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Original Research Article

Integrative chromatographic and spectrophotometric analysis of the phytoconstituents, microbial limit test and antioxidant activity of a polyherbal immune booster

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ABSTRACT

Despite the widespread use of herbal immune boosters in Nigeria, many lack rigorous quality evaluation to ensure consumer safety. As data on the quality and safety of many marketed polyherbal products remain scarce, this study aimed to evaluate BDP, a polyherbal immune-booster containing *Plukenetia conophora* Müll. Arg. (Euphorbiaceae) and *Terminalia catappa* L. (Combretacea), following pharmacopoeial standards. Qualitative phytochemical screening, spectrophotometric quantification, and determination of physicochemical parameters were performed. Bioactive compounds and vitamins were evaluated using High-Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS). Antioxidant potential was determined via 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and total antioxidant capacity (TAC) assays. Total aerobic microbial and total yeast/mould counts were also determined. Phytochemical analysis revealed phenolics (47.506 mg/g), steroids (45.469 mg/g), and flavonoids (14.097 mg/g). Total ash (8.37%), acid-insoluble (2.67%), and moisture content (7.80%) indicated acceptable purity and stability. The main compounds detected in the GC-MS analysis were Linolenic acid (69.73%), Stearic acid (5.46%), Linolenic acid methyl ester (4.86%), (Z)-3-(Heptadec-10-en-1-yl) phenol (3.95%), and Palmitic acid (2.99%). HPLC analysis showed high levels of quercetin, gallic acid, and luteolin, along with vitamins B3, B6, B9, and C, and the extract demonstrated potent concentration-dependent antioxidant activity. The microbiological results complied with British Pharmacopoeia limits. Findings from this study confirm that the polyherbal formulation meets several quality standards and shows significant antioxidant activity. Physicochemical and microbial parameters were also within the acceptable limits. However, further investigations are required to concretely establish the safety of this product.

Keywords: Polyherbal formulation, Immune booster, Standardisation, High Performance Liquid Chromatography, Gas Chromatography-Mass Spectrometry, Antioxidant activity

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Introduction

Herbal remedies are often composed of multiple plant components that act synergistically to enhance therapeutic efficacy.[1] With an estimated 80% of the population in developing countries relying on herbal medicinal products as their primary source of healthcare, the global use of herbal medicines is increasing rapidly.[2] This growth is largely driven by their perceived safety, accessibility, and efficacy, with plant-based immune boosters garnering significant attention following the COVID-19 pandemic.[3,4] Unlike conventional drugs, herbal products often lack consistent quality and standardisation due to variations in cultivation, collection, and processing.[5] Effective regulatory frameworks are therefore necessary to guarantee public health safety, as microbial and chemical contaminants pose serious health risks.[6]

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The World Health Organisation (WHO) has established essential quality control parameters, including ash content and microbial load assessments, to ensure product purity and safety.[7]

The commercial polyherbal product, BDP, evaluated in this study, is marketed as an immune booster containing *Plukenetia conophora* Müll. Arg. (Euphorbiaceae) and *Terminalia catappa* L. (Combretaceae). While these plants are recognised in traditional medicine for their antioxidant, anti-inflammatory, and immunomodulatory activities, limited scientific data exist on the safety profile and phytochemical consistency of the combined formulation.[8,9] Many polyherbal formulations on the market are produced with limited regulatory oversight,[5] leading to variability in composition and the potential for contamination. This lack of standardisation can lead to inconsistent therapeutic effects or adverse events.

The increasing reliance on herbal medicines necessitates rigorous scientific evaluation to ensure they meet established pharmacopoeial standards. However, comprehensive data linking the phytochemical composition, physicochemical quality, antioxidant activity, and microbial safety of commercially marketed polyherbal immune boosters such as BDP remain limited. By utilising validated techniques such as HPLC and GC-MS, this study aimed to establish a comprehensive quality and safety profile for the product. Consequently, the specific objectives were to identify and quantify the bioactive constituents, assess physicochemical parameters (ash and moisture levels), and evaluate the antioxidant capacity and microbial purity of BDP, to ensure its regulatory compliance and consumer safety.

