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Research Article

# Phytochemical profile, antioxidant capacity, and enzyme inhibitory properties of *Alkanna tubulosa* extract obtained via ultrasound-assisted extraction

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**Abstract:** Phytochemicals from under-explored Mediterranean taxa represent an untapped reservoir of multitarget agents for oxidative and metabolic stress management. Here, we characterised the antioxidant and enzyme-modulating potential of a methanolic ultrasound-assisted extract (UAE) prepared from the aerial parts of *Alkanna tubulosa*. Total phenolics reached 97.34 mg GAEs (gallic acid equivalents)/g and flavonoids 45.03 mg REs (Rutin equivalents)/g. LC-ESI-MS/MS (Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry) identified 17 metabolites, predominantly hesperidin (13626 µg/g) and rosmarinic acid (6637 µg/g). The extract exhibited potent radical-scavenging capacity-DPPH (2,2-Diphenyl-1-picrylhydrazyl): 181.43 mg TEs (Trolox equivalents)/g; ABTS [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]: 182.85 mg TEs/g-and high electron-donor power—phosphomolybdenum: 570.77 mg TEs/g; CUPRAC (Cupric Ion Reducing Antioxidant Capacity): 479.07 mg TE/g; FRAP (Ferric Reducing Antioxidant Power): 270.59 mg TE/g-but only moderate $Fe^{2+}$ chelation (6.63 mg EDTAEs (Ethylenediaminetetraacetic Acid equivalents)/g). Enzymatic assays revealed selective inhibition of carbohydrate-hydrolysing enzymes, with α-glucosidase and α-amylase activities equivalent to 598.36 and 299.50 mg ACEs (Acarbose equivalents)/g, respectively; tyrosinase suppression reached 57.30 mg KAEs (Kojic acid equivalents)/g, whereas cholinesterase inhibition was low [AChE (acetylcholinesterase) 2.07 mg GALAEs (Galanthamine equivalents)/g; BChE (butyrylcholinesterase) 1.05 mg GALAEs/g]. These findings position *A. tubulosa* as a rich source of phenolic acid and flavanone-glycoside-centred antioxidants with promising anti-hyperglycaemic and ancillary dermatoprotective properties. Future work should isolate dominant constituents, evaluate their synergistic interactions and validate efficacy in relevant cellular and in vivo models.

## INTRODUCTION

The search for multidimensional, low-toxicity therapeutics has renewed interest in plant-derived secondary metabolites. Polyphenols, flavonoids, alkaloids and terpenoids not only safeguard the producing organism but also act as radical scavengers, metal chelators, enzyme modulators and signal-transduction regulators in human systems (Hartavi, 2025; Hartavi *et al*., 2025). Their pleiotropic behaviour underlies a range of pharmacological indications, from cardioprotection to neuroprotection, and explains why several plant metabolites (e.g.,

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galantamine, paclitaxel) have become first-line drugs. Comprehensive reassessment of lesser-known taxa therefore remains a productive strategy for expanding the phytochemical toolbox (Atanasov *et al*., 2021; Chopra & Dhingra, 2021).

Oxidative stress generated by reactive oxygen and nitrogen species is a common pathological denominator in atherosclerosis, cancer, diabetes, and neurodegeneration. *In vitro* screening typically combines hydrogen-atom-transfer (HAT) and single-electron-transfer (SET)-based assays to capture different mechanistic facets of antioxidant action. DPPH• and ABTS•+ decolourisation reflect radical quenching capacity, whereas CUPRAC and FRAP quantify reducing power; the phosphomolybdenum assay gauges overall redox potential, and ferrous-ion chelation probes metal-sequestering ability. Consensus recommendations now stress the importance of using multi-assay panels to avoid over- or underestimating activity (Gulcin & Alwasel, 2023).

