

Assessment of Antioxidant Potential and Standardization of Fermented Biomedicine Balarista by High-Performance Thin-Layer Chromatography and Fourier-Transform Infrared Spectroscopy Fingerprint Analysis

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Das *et al.*: High-Performance Thin-Layer Chromatography and Fourier-Transform Infrared study of Balarista formulation

Balarista, a traditional fermented biomedicine, is prescribed for treating rheumatoid arthritis. The present work was undertaken to standardize the Balarista formulation using Fourier-transform infrared fingerprinting and high-performance thin-layer chromatography analysis and evaluate its antioxidant properties. The quality control of the in-house Balarista and marketed formulations was assessed by microbial count, aflatoxin content, high-performance thin-layer chromatography, and Fourier-transform infrared fingerprint analysis. The *in vitro* antioxidant properties of the formulation were studied by 2, 2-diphenyl-1-picrylhydrazyl, 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), hydrogen peroxide, nitric oxide scavenging and reducing power assay. Total bacterial count in in-house Balarista and M1 was found within the standard limit. Aflatoxin content was found less than 5.0 ppb in all formulations. Fourier-transform infrared fingerprint spectra of different fraction of M1 formulation revealed an excellent common peak ratio with in-house Balarista, suggesting the use of prescribed raw materials in the formulations. However, the poor common variation peak ratio with other marketed formulations indicates the use of improper raw materials. The high-performance thin-layer chromatography profile of chloroform fraction of in-house and marketed formulations revealed five spots each while ethyl acetate fraction of in-house Balarista and M3 showed six spots each. The highest 2, 2-diphenyl-1-picrylhydrazyl and nitric oxide radical scavenging activity was achieved by in-house Balarista, 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) by M4, hydrogen peroxide by M1 and reducing power by M2 formulation. The antioxidant activity of the formulation could be due to the presence of considerable amount of phenolic and flavonoid content. The formulation's comprehensive chemical analysis by Fourier-transform infrared and high-performance thin-layer chromatography profile can be used as standards for future reference.

Key words: Balarista, Fourier-transform infrared, High-performance thin-layer chromatography, aflatoxin, microbial count, antioxidant

Ayurveda has been practiced for thousands of years and has been estimated to meet 70 %-80 % of the healthcare needs in India due to its safety and efficacy. As there is increasing demand of Ayurvedic formulations in the present era, pharmacist and physicians must know about the safety and effectiveness of these preparations^[1]. Therefore, quality control tests have been prescribed by Ayurvedic pharmacies to make sure that the prepared formulations meet the

standard limits as mentioned in the Ayurvedic Pharmacopoeia of India (API). Different methods of evaluation mentioned in ancient literature need more scientific validation. Therefore, developing

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quality control parameters using modern analytical techniques to standardize Ayurvedic formulations is of prime importance^[2]. As the Ayurvedic formulations come under the Drugs and Cosmetics Act, there is a need to develop standards for the quality control by the drug control authorities as well as by the manufactures^[3].

Fourier-Transform Infrared Spectroscopy (FTIR) is getting advantages over other methods due to its high resolution, fast measurement, low cost, simultaneous determination of functional groups, phytochemicals, phytoelements and direct measurement of samples without sample preparation. Due to this, there is an increasing demand in agriculture, biology, ecology, biochemistry and medicine^[4]. FTIR spectroscopy has also been found successful in examining pure powdered compounds. But in the past decade, there has been a significant increase in the use of infra-red spectroscopy for the testing of both herbal extract and crude materials. Attenuated Total Reflection-Infrared (ATR-IR) and Near Infrared (NIR) are the most commonly applied techniques for the analysis of herbal medicines^[5]. High-Performance Thin-Layer Chromatography (HPTLC) is the most widely used analytical technique for the identification and quality assessment of herbal materials. Due to its automated procedure and excellent reproducibility of chromatographic conditions, there is an increased demand for HPTLC in standardizing herbal preparations^[6]. HPTLC fingerprints are essential in characterizing multiple compounds in the botanicals, detection of adulterants, and differentiation between closely related species. Analysis of numerous samples in a single assay in the industry is another advantage of HPTLC^[5].

Balarista, a traditional fermented biomedicine, consists of twelve ingredients, among which *Withania somnifera* and *Sida cordifolia* are the most important. This formulation is mainly prescribed for the management of digestive impairment, general weakness, and Rheumatoid Arthritis (RA)^[7]. Marketed herbal formulations are not standardized properly and also not assessed for their quality which leads to several issues^[8]. The quality control of raw materials (macroscopic, microscopic, scanning electron microscopy, physicochemical parameters, Gas Chromatography-Mass Spectrometry, and High-

Performance Layer Chromatography analysis using withanolides and vasicine as marker compounds) used in the preparation of Balarista formulation was carried out in our previous study^[9]. In the present investigation, an In-house Balarista Formulation (IBF) was prepared using various ingredients. The quality control was assessed by FTIR fingerprint and HPTLC analysis and compared with the marketed formulations.

MATERIALS AND METHODS

Plant materials:

The crude drug materials referred to in API were procured from the local market and authenticated by Prof. Dr. Arun Kumar Das, Department of Rasa Sastra Bhaisajya Kalpana, Gopabanhu College of Ayurveda, Puri, Odisha. The prepared herbariums (SPS/SOAU-22-33) were stored in the Department of Pharmacognosy as a reference.

Preparation of Balarista formulation:

Balarista formulation was formulated using the raw materials according to the standard procedure mentioned in API^[10] and referred to as IBF formulation. The root of *Sida cordifolia* (L) and *Withania somnifera* (L Dunal) were the primary ingredients of Balarista formulation, along with minor ingredients such as *Saccharum officinarum* (L), *Woodfordia fruticosa* flower (L) Kurz, *Ipomoea digitata* sub root (L), *Ricinus communis* root (L), *Alpinia galanga* root (L) Willd., *Elettaria cardamom* seed (L) Maton, *Paederia foetida* whole plant (L), *Syzygium aromaticum* flower buds (L) Merr and L. M. Perry, *Vetiveria zizanioids* root (L) Nash and *Tribulus terrestris* fruit (L). Four marketed formulations of different manufacturers were purchased from local markets and coded as M1 (B.N.517244), M2 (B.N.HA-071), M3 (B.N.SB0108) and M4 (B.N. RN-02/12).

Determination of total aerobic microbial count and aflatoxin content:

The total bacterial count was performed as per the method described in API^[11]. The quantitative estimation of total aflatoxin was carried out by using a complete aflatoxin assay kit (Helica Biosystems, USA).

FTIR spectroscopy analysis of formulation:

About 50 ml of IBF and marketed formulations were fractionated with n-hexane, chloroform, and

ethyl acetate in a separating funnel, finally, the remaining portion was filtered and concentrated on getting n-hexane, chloroform, ethyl acetate, and aqueous extract respectively^[12]. The different fractions of formulations were taken for FTIR analysis. All the spectra were recorded in 400 cm^{-1} - 4000 cm^{-1} using an ALPHA II FTIR spectrometer and a platinum ATR unit, using OPUS TOUCH software. The spectra were collected at a resolution of 4 cm^{-1} .

Quantitative evaluation of similarity between IBF and marketed formulation using FTIR spectrum:

The quantitative evaluation of similarity between the different fractions of IBF and marketed formulation in the Fingerprint Spectra (F.P.S) 400 cm^{-1} - 1500 cm^{-1} region was performed by the common peak ratio method described by Zou *et al.*^[13]. IBF was considered as a reference sample. The formulations were arranged according to common peak ratio values from high to low, and the relationship between IBF and marketed formulations was determined quantitatively. The common peak ratio, with a higher common peak ratio, indicates a closer similarity between the IBF and the marketed formulation.