Materials and Methods

Materials and Reagents

The powdered formulation (BDP), assigned the sample code BDP for this study, consisting of *Plukenetia conophora* and *Terminalia catappa*, prepared under a standard operating procedure by Hakmaz Superior Global Resources Ltd., 20, Ose-Olorun Street, Alagbado, Lagos State, Nigeria, was obtained on February 26, 2025. Analytical-grade solvents, including ethanol, methanol, chloroform, and ethyl acetate, were obtained from BDH (UK). Microbiological media, including Sabouraud Dextrose Agar (SDA), Tryptone Soya Agar (TSA), and MacConkey Agar, were sourced from Himedia Laboratories (India) and Oxoid (UK).

Extraction and Preparation of Samples

BDP powder (50 g) was extracted by maceration using 500 mL of 99% ethanol for 72 hours with intermittent agitation. The mixture was filtered using a filter cloth and subsequently with Whatman No. 42 filter paper. The filtrate was concentrated using a water bath at 42.5 ℃ to obtain the crude extract. The extractive value was calculated as a percentage of the starting material.

$$Extractive\ value(\%w/w) = \frac{weight\ of\ dry\ extract}{weight\ of\ powder\ sample} * 100$$

The extract was used for qualitative screening and quantification of phytoconstituents.

Qualitative Phytochemical Screening

The crude extract was screened for secondary metabolites using standard procedures described by Sofowora.[10] This included the Frothing test for saponins, Ferric chloride test for tannins, Lead acetate test for phenolics, and Fehling's test for reducing sugars. Specific tests for anthraquinones (Modified Borntrager's), cardiac glycosides (Keller Killiani), flavonoids, and steroids (Salkowski's). Tests for alkaloids (Dragendorff's, Mayer's, and Wagner's) were also performed, using the leaf of *Datura stramonium* L. (Solanaceae) and root bark of *Fagara zanthoxyloides* Lam. (Rutaceae) as positive control.

Quantification of Phytoconstituents

Total phenolic content (TPC) and total flavonoid content (TFC) were determined spectrophotometrically. TPC was measured at 765 nm using the Folin-Ciocalteu method, with gallic acid as the standard, while TFC was determined at 417 nm using the aluminium chloride method, with rutin as the standard.[11,12] Total steroid content (TSC) was quantified at 620 nm using the Liebermann-Burchard reagent, with cholesterol as a standard.[13]

Physicochemical Analysis

The physicochemical parameters of BDP, including moisture content, extractive yields (ethanol and water), total ash, acid-insoluble ash, and water-soluble ash, were determined in accordance with the standard methods for the quality control of medicinal plant materials as established by the World Health Organisation (WHO).[14] All analyses were performed in triplicate.

GC-MS Analysis

Samples were analysed using an Agilent 8860 Gas Chromatograph equipped with a 5977B Mass Spectrometry Detector (MSD), fitted with an Elite-5MS (5% diphenyl/95% dimethyl polysiloxane) fused capillary column (30 × 0.25 µm ID × 0.25 µm df). For GC-MS detection, an electron ionisation system was operated in electron impact mode at 70 eV. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 µL was used (split ratio of 10:1).

The injector temperature was maintained at 300 °C, the ion-source temperature was 250 °C, and the GC oven temperature was programmed from 110 °C (1 min), then ramped at 150 °C/min to 310 °C (2min). Mass spectra were obtained at 70 eV, with a scan interval of 0.5 s and fragment ions from 45 to 450 Da. The solvent delay was 0-3 min.