Enzymes that gatekeep neurotransmission, melanogenesis or carbohydrate digestion are validated targets for chronic disorders. Plant extracts rich in phenolics frequently inhibit acetyl- and butyryl-cholinesterase (AChE/BChE), offering symptomatic relief in Alzheimer's disease (ALNasser *et al*., 2025). Polyphenols and flavonoids can also modulate tyrosinase, mitigating hyperpigmentation, while inhibition of α-amylase and α-glucosidase slows post-prandial glycaemic excursions—an approach increasingly advocated for type-2 diabetes management (Farazi *et al*., 2023). Consequently, extracts that combine antioxidant power with a broad enzyme-inhibitory spectrum are highly sought after.

*Alkanna* Tausch (Boraginaceae) comprises about 60 taxa centred in the Mediterranean and Near East. Its roots accumulate naphthoquinone pigments (alkannin/shikonin) responsible for long-standing ethnomedical use in wound healing and more recently recognised for antimicrobial, anti-inflammatory and DNA-topoisomerase-I inhibitory effects (Papageorgiou *et al*., 1999; Sevimli-Gür *et al*., 2009). By contrast, aerial tissues harbour diverse phenolic acids and flavonoids whose bioactivities remain under-charted. *Alkanna tubulosa* Boiss. an Eastern-Mediterranean endemic with fragmented populations, has attracted limited phytochemical scrutiny. The first multi-assay investigation, performed on several extracts obtained by using solvents of different polarities, documented radical-scavenging and selective α-glucosidase/α-amylase inhibition but employed conventional maceration and covered a restricted metabolite panel (Zengin *et al*., 2024). Earlier, Sevimli-Gür *et al*. (2009) reported cytotoxicity of root extracts but did not address antioxidant or enzyme-inhibitory endpoints. No study has yet integrated modern green extraction for *A. tubulosa* aerial parts.

To bridge these gaps, we applied ultrasound-assisted extraction (UAE) with methanol—an energy- and solvent-efficient technique that preserves thermo-labile phenolics—to the aerial parts of *A. tubulosa*. The extract was (i) quantified spectrophotometrically for total phenolics and flavonoids; (ii) profiled by high-resolution LC-ESI-MS/MS; and (iii) evaluated through a six-assay antioxidant panel (DPPH, ABTS, CUPRAC, FRAP, phosphomolybdenum, ferrous-ion chelation) alongside a five-enzyme inhibition suite (AChE, BChE, tyrosinase, α-amylase, α-glucosidase). By juxtaposing our findings with prior data, we provide the most encompassing bioactivity map of *A. tubulosa* to date and assess its suitability as a multitarget lead for functional-food or phytotherapeutic development.

## MATERIAL AND METHODS

### Plant Material

Aerial parts of *A. tubulosa* were harvested on 17 May 2022 in Nebiler village, Kavaklıdere–Muğla, Türkiye (37° 27′ 35″ N, 28° 25′ 00″ E; 1060 m). Dr. Olcay Ceylan verified the species, and a voucher specimen (O.2154) was stored in the Muğla Sıtkı Koçman University Biology Department Herbarium. Freshly collected material was shade-dried for several weeks and pulverized to a fine powder with a laboratory mill.

### Preparation of Methanol Extract

Powdered plant material (1 g) was extracted in 20 mL methanol for 60 min in an ultrasonic bath (1:20 w/v) (Kargın Solmaz & Sarıkürkcü, 2025). After filtration, the filtrate was concentrated under reduced pressure using a rotary evaporator, and the residue was kept at 4 °C until analysis. The methanol extract yield was 7.40 % (w/w).

### Determination of Total Phenolics and Flavonoids

Total flavonoid content (TFC) was quantified using the aluminium-chloride colourimetric assay, whereas total phenolic content (TPC) was assessed with the Folin–Ciocalteu reagent, under conditions reported previously (Sarikurkcu *et al*., 2013). Results are expressed as rutin equivalents (RE) and gallic-acid equivalents (GAE), respectively. Full protocols are provided in the Supplementary File.

