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# Phytochemical screening, isolation, and antioxidant activity of a triterpenoid mixture ($\alpha$- and $\beta$-amyrin) from the methanol leaf extract of *Diospyros mespiliformis*

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*Diospyros mespiliformis*, commonly known as African ebony, is a medicinal plant widely used in traditional African medicine. This study aimed to investigate the phytochemical constituents, isolate bioactive compounds, and evaluate the antioxidant activity of the methanol leaf extract of *Diospyros mespiliformis*. Preliminary phytochemical screening revealed the presence of alkaloids, carbohydrates, cardiac glycosides, flavonoids, saponins, and tannins. Column chromatography and thin-layer chromatography (TLC) were employed to isolate and characterize a compound, identified as a triterpenoid mixture consisting of α-amyrin and β-amyrin. The antioxidant activity of the crude extract and the isolated compound was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The isolated compound exhibited significant antioxidant activity, with an $IC_{50}$ value of 8.5 µg/mL, compared to the crude extract ($IC_{50}$ = 10.5 µg/mL) and ascorbic acid ($IC_{50}$ = 4.25 µg/mL). These findings suggest that *Diospyros mespiliformis* is a rich source of bioactive compounds with potent antioxidant properties, supporting its traditional use in managing oxidative stress-related diseases.

***Keywords:*** *Diospyros mespiliformis, phytochemicals, α-amyrin, β-amyrin, antioxidant activity, DPPH assay*

## Introduction

Medicinal plants have been an integral part of traditional healthcare systems for centuries, particularly in developing countries where access to modern medicine is limited. According to the World Health Organization (WHO), approximately 80% of the global population relies on traditional medicine for their primary healthcare needs, especially in rural and remote areas (World Health Organization [WHO], 2023). Traditional medicine is often more accessible, affordable, and culturally acceptable, making it a vital resource for communities in Sub-Saharan Africa and other developing regions. Among the many medicinal plants used in traditional medicine, *Diospyros mespiliformis*, commonly known as African ebony, stands out for its wide range of therapeutic applications, such as anticancer and anti-inflammatory effects (Ahmed & Mahmud 2017). *Diospyros mespiliformis* Hochst. ex A.DC. is a large deciduous tree of the Ebenaceae family, predominantly occurring in savanna and woodland biomes (South African National Biodiversity Institute [SANBI], 2023). Characteristically found on termitaria, this species exhibits a wide distribution across eastern and southern Africa, extending from Ethiopia through to Eswatini (formerly Swaziland). Optimal growth conditions include areas with reliable water availability and frost-free environments (SANBI, 2023). The plant has a long history of use in traditional medicine, with various parts of the tree such as the leaves, bark, roots, and fruits, being used to treat different ailments. For example, leaf infusions are used as mild laxatives and hemostatics, while bark and root extracts are employed to treat infections like malaria, pneumonia, and syphilis (Ramadwa & Meddows-Taylor, 2023). The plant

