition (ZOI) Measurements against Gram-Negative Enterobacteriaceae

Sample	Vol (mL)	AA (mg)	GA (mg)	ZOI (mm)	Vol (mL)	AA (mg)	GA (mg)	ZOI (mm)
Amla Extract	0.05	0.00199	0.00031	10	0.10	0.00038	0.00062	20
Chyavanprash Extract	0.05	0.00007	0.00043	8	0.10	0.00014	0.00086	18
Tablet F05	0.05	0.00012	0.00038	15	0.10	0.00024	0.00077	25
Granules F06	0.05	0.00012	0.00038	16	0.10	0.00023	0.00077	27

One-Way ANOVA testing on the ZOI dataset (Table 5) yielded an F-statistic of 12.8977 ($F_{crit} = 5.9874$, $p = 0.011485$, $p < 0.05$), confirming that the observed increase in antibacterial activity at higher application concentration is statistically significant. Granules F06 yielded slightly larger zones than Tablet F05, likely owing to faster dispersion and drug release in agar media.

Table 5: One-Way ANOVA Summary Table for Zone of Inhibition Data

Source of Variation	SS	df	MS	F-Statistic	P-value	F Critical

Between Groups	210.125	1	210.125	12.8977	0.011485*	5.987378
Within Groups	97.750	6	16.292	—	—	—
Total	307.875	7	—	—	—	—

Group 1: ZOI (0.05 mL): Count = 4, Sum = 49, Mean = 12.25, Variance = 14.917
Group 2: ZOI (0.10 mL): Count = 4, Sum = 90, Mean = 22.50, Variance = 17.667
**Statistically significant difference at $p < 0.05$ level.*

3.3. Minimum Inhibitory Concentration (MIC) Results

Broth macrodilution testing established precise MIC cutoffs (Table 6 and Table 7). Single extracts of Amla (AE1–AE3) and Chyavanprash (CE1–CE3) failed to inhibit bacterial growth at 0.05 mL and 0.10 mL ('++++' and '++' turbidity), achieving complete inhibition ('-') only at 0.20 mL (MIC = 199 µg/mL). In contrast, Tablet Formulation F05 (TF1–TF3) and Granules Formulation F06 (GF1–GF3) achieved complete growth inhibition at 0.10 mL, establishing an MIC of 100 µg/mL. This two-fold increase in potency demonstrates that polyherbal combination provides superior antibacterial efficacy over individual extracts against Gram-negative pathogens.

Table 6: Minimum Inhibitory Concentration (MIC) Broth Macrodilution Assay Results

Sample Description	Code	Vol (mL)	AA (mg)	GA (mg)	MIC (µg/mL)	Turbidity	Interpretation
Amla Extract (58.82 %)	AE1	0.05	0.0019	0.0031	—	Turbid	Growth
Amla Extract (58.82 %)	AE2	0.10	0.0038	0.0062	—	Turbid	Growth
Amla Extract (58.82 %)	AE3	0.20	0.0076	0.0124	199	Non-turbid	No Growth (MIC)
Chyavanprash (18.82 %)	CE1	0.05	0.0007	0.0043	—	Turbid	Growth
Chyavanprash (18.82 %)	CE2	0.10	0.0014	0.0086	—	Turbid	Growth
Chyavanprash (18.82 %)	CE3	0.20	0.0027	0.0173	199	Non-turbid	No Growth (MIC)
Tablet F05	TF1	0.05	0.0012	0.0038	—	Turbid	Growth
Tablet F05	TF2	0.10	0.0024	0.0076	100	Non-turbid	No Growth (MIC)
Tablet F05	TF3	0.20	0.0047	0.0153	—	Non-turbid	No Growth
Granules F06	GF1	0.05	0.0012	0.0038	—	Turbid	Growth
Granules F06	GF2	0.10	0.0023	0.0077	100	Non-turbid	No Growth (MIC)
Granules F06	GF3	0.20	0.0046	0.0154	—	Non-turbid	No Growth

Table 7: Visual Turbidity Scoring for Relative Bacterial Density

Sample Code	Application Volume (mL)	Visual Turbidity / Growth Score

AE1 (Amla Extract)	0.05	++++ (Heavy Growth)
AE2 (Amla Extract)	0.10	++ (Moderate Growth)
AE3 (Amla Extract)	0.20	- (No Growth / MIC)
CE1 (Chyavanprash)	0.05	++++ (Heavy Growth)
CE2 (Chyavanprash)	0.10	++ (Moderate Growth)
CE3 (Chyavanprash)	0.20	- (No Growth / MIC)
TF1 (Tablet F05)	0.05	++ (Moderate Growth)
TF2 (Tablet F05)	0.10	- (No Growth / MIC)
TF3 (Tablet F05)	0.20	- (No Growth)
GF1 (Granules F06)	0.05	++ (Moderate Growth)
GF2 (Granules F06)	0.10	- (No Growth / MIC)
GF3 (Granules F06)	0.20	- (No Growth)

4. CONCLUSION

The present study successfully evaluated the in vitro antioxidant capacity and antimicrobial efficacy of polyherbal immunomodulatory formulations. Formulating Amla and Chyavanprash extracts together with Marking Nut tree extract (Tablet F05) or Gorakhmundi extract (Granules F06) produced marked synergistic enhancement in both antioxidant potential (FRAP > 60 µmol TE/g) and antibacterial activity (ZOI up to 27 mm; MIC of 100 µg/mL against Gram-negative Enterobacteriaceae). These findings demonstrate the therapeutic superiority of polyherbal dosage forms over individual botanical extracts, supporting their potential application as effective immunomodulatory and antimicrobial remedies.

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AUTHORS' CONTRIBUTIONS

T. Vyas conceptualized the study, performed experimental assays, and drafted the manuscript. U. Shah and J. Patel assisted in study design and data interpretation. M. Tiwari contributed to technical review and manuscript editing. All authors approved the final version.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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