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Optimization of microwave parameters to enhance phytochemicals, antioxidants and metabolite profile of de-oiled rice bran

Alonkrita Chowdhury[1], Alla Yaswanth Naveen Kumar[1], Rajesh Kumar[2], Vivek Kumar Maurya[3], M. S. Mahesh[4], Abhishek Kumar Singh[5], Pavan Kumar Yadav[6] & Mayukh Ghosh[6]✉

The current study explores the effects of microwave treatment at varying wattage and durations on the phytoconstituents, antioxidant status, anti-nutritional factors (ANFs), and metabolite profiles of de-oiled rice bran. The total phenolics and flavonoids showed both increases and decreases depending on specific microwave parameters, while flavonol content consistently increased across all treated groups compared to the control. The DPPH and ABTS free radical scavenging activity, total antioxidant capacity, FRAP, CUPRAC, metal chelating activity, and ascorbic acid content were enhanced in most of the microwaved samples; however, longer microwave exposure at higher wattage led to their reduction. A treatment-specific decrease in ANFs, including condensed tannins, oxalates, and phytates, was observed. HRMS-based untargeted metabolomics identified a diverse range of primary and secondary metabolites, which clustered in a group-specific manner, indicating notable group-wise metabolite variations. Analysis of discriminating metabolites revealed no significant differences in the overall levels of phenolics, flavonoids, vitamins and cofactors, sugars, amino acids, terpenoids, fatty acids, and their derivatives among the treated groups compared to the control; however, several individual metabolites within these metabolite classes differed significantly. These findings suggest that optimized microwaving of de-oiled rice bran can enhance phytochemicals and antioxidants while improving the metabolite profile.

Keywords De-oiled rice bran, Microwave, Phytoconstituents, Antioxidants, Metabolomics, High-resolution mass spectrometry (HRMS)

De-oiled rice bran (DORB) plays a crucial role in the livestock industry, providing a sustainable solution for enhancing animal nutrition and promoting economic efficiency. It is rich in essential nutrients, including protein, fiber, fatty acids, vitamins, and minerals[1,2]. With a high protein content (14–16%) and a balanced amino acid profile, it is a suitable dietary ingredient for poultry, cattle, swine, and aquaculture[3–5]. As a by-product of rice milling, DORB is readily accessible and serves as a cost-effective source of protein and energy in feed formulations, contributing to sustainable agricultural practices and maximal resource utilization. In addition

[1]Department of Plant Biotechnology, Institute of Agricultural Sciences, Banaras Hindu University, Rajiv Gandhi South Campus, Barkachha, Mirzapur, U.P. 231001, India. [2]Department of Veterinary Physiology and Biochemistry, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana 125004, India. [3]High-Resolution Mass Spectrometry Lab, Sophisticated Analytical & Technical Help Institute (SATHI), Central Discovery Center, Banaras Hindu University, Varanasi, U.P. 221005, India. [4]Livestock Farm Complex, Faculty of Veterinary and Animal Sciences, Institute of Agricultural Sciences, Banaras Hindu University, Rajiv Gandhi South Campus, Barkachha, Mirzapur, U.P. 231001, India. [5]Department of Animal Nutrition, Faculty of Veterinary and Animal Sciences, Institute of Agricultural Sciences, Banaras Hindu University, Rajiv Gandhi South Campus, Barkachha, Mirzapur, U.P. 231001, India. [6]Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary and Animal Sciences, Institute of Agricultural Sciences, Banaras Hindu University, Rajiv Gandhi South Campus, Barkachha, Mirzapur, U.P. 231001, India. ✉email: ghosh.mayukh87@gmail.com

to its nutritional value for livestock, DORB may offer potential health benefits, including antioxidant properties and support for gastrointestinal health, which can enhance productivity and overall well-being[6–8].

Stabilizing rice bran as soon as possible after it is separated from the kernel is crucial to prevent undesirable physicochemical changes caused by lipid deterioration, which occurs due to enzymes like lipase. Various pretreatment methods, such as extrusion cooking, ohmic heating, dry and moist heating, autoclaving, parboiling, roasting, enzymatic treatment, infrared radiations, microwave heating, exist for stabilizing rice bran, each with its own advantages and considerations[9–12]. Among these methods, dry heat treatment is often favored for its simplicity, practicality, and commercial potential. However, there are growing concerns about its effects on the functional properties of rice bran proteins. Such effects can influence further processing and utilization of rice bran protein, phytoconstituents, antioxidants and vitamins in the food industry, underscoring the need to carefully balance enzyme inactivation with the preservation of bioactive components during stabilization.

Microwave treatment is increasingly gaining popularity due to its time-efficient heating, operational convenience, cost-effectiveness, instantaneous control, and its ability to enhance phytoconstituents and antioxidant activity, making it a preferred stabilization method over conventional alternatives[10]. Microwave irradiation has been identified as a beneficial pretreatment method for stabilizing and enhancing the nutraceutical properties of diverse plant seeds, such as sunflower, apricot kernels, poppy seeds, rape oilseed, and pomegranate seeds[13–16].

The presence of anti-nutritional factors (ANFs) in DORB poses a challenge to its inclusion in animal feeds, despite its abundance and low cost. ANFs are compounds that interfere with nutrient digestion, absorption, or utilization, reducing the nutritional value of feed ingredients. These ANFs are primarily concentrated in the bran fraction, and in rice bran, the primary ANFs include phytate, oxalates, tannins, lectins, saponins, and various enzyme inhibitors[17,18]. Microwave heating has been shown to reduce ANFs, thus improving the bioavailability of nutrients in treated cereal brans[18,19].

Although microwave treatment of feed ingredients like DORB can positively affect phytoconstituents, antioxidant profiles, and ANFs, comprehensive metabolite analysis is essential to assess its overall impact on the nutritional profile and sensory qualities of the processed feed. Previous reports have noted changes in the phytochemical, antioxidant and ANF profiles of rice bran due to thermal processing[8,18,19]. However, the novelty of the current study lies in its first-time use of LC-HRMS-based metabolomics analysis of microwave-processed de-oiled rice bran, in addition to documenting changes in phytochemicals, antioxidants and ANFs.

Metabolite analysis is key to evaluating changes in essential nutrients like sugars, amino acids, and fatty acids, ensuring that the nutritional integrity of the feed is maintained or enhanced post-microwaving. It also enables the identification and quantification of secondary metabolites, offering insights into their potential effects on feed quality and consumer acceptance. By integrating metabolomics into research and development, stakeholders can make informed decisions to optimize feed processing techniques and improve the nutritional value and acceptability of animal feeds[1,20–22]. The combination of liquid chromatography (LC) or gas chromatography (GC) with mass spectrometry (MS) has revolutionized metabolite analysis in food grains, including rice bran, offering unparalleled sensitivity, resolution, and versatility for studying metabolic pathways, identifying bioactive compounds, and assessing the nutritional quality of grain-based foods[20,22–24]. These techniques remain central to current research aimed at understanding the impact of microwave processing with different power and duration combinations on phytoconstituents, antioxidant status, ANFs, and the metabolite profile of DORB. The findings of this study could demonstrate the utility of microwave technology, with potential benefits for the feed manufacturing, agriculture, and food processing sectors in leveraging DORB as a valuable commodity in the global market.

Materials and methods

Sample preparation and microwave treatment

The DORB was supplied by Aman Trading Company, Uttar Pradesh, India. It was prepared by solvent extraction of oil from the full-fat rice bran using hexane, followed by desolventizing, as reported by Bandyopadhyay et al.[25]. The DORB was finely ground using a coffee grinder and sieved to obtain a powder of uniform particle size. The powder was stored in amber glass vials and kept in a glass desiccator at room temperature until further analysis.

A microwave oven with a 20-litre capacity (MW73AD-B/XTL, Samsung, Malaysia), capable of generating a maximum output power of 800 watts at 2450 MHz, was used to treat the DORB samples. Equal amounts (10 g) of DORB powder were heated intermittently with varying wattage-time combinations to produce different treatment groups, while the control group consisted of raw DORB powders that were not subjected to microwave treatment. The treatment groups were as follows: 300 watts for 3 min (T-1), 6 min (T-2), and 9 min (T-3); 600 watts for 2 min (T-4), 4 min (T-5), and 6 min (T-6); and 800 watts for 1.5 min (T-7), 3 min (T-8), and 5 min (T-9). The dry matter (DM) content of the control and treated samples was determined gravimetrically and ranged from 81.94 ± 2.06% to 91.27 ± 1.36%. Phytochemicals and metabolites from 1 g DM equivalent DORB powders from the control and each treatment group were extracted by continuous shaking in an orbital shaker at 150 rpm for 14 h at room temperature using 20 mL of 70% methanol. This extraction solvent was chosen for its superior solvation potential and extraction efficiency for phenolics, anthocyanins, and antioxidants from rice bran and other fruits[26,27]. The extracts were then filtered through 0.45 µm nylon syringe filters and stored at -80 °C.

Phytochemical analysis

The total phenolic content in DORB extracts was estimated using the Folin-Ciocalteu method[28], with results expressed as micrograms of gallic acid equivalent (GAE)/g of DM. The total flavonoid content was determined using the aluminium chloride/sodium nitrite colorimetric assay[29], with results expressed as micrograms of quercetin equivalent (µg QE)/g of DM. The flavonol content was measured according to the method described by Quettier-Deleu et al.[30], with some modifications. Briefly, 50 µl of extract was mixed with 1 mL of 0.1% (w/v)

p-dimethylaminocinnamaldehyde in methanolic/HCl (3/1; v/v) reagent. After 10 min of incubation at room temperature, the absorbance was measured at 640 nm using a multimode reader (Tecan Infinite® 200 PRO M Nano+, Austria). The flavonol content was calculated from a standard curve prepared using catechin and expressed as micrograms of catechin equivalent (CE)/g of DM. Total soluble sugar was estimated using the anthrone method[31] and expressed as micrograms of dextrose equivalent (DE)/g of DM.

