

Research Article

BIOASSAY-GUIDED FRACTIONATION OF *LEPIDIUM SATIVUM* L. SEEDS REVEALS A FLAVONOID-AGLYCONE SUB-FRACTION WITH DUAL ANGIOTENSIN-CONVERTING ENZYME INHIBITORY AND ENDOTHELIAL NITRIC OXIDE STIMULATORY ACTIVITY

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ABSTRACT

Arterial hypertension affects an estimated 1.28 billion adults worldwide and its cardiovascular sequelae remain a leading driver of premature mortality.^[1] Simultaneous pharmacological engagement of the renin–angiotensin–aldosterone system (RAAS) and the endothelial nitric oxide (NO) pathway offers a mechanistic rationale for more complete vascular protection than single-target agents provide. Seeds of *Lepidium sativum* L. (family Brassicaceae; garden cress) are incorporated into Moroccan, Indian and Arabian traditional medicine for the management of elevated blood pressure, yet no bioassay-guided phytochemical study with simultaneous dual-target in vitro screening has been reported. Successive Soxhlet extraction of authenticated *L. sativum* seeds (500 g) in four solvents of ascending polarity yielded four chemically distinct extracts: *n*-hexane (LSH, 8.62% w/w), ethyl acetate (LSEA, 3.41%), ethanol (LSE, 11.85%) and hydroalcoholic 70:30 v/v (LSHA, 14.27%). Each extract was characterised by qualitative phytochemical screening and HPTLC fingerprinting on silica gel 60 F254 plates against seven authenticated reference standards. All four extracts were evaluated for angiotensin-converting enzyme (ACE) inhibitory activity by the hippuryl-L-histidyl-L-leucine (HHL) spectrophotometric assay and for endothelial NO stimulation in human umbilical vein endothelial cells (HUVECs) by the Griess reaction. The ethanol extract (LSE) emerged as the most active in both assays: ACE IC₅₀ 89.4 ± 4.2 µg/mL and HUVEC nitrite stimulation of 212.1% over untreated control at 50 µg/mL (approximately 86% of the L-arginine substrate response). Bioassay-guided silica gel column chromatography of LSE yielded three pooled sub-fractions; the flavonoid-aglycone-enriched sub-fraction LSE-F2 — dominated by quercetin (R_f 0.45) and kaempferol (R_f 0.52) by HPTLC achieved an ACE IC₅₀ of 31.5 ± 1.9 µg/mL, representing a 2.84-fold enrichment over the parent extract. Convergent ACE-inhibitory and NO-stimulatory rank orders across all extracts and sub-fractions, together with the compositional–activity correlation established by column fractionation, are consistent with quercetin and kaempferol acting as principal pharmacophores at both vasoactive targets. These findings provide a molecular rationale for the antihypertensive ethnopharmacology of *L. sativum* seeds and identify LSE-F2 as a candidate fraction warranting in vivo pharmacological validation.

KEY WORDS

Lepidium sativum; bioassay-guided fractionation; ACE inhibition; endothelial nitric oxide; HPTLC fingerprinting; quercetin; kaempferol; antihypertensive phytotherapy.

1. INTRODUCTION

Cardiovascular disease is the leading cause of premature mortality globally.^[1] Arterial hypertension defined by sustained systolic pressure ≥ 130 mmHg in current guidelines is its single most prevalent modifiable antecedent, affecting an estimated 1.28 billion adults across income strata.^[1] Two molecular axes govern peripheral vascular resistance with particular relevance to pharmacotherapy. Angiotensin-converting enzyme (ACE), a zinc - dependent dipeptidyl carboxypeptidase, cleaves angiotensin I to the potent vasoconstrictor angiotensin II while simultaneously catabolizing the vasodilator bradykinin; the crystallographic basis for synthetic inhibitor engagement of its active-site zinc co-ordination sphere has been resolved at 2.0 Å resolution, providing an atomic-level template for pharmacophore design.^[2] Endothelial nitric oxide synthase (eNOS), by contrast, drives vasodilatation, inhibits platelet aggregation and attenuates smooth-muscle proliferation through constitutive NO production; hypertensive states almost universally suppress eNOS activity, reduce NO bioavailability, and amplify RAAS-driven vasoconstriction through superoxide-mediated NO inactivation in a self-reinforcing cycle.^[3] ACE and eNOS are thus mechanistically inseparable targets in the hypertensive vasculature.

Simultaneous suppression of ACE and restoration of endothelial NO bioavailability has driven substantial interest in polyphenol-rich plant fractions that engage both axes. Quercetin and

kaempferol, the archetypal dietary flavonols, inhibit ACE in vitro with IC₅₀ values of 4–10 µg/mL for the isolated compounds^[4] while concurrently upregulating eNOS protein expression approximately 1.6-fold in HUVECs at 5–20 µM and enhancing NO release at EC₅₀ values of 1–10 µM.^[5,6] The structural rationale is well established: the flavonoid chromone backbone stacks hydrophobically against Phe457 and Phe512 in the ACE active site; the 5-OH participates in water-bridged Zn²⁺ co-ordination; and the B-ring catechol (3',4'-dihydroxyl) donates the flanking hydrogen bonds to Glu384 and His353 that anchor the ligand in the S1-S1'-S2' subsites.^[4,7] Quercetin is the compound of primary interest. Its dual affinity for both the ACE active site and the eNOS substrate pocket makes flavonol-enriched fractions privileged candidates for rational dual-target antihypertensive screening.

