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Research Article

Phytochemical Isolation of α-Linolenic Acid from Velvet Apple (*Diospyros blancoi*) Stem Bark and Assessment of Its Antibacterial, Antioxidant, and Cytotoxic Activities

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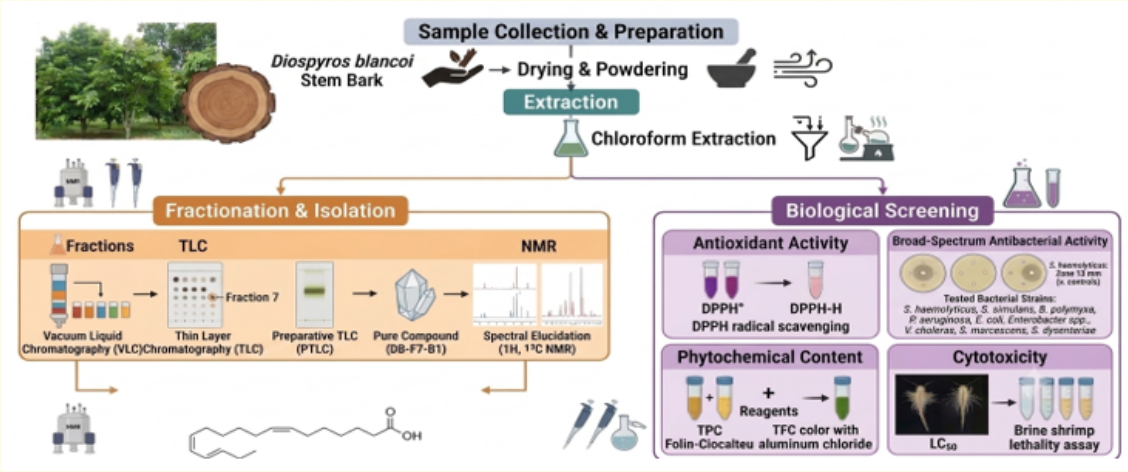
Abstract

Diospyros blancoi (velvet apple) is a medicinal plant whose stem bark remains relatively underexplored phytochemically. This study aimed to isolate and characterize a major constituent from the chloroform extract of *D. blancoi* stem bark and to evaluate its antibacterial, antioxidant, and preliminary cytotoxic activities. Fractionation of the chloroform extract by vacuum liquid chromatography followed by preparative thin-layer chromatography yielded compound DB-F7-B1, which was identified as α-linolenic acid based on its ^{1}H and ^{13}C nuclear magnetic resonance spectroscopic characteristics and comparison with reported literature data. The chloroform extract exhibited antibacterial activity against six of nine tested Gram-positive and Gram-negative bacterial strains, with inhibition zones ranging from 8 to 13 mm. The extract contained 11.86 mg gallic acid equivalents (GAE)/g of total phenolics and 4.91 mg quercetin equivalents (QE)/g of total flavonoids. Among the tested fractions, the chloroform-soluble fraction showed the strongest DPPH radical-scavenging activity, with an IC_{50} value of 41.0 µg/mL, whereas the crude extract exhibited preliminary cytotoxic activity in the brine shrimp lethality assay, with an LC_{50} value of 3.255 µg/mL. Overall, the findings contribute to the phytochemical characterization of *D. blancoi* stem bark and demonstrate its preliminary antibacterial, antioxidant, and cytotoxic potential. Further studies involving MIC/MBC determination, bioactivity-guided fractionation, and *in vivo* evaluation are warranted to establish the therapeutic relevance of its bioactive constituents.

Keywords: *Diospyros blancoi*; α-linolenic Acid; Phytochemical Isolation; Antibacterial Activity; Antioxidant Activity; Brine Shrimp Lethality Assay



Graphical abstract

Abbreviations

BHT: Butylated Hydroxytoluene; CCl4: Carbon Tetrachloride; DMSO: Dimethyl Sulfoxide; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; EA: Ethyl Acetate; GAE: Gallic Acid Equivalents; IC50: Half-Maximal Inhibitory Concentration; LC50: Lethal Concentration Required to Kill 50% of the Population; NMR: Nuclear Magnetic Resonance; PE: Petroleum Ether; PTLC: Preparative Thin-Layer Chromatography; QE: Quercetin Equivalents; TFC: Total Flavonoid Content; TLC: Thin-Layer Chromatography; TPC: Total Phenolic Content; UV: Ultraviolet; VLC: Vacuum Liquid Chromatography.

Introduction

Medicinal plants continue to represent an important source of structurally diverse bioactive compounds with significant pharmaceutical and nutraceutical potential. Their chemical diversity provides an important reservoir of natural molecules that may possess antioxidant, antimicrobial, anti-inflammatory, cytoprotective, and other pharmacological properties. Increasing antimicrobial resistance, together with the growing burden of oxidative stress-related and chronic diseases, has intensified the search for naturally occurring compounds with potential therapeutic value. Consequently, systematic phytochemical investigation of medicinal plants remains essential for the discovery, isolation, structural characterization, and biological evaluation of bioactive natural products that may serve as promising leads for future pharmaceutical development [1-4].