is also noted for its antimicrobial, anti-inflammatory, and pain-relieving properties, making it a valuable resource in traditional healthcare systems (Aminu et al., 2021). The therapeutic potential of *Diospyros mespiliformis* is mainly due to its rich phytochemical makeup. Prior studies have identified bioactive compounds such as flavonoids, tannins, triterpenes, and alkaloids in the plant (Shamsuddeen, 2023). These compounds are known to demonstrate a wide array of pharmacological activities, including antioxidant, antimicrobial, and anti-inflammatory effects. Among these, the antioxidant properties are especially significant since oxidative stress is linked to the development of numerous diseases, including cancer, heart disorders, and neurodegenerative conditions (Malgwi et al., 2024). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants. ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, can cause damage to cellular components, including DNA, proteins, and lipids, leading to chronic diseases and aging (Sies and Jones, 2020). Natural antioxidants, such as those found in medicinal plants, play a crucial role in scavenging ROS and mitigating oxidative damage. Therefore, the search for natural antioxidants from medicinal plants has gained significant attention in recent years, as they offer a safer and more sustainable alternative to synthetic antioxidants, which are often associated with adverse effects (Xu et al., 2021). In this study, we isolated a bioactive compound from the methanol leaf extract of *Diospyros mespiliformis* and identified it as a mixture of α-amyrin and β-amyrin. α-Amyrin and β-amyrin, pentacyclic triterpenes, exhibit diverse pharmacological properties including anti-inflammatory, antimicrobial, antioxidant, insecticidal, anti-arthritic, anti-nociceptive, antidepressant, anti-hyperglycemic, antiulcer, and gastroprotective effects as demonstrated in *Suaeda maritima* studies along India's south-east coast (Pati and Nandi, 2024). The isolation of these compounds from *Diospyros mespiliformis* provides further insight into the plant's medicinal potential and supports its traditional use in managing oxidative stress-related conditions. Despite the widespread use of *Diospyros mespiliformis* in traditional medicine, there is limited scientific research on its phytochemical composition and antioxidant potential. While some studies have explored the antimicrobial and anti-inflammatory properties of the plant, there is also limited data on its antioxidant activity, particularly from its leaf extracts. Given the plant's traditional use in managing diseases related to oxidative stress, there is a need for systematic studies to validate its antioxidant properties and identify the bioactive compounds responsible for these effects. This study, conducted at the Faculty of Pharmacy, University of Maiduguri, aims to address this gap by investigating the phytochemical constituents and antioxidant activity of the methanol leaf extract of *Diospyros mespiliformis*. The specific objectives of the study were to carry out preliminary phytochemical screening of the crude methanol leaf extract, isolate and identify bioactive compounds from the extract, and evaluate the antioxidant activity of the crude extract and the isolated compounds using the DPPH radical scavenging assay.

## Materials and Methods

### Plant material collection and extraction

Leaves of *Diospyros mespiliformis* were collected in Jama'are, Bauchi State, Nigeria, in October 2021. The plant was identified and authenticated by Dr. Cletus A. Ukwubile of the Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, Nigeria. A voucher specimen number of UMM/FPH/EBN/001 was deposited in the herbarium of the Department of Pharmacognosy. The leaves were air-dried, powdered, and 500 g of the dried material was extracted with 5 L of absolute methanol by maceration. The crude extract was concentrated under reduced pressure and subsequently subjected to phytochemical screening and further isolation of active constituents.

### Phytochemical screening

Preliminary phytochemical screening was performed to detect secondary metabolites, including alkaloids, carbohydrates, cardiac glycosides, flavonoids, saponins, and tannins using standardized qualitative methods (Shaikh and Patil, 2020).

### Isolation of bioactive compounds

The crude methanol extract was subjected to column chromatography on silica gel as the stationary phase. Elution was carried out using a gradient of *n*-hexane and chloroform as the mobile phase with increasing polarity, starting from *n*-hexane: chloroform (9:1), followed by 8:2, 7:3, 1:1, and finally 3:7 (v/v) at a flow rate of 1 ml/min. Fractions of 5 mL each were collected and monitored by thin-layer chromatography (TLC) using silica gel 60 F254 plates. Spots were visualized under UV light (254 and 365 nm) and by spraying with 5% ethanolic sulphuric acid, followed by gentle heating. Fractions with similar TLC profiles were pooled, concentrated and subjected to further purification and analysis. A compound, designated as DMLM73, which was identified as a mixture of α-amyrin and β-amyrin, was

successfully isolated. The structure of DMLM73 was characterized using $^{1}H$ and $^{13}C$ NMR spectroscopy, and its identity was confirmed by comparing the spectral data with relevant literature (s).

### Antioxidant activity assay

The antioxidant activity of the fractions was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay as described by Munteanu et al. (2021). Briefly, 2.0 mL of freshly prepared 0.1 mM DPPH solution was mixed with 0.9 mL of Tris-HCl buffer (pH 7.4) and 0.1 mL of the test sample. The mixture was incubated at room temperature for 30 minutes, and the absorbance was measured at 517 nm using a UV-visible spectrophotometer. The percentage inhibition of DPPH radicals was calculated, and the $IC_{50}$ values were determined. Ascorbic acid was used as a standard for comparison.

### Statistical analysis

All antioxidant assays were carried out in triplicate ($n = 3$), and results are expressed as mean ± standard error of the mean (SEM). Percentage inhibition was calculated for each replicate. The $IC_{50}$ values were determined by regression analysis of the dose–response curves using Microsoft Excel 2024.