Antioxidant activities

The antioxidant activity of the extracts, specifically the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, was estimated following the method described by Mohdaly et al.[32], with minor modifications. Briefly, 50 µL of extract was added to 5 mL of methanolic DPPH solution (6×10^{-5} M). The mixture was incubated for 30 min in the dark, and absorbance was recorded at 517 nm using a spectrophotometer (Shimadzu 1900 UV-VIS spectrophotometer, Japan). The results were expressed as micrograms of ascorbic acid equivalent (AAE)/g of DM.

The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical ($ABTS^{\cdot+}$) scavenging activity was determined according to the method of Re et al.[33], and expressed as micrograms of gallic acid equivalent (GAE)/g of DM.

Total antioxidant capacity (TAC) was determined using the phosphomolybdate assay[34], and the results were expressed as micrograms of ascorbic acid equivalent (AAE)/g of DM.

Ferric-reducing antioxidant power (FRAP) was determined following the method of Oyaizu[35], with slight modifications[36], and expressed as micrograms of ascorbic acid equivalent (AAE)/g of DM.

Cupric-reducing antioxidant capacity (CUPRAC) was determined using the method of Apak et al.[37] and expressed as micrograms of ascorbic acid equivalent (AAE)/g of DM.

The ferrous ion chelating activity was estimated using the method described by Dinis et al.[38], with minor modifications. Briefly, 2 mL of DORB extract was added to 50 µL of a 2 mM of ferrous chloride ($FeCl_2$) solution, followed by the addition of 200 µL of 5 mM ferrozine. The mixture was vortexed and incubated for 10 min at room temperature, after which the absorbance was measured at 562 nm using a spectrophotometer (Shimadzu 1900 UV-VIS spectrophotometer, Japan). The results were expressed as micrograms of EDTA equivalent/g of DM.

The ascorbic acid content was determined using the method of Jagota and Dani[39] and expressed as micrograms of ascorbic acid/g of DM.

Estimation of anti-nutritional factors (ANFs)

The phytate content in the extracts was estimated using Wade Reagent[40], with minor modifications[41], and expressed as micrograms of phytic acid equivalent (PAE)/g of DM. The condensed tannin content was determined using the vanillin reagent[42,43] and expressed as micrograms of catechin equivalent (CE)/g of DM. Oxalates were extracted with HCl and estimated by titration with potassium permanganate, following the method of Baker[44].

Statistical analysis of phytochemicals, antioxidants, and ANFs

All measurements of phytochemicals, antioxidants, and ANFs were expressed as mean ± standard error ($n = 6$) and statistically analysed using the R software. Results were visualized through Box-Whisker plots generated with MetaboAnalyst 6.0 (https://new.metaboanalyst.ca). Correlations between phytochemicals and antioxidant parameters were established using Pearson (r) analysis and represented via a heat map created with MetaboAnalyst 6.0 (https://new.metaboanalyst.ca). A significance level of $P < 0.05$ was considered.

Metabolomic analysis by liquid chromatography-high-resolution mass spectrometry (LC-HRMS)

The control and treated DORB samples were subjected to metabolomic analysis using an Orbitrap Eclipse™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific Inc., USA) coupled with a Dionex Ultimate 3000 RS UHPLC system. Separations were performed at 40 °C using a Hypersil GOLD™ C18 Selectivity HPLC Column (2.1 × 100 mm, particle size 1.9 µm, Thermo Fisher Scientific Inc., USA) at a constant flow rate of 0.3 mL/minute. The mobile phases were 0.1% formic acid in water (A) and methanol (C), with the following elution gradient: 5% (C) for 0–4 min, 20% (C) for 4–8 min, 35% (C) for 8–10 min, 45% (C) for 10–20 min, 90% (C) for 20–28 min, and 5% (C) for 28–30 min. Mass spectra were acquired using a heated electrospray ionization (H-ESI) source, operated in both positive and negative modes, with full-scan and $ddMS^2$ at a scan range of 100–1000 m/z. The other H-ESI parameters were as follows: spray voltage: static, positive ion: 3500 V, negative ion: 2500 V, sheath gas: 50Arb, aux gas: 10Arb, sweep gas: 1Arb, ion transfer tube temperature: 325 °C, vaporize temperature: 350 °C, filter intensity threshold: 2e5, mass tolerance: 5 ppm. Quality control samples (QCs) were injected at regular intervals to ensure the repeatability of the data set. Metabolite identification was conducted using the Compound Discoverer programme (Thermo Scientific, version 3.2.0.421) through searches in mzCloud (ddMS2 and/or DIA), ChemSpider (exact mass or formula), and local database searches against Mass Lists.

Univariate and multivariate statistical analysis

A metabolite data matrix was constructed after removing internal peaks based on the maximum peak area. The relative standard deviation (RSD = SD/mean) or coefficient of variation (CV) was set at 40% for the data filtering, followed by normalization using Pareto scaling. For univariate analysis, one-way ANOVA was performed with an adjusted p-value (FDR) cutoff of 0.05, followed by post-hoc analysis using Fisher's Least Significant Difference (LSD) for p-values < 0.05. Metabolite variations between different treatment groups and the control group were elucidated through volcano plot analysis, with a fold change (FC) threshold 2.0 and the FDR p-value threshold of 0.05. For multivariate analysis, chemometric evaluation using principal component analysis (PCA),

class discrimination using Sparse Partial Least Squares Discriminant Analysis (sPLS-DA), and Hierarchical Clustering Analysis (HCA) were conducted using the MetaboAnalyst 6.0 program (https://new.metaboanalyst.ca).

Identification and analysis of discriminating metabolites

Variable Importance in Projection (VIP) score plot for metabolites influencing group-wise variations was generated from the orthogonal partial least squares-discriminant analysis (OPLS-DA) model using the MetaboAnalyst 6.0 program (https://new.metaboanalyst.ca). Differential metabolites were selected based on a combination of VIP values ≥ 1, a log2(Fold Change) threshold ≥ 1 (for up-regulation) or ≤ -1 (for down-regulation), and a p-value < 0.05 from a two-tailed Student's t-test on the normalized peak areas. Variations in overall phenolics, flavonoids, amino acids, sugars, lipids, and terpenoids among different treatment and control groups were analysed using one-way ANOVA (p-value cutoff: 0.05), followed by Dunnett's multiple comparison post-test using GraphPad Prism 5.01, with significance set at $p < 0.05$. Group-wise differences in individual major discriminating metabolites from each chemical class were evaluated using two-way ANOVA in GraphPad Prism 5.01.

Results and discussion

Phytochemical analysis

The TPC values varied across the different treatment groups, ranging from 1743.69 ± 3.2 to 3879.31 ± 24.67 µg GAE/g of DM. The control group had a TPC of 2082.75 ± 5.58 µg GAE/g of DM. Most treatment groups showed an increase in phenolic content compared to the control group, with T-1 exhibiting the highest TPC at 3879.31 ± 24.70 µg GAE/g of DM, followed by T-2, T-7, T-5, T-4, T-3, T-6, and T-8 in decreasing order (Fig. 1a). In contrast, the T-9 group showed a significant decrease in phenolic content compared to the control. These results indicate that microwave parameters have a notable impact on the total phenolic content of the treated DORB samples, highlighting the importance of optimizing these parameters to enhance nutritive value. The treatment-specific influence on TPC aligns with the findings of Pokkanta et al.[8] in rice bran. They reported that microwaving at 260 watts for 0.5 to 3 min and at 440 watts for 0.5 to 2.5 min resulted in a maximum increase in phenolic content, while a decrease occurred at 880 watts. This study corroborates those findings, as the highest TPC was observed with the 300 watts for 3 min treatment (T-1), while a significant reduction was seen in the 800 watts for 5 min treatment (T-9). The reduction in T-9 might be due to the degradation of phenolics caused by prolonged exposure to high temperatures. The effectiveness of the 300 watts for 3 min microwave treatment in enhancing phenolic content could be attributed to factors such as the release of bound phenolics through the breakdown of cell walls and minimal thermal damage to bioactive compounds during the process[45].

The TFC varied among the different treatment groups, ranging from 482.73 ± 9.96 to 916.82 ± 16.29 µg QE/g of DM, with the control group having a TFC of 2373.64 ± 47.20 µg QE/g of DM. Most treatment groups showed a significant increase in flavonoid content compared to the control. The T-6 treatment group exhibited the highest TFC, followed by T-4, T-7, T-1, T-2, T-5, and T-3 (Fig. 1b). A significant decrease in flavonoid content was observed in the T-9 group, while the TFC of T-8 was comparable to the control. The decrease in T-9, which involved treatment at 800 watts for 5 min, is likely due to the degradation of flavonoids caused by prolonged exposure to high-intensity microwaves[13,46]. A similar wattage-time -dependent variation in TFC in microwaved rice bran was also reported by Pokkanta et al.[8] The flavonol content ranged from 14.30 ± 1.61 to 38.94 ± 1.94 µg CE/g of DM among the treated samples, whereas the control group had a much lower flavonol content of 10.07 ± 0.70 µg CE/g of DM. All treated samples exhibited a significant ($p < 0.05$) increase in flavonol content compared to the control, indicating that microwave treatment positively influenced flavonol levels in DORB. The highest flavonol content was observed in the T-7 group (800 watts for 1.5 min), which yielded a concentration of 38.94 ± 1.94 µg CE/g of DM, followed by T-6, T-5, T-4, T-3, T-1, T-2, T-8, and T-9 (Fig. 1c). Overall, short exposures at moderate to high microwave power enhanced the flavonol content in DORB, whereas longer exposure at high wattage led to flavonol degradation, as seen in the lowest levels for the 800 watts for 5 min (T-9) and 3 min (T-8) treatment groups. These findings align with the observations of Rodrigues et al.[47], who reported that moderate microwave roasting at 450 watts for 4 min did not affect flavonols such as quercetin glycosides and anthocyanins in onion bulbs, but intense microwaving at 750 watts for 4 min resulted in significant flavonol loss.

