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Antioxidant Potential Of Endophytic Fungal Extracts Isolated From Breonadia Salicina Assessed By DPPH And FRAP Assays

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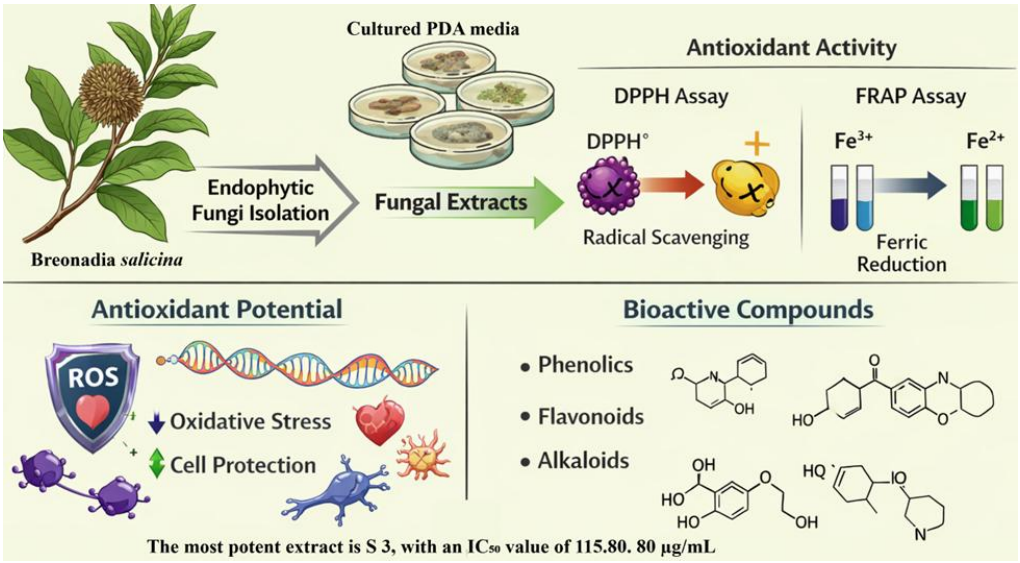
Abstract
Endophytic fungi are increasingly recognised as prolific producers of structurally diverse secondary metabolites with significant pharmacological potential. In the quest for novel bioactive agents from natural sources, this study investigates the antioxidant capacity of endophytic fungi isolated from the medicinal plant Breonadia salicina. Fungi were isolated from surface- sterilised leaf and stembark tissues and cultured on PDA media. Ethyl acetate extracts of the fungal isolates were screened for antioxidant activity using the DPPH radical scavenging assay, with IC_{50} values benchmarked against ascorbic acid (52. 73 µg/mL). FRAP analysis indicated a concentration-dependent ferric reducing capacity, with the same extracts demonstrating superior electron- donating ability. The strong correlation between DPPH and FRAP results suggests that the antioxidant activity of the most active extracts is mainly mediated through redox- based mechanisms. Phytochemical screening and chromatographic profiling (TLC, HPLC) were performed to assess metabolite richness and diversity. Seventeen fungal extracts displayed a wide spectrum of antioxidant activity. The most potent extract, S 3, exhibited an IC_{50} of 115.80. 80 µg/mL, followed by B 3 (178. 19 µg/mL) and L 1 (213. 98 µg/mL), classifying them as strong scavengers. These values indicate a high concentration of antioxidant compounds, likely phenolics, flavonoids, and triterpenoids. Moderate activity was recorded for S 2 and S 7, while B 2 and others showed weak or negligible effects. The chemical profile of active extracts revealed metabolite signatures consistent with known antioxidant scaffolds. This study confirms that endophytic fungi residing in B. salicina constitute a rich and underexplored reservoir of bioactive secondary metabolites. The antioxidant potency of selected isolates, particularly S 3, supports their candidacy for further compound isolation, structural elucidation, and pharmacological evaluation. These findings reinforce the importance of endophyte- focused bioprospecting as a sustainable and productive strategy for natural product drug discovery.

