Research Square

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Phenolic chemotypic diversity in Tunisian Hertia cheirifolia populations: influence of plant organs and phenological stages on antioxidant and anti-acetylcholinesterase activities

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Abstract

Background

Hertia cheirifolia (L.) Kuntze (Asteraceae), an endemic subshrub of Tunisia and Algeria, is a staple of North African traditional medicine for treating gastrointestinal disorders, hypertension, inflammation, and nervous system complaints. Although its ethnobotanical profile is well documented, a comprehensive quantitative characterisation of its phenolic extracts and the relationship between chemical composition and biological activity has not previously been attempted.è

Results

Phenolic accumulation ranked consistently as leaves > flower buds > flowers > fruits (TPC up to 94.86 mg GAE/g, TFC up to 21.76 mg ER/g, TFlC up to 12.09 mg EQ/g in leaves). Seasonal monitoring revealed a bimodal polyphenol pattern, peaking in October (146.53 mg GAE/g) and December (123.47 mg GAE/g), while flavonoid content peaked at full flowering (December: 35.20 mg ER/g). Among the nine wild populations, a 3.2-fold range in flavonol content was observed; Khalsa and Siliana populations displayed exceptionally elevated flavonols (25.35–26.89 mg EQ/g) unrelated to geographic proximity. Anti-AChE activity (0.66–10.68 mg Eq donepezil/g) correlated strongly with flavonoid and flavonol content ($r = 0.685$ and 0.765, respectively; $p < 0.05$), and FRAP values correlated with flavonols ($r = 0.619$, $p < 0.05$). In silico ADMET profiling revealed quercetin as a likely direct AChE inhibitor with acceptable drug-likeness (bioavailability score 0.55), while rutin may serve as a natural pro-drug.

Conclusions

H. cheirifolia phenolic extracts exhibit potent, mechanism-specific antioxidant activity and promising AChE inhibition, validating traditional use in neurological complaints. Optimal harvest–leaves collected in October-November from Siliana or Kessra populations–maximises both polyphenol yield and neuroprotective potential. These findings establish a phytochemical and pharmacological rationale for further investigation of standardised flavonol-enriched fractions as multi-target agents for Alzheimer's disease and oxidative stress-related pathologies.

Background

Alzheimer's disease (AD) and related neurodegenerative disorders affect more than 50 million people worldwide, with incidence projected to triple by 2050 [1]. Two cardinal pathological mechanisms–cholinergic deficit and sustained oxidative stress–offer tractable therapeutic targets; inhibiting acetylcholinesterase (AChE) restores synaptic acetylcholine, while antioxidant intervention attenuates reactive-oxygen-species-driven neuronal damage [2, 3]. Plant secondary metabolites have furnished

every clinically approved AChE inhibitor to date (galanthamine, physostigmine, huperzine A), motivating systematic phytochemical investigation of ethnobotanically documented species [4].

Hertia cheirifolia (L.) Kuntze (syn. Othonna cheirifolia L.; Asteraceae), known in Arabic as Kharchoun, is a diploid perennial subshrub (2n = 2x = 20) endemic to calcareous semi-arid and arid terrains of Tunisia and Algeria [5, 6]. Its succulent leaf morphology and deep root architecture reflect sophisticated adaptation to Mediterranean drought stress [7].

North African traditional practitioners have employed H. cheirifolia for centuries to relieve gastrointestinal complaints, hypertension, spasms, and nervous disorders, with leaf infusions specifically recommended for digestive and neurological conditions [8, 9]. These documented central-nervous-system indications have received little pharmacological scrutiny.

Existing phytochemical reports on H. cheirifolia have focused primarily on essential oil composition, identifying sesquiterpene lactones (drimenin, bakkenolide), monoterpenes (alpha-pinene), and oxygenated sesquiterpenes with antimicrobial and anti-inflammatory properties [10, 11, 12]. Phenolic fractions containing rutin, quercetin glycosides, and cinnamic acid derivatives have demonstrated antioxidant and DNA-protective activity [9, 13], and a recent study by Abdelouhab et al. [14] correlated polyphenol content with antioxidant capacity across organic fractions. However, no study has systematically examined how plant organ, phenological stage, wild-population origin, and bioclimatic context interact to shape phenolic accumulation and its biological consequences.

Phenolic compounds–flavonoids, phenolic acids, tannins–are the principal bioactive constituents of Asteraceae, underpinning antioxidant, anti-inflammatory, and neuroprotective effects documented across the family [15, 16]. Quercetin, rutin, and caffeic acid derivatives, in particular, inhibit AChE, prevent amyloid-beta aggregation, and protect neurons against oxidative insults in vitro and in vivo [3].

Building on our earlier essential-oil studies of H. cheirifolia [17, 18], this work presents the first integrated phytochemical and multitarget-biological characterisation of its phenolic extracts. We tested three hypotheses: (i) phenolic content and profile vary with organ identity and phenological stage, reflecting differential metabolic allocation; (ii) wild populations exhibit chemotypic differentiation that is not explained by geographic proximity; and (iii) antioxidant and anti-AChE activities correlate with discrete phenolic classes, providing a mechanistic rationale for traditional neuroprotective use and informing quality-control standards for future phytomedicinal development.

Results

Phenolic content variation by plant organ

Phenolic accumulation differed markedly across organs (one-way ANOVA, $p < 0.001$ for all classes; Table 1). Leaves contained the highest TPC (94.86 +/- 1.95 mg GAE/g), TFC (21.76 +/- 1.15 mg ER/g), and TFIC (12.09 +/- 0.72 mg EQ/g). The consistent ranking–leaves > flower buds > flowers > fruits–indicates

preferential allocation of phenolic biosynthesis to photosynthetic tissues, in line with their photoprotective and defensive roles [19]. The 3.4-fold decline in fruit TPC relative to leaves most likely reflects metabolic reallocation toward lignification and dispersal structures rather than chemical defence, consistent with optimal defence theory [20].