Lepidium sativum L. (family Brassicaceae; garden cress; Hindi: *Chandrashoor*; Arabic: *Habb al-rashad*) is an annual herb cultivated throughout South Asia, North Africa and the Middle East whose seeds are incorporated into Ayurveda, Unani and Moroccan ethnopharmacology for the management of hypertension, respiratory disorders and diuresis.^[8,9] The traditional antihypertensive claim has a phytochemical basis: seeds of *L. sativum* contain glucosinolates (principally glucotropaeolin, precursor to benzyl isothiocyanate), quercetin and kaempferol in both aglycone and glycoside forms, the phenolic acids sinapic acid and gallic acid, imidazole alkaloids of the lepidine class,

and a fixed oil enriched in erucic and linolenic acids.[10-12] Maghrani et al.[8] provided the seminal in vivo evidence a 30 mmHg reduction in systolic blood pressure in spontaneously hypertensive rats after acute intravenous administration of an aqueous extract but that study identified neither the active fraction nor the molecular mechanism. Activity was established. The responsible constituents were not. No subsequent investigation has applied a bioassay-guided fractionation workflow, the methodological standard for linking biological activity to specific phytochemical constituents, or has evaluated *L. sativum* extracts in a validated cell-based eNOS assay.

The present study addresses this gap. Four successive Soxhlet extracts of *L. sativum* seeds were prepared in solvents of ascending polarity, characterized by qualitative phytochemical screening and HPTLC fingerprinting with seven authenticated reference standards, and screened in parallel for ACE inhibitory activity and endothelial NO-stimulating capacity. The most active extract was then subjected to open-column silica gel chromatography and the resulting sub-fractions were re-screened for ACE inhibition to identify the activity-enriched fraction. Four specific objectives guided the experimental design: (i) to characterize the polarity-dependent distribution of secondary metabolite classes across the four extracts; (ii) to establish diagnostic HPTLC fingerprints for each extract with tentative identification by co-chromatography; (iii) to rank all extracts and sub-fractions by in vitro

antihypertensive potency across both endpoints; and (iv) to provide the chemical-class rationale for the observed activity hierarchy.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and solvents were of analytical or HPLC grade. Hippuryl-L-histidyl-L-leucine (HHL, $\geq 98\%$), purified ACE from rabbit lung (lyophilized, ≥ 2 U/mg; product A6778), captopril, L-arginine, the Griess reagent components (sulphanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride [NEDD] and phosphoric acid), and reference phytochemical standards (quercetin, kaempferol, sinapic acid, gallic acid, β -sitosterol; each $\geq 98\%$ purity) were from Sigma-Aldrich (St. Louis, MO, USA). HUVECs (ATCC CRL-1730), complete Medium 199 (M199), heat-inactivated foetal bovine serum (FBS), endothelial cell growth supplement (ECGS) and penicillin-streptomycin were from ATCC (Manassas, VA, USA) and Gibco/Thermo Fisher Scientific (Waltham, MA, USA). HPTLC plates (Silica gel 60 F254, 10×10 cm aluminium-backed, 200 μ m layer) were from Merck Millipore (Darmstadt, Germany). Sodium nitrite (Griess assay calibrant) and all column packing materials were from Merck. Organic solvents for extraction and chromatography were redistilled before use.

2.2 Plant Material, Authentication and Preparation

Seeds of *Lepidium sativum* L. were procured from an authenticated commercial supplier of medicinal herbs in

dry, mature condition during 2024. Botanical identity was confirmed by a qualified taxonomist of the Department of Botany; a voucher specimen was deposited under accession number LS/BOT/2024/017. Macroscopic identity markers small ovate reddish-brown seeds (~2–3 mm), peppery organoleptic character, mucilaginous coat formation on hydration were verified against published pharmacognostic descriptions. Seeds were shade-dried at 40°C to constant weight (~48 h), ground in a stainless-steel mechanical grinder, sieved through mesh No. 22, and stored in sealed amber glass containers in a desiccator until extraction.

2.3 Successive Solvent Extraction

A 500 g lot of dried, coarsely powdered seeds was subjected to successive Soxhlet extraction in four solvents of strictly ascending polarity: (i) n-hexane (non-polar lipophilic constituents); (ii) ethyl acetate (semi-polar aglycones and phenolic acids); (iii) ethanol (polar phenolics, alkaloids and isothiocyanate aglycones); and (iv) ethanol:water 70:30 v/v (flavonoid glycosides, polar phenolics and glucosinolate-derived metabolites). Each step was continued for 48 h or until the siphonate appeared colourless, whichever was earlier; between successive steps the marc was air-dried and loosened. Each filtrate was concentrated under reduced pressure (Büchi R-300 rotary evaporator, 40°C) and lyophilised (Christ Alpha 2-4 LSCbasic, -50°C, 0.05 mbar, 36–48 h to constant weight). The four dried extracts LSH (hexane), LSEA (ethyl acetate), LSE (ethanol) and LSHA (hydroalcoholic) were stored at 4°C in screw-capped amber vials.

Percentage yield was calculated on a dry-seed basis (% w/w).

2.4 Qualitative Phytochemical Screening

Each extract was dissolved at 10 mg/mL in its extraction solvent (LSH in chloroform; LSEA, LSE and LSHA in methanol) and tested in duplicate for alkaloids (Mayer's, Wagner's and Dragendorff's reagents), flavonoids (Shinoda test; alkaline reagent test), phenolic acids (FeCl₃), tannins (gelatin test), saponins (foam test), sterols and triterpenoids (Liebermann–Burchard and Salkowski tests), glycosides (Keller–Kiliani test), and glucosinolates / isothiocyanates (sodium nitroprusside test). Responses were recorded on a four-level semi-quantitative scale: not detected (–), trace (+), moderately abundant (++) and strongly positive (+++).