The total soluble sugar (TSS) content varied among the treated samples, ranging from 32625.63 ± 284.47 to 81634.404 ± 467.86 µg DE/g of DM. In comparison, the control group had a sugar content of 54156.88 ± 543.1 µg DE/g of DM. Most treatment groups showed a significant ($p < 0.05$) reduction in TSS content compared to the control group, in the following order: T-1, T-9, T-5, T-2, T-8, T-3, and T-7. Among the downregulated groups, the T-1 group (300 watts for 3 min) showed the least reduction in TSS content. However, a significant increase in TSS was observed in the T-4 and T-6 groups (Fig. 1d). The decrease in TSS content in most treated samples may be due to the breakdown of carbonyl and amino compounds through non-enzymatic browning reactions[48]. A similar reduction in carbohydrates and reducing sugars was observed in various cultivars of rice bran microwaved at 750 watts for 9, 12, and 15 min[49]. Conversely, an increase in soluble sugar content has been reported in persimmon slices microwaved at 280, 350, 420, 490, and 560 watts[50], and in sweet potatoes microwaved at 800 watts for 5 min, which led to an increase in reducing sugars and other soluble sugars[51]. This increase in soluble sugars may result from the degradation of polysaccharides and other macromolecules during thermal treatments[52]. The current findings suggest that the microwave parameters significantly influence the TSS content of DORB, with the 600 watts for 2 min treatment (T-4) producing the highest soluble sugar yield. Optimization of microwave parameters is therefore crucial to maximize TSS content.

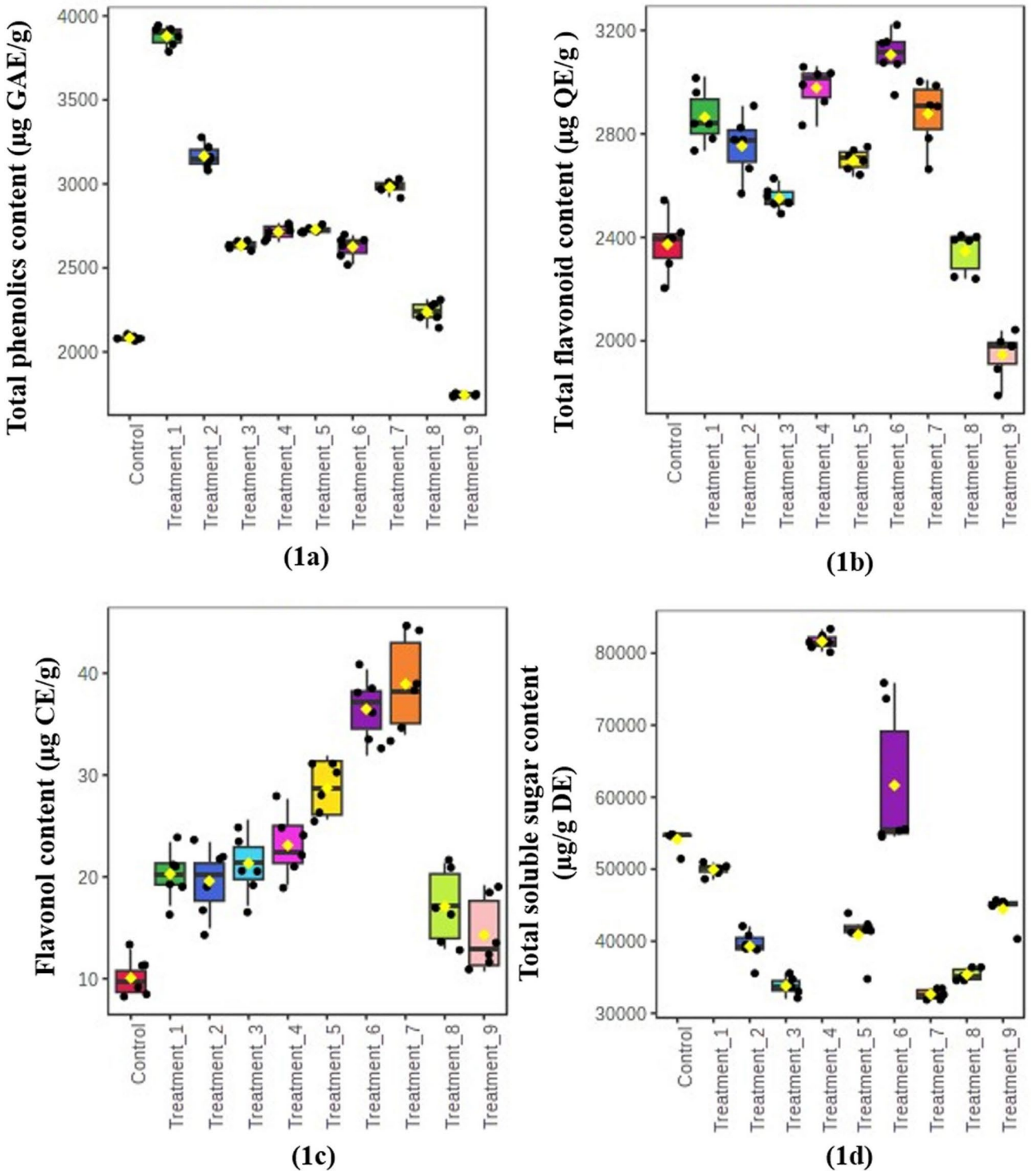

Fig. 1. Box-Whisker plot depicting different concentrations of phytochemicals and total soluble sugar content between control and microwave-treated DORB samples: (**a**) Total phenolic content (**b**) Total flavonoid content, (**c**) Flavonol content, and (**d**) Total soluble sugar content.

Antioxidant status

The DPPH free radical scavenging activity varied among the treated samples, ranging from 397.14 ± 25.42 to 2854.29 ± 49.62 µg AAE/g of DM, while the control group had a scavenging activity of 818.57 ± 29.51 µg AAE/g of DM. Most treatment groups showed a significant ($p < 0.05$) increase in DPPH free radical scavenging activity compared to the control. The highest activity was observed in treatment group T-1, with a value of 2854.29±49.62 µg AAE/g of DM, followed by T-2, T-3, T-7, T-4, T-6, and T-5 (Fig. 2a). The increase in antioxidant activity may be attributed to the release of bound phenolics and phytochemicals from cellular components due

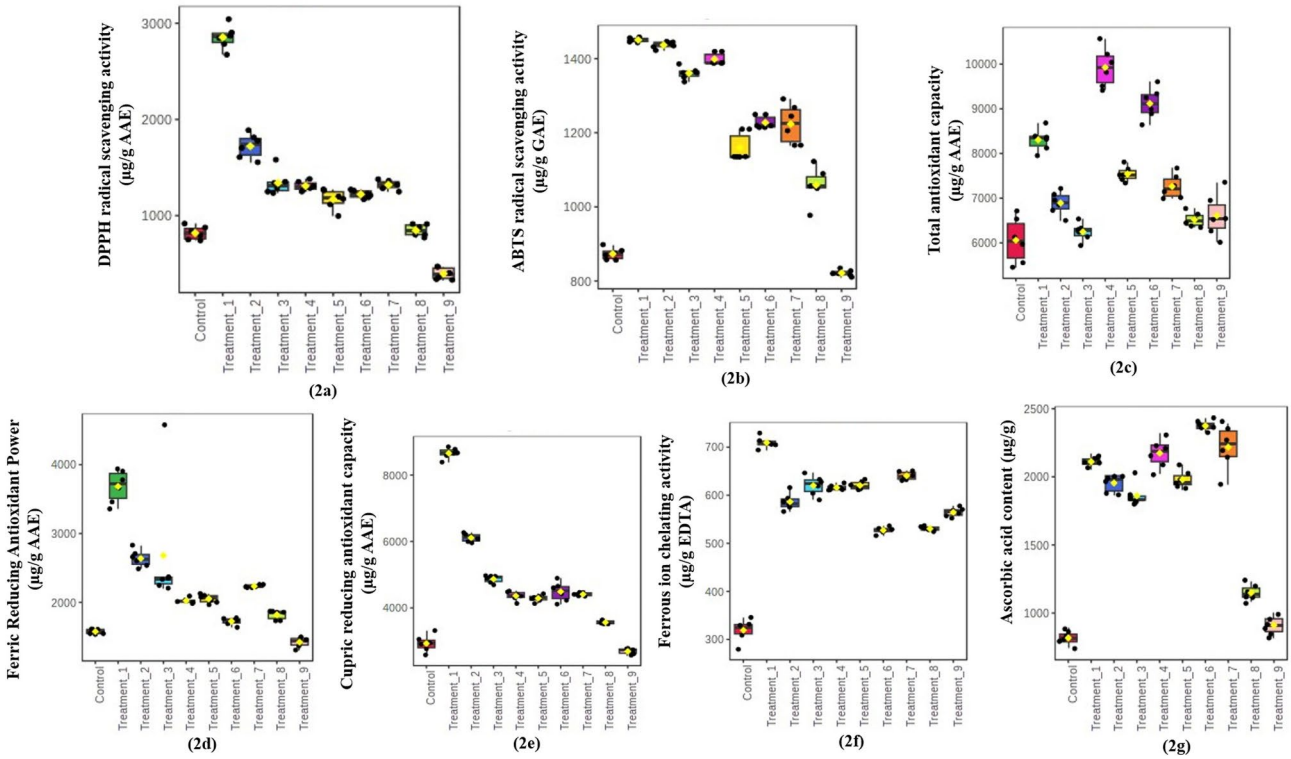

Fig. 2. Box-Whisker plot representing the antioxidant status of the control and microwave-treated DORB samples: (**a**) DPPH free radical scavenging activity (**b**) ABTS free radical scavenging activity, (**c**) Total antioxidant capacity, (**d**) Ferric reducing antioxidant power, (**e**) Cupric reducing antioxidant capacity, (**f**) Ferrous ion chelating activity, (**g**) Ascorbic acid content.

to microwave heating, as well as Maillard reaction products formed during browning reactions[53,54]. Similar findings have been reported in rice bran, where DPPH radical scavenging activity increased under most microwave treatments, with the highest activity observed at 440 watts for 2.5 min[8]. Additionally, infrared heating of rice bran under optimum conditions has also been shown to enhance DPPH free radical scavenging activity[55]. In this study, the highest activity was noted with the T-1 treatment (300 watts for 3 min). However, increasing the wattage resulted in a reduction in activity, with the lowest value recorded in the T-9 treatment (800 watts for 5 min). This suggests that exposure to high microwave power and longer heating can diminish DPPH radical scavenging activity, likely due to the degradation of phenolics and flavonoids during extended heating[14,56].