Diospyros blancoi A. DC. (Ebenaceae), commonly known as velvet apple or mabolo, is a tropical fruit-bearing species distributed across several regions of Southeast Asia, including Bangladesh. The plant has attracted attention because different parts have traditionally been used for the management of dysentery, diarrhea, fever, cough, hypertension, inflammatory disorders, and various skin-related conditions. Phytochemical investigations of the genus *Diospyros* have revealed a wide range of secondary metabolites and other bioactive constituents, including naphthoquinones, triterpenoids, flavonoids, tannins, phenolic compounds, and fatty acids [5-9]. These compounds have been associated with diverse biological activities, including antioxidant, antimicrobial, anti-inflammatory, and anticancer effects. Previous investigations have also demonstrated that *D. blancoi* possesses measurable antioxidant and cytotoxic potential, indicating that the species may contain pharmacologically relevant constituents. However, compared with several other members of the genus and other plant parts, the phytochemical profile of *D. blancoi* stem bark remains comparatively less explored. The stem bark represents an important but relatively underinvestigated plant material because it may contain lipophilic as well as polar secondary metabolites that contribute to its biological properties. Previous studies on *D. blancoi* have mainly focused on general phytochemical screening and biological evaluation of crude extracts or other plant parts, whereas information on the isolation and structural characterization of individual compounds from the stem bark

is still limited. In particular, the occurrence of specific bioactive fatty acids in the stem bark has received comparatively little attention. Detailed chromatographic separation and spectroscopic characterization of isolated constituents may therefore provide useful information for expanding the phytochemical profile of this medicinal species. α-Linolenic acid (ALA) is an essential omega-3 polyunsaturated fatty acid that has attracted considerable scientific interest because of its diverse biological activities and nutritional importance. As an essential fatty acid, ALA is involved in lipid metabolism and serves as a precursor for the biosynthesis of longer-chain omega-3 fatty acids. In addition to its nutritional significance, previous studies have reported antioxidant, antimicrobial, anti-inflammatory, cardioprotective, and cytoprotective properties of ALA [10-16]. Evidence from experimental studies also suggests that ALA may influence cellular oxidative processes, inflammatory responses, membrane-associated functions, and microbial viability. These biological properties have contributed to increasing interest in ALA in both nutritional and pharmaceutical research. Despite the well-documented biological importance of ALA, information regarding its occurrence in *D. blancoi*, particularly in the stem bark, remains limited [17]. The identification of ALA from an underexplored plant source could therefore provide additional information regarding the chemical diversity of *D. blancoi* and may help explain, at least partly, some of the biological properties associated with its extracts. However, because crude plant extracts contain complex mixtures of phytochemicals, the biological activities of the extract cannot necessarily be attributed to a single isolated compound. Therefore, combining phytochemical isolation with preliminary biological screening provides a useful approach for generating baseline information and identifying potential bioactive constituents for further investigation. Accordingly, the present study aimed to isolate and characterize α-linolenic acid from the stem bark of *Diospyros blancoi* using chromatographic and spectroscopic techniques and to evaluate the antibacterial, antioxidant, and preliminary cytotoxic activities of the plant extract. The isolated compound was characterized by comparing its spectroscopic data with previously reported reference data, while the biological properties of the extract were assessed using established *in vitro* assays. The findings are expected to contribute to the phytochemical characterization of *D. blancoi*, provide additional information on the occurrence of α-linolenic acid in its stem bark, and establish baseline biological evidence for future investigations of its individual bioactive constituents and possible mechanisms of action.

Materials and Methods

Experimental setup

The stem bark of Diospyros blancoi A. DC. was collected from Jhalakathi, Bangladesh, in May 2023 and authenticated by a qualified botanist. A voucher specimen was deposited in the institutional herbarium for future reference. The collected stem bark was air-dried at room temperature and ground into a coarse powder. Of the total dried material (1.5 kg), 1.0 kg of powdered stem bark was subjected to cold maceration with 1.0 L of chloroform for 5 days in the dark, with intermittent shaking. The extract was then filtered, and the solvent was removed under reduced pressure using a rotary evaporator at 40°C to obtain the crude chloroform extract. The extraction procedure was performed following commonly employed phytochemical extraction techniques, with slight modifications [18-22]. All subsequent experimental analyses were performed in triplicate (n = 3).

Isolation and structural characterization

The crude extract was fractionated by vacuum liquid chromatography (VLC) on a silica gel column using a stepwise gradient of petroleum ether, ethyl acetate, and methanol to yield 29 fractions. The crude extract was fractionated by vacuum liquid chromatography (VLC) on a silica gel column using a stepwise gradient of petroleum ether, ethyl acetate, and methanol to yield 29 fractions. The VLC fractions were screened by thin-layer chromatography to assess their chromatographic profiles. Based on these patterns, Fraction 7 (eluted with a petroleum ether to ethyl acetate ratio of 85:15, as summarized in supplementary Table S1) was selected for further purification via preparative thin-layer chromatography (PTLC). The isolated compound was identified by ^{1}H and ^{13}C NMR spectroscopy (600 and 150 MHz, respectively, in $CDCl_3$), and the resulting spectral data were compared with published literature values, supporting its identification as α-linolenic acid [23-28].