HPLC Quantification of flavonoids and phenolics

A methanolic extract of the sample was prepared by weighing 500 mg of the powdered material and extracting it in 5 mL of analytical-grade methanol. The mixture was transferred to a sample vial and sonicated for 10 minutes to ensure complete extraction. A 1:2 dilution of the extract was then prepared using 2 mL of methanol to obtain a final concentration of 50,000 µg/mL. The resulting solution was filtered through a 0.45 µm Millipore filter into an HPLC vial before analysis. HPLC analysis was performed using an Agilent Eclipse XDB C18 column (150 × 4.6 mm, 5 µm particle size) at ambient temperature. The mobile phase consisted of methanol:acetonitrile:water (40:15:45) containing 1% acetic acid, delivered at a flow rate of 1.0 mL/min. An injection volume of 10 µL was used for both samples and standards. Two injections were used for the samples, and one injection each for the standards. Detection was performed at 257 nm. For calibration, a mixed standard solution containing quercetin, gallic acid, luteolin, and formononetin was prepared at concentrations of 10, 20, 40, 60, 80, and 100 µg/mL. The concentrations of flavonoids and phenolics in the sample extract were quantified using the equation of the calibration curve: $y = mx + c$

HPLC Analysis of water-soluble vitamins

250 mg of the sample was extracted with 5 mL of analytical-grade methanol. The extract was filtered through a 0.45 µm filter and used directly for HPLC analysis. Chromatographic separation was performed using an Agilent Zorbax SB-C8 column (150 × 4.6 mm, 5 µm particle size) at ambient temperature. The mobile phase consisted of methanol: 10 mM sodium hexanesulfonate with 0.1% phosphoric acid (26:74, v/v), delivered at a flow rate of 1.0 mL/min. An injection volume of 20 µL was used, and detection was performed at 245 nm. A mixed standard solution containing vitamins B1, B3, B6, B9, and C at 200 µg/mL each was used to identify vitamins in the sample by comparing the sample peaks' retention times with those of the standards.

Antioxidant Capacity Assays

The antioxidant potential of BDP was evaluated using the DPPH radical scavenging method at 517 nm. The free radical scavenging activity of BDP was evaluated using ascorbic acid as the reference standard according to the method of Blois.[15] The assay relies on the reduction of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, which is a stable, dark-purple free radical by an antioxidant to its non-radical form (1,1-diphenyl-2-picrylhydrazine), resulting in a visible colour change from deep purple to pale yellow. The total antioxidant capacity (TAC) of the BDP was determined at 765 nm, using the Phosphomolybdenum method as described by Prieto *et al*.[16] The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green Phosphate/Mo (V) complex at acidic pH. Ascorbic acid was used as the reference standard. The ferric reducing antioxidant power (FRAP) was assessed at 593 nm according to the procedure outlined by Benzie and Strain.[17] This method measures the ability of the sample to reduce a ferric tripyridyltriazine complex to its ferrous form, resulting in an intense blue colour measured spectrophotometrically.

Microbiological Evaluation

Microbial quality was assessed in accordance with British Pharmacopoeia standards.[18] Total Aerobic Microbial Count (TAMC) and Total Combined Yeasts and Moulds Count (TYMC) were determined using TSA and SDA media, respectively. Specific pathogens, including *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*, were screened using selective media such as Eosine Methylene Blue (EMB) and Mannitol Salt Agar (MSA).

Statistical Analysis

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Statistical analysis was carried out using Microsoft Excel Version 2108 [Build 14332.20435]. Quantitative values were measured in triplicate. The mean value (X) was recorded as X±SEM, where X = mean and SEM = Standard error of mean.

Results and Discussion

Qualitative phytochemical evaluation identified various phytochemicals of therapeutic importance, including tannins, saponins, flavonoids, terpenoids, reducing sugars, glycosides, steroids, and phenols in the ethanolic extract of BDP (Table 1). This is consistent with existing literature on the phytochemistry of *Plukenetia conophora* and *Terminalia catappa*; however, no alkaloids were detected (Table 1).

Table 1: Phytochemical composition of BDP extract

Phytoconstituent	Result[a]
Saponins	+
Phenolics	+
Tannins	+
Reducing Sugars	+
Combined Anthraquinones	-
Cardiac Glycosides	+
Alkaloids	-
Flavonoids	+
Steroids	+
Triterpenoids	+

[a] Key: -, not detected; +, present.