### Bioactivity Assays

Antioxidant capacity (phosphomolybdenum, DPPH and ABTS radical scavenging, CUPRAC and FRAP reducing power, and ferrous ion chelating assays) (Apak *et al*., 2006; Kocak *et al*., 2016; Sarikurkcu *et al*., 2020; Tepe *et al*., 2011; Zengin *et al*., 2017) and enzyme-inhibitory effects (Ozer *et al*., 2018) were evaluated following the literature methods detailed in the Supplementary File.

### Statistical Treatment

All measurements were performed in triplicate, and the data are presented as mean ± standard deviation (SD).

## RESULTS and DISCUSSION

### Chemical Composition

Ultrasound-assisted extraction (UAE) of the aerial parts of *A. tubulosa* yielded a methanol extract [yield: 7.40% (w/w)] exceptionally rich in polyphenols. The total phenolic content reached 97.34 mg gallic acid equivalents (GAEs)/g extract, more than double the 45.03 mg rutin equivalents (REs)/g extract recorded for total flavonoids (Figure 1). These values were calculated from three independent determinations and expressed as mean ± SD. The high phenolic-to-flavonoid ratio (~2.2:1) indicates that non-flavonoid phenolics dominate the extract's low-molecular-weight antioxidant pool.

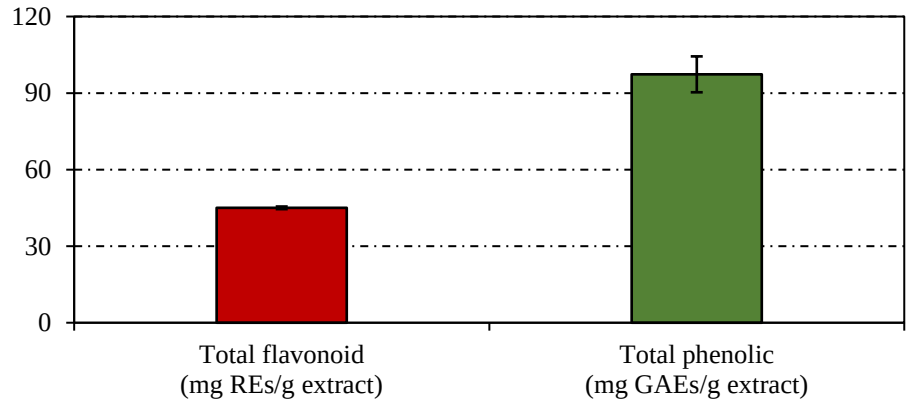


**Figure 1.** Total phenolic and flavonoid contents of the methanol extract from *A. tubulosa*. REs and GAEs means rutin and gallic acid equivalents, respectively. Values expressed are means ± SD of three parallel measurements.

Targeted LC-ESI-MS/MS analysis quantified 31 reference phenolics; 17 were detected above the limit of quantification (Table 1). Flavonoid glycosides prevailed, with hesperidin (13626 µg/g) constituting ~65 % of the quantified phenolic fraction. The next most abundant metabolites were rosmarinic acid (6637 µg/g) and hyperoside (913 µg/g). Among simple phenolic acids, caffeic acid (412 µg/g), *p*-coumaric acid (52.3 µg/g), 3-hydroxybenzoic acid

(129 µg/g), and 4-hydroxybenzoic acid (128 µg/g) were prominent. Protocatechuic acid, 2,5-dihydroxybenzoic acid, ferulic acid and gallic acid appeared in the mid-to-low µg/g range, whereas several naphthoquinones (e.g., alkannin/shikonin derivatives) were absent, consistent with their preferential accumulation in roots rather than aerial tissues.