The ABTS free radical scavenging activity varied among the treated samples, ranging from 821.42 ± 3.46 to 1450.43 ± 2.37 µg GAE/g of DM, compared to 873.04±6.40 µg GAE/g of DM in the control group. Most treatment groups showed a significant ($p < 0.05$) increase in ABTS free radical scavenging activity relative to the control. The highest scavenging activity was observed in the T-1 group (300 watts for 3 min), with a value of 1450.43 ± 2.37 µg AAE/g of DM, followed by T-2, T-4, T-3, T-6, T-7, T-5, and T-8 (Fig. 2b). In contrast, the T-9 group displayed a significant ($p < 0.05$) decrease in ABTS scavenging activity compared to the control. Overall, these findings suggest that microwave treatments enhance ABTS scavenging activity in DORB, though prolonged exposure at high microwave power can negatively impact the activity, as seen in the 800 watts for 5 min treatment (T-9). This aligns with previous research showing that roasting can enhance ABTS free radical scavenging activity in maize, soybeans, and rice bran[18,57,58]. However, longer microwave exposures at higher wattages, such as 900 watts for 2–5 min, have been reported to reduce the scavenging activity in quinoa grains[59]. This indicates that the effect of microwave treatment on the antioxidant activity varies based on the feed type and specific treatment parameters used.

The total antioxidant capacity (TAC) varied among the treated samples, ranging from 6246.25 ± 81.46 to 9927.50 ± 179.34 µg AAE/g of DM, while the control group had a TAC of 6059.53 ± 102.01 µg AAE/g of DM. Most treatment groups showed a significant ($p < 0.05$) increase in TAC compared to the control. The highest TAC was observed in treatment group T-4, with a value of 9927.50 ± 179.34 µg AAE/g of DM, followed by T-6, T-1, T-5, T-7, T-2, T-9, and T-8, and T-8 (Fig. 2c). Treatment group T-3 showed a slight increase (6246.25 ± 81.45 µg AAE/g of DM), although it was comparable to the control. Prolonged exposure or higher microwave power appeared to negatively affect TAC, as seen in the lowest values obtained in treatments of 300 watts for 9 min (T-3) and 800 watts for 5 min (T-9). These findings align with previous research, which highlights the importance of optimizing microwave parameters to maximize the release of bioactive compounds and antioxidant activity in different vegetables, fruits, and grains[46,60–62].

The ferric reducing ability varied among the treated samples, ranging from 1420.45 ± 29.87 to 3684.29 ± 96.55 µg AAE/g of DM, compared to 1574.91 ± 15.08 µg AAE/g of DM in the control group. Significant ($p < 0.05$) upregulation was observed in treatment groups T-1, T-3, T-2, T-7, T-5, and T-4 (Fig. 2d). The highest increase in ferric reducing ability was seen in T-1 (300 watts for 3 min), with a value of 3684.29 ± 96.55 µg AAE/g of DM. Treatment groups T-8 and T-6 showed slight increases, while T-9 showed a non-significant ($p < 0.05$) decrease in FRAP compared to the control group. Similar increases in FRAP have been reported in rice bran treated with microwaves at 600 watts, 700 watts, 800 watts and 900 watts for 2.5 min[63,64]. However, in this study, the 800 watts for 5 min treatment (T-9) resulted in a reduction in FRAP, suggesting that prolonged exposure to higher microwave wattage should be avoided to preserve or enhance the ferric reducing ability.

The cupric reducing antioxidant capacity (CUPRAC) ranged from 2694.58 ± 35.12 to 8657.08 ± 65.46 µg AAE/g of DM across the treatment groups. In comparison, the control group had a CUPRAC of 2927.92 ± 102.54 µg AAE/g of DM. Most treatment groups showed a significant ($p < 0.05$) increase in CUPRAC, with the highest value observed in the 300 watts for 3 min treatment (T-1), at 8657.08 ± 65.46 µg AAE/g of DM, followed by T-2, T-3, T-6, T-7, T-4, T-5, and T-8 (Fig. 2e). However, treatment group T-9 showed a significant ($p < 0.05$) decrease in CUPRAC compared to the control. These findings are consistent with several other studies, which report that microwave processing increases CUPRAC in various fruits and grains[65–67]. However, as with the other antioxidant parameters, prolonged microwave heating at higher wattages, such as the 800 watts for 5 min treatment (T-9), led to reduced values, likely due to the degradation of phytochemicals with antioxidant properties.

The ferrous ion chelating activity varied across the treatment groups, ranging from 527.16 ± 2.80 to 709.41 ± 4.75 µg EDTA equivalent/g of DM. In comparison, the control group had the lowest chelating activity, at 319.31 ± 9.38 µg EDTA equivalent/g of DM. All treatment groups showed a significant ($p < 0.05$) increase in ferrous ion chelating activity compared to the control group. The highest activity was observed in the 300 watts for 3 min treatment (T-1), with a value of 709.41 ± 4.75 µg EDTA equivalent/g of DM, followed by T-7, T-5, T-3, T-4, T-2, T-9, T-8, and T-6 (Fig. 2f). These findings are consistent with previous research, where microwaving at 900 watts for 3.5 and 5 min has been shown to enhance metal chelating activity in quinoa grains[59]. A similar increase in metal chelating activity has been reported in barley microwaved at 900 watts for 2 min[68]. The microwave-induced release of bound phenolics and the formation of melanoids may have contributed to the increase in metal chelating activity[68].

The ascorbic acid content in the treated DORB groups ranged from 911.25 ± 28.64 to 2373.75 ± 16.06 µg/g of DM, while the control group having an ascorbic acid content of 817.50 ± 21.6540.4 µg/g of DM. All treatment groups showed an increase in ascorbic acid content compared to the control. The highest value was observed in treatment group T-6 (2373.75 ± 16.06 µg/g of DM), followed by significant ($p < 0.05$) increases in T-7, T-4, T-1, T-5, T-2, T-3, and T-8 (Fig. 2g). Although treatment group T-9 showed an increase in ascorbic acid content compared to the control, the difference was not statistically significant ($p < 0.05$). These findings suggest that prolonged microwave heating at higher wattage significantly reduces the ascorbic acid content in DORB samples, with the lowest values recorded in the 800 watts for 3 min (T-8) and 800 watts for 5 min treatment (T-9). Therefore, optimizing microwave parameters specific to the feed is crucial to achieve the ideal ascorbic acid content in treated samples.

Correlation analysis between phytochemicals and antioxidant parameters revealed strong positive correlations among DPPH and ABTS free radical scavenging activity, CUPRAC, FRAP, ferrous ion chelating activity and total phenolic content. Total flavonoid content also showed significant positive correlations with most antioxidant activities, except for FRAP and ferrous ion chelating activity. Ascorbic acid content displayed a strong positive correlation with most antioxidant activities, except FRAP. Flavonols were moderately correlated with total antioxidant capacity, ABTS free radical scavenging activity, and ferrous ion chelating activity, but showed weaker correlations with other antioxidant parameters such as DPPH free radical scavenging activity, CUPRAC, and FRAP (Fig. 3). This suggests that phenolics, flavonoids and ascorbic acid play a key role in eliciting antioxidant responses in DORB. Several studies have also reported positive correlations between total phenolic content and antioxidant activity in various food grains, vegetables, and fruits[69–72]. Flavonoids, particularly flavones and catechins with multiple hydroxyl groups, are recognized as powerful antioxidants in this phytochemical class. This explains the strong positive correlations observed between DORB flavonoids and antioxidant activities in the current study, aligning with existing literature[73]. While ascorbic acid is a well-known antioxidant, its contribution to overall antioxidant activity can range from significant to negligible. Therefore, the strong positive correlation observed between ascorbic acid content and antioxidant activities in DORB extracts is consistent with previous reports[74].