Table 2: Extractive value of BDP

Extractive value (%w/w)	21.14

Quantitatively, the total flavonoid, phenolic, and steroid concentrations were 14.097 ± 0.860, 47.506 ± 0.121, and 45.469 ± 0.193 mg/g, respectively (Figure 1). Phenolic and flavonoid contents are typically linked to antioxidant activity[19], while steroidal components relate to anti-inflammatory effects, which may contribute to the immune-boosting action of the formulation. Phytosteroids possess various medicinal activities, including anti-tumour, hepatoprotective, and antibacterial properties.[20] Plant-derived phenolics influence the non-specific immune response by enhancing phagocytosis and macrophage proliferation, while flavonoids exert antioxidant and anti-inflammatory effects.[21,22] The calibration curves for rutin, gallic acid, and cholesterol demonstrated excellent linearity (R^2 = 0.9999, 0.9999, and 0.9944, respectively), confirming the validity and precision of the quantitative methods employed.

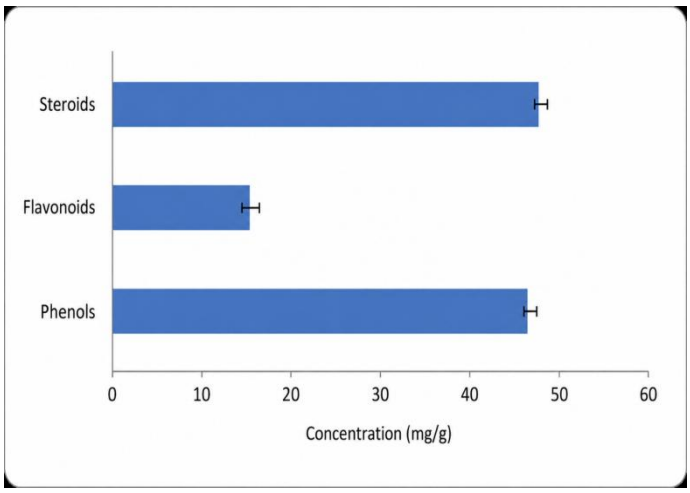

Figure 1: Quantitative comparison of phytochemicals (mg/g) present in BDP sample.

The extractive value of the sample was determined to be 21.14% (Table 2). The total ash (8.37 ± 0.125%), acid-insoluble ash (2.67 ± 0.165%), and water-soluble ash (3.60 ± 0.420%) of BDP show expected levels of intrinsic mineral content and low siliceous contamination (Table 3). Values of acid-insoluble ash under 3–5% indicate minimal soil or sand contamination in well-prepared herbal materials.[23,24] A moisture content of 7.80 ± 0.600% supports product stability and is consistent with the favourable microbial profile obtained. Lower moisture levels are critical for preventing microbial proliferation during storage.[25-27]

The GC–MS of fatty acid methyl esters (FAME) of BDP revealed the presence of several types of fatty acids, such as saturated, monounsaturated, and polyunsaturated fatty acids (PUFA) and their esters, with a high abundance of 9,12,15-octadecatrienoic acid (alpha-linolenic acid, ALA) at 69.73% (Table 4; Figure 2). ALA is an omega-3 fatty acid known to lower blood pressure and exert strong immunomodulatory effects by reducing cytokines such as IL-6 and TNF-alpha.[28,29] The identification of dibutyl phthalate is concerning; as a synthetic plasticiser, its presence likely indicates environmental or laboratory contamination, raising safety concerns regarding potential endocrine disruption.[30,31]

HPLC analysis confirmed quercetin as the most abundant flavonoid (307.87 mug/mL) (Table 5), with a highly linear calibration curve (R^2 = 0.9954). The co-occurrence of polyphenols with detected vitamins B3, B6, B9, and C (Table 6; Figures 3-4) suggests synergistic antioxidant and immunomodulatory effects. The extract demonstrated significant antioxidant activity across DPPH, TAC, and FRAP assays. The IC_{50} for DPPH was 38 mug/mL, indicating superior radical scavenging activity compared to the standard under the assay conditions (Figure 5).

