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# Optimization of Extraction Conditions to Enhance Polyphenol Content and Antioxidant Activity İn *Orthosiphon Aristatus* Leaves

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**ABSTRACT.** *Orthosiphon aristatus* (Java tea) is a medicinal plant known for its high polyphenol content and antioxidant properties. Unlike previous studies that focused solely on solvent type or temperature, this study simultaneously optimizes temperature and pH extraction parameters to achieve maximal bioactive compound recovery. This study aimed to optimize extraction parameters—temperature and pH—to enhance polyphenol yield and antioxidant activity in methanolic extracts of Java tea leaves. Samples were extracted at temperatures ranging from 30 °C to 80 °C (pH 7) and pH levels from 2 to 6 (at room temperature). Total phenolic content (TPC) and total flavonoid content (TFC) were determined to quantify polyphenol levels, and antioxidant activity was evaluated using four complementary in vitro assays: DPPH, ABTS, FRAP, and CUPRAC. The highest TPC (9.68 mg GAE/g DW) and TFC (6.53 mg QE/g DW) were observed at 70 °C and 80 °C, respectively. Maximum antioxidant activities were observed at 70–80 °C and pH 2–3, with peak values of 102.71 μmol TE/g DW (ABTS), 101.9 μmol TE/g DW (FRAP), and 56.12 μmol TE/g DW (CUPRAC). A strong correlation was found between phenolic content and antioxidant capacity. These findings highlight the critical role of extraction conditions in maximizing the biofunctional potential of *O. aristatus* and support its application in pharmaceutical and nutraceutical formulations. The study provides a scientific basis for developing standardized extraction protocols to improve functional ingredient consistency in herbal product development.

**Keywords:** Antioxidant activity, extraction optimization, *Orthosiphon aristatus*, pH effect, temperature

## INTRODUCTION

Over the past few decades, there has been a global shift in disease prevalence from infectious diseases to chronic degenerative conditions. This epidemiological transition has prompted heightened interest in understanding the underlying mechanisms of chronic diseases, with oxidative stress emerging as a central pathogenic factor. Oxidative stress is caused by an imbalance between the excessive generation of reactive oxygen species (ROS) and the body's antioxidant defense systems, leading to cumulative damage at the cellular and molecular levels. Numerous studies have established that oxidative stress contributes to the pathogenesis of a wide range of degenerative diseases, including cardiovascular disorders, type 2 diabetes mellitus, neurodegenerative diseases such as Alzheimer's and Parkinson's, certain cancers, and dysfunctions of the liver and kidneys (Baccouri & Rajhi, 2021; Korovesis et al., 2023; Prata et al., 2024; Kumari, 2023).

To counteract oxidative stress and mitigate its deleterious consequences, exogenous antioxidants are often required to complement endogenous systems. Although synthetic antioxidants are readily available and often highly potent, they are increasingly associated with toxicological concerns and bioavailability limitations, raising questions about their long-term safety and therapeutic applicability (Zahra et al., 2024; Prata et al., 2024). Consequently, scientific and industrial communities have turned to natural antioxidants derived from plants, especially polyphenols and flavonoids, which are considered safer and offer broader health-promoting properties with fewer side effects (Hassanpour & Doroudi, 2023; Baccouri & Rajhi, 2021; Nardini, 2022; Zahra et al., 2024).

Among the wide array of medicinal plants investigated for their antioxidant potential, *Orthosiphon aristatus*, commonly known as Java tea, has garnered particular interest. This plant is native to Southeast Asia and has been traditionally used for its diuretic, anti-inflammatory, and antidiabetic properties. Its pharmacological activity has been attributed to the high concentrations of polyphenolic compounds such as rosmarinic acid, caffeic acid, sinensetin, eupatorin, and cirsimaritin (Guo et al., 2019; Kusmala et al., 2023; Sari et al., 2023). In addition, *O. aristatus* contains orthosiphol A, a diterpene compound that has demonstrated strong α-glucosidase inhibitory activity, indicating its

potential role in glycemic regulation and diabetes management (Damsud et al., 2014; Wang et al., 2022). These bioactive constituents contribute synergistically to the plant's antioxidant, anti-inflammatory, and hypoglycemic effects (Rekasih et al., 2021; Faramayuda et al., 2025).

