

Pharmacognostical, Phytochemical, In Vitro Antioxidant And Immunomodulatory Evaluation Of Vetiveria Zizanioides (Linn.) Leaves

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ABSTRACT

Vetiveria zizanioides (VZ), a perennial aromatic grass long employed in Ayurvedic and traditional systems of medicine, was systematically investigated for its pharmacognostical, phytochemical, antioxidant, and immunomodulatory properties. Macroscopic and quantitative microscopic evaluation of the leaves established key diagnostic characters, while physicochemical parameters (loss on drying $2.89 \pm 0.22\%$ w/w, total ash $8.88 \pm 0.35\%$ w/w, acid-insoluble ash $2.17 \pm 0.33\%$ w/w, water-soluble ash $5.72 \pm 0.45\%$ w/w, and ethanol-soluble extractive $6.30 \pm 0.90\%$ w/w) confirmed the quality of the raw material. Preliminary phytochemical screening of the ethanolic extract of VZ leaves (EEVZL) revealed carbohydrates, amino acids, steroids, saponin glycosides, alkaloids, and tannins/phenolics, findings corroborated by thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) fingerprinting against quercetin, cholesterol, and amino acid reference standards. Quantitative estimation revealed a total phenolic content of 85.33 mg gallic acid equivalent/g and a total flavonoid content of 68.29 mg quercetin equivalent/g. EEVZL exhibited potent free-radical-scavenging activity in the DPPH assay ($IC_{50} = 5.39 \mu\text{g/mL}$, comparable to ascorbic acid, $IC_{50} = 5.06 \mu\text{g/mL}$) and strong ferric-reducing power ($IC_{50} = 58.9 \mu\text{g/mL}$ versus $45.07 \mu\text{g/mL}$ for ascorbic acid). In vitro immunomodulatory evaluation demonstrated concentration-dependent stimulation of neutrophil locomotion/chemotaxis and phagocytosis of *Candida albicans*, with a maximal stimulation index of $140.13 \pm 8.06\%$ relative to the pooled-serum positive control. These findings validate the traditional ethnomedicinal use of VZ leaves and identify the plant as a promising source of natural antioxidant and immune-enhancing agents. Further bioassay-guided isolation, in vivo immunological studies, and formulation development are recommended to translate these findings into a standardized therapeutic product.

Keywords: Vetiveria zizanioides; Vetiver; Pharmacognosy; Phytochemical screening; HPTLC; DPPH assay; Immunomodulatory activity; Neutrophil chemotaxis; Phagocytosis

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INTRODUCTION

Herbal medicine has become an increasingly prominent component of complementary and alternative medicine owing to its affordability, natural origin, comparatively low toxicity, and demonstrated efficacy against several difficult-to-manage conditions [1,2]. Plant-derived compounds constitute one of the largest reservoirs of immunomodulatory agents known to humankind, and a growing body of evidence indicates that supporting the body's own immune defences, rather than merely suppressing symptoms, offers a more sustainable therapeutic strategy [3]. Ayurveda, the most ancient of the Indian systems of medicine, frames disease treatment and prevention around the normalisation of

cellular activity and the correction of doshic imbalance rather than the symptomatic suppression that characterises much of modern pharmacotherapy [4,5]. Well-known examples of Ayurvedic leads that matured into modern drugs include reserpine from *Rauwolfia serpentina* and curcumin from *Curcuma longa*, underscoring the continuing translational value of traditional plant knowledge.

Roughly a quarter of pharmaceutical prescriptions dispensed in the United States contain at least one plant-derived component, and traditional or folk medicine remains the first line of primary healthcare for approximately 80% of the world's population [6,7]. India alone harbours an estimated 20,000–40,000

species of medicinal plants, of which several hundred are in regular use by traditional communities. Bioactive phytoconstituents such as alkaloids, flavonoids, tannins, terpenoids, steroids, and polysaccharides are frequently implicated in immune stimulation, and interest in isolating and characterising these compounds has intensified over the past two decades [7,8].