HPTLC analysis:

For HPTLC analysis, pre-coated aluminium plates ($10\times 10\text{ cm}$) of silica gel 60 F₂₅₄ of 0.2 mm thickness were taken and activated at 60° for 5 min. Chloroform and ethyl acetate fractions of IBF and marketed formulations were dissolved in respective solvents and passed through a 0.45 millipore filter. The sample concentration was taken at 10 mg/ml and applied as a band of 10 μl using CAMAG LINOMAT 5 applicator. The plates were developed in the CAMAG glass chamber ($20\times 10\text{ cm}$) saturated with mobile phase, toluene:ethyl acetate:glacial acetic acid (55:45:3) for chloroform fraction, and toluene:ethyl acetate:formic acid (15:4.5:1.5) for ethyl acetate fraction for 20 min. The image of the developed plate was observed under daylight, 254 nm and 366 nm. Methanolic sulphuric acid (10 %) was used for derivatization of plates. The plates were heated at 100° for 3 min till the spots were visible. The developed plates were dried at room temperature and observed under daylight and at 366 nm using CAMAG SCANNER 4. The R_f values were recorded using Vision CATS, version 2.5.18262.1 software.

Determination of total phenolic and flavonoid content:

The IBF and marketed formulations were dried in the water bath to remove the alcohol and water. The obtained solid mass was suspended in methanol, filtered and allowed to dry in the water bath to get the methanol extract of the formulation^[12]. Folin-Ciocalteu reagent was used to determine of total phenolic content^[14]. The gallic acid content was calculated by plotting the standard curve of gallic acid (5-100 μg) and expressed as mg of Gallic Acid Equivalent (GAE) per g of extract. The total flavonoid content was determined following the method of Meda *et al.*^[15]. The content of total flavonoid was estimated from the standard curve of quercetin (5-100 μg) and expressed as mg of Quercetin Equivalent (QE).

Determination of *in vitro* antioxidants:

The methanolic fraction of IBF and marketed formulation (25-400 $\mu\text{g/ml}$) was subjected to 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH)^[16], 2, 2'-Azino-Bis-(3-ethylbenzothiazoline-6-Sulfonic acid) (ABTS)^[17], Hydrogen peroxide (H_2O_2)^[18], nitric oxide^[19] radical scavenging activity and reducing power assay^[17] using ascorbic acid as standard. The total phenolic content and flavonoid content were expressed as milligrams of GAE/g and mg of QE/g respectively.

Calculation of half-maximal Inhibitory Concentration (IC_{50}) value:

It is defined as the concentration of antioxidants required to scavenge 50 % of the free radicals, which was determined by plotting the graph, scavenging activity versus the concentration of formulation^[16].

Statistical analysis:

All the data was performed in triplicate and subjected to statistical analysis by One-way Analysis of Variance (ANOVA), and posthoc Tukey honest significant difference test using Graphpad Prism 6.01 software. The results were expressed as mean \pm standard error mean (SEM). $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Microbial contamination not only affects the chemical composition but also causes a decrease in the therapeutic potency of herbal drugs.

Therefore, the raw materials should be prevented from microbial contamination before processing to formulation^[20]. The total aerobic count of IBF and marketed formulations is depicted in fig. 1 and Table 1. IBF and M1 were found within the pharmacopoeial limit, while M2 and M3 were overloaded with bacteria. M4 formulation was observed just above the pharmacopoeial limit. The total bacterial count in IBF and M1 formulation was found within the API limit. Mycotoxins are the toxic secondary metabolites produced by the eukaryotic organisms' fungi under favorable temperature and humidity conditions. As the herbal drug materials used in the preparation of herbal formulations are mostly contaminated with these fungi due to improper handling, there is a probability of getting contamination with

mycotoxins during pre-harvest or post-harvest^[21]. About 300 mycotoxins, such as aflatoxins, ochratoxins, patulin, zearalenone, fumonisins, and trichothecenes have been identified^[22]. Among these, aflatoxins are produced from *Aspergillus* species, such as *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and *Aspergillus niger*^[23], which is considered as group 1 human carcinogen according to 'The International Agency of Research on Cancer' (IARC)^[24]. The detection of aflatoxin in the herbal materials indicates contamination by fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) which produce aflatoxins. In the present study, the aflatoxin content in IBF, M1, M2, M3 and M4 formulations was found to be less than 5.0 ppb, indicating less contamination of raw materials (Table 1).

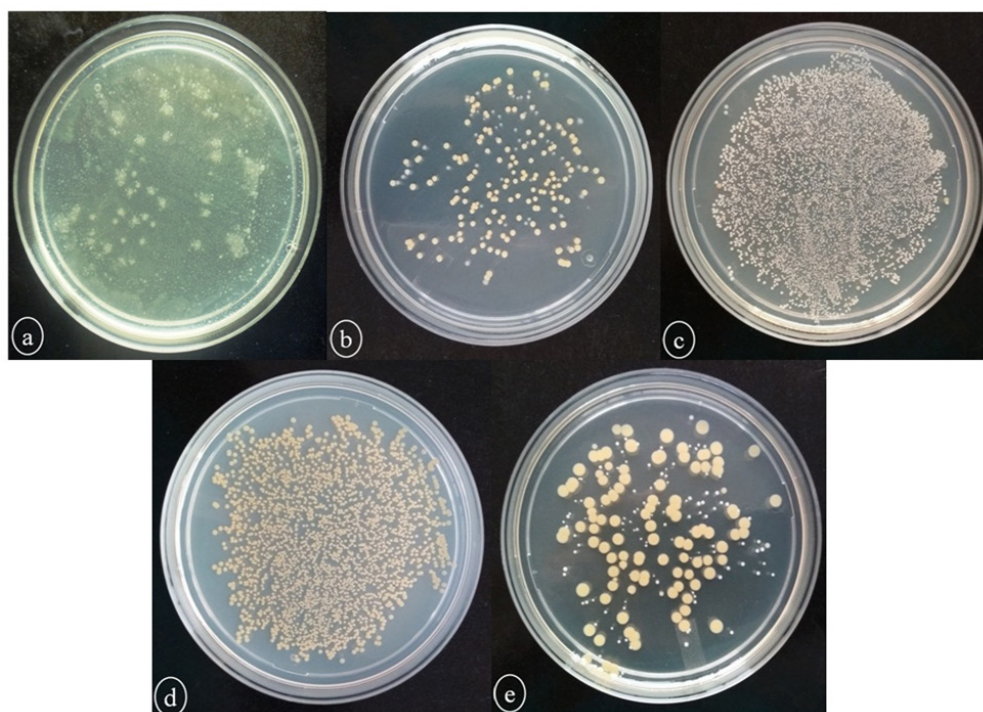


Fig. 1: Total bacterial count, (a) IBF; (b) M1; (c) M2; (d) M3 and (e) M4 formulation

TABLE 4: DETERMINATION OF TOTAL AEROBIC COUNT AND AFLATOXIN CONTENT IN IBF, M1, M2, M3 AND M4 FORMULATION

Formulation	Total aerobic count	Pharmacopoeial limit	Total aflatoxin (ppb)
IBF	12.9×10^7 CFU/ml	Not more than 300 CFU/ml	<5.0
M1	16.8×10^7 CFU/ml	Not more than 300 CFU/ml	<5.0
M2	81.9×10^7 CFU/ml	Not more than 300 CFU/ml	<5.0
M3	60.1×10^7 CFU/ml	Not more than 300 CFU/ml	<5.0
M4	33.3×10^7 CFU/ml	Not more than 300 CFU/ml	<5.0