Despite its promising phytochemical profile, the practical application of *O. aristatus* in nutraceuticals and pharmaceuticals is challenged by the need for effective extraction methods that ensure both high yield and preservation of antioxidant bioactivity. The efficiency of extracting polyphenols from plant matrices is highly dependent on process parameters such as solvent type, extraction temperature, and pH. Elevated temperatures generally improve solubility and facilitate the breakdown of plant cell walls, thereby enhancing polyphenol release (Vuong et al., 2010). However, excessive heat may lead to the degradation or oxidation of thermolabile compounds such as certain phenolic acids and flavonoids (Artika et al., 2018; Hwang & Nhuan, 2014). Likewise, solvent pH influences polyphenol solubility, ionization state, and oxidation–reduction balance, which can alter both yield and bioactivity (Antony & Farid, 2022; Hamad & Hartanti, 2023).

Given the significant impact of these parameters, the optimization of extraction conditions is essential to maximize the recovery of functional compounds while maintaining their stability and biological effectiveness. However, most prior studies have examined extraction parameters such as temperature or pH in isolation. For example, studies have either focused on solvent composition and concentration, or temperature gradients, without considering interactive effects. Moreover, limited attention has been given to how pH modulation might affect the solubility and oxidative stability of key polyphenolic compounds (Motikar et al., 2020; Purnomo et al., 2023).

Emerging research highlights the need for more integrative approaches to extraction optimization. Recent findings suggest that the combination of high temperature and acidic pH could be particularly effective in enhancing polyphenol extraction, provided degradation is minimized (Mai et al., 2020; Faramayuda et al., 2025). Nevertheless, few studies have systematically investigated these parameters together in a controlled and comparative manner, which restricts the ability to establish standardized protocols for industrial or pharmaceutical use. Addressing these knowledge gaps is essential for improving the consistency, efficacy, and scalability of *O. aristatus* as a functional ingredient in health-promoting formulations.

In light of these considerations, the present study was designed to evaluate the combined effects of extraction temperature and pH on the total phenolic and flavonoid content, as well as antioxidant activity, of methanolic extracts from *O. aristatus* leaves. By simultaneously optimizing two critical parameters under controlled experimental conditions, this study aims to establish a scientific foundation for the development of standardized extraction protocols. Furthermore, it seeks to validate the correlation between polyphenol content and antioxidant activity using four complementary antioxidant assays: DPPH, ABTS, FRAP, and CUPRAC. The findings are expected to support the practical application of *O. aristatus* in the formulation of high-quality nutraceuticals and plant-based therapeutics.

## EXPERIMENTAL SECTION

### Chemicals and Reagents

All analytical grade chemicals were used as obtained from the suppliers. Methanol (pro-analysis), Folin–Ciocalteu reagent (a mixture of phosphomolybdic-phosphotungstic acid complexes), gallic acid, ammonium acetate buffer, $CuCl_2$, $K_2S_2O_8$, neocuproine, $AlCl_3$, $FeCl_3$, HCl, NaOH, and quercetin were obtained from Merck-Millipore (Darmstadt, Germany). Trolox, ABTS, DPPH, sodium carbonate, and glacial acetic acid were procured from Sigma-Aldrich (St. Louis, MO, USA). Additional reagents such as 2,4,6-tripyridyl-s-triazine (TPTZ) and acetic acid were purchased from Sisco Research Laboratories Pvt. Ltd. (Maharashtra, India).

### Sample Preparation

Leaves of *O. aristatus* were sourced from the Tropical Biopharmaca Research Center at IPB University, Bogor, Indonesia. The plant material was dried at 50 °C for 48 hours and then ground to a fine powder, which was subsequently passed through an 80-mesh sieve for uniformity.