Immune system and immunomodulation

The vertebrate immune system comprises innate (non-specific) and adaptive (specific) arms that act in concert to recognise and eliminate foreign antigens while sparing self-tissue. Innate immunity is mediated by physical barriers, phagocytic cells (neutrophils, monocytes/macrophages), natural killer cells, and soluble factors such as complement and interferons, whereas adaptive immunity depends on the clonal expansion of antigen-specific T- and B-lymphocytes and confers immunological memory. Amino acids and their metabolic derivatives are essential substrates for antibody and cytokine synthesis, linking nutritional and phytochemical status directly to immune competence [9]. CD4⁺ helper T-cells differentiate into TH1, TH2, and TH3 subsets that secrete distinct cytokine profiles governing cell-mediated immunity, humoral immunity, and mucosal tolerance, respectively [10].

Immunomodulation refers to any process that induces, amplifies, or suppresses an aspect of the immune response, and immunomodulatory agents are broadly classed as immunoadjuvants, immunostimulants, or immunosuppressants depending on their net effect [11–13]. Numerous plant-derived immunostimulants — including polysaccharides, lectins, saponins, and phenolic compounds have been shown to enhance macrophage and neutrophil function, lymphocyte proliferation, and antibody production, providing a pharmacological rationale for the ethnomedicinal use of Rasayana (rejuvenative) plants in Ayurveda [14–16]. Herbal antioxidants in particular have attracted attention because oxidative stress and dysregulated immune signalling are mechanistically intertwined: reactive-oxygen-species scavenging by phenolics and flavonoids can indirectly normalise phagocyte function and cytokine output [17,18]. This principle has practical significance in oncology and chronic infection, where conventional immunosuppressive chemotherapeutics and radiotherapy frequently compromise host defence, and adjunct herbal immunomodulators are being explored to counteract treatment-related immunosuppression [19–22]. Polysaccharide-based immunomodulators, in particular, have shown promise as both prophylactic and therapeutic adjuvants in infectious and neoplastic disease models [22, 23].

Botanical profile of *Vetiveria zizanioides*

Vetiveria zizanioides (Linn.) Nash (synonym *Chrysopogon zizanioides* (Linn.), family Poaceae), popularly known as khas or vetiver, is a tall, tufted, aromatic perennial grass with a deep, fibrous, aromatic root system. In India it is cultivated widely across Haryana, Uttar Pradesh, Rajasthan, Gujarat, Bihar,

Odisha, Madhya Pradesh, and throughout the southern states. The leaves are long, narrow, and parallel-veined; the stem is erect and stiff; the inflorescence bears brownish-purple spikelets; and the root system is spongy, highly branched, and may extend 4–7 m into the soil. Reported phytoconstituents include khusenic acid, khusimol, sesquiterpenoids (2-vetivenene, vetiselinol, isovalencenol, vetivenic acid, vetivone, prezizaene, khusimene), alkaloids, flavonoids, tannins, phenolic acids, terpenoids, and saponins. Ethnomedicinally, vetiver has been used as an expectorant, diuretic, and antispasmodic agent and in the management of insomnia, skin disease, asthma, amenorrhoea, renal calculi, rheumatism, fever, inflammation, and anxiety, and it is credited with antioxidant, anti-gout, antifungal, anti-mycobacterial, and antimicrobial properties.