The microbial quality assessment showed a total plate count of 1425 CFU/g and a total yeast and mould count of 3840 CFU/g, as shown in Table 7, both within British Pharmacopoeia limits.[18] The absence of objectionable microorganisms like *E. coli* and *Salmonella* spp. indicates good hygiene during processing, which is essential for consumer protection.[32]

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Table 3: Total, water-soluble ash, acid-insoluble ash, and moisture content of BDP

Parameter	Mean ± SEM (%)
Total ash	8.37 ± 0.125
Water-soluble ash	3.60 ± 0.420
Acid-insoluble ash	2.67 ± 0.165
Moisture content	7.80 ± 0.600

n = 3

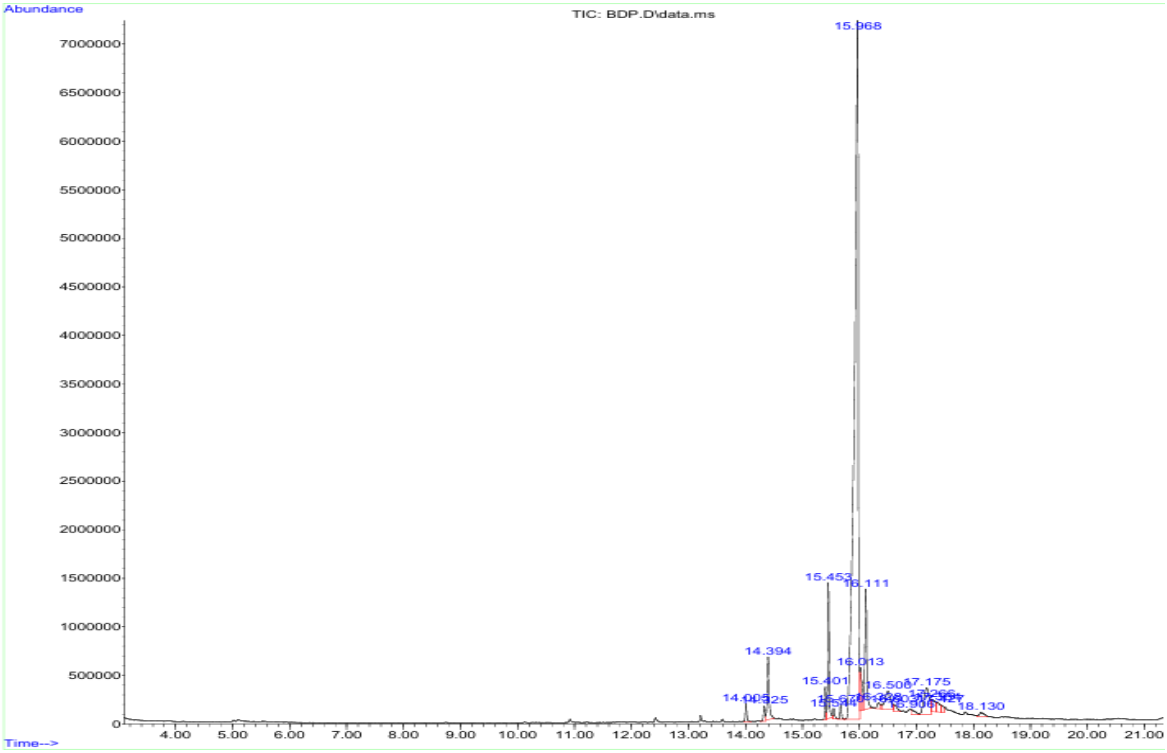

Figure 2: Chromatogram of BDP extract using GC-MS

Table 4: Phytoconstituents detected in the BDP extract using gas chromatography-mass spectrometry

S/N	RT (min)	% Area	Name	Molecular Weight (amu)	Compound nature
1	14.005	0.65	Hexadecanoic acid, methyl ester	270	Fatty acid methyl ester
2	14.325	0.67	Dibutyl phthalate	278	Phthalate (plasticiser, possible contaminant)
3	14.394	2.99	n-Hexadecanoic acid (Palmitic acid)	256	Saturated fatty acid
4	15.401	1.15	9,12-Octadecadienoic acid, methyl ester (Linoleic acid methyl ester)	294	Polyunsaturated fatty acid ester
5	15.453	4.86	9,12,15-Octadecatrienoic acid, methyl ester (Linolenic acid methyl ester)	292	Polyunsaturated fatty acid ester
6	15.544	0.47	Neophytadiene	278	Diterpene hydrocarbon
7	15.670	0.54	Methyl stearate	298	Fatty acid methyl