### Extraction Conditions and Design

Five grams of the dried Java tea leaf powder were extracted using maceration with 50 mL of 50% methanol in a ratio of 1:10 (w/v) for 24 hours at various temperatures (30, 40, 50, 60, 70, and 80 °C) at neutral pH in a water bath shaker (Memmert, Schwabach, Germany). For pH optimization, maceration was conducted at room temperature using 0.1 M HCl to achieve the desired pH levels (2, 3, 4, 5, and 6). Post-extraction, the mixture was filtered through Whatman No.1 filter paper and adjusted to a final volume of 50 mL (0.1 g/mL extract concentration).

### Total Phenolic Content (TPC)

TPC was determined using the Folin–Ciocalteu method with gallic acid as the standard, as described by Khumaida et al. (2019). A standard curve (25–100 ppm gallic acid) was prepared. In a 96-well microplate, 20 µL of extract was added to 120 µL of 10% Folin–Ciocalteu reagent and incubated for 5 minutes in the dark. Then, 80 µL of 10% $Na_2CO_3$ was added, followed by 30 minutes of incubation at room temperature. Absorbance was measured at 750 nm using a SPECTROstarNano spectrophotometer (BMG LABTECH, Germany). Results were expressed as mg

gallic acid equivalents per gram dry weight (mg GAE/g DW).

### Total Flavonoid Content (TFC)

TFC was assessed using a modified method from Khumaida et al. (2019) with quercetin as the standard (100–900 ppm). In a 96-well plate, 10 µL of extract was mixed with 60 µL methanol, 10 µL of 10% $AlCl_3$, 10 µL of 1 M glacial acetic acid, and 120 µL distilled water. After 30 minutes of incubation at room temperature, absorbance was recorded at 415 nm. Results were expressed in mg quercetin equivalents per gram dry weight (mg QE/g DW).

### DPPH Radical Scavenging Assay

DPPH antioxidant capacity was determined based on Arista et al. (2022), using Trolox as standard (10–50 µM). In a 96-well plate, 100 µL of extract or Trolox solution was added to 100 µL of 125 µM DPPH solution. After 30 minutes of incubation in the dark, absorbance was measured at 517 nm. Results were expressed in µmol Trolox equivalents per gram dry weight (µmol TE/g DW).

### ABTS Radical Scavenging Assay

Following Nurcholis et al. (2022), ABTS solution was mixed with potassium persulfate and incubated overnight. In a 96-well plate, 20 µL of extract or Trolox (50–500 µM) was added to 180 µL of ABTS solution. After 6 minutes incubation in the dark, absorbance was read at 734 nm. Results were reported as µmol TE/g DW.

### FRAP Assay

FRAP capacity was assessed as per Arista et al. (2022) using Trolox standards (100–700 µM). A mixture of 10 µL sample and 300 µL FRAP reagent was incubated for 30 minutes. Absorbance was measured at 593 nm. Results were expressed in µmol TE/g DW.

### CUPRAC Assay

Following Nurcholis et al. (2021), CUPRAC antioxidant activity was measured by mixing 50 µL of sample or Trolox (50–600 µM) with 50 µL $CuCl_2$, 50 µL neocuproine, and 50 µL ammonium acetate buffer. After 30 minutes, absorbance was read at 450 nm. Results were expressed in µmol TE/g DW.

### Statistical Analysis

All data were expressed as mean ± standard deviation (n = 3) and analyzed using IBM SPSS version 25. One-way ANOVA and Tukey's post hoc test ($\alpha$ = 0.05) were used to identify significant differences. Pearson correlation analyses between polyphenol content and antioxidant activity were conducted using GraphPad Prism version 9.