Keywords: *Endophytic fungi; Breonadia salicina; Antioxidant activity; DPPH; FRAP; Secondary metabolites*

Graphical Abstract



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I. Introduction

The burden of chronic diseases is rapidly increasing worldwide. Diet and nutrition play vital roles in promoting and maintaining good health throughout life. Physiological and biochemical changes in the human body can lead to an overproduction of free radicals, causing oxidative damage to biomolecules such as lipids, proteins, and DNA (Jayathilake et al., 2016). Free radicals produced during aerobic metabolism participate in regulatory processes like cell proliferation, apoptosis, and gene expression. However, when produced in excess, free radicals can weaken the body's antioxidant defence system, damaging essential biomolecules by oxidising membrane lipids, proteins, carbohydrates, DNA, and enzymes (Pisoschi et al., 2016). These highly reactive molecules initiate chain reactions that result in cellular damage (Lawal et al., 2017). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's capacity to neutralise them. Persistent oxidative stress has been linked to many chronic and degenerative diseases, including cancer, cardiovascular conditions, diabetes, neurodegenerative disorders, and inflammatory states (Pisoschi & Pop, 2015). Antioxidants are free radical scavengers that donate electrons to electron-deficient free radicals, thus neutralising them. These free radicals include reactive oxygen species (ROS), such as the superoxide anion radical and hydroxyl radical, hydrogen peroxide, and singlet oxygen, produced from metabolism, as well as reactive nitrogen species like nitric oxide and peroxynitrite (Nyero et al., 2023). Antioxidants halt this chain of reactions by removing free radical intermediates, thus preventing further oxidation reactions (Lawal et al., 2017). Although synthetic antioxidants have been used to reduce oxidative damage, concerns about their safety and long-term toxicity have increased interest in natural, sustainable sources of antioxidants (Prior et al., 2005). Plant phenols have attracted scientific interest for decades, particularly for their health benefits. Herbs have been utilised as food and medicine for centuries.

Over the past twenty years, there has been a significant resurgence in the use of medicinal plant products and a keen interest in "nutraceuticals" or "functional foods," where phytochemicals potentially offer long-term health benefits or medicinal effects (Jayathilake et al., 2016). Endophytic fungi are microorganisms residing within the internal tissues of plants without causing harm. These fungi are of substantial scientific interest because of their ability to produce structurally diverse secondary metabolites with a broad spectrum of biological activities (Strobel & Daisy, 2003; Hyde et al., 2019). Many endophytic fungal metabolites demonstrate potent antioxidant properties, often comparable to or exceeding those of compounds directly derived from their host plants. This biosynthetic ability is believed to result from long-term co-evolution between endophytes and their hosts, encouraging the production of metabolites involved in stress tolerance and defence (Zhang et al., 2006; Newman & Cragg, 2020). Medicinal plants are especially valuable ecological niches for endophytic fungi; their complex phytochemical environments influence endophytic metabolic pathways, leading to the production of phenolics, flavonoids, terpenoids, polyketides, and alkaloids with notable antioxidant, antimicrobial, anticancer, and anti-inflammatory properties (Kharwar et al., 2011; Asomadu et al., 2024). Crucially, endophytic fungi offer a sustainable alternative to direct plant harvesting, enabling continuous metabolite production under controlled conditions while supporting biodiversity conservation (Oktiansyah et al., 2024). Breonadia *salicina* (Rubiaceae), a medicinal plant traditionally employed in African ethnomedicine for treating infections, inflammation, and various ailments, has shown some pharmacological potential. However, the antioxidant capacity of its associated endophytic fungi remains largely unexplored. Investigating the endophytic fungal community of B. salicina thus presents a promising avenue for discovering novel natural antioxidants (Oktiansyah et al., 2024). In vitro antioxidant assays provide a rapid and reliable means of screening bioactive extracts. The DPPH radical scavenging assay evaluates the ability of compounds to neutralize free radicals through hydrogen or electron donation, whereas the FRAP assay measures antioxidant reducing power via electron-transfer mechanisms (Apak et al., 2016). The combined use of these assays offers a more comprehensive assessment of antioxidant potential. Accordingly, the present study aimed to isolate endophytic fungi from leaf and stembark tissues of *B. salicina* and evaluate the antioxidant activities of their crude extracts using DPPH and FRAP assays.

II. Materials And Methods

Plant Material Collection and Authentication

Healthy, disease-free leaves and stembark of Breonadia *salicina* were collected from Bununu LGA in Bauchi, Nigeria. The selected plant parts showed no signs of infection or physical damage, ensuring the isolation of authentic endophytic microorganisms. The plant was identified by a qualified taxonomist at Bayero University, and a voucher specimen was stored in the institutional herbarium for future reference.