Note: CFU: Colony Forming Unit and PPB: Parts Per Billion

FTIR spectral analysis of n-hexane fraction of formulations presented in fig. 2. In IBF, M1 formulations, the aliphatic primary amine was identified by the absorbance peak at 3390.21 and 3394.26 cm^{-1} , which represented N-H stretching vibration. The absorbance peak at 2930.27, 2933.77 cm^{-1} due to asymmetric C-H stretching vibration signified the occurrence of an acyclic compound in IBF, M1 formulations. The strong C=O stretching vibration at peaks 1707.85, 1710.01 cm^{-1} revealed a carboxylic group in IBF and M1 formulation. The cyanovinyl group, due to strong C=C stretching vibration at peak 1604.00, 1603.52 cm^{-1} appeared in IBF and M1 formulation. The strong asymmetric NO₂ stretching vibration at peak 1511.29, 1513.03 cm^{-1} portrayed α , β -unsaturated nitro compounds in IBF, M1 formulation. The absorbance peak at 1455.27, 1455.70, 1452.12 cm^{-1} due to N=N stretching vibration observed azothio compound in IBF, M1, M3 formulation. Due to C-H deformation

vibration at peak 1374.28, 1371.10 cm^{-1} , the aldehyde group was found in IBF, M1 formulation. The C-H symmetric deformation vibration at peaks 1269.74, 1271.78, and 1259.87 cm^{-1} implied methyl silane in IBF, M1, and M3 formulations. The CH₃ rocking vibration at peak 1170.37, 1171.96, 1176.64, 1177.50, 1177.59 cm^{-1} portrayed 1-methoxy phosphane group in IBF, M1, M2, M3, M4 formulations. The absorbance peak at 1113.62, 1113.81, 1117.34, and 1120.82 cm^{-1} due to strong C-O stretching vibration depicted secondary alcohol in IBF, M1, M2, and M4 formulations. The primary aliphatic amine due to C-N stretching vibration at 1032.30, 1032.78, 1025.92, 1023.80, 1021.63 cm^{-1} was noticed in IBF, M1, M2, M3, and M4 formulation. The sharp and strong absorbance peak at 690.82, 690.59, 698.48, 702.62, 698.61 cm^{-1} due to ring deformation vibration implied the presence of 1-alkylbenzene in IBF, M1, M2, M3, M4 formulation.

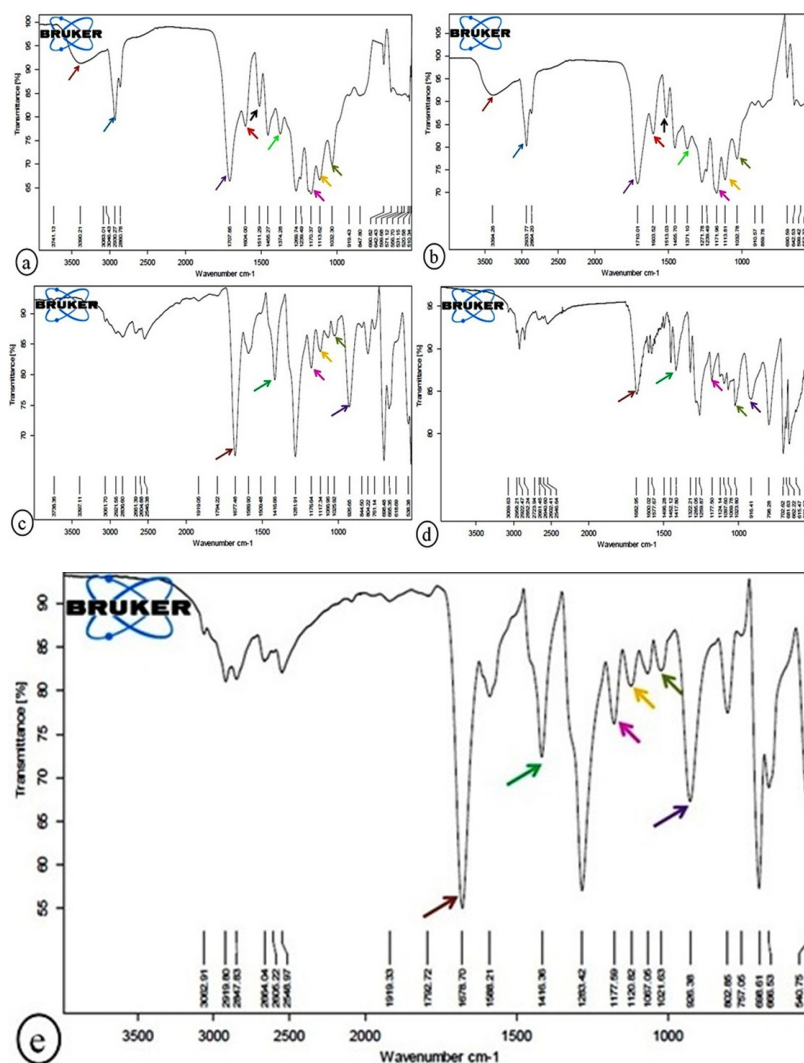


Fig. 2: FTIR spectral analysis of n-hexane fraction, (a) IBF; (b) M1; (c) M2; (d) M3 and (e) M4 formulation

FTIR spectral analysis of chloroform fraction of formulation (fig. 3) revealed the strong C=O stretching vibration at absorption band 1673.24, 1689.10 1682.44, 1676.51-1 cm displayed ketone group in IBF, M2, M3, and M4 formulation. Due to N-O stretching vibration, the absorbance band at 1519.21, 1514.26, 1512.75, 1517.49 and 1520.75 cm^{-1} revealed nitro compound in IBF, M1, M2, M3, and M4 formulation. The vinyl ether group due to C-O stretching vibration at 1213.89, 1213.53, 1215.57, 1217.83, 1215.34 cm^{-1} was depicted in

IBF, M1, M2, M3 and M4. The absorption peak at 1022.61, 1027.24, 1022.91, 1023.32, 1021.32 cm^{-1} due to C-N stretching vibration revealed primary aliphatic amine in IBF, M1, M2, M3, M4 formulations. The observed secondary amine in IBF, M1, M2 and M4 formulations was due to strong N-H wagging vibrations at 741.20, 746.11 and 742.85, 750.48 cm^{-1} . The display of aliphatic aldehyde compound in IBF, M1, M2, M3, M4 formulations was due to C-C-CO wagging vibration at 668.91, 668.32, 669.54, 670.57, 666.30 cm^{-1} .