## RESULTS AND DISCUSSION

### Effect of Extraction Temperature and pH on Phenolic and Flavonoid Contents

As shown in **Figure 1**, the total phenolic content (TPC) and total flavonoid content (TFC) of *O. aristatus* leaf extracts were significantly influenced by extraction temperature. The TPC increased with temperature and reached its maximum value of 9.68 mg GAE/g DW at 70 °C, followed by a slight decline at 80 °C. In contrast, the TFC continued to increase and peaked at 80 °C with a value of 6.53 mg QE/g DW. This difference indicates that certain flavonoid compounds exhibit higher thermal stability than some phenolic acids, which are more susceptible to heat-induced degradation. Elevated extraction temperatures enhance the solubility of phenolic and flavonoid compounds, promote disruption of plant cell wall structures, and reduce solvent viscosity, thereby improving mass transfer efficiency from the plant matrix into the solvent (Liao et al., 2021; Supasatyankul et al., 2022; Safrina et al., 2022). These mechanisms explain the enhanced recovery of polyphenols observed at 70–80 °C. Nevertheless, temperatures exceeding optimal levels may negatively affect compound stability. Several major phenolic constituents, including chlorogenic acid and gallic acid, have been reported to undergo significant thermal degradation at temperatures above 60 °C (Panyatip et al., 2022; Vargas-Sánchez et al., 2021), which likely accounts for the reduction in TPC at 80 °C. These findings are consistent with previous studies demonstrating that moderate heating improves extraction efficiency, whereas excessive thermal exposure can compromise the integrity of thermolabile bioactive compounds. Therefore, precise control of extraction temperature is crucial to achieving a balance between maximizing polyphenol yield and preserving antioxidant stability in *O. aristatus* extracts intended for nutraceutical and pharmaceutical applications.

The pH of the extraction solvent significantly influenced both TPC and TFC in *O. aristatus* leaf extracts, as shown in **Figure 2**. The highest values were observed at pH 2, with TPC reaching 5.62 mg GAE/g DW and TFC at 1.79 mg QE/g DW. This enhancement under acidic conditions can be attributed to the protonation of hydroxyl groups in phenolic and flavonoid structures, which improves solubility and promotes stronger interactions with the solvent (Mikucka et al., 2022; Zhai et al., 2024). In addition, low pH facilitates the hydrolysis of ester bonds in phenolic glycosides and stabilizes these compounds by minimizing oxidative degradation (Motikar et al., 2020; Mai et al., 2020). In contrast, TPC and TFC declined significantly above pH 3, likely due to increased oxidation, polymerization, or precipitation of phenolics in their deprotonated state, which reduces solubility and structural stability (Li et al., 2025). Although some reports suggest that pH values around 5 may optimize antioxidant activity due to reduced polyphenol oxidase activity and improved compound stability (Lee et al., 2025), this study demonstrates that for *O. aristatus*, maximum extraction efficiency occurs under more acidic conditions. Compared to a previous study using Java tea genotypes without pH control,

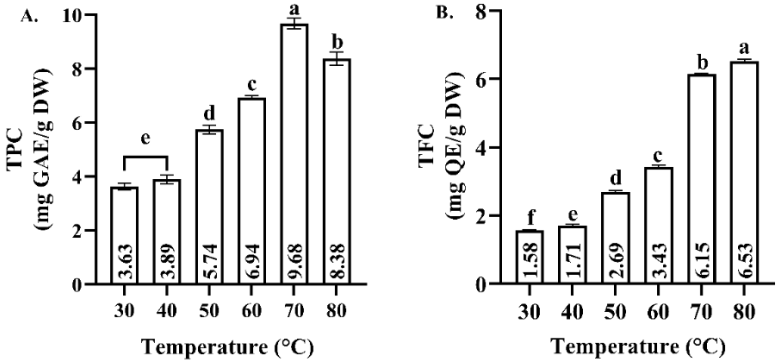


**Figure 1.** Total phenolic content (A) and total flavonoid content (B) in methanolic extracts of *O. aristatus* (Java tea) leaves extracted at different temperatures (30–80 °C). Different letters (a–f) indicate significant differences according to Tukey's post hoc test at $p < 0.05$.