Isolation of Endophytic Fungi

Endophytic fungi were isolated following standard surface-sterilisation procedures (Strobel & Daisy, 2003). Plant tissues were washed under running tap water, cut into 1–2 cm segments, and sequentially surface-sterilised in 70% ethanol and sodium hypochlorite, then rinsed with sterile distilled water. The final rinse water was plated onto potato dextrose agar (PDA) to confirm the effectiveness of the surface sterilisation. Sterilised

tissue segments were aseptically placed on PDA plates supplemented with antibiotics to suppress bacterial growth and incubated at 25–28 °C for up to 14 days. Emerging fungal colonies were subcultured repeatedly to obtain pure isolates. Isolates were coded according to tissue origin (leaf and stembark) and maintained on PDA slants at 4 °C.

Fermentation and Extraction of Fungal Metabolites

Pure fungal isolates were cultured in potato dextrose broth (PDB) under static conditions at room temperature for 21 days to encourage secondary metabolite production. After incubation, cultures were filtered to separate mycelial biomass from the culture broth. The filtrates were extracted with ethyl acetate, and the organic phase was concentrated under reduced pressure using a rotary evaporator to produce crude fungal extracts. Extracts were stored at 4 °C until further analysis (Kubayi et al., 2025; El-Bialy & El-Baky, 2023).

DPPH Radical Scavenging Assay

The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay with ascorbic acid standard according to the method described by Nyero et al., (2023) with minor modifications. The sample solutions (100, 200, 300, 400, and 500 µl) and 5 ml of 0.0039% DPPH were rapidly mixed in the test tubes. After vigorous shaking, the reaction mixture was incubated in the dark at 37°C for 30 min, and the absorbance was measured at 517 nm against a blank using a UV–Vis. Spectrophotometer. The ascorbic acid standard curve (Y = MX + C;) made from serial concentrations (5, 10, 15, and 20 µg/ml) was used as a positive control. The DPPH free radicals scavenging activity of the fungal extract was expressed in terms of the effective concentration in parts per million of ascorbic acid standard and samples required to scavenge 50% of DPPH free radicals in vitro, IC_{50}, using the equation (1):

DPPH free radical scavenging activity (%) = $\frac{Ac-As}{Ac} \times 100$ Eq. 1

Where Ac is the absorbance of the blank or control solution (i.e., the absorbance of DPPH + methanol); As is the absorbance of DPPH radical plus the sample (i.e., the absorbance of extract or standard). The 1C50 values were determined from the plotted graph of scavenging activity as a percentage against the concentration of different extracts from the three replicates. A lower IC50 value indicates greater antioxidant activity in the sample (Prior et al., 2005).

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP analysis was conducted to evaluate the extracts' reducing ability by measuring the reduction of Fe^{3+} to Fe^{2+} (Apak et al., 2016). Fresh FRAP reagent was prepared, mixed with extract solutions at different concentrations, and incubated at room temperature. Absorbance was read at 593 nm, with higher absorbance indicating stronger reducing power. Ascorbic acid was used as the positive control.

Statistical Analysis

All experiments were conducted in triplicate, and results are expressed as mean ± standard deviation.

III. Results

DPPH Radical Scavenging Activity

DPPH radical scavenging activity varied significantly among the fungal extracts, with IC_{50} values ranging from 115.80 to 3271.68 µg/mL (**Table 1**). Extract S3 exhibited the strongest activity, followed by B3 and L1. Moderate activity was observed for S2, while the remaining extracts showed weak to negligible scavenging capacity.

Table 1: DPPH Radical Scavenging Activity of Endophytic Fungal Extracts Isolated from Breonadia *salicina*

Sample	IC_{50} (µg/mL)	Antioxidant Activity
S3	115.80	Very strong
B3	178.19	Strong
L1	213.98	Strong
S2	270.26	Moderate
S7	568.80	Weak–moderate
L2	697.66	Weak
S1	885.35	Weak
B7	938.32	Very weak
S6	1050.87	Very weak
S4	1136.78	Very weak
B8	1233.87	Weak
B4	1274.73	Weak
B1	1278.16	Weak
B6	1565.37	Very poor