Antinutritional factors (ANFs)

Condensed tannin, an important ANF, ranged from 233.90 ± 9.24 to 571.71 ± 10.16 µg CE/g of DM in treated DORB samples. Compared to the control group (347.93 ± 7.48 µg CE/g of DM), a significant ($p < 0.05$) downregulation was observed only in T-2, T-4 and T-8, while the reduction in T-3 was comparable. However, significant ($p < 0.05$) upregulation was noted in T-1, T-5, T-6, T-7 and T-9 (Fig. 4a). The decrease in condensed tannin content was found to depend on specific microwave wattage-time combinations, with 300 watts for 6 min treatment (T-2) being the optimal condition for reducing condensed tannin in DORB. The heat lability of DORB-derived condensed tannins and their degradation under optimal microwave conditions likely contributed to this reduction. Similarly, microwave treatment at 850 watts for 3 min has been reported to decrease tannin content in soybeans[75]. However, Irakli et al.,[18] found no significant reduction in the total tannin content of rice bran processed at 650 watts for 2 min. Additionally, Osman reported an increase in tannin content in roasted or cooked lablab beans[76].

Oxalate is another important ANF found in various cereal brans, which can limit mineral bioavailability. In this study, the oxalate content was significantly upregulated in all the microwaved DORB extracts, ranging from

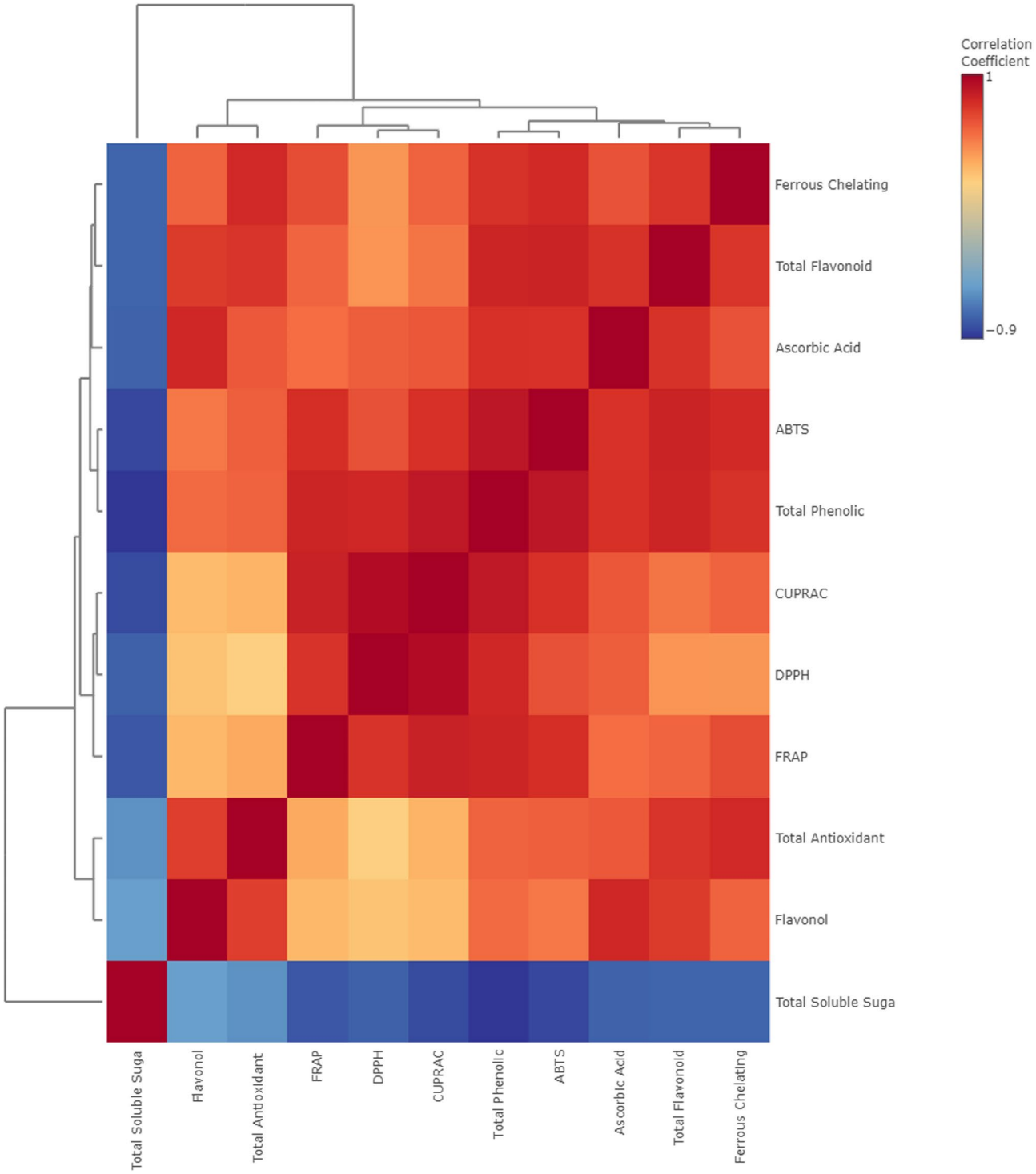

Fig. 3. Correlation analysis between the phytochemicals and antioxidants: the heat map of the different parameters generated by using the MetaboAnalyst 6.0 program (https://new.metaboanalyst.ca) portrays high to low correlation on the basis of correlation coefficient ranging from −0.9 to 1. Shades of red color represent high correlation whereas the blue colors denote lowly correlated parameters.

0.562 ± 0.029 to 1.144 ± 0.016 mg/g of DM, compared to the control samples, which contained 0.469 ± 0.023 mg/g of DM. The highest oxalate content was observed in T-2, followed by T-3, T-6, T-1, T-9, T-4, T-7, T-5 and T-8 (Fig. 4b). While reductions in oxalate content in cereal brans after various thermal treatments, including microwaving, have been reported in previous studies[18,19], no such decrease was observed in the current study. Instead of decomposing oxalates, microwaving likely facilitated the leaching of oxalate from the DORB, leading to an increase in oxalate concentration in the extracts.

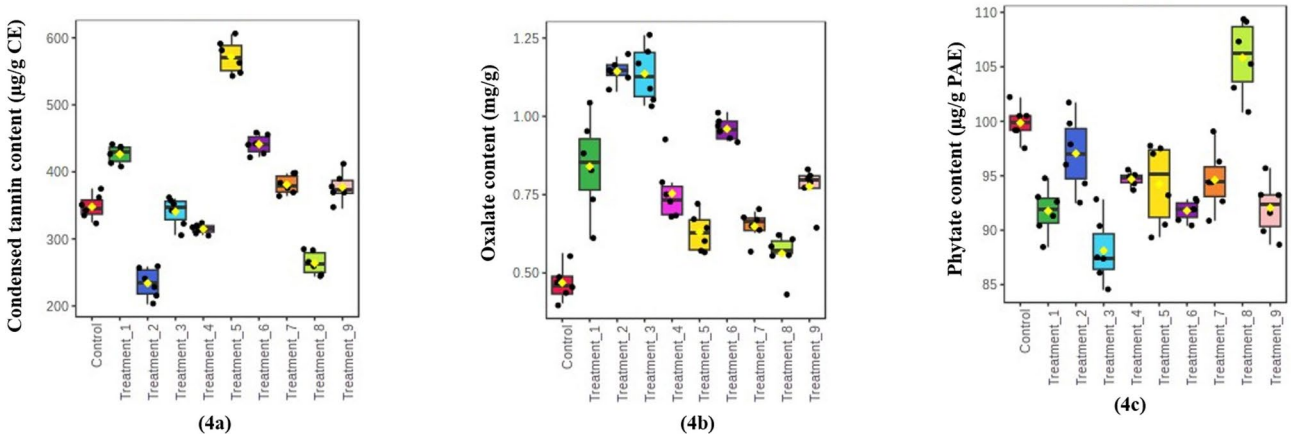

Fig. 4. Box-Whisker plot signifying different concentrations of anti-nutritional factors between control and microwave-treated DORB samples: (**a**) Condensed Tannin Content, (**b**) Oxalate content, and (**c**) Phytate content.

The phytate content in the treated DORB samples ranged from 88.13 ± 1.23 and 105.84 ± 1.39 µg PAE/g of DM, while the control sample contained 99.86 ± 0.65 µg PAE/g of DM. A significant ($p < 0.05$) reduction in phytate content was observed in most of the microwaved samples, with the greatest reduction occurring in the 300 watts for 9 min treatment (T-3) compared to the control, followed by treatments T-1, T-6, T-9, T-5, T-7, and T-4. In T-2, the reduction was comparable to the control, whereas a significant ($p < 0.05$) increase was noted in T-8 (Fig. 4c). Thus, phytate reduction in DORB was dependent on specific microwave power-time combinations. The reduction in phytic acid content due to microwave treatments can likely be attributed to its heat sensitivity, consistent with similar findings in other cereal brans and vegetable pea seeds[18,77,78].

Furthermore, correlation analysis revealed no significant positive relationships between the ANFs studied (data not shown), suggesting that no single microwave treatment is universally effective in reducing all three ANFs in DORB. This highlights the necessity for feed-specific optimization of microwave parameters to achieve targeted reductions in different ANFs.

LC-HRMS-based metabolite analysis

The results of the phytochemical and antioxidant analyses indicated that short microwave exposure led to better retention of active ingredients. For example, the highest values for total phenolics, DPPH and ABTS radical scavenging activities, FRAP, CUPRAC, and ferrous ion chelating activities were observed in the 300 watts for 3 min treatment group (T-1), while the highest total antioxidant capacity and soluble sugar content were found in the 600 watts for 2 min treatment group (T-4). Based on these observations, three groups of microwaved DORB samples with the shortest treatment durations from each wattage (T1, T4, and T7), along with the control, were subjected to HRMS-based metabolite analysis. An initial data matrix containing 2373 metabolite features was generated from mass spectral acquisition in both positive and negative ion modes using ThermoXcalibur™ 3.1 software, and further processed by the Compound Discoverer programme (Thermo Scientific, version 3.2.0.421). A final data matrix of 1550 metabolites was created after removal of the repeats, based on their maximum group area obtained in either ion modes of detection (Supplementary Table S1). After applying a statistical data filter (RSD at 40%) to the final data matrix, 930 metabolites remained for univariate and multivariate analyses. These metabolites encompassed a wide range of primary and secondary metabolites, including sugars and their derivatives, organic acids, amino acids, lipids, fatty acids, nucleotides, vitamins and cofactors, phenolics, flavonoids, terpenoids, and more, consistent with previous MS-based rice bran metabolome analyses[1,21,22].