## Results

### Phytochemical screening

Phytochemical screening of the crude methanol extract was conducted to identify the presence of various secondary metabolites. The results are summarized in Table 1.

**Table 1. Phytochemical Screening Results of Crude Methanol Extract**

| Phytochemicals | Test/Reagent Used | Observation | Result |
|---|---|---|---|
| Carbohydrates | Molisch's test | Purple ring at the interface | Positive |
| Alkaloids | Mayer's reagent | White precipitate | Positive |
| Flavonoids | Shinoda test | Red or pink colour | Positive |
| Terpenoids | Salkowski test | Reddish-brown layer | Positive |
| Saponins | Froth test | Persistent froth formation | Positive |
| Tannins | Ferric chloride test | Blue-black coloration | Positive |
| Steroids | Liebermann-Burchard test | Greenish coloration | Positive |
| Phenolic compounds | Folin-Ciocalteu reagent | Blue coloration | Positive |

### 3.2 Isolation and Characterization of DMLM73

The crude methanol extract was purified using column chromatography with silica gel (60–120 mesh) as the stationary phase and a gradient elution system of *n*-hexane and chloroform as the mobile phase, starting with 100% n-hexane and gradually increasing to 100% chloroform. Fractions of 10 mL each were collected and monitored using thin-layer chromatography (TLC) with a solvent system of n-hexane: ethyl acetate (7:3, v/v). The TLC plates were air-dried, visualized under UV light at 254 nm and 366 nm, and then sprayed with 5% sulfuric acid in ethanolic solution and heated at 110°C for 5–10 minutes, revealing distinct spots. Fractions 1–10, eluted with 100% n-hexane, showed no significant spots, while fractions 11–25, eluted with 90% n-hexane and 10% chloroform, displayed faint spots with $R_f$ values of 0.12 and 0.18, indicating minor non-polar compounds. Fractions 26–40, eluted with 80% n-hexane and 20% chloroform, showed two distinct spots with $R_f$ values of 0.45 and 0.52, which turned violet. respectively, after visualization, suggesting the presence of triterpenoids. Fractions 41–55, eluted with 70% n-hexane and 30% chloroform, exhibited a single dominant spot with an $R_f$ value of 0.52, which turned brown and was identified as the target compound. Fractions 56–70, eluted with 50% n-hexane and 50% chloroform, showed no significant spots and were discarded. The combined fractions (26–40 and 41–55) were further purified using preparative TLC with the same solvent system, and the band corresponding to the $R_f$ value of 0.52 was scraped off, extracted with chloroform, and evaporated under reduced pressure to yield a white crystalline solid, designated as DMLM73. The TLC plate of fractions collected after column chromatography are presented in Figure 1.