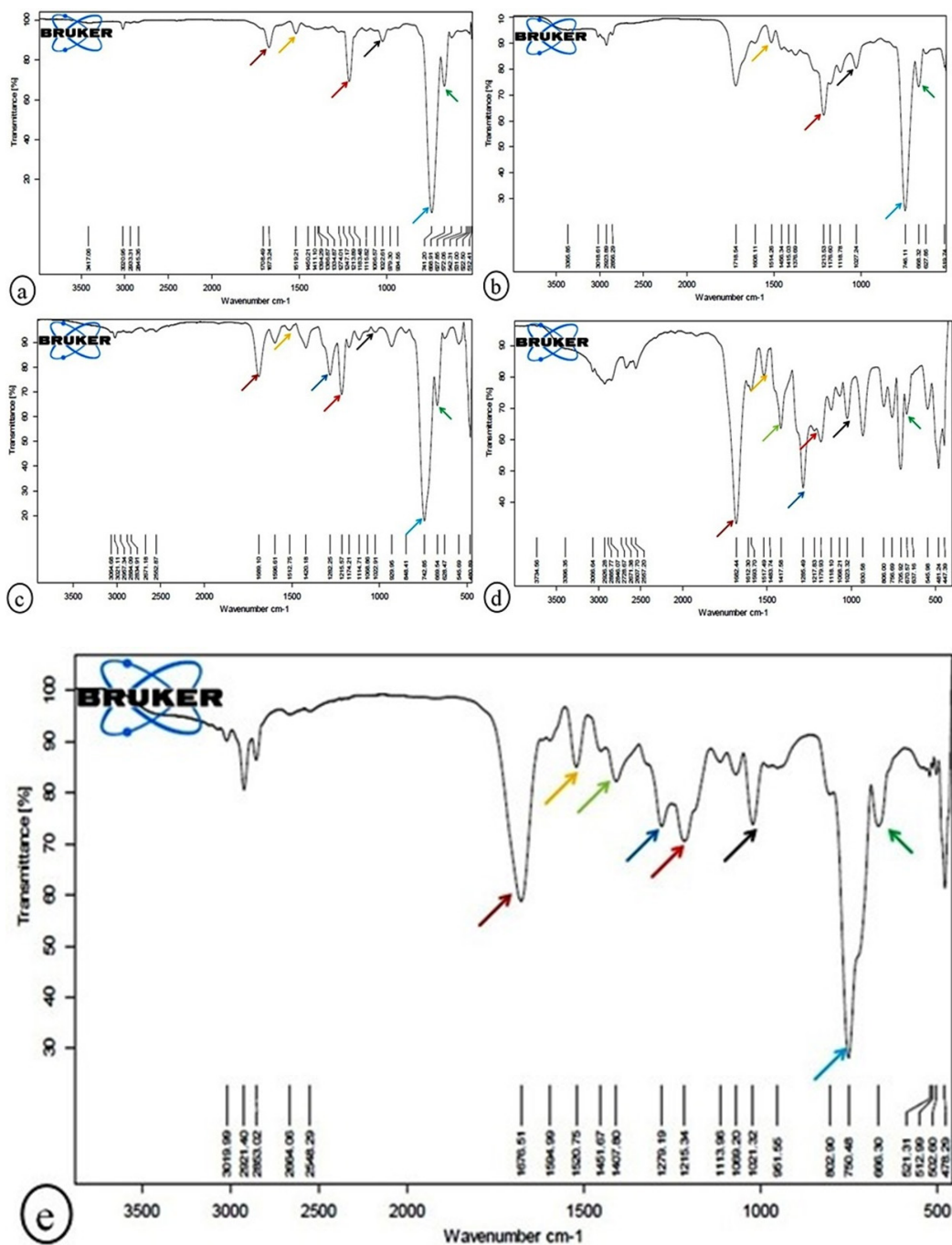


Fig. 3: FTIR spectral analysis of chloroform fraction, (a) IBF; (b) M1; (c) M2; (d) M3 and (e) M4 formulation

Fig. 4 displayed the FTIR spectral study of the ethyl acetate fraction of formulation. The absorption band at 3326.31, 3331.77, 3325.12, 3325.29, 3327.92 cm^{-1} due to the strong and broad O-H stretching vibration represented alcohol group in IBF, M1, M2, M3, M4 formulations. The 1-methyl argon group in IBF, M1, M2, M3, M4 formulations was due to C-H asymmetric stretching vibration at 2943.76, 2945.07, 2942.25, 2943.25 and 2942.18 cm^{-1} . The sharp C-H symmetric stretching vibration at 2832.93, 2834.60, 2832.34, 2832.35, and 2832.70 cm^{-1} depicted ether group in IBF, M1, M2, M3, M4 formulations. The ethylene group in IBF, M1, M2, M3, and M4 formulation was due to C=C stretching vibration at peak 1655.79,

1647.39, 1659.63, 1659.29, 1660.43 cm^{-1} . The N=N stretching vibration at wave length 1451.68, 1451.04, 1450.38, 1450.13, 1449.74 cm^{-1} resulted azothio compound in IBF, M1, M2, M3, M4 formulations. C-H in-plane deformation vibration at 1409.09, 1407.74, 1410.65, 1411.37, and 1411.42 cm^{-1} displayed vinylene in IBF, M1, M2, M3, M4 formulations. Due to C-O stretching vibration, the absorption band at 1121.87, 1121.41, 1114.93, 1115.41, and 1112.25 cm^{-1} revealed saturated secondary alcohol in IBF, M1, M2, M3, and M4 formulations. The presence of monothio ester group in IBF, M1, M2, M3, M4 formulations was due to strong C-S stretching vibration at 1018.22, 1017.85, 1018.97, 1018.89, 1018.99 cm^{-1} .