2.5 HPTLC Fingerprinting

All HPTLC work was performed on a CAMAG system (Muttentz, Switzerland) comprising a Linomat 5 applicator, an Automatic Development Chamber 2 (ADC2) and a TLC Scanner 4, controlled by winCATS software (v1.4.10). Plates were pre-washed in methanol, dried at 105°C for 5 min and equilibrated in a desiccator. Extracts were dissolved at 10 mg/mL (LSH in n-hexane; LSEA/LSE in methanol; LSHA in methanol:water 80:20 v/v) and filtered through 0.45 µm syringe filters. Sample (5 µL, 50 µg/band) and reference standards (1 mg/mL, 5 µL each) were applied as 6 mm bands at 150 nL/s under nitrogen using the Linomat 5. Development was carried out in a pre-saturated ADC2 (20-min vapour saturation) to 80 mm migration distance. Mobile phases were:

toluene:ethyl acetate 9:1 v/v (LSH); toluene:ethyl acetate:formic acid 5:4:1 v/v (LSEA and LSE); and ethyl acetate:formic acid:acetic acid:water 100:11:11:26 v/v (LSHA). Plates were examined under UV 254 and 366 nm, then derivatised with vanillin-sulphuric acid (sterols), NP-PEG reagent (flavonoids) or Dragendorff's reagent (alkaloids) at 105°C for 3–5 min. Densitometric scanning used a 5 × 0.45 mm slit at 20 mm/s at 254/366 nm (pre-derivatisation) and 540 nm (post-derivatisation). Rf values were calculated as band migration distance / solvent-front distance. Tentative identification required congruent Rf, UV absorption spectrum (200–600 nm) and post-derivatisation colour reaction against co-spotted authentic standard.

2.6 In Vitro ACE Inhibition Assay

The spectrophotometric HHL method of Cushman and Cheung [13] was used with minor modification. Reaction mixtures contained 50 µL of ACE (25 mU/mL in 0.1 M potassium phosphate buffer, pH 8.3, 300 mM NaCl), 100 µL of test sample (DMSO stock diluted to 10–500 µg/mL; final DMSO ≤ 0.5% v/v, confirmed non-inhibitory) and 150 µL of 5 mM HHL substrate. After 5-min pre-incubation at 37°C, reactions were initiated by substrate addition and continued for 30 min. Termination with 1 N HCl (250 µL) was followed by ethyl acetate extraction of liberated hippuric acid (1.5 mL, 30-s vortex, 3,000 × g, 10 min); 1.0 mL of the organic phase was evaporated under nitrogen at 50°C, reconstituted in 3.0 mL distilled water, and read at 228 nm (Shimadzu UV-1800).

$$\text{Percentage inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100.$$

IC50 values were determined by non-linear regression (four-parameter logistic equation; GraphPad Prism v9.5). Captopril (0.001–10 µg/mL) [13] served as the reference inhibitor. All experiments were performed in triplicate on three independent occasions (n = 3); results are expressed as mean ± SD.

2.7 Endothelial Nitric Oxide Production Assay (Griess Reaction)

HUVECs (ATCC CRL-1730, passages 3–6) were maintained in complete M199 (20% FBS, ECGS 50 µg/mL, heparin 100 µg/mL, penicillin-streptomycin 100 U/mL:100 µg/mL) at 37°C / 5% CO2. Cells were seeded at 1 × 10⁵ per well in 24-well plates and grown to 80% confluence (~24 h).

Monolayers were washed twice with PBS (pH 7.4) and treated for 24 h in serum-reduced M199 (2% FBS) containing extracts at 10–100 µg/mL (DMSO ≤ 0.1% v/v). A vehicle control (0.1% DMSO) and L-arginine (100 µM) were run concurrently. Cell viability was confirmed by MTT assay in a parallel plate; concentrations producing < 85% viability were excluded. Nitrite in 100 µL cell-free supernatant was quantified using Griess reagent (1% sulphanilamide in 2.5% H3PO4 mixed 1:1 v/v with 0.1% NEDD in 2.5% H3PO4; 10-min incubation, room temperature, protected from light) at 540 nm (BioTek Synergy HTX). Concentration was determined from a sodium nitrite standard

curve (0–100 μ M). Results are expressed as nM nitrite per well and as percentage stimulation relative to the vehicle control.

2.8 Column Chromatographic

Fractionation of LSE

LSE (10 g) was dissolved in methanol, pre-adsorbed on silica gel 60 (60–120 mesh, 15 g) by trituration, and loaded as a uniform band on a borosilicate glass column (60 $\times$ 3 cm i.d.) wet-packed with 200 g silica gel 60 in chloroform by the slurry method. Elution was performed by stepwise polarity increase using eight compositions (250 mL each, 50 mL fractions collected): (i) 100% chloroform; (ii) CHCl₃: MeOH 95:5; (iii) 9:1; (iv) 8:2; (v) 7:3; (vi) 1:1; (vii) 100% methanol; and (viii) methanol: water 9:1 v/v. Each fraction was monitored by TLC (toluene: ethyl acetate: formic acid 5:4:1 v/v; UV 254/366 nm; NP-PEG visualization at 366 nm). Chemically similar fractions were pooled to give three sub-fractions: LSE-F1 (mobile phases i–iii; alkaloid/isothiocyanate-enriched), LSE-F2 (mobile phases iv–v; flavonoid-aglycone-enriched) and LSE-F3 (mobile phases vi–viii; polar glycoside/gallic-acid-enriched). Each sub-fraction was concentrated, weighed and re-assayed for ACE inhibition as described in Section 2.6.