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					ester
8	15.968	69.73	9,12,15-Octadecatrienoic acid (Linolenic acid)	278	Polyunsaturated fatty acid
9	16.013	2.38	Methyl 8,11,14-heptadecatrienoate	278	Polyunsaturated fatty acid ester
10	16.111	5.46	Octadecanoic acid (Stearic acid)	284	Saturated fatty acid
11	16.328	0.50	Methyl 8,11,14-heptadecatrienoate	278	Polyunsaturated fatty acid ester
12	16.500	2.67	cis,cis,cis-7,10,13-Hexadecatrienal	236	Unsaturated aldehyde
13	16.603	0.54	9,12,15-Octadecatrienoic acid, 1-methylethyl ester	320	Polyunsaturated fatty acid ester
14	16.906	0.45	9,12,15-Octadecatrienoic acid (Linolenic acid)	278	Polyunsaturated fatty acid
15	17.175	3.95	(Z)-3-(Heptadec-10-en-1-yl)phenol	330	Alkylphenol derivative
16	17.266	1.17	(Z)-3-(Heptadec-10-en-1-yl)phenol	330	Alkylphenol derivative
17	17.364	0.71	Phenol, 2-methyl-	108	Phenolic compound
18	17.427	0.56	Ethyl 6,9,12-hexadecatrienoate	292	Fatty acid ester
19	18.130	0.53	cis-Vaccenic acid	282	Monounsaturated fatty acid

Table 5: Peak area and concentration (mg/mL) of flavonoids and phenolic compounds in BDP

Compound	Peak Area (mAU*S) ± SEM	Concentration (µg/mL) ± SEM
Quercetin	8719.33 ± 211.40	307.87 ± 7.465
Gallic Acid	3788.84 ± 60.010	322.40 ± 5.106
Luteolin	7308.18 ± 232.69	270.60 ± 8.616
Formononetin	-	-

n = 2

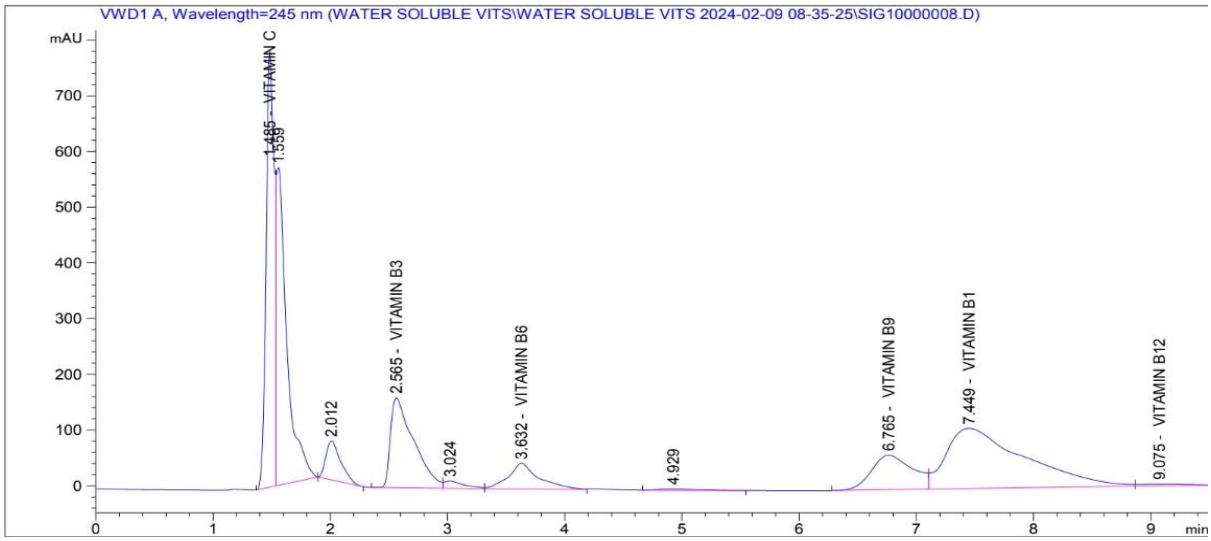

Figure 3: HPLC chromatogram of standard mixture of water-soluble vitamins showing retention times