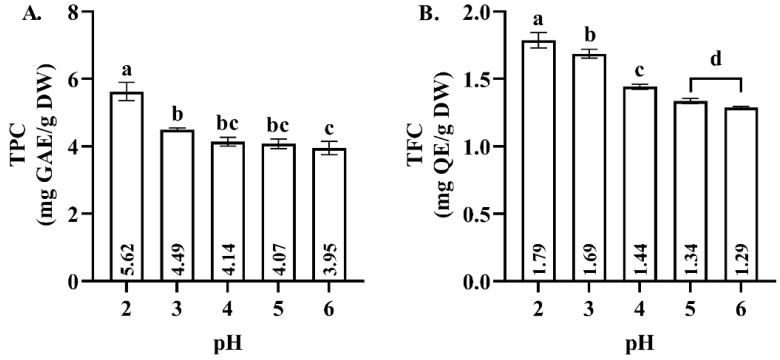


**Figure 2.** Total phenolic content (**A**) and total flavonoid content (**B**) in methanolic extracts of *O. aristatus* leaves at various pH levels (2–6). Different letters (a–d) represent significant differences based on Tukey's post hoc test ($p < 0.05$).

which reported TPC and TFC ranges of 1.79–8.46 mg GAE/g DW and 0.20–1.46 mg QE/g DW, respectively (Bovani et al., 2024), the current results indicate superior yields. These findings reinforce the importance of pH control as a critical parameter in designing extraction protocols aimed at maximizing polyphenol yield and stability in *O. aristatus*.

## Antioxidant Activity under Different Extraction Conditions

The antioxidant activity of *O. aristatus* leaf extracts, evaluated through DPPH, ABTS, FRAP, and CUPRAC assays, showed significant variation across different extraction temperatures and pH conditions (**Figures 3** and **4**). The highest DPPH and ABTS values were recorded at 80 °C, with activities reaching 8.95 μmol TE/g DW and 102.71 μmol TE/g DW, respectively. In contrast, maximum FRAP and CUPRAC activities were observed at 70 °C, with values of 101.9 μmol TE/g DW and 56.12 μmol TE/g DW. These discrepancies are closely related to the underlying mechanisms of each assay. While DPPH and ABTS evaluate radical scavenging through hydrogen atom or electron donation to stabilize free radicals, FRAP and CUPRAC measure reducing power and electron transfer capability (Munteanu et al., 2021). The rise in antioxidant activity with increased temperature is largely attributable to enhanced extraction of phenolic compounds, facilitated by increased solubility and mass transfer at elevated thermal conditions (Ickovski et al., 2022; Irakli et al., 2021). These results corroborate prior evidence suggesting that phenolic-rich extracts exhibit high antioxidant potential due to their redox-active hydroxyl groups and conjugated structures (Moreira et al., 2017).

Under varying pH conditions, DPPH activity was highest at pH 3, with slightly lower but comparable values at pH 2 and 4. FRAP and CUPRAC assays showed optimal results at lower pH (2–3), indicating that acidic environments favor the extraction and stabilization of antioxidant constituents. Conversely, ABTS activity peaked at pH 6, likely due to the greater stability of the ABTS radical in near-neutral conditions, whereas its performance decreases in strongly acidic environments because of radical instability and spectral degradation (Tang & Zhuang, 2015; Ferri et al., 2013). These findings highlight the pH-dependent behavior of different assay systems and their interactions with extracted compounds. As the ionization state of phenolics changes with pH, their chemical reactivity and interaction with assay radicals also shift (Ronca et al., 2024; Uwineza et al., 2021). Additionally, structural features of individual phenolic compounds such as rutin and chlorogenic acid greatly influence assay responses, with certain compounds exhibiting superior scavenging or reducing power depending on the test used (Janiak et al., 2023; Mihai et al., 2025).