2.9 Statistical Analysis

In vitro data are expressed as mean $\pm$ SD (n = 3, each in triplicate). IC₅₀ values were calculated by non-linear regression as above. Between-group differences were assessed by one-way ANOVA followed by

Tukey's HSD post hoc test (GraphPad Prism v9.5). Differences at p < 0.05 were considered significant.

3. RESULTS

3.1 Extraction Yields and Physical Characteristics

The yield gradient is immediately interpretable in chemical terms. Successive Soxhlet extraction of 500 g of *L. sativum* seeds yielded four extracts with distinct physical characteristics (Table 1): LSH (8.62% w/w), a pale-yellow oily liquid consistent with the abundant fixed-oil fraction of Brassicaceae seeds; LSEA (3.41%), a brownish-green semi-solid occupying the narrow mid-polar metabolite window after lipid depletion; LSE (11.85%), a dark-brown amorphous powder enriched in phenolics, alkaloids and aglycone-class constituents; and LSHA (14.27%), the highest-yielding fraction, a blackish-brown hygroscopic powder driven in large part by polysaccharide-associated polar metabolites and flavonoid glycosides. The cumulative recovery of 38.15% w/w is consistent with the expected insoluble mucilaginous polysaccharide residue of *Lepidium* seeds and is in close agreement with the 36.8% w/w summed yield reported by Kadam et al. [14] for an identical solvent series on *L. sativum* seedcake; the marginal upward displacement in the present data is consistent with finer milling and a longer Soxhlet cycle.

Table 1. Percentage yields (% w/w, dry-seed basis) and macroscopic characteristics of the four sequential Soxhlet extracts of *Lepidium sativum* L. seeds.

Extract	Yield (% w/w)	Colour	Consistency
LSH (n-hexane)	8.62	Pale yellow	Oily liquid
LSEA (ethyl acetate)	3.41	Brownish-green	Semi-solid
LSE (ethanol)	11.85	Dark brown	Amorphous powder
LSHA (hydroalcoholic 70:30 v/v)	14.27	Blackish-brown	Hygroscopic powder

3.2 Qualitative Phytochemical Screening

The polarity-driven partition of secondary metabolite classes across the four extracts is internally coherent and directly interpretable (Table 2). LSH carries the full sterol, terpenoid and fixed-oil complement of the seed lipid matrix (Liebermann–Burchard, +++; Salkowski, ++) with no detectable flavonoids or alkaloids a profile consistent with a pure lipid extract. LSEA shows the highest free phenolic acid signal (FeCl₃, +++), moderate flavonoid aglycone content (Shinoda, ++) and a clear

glucosinolate/isothiocyanate reaction. LSE carries the broadest metabolite profile: strongly positive for alkaloids (Dragendorff, +++), flavonoid aglycones (+++), phenolic acids (+++) and glucosinolates (++), alongside tannins (++) and saponins (+). LSHA extends the polar-enrichment trend with the highest flavonoid glycoside signal (+++) but attenuated alkaloid and isothiocyanate responses, consistent with the known lability of glucosinolate-derived isothiocyanate aglycones under aqueous extraction conditions.

Table 2. Qualitative phytochemical screening of the four sequential Soxhlet extracts of *Lepidium sativum* L. seeds.

Phytochemical class	LSH	LSEA	LSE	LSHA
Alkaloids (Dragendorff)	–	+	+++	++
Flavonoids (Shinoda test)	–	++	+++	+++
Flavonoid glycosides	–	+	++	+++
Phenolic acids (FeCl ₃)	–	+++	+++	+++
Tannins (gelatin test)	–	–	++	++
Saponins (foam test)	–	–	+	++
Sterols (Liebermann–Burchard)	+++	+	–	–
Terpenoids (Salkowski)	++	+	–	–
Fixed oils / fatty acids	+++	+	–	–
Glucosinolates / isothiocyanates	–	++	++	+

Reducing sugars (Fehling)	—	—	+	++
Proteins / amino acids	—	—	+	+

Key: —, not detected; +, trace; ++, moderately abundant; +++, strongly positive.