S5	2230.12	Poor
B5	2667.39	Negligible
B2	3271.68	Poorest

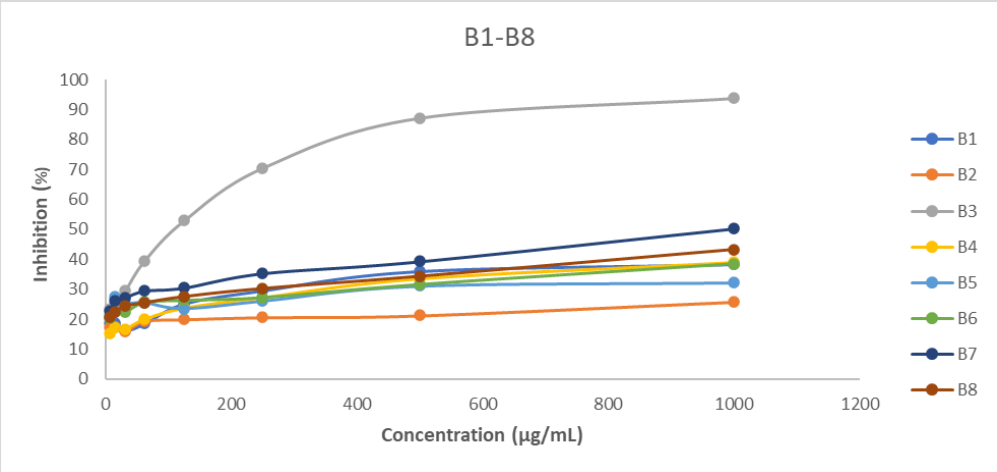

Fig 1: Chart of DPPH assay of B1-B8

CONC µg/	B1	B2	B3	B4	B5	B6	B7	B8
1000	38.30156	25.72501	93.92259	38.93125	32.24726	38.43443	50.28307	43.32756
500	35.97343	21.17851	87.29636	33.48931	31.12652	31.55402	39.30098	34.34431
250	29.52051	20.57192	70.53148	27.1346	26.04275	27.22704	35.30907	30.23108
125	25.15309	19.88446	52.9405	23.5933	23.49509	26.30849	30.48527	27.52166
62.5	18.50376	19.34142	39.5725	19.90179	25.54593	25.62103	29.46852	25.4766
31.25	16.24495	15.85211	29.35875	16.61467	25.28018	22.35702	27.25592	24.30965
15.625	18.59619	17.38879	24.30387	17.19237	27.49278	22.31658	26.10052	22.35702
7.8125	18.45754	17.24437	23.35644	15.31485	21.09185	20.25419	22.68631	20.77412

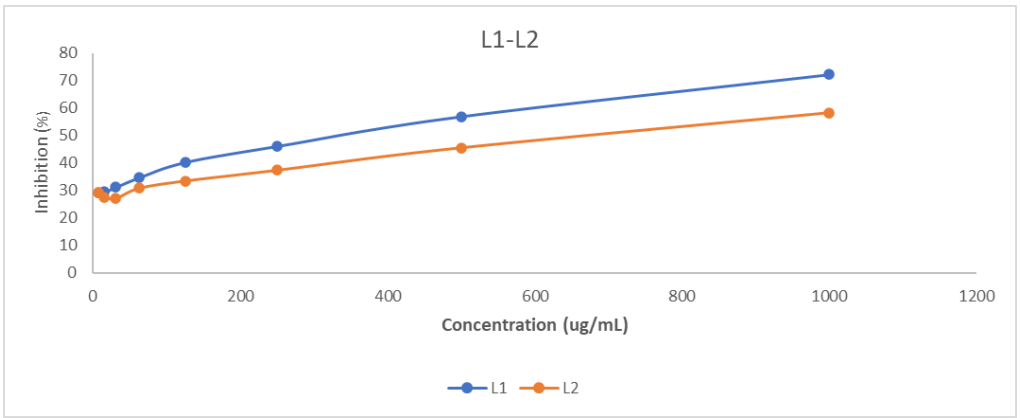

Fig 2: Result of the DPPH assay for L1-L2

CONC µg/ml	L1	L2
1000	72.29636	58.24957
500	57.00173	45.51993
250	46.16984	37.37435
125	40.31196	33.47487
62.5	34.71404	30.82322
31.25	31.24783	27.19237
15.625	29.4714	27.44367
7.8125	29.39341	29.21144