Antibacterial assay

Antibacterial activity of the crude extract was evaluated against nine Gram-positive and Gram-negative bacterial strains using the disk diffusion method following a published protocol. Sterile paper disks containing the extract (400 µg/mL) were placed on Mueller-Hinton agar plates inoculated with the test organisms. Ciprofloxacin (5 µg/disk), azithromycin (15 µg/disk), and ampicillin (10 µg/disk) served as positive controls, while chloroform served as the

negative control. Following incubation at 37°C for 20 h, inhibition zones were measured in millimeters [29,30].

$$\text{Inhibition zone width} = \frac{\text{Dtotal} - \text{Ddisk}}{2}$$

Determination of total phenolic and flavonoid contents

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method with gallic acid as the reference standard and expressed as mg gallic acid equivalents (GAE)/g extract. Total flavonoid content (TFC) was determined using the aluminum chloride colorimetric assay with quercetin as the reference standard and expressed as mg quercetin equivalents (QE)/g extract [31,32]. The concentrations of phenolic and flavonoid compounds were calculated from the respective calibration curves of gallic acid and quercetin, respectively.

DPPH radical scavenging assay

Antioxidant activity was assessed using the DPPH free radical scavenging assay. The extract and the reference antioxidant butylated hydroxytoluene (BHT) were evaluated over a range of concentrations, and absorbance was measured at 517 nm after 30 min of incubation in the dark. Radical scavenging activity was expressed as IC_{50} values. The assay was conducted according to established DPPH procedures [33,34]. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_s) / A_0] \times 100$$

Where A_0 represents the absorbance of the DPPH control and A_s represents the absorbance of the sample or standard. The IC_{50} value, defined as the concentration required to inhibit 50% of the DPPH radicals, was determined from the concentration-response curve.

Brine shrimp lethality assay

Preliminary cytotoxic activity was evaluated using the brine shrimp (*Artemia salina*) lethality assay. Nauplii were exposed to serial concentrations of the crude extract, with vincristine sulfate and dimethyl sulfoxide (DMSO) serving as positive and negative controls, respectively. Mortality was recorded after 24 h. The percentage mortality was calculated using the following equation:

$$\text{Mortality (\%)} = (\text{Number of dead nauplii} / \text{Total number of nauplii exposed}) \times 100$$

The LC_{50} value, defined as the concentration of the crude extract required to cause 50% mortality of the exposed nauplii, was determined from the concentration-mortality relationship. The assay followed a widely used preliminary cytotoxicity screening procedure [35].

Statistical analysis

Experimental data were obtained from replicate measurements where applicable and expressed as mean values. IC_{50} and LC_{50} values were calculated from dose-response curves generated using the experimental data [36]. Data were further evaluated using analysis of variance (ANOVA), and a probability value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Isolation and structural characterization of α-linolenic acid

Chromatographic separation of the chloroform extract was performed by vacuum liquid chromatography (VLC), and thin-layer chromatography (TLC) screening identified Fraction 7 as the most suitable fraction for further purification. Preparative TLC (PTLC) of Fraction 7 yielded a single yellow band (DB-F7-B1) with an Rf value of 0.52, >95% purity, as illustrated in (Figure 1), indicating successful isolation of a relatively pure compound. The combination of VLC and PTLC represents an effective and widely employed strategy for the separation and purification of bioactive plant metabolites, particularly when targeting individual constituents from complex crude extracts [37,38]. The isolated compound was structurally characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The spectral assignments are summarized in Tables 1 and 2, while the representative spectra are presented in (Figure 2), with the raw, uncropped BCSIR acquisition spectra provided in Supplementary Figures S2 and S3. The ^{1}H NMR spectrum exhibited characteristic resonances of an unsaturated fatty acid, including olefinic proton signals at δ 5.33 ppm, methylene protons adjacent to the carboxyl group at δ 2.35 ppm, and a terminal methyl triplet at δ 0.88 ppm. Similarly, the ^{13}C NMR spectrum showed a carboxyl carbon resonance at δ 178.82 ppm, olefinic carbon signals between δ 126.0 and 130.0 ppm, and a terminal methyl carbon at δ 14.23 ppm. Comparison of these spectral data with published literature confirmed compound DB-F7-B1 as α-linolenic acid, thereby expanding the phytochemical profile of the stem bark of *Diospyros blancoi* [39]. While previous pharmacological investigations on *D. blancoi* and related species have largely focused on the leaves

or fruits, our findings demonstrate that the stem bark is also a rich source of bioactive constituents. Furthermore, these results corroborate broader phytochemical and pharmacological reviews indicating that as α-linolenic acid serves as a versatile bioactive constituent across diverse plant matrices, exhibiting notable membrane-modulating, antimicrobial, and free-radical-scavenging properties [39].