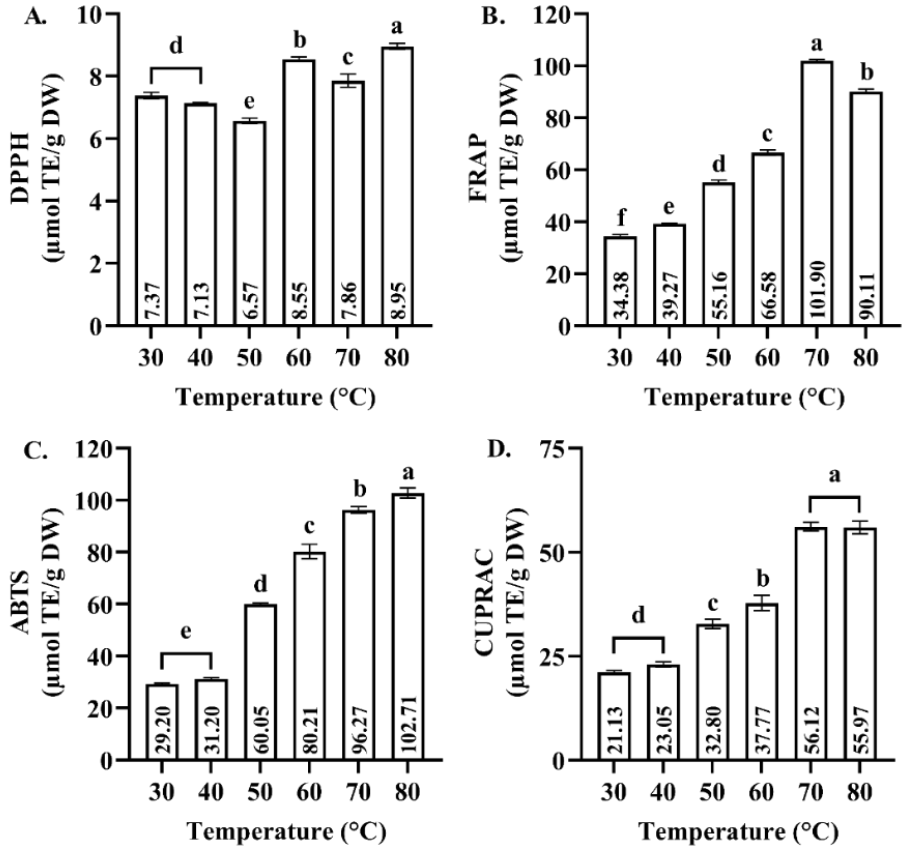


Figure 3. Antioxidant activity of *O. aristatus* leaf extracts under different temperature treatments (30–80 °C) evaluated using: DPPH (A), FRAP (B), ABTS (C), and CUPRAC (D) assays. Different letters (a–f) indicate statistically significant differences based on Tukey's post hoc test ($p < 0.05$).