Review of previous studies on *Vetiveria zizanioides*

A substantial and growing body of literature supports the pharmacological versatility of *V. zizanioides*. Gavle et al. [24] conducted a preliminary phytochemical screening of Usheer (*V. zizanioides*) using a non-polar benzene extraction system and reported alkaloids, steroids, saponins, and reducing sugars, attributing the profile to the lipophilic selectivity of the solvent. Gunasekar et al. [25] reviewed the preclinical evidence for *Chrysopogon zizanioides*, highlighting anti-inflammatory, analgesic, antimicrobial, anticancer, and anti-diabetic activity across multiple extract types. Muthuviveganandavel et al. [26] compared aqueous and ethanolic root extracts and found the ethanolic fraction markedly more antibacterial, consistent with the polarity-dependent extraction of antimicrobial phenolics. Kumare et al. [27] and Borde et al. [28] both reviewed the antioxidant, antibacterial, antifungal, and anticancer potential of vetiver essential oil and extracts, while Dutta et al. [29] established pharmacognostic and phytochemical standards for the roots (*Usira*) to support authentication of the crude drug.

Of particular relevance to the present work, Sunitha et al. [30] demonstrated that a valencene-rich sesquiterpenoid fraction isolated from *V. zizanioides* possessed significant immunomodulatory activity, increasing antibody titres and delayed-type hypersensitivity responses in animal models — direct pharmacological precedent for the immune-stimulating potential explored here. David et al. [31] characterised vetiver essential oil obtained by a green Carbon-Dioxide-Expanded-Ethanol extraction technique, while Muthukrishnan et al. [32] and Zahoor et al. [33] documented the antioxidant and broader pharmacological profile of the plant. Savanur et al. [34] and Mishra [35] established phytochemical and physicochemical quality-control benchmarks for Ayurvedic vetiver raw material, Bhushan et al. [36] reviewed its use in inflammatory and infectious conditions, Narkhede et al. [37] confirmed antipyretic activity of hexane and methanol root extracts, and Gupta et al. [38] reported anti-mycobacterial activity of

the essential oil against drug-resistant *Mycobacterium smegmatis*. Collectively, these studies establish a strong ethnopharmacological and preliminary experimental basis for *V. zizanioides* as an antioxidant and immunomodulatory agent, but comprehensive pharmacognostic standardisation of the leaves, combined with functional In vitro immune bioassays, has remained comparatively limited — the gap that the present study addresses.

MATERIALS AND METHODS

Plant material and authentication

Fresh leaves of *Vetiveria zizanioides* (Linn.) Nash were collected from local cultivated stands in Chennai, Tamil Nadu, India. The plant material was identified and authenticated by a qualified botanical taxonomist. Voucher material was retained for reference. Collected leaves were washed thoroughly, shade-dried at room temperature for five days, coarsely powdered, passed through a No. 50 mesh sieve, and stored in tightly sealed sterile glass containers protected from light and moisture until use.

Pharmacognostical studies

Macroscopy: Fresh leaves were evaluated for colour, odour, taste, shape, margin, apex, base, and venation according to standard pharmacognostic protocol [39,40].

Quantitative microscopy: Vein-islet number, vein-termination number, stomatal number, stomatal index, and palisade ratio were determined on cleared leaf preparations mounted in chloral hydrate, using a stage micrometer and camera lucida at standard magnification. Stomatal index was calculated as $S/(S+E) \times 100$, where S is the number of stomata and E the number of epidermal cells per unit area.

Powder microscopy: Powdered leaf material was mounted in phloroglucinol-concentrated HCl and glycerol and examined under bright-field illumination using a trinocular microscope fitted with a digital camera; diagnostic cellular elements were photo-documented.

Physicochemical parameters: Loss on drying, total ash, acid-insoluble ash, water-soluble ash, and ethanol-soluble extractive values were determined in accordance with World Health Organization guidelines for the quality control of medicinal plant materials [40]. Loss on drying was calculated from the weight loss of a 2 g air-dried sample dried at 100–105°C to constant weight. Total ash was determined by incineration of powdered drug to a carbon-free residue; the insoluble residue obtained after boiling total ash in dilute hydrochloric acid gave the acid-insoluble ash, and the difference between total ash and the residue remaining after boiling in water gave the water-soluble ash. Ethanol-soluble extractive was determined by cold maceration of 5 g powdered drug in 90% ethanol with periodic shaking, followed by filtration, evaporation, and gravimetric estimation.