Antibacterial activity

The antibacterial activity of the chloroform stem bark extract was evaluated against nine clinically relevant Gram-positive and Gram-negative bacterial strains using the disc diffusion method. The inhibition zones are summarized in Table 3, while representative antibacterial activity is illustrated in Figure 3. The

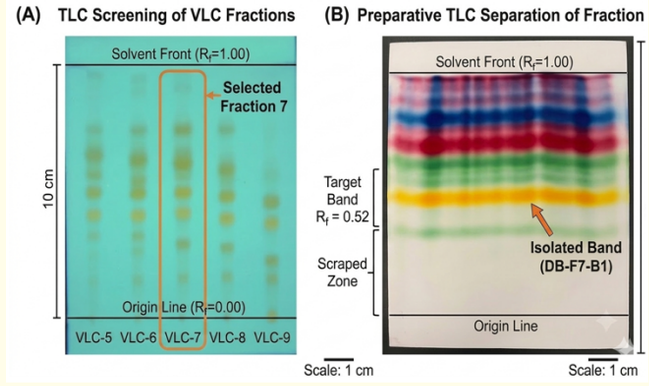


Figure 1: TLC Screening and Preparative Separation of VLC Fractions. 1(A) TLC screening of VLC fractions, identifying Fraction 7 for further purification; (B) Preparative TLC separation of Fraction 7 to isolate the target band (DB-F7-B1) at Rf = 0.52.

Position of Hydrogen	δH-value of isolated compound (DB-F7-B1)	Chemical shift value from literature [36]	ΔδH
H9, H10, H12, H13, H15, H16	5.33(m)	5.36(m)	0.03
H11, H14	2.76(t)	2.8(t)	0.04
H2	2.32(t)	2.35(t)	0.03
H8, H17	2.04(m)	2.04(m)	0.00
H3	1.64(m)	1.61(m)	0.03
H4, H5, H6, H7	1.37(m)	1.31(mc)	0.06
H18	0.88(t)	0.88(t)	0.00

Table 1: Comparison of ^{1}H NMR data of isolated compound DB-F7-B1 with reference data of α-linolenic acid from literature.

ΔδH = |δH observed − δH literature|. A paired *t*-test showed no statistically significant difference between the observed and literature values (t = −0.104, df = 6, p = 0.920).

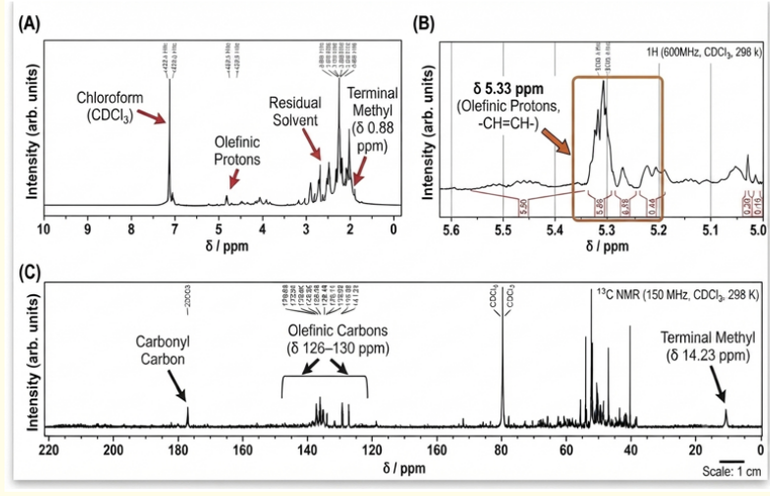


Figure 2: Key NMR Spectra of DB-F7-B1. 2(A)1H NMR spectrum of DB-F7-B1. 2(B) 1H NMR spectrum showing the olefinic proton signals. 2(C) 1C NMR spectrum of speromhs solutions from DB-F7-B1.

Position of carbon	δC-value of isolated compound (DB-F7-B1)	Chemical shift value from literature [35]	ΔδC
1	178.82	180.43	1.61
2	34.11	34.13	0.02
3	24.6	24.69	0.09
4	29.03	29.09	0.06
5	29.14	29.13	0.01
6	29.63	29.62	0.01
7	29.21	29.21	0.00
8	27.33	27.23	0.10
9	130.66	131.85	1.19
10	126.77	127.16	0.39
11	25.74	25.56	0.18
12	128.17	128.12	0.05
13	128.4	128.22	0.18
14	25.87	25.65	0.22
15	128.01	127.8	0.21
16	130.13	130.14	0.01
17	22.79	20.56	2.23
18	14.23	14.26	0.03

Table 2: Comparison of chemical shift values of ^{13}C NMR spectrum between isolated compound DB-F7-B1 and reference value of α-linolenic acid.