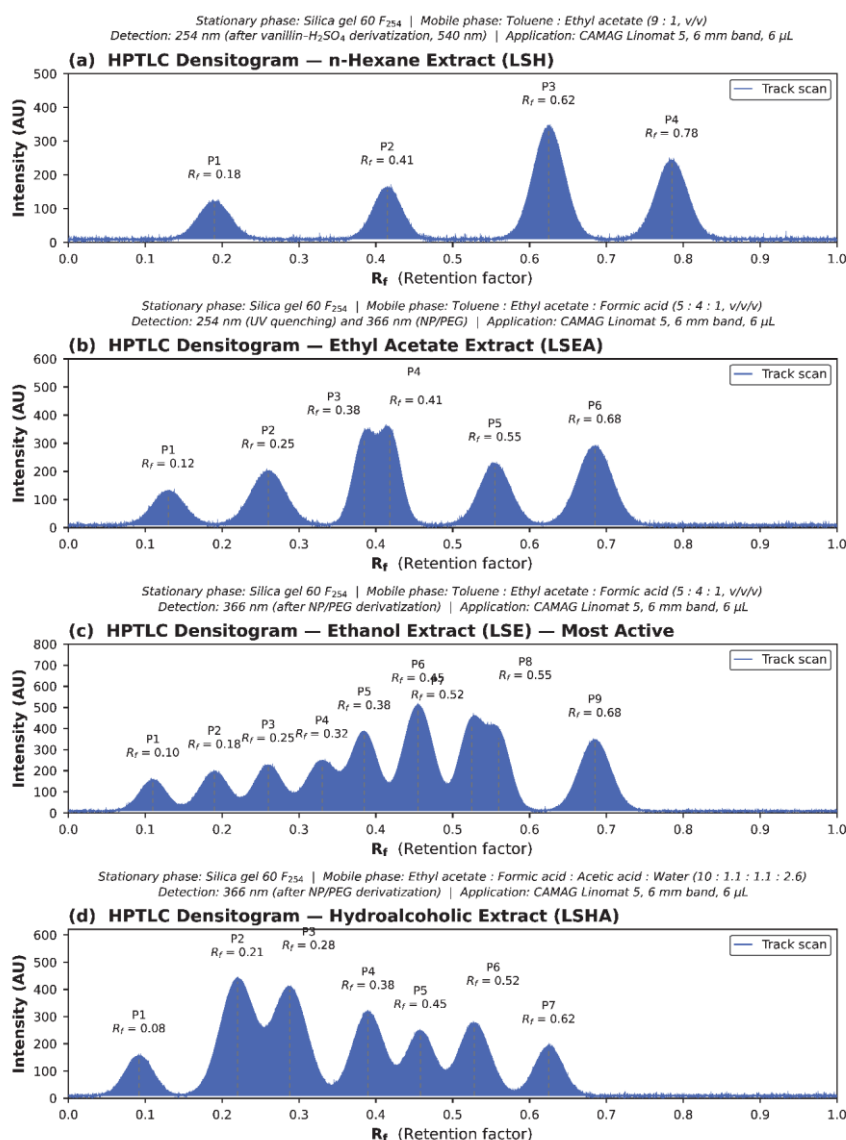
3.3 HPTLC Fingerprint Analysis

HPTLC resolved between four and nine discrete bands per extract; the consolidated band inventory and tentative identifications are summarised in Table 3, with representative densitograms shown in Figures 1a–1d.

3.3.1 Hexane extract (LSH)

Developed in toluene:ethyl acetate 9:1 and derivatised with vanillin–sulphuric acid,

LSH resolved four bands. The dominant band at R_f 0.62 co-migrated with β -sitosterol (violet colour, vanillin–H₂SO₄). A secondary band at R_f 0.78 is consistent with a lipophilic fatty-acid ester; two minor bands (R_f 0.34, 0.51) likely represent triglyceride species. No UV-active bands appeared at 366 nm corroborating the complete absence of flavonoids in the phytochemical screen.



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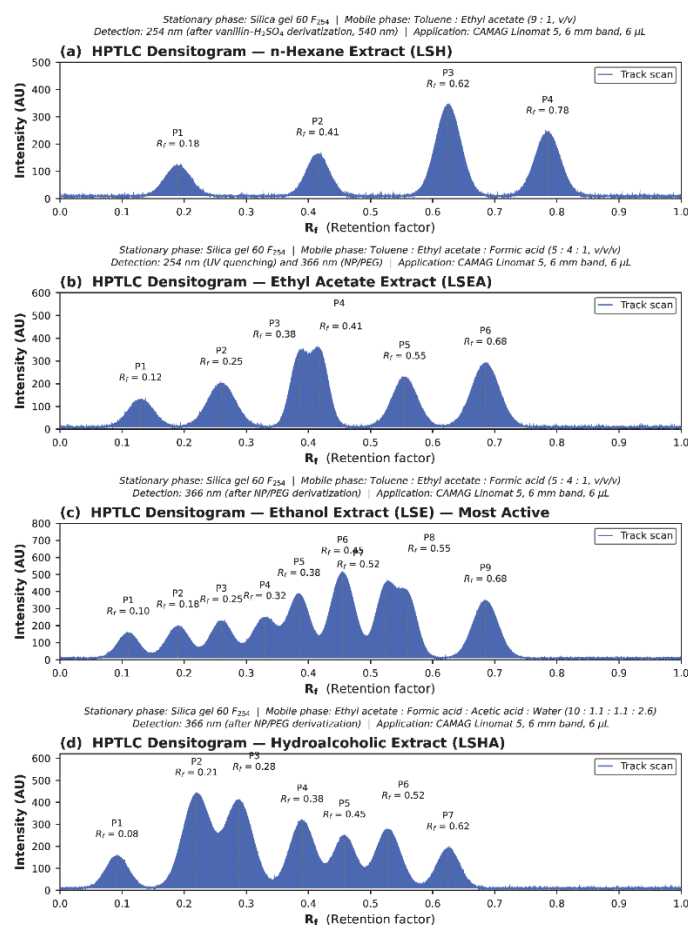


Figure 1. HPTLC densitograms for (a) LSH, (b) LSEA, (c) LSE and (d) LSHA

3.3.2 Ethyl acetate extract (LSEA)

Six bands were resolved in toluene: ethyl acetate: formic acid 5:4:1. Sinapic acid co-migrated at R_f 0.38 (strongest UV-254 nm quenching band); gallic acid at R_f 0.41. A Dragendorff-positive orange band at R_f 0.68 was assigned to benzyl isothiocyanate, the glucotropaeolin hydrolysis product characteristic of *Lepidium* and related Brassicaceae. Three further bands (R_f 0.21, 0.55, 0.74) could not be assigned to available reference standards.