**Table 1.** Concentration of selected phenolic compounds in the methanolic extract of *A. tubulosa*.

| Compounds | Concentration (µg/g extract) |
|---|---|
| Gallic acid | 16.5±0.6 |
| 2,5-Dihydroxybenzoic acid | 28.7±0.1 |
| Protocatechuic acid | 27.2±0.3 |
| 3,4-Dihydroxyphenylacetic acid | 4.35±0.15 |
| Pyrocatechol | nd |
| (+)-Catechin | 0.60±0.02 |
| Chlorogenic acid | nd |
| 3-Hydroxybenzoic acid | 129±2 |
| 4-Hydroxybenzoic acid | 128±1 |
| (-)-Epicatechin | nd |
| Caffeic acid | 412±1 |
| Syringic acid | nd |
| Vanillin | 7.34±0.43 |
| Verbascoside | nd |
| Sinapic acid | nd |
| p-Coumaric acid | 52.3±2.6 |
| Taxifolin | nd |
| Ferulic acid | 16.81±0.19 |
| Luteolin 7-glucoside | nd |
| Hesperidin | 13626±50 |
| Hyperoside | 913±4 |
| Rosmarinic acid | 6637±130 |
| 2-Hydroxycinnamic acid | 1.14±0.06 |
| Apigenin 7-glucoside | 9.59±0.02 |
| Ellagic Acid | nd |
| Pinoresinol | nd |
| Eriodictyol | nd |
| Quercetin | nd |
| Luteolin | nd |
| Kaempferol | nd |

Values expressed are means ± SD of three parallel measurements.

Most flavonoid aglycones (quercetin, kaempferol, luteolin) and several lignans (pinoresinol) were below detection limits, suggesting that glycosylated and esterified phenolics represent the principal antioxidant reservoir in *A. tubulosa*. Collectively, these findings highlight a distinctive phenolic fingerprint dominated by citrus-type flavanone glycosides (hesperidin) and caffeic-acid-derived esters (rosmarinic acid), providing a chemical rationale for the potent antioxidant activity documented in subsequent bioassays.

The phytochemical constellation documented for the ultrasound-assisted methanolic extract of *A. tubulosa* both corroborates and meaningfully extends earlier reports. Zengin *et al*. (2024) characterised ethanolic and ethyl-acetate extracts from the same species and confirmed the presence of rosmarinic acid, hyperoside and several simple phenolic acids, yet hesperidin was not highlighted, and the authors detected barely half of the compounds captured by the LC-ESI-MS/MS panel used here. Our data therefore introduce flavanone glycosides—led by hesperidin—as previously overlooked, high-abundance markers of the aerial chemotype and

reveal a markedly higher phenolic diversity, an outcome that can be ascribed to the improved mass-transfer efficiency of UAE and the broader set of reference standards employed.

Historical studies on *Alkanna* chemistry have centred on the naphthoquinone pigments of the roots. Assimopoulou *et al*. (2009) quantified alkannin and shikonin derivatives in root material but recorded no polyphenol; Pekin *et al*. (2007) later confirmed that rosmarinic acid is minor in roots of *A. tinctoria*, whereas naphthoquinone pigments (alkannin/shikonin derivatives) dominate. The total absence of these pigments in our aerial-part extract underscores pronounced tissue specificity within the genus and eliminates a class of compounds known to complicate stability and safety assessments. At the same time, the unusually high phenolic-to-flavonoid ratio (~2.2) places *A. tubulosa* at the upper end of the spectrum reported for its congeners, where flavonoid aglycones or hydroxy-cinnamic acids typically prevail.

The pronounced enrichment of hesperidin and rosmarinic acid defines an aerial part-specific chemotype markedly different from the naphthoquinone-dominated profile of *Alkanna* roots. As a glycosylated flavanone and an esterified caffeic-acid derivative, respectively, hesperidin and rosmarinic acid are markedly more polar than the hydrophobic alkannin/shikonin pigments characteristic of subterranean tissues; rosmarinic acid is freely soluble in water and shows good light-stability, whereas shikonin dissolves almost exclusively in organic solvents and is classed as a lipophilic metabolite (Aydi *et al*., 2016; Tatsumi *et al*., 2016; Malik *et al*., 2023). This compositional polarity suggests that extracts from aerial parts may be more amenable to water-based nutraceutical or cosmeceutical formulations and less prone to the colour instability and oxidative degradation commonly associated with quinonoid constituents.