Here, ΔδC = |δC observed − δC literature|. A paired *t*-test showed no statistically significant difference between the observed and literature values ($t = -0.067$, df = 17, $p = 0.947$).

extract demonstrated antibacterial activity against six of the nine tested microorganisms, producing inhibition zones ranging from 8 to 13 mm. Among the Gram-positive bacteria, the highest inhibitory activity was observed against *Staphylococcus haemolyticus* (13 mm), followed by *Bacillus polymyxa* (9 mm), whereas no inhibition was detected against *Staphylococcus simulans*. Among the Gram-negative bacteria, the extract exhibited the greatest activity against *Vibrio cholerae* (12 mm), followed by *Pseudomonas aeruginosa* (11 mm), *Shigella dysenteriae* (10 mm), and *Escherichia coli* (8 mm). No detectable antibacterial activity was observed against *Enterobacter* spp. or *Serratia marcescens*. The reference antibiotics produced larger inhibition zones than the plant extract and served as positive controls to validate the assay. Considering that the plant extract was evaluated at a substantially higher loading than the antibiotics, these comparisons should be interpreted qualitatively rather than quantitatively. Nevertheless, the observed antibacterial activity indicates that the chloroform extract contains bioactive constituents capable of inhibiting both Gram-positive and Gram-negative bacteria. Because the assay was performed using the crude extract, the antibacterial activity is likely attributable to the combined effects of multiple phytochemicals rather than a single constituent. These findings support the ethnomedicinal relevance of *D. blancoi* and align with recent antimicrobial investigations highlighting the direct bactericidal and synergistic properties of plant-derived polyunsaturated constituents, such as α-linolenic acid, against clinical pathogens [22].

While the disk diffusion assay clearly demonstrates broad-spectrum activity through prominent inhibition zones, a limitation of the present study is the qualitative nature of this screening; future

Type of Bacteria	Bacterial strains	Diameter of clear zone (mm)				
		Blank disk (Chloroform)	Plant sample (400µg/ml)	Antibiotic disks		
				Ciprofloxacin (5µg/disk)	Azithromycin (15µg/disk)	Ampicillin (10µg/disk)
Gram Positive Bacteria	*Staphylococcus haemolyticus*	7	13	8	6	6
	Staphylococcus simulans	0	0	N/A	22	16
	Bacillus polymyxa	6	9	28	23	N/A
Gram Negative Bacteria	*Pseudomonas aeruginosa*	7	11	30	12	7
	Escherichia coli	6	8	N/A	7	7
	Enterobacter spp	0	0	20	8	N/A
	Vibrio cholerae	6	12	22	19	12
	Serratia marcescens	0	0	10	N/A	8
	Shigella dysenteriae	0	10	30	28	N/A

Table 3: Determination of zone of inhibition around sample and control disks.

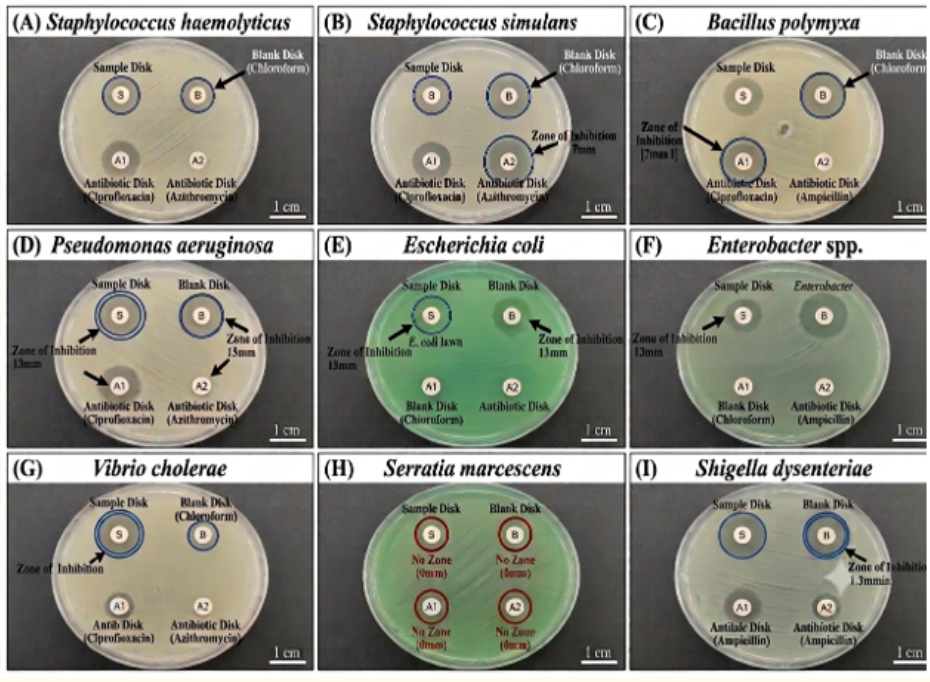
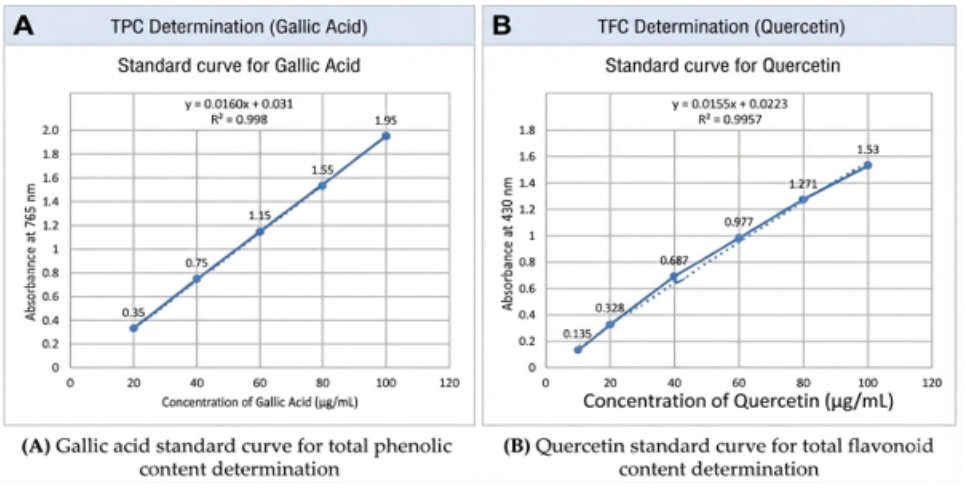