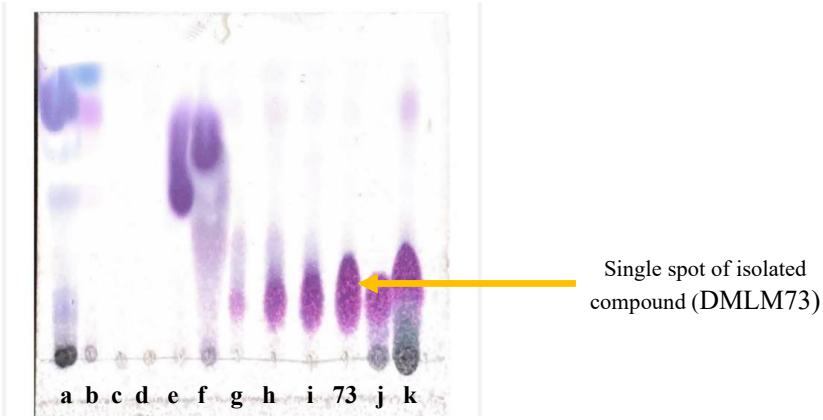


**Figure 1. TLC profile of fractions collected after column chromatography**

**Characterization of DMLM73 by NMR spectroscopy**

The $^{1}H$ NMR spectrum (500 MHz, $CDCl_3$) of compound DMLM73 (Figure 2) revealed characteristic signals consistent with a mixture of α-amyrin and β-amyrin. Two triplets were observed at δ 5.16 ppm (H-12, 1H) and δ 5.10 ppm, corresponding to the olefinic protons (H-12) of α-amyrin and β-amyrin, respectively. Additionally, eight singlets corresponding to methyl ($CH_3$) protons were identified in the range of δ 1.28–0.89 ppm. Key signals included singlets at δ 0.89 ppm (3H) and δ 0.88 ppm (3H), indicating the presence of methyl group -28. A characteristic oxygenated proton signal at position C-3 was observed at δ 3.22 ppm (1H, s; CH-3). The methyl groups of α-amyrin were identified at δ 1.16 ppm (singlet; CH3-27), δ 0.93 ppm (doublet-doublet; J = 3.2 Hz; CH3-30), and δ 0.82 ppm (singlet; CH3-29). For β-amyrin, the methyl groups CH3-27, CH3-29, and CH3-30 exhibited peaks at δ 1.28 ppm (singlet; 3H; CH3-27) and δ 0.95 ppm (singlet; 6H; CH3-29 and CH3-30). Other signals common to both α-amyrin and β-amyrin included: δ 1.11 ppm (6H, s; CH3-26), δ 1.01 ppm (6H, s; CH3-28), δ 0.78 ppm (6H, s; CH3-25), δ 0.98 ppm (6H, s; CH3-23), and δ 0.98 ppm (6H, s; CH3-24). The $^{13}C$ NMR spectrum (500 MHz, $CDCl_3$) of DMLM73 (Figure 3) displayed four peaks in the range of δ 121.7–145.2 ppm, characteristic of the olefinic carbons (C-12 and C-13) in the olean-12-ene and urs-12-ene skeletons. Specifically, signals at δ 145.2 ppm and δ 121.7 ppm were attributed to β-amyrin, while signals at δ 139.6 ppm and δ 124.4 ppm were assigned to α-amyrin. Additionally, signals corresponding to C-19 and C-20 were observed at δ 47.3 ppm and δ 31.21 ppm for β-amyrin and δ 39.68 ppm for α-amyrin. A distinct signal at δ 79.1 ppm, characteristic of the hydroxyl group at C-3, was present for both α-amyrin and β-amyrin. The combined $^{1}H$ and $^{13}C$ NMR data enabled the identification of DMLM73 as a mixture of α-amyrin and β-amyrin (Figure 4). This identification was further confirmed by direct comparison of the spectral data with published literature (Viet *et al*., 2021). The complete $^{1}H$ and $^{13}C$ NMR data are shown in Table 3. In summary, the combined $^{1}H$ and $^{13}C$ NMR spectral data of compound DMLM73, along with comparison to reported literature values (Viet *et a*l., 2021), confirmed its identity as a mixture of α-amyrin and β-amyrin.