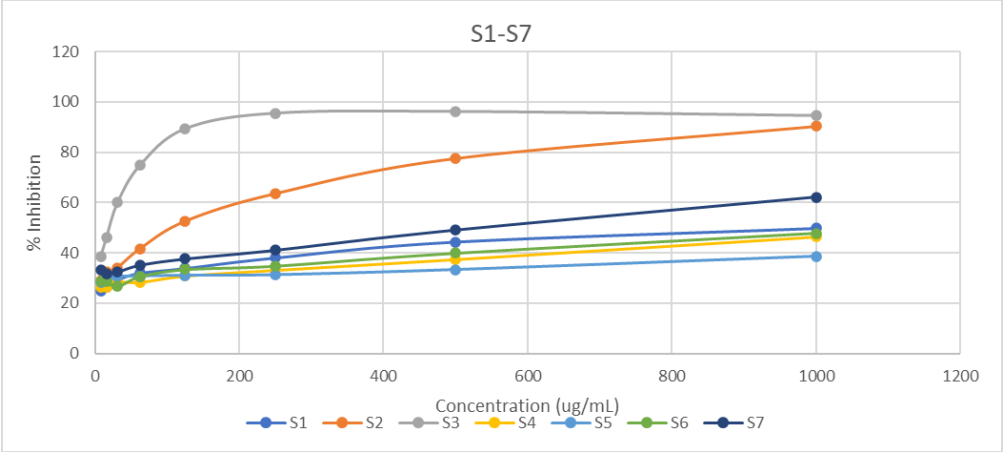

Fig 3: Result of DPPH assay for S1-S7

Conc µg/mL	S1	S2	S3	S4	S5	S6	S7
1000	49.74581	90.28307	94.55806	46.41248	38.65396	47.78163	62.20104
500	44.35009	77.49278	96.18718	37.39457	33.28134	39.86713	49.12478
250	38.05315	63.49509	95.49393	33.07915	31.20739	34.77181	41.11785
125	33.88215	52.53033	89.32987	30.7279	30.99365	33.38533	37.64298
62.5	31.99884	41.61179	74.88157	28.24957	30.71635	30.58348	35.18198
31.25	29.35875	34.10168	60.04622	28.46332	30.53726	26.62045	32.46101
15.625	26.17562	32.27614	45.94454	26.54535	30.06355	28.74061	31.71577
7.8125	24.84691	29.10456	38.42288	26.23917	28.27267	28.65396	33.02426

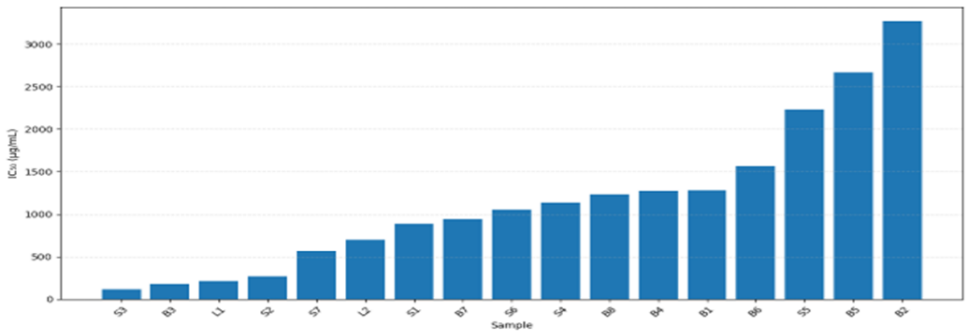

Fig 4: Result of IC_{50} values for the fungal extracts

Ferric Reducing Antioxidant Power (FRAP)

All extracts demonstrated concentration-dependent ferric reducing capacity. Consistent with DPPH results, extracts S3, B3, and L1 exhibited the highest reducing power. Ascorbic acid showed the strongest FRAP response, confirming the reliability of the assay.

