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Gas Chromatography–Mass Spectrometry Profiling and DPPH Radical-Scavenging Capacity of the N-Hexane Extract of Arnebia Linearifolia A.DC. (Boraginaceae): First Report of Shikonin and Its Esters in the Species

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ABSTRACT: The genus Arnebia (Boraginaceae) is a recognised source of shikonin-type naphthoquinones, but pharmacognostic attention has concentrated on a small number of slow-growing perennials, several of which are now threatened in the wild. Arnebia linearifolia A.DC., an annual with a wide native distribution across Iraq and South-West Asia, has not previously been chemically profiled. In this study the lipophilic constituents of A. linearifolia were recovered by cold maceration in n-hexane and characterised by gas chromatography–mass spectrometry (GC-MS), and the radical-scavenging capacity of the resulting extract was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Maceration of 20.0 g of dried plant powder in n-hexane (1:10 w/v, 24 h, 22–25 °C) gave 0.770 g of a lipophilic extract, a yield of 3.85% (w/w). GC-MS resolved twelve constituents accounting for 94.62% of the total integrated peak area. Fatty acids and their esters formed the largest fraction (47.10%; linoleic acid 18.65%, palmitic acid 14.20%, oleic acid 11.40%, ethyl palmitate 2.85%), followed by naphthoquinones (31.00%; shikonin 15.80%, acetylshikonin 9.75%, isobutyrylshikonin 5.45%), phytosterols (9.25%; β-sitosterol 7.10%, stigmasterol 2.15%), squalene (4.30%), α-tocopherol (1.72%) and tetracosane (1.25%). Combining the extraction yield with the peak-area data places the shikonin content of the dried plant material at approximately 6.08 mg g−1 and total naphthoquinone at approximately 11.93 mg g−1. The extract scavenged DPPH radicals in a concentration-dependent manner, from 18.42 ± 1.15% at 6.25 µg mL−1 to 91.15 ± 1.65% at 200 µg mL−1, giving a half-maximal inhibitory concentration (IC50) of 26.85 ± 1.12 µg mL−1 against 8.82 ± 0.35 µg mL−1 for ascorbic acid. These results constitute the first report of shikonin, acetylshikonin and isobutyrylshikonin in A. linearifolia and identify the species as a potentially sustainable annual source of shikonin-type naphthoquinones. Because these compounds are thermolabile and only semi-quantitatively determined by GC-MS, the reported proportions require confirmation by HPLC with photodiode-array detection.

1. INTRODUCTION

The genus *Arnebia* Forssk. (Boraginaceae) comprises annual and perennial herbs distributed across the arid and semi-arid belt from North Africa to the western Himalaya. Its roots have a long history of traditional use in the Middle East, Central Asia,

India and China, principally in the treatment of burns, wounds, ulcers and inflammatory skin conditions, and the genus has been the subject of a substantial modern pharmacological literature (Hosseini *et al.*, 2018; Bao *et al.*, 2025).

The chemistry underlying that reputation is dominated by the naphthazarin pigments alkannin and shikonin and their side-chain esters. These compounds combine a 5,8-dihydroxy-1,4-naphthoquinone core with an isohexenyl side chain, and their chemistry and biology have been reviewed comprehensively (Papageorgiou *et al.*, 1999). Interest in shikonin has intensified as its antitumour, antimicrobial and anti-inflammatory activities have been characterised in more detail (Zhang, X. *et al.*, 2018; Zhu *et al.*, 2021), and the biosynthetic route to the naphthoquinones and the enzymes that partition flux between the shikonin and shikonofuran branches have been progressively resolved in *Arnebia euchroma* (Wang, R. *et al.*, 2023; Wang, S. *et al.*, 2023).