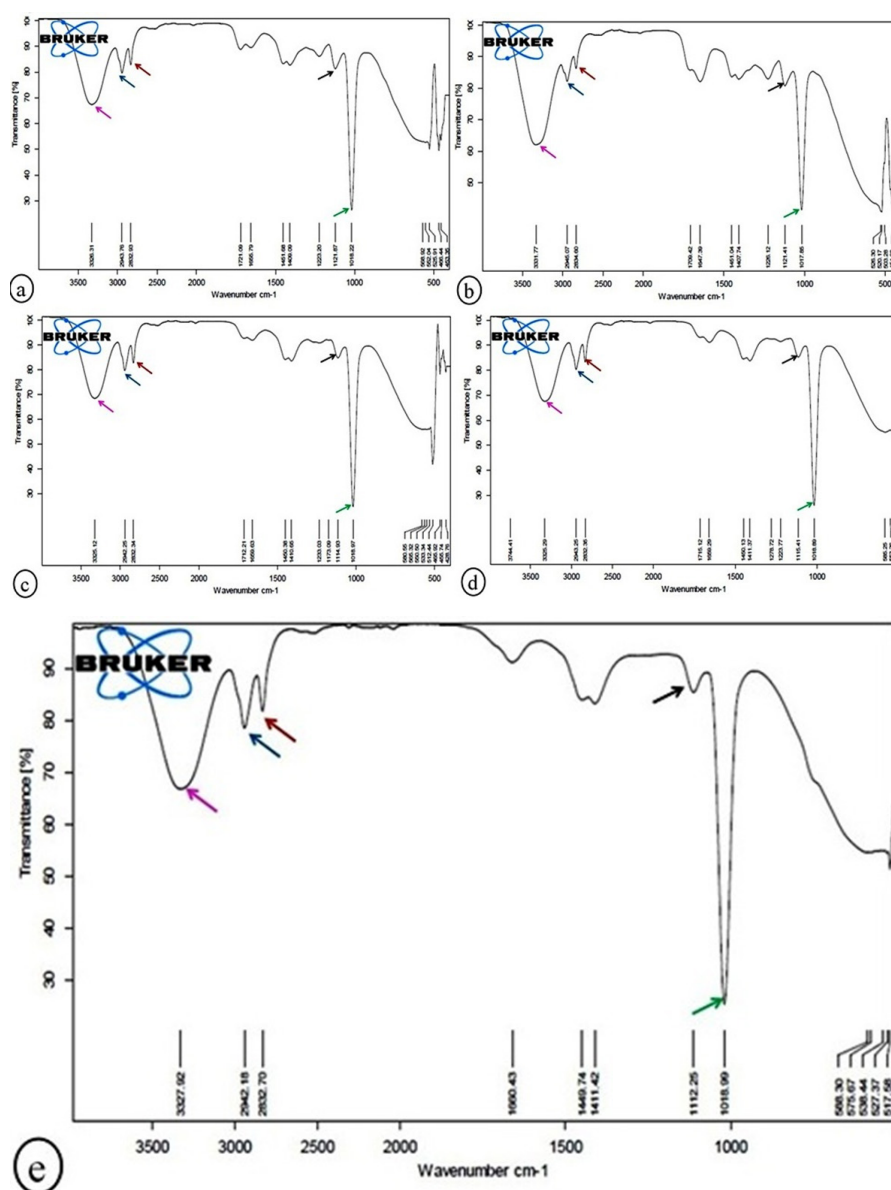


Fig. 4: FTIR spectral analysis of ethyl acetate fraction, (a) IBF; (b) M1; (c) M2; (d) M3 and (e) M4 formulation

FTIR analysis of the aqueous fraction of formulation was given in fig. 5. The absorption peak at 3271.65, 3281.43, 3274.25, 3283.83, and 3264.27 cm^{-1} represented alcohol in IBF, M1, M2, M3, and M4 formulation due to the strong and broad O-H stretching vibration. The absorption band at 1635.33, 1634.62, 1636.09, and 1639.99 cm^{-1} implied an alkene group in IBF, M1, M3, and M4 formulations due to C=C stretching vibration. C-H rocking vibration at 1249.55, 1240.47, 1249.77, 1243.08, and 1252.66 cm^{-1} presented vinyl groups in IBF, M1, M2, M3, and M4. Strong C-O stretching vibration at 1020.91, 1025.77, 1028.17, 1031.82, 1032.21 cm^{-1} revealed acyclic secondary alcohol group in IBF, M1, M2, M3, and M4 formulation.

The absorbance of IR frequency depends upon the chemical nature of the sample. It thus helps in the identification of functional groups (e.g., carboxylic acid, aldehyde, amines, alcohol, etc.) and compounds^[25]. The present study recorded FTIR spectra in the fingerprint region, 400-1500 cm^{-1} , to examine the chemical compositions of different fractions of IBF, M1, M2, M3, and M4 formulations. The common variation peak ratios were performed between IBF and marketed formulations to mark the characteristic peaks and check their presence in the marketed formulations. For this, some sharp, intense, and well-defined peaks in FTIR FPS were selected from each fraction of IBF formulation to characterize their presence in the respective fractions of marketed formulations (Table 2).

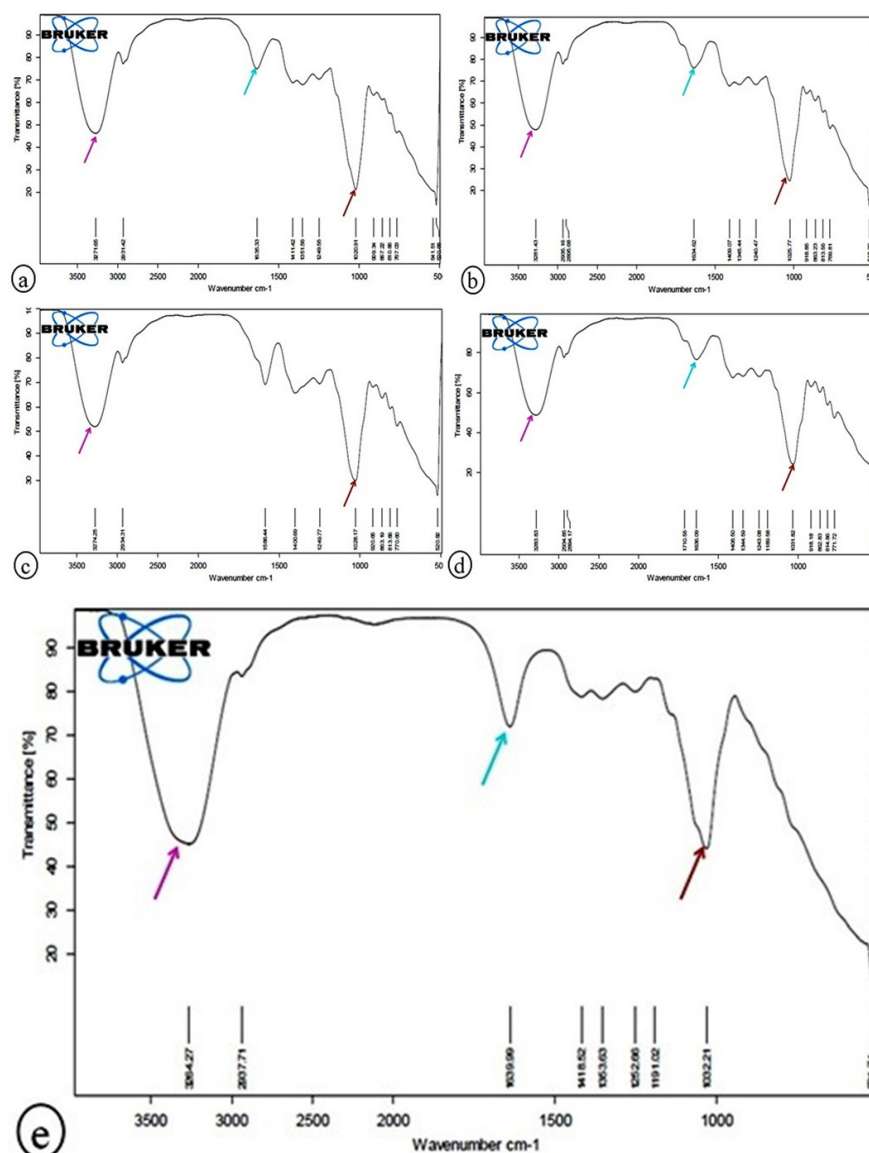


Fig. 5: FTIR spectral analysis of aqueous extract, (a) IBF; (b) M1; (c) M2; (d) M3 and (e) M4 formulation

TABLE 2: SPECIFIC PEAKS IN FOURIER TRANSFORM INFRARED-FINGERPRINT SPECTRA OF DIFFERENT FRACTIONS OF IBF, M1, M2, M3 AND M4 FORMULATIONS FOR THEIR CHARACTERIZATION WITH CORRESPONDING PEAK RATIO