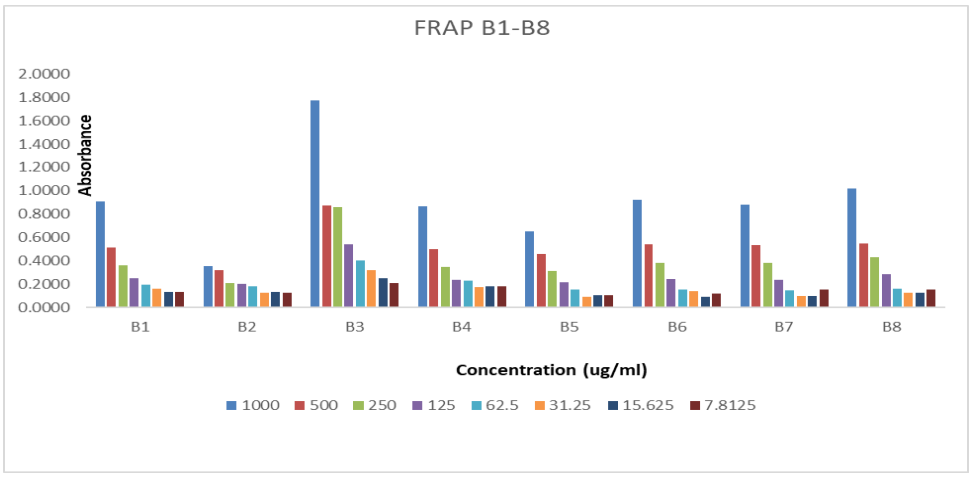

Fig. 5. FRAP of bark-derived extracts (B1–B8).

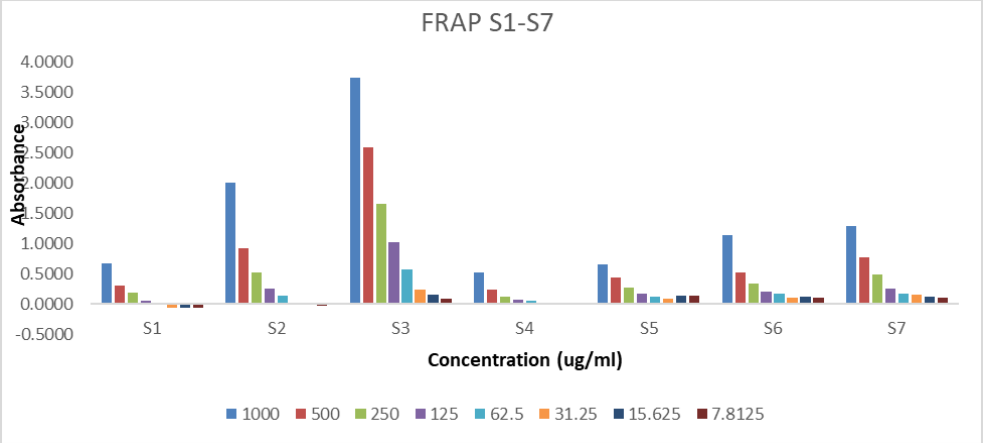

Fig. 6. FRAP of stem-derived extracts (S1–S7).