Extraction of plant material

Dried, powdered leaf material (50 g) was extracted with ethanol (solvent-to-drug ratio 6:1) by hot continuous (Soxhlet) extraction for approximately 40–50 cycles at 45–50°C to yield the ethanolic extract of *Vetiveria zizanioides* leaves (EEVZL), which was concentrated and stored at 4°C protected from light until further use.

Qualitative phytochemical screening

EEVZL was subjected to standard qualitative tests [41,42] for carbohydrates (Fehling's, Tollen's/pentose, phloroglucinol/hexose, and cobalt-chloride tests), proteins and amino acids (Biuret, Xanthoproteic, Ninhydrin, and cysteine tests), steroids (Salkowski and Liebermann–Burchard reactions), glycosides (Legal's and Keller–Killiani tests for cardiac glycosides; foam test for saponin glycosides), alkaloids (Dragendorff's, Wagner's, Mayer's, Murexide, and tannic acid tests), flavonoids (Shinoda test, sulphuric acid test, lead acetate and zinc–HCl tests), tannins/phenolics (ferric chloride, lead acetate, potassium dichromate, iodine, and silver nitrate tests), and triterpenes.

Chromatographic studies (TLC and HPTLC)

Thin-layer chromatography was performed on precoated silica gel 60 F254 aluminium sheets (10 × 10 cm, Merck) using dioxane:ammonia (18:2, v/v) as the mobile phase. Spots were visualised under short-wave (254 nm) and long-wave (366 nm) UV light before and after derivatisation with anisaldehyde–sulphuric acid reagent, and retardation factor (R_f) values were calculated as the ratio of the distance travelled by the solute to that travelled by the solvent front [43]. High-performance TLC was carried out on a CAMAG system (Linomat 5 applicator, TLC Scanner 4, winCATS software v.1.4.7) using silica gel 60 F254 plates (11 × 10 cm); flavonoids were resolved with cyclohexane:ethyl acetate:formic acid (6:4.5:0.2, v/v/v) against quercetin and kaempferol standards, steroids with toluene:methanol (8:1, v/v) against cholesterol using 10% methanolic sulphuric acid derivatisation, and amino acids with n-butanol:acetic acid:water (6.5:3.5:2, v/v/v) against glutamic acid, tyrosine, leucine, valine, isoleucine, and threonine using 0.3% ninhydrin reagent.

Quantitative estimation of phytoconstituents

Total phenolic content (TPC) was determined spectrophotometrically by the Folin–Ciocalteu method [44] using gallic acid (10–50 µg/mL) as the reference standard; absorbance was recorded at 760 nm on a UV-visible spectrophotometer (Shimadzu, model 1800) and TPC expressed as mg gallic acid equivalent (GAE)/g extract. Total flavonoid content (TFC) was determined by the aluminium chloride colorimetric method [45] using quercetin (10–50 µg/mL) as the reference standard, with absorbance recorded at 510 nm, and expressed as mg quercetin equivalent (QE)/g extract.

In vitro antioxidant assays

DPPH radical-scavenging assay: The scavenging activity of EEVZL against the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was assessed spectrophotometrically at 517 nm against ascorbic acid as reference standard [46], with percentage radical-

scavenging activity calculated as $[(\text{Abs control} - \text{Abs test})/\text{Abs control}] \times 100$ and IC₅₀ values derived from the concentration–response curve.

Reducing-power assay: Ferric-reducing power was determined by monitoring the $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ Prussian-blue complex formed at 700 nm following incubation of the extract with potassium ferricyanide and trichloroacetic acid [47], with ascorbic acid as reference standard and IC₅₀ defined as the extract concentration producing an absorbance of 0.5.