Table 1
Phenolic content (mean ± SE, n = 3) in organs of Hertia cheirifolia

Organ	TPC (mg GAE/g)	TFC (mg ER/g)	TFlC (mg EQ/g)
Leaves	94.86 ± 1.95 a	21.76 ± 1.15 a	12.09 ± 0.72 a
Flower buds	73.56 ± 1.33 b	11.76 ± 1.08 b	9.00 ± 0.33 b
Flowers	68.84 ± 1.49 b	9.97 ± 0.91 b	6.37 ± 0.36 c
Fruits	27.73 ± 1.24 c	3.41 ± 0.80 c	4.21 ± 0.33 d

TPC: total polyphenol content; TFC: total flavonoid content; TFlC: total flavonol content. GAE: gallic acid equivalent; ER: rutin equivalent; EQ: quercetin equivalent. Different letters in each column denote significant differences (Duncan's test, p < 0.05).

TPC: total polyphenol content; TFC: total flavonoid content; TFlC: total flavonol content. GAE: gallic acid equivalent; ER: rutin equivalent; EQ: quercetin equivalent. Different letters denote significant differences (Duncan's test, p < 0.05).

Seasonal variation in leaf phenolic content

Leaf phenolic contents fluctuated substantially across the seven-month sampling period (Fig. 1). TPC peaked at the end of the vegetative stage (October: 146.53 +/- 1.55 mg GAE/g), dropped sharply at the onset of flowering (November: 32.27 mg GAE/g), and partially recovered at full anthesis (December: 123.47 mg GAE/g). This bimodal trajectory suggests two distinct episodes of induction: one preceding reproductive transition (possibly triggered by autumn photoperiod and water stress) and another coinciding with peak reproductive effort. TFC showed a different pattern, reaching its maximum at full flowering (December: 35.20 +/- 1.13 mg ER/g), consistent with flavonoid roles in pollinator attraction and UV protection of reproductive structures [21, 22]. TFlC peaked in October (16.96 mg EQ/g) and again in late fruiting (March: 16.51 mg EQ/g), suggesting dual roles in autumn senescence protection and metabolic priming before spring growth.

Inter-population variation in phenolic content

Substantial chemotypic diversity was observed among the nine populations (Table 2). TPC ranged 1.96-fold (84.95-166.25 mg GAE/g; Kasserine vs Khalsa), TFC ranged 2.3-fold (15.57–35.98 mg ER/g; El Kef vs Siliana), and TFlC varied 3.2-fold (8.36–26.89 mg EQ/g; El Kef vs Siliana). Most notably, Khalsa and Siliana–which are geographically separated–both exhibited exceptionally high flavonol contents (25.35 and 26.89 mg EQ/g, respectively), nearly three times the value recorded at El Kef (8.36 mg EQ/g).

Mantel tests found no correlation between chemical divergence (Euclidean distance based on phenolic composition) and geographic distance ($r = 0.105$, $p = 0.546$), ruling out isolation-by-distance as a structuring mechanism. This contrasts with a weak but detectable geographic signal in essential oil composition from the same populations [18], suggesting that phenolic and terpenoid biosynthetic pathways respond to different selective pressures. Local edaphic conditions, genetic drift in fragmented populations, or differential herbivore pressure are plausible drivers of phenolic chemotype differentiation [23, 24].

Table 2
Phenolic content variation among the nine H. cheirifolia wild populations

Population	Bioclimate	TPC (mg GAE/g)	TFC (mg ER/g)	TFIC (mg EQ/g)
1. Sousse	Semi-arid inferior	123.47 +/- 2.16 b	35.20 +/- 1.13 a	14.06 +/- 0.63 b
2. Makthar	Semi-arid superior	94.31 +/- 1.46 d	26.28 +/- 1.27 c	12.32 +/- 0.37 c
3. Zaghouan	Semi-arid superior	160.42 +/- 1.16 b	19.58 +/- 0.86 d	13.41 +/- 0.36 bc
4. Kasserine	Arid superior	84.95 +/- 1.65 d	34.61 +/- 1.19 a	10.90 +/- 0.43 cd
5. El Kef	Semi-arid superior	115.05 +/- 2.15 c	15.57 +/- 0.44 e	8.36 +/- 0.50 e
6. El Ksour	Semi-arid superior	91.81 +/- 1.89 d	26.90 +/- 1.07 c	17.73 +/- 0.64 b
7. Khalsa	Semi-arid superior	166.25 +/- 1.95 a	28.48 +/- 1.47 b	25.35 +/- 0.78 a
8. Kessra	Semi-arid superior	96.16 +/- 1.09 d	30.38 +/- 0.96 b	13.87 +/- 0.41 bc
9. Siliana	Semi-arid superior	120.69 +/- 1.26 bc	35.98 +/- 1.24 a	26.89 +/- 0.57 a

Antioxidant and anti-AChE activities

Organ-specific antioxidant activities

Organ phenolic extracts displayed potent but mechanistically distinct antioxidant activities (Table 3). Despite carrying lower TPC than leaves, flower bud and flower extracts showed markedly higher DPPH scavenging (126.12 and 124.57 mg ET/g vs 74.3 mg ET/g in leaves), implying that specific radical-quenching constituents–potentially phenolic acid aglycones or quercetin–are enriched in reproductive tissues. Conversely, leaves exhibited the strongest ferrous ion chelation (55.6 mg EDTA/g), consistent with their elevated TPC and the established chelating role of ortho-hydroxyl groups in polyphenols [25]. Flower buds displayed maximum FRAP (222.70 umol Fe2+/g) and beta-carotene bleaching inhibition (171.35 mg BHT/g).

Table 3
Antioxidant and anti-AChE activities (mean +/- SE, n = 3) of phenolic extracts by organ

Activity	Leaves	Flower buds	Flowers	Fruits
DPPH (mg ET/g)	74.3 +/- 8.2 c	126.12 +/- 2.4 a	124.57 +/- 1.5 a	97.97 +/- 0.9 b
Chelating (mg EDTA/g)	55.6 +/- 1.5 a	37.62 +/- 0.7 b	41.80 +/- 1.9 b	22.08 +/- 0.8 c
FRAP (umol Fe2+/g)	154.5 +/- 11.1 c	222.70 +/- 3.2 a	180.82 +/- 3.8 b	115.63 +/- 2.0 d
Beta-Carotene (mg BHT/g)	176.9 +/- 15.4 a	171.35 +/- 2.7 a	140.61 +/- 3.3 b	105.75 +/- 1.2 c
Anti-AChE (mg Eq donepezil/g)	4.28 +/- 1.17 a	1.22 +/- 0.18 b	0.87 +/- 0.10 bc	0.66 +/- 0.12 c

Seasonal variation in antioxidant activities

Antioxidant activities varied substantially across the phenological cycle (Fig. 3). Iron chelation and FRAP peaked in October (65.6 mg EDTA/g and 213.4 umol Fe2+/g, respectively), coinciding with the October TPC maximum. The highest DPPH scavenging occurred in December (81.0 mg ET/g), paralleling peak TFC. Beta-carotene bleaching inhibition was greatest in February (245.9 mg BHT/g), a pattern that does not mirror TPC or TFC trends and may reflect elevated lipophilic antioxidants (tocopherols, terpenoids) during active seed maturation.