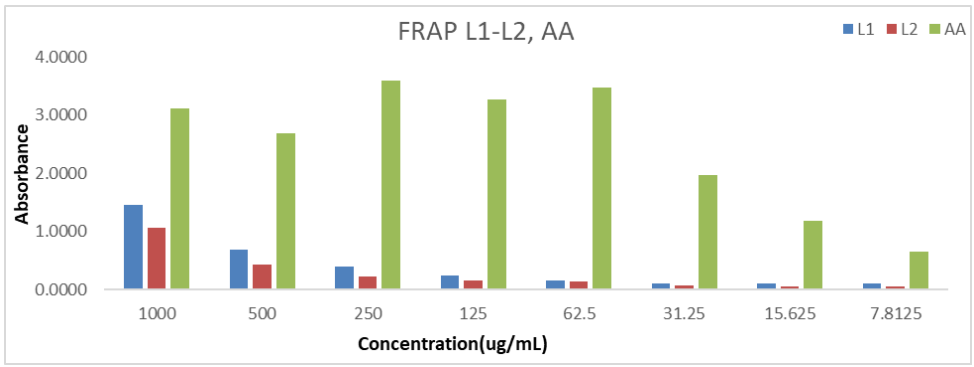

Fig. 7. FRAP of leaf-derived extracts (L1–L2) compared with ascorbic acid

IV. Discussion

The comparative analysis of antioxidant activities based on IC_{50} values showed considerable variation across the endophytic fungal extracts. The DPPH radical scavenging assay is a widely accepted and reliable method for evaluating the antioxidant potential of crude extracts and purified metabolites by donating hydrogen or electrons to the DPPH radical (Blois, 1958; Brand-Williams et al., 1995). In this study, antioxidant activity was expressed as IC_{50} values, with lower IC_{50} values indicating greater free-radical scavenging capacity (Molyneux, 2004). As shown in Figure 3, extract S3 displayed the lowest IC_{50} value (115.80 µg/mL), indicating the highest antioxidant potential among all samples. This was followed by B3 (178.19 µg/mL) and L1 (213.98 µg/mL). These findings imply that the corresponding endophytic fungi may produce potent antioxidant secondary metabolites such as phenolics, polyketides, quinones, and terpenoid derivatives, which are commonly reported in endophytic fungi (Strobel & Daisy, 2003; Aly et al., 2011). Endophytes are well known to biosynthesise pharmacologically relevant metabolites, sometimes similar to those produced by their host plants, making them valuable sources of novel bioactive compounds (Schulz et al., 2002; Strobel & Daisy, 2003). Moderate antioxidant activity was observed in S2 (270.26 µg/mL), while several extracts displayed weak to very weak scavenging effects with IC_{50} values above 500 µg/mL (e.g., S7, L2, S1, B7, S6, and S4). This wide variability may be attributed to differences in fungal species, gene expression, fermentation conditions, and metabolite profiles, as fungal secondary metabolite production is strongly influenced by growth media composition, incubation period, aeration, and culture conditions (Bérdy, 2005; Demain & Sanchez, 2009). The least active samples were B5 (2667.39 µg/mL) and B2 (3271.68 µg/mL), suggesting negligible radical scavenging potential. Such low activity may reflect either the absence of antioxidant metabolites or production at concentrations insufficient to exert measurable scavenging activity in the DPPH system (Molyneux, 2004; Kedare & Singh, 2011). Furthermore, the grouped bar chart (Figure 1) revealed a clear trend in which antioxidant activity classes corresponded directly to mean IC_{50} values, confirming that extracts categorized as very strong/strong had the lowest mean IC_{50} values, while extracts classified as negligible/poorest recorded the highest mean IC_{50} values. Overall, these findings support the hypothesis that certain endophytic fungi associated with Breonadia *salicina* possess significant antioxidant potential and represent promising candidates for bioassay-guided purification and structural characterization of antioxidant lead compounds (Strobel & Daisy, 2003; Newman & Cragg, 2020).

The Ferric Reducing Antioxidant Power (FRAP) assay is a common method used to assess antioxidant capacity. It measures how antioxidants in a sample can reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This

reduction results in a bright blue Fe^{2+}–TPTZ complex that can be detected spectrophotometrically at 593 nm (Benzie & Strain, 1996). Unlike radical scavenging tests such as DPPH, FRAP mainly indicates the electron-donating or reducing ability of antioxidant compounds, especially phenolics, flavonoids, and other redox-active metabolites (Prior et al., 2005; Apak et al., 2016). Therefore, higher FRAP values indicate the presence of secondary metabolites that function as reducing agents, which is a key mechanism in antioxidant activity. The FRAP B1–B8 chart demonstrates a concentration-dependent decline in the FRAP response: higher concentrations (1000 µg/mL) generally show the strongest reducing power, whereas lower concentrations (7.8125–31.25 µg/mL) exhibit minimal activity. This pattern aligns with the principle of the FRAP assay, where antioxidant-driven reduction of Fe^{3+} increases proportionally with the analyte concentration (Benzie & Strain, 1996). Among the B extracts, B3 exhibited the highest FRAP activity, particularly at 1000 µg/mL, indicating that it contains the greatest abundance or strongest electron-donating metabolites within the B-series. In natural product research, such strong reducing capacity is often linked to phenolic-rich or quinone-like metabolites produced by fungi, including polyketides and aromatic compounds that act effectively as reductants (Strobel & Daisy, 2003; Aly et al., 2011). The heightened FRAP activity of B3 supports its selection for bioassay-guided fractionation, purification, and structural analysis.