Figure 3: Antimicrobial susceptibility testing of a sample against various bacterial pathogens.

investigations will incorporate quantitative broth microdilution assays to determine precise Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values.

Total phenolic and flavonoid contents

The calibration curves generated using gallic acid and quercetin standards (Figure 4) showed good linear relationships and were used for the quantitative determination of total phenolic content (TPC) and total flavonoid content (TFC), respectively. The chloroform stem bark extract contained 11.86 mg gallic acid equivalents (GAE)/g crude extract of total phenolics and 4.91 mg quercetin equivalents (QE)/g crude extract of total flavonoids. Phenolic compounds, including flavonoids, are recognized as important natural antioxidants because of their ability to donate hydrogen atoms or electrons and neutralize reactive oxygen species. Therefore, the presence of these phytochemicals may contribute to the biological activities observed for the extract. Differences in phenolic and flavonoid contents reported among studies may arise from variations in extraction solvent, plant material, geographical origin, and environmental conditions.



(A) Gallic acid standard curve for total phenolic content determination

(B) Quercetin standard curve for total flavonoid content determination

Figure 4: Caliberation curves for gallic and quercetin used in TPC and TFC analysis.

Antioxidant activity

The antioxidant potential of the different solvent fractions was evaluated using the DPPH free radical scavenging assay. The IC_{50} values are summarized in Table, while the corresponding dose-response curves are presented in (Figure 5). Among the tested fractions, the chloroform-soluble fraction exhibited the strongest antioxidant activity with an IC_{50} value of 41.0 µg/mL, followed by the methanolic extract (58.5 µg/mL) and the carbon tetrachloride-soluble fraction (87.0 µg/mL). As expected, the reference antioxidant, butylated hydroxytoluene (BHT), displayed the highest radical scavenging activity with an IC_{50} value of 22.0 µg/mL. The comparatively lower IC_{50} value of the chloroform fraction indicates greater antioxidant capacity than the other solvent fractions. This observation is consistent with its measurable phenolic and flavonoid contents, which are known to act as effective free radical scavengers through hydrogen atom donation, electron transfer, and stabilization of reactive oxygen species. In addition, α-linolenic acid has been reported to exhibit antioxidant and cytoprotective properties, suggesting that it may also contribute to the overall antioxidant activity of the extract. Nevertheless, because the antioxidant assay was performed using crude fractions, the relative contribution of individual phytochemicals cannot be determined.

Preliminary cytotoxic activity

The cytotoxic potential of the chloroform stem bark extract was investigated using the brine shrimp (*Artemia salina*) lethality assay. The concentration-mortality relationship and calculated LC_{50} values are presented in Table and Figure 6, respectively.

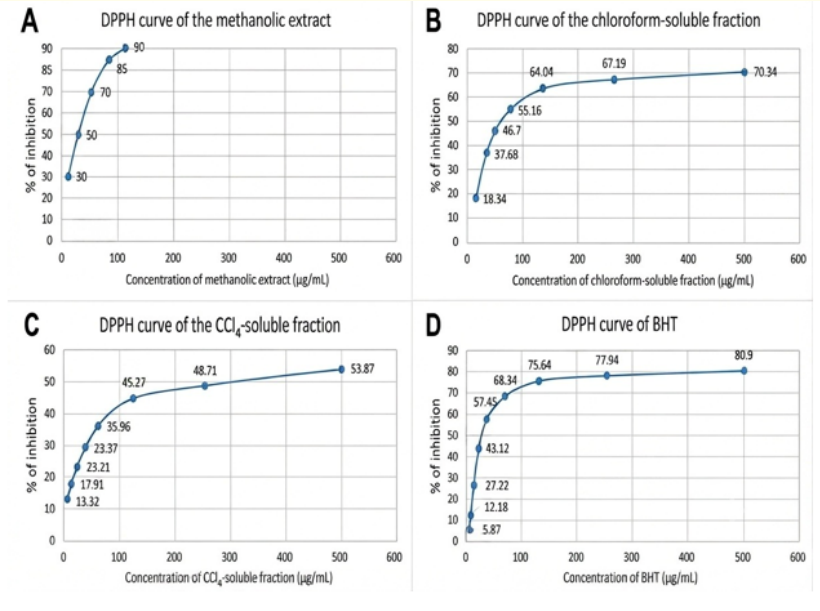


Figure 5: DPPH radical scavenging activity and IC50 determination for different extracts and the standard antioxidant BHT.