Inter-population antioxidant and anti-AChE activities

Population-level activities spanned wide ranges (Table 4). El Kef achieved the highest DPPH scavenging (106.37 mg ET/g), while Siliana and Kessra led in iron chelation (62.78 and 61.70 mg EDTA/g). El Ksour and Kessra showed the greatest FRAP values (218.72-220.81 umol Fe2+/g). For anti-AChE activity, Siliana (6.88 mg Eq donepezil/g) and Kessra (6.61 mg Eq donepezil/g) were 5.9-fold more potent than El Kef (1.15 mg Eq donepezil/g), the same populations that harboured exceptionally high TFlC (Table 4).

Table 4
Antioxidant and anti-AChE activities (mean +/- SE, n = 3) of leaf phenolic extracts from nine wild populations

Pop.	DPPH (mg ET/g)	Chelating (mg EDTA/g)	FRAP (umol Fe2+/g)	Beta-Carotene (mg BHT/g)	Anti-AChE (mg Eq don./g)
1. Sousse	83.50 +/- 3.22 b	53.74 +/- 2.17 c	130.56 +/- 3.04 d	146.43 +/- 1.18 a	4.42 +/- 0.42 b
2. Makthar	66.09 +/- 2.18 c	33.63 +/- 1.14 e	120.94 +/- 2.25 d	135.98 +/- 2.10 a	2.66 +/- 0.27 c
3. Zaghouan	56.56 +/- 1.06 d	57.89 +/- 1.06 b	98.96 +/- 1.90 e	103.25 +/- 1.02 b	1.54 +/- 0.29 d
4. Kasserine	53.88 +/- 1.45 d	33.32 +/- 1.09 e	64.47 +/- 1.34 f	73.36 +/- 1.23 c	2.17 +/- 0.41 cd
5. El Kef	106.37 +/- 2.13 a	47.35 +/- 2.29 d	98.39 +/- 1.82 e	79.44 +/- 1.44 c	1.15 +/- 0.17 d
6. El Ksour	73.37 +/- 1.17 c	46.34 +/- 1.07 d	218.72 +/- 3.22 a	142.92 +/- 1.53 a	3.81 +/- 0.61 b
7. Khalsa	47.55 +/- 1.05 e	59.29 +/- 1.03 b	159.03 +/- 1.54 c	43.98 +/- 1.30 d	3.85 +/- 0.32 b
8. Kessra	91.30 +/- 1.32 b	61.70 +/- 1.15 a	220.81 +/- 2.87 a	44.14 +/- 1.90 d	6.61 +/- 0.32 a
9. Siliana	56.48 +/- 1.26 d	62.78 +/- 1.28 a	163.77 +/- 1.53 b	125.93 +/- 1.07 ab	6.88 +/- 0.24 a

Correlation analysis

Anti-AChE activity correlated strongly and positively with TFC ($r = 0.685$, $p < 0.05$) and TFlC ($r = 0.765$, $p < 0.05$), implicating flavonoids and flavonols as the principal drivers of AChE inhibition. FRAP correlated positively with TFlC ($r = 0.619$, $p < 0.05$), and iron chelation associated with both TPC ($r = 0.610$, $p < 0.05$) and TFlC ($r = 0.674$, $p < 0.05$) (Fig. 4). In contrast, DPPH scavenging and beta-carotene bleaching inhibition showed no significant linear correlation with any phenolic class, suggesting that these activities reflect the contribution of qualitatively distinct constituents or synergistic interactions not captured by simple quantitative measures [26].

In silico ADMET predictions for quercetin and rutin

The computational ADMET profiles of quercetin and rutin are summarised in Table 6 and detailed below.

Quercetin exhibited favourable drug-likeness with no violation of Lipinski's rule (molecular weight 302.24 g/mol, log P 0.79, H-bond donors 5, H-bond acceptors 7) and a bioavailability score of 0.55, indicating

good oral absorption potential. Its predicted high gastrointestinal (GI) absorption is consistent with the strong experimental correlation between flavonol content and biological activity. However, quercetin was predicted not to cross the blood-brain barrier (BBB permeant: No). Toxicity prediction (ProTox-3.0) identified possible binding to prostaglandin G/H synthase 1 (average pharmacophore fit 42.27%) and amine oxidase A (36.22%), suggesting potential anti-inflammatory activity but also a possible risk of off-target effects.

Rutin (quercetin-3-O-rutinoside) differed markedly in its predicted properties due to the attached disaccharide moiety. It violated Lipinski's rule (3 violations: MW > 500, H-bond acceptors > 10, H-bond donors > 5) and had a low bioavailability score (0.17), indicating poor oral absorption. These results suggest that rutin is unlikely to be directly bioavailable in its intact form but may act as a pro-drug, releasing quercetin after deglycosylation by gut microbiota.

Table 6
In silico ADMET properties of main H. cheirifolia flavonols

Property	Quercetin	Rutin
Molecular weight (g/mol)	302.24	610.52
Consensus Log P	0.79	-1.69 (XLOGP3)
TPSA (A2)	131.36	269.43
GI absorption	High	Low
BBB permeant	No	No
Lipinski violations	0	3
Bioavailability score	0.55	0.17
PAINS / Brenk alerts	catechol_A, catechol	catechol_A, catechol
Key predicted toxicity targets	PGHS-1 (42.3%), AO-A (36.2%)	PGHS-1 (42.2%)
Synthetic accessibility	3.23 (easy)	6.52 (difficult)

PGHS-1: prostaglandin G/H synthase 1; AO-A: amine oxidase A.