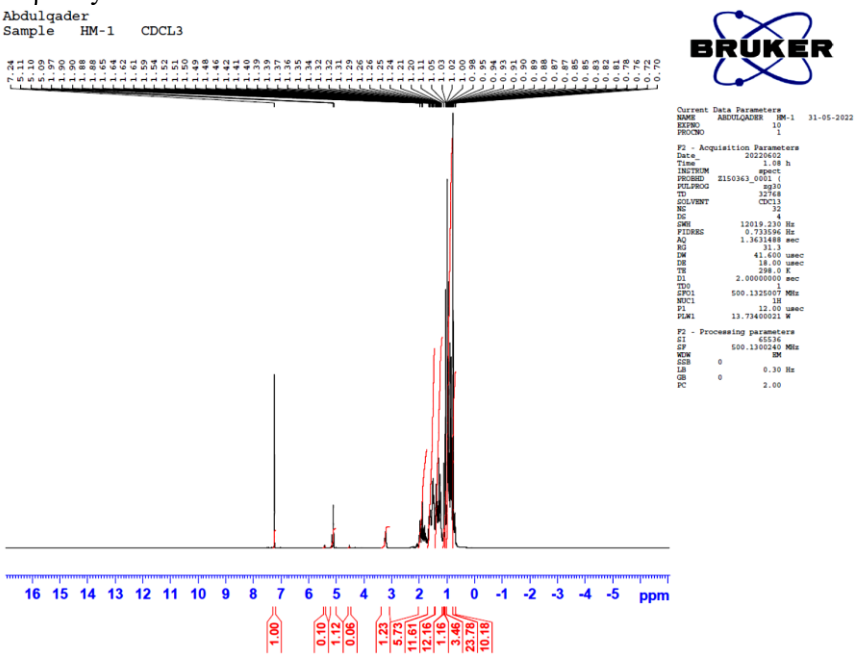


**Figure 2. $^{1}H$ NMR spectrum of DMLM73**

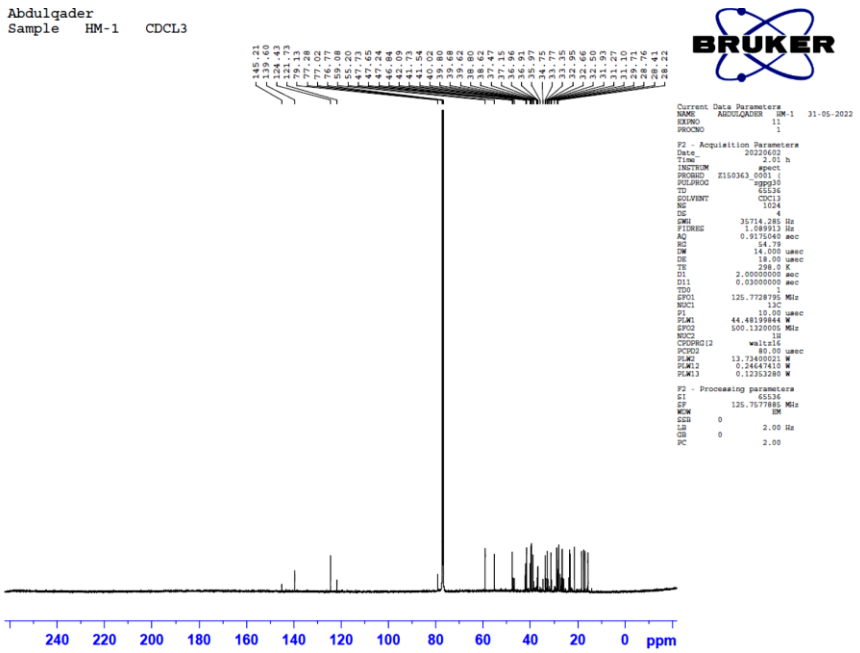


**Figure 3. $^{13}$C NMR spectrum of DMLM73**

**Table 3. Chemical shift data obtained from DMLM73 with literature data of α, β-amyrin**

| Position | DMLM73 | | α-amyrin (λ) | | DMLM73 | | β-amyrin (λ) | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 1.46-1.54 | 38.80 | | 38.80 | 1.46-1.54 | 38.62 | 1.55, 1.49 | 38.60 |
| 2 | 1.46-1.54 | 27.40 | 1.61 | 27.30 | 1.46-1.54 | 27.40 | 1.52, 1.55 | 27.20 |
| 3 | 3.22 | 79.10 | 3.24 | 79.10 | 3.22 | 79.10 | 3.20 | 79.00 |
| 4 | | 38.80 | 58.2 | 38.80 | | 38.80 | | 39.99 |
| 5 | 0.81 | 55.20 | 0.81 | 55.20 | 0.72 | 55.20 | 0.71 | 55.20 |
| 6 | | 18.37 | | 18.58 | | 18.37 | | 18.44 |
| 7 | | 32.95 | | 32.90 | | 32.50 | | 32.50 |
| 8 | | 40.01 | | 40.00 | | 41.73 | | 41.70 |
| 9 | 1.54 | 47.73 | 1.54 | 47.70 | 1.90 | 47.43 | 1.95 | 47.60 |
| 10 | | 36.91 | | 36.90 | | 37.15 | | 37.00 |
| 11 | 1.84 | 23.28 | 1.89 | 23.30 | 1.83 | 23.70 | 1.84 | 23.70 |
| 12 | 5.16 | 124.43 | 5.10 | 124.40 | 5.16 | 121.73 | 5.16 | 121.70 |
| 13 | | 139.60 | | 139.60 | | 145.21 | | 145.20 |
| 14 | | 41.54 | | 41.50 | | 42.09 | | 42.80 |
| 15 | | 28.11 | | 28.10 | | 26.95 | | 26.90 |
| 16 | | 26.63 | | 26.60 | | 26.17 | | 26.20 |
| 17 | | 33.77 | 30.1 | 33.80 | | 32.66 | | 32.70 |
| 18 | 1.29 | 59.08 | 1.29 | 59.10 | 1.88 | 47.24 | 1.89 | 47.20 |
| 19 | 1.39 | 39.68 | 1.38 | 39.70 | 1.22-1.37 | 47.73 | 1.59 | 46.80 |
| 20 | 1.98 | 39.68 | 1.98 | 39.60 | | 31.27 | | 31.30 |
| 21 | | 31.27 | | 31.30 | 1.46- 1.54 | 34.75 | 1.66 | 34.70 |
| 22 | | 40.01 | | 40.00 | | 37.15 | | 37.10 |
| 23 | 0.98 | 28.11 | 0.85 | 28.10 | 0.78 | 28.11 | 0.77 | 28.10 |
| 24 | 0.98 | 15.75 | 0.84 | 15.70 | 0.98 | 15.75 | 0.98 | 15.60 |
| 25 | 0.95 | 15.70 | 0.96 | 15.60 | 0.93 | 15.70 | 0.92 | 15.50 |
| 26 | 0.98 | 17.49 | 0.98 | 17.40 | 0.94 | 16.88 | 0.94 | 16.80 |
| 27 | 1.05 | 23.38 | 1.04 | 23.40 | 1.11 | 26.01 | 1.11 | 26.00 |
| 28 | 0.78 | 28.78 | 0.78 | 28.80 | 0.81 | 28.41 | 0.81 | 28.40 |
| 29 | 0.76 | 16.82 | 0.77 | 16.90 | 0.85 | 33.56 | 0.85 | 33.30 |
| 30 | 0.82 | 21.41 | 0.83 | 21.40 | 0.85 | 23.54 | 0.85 | 23.50 |