The extract exhibited an LC_{50} value of 3.255 µg/mL, whereas the reference standard, vincristine sulfate, showed a markedly lower LC_{50} value of 0.252 µg/mL, indicating greater cytotoxic potency. Although the extract was less active than the standard drug, its relatively low LC_{50} value suggests the presence of biologically active constituents with promising cytotoxic potential. Because the brine shrimp lethality assay is widely accepted as a rapid and inexpensive preliminary screening method for identifying plant extracts containing potentially cytotoxic or biologically active compounds [40-44]. However, this assay does not distinguish between general toxicity and selective anticancer activity. Therefore, the present findings should be interpreted as preliminary evidence of bioactivity rather than direct evidence of anticancer efficacy. Future studies should employ human cancer and normal cell lines to evaluate cytotoxic selectivity, isolate the active constituents through bioactivity-guided fractionation, and investigate their underlying molecular mechanisms. Given the reported anticancer activities of α-linolenic acid and several *Diospyros*-derived phytochemicals, these investigations may provide valuable insight into the therapeutic potential of the stem bark of *D. blancoi* [45-47]. The observed ^{1}H NMR chemical shifts of the isolated compound DB-F7-B1 showed close agreement with the corresponding literature values for α-linolenic acid (Table 1). The absolute differences (ΔδH) between the observed and reported chemical shifts ranged from 0.00 to 0.06 ppm, indicating a high degree of agreement between the isolated compound and the reference data. The smallest differences (0.00 ppm) were observed for H8/H17 and H18, whereas the largest difference (0.06 ppm) was observed for H4-H7 (Table 1). Overall, the close correspondence of the ^{1}H NMR chemical shifts supports the identification of DB-F7-B1 as α-linolenic acid. The ^{13}C NMR chemical shifts of the isolated compound DB-F7-B1 were compared with the reported values for α-linolenic acid (Table 2). The absolute differences (ΔδC) between the observed and literature values ranged from 0.00 to 2.23 ppm, with a mean absolute difference of approximately 0.38 ppm across the 18 carbon signals. Most carbon signals showed relatively small differences from the reported values, particularly C2-C8, C12, C16, and C18. The largest differences were observed for C17 (2.23 ppm), C1 (1.61 ppm), and C9 (1.19 ppm). Despite these variations, the overall ^{13}C NMR profile, including the characteristic carbonyl, olefinic, aliphatic, and terminal methyl carbon signals, showed correspondence with the reported spectral data for α-linolenic acid. Together with the ^{1}H NMR data, these findings support the proposed identification of DB-F7-B1 as α-linolenic acid (Table 2). The present study demonstrated that *Diospyros blancoi* stem bark possesses measurable phenolic and flavonoid contents and

exhibits antioxidant activity. These findings are consistent with Khan., *et al*. (2016), who reported that *D. blancoi* stem bark showed strong free-radical-scavenging activity and relatively high phenolic and flavonoid contents, suggesting an important contribution of phytochemicals to its antioxidant potential. The crude stem bark extract also showed variable antibacterial activity against the tested bacterial strains. Similar antibacterial activity of *D. blancoi* bark extracts has recently been reported by [48], although the authors described the activity as relatively mild and recommended further phytochemical investigation to identify the responsible bioactive constituents [49]. The variation in antibacterial response among bacterial strains in the present study may therefore reflect differences in their susceptibility to the phytochemicals present in the crude extract. The preliminary cytotoxic activity observed in the present study is also comparable with the findings of [49], who reported moderate cytotoxic and anticancer properties of *D. blancoi* stem bark extract using brine shrimp and cancer-cell models [49-51]. Overall, these findings support the potential of *D. blancoi* stem bark as a source of biologically active phytochemicals, while further studies are required to determine the specific contribution of isolated α-linolenic acid and other constituents to the observed biological activities. The correlation analysis revealed an extremely strong positive relationship between the observed and literature ^{13}C NMR chemical shift values of the isolated compound DB-F7-B1. Pearson's correlation analysis showed a very high correlation coefficient (r = 0.99994, R^2 = 0.99987, p < 0.001; n = 18), indicating a highly significant and close correspondence between the experimental and reported chemical shift values. This strong correlation further supports the proposed identification of DB-F7-B1 as α-linolenic acid (Figure 7).