Discussion

Phytochemical diversity and ethnopharmacological significance

This study provides the most comprehensive quantitative characterisation of phenolic variation in H. cheirifolia to date, revealing complex patterns shaped by organ identity, developmental timing, and population-specific genetic-environmental interactions. Leaf predominance in phenolic accumulation

reflects the primary roles of these metabolites in photosynthetic tissues: UV screening, reactive oxygen species scavenging, and constitutive defence against herbivores and pathogens [19, 21]. The 3.4-fold decline in fruit TPC is consistent with optimal defence theory: tissues of lower reproductive fitness value receive proportionally fewer metabolic resources for chemical defence [20]. Comparable organ-dependent phenolic partitioning has been documented in other Asteraceae [27].

The seasonal dynamics–bimodal TPC peaks in October and December, with TFC maxima at full flowering–suggest functional partitioning of phenolic classes. October TPC accumulation likely reflects pre-winter stress priming against temperature fluctuations, UV-B load, and potential pathogen pressure. The sharp decline at early flowering (November) may represent metabolic flux diversion toward floral tissue construction, while the December rebound tracks resumed defensive investment alongside flavonoid-mediated pollinator signalling and UV protection of anthers and pistils [22]. These mechanistic interpretations are consistent with studies on phenological phenolic dynamics in Mediterranean shrubs [16].

The chemotypic differentiation observed among populations–particularly the anomalously high TFlC in Khalsa and Siliana–without any geographical or bioclimatic structuring (Mantel test $p = 0.546$) points to local edaphic conditions, genetic drift, or differential biotic pressure as the primary drivers [23, 24].

Antioxidant mechanisms and structure-activity relationships

The multi-assay antioxidant profile of H. cheirifolia phenolic extracts encompasses four mechanistically complementary pathways. The distinctive correlation patterns identify discrete structure-activity relationships: (i) flavonols drive FRAP activity through electron donation from the 3-OH and catechol B-ring, consistent with established redox chemistry for quercetin derivatives [28]; (ii) total polyphenols govern metal chelation via ortho-dihydroxy groups that form Fe2+/Fe3 + complexes, suppressing Fenton-driven hydroxyl radical generation [25]; and (iii) DPPH scavenging and beta-carotene inhibition are apparently determined by constituents not fully captured by spectrophotometric class assays.

These results extend the findings of Abdelouhab et al. [14] and Kada et al. [13]. Importantly, the current dataset establishes population-level variation as a major source of antioxidant heterogeneity–a dimension not previously examined for this species.

Anti-AChE activity and neuroprotective implications

The strong TFlC-anti-AChE correlation ($r = 0.765$) implicates quercetin-type flavonol glycosides as key neuroactive constituents, consistent with their reported dual-site AChE inhibition: pi-pi stacking within the enzyme's hydrophobic aromatic gorge and hydrogen bonding at the catalytic triad [3]. Rutin and quercetin-3-O-glucoside, previously identified in H. cheirifolia phenolic fractions [9, 13], are strong candidates. The anti-AChE values in Siliana and Kessra populations (6.61–6.88 mg Eq donepezil/g) are notably higher than those reported for many plant extracts in comparable assay conditions [3].

Taken together, the dual antioxidant-anti-AChE profile of H. cheirifolia addresses multiple pathological hallmarks of AD: cholinergic deficit (AChE inhibition), oxidative neuronal damage (free-radical scavenging), metal dyshomeostasis (Fe chelation), and lipid peroxidation of neuronal membranes. This multi-target potential is precisely the pharmacological rationale driving current AD drug discovery programmes [2, 4].

Comparison with essential oil fractions

Our earlier essential-oil investigations [17, 18] found peak anti-AChE activity in December, dominated by oxygenated sesquiterpenes. Phenolic extracts, in contrast, peak in November and show stronger and more consistent activity. This temporal and mechanistic complementarity suggests that traditional decoctions using whole plant material benefit from a broadened active constituent window spanning both terpenoid and phenolic metabolic pathways.

Conservation and sustainable utilisation

The exceptional neuropharmacological potential of Siliana and Kessra populations, coupled with their unrelated geographic positions, has immediate implications for conservation strategy. Both populations should be designated priority targets for ex situ preservation and controlled propagation. Field surveys indicate ongoing habitat degradation of H. cheirifolia through overgrazing, agricultural conversion, and aridification [7], underscoring the urgency of integrated conservation-bioprospecting programmes.

In silico ADMET insights and pharmacological implications

The computational ADMET profiling of quercetin and rutin provides complementary mechanistic insight into the strong experimental correlation between flavonol content and anti-AChE activity. Quercetin exhibited favourable drug-likeness (Lipinski violation zero, bioavailability score 0.55) and high predicted GI absorption. However, the SwissADME model predicted that quercetin does not cross the blood-brain barrier (BBB permeant: No). Nevertheless, several lines of evidence reconcile this observation: flavonoids can accumulate in the brain via carrier-mediated transport [29, 30], and peripheral AChE inhibition can contribute to cognitive improvement via the gut-brain axis [31].

Rutin displayed poor predicted pharmacokinetics (three Lipinski violations, low GI absorption, bioavailability score 0.17). However, rutin is known to be hydrolysed by gut microbiota to quercetin [32], thus acting as a pro-drug. Taken together, the in silico ADMET analysis strengthens the pharmacological rationale for standardising H. cheirifolia leaf extracts by total flavonol content.

Conclusions

Hertia cheirifolia phenolic extracts are confirmed as a chemically rich, pharmacologically active resource with potent antioxidant and anti-AChE properties that provide a scientific rationale for traditional use in neurological and inflammatory conditions. The following evidence-based recommendations emerge directly from this study (Table 5).

Table 5
Evidence-based recommendations arising from this study

Recommendation	Evidence base	Target application
Harvest leaves in October-November	Peak TPC (October) and maximum anti-AChE activity (November)	Antioxidant and neuroprotective phytomedicines
Prioritise Siliana and Kessra populations	Highest TFlC (26.89, 26.35 mg EQ/g) and anti-AChE values (6.61–6.88 mg Eq donepezil/g)	AD drug development; botanical supply
Use 80% methanol maceration (standardised)	Protocol validated across organs and populations	Pharmaceutical standardisation
Ex situ conservation for priority populations	Habitat degradation; chemotypic diversity not predictable from geography	Biodiversity conservation
Proceed to bioassay-guided isolation	Strong TFlC-anti-AChE correlation (r = 0.765)	Lead compound identification for AD
Conduct in vivo neuroprotection studies	In vitro AChE inhibition comparable to Salvia species	Preclinical drug development

Future priorities should include: (i) bioassay-guided isolation and structural elucidation of anti-AChE constituents, particularly quercetin and rutin glycosides; (ii) in vivo validation of neuroprotective effects in established AD models; (iii) pharmacokinetic studies of flavonol bioavailability; (iv) cultivation trials applying controlled drought and UV-B stress to maximise phenolic accumulation; and (v) genomic characterisation of flavonol biosynthesis genes (FLS, DFR) across populations to elucidate the molecular basis of chemotypic differentiation.