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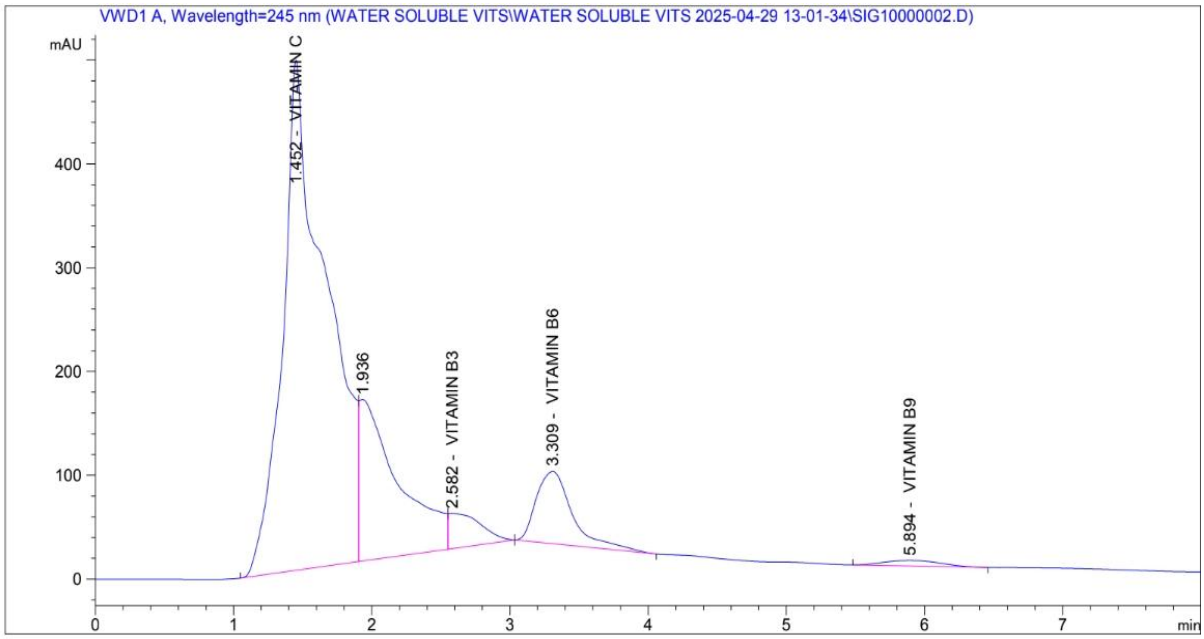

Figure 4: HPLC chromatogram of BDP sample extract showing matching retention times.

Table 6: Comparison of retention times of vitamin standards with those observed in BDP sample (qualitative detection)

Vitamin	Standard Retention Time (min)	Sample Retention Time (min)
B1 (Thiamine)	7.45	-
B3 (Niacin)	2.57	2.58
B6 (Pyridoxine)	3.63	3.31
B9 (Folate)	6.77	5.89
B12 (Cobalamin)	9.08	-
C (Ascorbic acid)	1.49	1.45