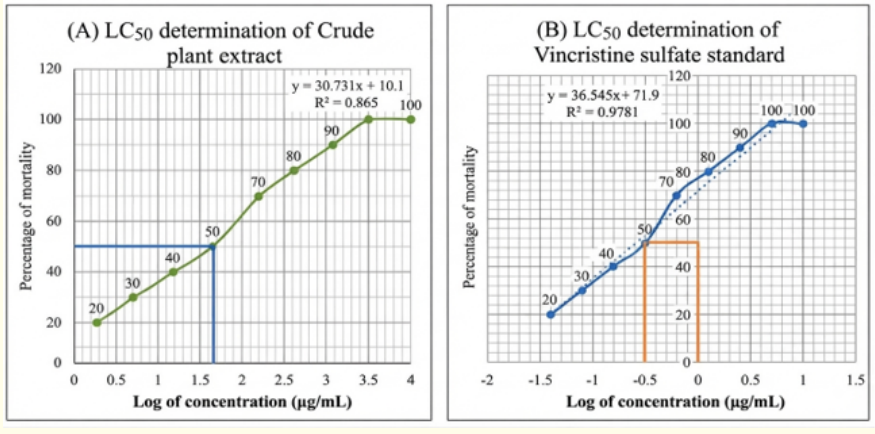


Figure 6: (A) Percentage mortality of Artemia salina nauplii at different concentrations of the test extract and vincristine sulfate. (B) Relationship between log of concentration (µg/mL) and percentage mortality used for LC_{50} estimation.

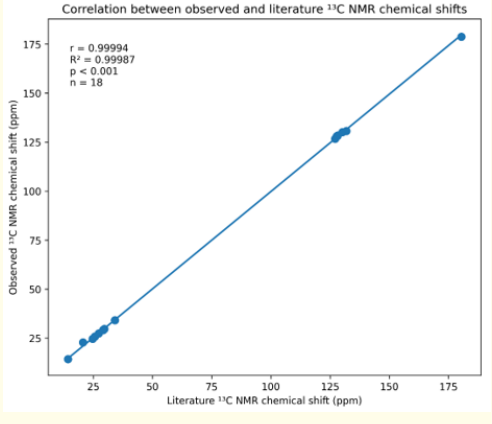


Figure 7: Pearson correlation between the observed ^{13}C NMR chemical shift values of DB-F7-B1 and the corresponding literature values of α-linolenic acid.

Conclusions

The present study successfully isolated and structurally characterized α-linolenic acid from the chloroform stem bark extract of *Diospyros blancoi* using chromatographic purification followed by ^{1}H and ^{13}C NMR spectroscopic analyses. In addition to its phytochemical characterization, the extract exhibited moderate antibacterial activity against several Gram-positive and Gram-negative bacterial strains, appreciable antioxidant activity, and promising preliminary cytotoxicity in the brine shrimp lethality assay. The presence of measurable amounts of total phenolic and flavonoid constituents further supports the observed antioxidant potential of the extract. Although the biological activities were lower than those of the corresponding reference standards, the results demonstrate that *D. blancoi* stem bark is a valuable source of bioactive phytochemicals with potential pharmaceutical relevance. Because the antibacterial, antioxidant, and cytotoxic assays were performed using the crude extract or solvent fractions, the contribution of individual constituents, including α-linolenic acid, remains to be established through further investigation. Future studies should focus on bioactivity-guided fractionation to identify the principal active constituents, quantitative antimicrobial evaluation through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, and comprehensive cytotoxic assessment using mammalian normal and cancer cell lines. Mechanistic investigations, together with *in vivo* pharmacological studies and safety evaluations, will be essential to validate the therapeutic potential of *Diospyros blancoi* and facilitate the development of natural product-based pharmaceutical candidates.

Credit Authorship Contribution Statement

Conceptualization: B.P. Formal analysis and validation: S.N., B.P., M.M. and M.E.H. Writing original draft: B.P., S.N., M.M. and M.E.H., Writing Editing: S.N., B.P., N.C.H and M.E.H. Project administration and Supervision: S.N., B.P., M.M. and M.E.H. All authors read and agreed to submit the final version of this manuscript for publication.

Ethical Statement

Not applicable to this study.

Clinical Trial Registration

Not applicable.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent to Participate

Not applicable.

Conflicts of Interest

The authors declare they have no conflicts of interest.

Consent to Publish

Not applicable.

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Conflict of Interest

All authors declare that there is no conflict of interest regarding this study.

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Study Limitations

Despite these promising findings, several limitations of the present study should be acknowledged. *In Vitro* Nature: The biological evaluations were conducted strictly *in vitro*; therefore, the observed antioxidant, cytotoxic, and antibacterial activities may not directly translate to *in vivo* efficacy or physiological conditions. Qualitative Screening Boundaries: Although the disk diffusion assay provided preliminary evidence of antibacterial activity, the absence of quantitative MIC/MBC measurements limits precise

assessment of the antibacterial potency of the extract. Future studies should determine MIC and MBC values using standardized broth microdilution methods. Synergistic Complexities: Although α-linolenic acid was isolated and structurally characterized, the overall bioactivity of the crude stem bark extract may also arise from synergistic or additive interactions among other, as yet uncharacterized, phytochemicals. Therefore, the contribution of α-linolenic acid to the observed activities of the crude extract cannot be considered definitive based on the present findings alone.

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