Fraction	Formulation	Peak (cm ⁻¹)	Peak ratio of marketed formulation
n-Hexane	IBF	1455.27, 1374.28, 1269.74, 1239.49, 1170.37, 1113.62, 1032.30, 919.43, 847.80, 690.82, 642.43, 531.15, 520.58, 510.34	
	M1	1455.70, 1371.10, 1271.78, 1213.49, 1171.96, 1113.81, 1032.78, 910.57, 690.59, 642.53, 529.66	55
	M2	1117.34, 844.50, 698.48, 665.35	14.81
	M3	1452.12, 1259.87, 1023.80, 702.62, 526.07, 517.63	20
	M4	1021.63, 926.38, 698.61, 666.53, 540.75, 522.48, 1177.59	30.43
Chloroform	IBF	1450.21, 1411.10, 1394.29, 1274.01, 1213.89, 1183.48, 1115.82, 1066.57, 1022.61, 979.30, 741.20, 668.91, 627.85, 542.31, 522.50, 512.41	
	M1	1456.34, 1415.03, 1376.69, 1213.53, 1176.60, 1118.78, 1027.24, 746.11, 668.32, 627.85, 519.74	50
	M2	1420.18, 1282.25, 1215.57, 1114.71, 1068.86, 1022.91, 742.85, 669.54, 628.47, 545.69	38.46
	M3	1217.83, 1068.21, 1023.32, 670.57, 637.16	14.7
	M4	1451.67, 1215.34, 1113.96, 1069.20, 1021.32, 951.55, 750.48, 666.30, 521.31, 512.99	37.03
Ethyl acetate	IBF	1451.68, 1409.09, 1121.87, 1018.22, 525.91, 466.44	
	M1	1451.04, 1407.74, 1121.41, 1017.85, 441.88	33.33
	M2	1450.38, 1410.65, 1114.93, 1018.97, 512.44	26.31
	M3	1450.13, 1411.37, 1115.41, 1018.89	28.57
	M4	1449.74, 1411.42, 1112.25, 1018.99	28.57
Aqueous	IBF	1411.42, 1351.56, 1249.55, 1020.91, 909.34, 857.22, 810.66, 767.72	
	M1	1409.07, 1345.44, 1240.47, 1025.77, 918.85, 863.23, 813.55, 769.81	80
	M2	1400.69, 1249.77, 1028.17, 920.65, 863.19, 813.86, 770.60	70
	M3	1408.50, 1344.59, 1243.08, 1031.82, 918.18, 862.83, 814.86, 771.72	72.72
	M4	1418.52, 1353.63, 1252.66, 1032.21	36.36

FTIR spectral analysis revealed the differences in the absorbance between the same fractions of the different formulations, which could result from using different raw materials or adding organic substances by different manufacturers^[26]. It is observed that, the intensity and the area of these characteristic peaks in the same fraction obtained from different formulation showed significant changes, however, their peak positions remained unaltered. This study revealed that all fractions except ethyl acetate of the M1 showed a very good common variation peak ratio with IBF formulation

(Table 2). The strong common variation peak ratio observed with M1 formulation suggests using of prescribed raw materials in the formulation. However, poor common variation peak ratios were noticed in M2, M3 and M4 formulation. This confirms that although the label of all marketed formulations represents the ingredient as per API, but the lower common variation peak ratio indicates that there may be use of improper raw materials or different methods of preparation in the formulation, which were responsible for new peaks or a shift in peaks, giving rise to a poor

common variation peak ratio. Thus, the common peak ratio could be used to judge the quantitative similarity and quality control of the formulations.

The photo images of the developed HPTLC chromatogram of chloroform and ethyl acetate fraction of IBF, M1, M2, M3, and M4 formulation were taken under daylight, at 254 and 366 nm before and after derivatization with 10 % methanolic sulphuric acid (fig. 6). The HPTLC fingerprint profile of chloroform fraction of IBF, M1, M2, M3 and M4 formulation after post derivatization revealed five spots each. Few spots were found common between the formulations; R_f 0.22 in M1, M2, and M3; R_f 0.32 in M1 and M3; R_f 0.23 in IBF and M4; R_f 0.41 in IBF, M1, and M4; R_f 0.89 in IBF, M1, and M2. From this result, it was observed that spots with R_f values of 0.23, 0.41, and 0.89 in the IBF formulation were found in the marketed formulation, indicating the presence of these constituents in the marketed preparation. However, the R_f value of 0.09 and 0.49 did not notice in any marketed formulations. Similarly, the ethyl acetate fraction of IBF and M3 showed six spots each, M1 displayed four spots, and M2 and

M4 revealed three. Spot with R_f 0.02 was found common in M1, M2, and M3; R_f 0.08 in M1 and M3; R_f 0.14 in IBF and M4; R_f 0.58 in IBF and M3. All marketed formulations except IBF were noticed with a common R_f value 0.19. R_f values 0.20, 0.33, 0.42 and 0.93 of IBF formulations did not found in marketed formulations.

Phenolic compounds distributed in plants are given much importance due to their antioxidant and free radical scavenging properties^[27]. This activity is believed to be mainly due to their redox property, which plays an important role in adsorbing and neutralizing the free radicals, quenching of singlet and triplet oxygen or decomposing the peroxide and act as reducing agent, hydrogen donors and also have metal chelating properties^[28,29]. Flavonoids are the most important natural phenolic compounds, which possess a broad spectrum of chemical and biological activities, including free radical scavenging properties^[30]. The total phenolic and flavonoid content of IBF, M1, M2, M3 and M4 was determined by UV spectroscopy (fig. 7). The, high phenolic content in IBF and flavonoid content in M1 might be responsible for its good antioxidant activity.

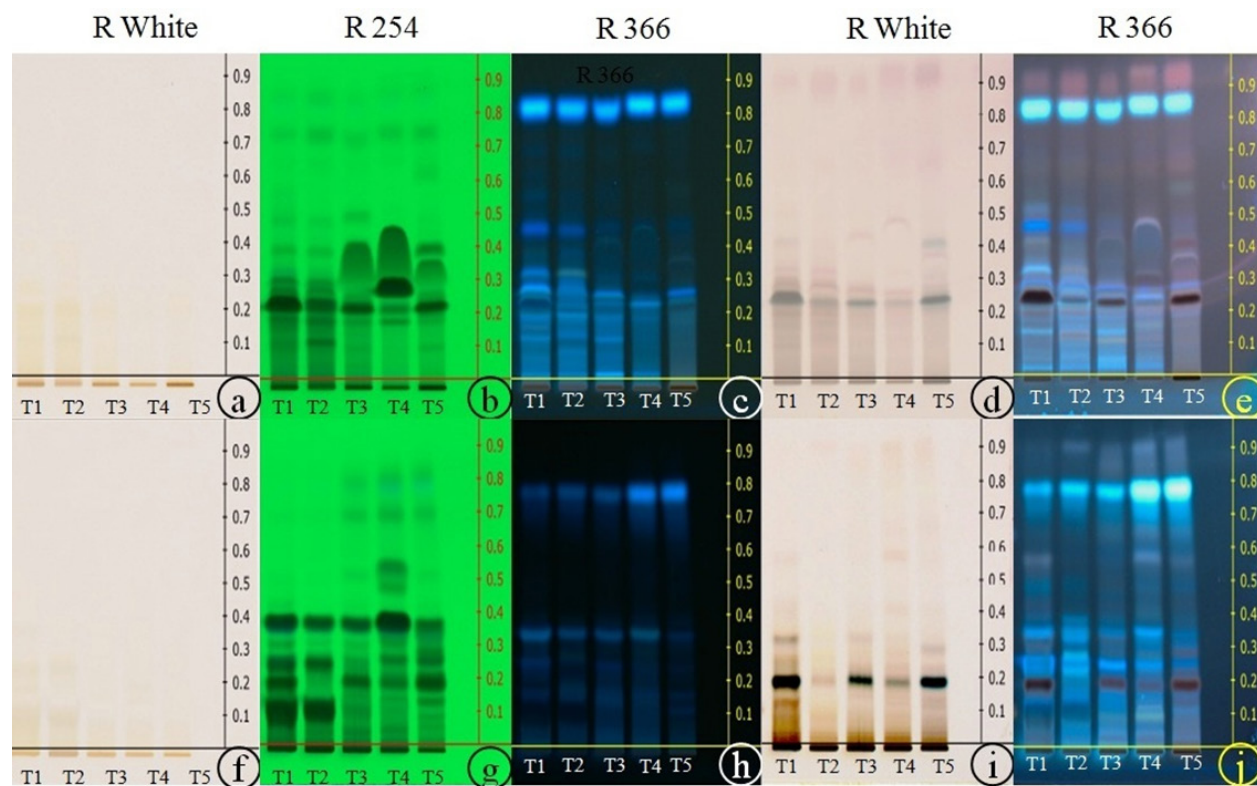


Fig. 6: Photo image of developed HPTLC plate of chloroform fraction, (a) IBF; (b) M1; (c) M2; (d) M3; (e) M4 and ethyl acetate fraction of; (f) IBF; (g) M1; (h) M2; (i) M3 and (j) M4 balarista formulation before derivatization under daylight, 254 nm, 366 nm and; after derivatization under daylight and 366 nm