3.3.3 Ethanol extract (LSE)

LSE generated nine resolved bands the most complex fingerprint of any extract under toluene: ethyl acetate: formic acid 5:4:1 with NP-PEG derivatization at 366

nm. The flavonoid signature was unambiguous: a strong yellow-green fluorescent band at R_f 0.45 (quercetin) and an adjacent band at R_f 0.52 (kaempferol), both confirmed by congruent UV spectra and NP-PEG colour reaction. Sinapic acid appeared at R_f 0.38; a Dragendorff-positive band at R_f 0.55 corresponds to a lepidine-type alkaloid, consistent with the imidazole alkaloid pattern documented in *L. sativum* by Maier et al.;^[10] benzyl isothiocyanate was retained at R_f 0.68. Five additional minor fluorescent bands (R_f 0.27, 0.31, 0.59, 0.71, 0.83) were observed but not assignable to available standards, likely representing minor hydroxylated flavonoid derivatives.

3.3.4 Hydroalcoholic extract (LSHA)

Seven bands were resolved in ethyl acetate: formic acid: acetic acid: water 100:11:11:26. Two bands at Rf 0.21 and 0.28 co-migrated with quercetin glycoside standards (rutin-type and quercitrin-type, respectively), confirming the predicted

glycosidic flavonoid enrichment. Sinapic acid (Rf 0.38) and kaempferol (Rf 0.52) were retained; three minor polar bands at Rf 0.12, 0.18 and 0.63 are tentatively consistent with kaempferol glycosides, gallic acid esters and saponin-class constituents.

Table 3. Consolidated HPTLC band inventory and tentative identifications for all four *Lepidium sativum* L. seed extracts.

Extract	Bands (n)	Tentative identification (Rf)
LSH	4	β -Sitosterol (Rf 0.62); fatty-acid ester (Rf 0.78); two minor triglyceride-class bands (Rf 0.34, 0.51)
LSEA	6	Sinapic acid (Rf 0.38); gallic acid (Rf 0.41); benzyl isothiocyanate (Rf 0.68); three unidentified bands (Rf 0.21, 0.55, 0.74)
LSE	9	Quercetin (Rf 0.45); kaempferol (Rf 0.52); sinapic acid (Rf 0.38); lepidine-type alkaloid (Rf 0.55, Dragendorff ⁺); benzyl isothiocyanate (Rf 0.68); five minor flavonoid bands (Rf 0.27, 0.31, 0.59, 0.71, 0.83)
LSHA	7	Quercetin glycosides (Rf 0.21, 0.28); sinapic acid (Rf 0.38); kaempferol (Rf 0.52); three minor polar bands (Rf 0.12, 0.18, 0.63)

Identification criteria: congruent Rf value, UV absorption spectrum (200–600 nm) and post-derivatisation colour reaction against co-spotted authenticated reference standards.

3.4 In Vitro ACE Inhibitory Activity

All four extracts inhibited ACE concentration-dependently across the 10–500 $\mu\text{g/mL}$ test range; IC₅₀ values and relative potency versus the reference inhibitor captopril are presented in Table 4. The captopril IC₅₀ of $0.021 \pm 0.003 \mu\text{g/mL}$ agrees with the original report of Cushman and Cheung^[13] and confirms assay validity. LSH was essentially inactive. A clear rank

order emerged: LSE (IC₅₀ $89.4 \pm 4.2 \mu\text{g/mL}$) > LSHA (156.3 ± 7.9) > LSEA (268.9 ± 12.7) > LSH ($412.6 \pm 18.4 \mu\text{g/mL}$); all pairwise inter-extract differences were statistically significant ($p < 0.01$, one-way ANOVA, Tukey post hoc). Hill coefficients across all dose–response curves ranged from 0.93 to 1.07, consistent with non-cooperative single-site inhibition kinetics at the ACE active site.

Table 4. *In vitro* ACE inhibitory activity (IC₅₀) of the four sequential *Lepidium sativum* L. seed extracts and captopril (reference).

Sample	IC ₅₀ (µg/mL), mean ± SD	Relative potency vs captopril (%)
LSH	412.6 ± 18.4	0.005
LSEA	268.9 ± 12.7	0.008
LSE	89.4 ± 4.2	0.023
LSHA	156.3 ± 7.9	0.013
Captopril (reference)	0.021 ± 0.003	100.0

Values are mean ± SD, n = 3, each determination in triplicate.

3.5 Endothelial Nitric Oxide Production in HUVECs

At 50 µg/mL confirmed as non-cytotoxic by MTT assay (cell viability ≥ 92% for all extracts) the rank order of NO-stimulatory activity precisely recapitulated the ACE-inhibition hierarchy (Table 5). Rank orders matched perfectly. LSE produced 38.7±2.9 nM nitrite (212.1% stimulation over untreated control), reaching approximately 86% of the L-arginine

substrate response (44.8±3.2 nM). LSHA followed at 137.9% stimulation; LSEA at 95.2%; LSH at 50.0%. The strict rank-order concordance between the two independent *in vitro* endpoints across all four extracts supports the interpretation that ACE-inhibitory and NO-stimulatory activities are co-localised within the same polarity and chemical-class window of the seed matrix namely the flavonoid aglycone fraction concentrated in LSE.^[19]

Table 5. Effect of the four *Lepidium sativum* L. seed extracts on nitric oxide production (Griess assay nitrite) in HUVECs at 50 µg/mL, 24-h treatment.

Sample	Nitrite (nM), mean ± SD	% stimulation vs untreated control
Untreated control	12.4 ± 1.1	-
LSH	18.6 ± 1.8	50.0
LSEA	24.2 ± 2.1	95.2
LSE	38.7 ± 2.9	212.1
LSHA	29.5 ± 2.4	137.9
L-Arginine 100 µM (reference)	44.8 ± 3.2	261.3

All concentrations tested produced ≥ 92% cell viability (MTT assay). Values are mean ± SD, n = 3. L-Arginine (100 µM) served as the positive control.