**Key**: (λ)= Viet *et al*., (2021), ($\delta_H$, $\delta_C$) = $^1$H and $^{13}$C NMR chemical shift values

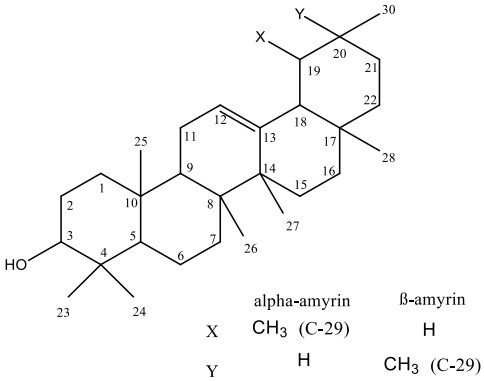


**Figure 4. Chemical structure of α, β-amyrin isolated from *D. mespiliformis* leaves**

### Antioxidant Activity

The *in vitro* antioxidant activities of *Diospyros mespiliformis* leaf extract, the isolated compound (DMLM73), and ascorbic acid were evaluated using the DPPH radical scavenging assay, as presented in Table 4. Ascorbic acid, the positive control, exhibited the highest potency, with an $IC_{50}$ value of 4.25 µg/mL, indicating strong free radical scavenging activity even at low concentrations. The leaf extract demonstrated moderate antioxidant activity, with an $IC_{50}$ of 10.5 µg/mL, likely due to the presence of bioactive compounds such as phenolics and flavonoids. The isolated compound, DMLM73, showed superior activity compared to the crude extract, with an $IC_{50}$ of 8.5 µg/mL, suggesting that the isolation process concentrated antioxidant components like triterpenoids.

**Table 4: *In-vitro* antioxidant activities of *Diospyros mespiliformis* leaf extract, isolated compound (DMLM73), and ascorbic Acid**

| Concentration (µg/mL) | % DPPH Scavenging Activity | | |
|---|---|---|---|
| | Ascorbic Acid | *D. mespiliformis* Leaf Extract | Isolated Compound (DMLM73) |
| 6.25 | 84.32 | 43.10 | 26.88 |
| 12.5 | 89.10 | 52.40 | 75.33 |
| 25 | 96.40 | 54.90 | 86.50 |
| 50 | 98.50 | 55.90 | 80.54 |
| 100 | 98.80 | 65.20 | 80.44 |
| **$IC_{50}$ (µg/mL)** | **4.25** | **10.5** | **8.5** |