Note: T: Track (T1: IBF, T2: M1, T3: M2, T4: M3, T5: M4)

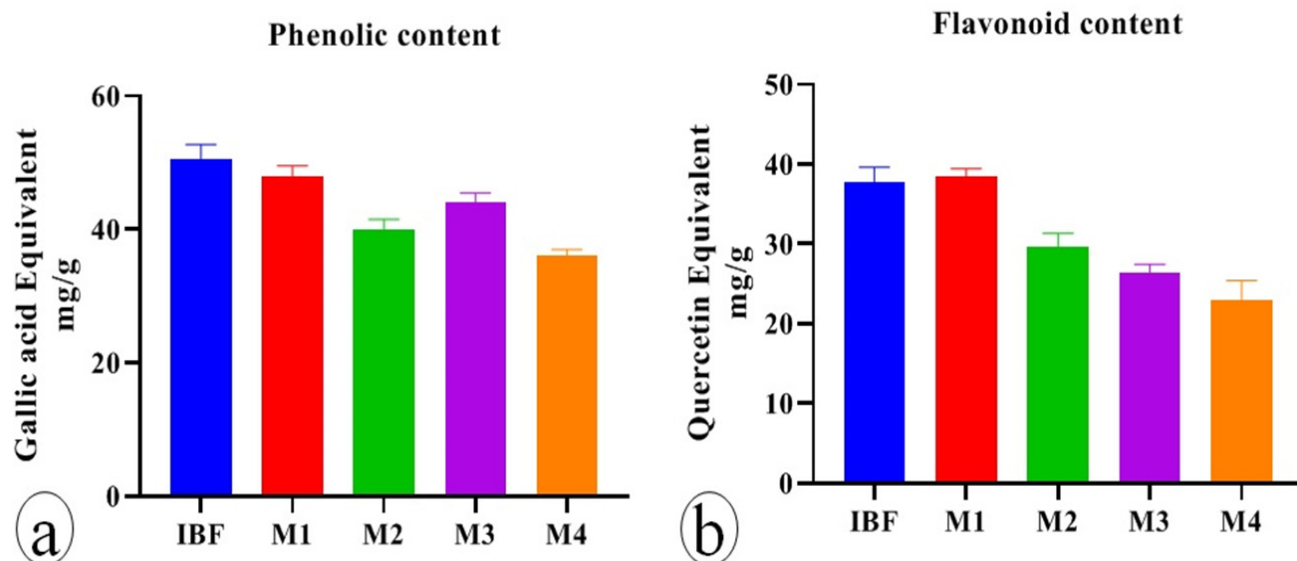


Fig. 7: (a) Total phenolic content and (b) Total flavonoid content in methanolic extract of IBF, M1, M2, M3 and M4 formulation. The data were taken in triplicate and expressed as mean \pm SEM, n=3

The percentages of DPPH, ABTS, H_2O_2 , nitric oxide radical scavenging activity, and reducing power assay of ascorbic acid, IBF, M1, M2, M3, and M4 were recorded at higher concentration with corresponding IC_{50} values.

DPPH is a stable nitrogen-centered free radical. DPPH stabilizes as free radical molecules due to the delocalization of odd electrons throughout the molecules. This stabilized DPPH produces intense violet color in methanol solution. The antioxidant reacts with DPPH free radical solution and converts them into reduced form diphenyl picryl hydrazine either by donating hydrogen atom or transferring electron followed by proton. This oxidation reaction was accompanied by a loss of violet color, which can be measured quantitatively at 517 nm^[31]. The percentages of DPPH radical scavenging activity of ascorbic acid, IBF, M1, M2, M3, and M4 were recorded at higher concentrations with corresponding IC_{50} values (fig. 8a and fig. 9a). The methanolic extract of the IBF and marketed formulations significantly scavenged DPPH radical in a concentration-dependent manner compared to standard ascorbic acid. Formulation IBF exhibited the highest DPPH radical scavenging activity, while M2 and M4 showed the least radical scavenging activity.

Proton radical scavenging is regarded as a characteristic of antioxidant activity. ABTS, a protonated radical, was generated during the incubation period through the reaction. The

reduction of radical cation was indicated by the decolorization of ABTS radical, which was measured at 734 nm in a UV-visible spectrophotometer^[32]. The capacity of methanolic extracts of the formulation for scavenging ABTS radical was examined by ABTS radical decolorization method and expressed as a percentage of ABTS radical scavenged. The ascorbic acid, IBF, M1, M2, M3, and M4 exhibited significant inhibition of ABTS free radicals at higher concentrations with corresponding IC_{50} values (fig. 8b and fig. 9b). The M4 formulation showed the highest ABTS radical scavenging activity, followed by IBF and M1, while M2 and M3 observed the most minor ABTS activity. The presence of a bioactive principle in the formulation might be inhibited the activity of potassium persulfate and thereby decrease the production of ABTS radical.

The ultraviolet radiation was absorbed by hydrogen peroxide at 230 nm. The decrease in hydrogen peroxide concentration in the reaction mixture was achieved by adding a scavenger, measured at 230 nm^[33]. The percentages of H_2O_2 scavenging activity and IC_{50} values of ascorbic acid, IBF, M1, M2, M3, and M4 were depicted in fig. 8c and fig. 9c. In contrast to standard ascorbic acid, the methanolic extract of IBF and marketed formulation exhibited pronounced dose-dependent hydrogen peroxide scavenging activity from 25-400 μ m. Hydrogen peroxide scavenging activity was best achieved by M1, followed by IBF and M2.