In the univariate analysis of the 930 metabolites, 822 showed significant variation ($p < 0.05$) across the different treatment and control groups (T-1, T-4, T-7, and control), based on one-way ANOVA analysis followed by post-hoc analysis using Fisher's LSD (Supplementary Table S2). Metabolite variations between the treatment groups (T-1, T-4, and T-7) and the control were further illustrated using volcano plot analysis. In the comparison of T-1 vs. control, 232 metabolites were upregulated, and 179 were downregulated, using a fold change (FC) threshold of 2.0 and an FDR p-value threshold of 0.05 (Fig. 5a). In T-4 vs. control, 131 metabolites were upregulated and 207 downregulated (Fig. 5b), while the T-7 vs. control comparison revealed 146 upregulated and 212 downregulated metabolites (Fig. 5c). Among the three treatment groups, the T-1 group (300 watts for 3 min) exhibited the highest upregulation and the least downregulation in the overall metabolite profile. This result aligns with the phytochemical and antioxidant analyses, where T-1 showed the highest total phenolic content along with significant increases in total flavonoid and flavonol content. The T-1 samples also exhibited the highest DPPH and ABTS free radical scavenging activities, FRAP and CUPRAC values, and metal chelating activity, alongside significant upregulation in total antioxidant capacity and ascorbic acid content. Therefore, the 300 watts–3 min microwave treatment of DORB may be recommended as the optimal condition for enhancing phytochemicals and antioxidants without adversely impacting the major metabolite profile.

Chemometric analysis using PCA revealed five principal components: PC1 (51.3%), PC2 (22.5%), PC3 (17.6%), PC4 (2.1%), and PC5 (1.6%), which explained the overall metabolic differences between the groups

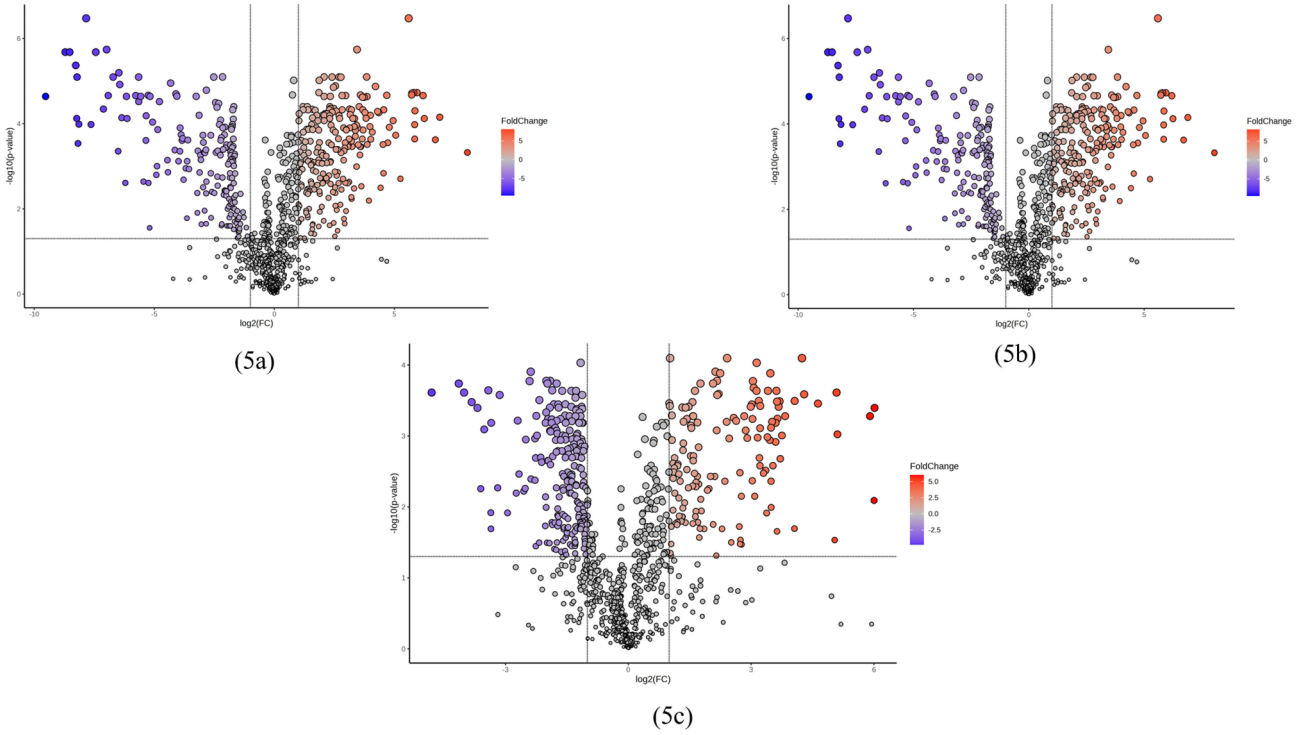

Fig. 5. Volcano plot denotes the univariate analysis of significantly different ($p < 0.05$) metabolites obtained through one-way ANOVA and Post-hoc analysis (Fisher's LSD). The X-axis and Y-axis represent Fold Change (log2FC) and T-test (-log10 p-value) respectively. Shades of red represent significant upregulations whereas the blue shades symbolize significant downregulations. The alterations in metabolites between different treatments and control group have been graphically represented through volcano plot as (**a**) T-1 vs. control group, (**b**) T-4 vs. control group, and (**c**) T-7 vs. control group.

(T-1, T-4, T-7, and control). Metabolites from the different groups formed distinct clusters, indicating significant inter-group variations in the PCA-synchronized 3D scatter plot (Fig. 6a). Similarly, the 2D score plot from sPLS-DA analysis, which incorporated Component 1 (40%) and Component 2 (25.9%), mirrored the pattern observed in PCA, producing clearly distinct group-specific clusters (Fig. 6b). The cluster representing the T-1 group was positioned furthest from the control group, highlighting the substantial upregulation of metabolites in T-1 compared to the control. The top ten metabolites with the highest loading weights in Component 1 are displayed in Fig. 6c. Hierarchical clustering based on group-specific metabolite profiles further demonstrated that samples from the same group clustered together, while samples from different groups formed distinct clades in the dendrogram (Fig. 6d). The T-1 group, with the most upregulated metabolites, appeared in the most distant clade from the control group, consistent with the sPLS-DA results.

Identification and analysis of discriminating metabolites

Supervised OPLS-DA was used to distinguish the metabolic profiles of the treatment groups (T-1, T-4, and T-7) from the control group. The selection criteria for identifying discriminating metabolites were OPLS-DA-derived VIP values ≥ 1, log2FC ≥ 1 or $\leq$ -1, and P-value < 0.05 (Supplementary Table S3). The score plot of the OPLS-DA analysis for T-1 vs. control, T-4 vs. control, and T-7 vs. control revealed significant variation in metabolite profiles, with no overlap between the ellipses representing each group (Fig. 7a and c). Permutation validation indicated no over fitting of the OPLS-DA models: [(T-1 vs. Control: Q2 of 0.986 and R2Y of 1.00); (T-4 vs. Control: Q2 of 0.981 and R2Y of 1.00); (T-7 vs. Control: Q2 of 0.98 and R2Y of 1.00)]. Venn diagram analyses identified 21 common significantly upregulated metabolites across all three microwave-treated groups (T-1, T-4, and T-7) compared to the control, while 29 common metabolites were significantly downregulated in all the treated groups (Fig. 8a and b) (Supplementary Tables S4 and S5). Additionally, the T-1 group had the highest number of unique upregulated metabolites (138) and the fewest unique downregulated metabolites (49).

According to a search of the Pubchem Database (https://pubchem.ncbi.nlm.nih.gov), 220 compounds were identified as lipids among the 930 metabolites in the data matrix. Additionally,143 flavonoids were detected through searching in Arita Lab 6549 Flavonoid Structure Database and the Pubchem Database (https://pubchem.ncbi.nlm.nih.gov). Furthermore, 59 phenolic compounds, 28 terpenoids, 23 sugars and their derivatives, 18 amino acids, and 14 vitamins and cofactors were identified using the Pubchem Database (https://pubchem.ncbi.nlm.nih.gov) searches. The relative abundance of these metabolites was represented by their respective peak areas obtained in LC-HRMS analysis. A one-way ANOVA (P value cutoff: 0.05, and Post Test: Dunnett's Multiple Comparison Testusing GraphPad Prism 5.01) on the cumulative peak areas of metabolites from each chemical class revealed prominent variations in overall lipids, flavonoids, phenolics, terpenoids, sugars and their