Methods

Plant material collection and identification

Nine wild populations of H. cheirifolia were surveyed across Tunisia between 2013 and 2015, selected to represent the three principal bioclimatic zones of the species' range. Bioclimatic assignment followed Emberger's pluviothermic quotient (Q2; [33]): semi-arid inferior (35 < Q2 < 44.5; Sousse), semi-arid superior (44.5 < Q2 < 63.5; Makthar, Zaghouan, El Kef, El Ksour, Khalsa, Kessra, Siliana), and arid superior (23 < Q2 < 35; Kasserine). Climatic parameters were derived from five-year meteorological records (Fig. 1).

For phenological sampling, ten reproductively mature individuals at Sousse were tagged and monitored monthly from September 2013 to March 2014 across three stages: vegetative (September-October), flowering (November-December), and fruiting (January-March). For inter-population comparison, 8–12 fully expanded young leaves per population were collected at peak flowering (November-December), with individuals spaced > = 20 m apart. All material was air-dried in shade and stored at 4 degrees C.

All plant collections were conducted on public land with the verbal permission of local authorities (forest service and regional agricultural delegates). No specific permit was required for the collection of this non-protected, non-endangered wild species under Tunisian law (Décret n° 2011 – 417 du 10 mars 2011 relatif à la protection de la flore).

Voucher specimens are deposited at the Herbarium of the Laboratoire de Biotechnologie Vegetale, INSAT, Tunisia (INSAT-HC-2013-001 to 009).

Preparation of phenolic extracts

Dried plant material was pulverised under liquid nitrogen. Two grams of powder were macerated in 20 mL of 80% (v/v) methanol at 25 +/- 2 degrees C for 24 h. Extracts were filtered through Whatman No. 4 paper, concentrated under reduced pressure (40 degrees C), and reconstituted in methanol to 10 mg/mL. Aliquots were stored at -20 degrees C. All determinations were performed in triplicate.

Quantification of phenolic compounds

Total polyphenol content (TPC) was quantified by the Folin-Ciocalteu method [34]. Total flavonoid content (TFC) was measured by the aluminium chloride method [35]. Total flavonol content (TFlC) was determined by the sodium acetate/aluminium chloride method [36]. All results are expressed per gram of dry residue.

Antioxidant activity assays

Four mechanistically complementary assays: DPPH radical scavenging [37], ferrous ion chelation [38], FRAP [39], and beta-carotene/linoleic acid bleaching inhibition [40].

Acetylcholinesterase inhibitory activity

AChE inhibition was quantified by Ellman's colorimetric method [41] adapted according to Eldeen et al. [42]. Donepezil hydrochloride served as positive control; results are expressed as mg donepezil equivalent per gram of dry residue.

Statistical and chemometric analyses

Data: mean +/- SE (n = 3). One-way ANOVA followed by Duncan's test (alpha = 0.05). Pearson correlations, PCA, UPGMA clustering, and Mantel test (1000 permutations).

In silico ADMET profiling

ADMET predictions for quercetin (CID: 5280343) and rutin (CID: 5280805) using SwissADME [43] and ProTox-3.0 [44].

Declarations

Ethics approval and consent to participate

Not applicable. This study did not involve human participants, human tissue, or vertebrate animals. Plant material was collected from wild populations with appropriate permissions as described in the Methods.

Consent for publication

Not applicable. This manuscript does not contain any individual person's data.

Availability of data and materials

All data generated or analysed during this study are included in this published article. Raw datasets are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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NR performed the laboratory works and wrote the draft. IS contributed to revision and interpretation. CM conducted the data analysis and reviewed the manuscript.

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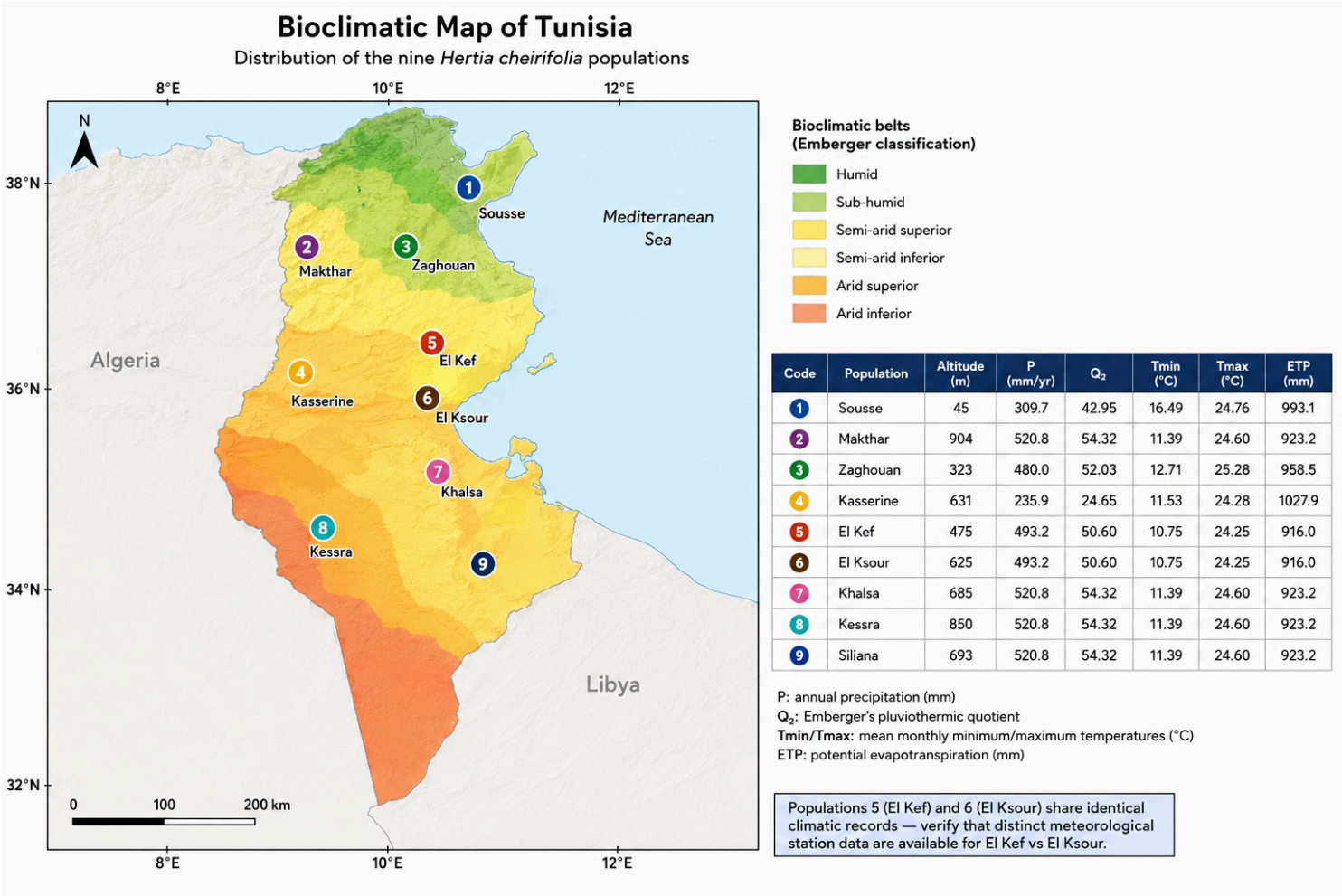
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Figures