## Antioxidant Activity

The methanol extract of *A. tubulosa* aerial parts exhibited a strong antioxidant profile across all *in vitro* tests (Figures 2 and 3). In free-radical–scavenging assays, the extract neutralised DPPH• and ABTS•$^{+}$ with almost identical efficiencies, recording 181.43 mg TE/g and 182.85 mg TE/g, respectively. By contrast, its ferrous-ion–chelating capacity was considerably lower, reaching 6.63 mg EDTAE/g, indicating that radical quenching rather than metal sequestration represents the dominant antioxidant mechanism.

Electron-transfer–based assays corroborated these findings. The phosphomolybdenum method yielded the highest value at 570.77 mg TE/g, followed by CUPRAC at 479.07 mg TE/g and FRAP at 270.59 mg TE/g. The hierarchy phosphomolybdenum > CUPRAC > FRAP suggests that the extract donates electrons readily to both molybdenum(VI) and copper(II) centres, while its interaction with ferric ions is comparatively moderate. Taken together, these results demonstrate that the ultrasound-assisted methanol extract possesses potent, multi-mechanistic antioxidant capacity characterised by efficient radical scavenging and robust reducing power, complemented by a modest ability to chelate transition metals.

The antioxidant profile obtained for the ultrasound-assisted methanolic extract of *A. tubulosa* aligns with, yet also extends, the emerging evidence for this endemic species. Zengin and co-workers showed that ethanol- and ethyl-acetate-based extracts quench DPPH and ABTS radicals and reduce ferric and cupric ions, but relied on conventional maceration (Zengin *et al*., 2024). By applying UAE—a technique that improves polyphenol recovery while lowering thermal stress—we recorded comparable radical-scavenging efficiencies and, importantly, demonstrated markedly higher electron-transfer capacity in the phosphomolybdenum and CUPRAC systems, indicating that extraction mode critically modulates redox responsiveness.

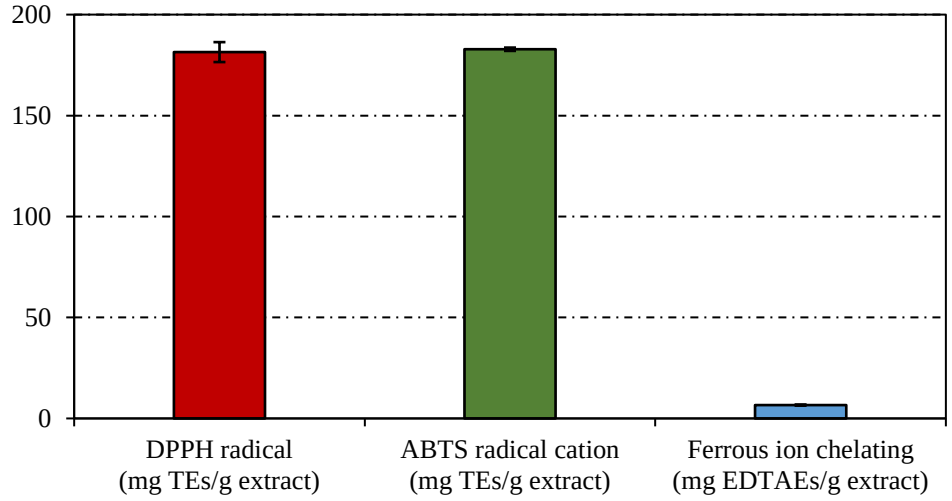


**Figure 2.** DPPH radical scavenging, ABTS radical cation scavenging, and ferrous-ion chelating activities of the methanolic extract of *A. tubulosa*. TEs and EDTAEs means trolox and ethylenediaminetetraacetic acid (disodium salt) equivalents, respectively. Values expressed are means ± SD of three parallel measurements.