Supply, however, has become a limiting concern. The species that have attracted most pharmacognostic effort — *A. benthamii*, *A. euchroma*, *A. nobilis* — are slow-growing perennials harvested for their roots, and *A. benthamii* is now regarded as critically endangered in the north-western Himalaya. This has driven sustained interest in cell-culture and biotechnological routes to shikonin production (Malik *et al.*, 2016). An alternative and much less explored approach is to look for comparable naphthoquinone content in fast-growing annual congeners with wide distributions.

Solvent choice determines whether such a survey can succeed at all. Bhat *et al.* (2024) showed that shikonin was undetectable by HPLC, LC-MS, GC-MS or normal-phase HPTLC in aqueous-ethanolic extracts of *A. benthamii* roots, yet was recovered at 4.55 mg g^{-1} when hexane was used. Polar solvents recover a far greater total mass — sugars, polar phenolics, tannins — but leave the lipophilic naphthazarins in the marc. Any screen for shikonin-type compounds that relies on methanolic or aqueous extraction is therefore liable to return a false negative.

Arnebia linearifolia A.DC. is an annual herb native to Iraq and much of South-West Asia (Plants of the World Online, 2026). Despite this accessibility, and despite work on the congeneric Iraqi species *A. decumbens* (Al-Moosawy, 2017), no phytochemical profile of *A. linearifolia* appears to have been published, and its naphthoquinone content is unknown.

The present study was therefore undertaken to characterise the lipophilic constituents of *A. linearifolia* by GC-MS following *n*-hexane maceration, to estimate the naphthoquinone content of the dried plant material, and to determine the radical-scavenging capacity of the extract by the DPPH assay. The antileishmanial activity of the same extract is reported separately (Nasser and Rahi, companion manuscript).

2. MATERIALS AND METHODS

2.1 Plant material

Fresh specimens of *Arnebia linearifolia* (roots and aerial parts) were collected during the flowering season. [DATA NEEDED: collection locality, GPS coordinates, altitude and exact collection date; whether roots and aerial parts were extracted together or separately, and in what proportion by mass.] Specimens were authenticated by a taxonomist [DATA NEEDED: name of the taxonomist, institution, and the herbarium accession/voucher number under which a specimen was deposited — most journals will not proceed without a voucher].

Plant material was washed in distilled water to remove adhering debris, shade-dried at room temperature (22–25 °C) for 10–14 days and then oven-dried at 40 °C for 12 h to a moisture content below 5%. The dried material was milled to a homogeneous powder (particle size < 0.5 mm) in a mechanical grinder and stored in airtight containers at −20 °C until extraction.

2.2 Extraction

Dried powder (20.0 g) was macerated in *n*-hexane (≥95%, analytical grade, Sigma-Aldrich, USA) at a 1:10 (w/v) ratio (200 mL) in a sealed conical flask, agitated on an orbital shaker (Labnet International, USA) at 160 rpm for 24 h at 22–25 °C. The mixture was filtered through Whatman No. 1 paper and the residue re-extracted with a further 100 mL of *n*-hexane for 2 h. The combined filtrates were concentrated on a rotary evaporator under reduced pressure at 40 °C, dried under a stream of nitrogen, weighed and stored in amber vials at −20 °C. Extraction yield was expressed as a percentage of the dry weight of starting material. [DATA NEEDED: number of independent extraction batches performed; the reported yield is presented as a single determination.]

2.3 GC-MS analysis

Analysis was performed on a Shimadzu GC-MS system fitted with a capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) [DATA NEEDED: stationary phase and column trade name, e.g. Rtx-5MS]. Helium (99.999%) was the carrier gas at a constant flow of 1.0 mL min^{-1}. The injector was held at 250 °C in split mode (1:10). The oven programme began at 60 °C (2 min hold), rose at 10 °C min^{-1} to 280 °C and was held for 10 min. Mass spectra were acquired in electron-impact mode at 70 eV over m/z 40–550. Constituents were identified by comparison of their mass spectra and retention behaviour with the NIST and Wiley reference libraries. Relative abundance is expressed as the percentage of total integrated peak area. [DATA NEEDED: injection volume, sample concentration and solvent, and the number of injections analysed.]