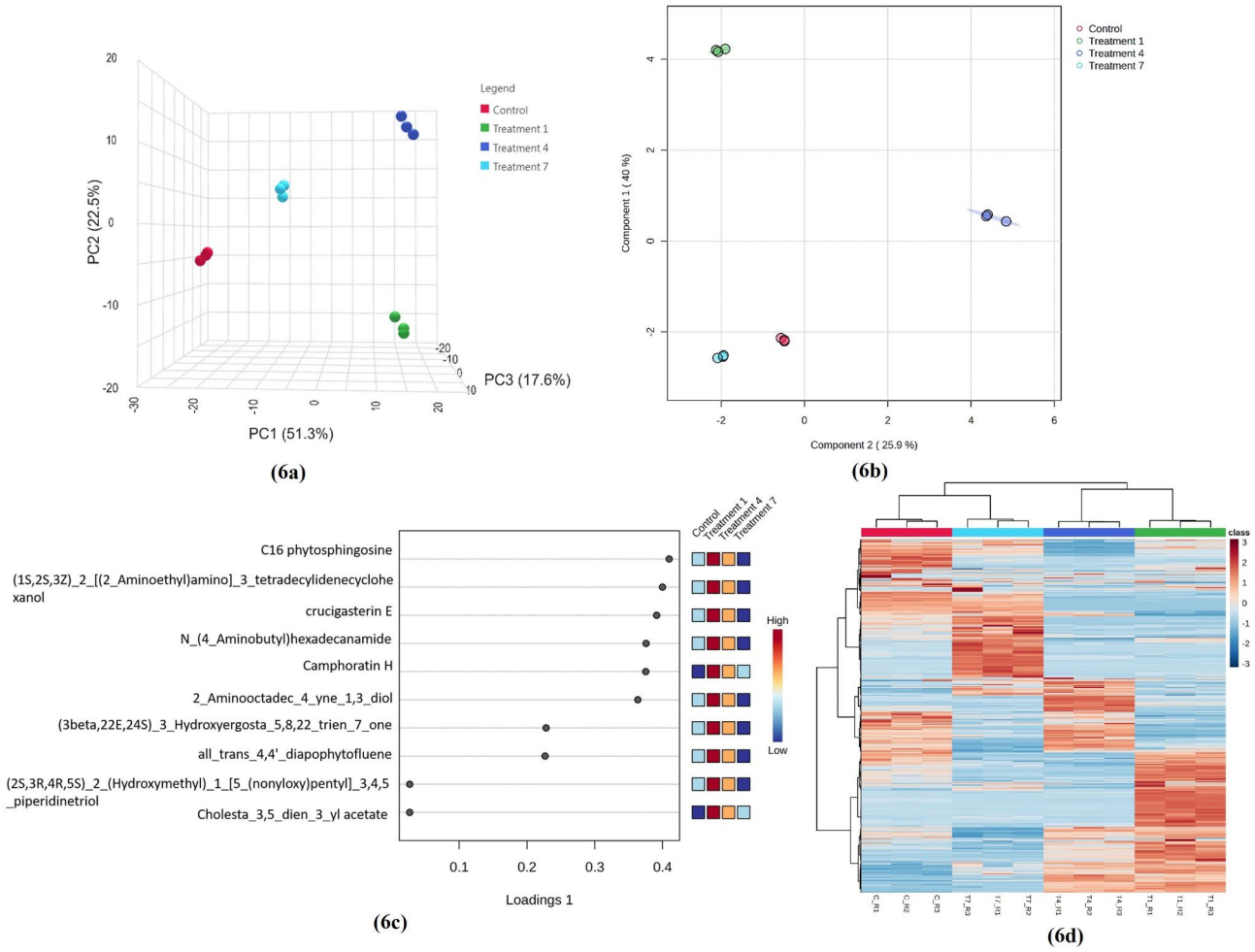

Fig. 6. (**a**) PCA synchronized 3D plot employing PC1 (51.3%), PC2 (22.5%), and PC3 (17.6%) represents groupwise distinct clusters signifying intrinsic variation in the metabolite data set and similarities in variables. (**b**) The 2D score plot of sPLS-DA analysis incorporating Component 1 (40%) and Component 2 (25.9%) yielded segregated clusters of control and different treatment groups maximizing the class discrimination. (**c**) Loading plots of sPLS-DA analysis represents the top ten metabolites with the highest loading weights in the first component. (**d**) The hierarchical clustering analysis of metabolites from control and treated groups depicted in the heat map represents that samples from the same group lied together while different group-specific samples orient them in distant clads in the dendrogram. The heat map was generated by using the MetaboAnalyst 6.0 program (https://new.metaboanalyst.ca).

derivatives, amino acids, vitamins and cofactors among the control and different treatment groups. However, these differences were statistically non-significant ($p < 0.05$) (Fig. 9). Additionally, mass spectrometry-based metabolite analysis indicated that most microwaved samples contained higher amounts of phytoconstituents and primary metabolites compared to control samples, aligning with the results of the phytochemicals and antioxidant analyses. Two-way ANOVA of discriminating metabolites revealed significant ($p < 0.05$) group-wise differences in individual metabolites within each chemical class. PLS-DA analysis identified the major discriminating lipids (VIP score ≥ 2.0), flavonoids (VIP score ≥ 1.5), phenols (VIP score ≥ 1.2), amino acids (VIP score ≥ 0.8), sugars and derivatives (VIP score ≥ 1.0), terpenoids (VIP score ≥ 1.0), and vitamins and cofactors (VIP score ≥ 1.0), highlighting significant ($p < 0.05$) group-wise variations in their relative abundance (Fig. 10). These variations among individual metabolites within a chemical class, based on wattage-time combinations, may be due to differences in the thermal responsiveness of the corresponding components, as suggested by findings in other related studies[8,13,79].

Conclusions

The current study aimed to explore the impact of microwave treatment at various wattage and duration combinations on the phytoconstituents, antioxidant status, ANFs, and metabolite profiles of de-oiled rice bran. The overall findings suggest that shorter microwave exposure led to better retention of phytochemicals and antioxidants, while prolonged exposure at higher wattage decreased most of these bioactive components. The 300 watts for 3 min microwave treatment was found to be optimal for enhancing most antioxidant parameters

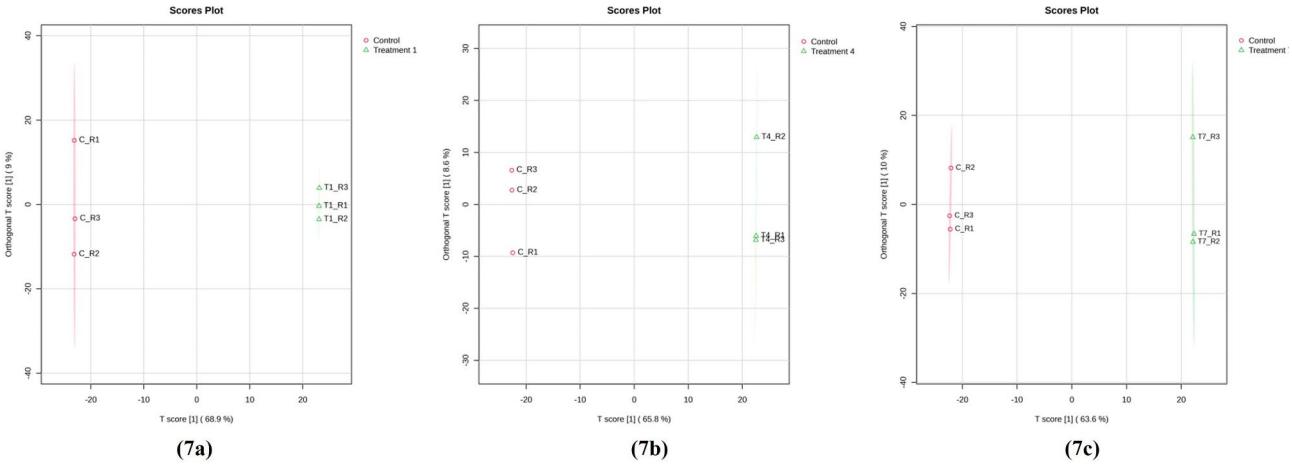

Fig. 7. The score plot of the OPLS-DA analysis between control and different treated groups represents prominent variations in differential metabolites (VIP values ≥ 1, ≥ 1 log2FC ≤ -1 and P-value < 0.05). (**a**) Score plot for T-1 vs. control group where X-axis and Y-axis represents T score (68.9%) and orthogonal T score (9%) respectively, (**b**) Score plot for T-4 vs. control group where X-axis and Y-axis represents T score (65.8%) and orthogonal T score (8.6%) respectively, (**c**) Score plot for T-7 vs. control group where X-axis and Y-axis represents T score (63.6%) and orthogonal T score (10%) respectively.