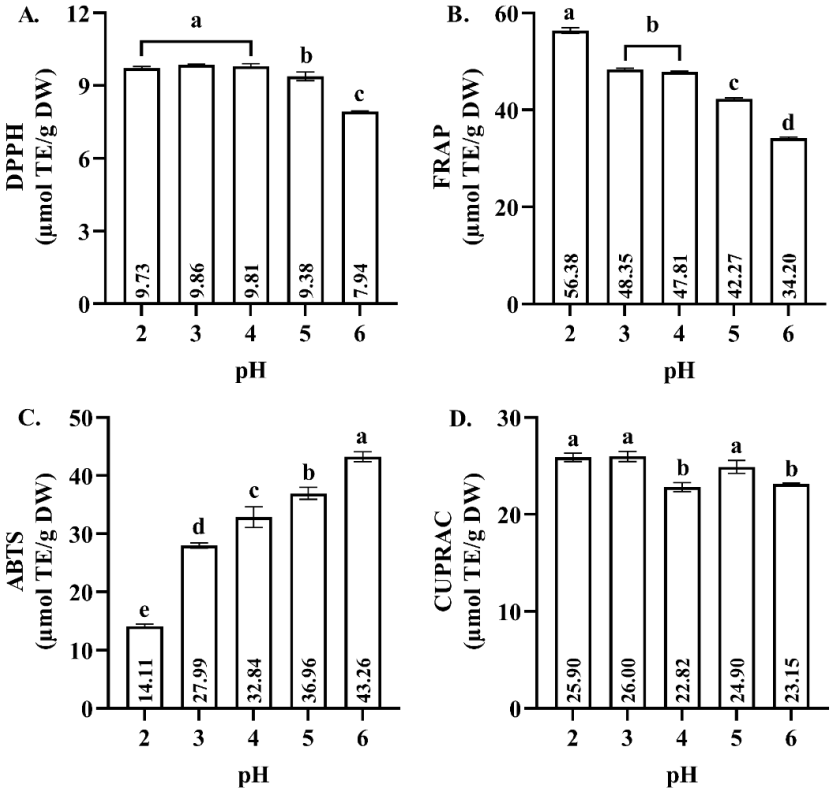


Figure 4. Antioxidant activity of *O. aristatus* leaf extracts at varying pH levels (2–6) measured using DPPH (A), FRAP (B), ABTS (C), and CUPRAC (D) assays. Different letters (a–e) denote statistically significant differences based on Tukey's post hoc test ($p < 0.05$).

Compared to previous studies using conventional or non-optimized methods (Chandra et al., 2023; Ho et al., 2014), the antioxidant activities recorded in this study were markedly higher, reflecting improved extraction efficiency through simultaneous optimization of temperature and pH. Although CUPRAC activity was slightly lower than that reported in phenotypic variation studies (Nurcholis et al., 2022), this variation may stem from differences in genotype, plant age, or solvent composition. Ultimately, these results affirm that carefully optimized extraction conditions are vital for maximizing antioxidant recovery from *O. aristatus*, and they offer a scientific basis for developing standardized procedures in herbal product formulation.

## Correlation Analysis

Correlation analysis revealed substantial relationships between polyphenol content and antioxidant activity in *O. aristatus*extracts under both temperature and pH variations (**Figures 5** and **6**). Under temperature treatments, strong positive correlations were observed between TPC and FRAP (r = 0.99), TFC and FRAP (r = 0.97), as well as between TPC and CUPRAC (r = 0.97) and TFC and CUPRAC (r = 0.99). These results suggest that electron transfer-based assays such as FRAP and CUPRAC are highly responsive to the total electron-donating capacity of phenolic compounds, particularly those with polyhydroxylated structures that facilitate redox activity (Apak et al., 2016; Rainatou et al., 2023). In contrast, DPPH showed only moderate correlations with TPC (r = 0.61) and TFC (r = 0.69), indicating that steric accessibility, radical type, or specific compound reactivity may limit its sensitivity (Sadowska-Bartosz & Bartosz, 2022; Akpotu et al., 2023).

Under pH variation, ABTS displayed a strong negative correlation with both TPC and TFC (r = –0.93), likely due to the known instability of ABTS radicals in acidic media, which may compromise accurate measurement of scavenging activity (Ferri et al., 2013). Meanwhile, FRAP and CUPRAC maintained high positive correlations with polyphenol content under acidic conditions, underscoring their robustness in such environments. Additionally, the strong correlation between TPC and TFC under both temperature (r = 0.95) and pH (r = 0.85) conditions supports the notion that flavonoids constitute a major subclass within the broader group of phenolic compounds (Singh et al., 2016; Seleshe et al., 2022). Similar findings have been widely reported in the literature across different plant species, where TPC and TFC are consistently linked with antioxidant performance across multiple assay types (Al-Hajj, 2021; Puangpronpitag et al., 2021; Monteiro et al., 2022).

For instance, studies on papaya seed and wild plant extracts reported near-perfect correlations (r ≈ 0.999) between phenolic content and antioxidant activity across DPPH, ABTS, and FRAP assays (Al-Hajj, 2021; Seleshe et al., 2022). These findings emphasize that both the concentration and chemical structure of polyphenolic compounds significantly affect antioxidant performance, further validating the use of multi-assay approaches for comprehensive evaluation (Elejalde et al., 2022). The current study aligns with this broader evidence, reinforcing the pivotal role of phenolic and flavonoid compounds in determining antioxidant capacity and the necessity of optimized extraction strategies to capture their full functional potential.