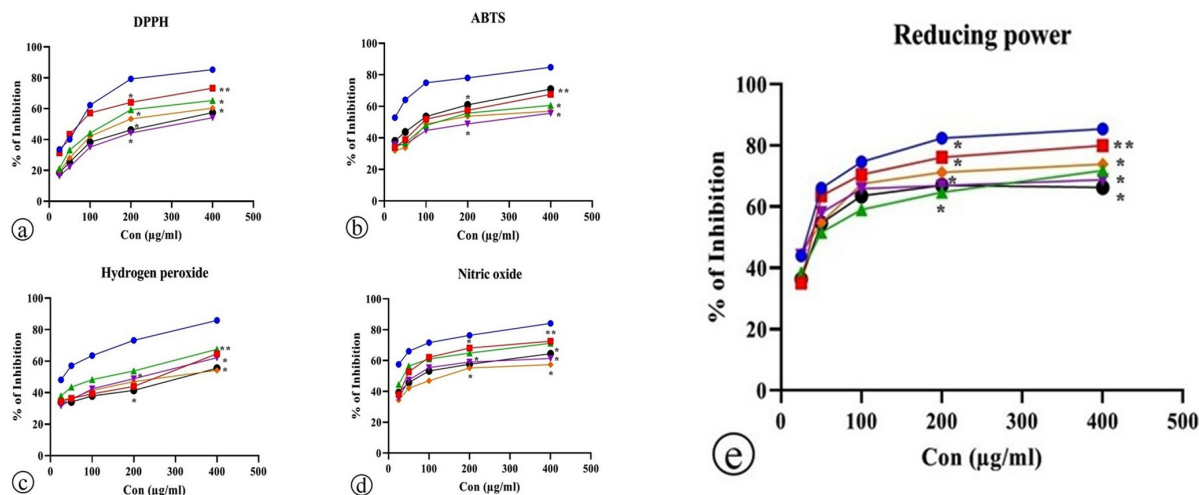


Fig. 8: Percentage of inhibition of ASC and methanol extract of IBF, M1, M2, M3, M4 on, (a) DPPH radical scavenging assay; (b) ABTS radical scavenging assay; (c) Hydrogen peroxide scavenging assay; (d) Nitric oxide radical scavenging assay and (e) Reducing power assay. The data were taken in triplicate and expressed as mean \pm SEM, $n=3$, and was estimated by One-way ANOVA followed by posthoc Tukey HSD test

Note: $p < 0.05$ is considered as significant; (●): ASC; (■): IBF; (▲): M1; (▼): M2; (◆): M3 and (●): M4

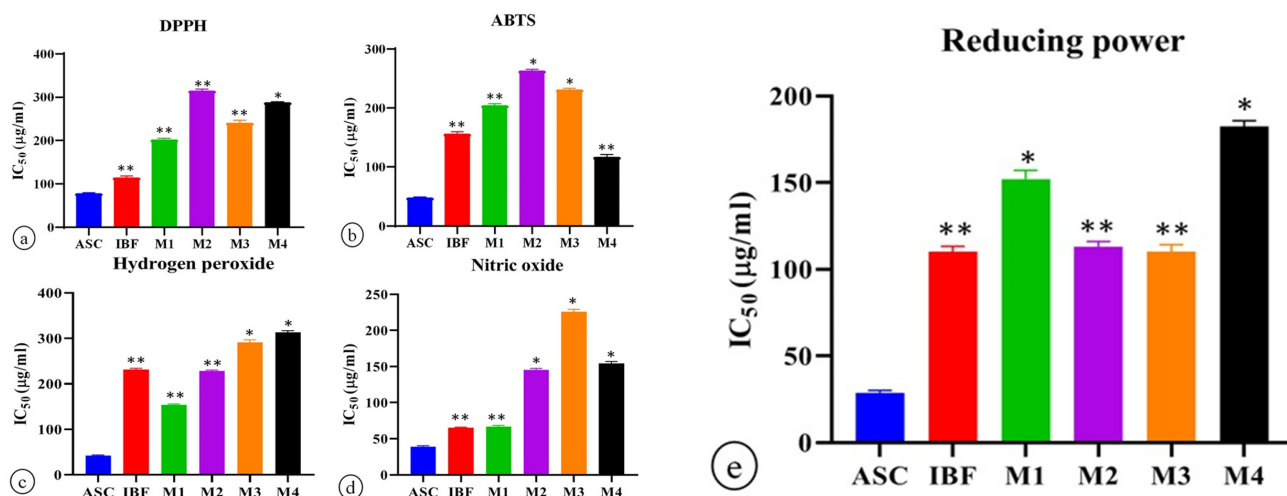


Fig. 9: IC_{50} values of ASC, IBF, M1, M2, M3, and M4 for, (a) DPPH radical scavenging assay; (b) ABTS radical scavenging assay; (c) Hydrogen peroxide scavenging assay; (d) Nitric oxide radical scavenging assay and (e) Reducing power assay. The data were taken in triplicate and expressed as mean \pm SEM, $n=3$, and were estimated by One-way ANOVA followed by a post hoc Tukey HSD test

Note: $p < 0.05$ is considered as significant

In an aqueous solution, nitrous oxide from sodium nitroprusside reacts with oxygen, forming stable nitrite and nitrate ion. The extract containing free radical scavenger competes with oxygen and decreases the concentration of nitrite ions. Further, the nitrite ion reacts with sulphanilamide of Griess reagent in aqueous solution and produces diazotized molecule, which was monitored at 546 nm^[34]. The NO radical scavenging activity of ascorbic acid and IBF, M1, M2, M3, M4, and characteristic IC_{50} values are shown in fig. 8d and fig. 9d. Formulation IBF exhibited the highest percentage of inhibition among the formulations. All the formulations demonstrated significant NO radical scavenging activity in a concentration-

dependent manner compared to ascorbic acid.

The reducing power of antioxidants is associated with the presence of reductions which exert antioxidant activity by breaking the free radical chain by donating hydrogen atoms^[35]. In this assay, the Fe^{3+} /ferric cyanide was reduced to a complex ferrous form, and the appearance of a navy blue color was measured at 700 nm^[36]. Fig. 8e and fig. 9e demonstrated the reducing power of the ascorbic acid, IBF, M1, M2, M3, and M4 at a concentration of 400 μ g/ml and corresponding IC_{50} . In this study, the M2 formulation exhibited the highest reducing power. However, all the formulations were observed with good reducing power in a concentration-dependent manner

compared to ascorbic acid. Thus, they proved an excellent electron donor to scavenge the radical chain reaction.

From the above result, we can assume that the antioxidant activity of the IBF and marketed Balarista formulation may be attributed to the presence of an appreciable quantity of phenolic and flavonoid compounds and the free radical scavenging property of the *Withania somnifera* and *Sida cordifolia*. The presence of phenolics, flavonoids, alkaloids, saponins, steroids, glycosides, tannins, protein, and amino acids^[37] in the root of *Withania somnifera* exhibited significant DPPH radical, hydrogen peroxide radical, reducing power^[38], ABTS radical^[39] and nitric oxide radical^[40] scavenging property. The antioxidant activity of *Withania somnifera* due to its active principles, like withanolides, withaferin-A, alkaloids, and flavonoids, has been reported by Jovanovic *et al.*^[41]. Besides this, sitoindosides VII-X, polyphenols, flavonoids, and vitamin C isolated from the root of *W. somnifera* also possess antioxidant property^[42-44]. The root of *Sida cordifolia* exhibited DPPH radical, reducing power, nitric oxide radical, hydrogen peroxide radical^[45], and ABTS radical^[46] scavenging activities. The antioxidant activity of the Balarista can also be supported by the free radical scavenging property of *Woodfordia fruticosa*^[47], *Alpinia galanga*^[48], *Elettaria cardamomum*^[49], *Syzygium aromaticum*^[50], *Ipomoea digitata*^[51], *Paederia foetida*^[52], *Ricinus communis*^[53], *Tribulus terrestris*^[54], *Vetiveria zizanioides*^[55].

In this study, the authors have described a comprehensive chemical analysis of different fractions of IBF and marketed Balarista formulation by FTIR fingerprint analysis and created a spectral library for quality control. The developed HPTLC profiling could also be used for quality control of formulation. As there was no information available on the aflatoxin content in API, the present data can be used as standard. Low bacterial count in IBF and M1 formulation indicates use of good quality raw materials. All the formulations were proven as good scavengers of free radicals.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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