Code	Population	Altitude (m)	P (mm/yr)	Q_2	Tmin (°C)	Tmax (°C)	ETP (mm)
1	Sousse	45	309.7	42.95	16.49	24.76	993.1
2	Makthar	904	520.8	54.32	11.39	24.60	923.2
3	Zaghouan	323	480.0	52.03	12.71	25.28	958.5
4	Kasserine	631	235.9	24.65	11.53	24.28	1027.9
5	El Kef	475	493.2	50.60	10.75	24.25	916.0
6	El Ksour	625	493.2	50.60	10.75	24.25	916.0
7	Khalsa	685	520.8	54.32	11.39	24.60	923.2
8	Kessra	850	520.8	54.32	11.39	24.60	923.2
9	Siliana	693	520.8	54.32	11.39	24.60	923.2

Figure 1

Ecological and climatic parameters of the nine H. cheirifolia populations. Populations: 1 = Sousse, 2 = Makthar, 3 = Zaghouan, 4 = Kasserine, 5 = El Kef, 6 = El Ksour, 7 = Khalsa, 8 = Kessra, 9 = Siliana.

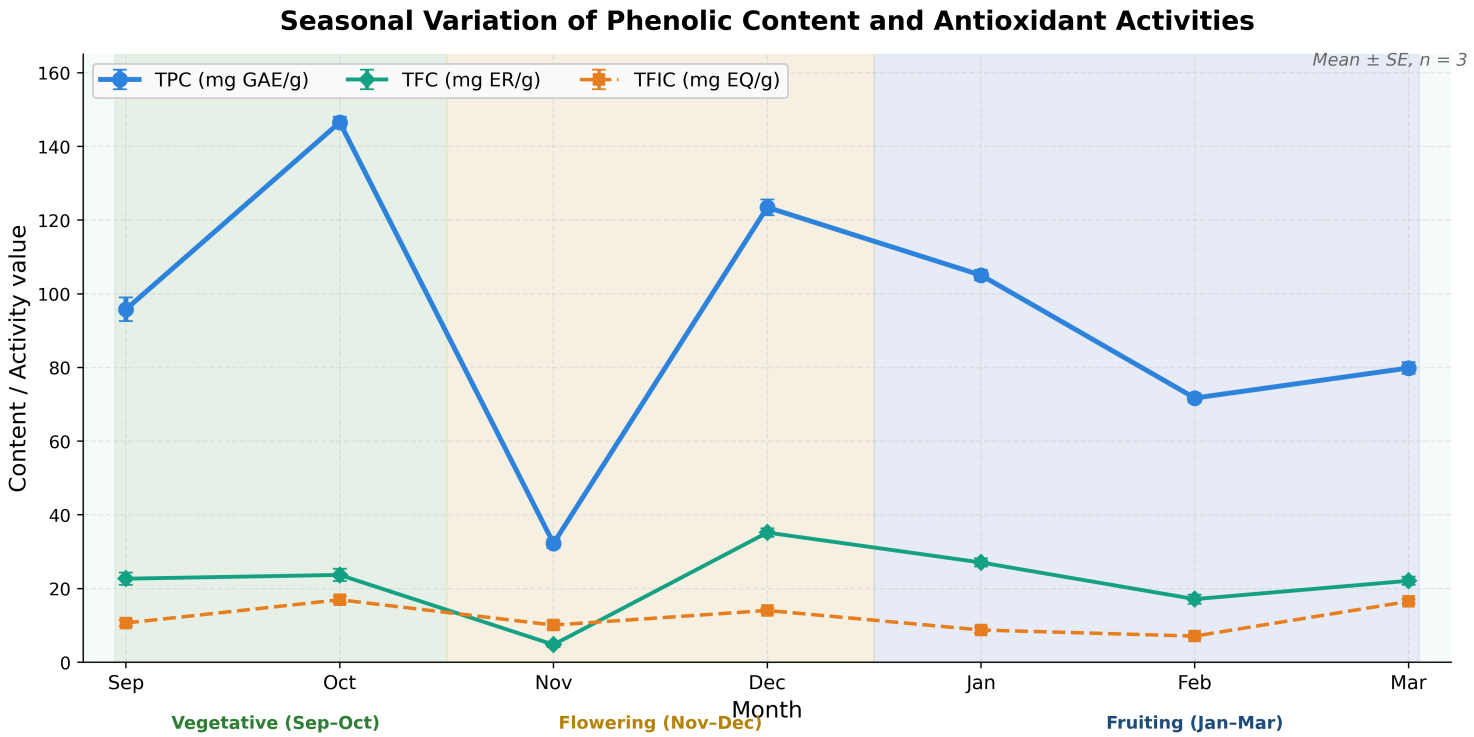


Figure 2

Seasonal variation in leaf phenolic content of H. cheirifolia (Sousse population, September 2013-March 2014). (A) Total polyphenol content (TPC); (B) Total flavonoid content (TFC); (C) Total flavonol content (TFlC). Values are mean +/- SE (n = 3). Different letters indicate significant differences (Duncan's test, $p < 0.05$).