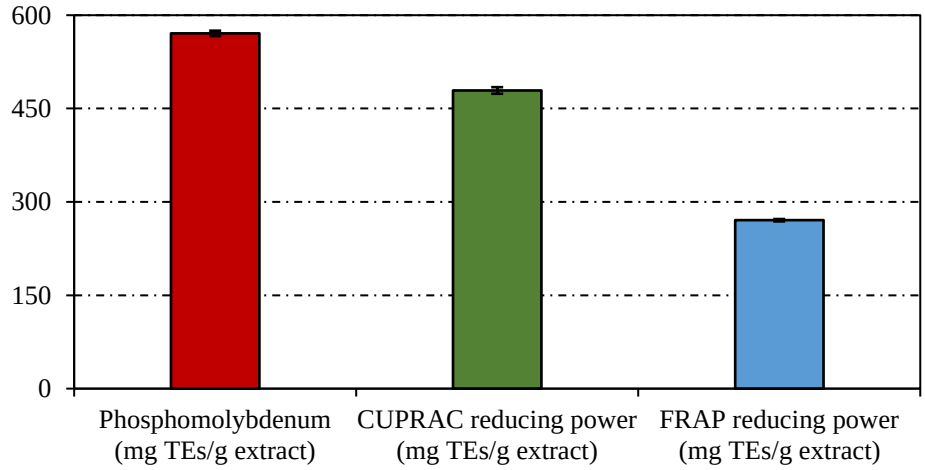


**Figure 3.** Phosphomolybdenum, CUPRAC and FRAP reducing power potential of the methanol extract from *A. tubulosa*. TEs means trolox equivalents. Values expressed are means ± SD of three parallel measurements.

Comparison with other members of the genus corroborates the high intrinsic antioxidant aptitude of *Alkanna*. Methanolic root and aerial-part extracts of *A. tinctoria* subsp. *tinctoria* consistently display strong DPPH scavenging, β-carotene bleaching as well as metal-reducing activity (Sabih Ozer *et al*., 2010), while butanol fractions from *A. orientalis* outperform reference antioxidants in multiple *in vitro* assays (Salimikia *et al*., 2015). Nevertheless, unlike those root-centred studies, our work elucidates the aerial part chemotype, revealing a hesperidin/rosmarinic-acid signature that sets *A. tubulosa* apart chemically and functionally.

Literature on the dominant constituents supports their decisive contribution. Hesperidin acts as a chain-breaking antioxidant and efficiently intercepts both alkyl and peroxyl radicals, although its stoichiometric factor is modest compared with trolox (Hirata *et al*., 2005). Rosmarinic acid, in turn, combines radical scavenging with metal-chelating and lipid-peroxidation-suppressing properties, features credited for its broad pharmacological versatility (Fadel *et al*., 2011; Topal & Gulcin, 2022; Vo *et al*., 2023). Their co-occurrence in the current extract offers a mechanistic rationale for the pronounced electron-donor hierarchy (phosphomolybdenum > CUPRAC > FRAP) and for the dominance of radical quenching over ferrous-binding observed.

Despite these strengths, two important considerations warrant further investigation. First, the extract's relatively modest iron-chelating capacity may limit its effectiveness in biological settings dominated by metal-catalyzed oxidative stress; co-formulation with potent chelators could improve therapeutic potential. Second, as all current results stem from cell-free systems, in vivo studies are needed to assess whether the high hesperidin content compensates for its limited bioavailability and to determine the metabolic fate of rosmarinic acid. Future research should prioritize fractionation-guided synergy analyses and pharmacokinetic profiling to support the development of *A. tubulosa* as a candidate for functional-food or dermo-protective applications.