In vitro immunomodulatory assays

Assays were performed on venous blood obtained, after informed consent, from 20 healthy volunteers. Neutrophils were isolated by sequential dextran sedimentation, hypotonic lysis, and Ficoll density-gradient centrifugation and adjusted to 10^6 cells/mL.

Neutrophil locomotion and chemotaxis assay: A modified Boyden micro-chamber technique was used, in which neutrophil suspension was placed in the upper chamber and EEVZL (5, 20, 40, 80, and 100 $\mu\text{g/mL}$) or casein (100 $\mu\text{g/mL}$, positive control) in the lower chamber, separated by a Millipore filter [48]. After 3 h incubation at 37°C, filters were fixed, stained with haematoxylin, and the number of migrated neutrophils per field was counted microscopically (400 $\times$).

Table: 1 Macroscopical characters of *Vetiveria zizanioides* leaves

S. No.	Character	Observation
1	Colour	Green to greyish-green
2	Odour	Characteristic aromatic odour
3	Taste	Slightly bitter
4	Surface	Rough, glabrous, parallel venation



Table: 2 Quantitative microscopy of *Vetiveria zizanioides* leaf

Phagocytosis assay: Polymorphonuclear leucocytes adherent to grease-free slides were incubated with EEVZL (5–100 $\mu\text{g/mL}$) and a standardised suspension of *Candida albicans* (MTCC-227; 1×10^6 cells/mL) for 60 min at 37°C [49]. Slides were stained with dilute Giemsa, and phagocytic index (number of neutrophils phagocytosing *Candida* per 100 neutrophils counted) was determined by oil-immersion microscopy (1000 $\times$) and compared with pooled-serum positive control. Percentage stimulation was calculated as $[(\text{PI}_{\text{test}} - \text{PI}_{\text{control}})/\text{PI}_{\text{control}}] \times 100$.

Statistical analysis

All experiments were performed in triplicate unless otherwise specified, and results are expressed as mean $\pm$ standard error (S.E.) or mean $\pm$ standard deviation (S.D.) as indicated. Group comparisons were performed by one-way ANOVA followed by Tukey's multiple-comparison post-test using GraphPad Prism version 5.00 [50]; a value of $P < 0.001$ was taken as statistically significant.

RESULTS

Pharmacognostical studies

Macroscopic characters

The macroscopic characters of *V. zizanioides* leaves are summarised in Table 1.

5	Shape	Linear-lanceolate
6	Length	30–100 cm
7	Width	0.5–1.5 cm
8	Apex	Acute
9	Base	Sheathing, overlapping
10	Margin	Entire, scabrid
11	Petiole	Sessile (absent)
12	Lateral nerves	Parallel, prominent



Figure: 1 *Vetiveria zizanioides* Linn whole plant showing the aerial shoot and fibrous root system

Quantitative microscopy

Parameter	Upper (adaxial)	Lower (abaxial)
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	epidermis	epidermis
Epidermal number (per mm ²)	520–560	580–600
Stomatal number (per mm ²)	190–210	480–500

The adaxial and abaxial epidermal cell counts (520–560 and 580–600 per mm², respectively) and the markedly higher abaxial stomatal index (44–45 versus 25–27) reflect the typical amphistomatic, xeromorphic architecture of Poaceae leaves adapted to high transpirational demand.

Powder microscopy

Powder microscopy of the leaves revealed multicellular trichomes, sclerenchymatous fibres, parenchyma cells, dumbbell-shaped stomata, and reticulate vessels, consistent with the diagnostic anatomy of Poaceae foliage (Figure 2).