2.4 DPPH radical-scavenging assay

Radical-scavenging capacity was determined by the DPPH method. DPPH solution (1.0 mL, 0.1 mM in methanol) was mixed with 1.0 mL of extract at concentrations of 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 µg mL^{-1}. [DATA NEEDED: solvent used to prepare and dilute the *n*-hexane extract for this methanolic assay, and the composition of the solvent blank against which absorbance was referenced.] Reaction mixtures were held in the dark at room temperature for 30 min and absorbance read at 517 nm. Ascorbic acid served as the reference standard across the same concentration range. Scavenging activity was calculated as:

$$\% \text{ scavenging} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.5 Statistical analysis

DPPH measurements were performed in triplicate in three independent experiments (n = 3) and are expressed as mean ± standard deviation. IC_{50} values were obtained by non-linear regression of scavenging activity on $\log_{10}$ concentration in GraphPad Prism (v9.0).

3. RESULTS

3.1 Extraction yield

Maceration of 20.0 g of dried plant powder yielded 0.770 g of a dark, oily extract, corresponding to 3.85% (w/w). The extract was completely soluble in both *n*-hexane and dimethyl sulfoxide.

3.2 Chemical composition

GC-MS analysis resolved twelve constituents accounting for 94.62% of the total integrated peak area (Table 1, Figure 1). [DATA NEEDED: the total ion chromatogram itself should be included as a figure or as supplementary material; reviewers of phytochemical work routinely request it.]

Table 1. Constituents of the *n*-hexane extract of *Arnebia linearifolia* identified by GC-MS.

Peak	Compound	RT (min)	Area (%)	Formula	Chemical class
1	Tetracosane	14.22	1.25	$C_{24}H_{50}$	Alkane hydrocarbon
2	*n*-Hexadecanoic acid (palmitic acid)	16.42	14.20	$C_{16}H_{32}O_2$	Saturated fatty acid
3	Hexadecanoic acid, ethyl ester	17.10	2.85	$C_{18}H_{36}O_2$	Fatty acid ethyl ester
4	9,12-Octadecadienoic acid (linoleic acid)	18.85	18.65	$C_{18}H_{32}O_2$	Polyunsaturated fatty acid
5	9-Octadecenoic acid (oleic acid)	19.02	11.40	$C_{18}H_{34}O_2$	Monounsaturated fatty acid
6	Shikonin	21.15	15.80	$C_{16}H_{16}O_5$	Naphthoquinone pigment
7	Acetylshikonin	22.48	9.75	$C_{18}H_{18}O_6$	Naphthoquinone ester
8	Stigmasterol	24.95	2.15	$C_{29}H_{48}O$	Phytosterol
9	β-Sitosterol	25.30	7.10	$C_{29}H_{50}O$	Phytosterol

Peak	Compound	RT (min)	Area (%)	Formula	Chemical class
10	Isobutyrylshikonin	26.12	5.45	$C_{20}H_{22}O_6$	Naphthoquinone ester
11	Squalene	27.05	4.30	$C_{30}H_{50}$	Triterpenoid / isoprenoid
12	α-Tocopherol	28.40	1.72	$C_{29}H_{50}O_2$	Tocopherol

Grouped by class, the profile comprised a fatty-acid fraction of 47.10% (of which 30.05% was unsaturated), a naphthoquinone fraction of 31.00%, a phytosterol fraction of 9.25%, and minor contributions from squalene, α-tocopherol and a long-chain alkane (Figure 1a).