## Enzyme Inhibitory Activity

Consistent with its polyphenol-rich profile, the methanolic ultrasound-assisted extract displayed selective but noteworthy enzyme-inhibitory activity (Figures 4 and 5). Against carbohydrate-hydrolysing enzymes, the extract produced a strong, concentration-independent effect, reaching 598.36 mg ACEs/g for α-glucosidase and 299.50 mg ACEs/g for α-amylase; the two-fold higher potency toward α-glucosidase suggests a favourable anti-hyperglycaemic profile with reduced risk of gastrointestinal side effects typically linked to non-selective amylase inhibition. Tyrosinase was inhibited to a moderate extent, with an activity equivalent to 57.30 mg KAEs/g, a level that, although lower than kojic acid, may translate into ancillary dermatoprotective benefits. In contrast, cholinesterase inhibition was modest: activities corresponded to 2.07 mg GALAEs/g for AChE and 1.05 mg GALAEs/g for BChE, indicating that the extract is a relatively weak modulator of cholinergic enzymes. Overall, the data highlight pronounced anti-diabetic potential, complementary antimelanogenic activity and limited but measurable neuroprotective capacity.

The enzyme-modulating profile obtained here positions the aerial-part extract of *A. tubulosa* as a distinct antidiabetic and dermato-protective candidate within the genus. Selective suppression of α-glucosidase—twice as pronounced as α-amylase inhibition—mirrors the pattern previously reported for conventionally macerated ethanolic extracts of the same species, but the magnitude observed after ultrasound-assisted extraction (UAE) is appreciably higher and remains stable across the concentration range tested (Zengin *et al*., 2024). In contrast, earlier work on *A. tinctoria* and *A. kotschyana* showed either comparable inhibition of both carbohydrase targets or, in those species, cholinesterase suppression exceeding 1 mg GALAE/g (Ganos *et al*., 2024). The comparatively weak AChE/BChE activity measured for *A. tubulosa* therefore appears to be species-specific rather than methodology-driven and underlines a pharmacological niche focused on post-prandial glycaemic control rather than neuroprotection.

Chemical context substantiates this selectivity. Hesperidin—quantified here as the dominant metabolite—has documented affinity for α-glucosidase that surpasses its interaction with α-amylase and extends, albeit more modestly, to tyrosinase and AChE inhibition (Si *et al*., 2012; Kaliaperumal *et al*., 2023; Mir *et al*., 2024). Rosmarinic acid, the second major constituent, is likewise recognised as a non-competitive inhibitor of α-glucosidase and of tyrosinase, and it displays only marginal cholinesterase activity (Fujimoto *et al*., 2010; Zhu *et al*., 2014; Kamli *et al*., 2022). The co-abundance of these two polyphenols therefore offers a plausible mechanistic explanation for the pronounced α-glucosidase and moderate tyrosinase effects, while simultaneously accounting for the subdued cholinergic response.