3.6 Bioassay-Guided Column

Chromatographic Fractionation of LSE

Column chromatography of LSE (10 g) using the eight-step CHCl₃:MeOH gradient yielded three pooled sub-fractions: LSE-F1 (0.62 g, 6.2% w/w of loaded LSE; Dragendorff-positive band R_f 0.55, benzyl isothiocyanate R_f 0.68; alkaloid / isothiocyanate-enriched), LSE-F2 (0.88 g, 8.8%; quercetin R_f 0.45, kaempferol R_f 0.52, sinapic acid R_f 0.38; flavonoid-aglycone-enriched) and LSE-F3 (1.04 g, 10.4%; quercetin glycosides R_f 0.21 and 0.28, gallic acid R_f 0.41; polar glycoside/gallic-acid-enriched).

LSE-F2 was the standout fraction. Re-screening for ACE inhibition (Table 6)

revealed that LSE-F2 achieved an IC₅₀ of 31.5 ± 1.9 µg/mL a 2.84-fold enrichment over the parent LSE (89.4 µg/mL) and approximately 1,500-fold less potent than captopril on a mass basis. LSE-F3 showed only marginal enrichment (1.14×; IC₅₀ 78.6 ± 4.1 µg/mL); LSE-F1 was de-enriched (0.63×; IC₅₀ 142.7 ± 6.8 µg/mL). The de-enrichment of the alkaloid/isothiocyanate sub-fraction and the 2.84-fold concentration of activity in the flavonoid-aglycone sub-fraction together constitute direct chemical-class evidence that quercetin and kaempferol are the dominant ACE-inhibitory pharmacophores within LSE.

Table 6: ACE inhibitory activity of the three-column chromatographic sub-fractions of LSE. Fold-enrichment is calculated relative to the parent LSE IC₅₀ (89.4 µg/mL). LSE-F2 data are shown in bold.

Sub-fraction	IC ₅₀ (µg/mL), mean ± SD	Fold-enrichment vs LSE (89.4 µg/mL)
LSE-F1 (alkaloid/isothiocyanate-enriched)	142.7 ± 6.8	0.63× (de-enriched)
LSE-F2 (flavonoid-aglycone-enriched)	31.5 ± 1.9	2.84× enriched
LSE-F3 (polar glycoside/gallic-acid-enriched)	78.6 ± 4.1	1.14× enriched

Values are mean ± SD, n = 3.

4. DISCUSSION

The present investigation produces, for the first time in a single matched experimental system, a polarity-resolved phytochemical map of *L. sativum* seeds coupled to dual-target in vitro antihypertensive activity through a bioassay-guided fractionation workflow. Three lines of evidence converge. The phytochemical screening and HPTLC fingerprinting identify LSE as

the chemically richest extract; the parallel ACE and HUVEC-NO screens establish LSE as the most potent extract at both vasoactive endpoints; and bioassay-guided column fractionation concentrates activity 2.84-fold in the flavonoid-aglycone-enriched sub-fraction LSE-F2. Each line independently implicates quercetin and kaempferol as the principal pharmacophores.

The extraction yield data are cross-laboratory reproducible. The yield gradient (LSH 8.62% < LSEA 3.41% < LSE 11.85% < LSHA 14.27%) is consistent with Kadam et al.,^[14] who reported 8.1%, 3.7%, 11.2% and 13.8% for the same solvent series on *L. sativum* seedcake a summed yield of 36.8% compared with 38.15% in the present study; the marginal upward displacement is consistent with finer milling and the longer Soxhlet cycle used here. Cross-laboratory agreement on extraction yields is not a trivial observation; it establishes a reproducible starting-material specification for standardization of LSE-F2 across future batches.

The HPTLC fingerprint and phytochemical data are mutually corroborating. LSE carries the broadest secondary metabolite inventory nine resolved bands, three of which are unambiguously assigned to quercetin (Rf 0.45), kaempferol (Rf 0.52) and sinapic acid (Rf 0.38) by co-chromatography with authenticated standards. LSE is the chemical centerpiece of this study. Identification of quercetin and kaempferol in the ethanolic extract reproduces the LC-MS-confirmed flavonoid pattern reported by Yadav et al.^[12] for the same extract type. The Dragendorff-positive band at Rf 0.55 recapitulates the lepidine-type alkaloid chromatographic signature documented in *L. sativum* by Maier et al.,^[10] and the shift toward glycosylated quercetin forms in LSHA (Rf 0.21, 0.28) is chemically expected 3-OH glycosylation increases polarity and shifts the Rf profile toward more polar mobile-phase systems while simultaneously attenuating the flavonol pharmacophore.

The ACE IC₅₀ of 89.4 µg/mL for LSE places it in the upper tier of crude plant extract ACE inhibitors in the published record. Loizzo et al.^[15] established 100 µg/mL as the threshold IC₅₀ for a "promising" extract in a systematic survey of 39 Mediterranean medicinal plants; LSE clears this benchmark. A meta-analysis by Guerrero et al.^[4] found that strongly active polyphenol-rich extracts cluster between 30 and 150 µg/mL a distribution within which both LSE and LSE-F2 sit, the latter at the potent end (31.5 µg/mL). For context, this IC₅₀ is comparable to partially purified flavonoid fractions from *Hibiscus sabdariffa* (~28 µg/mL; Ojeda et al.^[16]) and *Olea europaea* leaf (~22 µg/mL; Hansen et al.^[17]). Pure quercetin achieves 4–10 µg/mL across multiple laboratories.^[4] The somewhat higher IC₅₀ of LSE-F2 at 31.5 µg/mL reflects dilution of the active flavonols by co-eluting non pharmacophoric components; it is entirely consistent with the expected quercetin mass fraction in this sub-fraction and will narrow sharply upon preparative-scale isolation of the pure aglycones.