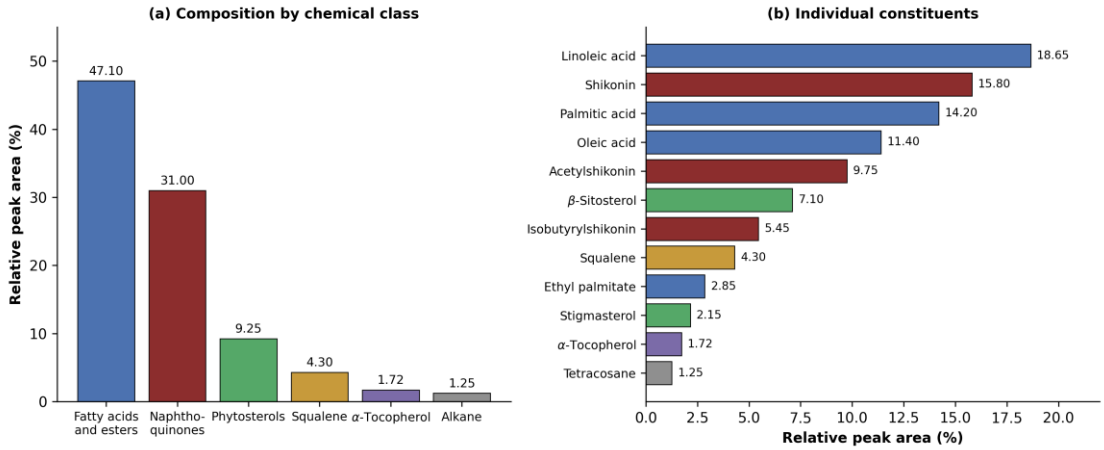


Figure 1. Composition of the *n*-hexane extract of *Arnebia linearifolia* determined by GC-MS, expressed as percentage of total integrated peak area: (a) by chemical class; (b) by individual constituent. Bars in (b) are coloured by the class assignment used in (a).

Combining the extraction yield of 3.85% with the peak-area percentages gives an estimated shikonin content of approximately 6.08 mg g^{-1} of dried plant material and a total naphthoquinone content of approximately 11.93 mg g^{-1}.

3.3 DPPH radical-scavenging activity

The extract scavenged DPPH radicals in a concentration-dependent manner across the range tested, rising from 18.42 ± 1.15% at 6.25 μg mL^{-1} to 91.15 ± 1.65% at 200 μg mL^{-1} (Table 2, Figure 2). Ascorbic acid was more potent at every concentration, beginning at 42.10 ± 1.85% at 6.25 μg mL^{-1} and exceeding 96% by 100 μg mL^{-1}. The IC_{50} was 26.85 ± 1.12 μg mL^{-1} for the extract and 8.82 ± 0.35 μg mL^{-1} for ascorbic acid, a difference of approximately 3.0-fold.

Table 2. DPPH radical-scavenging activity of the *n*-hexane extract of *Arnebia linearifolia* and of ascorbic acid (mean ± SD, n = 3).

Concentration (μg mL^{-1})	*A. linearifolia* extract, scavenging (%)	Ascorbic acid, scavenging (%)
6.25	18.42 ± 1.15	42.10 ± 1.85
12.50	31.05 ± 1.48	61.35 ± 2.10
25.00	47.80 ± 2.05	78.90 ± 1.75
50.00	68.25 ± 1.90	91.20 ± 1.40
100.00	82.40 ± 2.30	96.50 ± 0.85
200.00	91.15 ± 1.65	98.80 ± 0.42
IC_{50}	**26.85 ± 1.12**	**8.82 ± 0.35**