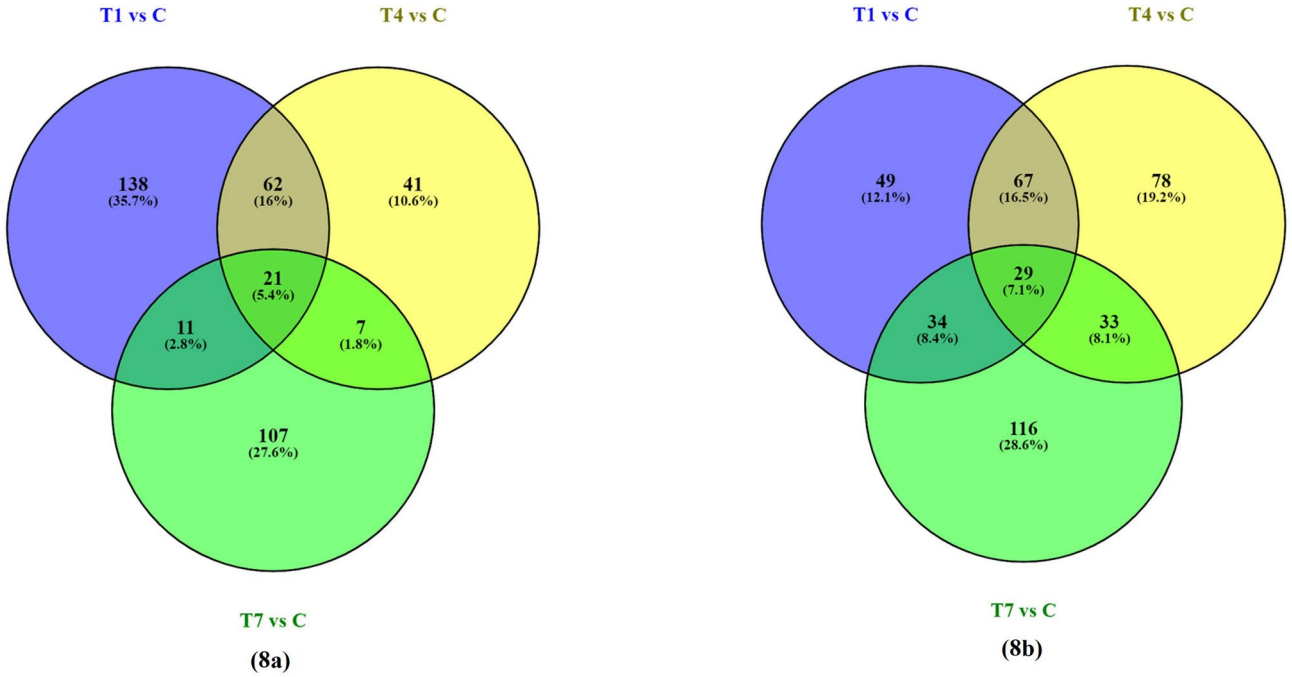

Fig. 8. Venn diagram analysis of discriminating metabolites based on OPLS-DA derived VIP values ≥ 1, ≥ 1 log2FC ≤ -1 and P-value < 0.05 depicting coinciding metabolites in different treatment. (**a**) Common upregulated metabolites in all the treated groups with respect to the control, (**b**) common downregulated metabolites in all the treated groups with respect to the control.

and total phenolic content in de-oiled rice bran. The ANFs exhibited treatment-specific upregulations and downregulations, with no single universal power-time combination effective in reducing all three ANFs studied.

Various primary and secondary metabolites were identified in both control and microwave-treated de-oiled rice bran samples through LC-HRMS analysis. Significant metabolite variation was observed between microwave-treated and control samples, with the 300 watts for 3 min treatment resulting in the most upregulated and fewest downregulated metabolites, consistent with the phytochemical and antioxidant analyses. Although overall levels of each metabolite class showed non-significant differences between control and microwave-treated groups, several individual metabolites within each class exhibited significant group-wise variations. The

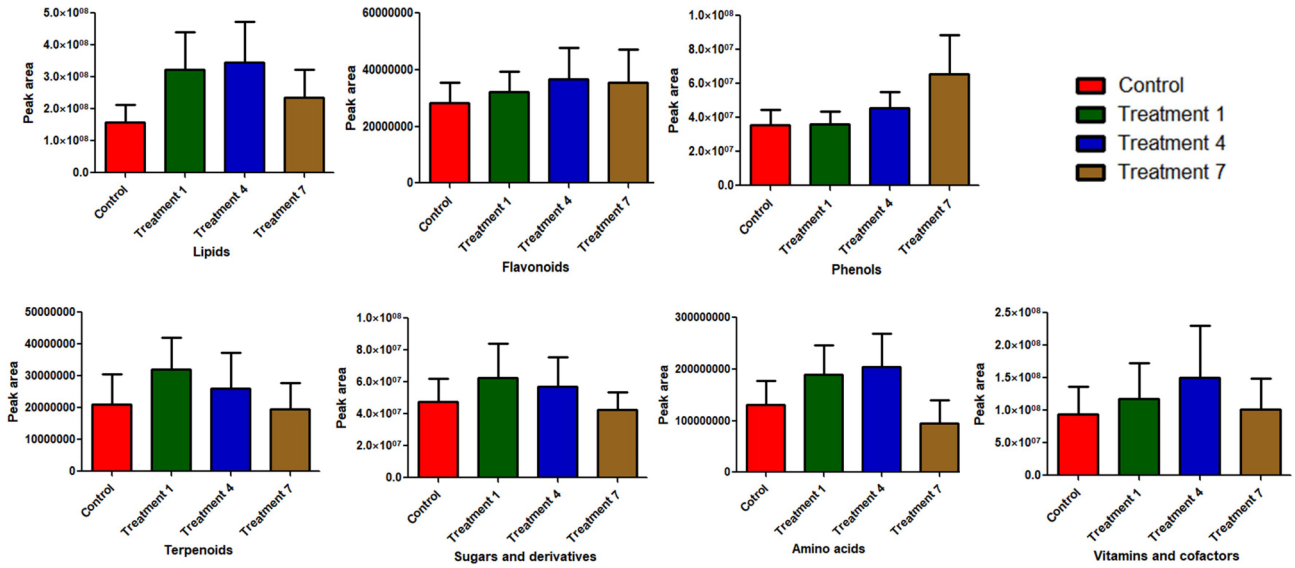

Fig. 9. One-way ANOVA analysis illustrates metabolic content in different treated groups with respect to the control using P-value cut off < 0.05 and Dunnett's Multiple Comparison Post Test through GraphPad Prism 5.01.

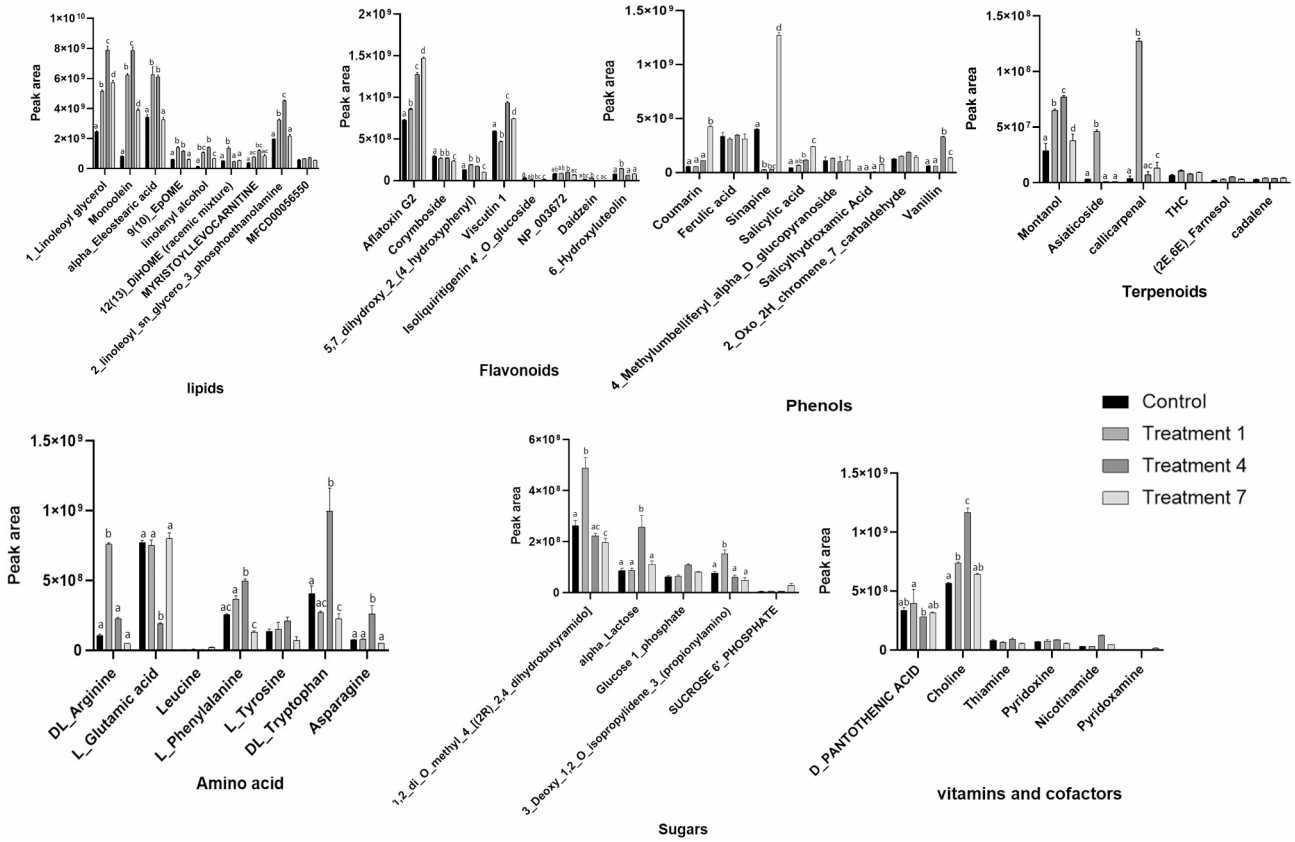

Fig. 10. Two-way ANOVA analysis using GraphPad Prism 5.01 depicting significant ($p < 0.05$) group-wise difference in major discriminating metabolites. Abundance of individual metabolites differed significantly between the groups are expressed by using different superscript letters (a–d).

current findings highlight the importance of optimized microwave treatment with appropriate wattage and time combinations to enhance the phytochemical and antioxidant status, as well as improve the metabolite profile, in de-oiled rice bran. However, in-vivo studies are necessary to confirm better nutrient utilization from the microwaved feed samples.

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

Mayukh Ghosh and Rajesh Kumar: Contributed to conceptualize and design the study. Alonkrita Chowdhury, Alla Yaswanth Naveen Kumar, M. S. Mahesh, Abhishek Kumar Singh, Pavan Kumar Yadav and Mayukh Ghosh: Experimentation, data collection, and data analysis. Vivek Kumar Maurya: Liquid Chromatography-High Resolution Mass Spectrometry. Alonkrita Chowdhury and Mayukh Ghosh: Manuscript preparation and editing.

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The authors declare no competing interests.

Ethical approval

This article does not contain any experimentation involving human subjects or animals carried out by any of the authors.

Additional information

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Correspondence and requests for materials should be addressed to M.G.