Rt = retention time

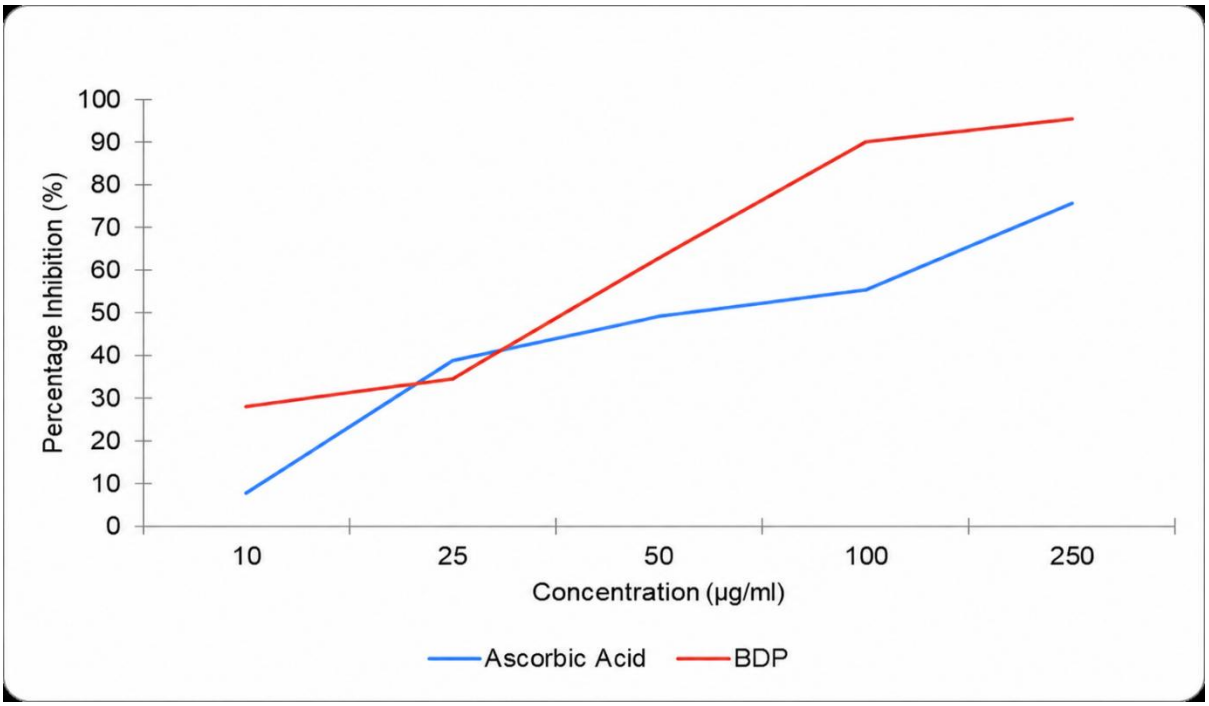

Figure 5: Comparison of DPPH scavenging activity of BDP against ascorbic acid

Table 7: Microbial quality assessment of the sample compared to British Pharmacopoeia (BP) limits

Microbial Parameter	Parametric Count	BP Limit (BP, Appendix XVI G Category C	Unit
Total Plate Count	1425	10^5	CFU/g
Total Yeast and Mold Count	3840	10^4	CFU/g
Bile Tolerant Gram Negative Bacteria	Not detected	10^4	CFU/g
Escherichia coli	Not detected	Absent	CFU/g
Salmonella spp.	Not detected	Absent	CFU/25g
Objectionable Organisms			
Vibrio spp.	Not detected	Absent	CFU/g
Shigella spp.	Not detected	Absent	CFU/g
Pseudomonas aeruginosa	Not detected	Absent	CFU/g
Staphylococcus aureus	Not detected	Absent	CFU/g

Conclusion

This study successfully evaluated the BDP formulation through a series of phytochemical, physicochemical, microbial, and instrumental analyses. Phytochemical chromatographic techniques confirmed the presence of bioactive compounds with potential therapeutic relevance. Physicochemical parameters, including ash values and moisture content, were within acceptable limits for quality control. The microbial assay showed compliance with pharmacopoeial standards, confirming safety against objectionable organisms. Chemical-based antioxidant assays demonstrated significant free radical scavenging activity, supporting the product's functional potential.

Overall, BDP meets several quality standards; however, further assessment and refinement are required to ensure full regulatory compliance and consumer safety. Further pharmacological and toxicological investigations are recommended to better establish the safety profile, therapeutic effectiveness, and mechanisms of action of the formulation. The identified bioactive constituents should be standardised to ensure purity and batch-to-batch consistency, and formulated into suitable dosage forms to improve therapeutic application and patient compliance.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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