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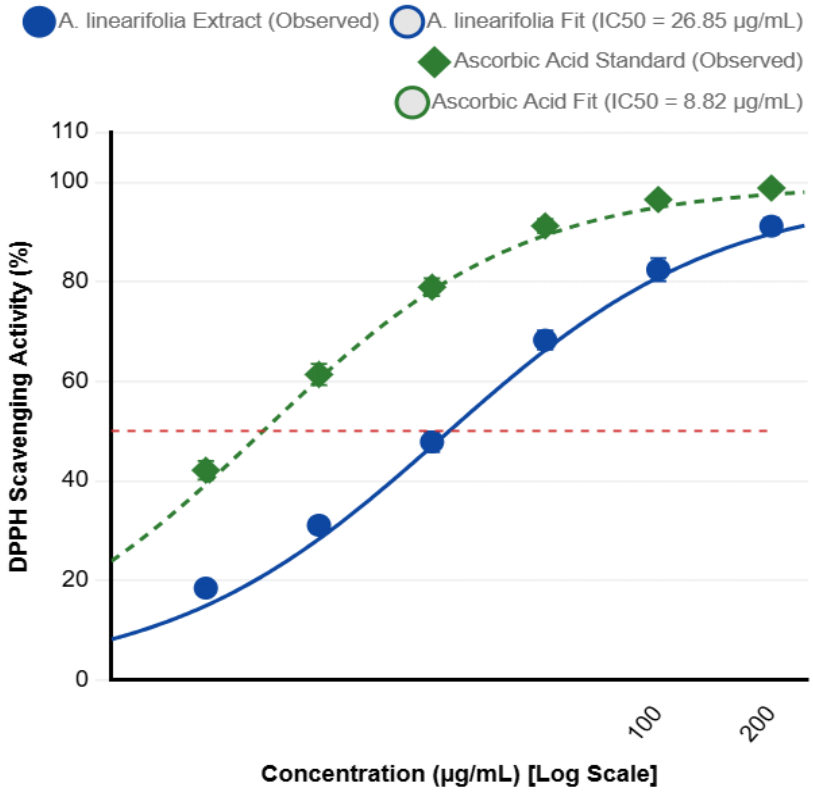


Figure 2. Concentration–response curves for DPPH radical scavenging by the *n*-hexane extract of *Arnebia linearifolia* and by ascorbic acid. Points are mean ± SD of three independent experiments; curves are non-linear regression fits. The dashed horizontal line marks 50% scavenging.

4. DISCUSSION

4.1 Yield reflects chemical selectivity rather than inefficiency

A yield of 3.85% is low against what polar extraction of comparable material typically returns; Al-Ogaili (2025), for example, obtained 6.93% by conventional methanolic extraction of Iraqi *Cordia myxa* and 14.8% by an optimised ultrasound-assisted method. Read as a measure of efficiency, that comparison is unfavourable. Read as a measure of selectivity, it is the point of the method.

The finding of Bhat *et al.* (2024) that shikonin is recoverable from *A. benthamii* by hexane but not detectable in aqueous-ethanolic extracts of the same roots establishes the principle directly. Non-polar extraction sacrifices gross mass in order to concentrate the naphthazarin fraction, and the data in Table 1 show what that trade purchased: 31.00% of the total integrated peak area of a 3.85% yield consists of shikonin and two of its esters.

4.2 A naphthoquinone-rich annual

The naphthoquinone content estimated here — approximately 6.08 mg g^{-1} shikonin and 11.93 mg g^{-1} total naphthoquinone in dried material — exceeds the 4.55 mg g^{-1} reported by Bhat *et al.* (2024) for hexane-extracted *A. benthamii*. Given that *A. benthamii* is a slow-growing perennial of restricted and declining distribution, while *A. linearifolia* is an annual distributed across Iraq and much of South-West Asia (Plants of the World Online, 2026), this comparison has a practical implication. If it survives quantitative validation, *A. linearifolia* would represent a more accessible and more readily renewable source of shikonin-type naphthoquinones than the species on which the field has concentrated, and a complement to the cell-culture routes reviewed by Malik *et al.* (2016).

Two qualifications are needed before that inference is pressed. The first is analytical and is set out in Section 4.5. The second is that the present comparison is between a single batch of *A. linearifolia* and a published value for *A. benthamii* obtained in a

different laboratory; naphthoquinone content in *Arnebia* varies with organ, phenological stage, provenance and season, and a proper comparison requires parallel analysis of both species under identical conditions.