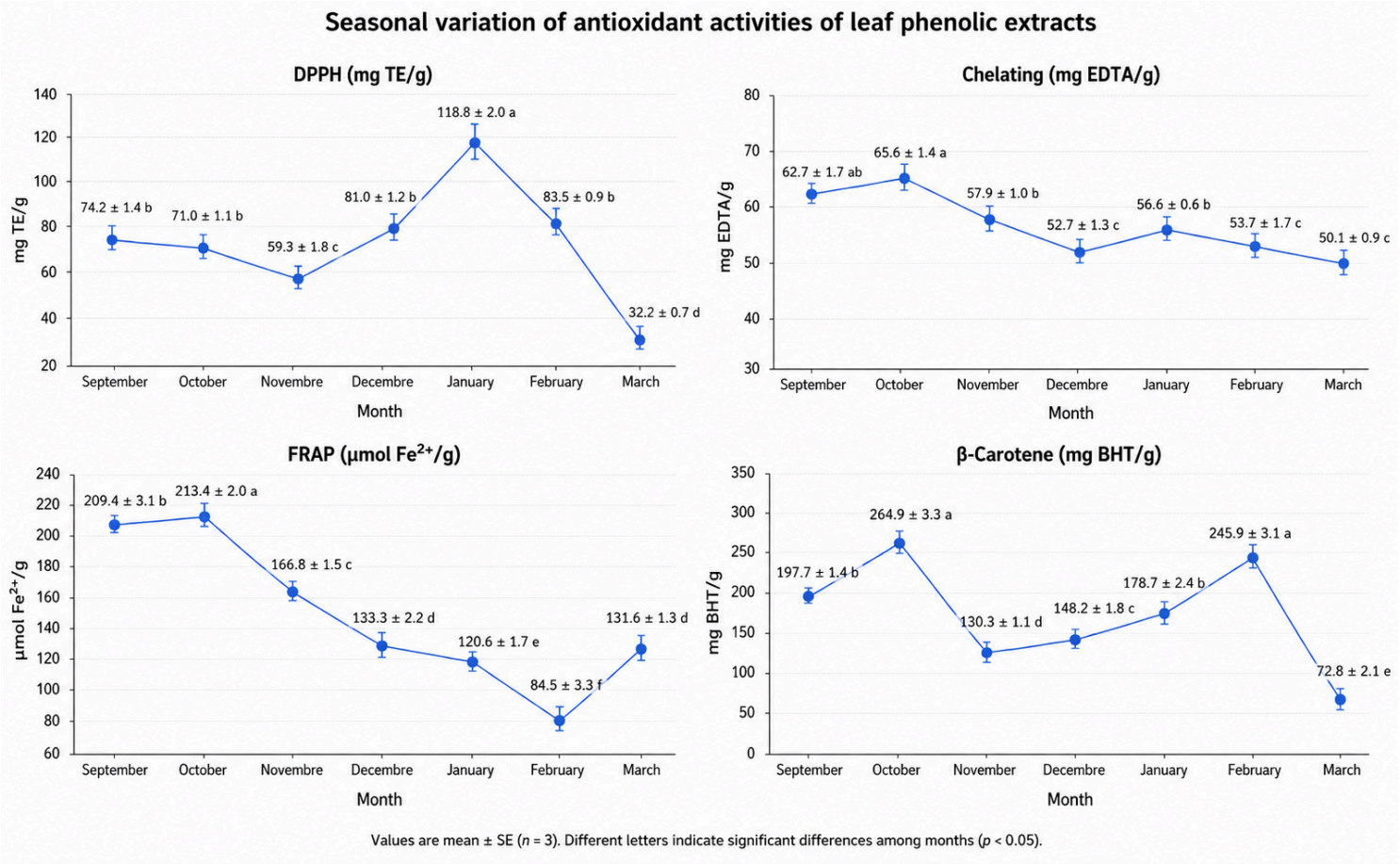


Figure 3

Seasonal variation in antioxidant activities of H. cheirifolia leaf phenolic extracts (Sousse population). (A) DPPH radical scavenging; (B) Ferrous ion chelation; (C) FRAP; (D) Beta-carotene bleaching inhibition. Values are mean +/- SE (n = 3). Different letters indicate significant differences (Duncan's test, $p < 0.05$).