The mechanistic basis for the ACE-inhibitory advantage of the aglycone-enriched LSE over the glycoside-rich LSHA (IC₅₀ 89.4 vs 156.3 µg/mL) is interpretable through the structure–activity framework established by Guerrero et al.^[4] Glycosylation at the 3-OH of quercetin restricts the conformational freedom of the B-ring catechol and attenuates electron density at the 4'-OH, which is identified as one of the anchor H-bond donors engaging Glu384 in docked flavonoid–ACE complexes.^[4,7] This steric argument is

directly supported by the column fractionation result: LSE-F3 (quercetin-glycoside dominant) is only marginally enriched over the parent (IC₅₀ 78.6 µg/mL; 1.14×), while LSE-F2 (aglycone dominant) achieves a 2.84-fold enrichment (IC₅₀ 31.5 µg/mL). Two independent experimental levels extract comparison and sub-fraction comparison confirm the same structure–activity principle.

The eNOS data add a mechanistically orthogonal dimension. The 212.1% stimulation of HUVEC nitrite production by LSE at 50 µg/mL places this extract within the upper quartile of polyphenol-extract responses surveyed by Schmitt and Dirsch^[18] across multiple cell models of endothelial NO regulation. The eNOS result is strong. Quercetin at 5–20 µM upregulates eNOS protein expression approximately 1.6-fold in HUVECs^[5] and kaempferol produces a mechanistically parallel eNOS-activating effect,^[6] and the co-presence of both flavonols in LSE (confirmed at R_f 0.45 and 0.52 by HPTLC) provides a direct chemical explanation for the sustained, concentration-dependent NO stimulation observed. The strict rank-order concordance between ACE-inhibitory and NO-stimulatory endpoints across all four extracts is consistent with the dual-action mechanism described for flavonoid-rich fractions,^[19] because both activities converge on the same chemical-class window the flavonoid aglycones that dominates LSE.

The de-enrichment observed in LSE-F1 represents a critical negative outcome of the fractionation process. Although LSE-F1 predominantly contains alkaloids and

benzyl isothiocyanate, its IC₅₀ value (142.7 µg/mL) is notably higher than that of the parent LSE (89.4 µg/mL), indicating reduced activity. This finding confirms that the major constituents of LSE-F1, namely lepidine-type alkaloids and benzyl isothiocyanate, do not significantly contribute to the ACE inhibitory effect. Importantly, this de-enrichment provides mechanistic insight: as biological activity diminishes within the alkaloid-rich fraction and becomes concentrated in fractions enriched with flavonoid aglycones, the assignment of the pharmacophore becomes clear and well-defined.

Three limitations merit explicit acknowledgement. The ACE inhibition mechanism was characterized only through IC₅₀ slope factors (Hill coefficients 0.93–1.07, consistent with single-site kinetics); kinetic mode classification competitive, non-competitive or mixed requires Dixon plot or Lineweaver Burk analysis at multiple substrate concentrations, which will be undertaken for the isolated pure flavonols. HPTLC identification rests on R_f co-migration and post-derivatization colour matching, not mass spectrometric confirmation; while this is concordant with independent LC-MS analyses of *L. sativum* ethanolic extracts,^[11,12] unambiguous structural confirmation by validated HPLC-DAD or LC-ESI-MS/MS is required before LSE-F2 advances to regulated preclinical studies. Third, total supernatant nitrite was measured as the NO surrogate; iNOS induction is an unlikely confound given the absence of inflammatory stimulation and confirmed

cell viability $\geq 92\%$, but selective eNOS inhibition with L-NIO would formally establish eNOS attribution. These limitations do not compromise the principal conclusion they define its scope.

5. CONCLUSION

Systematic polarity-staged Soxhlet extraction of *Lepidium sativum* seeds, HPTLC fingerprinting with seven authenticated reference standards, and dual-target in vitro pharmacological screening establish that the ethanol extract (LSE) is the chemically richest and biologically most active fraction of the seed matrix. Bioassay-guided silica gel column chromatography concentrates ACE-inhibitory activity 2.84-fold in the flavonoid-aglycone-enriched sub-fraction LSE-F2 (IC₅₀ 31.5 $\mu\text{g/mL}$; quercetin and kaempferol dominant by HPTLC), whose ACE-inhibitory and NO-stimulatory activities are consistent with simultaneous dual-target engagement of the RAAS and endothelial NO axes. The strict rank-order concordance of both endpoints across all extracts and sub-fractions, together with the de-enrichment of the alkaloid / isothiocyanate sub-fraction LSE-F1 and the 2.84-fold enrichment of the flavonoid-aglycone sub-fraction LSE-F2, constitutes multi-level chemical-class-resolved evidence implicating quercetin and kaempferol as the dominant pharmacophores. The diagnostic HPTLC fingerprints with tentative identification of seven co-chromatographed band markers provide a reproducible analytical framework for *L. sativum* seed extract standardisation. LSE-F2 is identified as a

candidate fraction warranting in vivo pharmacological validation, and the flavonol B-ring catechol motif is identified as the structural feature common to both target engagements and deserving priority in structure-guided lead development.

Funding

None

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

All data are contained within the manuscript or available upon reasonable request.

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