4.3 The naphthoquinone sub-profile

The co-occurrence of the free naphthoquinone with two of its side-chain esters is chemically coherent and characteristic of the genus. Esterification takes place at the hydroxyl of the isohexenyl side chain, leaving the naphthazarin core and its two phenolic hydroxyls — the moiety on which redox and antimicrobial activity depend — intact in all three molecules (Papageorgiou *et al.*, 1999; Al-Moosawy, 2017). The three constituents therefore share a pharmacophore while differing in lipophilicity. Simultaneous determination of shikonin, acetylshikonin and β-acetoxyisovalerylshikonin across four *Arnebia* species by densitometric TLC (Sharma *et al.*, 2009) indicates that homologous series of this kind are the norm rather than the exception in the genus.

4.4 Fatty acids, sterols and tocopherol

Fatty acids and their esters formed the largest single fraction of the extract, as would be expected of any non-polar extract of plant material, with linoleic and oleic acids contributing 30.05% between them. Unsaturated fatty acids of this class perturb membranes and have documented antimicrobial properties (Das, 2018), although their membrane-remodelling effects are characteristically expressed above their critical micelle concentrations, reported as 20 μM for oleic acid and 60 μM for linoleic acid (Shin *et al.*, 2023).

β-Sitosterol (7.10%) and stigmasterol (2.15%) are widely distributed phytosterols with their own reported bioactivities, β-sitosterol having been shown to inhibit trypanothione reductase in *Leishmania donovani* (Pramanik *et al.*, 2020) and to dock to the same enzyme and to leishmanolysin in *L. tropica* (Shah *et al.*, 2019). Squalene is an isoprenoid precursor in sterol biosynthesis, a pathway that differs materially between trypanosomatids and their mammalian hosts (Feng *et al.*, 2022; Visbal *et al.*, 2025). α-Tocopherol, at 1.72%, is a chain-breaking lipophilic antioxidant and is relevant both to the DPPH result discussed below and, as a possible internal antagonist of pro-oxidant constituents, to the biological activity of the extract reported in the companion manuscript.

4.5 Interpretation of the antioxidant data

An IC_{50} of 26.85 μg mL^{-1} places the extract approximately 3.0-fold below ascorbic acid, a pure and highly water-soluble reference antioxidant. Two features make that comparison more favourable to the extract than the ratio alone suggests.

First, DPPH is a methanolic assay, and non-polar extracts commonly perform poorly in it for reasons of miscibility and radical accessibility rather than intrinsic reducing capacity. That a hexane extract reaches an IC_{50} below 30 μg mL^{-1} is therefore notable, and it is consistent with the observation of Bhat *et al.* (2024) that the hexane extract of *A. benthamii* showed the highest DPPH activity of all solvent extracts tested — a consequence of, rather than despite, its naphthazarin enrichment. Radical-scavenging capacity has likewise been demonstrated for shikonin isolated from *A. benthamii* (Parray *et al.*, 2015).

Second, the shapes of the two curves differ informatively (Figure 2). Ascorbic acid starts high and saturates rapidly; the extract rises more gradually across the full 32-fold range. A graded response of this kind is the expected signature of a multi-component antioxidant system in which constituents of differing reducing potential contribute across overlapping concentration ranges — here, the two free phenolic hydroxyls of the naphthazarin core in each of three naphthoquinones, supplemented by the chain-breaking activity of α-tocopherol and a minor phytosterol contribution.

It should be emphasised that radical-scavenging capacity measured against a stable synthetic radical in methanol is a chemical property and does not license conclusions about antioxidant behaviour in a biological system. The naphthazarin scaffold is redox-ambivalent: the phenolic hydroxyls donate hydrogen atoms and quench radicals, which is what DPPH measures, while the quinone carbonyls accept electrons and can drive superoxide-generating redox cycling in a cellular environment (Papageorgiou *et al.*, 1999; Zhang, X. *et al.*, 2018). The DPPH result therefore characterises one face of the chemistry only.