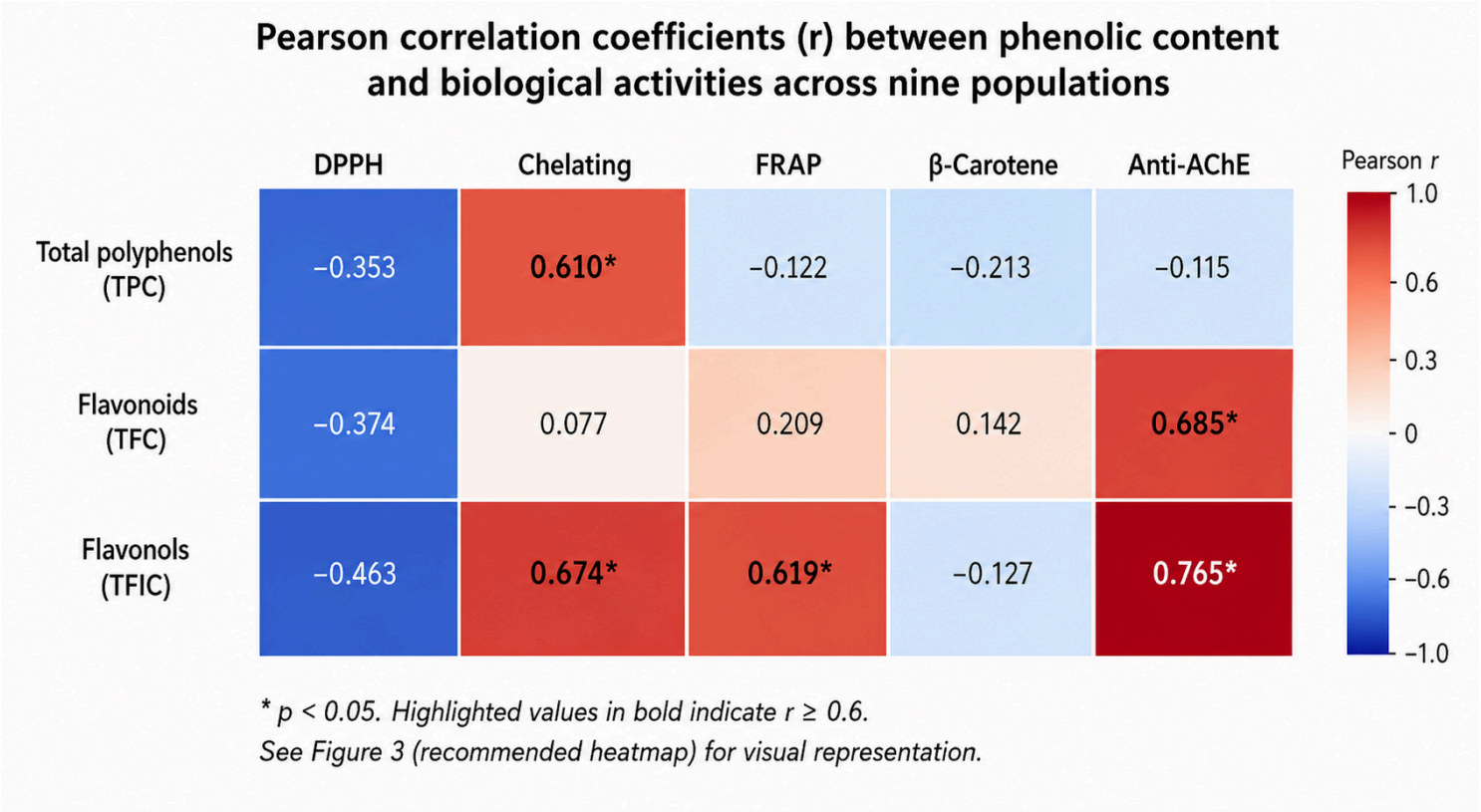


Figure 4

Pearson correlation coefficients (r) between phenolic content and biological activities across nine H. cheirifolia populations. *p < 0.05, **p < 0.01. TPC: total polyphenol content; TFC: total flavonoid content; TFlC: total flavonol content.

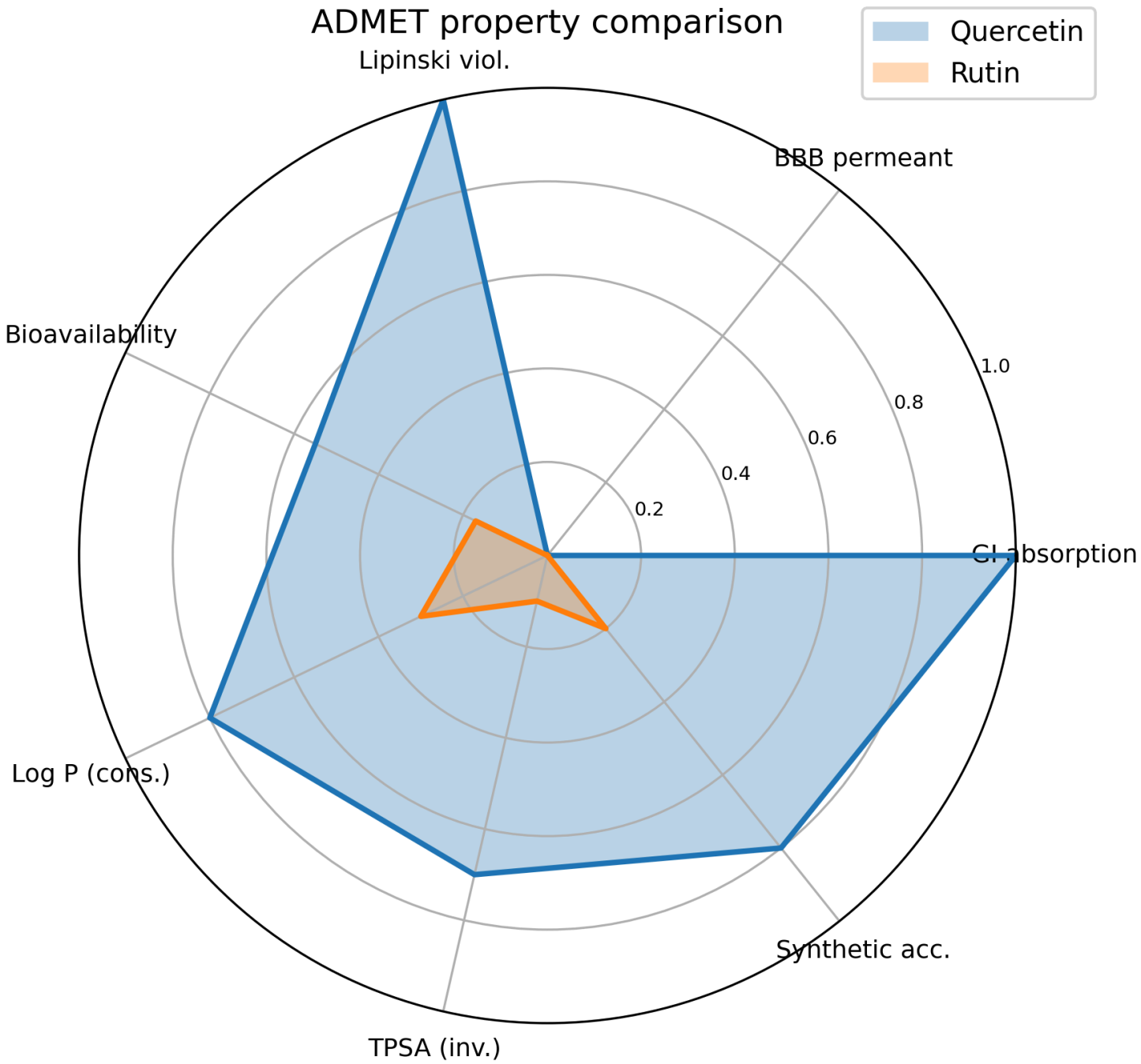


Figure 5

In silico ADMET profiling of quercetin and rutin. (A) Bioavailability radar; (B) Drug-likeness predictions; (C) Toxicity prediction summary from ProTox-3.0.