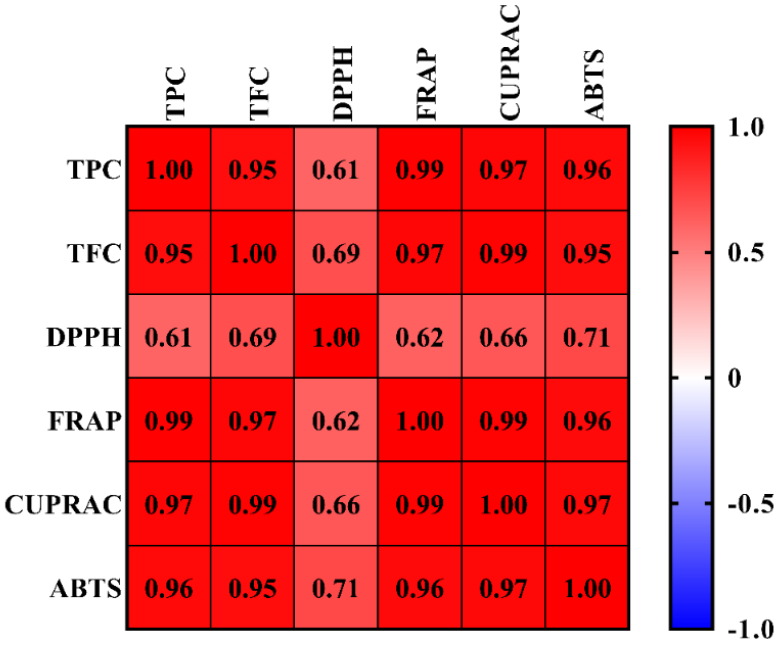


**Figure 5.** Correlation matrix of polyphenol content and antioxidant capacity of methanol extract of Java tea leaves with different temperature treatments.

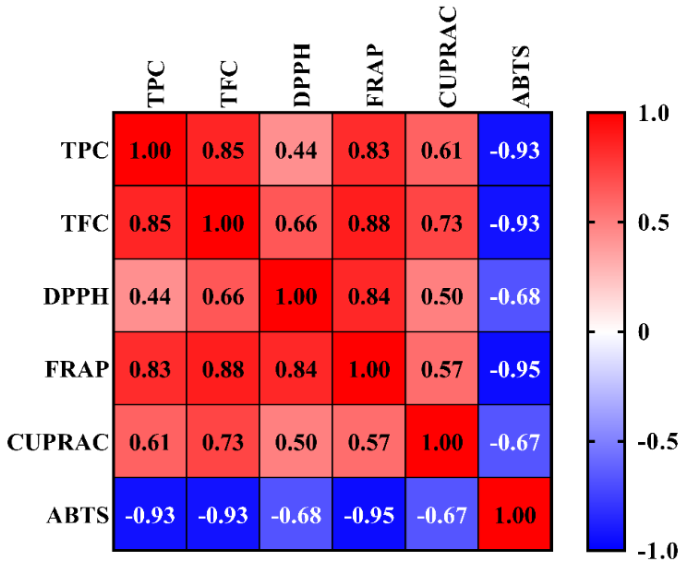


**Figure 6.** Correlation matrix of polyphenol content and antioxidant capacity of methanol extract of Java tea leaves with different pH treatments.

## CONCLUSIONS

This study demonstrated that extraction temperature and pH significantly affect the phenolic and flavonoid contents as well as the antioxidant capacities of *O. aristatus* leaf extracts. Optimal results were observed at higher temperatures (70–80 °C) and acidic pH levels (2–3), leading to increased polyphenol yield and antioxidant activity, which are essential for ensuring extract quality and consistency. The correlation analysis confirmed a strong relationship between polyphenol content and antioxidant performance, particularly in FRAP and CUPRAC assays. These findings highlight the critical role of extraction parameters in enhancing the functional properties of Java tea and provide a scientific foundation for developing standardized protocols for industrial-scale extraction in nutraceutical and pharmaceutical formulations.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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