4.6 Analytical limitations

Two limitations bear directly on the quantitative claims made here.

Peak-area percentage is not mass percentage. Mass-spectrometric response factors differ between compound classes, and polyoxygenated naphthoquinones and long-chain fatty acids differ appreciably in ionisation efficiency; day-of-analysis calibration is required before relative response factors can be treated as reliable (Rush *et al.*, 2025). Area percentages are best read as rank order and approximate magnitude.

More specifically, shikonin and its esters are thermally labile, relatively involatile, polyhydroxylated compounds that are not ideal GC analytes without derivatisation, and they tend to polymerise at elevated temperature (Papageorgiou *et al.*, 1999; Koletti *et al.*, 2014). The established methods for their quantification are HPLC and HPTLC with detection at 516–520 nm (Hu *et al.*, 2006; Sharma *et al.*, 2009; Bhat *et al.*, 2024). Library matching against NIST and Wiley spectra is sound for compound assignment, but the percentages reported for the naphthoquinone fraction, and the derived figures of 6.08 mg g^{-1} shikonin and 11.93 mg g^{-1} total naphthoquinone, should be regarded as provisional pending HPLC-PDA quantification against authentic standards. That measurement is the single most valuable analytical step to take next, and it would also settle the comparison with *A. benthamii* drawn in Section 4.2.

Two further limitations should be recorded. The material analysed came from a single collection at a single site and season, so nothing can be said here about how naphthoquinone content varies with provenance or phenology. And roots and aerial parts were processed together, which precludes attribution of the naphthoquinone fraction to a particular organ — a relevant point, since in *Arnebia* the naphthazarins are predominantly root pigments and organ-separated extraction would be expected to enrich them substantially.

5. CONCLUSIONS

The *n*-hexane extract of *Arnebia linearifolia* is a naphthoquinone-rich preparation in which shikonin, acetylshikonin and isobutyrylshikonin together account for 31.00% of the identified profile, alongside a fatty-acid fraction of 47.10% and a phytosterol fraction of 9.25%. This is the first report of these naphthoquinones in the species. The derived shikonin content of approximately 6.08 mg g^{-1} of dried plant material is comparable with, and on these figures exceeds, values reported for the extensively studied and now threatened *A. benthamii*, which identifies this widely distributed annual as a candidate sustainable source of shikonin-type compounds. The modest 3.85% (w/w) extraction yield reflects the chemical selectivity of non-polar extraction rather than inefficiency. The extract possesses moderate radical-scavenging capacity, approximately 3.0-fold weaker than ascorbic acid, attributable to the phenolic hydroxyls of the naphthazarin core together with co-extracted α-tocopherol. Confirmation of the naphthoquinone content by HPLC-PDA against authentic standards, and organ-separated analysis across sites and seasons, are the necessary next steps.

Declarations

Ethics approval. This study involved neither human participants nor animals. [DATA NEEDED: confirmation that any collection permit required for wild plant material was obtained, and the permit number.]

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Conflicts of interest. The authors declare no competing interests. [Confirm.]

Author contributions (CRediT). Sara Abdul Razzaq Nasser: Conceptualisation (supporting); Methodology (equal); Investigation (lead) — plant collection, extraction, GC-MS sample preparation and DPPH assays; Formal analysis (equal); Data curation (lead); Visualisation (equal); Writing – original draft (lead); Writing – review and editing (equal). **Abdulsadah A. Rahi:** Conceptualisation (lead); Methodology (equal); Validation (lead); Formal analysis (equal); Resources (lead); Visualisation (equal); Supervision (lead); Project administration (lead); Funding acquisition (lead); Writing – review and editing (equal). Both authors contributed substantially to the work, read the final manuscript and approved it for submission, and accept accountability for its accuracy and integrity.

[CONFIRM: this statement was drafted from the roles implied by the source thesis. Both authors must verify that it describes what each of them actually did before submission; several journals now require each author to confirm their own CRediT roles at the point of submission.]

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