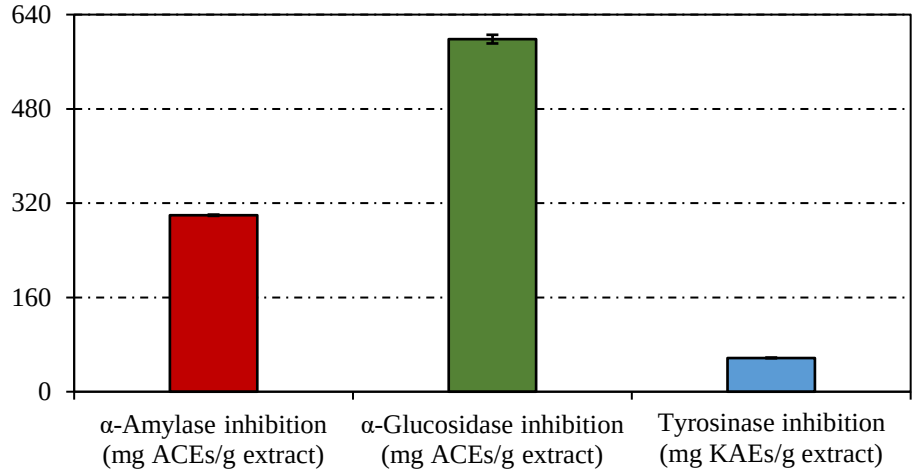


**Figure 4.** Tyrosinase, α-amylase and α-glucosidase enzyme inhibition activity of the methanol extract from *A. tubulosa*. KAEs and ACEs mean kojic acid and acarbose equivalents, respectively. Values expressed are means ± SD of three parallel measurements.

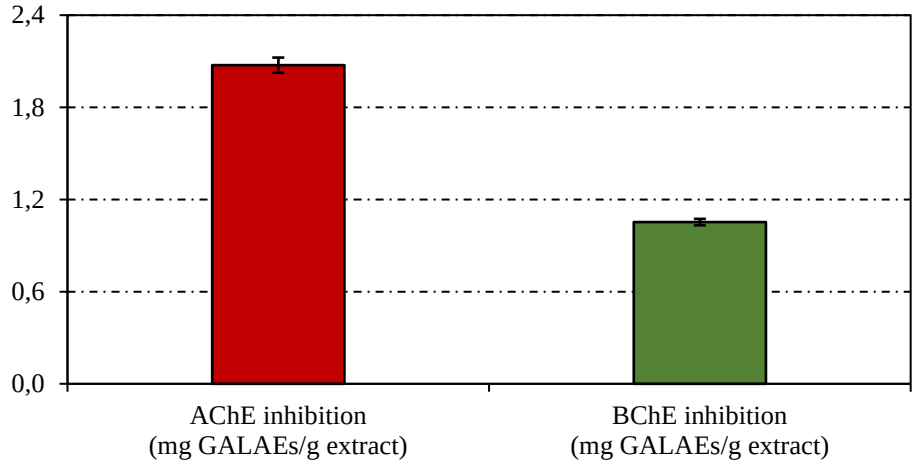


**Figure 5.** AChE and BChE enzyme inhibition activity of the methanol extract from *A. tubulosa*. GALAEs means galanthamine equivalents. Values expressed are means ± SD of three parallel measurements.

Compared with other *Alkanna* taxa, the present extract also benefits from the absence of naphthoquinone pigments that can attenuate carbohydrase inhibition via assay interference, a complication reported in root-based studies of *A. tinctoria* (Ganos *et al*., 2024). The UAE protocol, by enhancing recovery of thermolabile glycosides, further amplifies the bioactivity relative to solvent-intensive maceration strategies cited above.

Two limitations merit consideration. First, the modest ferrous-chelating capacity observed in antioxidant assays raises the possibility that metal-dependent pro-oxidant pathways could blunt enzyme inhibition under physiological conditions; co-formulation with stronger chelators might therefore be advantageous. Second, hesperidin's low oral bioavailability could dampen *in vivo* efficacy, although microencapsulation or co-delivery with permeability enhancers has been shown to improve systemic exposure and warrants evaluation in follow-up studies.

## DISCUSSION and CONCLUSION

This study identifies the aerial parts of *A. tubulosa* as a rich source of hesperidin- and rosmarinic acid–based phytochemicals, with a distinct phenolic-to-flavonoid ratio that underpins its strong radical scavenging and α-glucosidase-selective inhibitory activities. While the extract shows potential for glycaemic and dermal protection, limited iron-chelation and weak cholinesterase inhibition suggest specific rather than broad-spectrum benefits. Given the absence of root-

derived pigments and the reliance on cell-free assays, future research should explore *in vivo* efficacy, fractionation-guided synergy, and sustainable sourcing strategies to unlock the plant's full therapeutic value.

### Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

### Data Availability Statement

Research data is available and can be sent with e-mail upon request.

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