## Discussion

The findings of this study highlight the significant antioxidant potential of *Diospyros mespiliformis* leaf extract and the isolated compound, DMLM73, a mixture of α-amyrin and β-amyrin. The DPPH radical scavenging assay revealed that the isolated compound exhibited strong antioxidant activity, with an $IC_{50}$ value of 8.5 µg/mL, which is more potent than the crude extract ($IC_{50}$ = 10.5 µg/mL) but less potent than the standard antioxidant, ascorbic acid ($IC_{50}$ = 4.25 µg/mL). This suggests that the isolation process concentrated the bioactive components responsible for the antioxidant activity, likely the triterpenoids α-amyrin and β-amyrin, which are known for their free radical scavenging properties. The crude methanol extract also demonstrated notable antioxidant activity, albeit less potent than the isolated compound. This activity can be attributed to the presence of various phytochemicals, including flavonoids, tannins, and phenolics, which are known to contribute to antioxidant effects. The results align with previous studies that have reported the antioxidant potential of *Diospyros mespiliformis*, further validating its traditional use in managing oxidative stress-related conditions. The isolation of α-amyrin and β-amyrin from *Diospyros mespiliformis* in this study is consistent with previous research on the plant's phytochemical composition. Ramadwa & Meddows-Taylor, (2023) reported the presence of triterpenoids, including α-amyrin and β-amyrin, in *Diospyros mespiliformis*, highlighting their anti-inflammatory and antimicrobial properties. Similarly, Rosas-Viet et al. (2021) previously isolated these bioactive compounds from *Diospyros mespiliformis* and characterized their antioxidant and anti-inflammatory properties. Our findings corroborate these observations while demonstrating significantly enhanced antioxidant activity, as evidenced by a lower IC50 value of 8.5 µg/mL compared to previous reports. This increased potency may be attributed to differences in compound concentration, purity, or chemotypic variation due to geographic origin (Misra et al., 2021).

The strong antioxidant activity of DMLM73 suggests its potential for use in pharmaceutical and nutraceutical applications, particularly in formulations aimed at reducing oxidative stress and preventing free radical-mediated damage. The crude methanol extract, while less potent than the isolated compound, still holds promise as a source of natural antioxidants, especially in traditional medicine or as a starting material for further isolation and purification of bioactive compounds. Despite these promising results, further studies are needed to explore the mechanisms of action of the isolated compounds and their potential synergistic effects with other bioactive compounds in the crude extract. Additionally, in vivo studies are necessary to evaluate the bioavailability, safety, and efficacy of these compounds in biological systems. Comparative studies with other medicinal plants known for their antioxidant properties could also provide insights into the relative efficacy of *Diospyros mespiliformis* as a natural antioxidant source.

## Conclusion

This study demonstrates that *Diospyros mespiliformis* is a valuable source of bioactive compounds with significant antioxidant potential. Through column chromatography and thin-layer chromatography (TLC), a bioactive compound, DMLM73, was isolated from the methanol leaf extract. The compound was characterized using $^{1}H$ and $^{13}C$ NMR spectroscopy and identified as a mixture of α-amyrin and β-amyrin, two triterpenoids known for their antioxidant properties. The findings validate the traditional use of *Diospyros mespiliformis* in managing oxidative stress-related conditions and highlight its potential for pharmaceutical and nutraceutical applications. The superior antioxidant activity of the isolated compound compared to the crude extract underscores the importance of further research into its therapeutic potential. Future studies should focus on elucidating the mechanisms of action, conducting *in vivo* evaluations, and exploring the synergistic effects of these compounds. In addition, formulation studies and stability assessments are recommended to support the potential development of natural antioxidant-based therapeutic products.

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## Author contributions

Abdulqadir Bukar Bababe: Conceptualization, project administration, supervision, writing, original draft, writing review, and editing.
Hauwa Modu Mustapha: Methodology, investigation, data curation, writing, original draft.
Ibrahim Iliya: Methodology, investigation, data curation, supervision, formal analysis, validation.
Falmata Madu Aliyu: Investigation, data curation, resources, validation.
Hassan Braimah Yesufu: Investigation, resources, validation, writing review, and editing.
Hananiya Milagawanda Hamza: Investigation, data curation, resources, and editing.

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## Conflict of interest

The authors declare no conflict of interest.

## Ethics approval

Not applicable.

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