In contrast, samples like B2 and B5 exhibited noticeably lower FRAP responses across most concentrations, suggesting weaker reducing capacity or a lower concentration of redox-active metabolites. Variability in FRAP activity among isolates is expected, as fungal secondary metabolite production heavily depends on strain genetics, fermentation conditions, and metabolite expression (Bérdy, 2005; Demain & Sanchez, 2009). The FRAP S1–S7 chart similarly shows a strong concentration-dependent effect, with the highest antioxidant reducing power generally observed at 1000 µg/mL and decreasing progressively at lower concentrations. Notably, S3 exhibited the strongest reducing activity across concentrations, producing the highest FRAP signal within the S-series. This indicates that S3 produces metabolites with strong electron transfer capability, which correlates with potent antioxidant potential (Benzie & Strain, 1996; Prior et al., 2005). The significance of S3 in the FRAP assay is especially notable because FRAP complements DPPH results: while DPPH measures radical scavenging through hydrogen/electron transfer, FRAP highlights reducing power in an acidic medium (Apak et al., 2016). When a sample performs well in both FRAP and DPPH assays, it is more likely to contain strong antioxidants capable of acting through multiple mechanisms (Prior et al., 2005). Therefore, the strong FRAP performance of S3 reinforces its suitability as a candidate for further purification and identification of key antioxidant metabolites.

Moderately active samples such as S2, S6, and S7 showed intermediate FRAP values, indicating moderate levels of redox-active compounds. Meanwhile, S4 and S5 exhibited lower FRAP responses, suggesting weaker reducing capabilities. Such differences can arise from variability in metabolite classes (e.g., phenolics versus non-redox terpenoids) and variations in metabolite concentrations (Aly et al., 2011; Demain & Sanchez, 2009). The FRAP L1–L2, AA chart assesses the reducing power of extracts L1 and L2 compared to ascorbic acid (AA), a common reference antioxidant known for its strong electron-donating properties (Benzie & Strain, 1996). As anticipated, AA showed markedly higher FRAP values at all concentrations, validating the assay's reliability. Both L extracts exhibited lower FRAP values compared to AA, indicating weaker reducing power relative to the reference standard. However, the extracts still demonstrated dose-dependent reducing activity, suggesting the presence of antioxidant metabolites. In microbial natural products, crude extracts often show lower reducing power than purified standards because they contain complex mixtures, including non-antioxidant constituents that dilute the effect (Sarker & Nahar, 2012). Therefore, purification of L extracts could produce fractions or pure compounds with enhanced FRAP performance. Notably, the differences between L1 and L2 indicate possible strain-specific metabolite variation. Where one shows higher FRAP values, it implies a greater abundance of redox-active metabolites and should be prioritized for fractionation (Strobel & Daisy, 2003; Bérdy, 2005). The combined DPPH and FRAP assays showed that endophytic fungi associated with Breonadia *salicina* produce metabolites with notable antioxidant activity. The strong link between low DPPH IC_{50} values and high FRAP responses suggests that antioxidant effects are mainly driven by electron-transfer mechanisms typical of phenolic and flavonoid compounds (Apak et al., 2016; Pisoschi & Pop, 2015). Extract S3 consistently demonstrated superior antioxidant performance, indicating a high concentration of redox-active secondary metabolites and justifying its selection for bioassay-guided fractionation. Similar results have been reported for potent endophytes isolated from other medicinal plants (Tejesvi et al., 2008; Okezie et al., 2022). The robust activity of bark-derived extract B3 aligns with reports that bark-associated endophytes often synthesise chemically defensive metabolites due to the protective role of bark tissues (Chandra, 2024; Helaly et al., 2020). Variability among leaf-derived extracts underscores the metabolic diversity of endophytic fungi and points to differences in biosynthetic potential even within the same host tissue (Govindappa et al., 2013; Jia et al., 2025). Extracts with weaker activity may still exert antioxidant effects through alternative mechanisms not fully captured by DPPH or FRAP assays.

V. Conclusion

Antioxidant evaluation using DPPH radical scavenging assay (IC_{50} determination) and Ferric Reducing Antioxidant Power (FRAP) assay revealed significant variation in activity among the fungal extracts. Several isolates demonstrated notable antioxidant potential, particularly S3 (lowest IC_{50} and highest FRAP response), followed by B3 and L1. This indicates that these isolates contain metabolites with strong radical scavenging and reducing abilities. The consistent performance of S3 and B3 in both assays suggests that the antioxidants produced by these endophytes may operate through multiple mechanisms, including electron donation and radical stabilisation. Moreover, these findings support the hypothesis that endophytic fungi can biosynthesise metabolites with functional properties comparable to, or possibly superior to, plant-derived antioxidants. Overall, this research provides scientific evidence that Breonadia *salicina* endophytic fungi hold considerable potential for discovering natural antioxidant lead compounds.

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