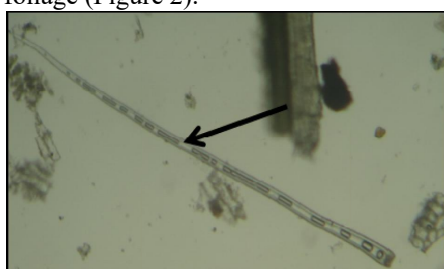


Table: 3 Physicochemical characters of *V. zizanioides* leaf powder

S. No.	Parameter	Result (% w/w)
1	Loss on drying	2.89 ± 0.22

The low loss-on-drying value (2.89 ± 0.22%) indicates a low moisture burden favouring microbial stability and shelf-life, while the low acid-insoluble ash (2.17 ± 0.33%) indicates minimal soil/sand contamination. The moderate ethanol-soluble extractive (6.30 ± 0.90%) is consistent with the presence of alkaloids, glycosides, and other ethanol-soluble phytoconstituents identified on qualitative screening.

Chemical class	Representative tests	Inference
Carbohydrates – reducing sugars	Fehling's, pentose, hexose, cobalt-chloride	+ve
Carbohydrates – non-reducing sugars	Fehling's (test solution)	–ve
Non-reducing polysaccharides	Iodine test / tannic acid test	–ve / +ve
Proteins	Biuret, Xanthoproteic, sulphur-precipitation	–ve (trace +ve with lead acetate)
Amino acids	Ninhydrin, cysteine	+ve
Steroids	Salkowski,	+ve

Stomatal index	25–27	44–45
Palisade ratio	10–12	—
Vein islet number	10	—
Vein termination number	41	—

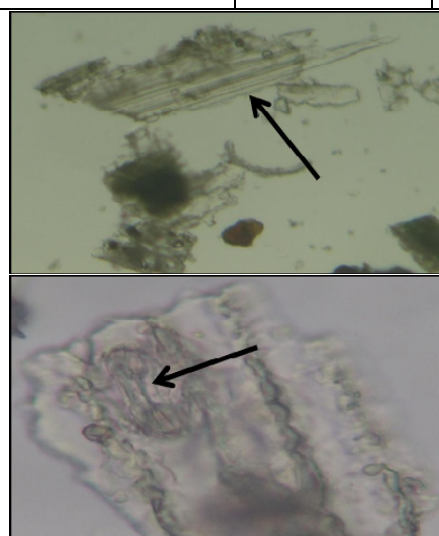


Figure: 2 Powder-microscopic diagnostic elements of *V. zizanioides* leaves: (a) multicellular trichome, (b) sclerenchymatous fibre, (c) parenchyma cells with dumbbell stomata

Physicochemical parameters

2	Total ash value	8.88 ± 0.35
3	Acid-insoluble ash	2.17 ± 0.33
4	Water-soluble ash	5.72 ± 0.45
5	Ethanol-soluble extractive	6.30 ± 0.90

Phytochemical studies

Qualitative phytochemical screening

	Liebermann–Burchard	
Cardiac glycosides	Legal's, Keller–Killiani	–ve
Saponin glycosides	Foam test	+ve
Alkaloids	Dragendorff's, Wagner's, Murexide, Mayer's, tannic acid	Mixed (+ve: Dragendorff, tannic acid, Mayer's)
Flavonoids	Shinoda, sulphuric acid, lead acetate, zinc–HCl, NaOH/acid	Mixed (+ve: lead acetate, NaOH/acid)
Tannins and phenolics	FeCl ₃ , lead acetate, K ₂ Cr ₂ O ₇ , iodine, K ₃ Fe(CN) ₆ , AgNO ₃	Mixed (+ve: FeCl ₃ , lead acetate)

Triterpenes	Chloroform-H ₂ SO ₄	-ve
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The screening profile confirms a chemically diverse extract dominated by reducing sugars, amino acids, steroids, saponin glycosides, and select alkaloid, Table: 5 TLC analysis of EEVZL (solvent system: dioxane-ammonia, 18:2 v/v)

Spot	Rf (254 nm, pre-derivatisation)	Rf (366 nm, pre-derivatisation)	Rf (visible light, post-derivatisation)
1	0.15	0.01	0.02
2	0.42	0.12	0.07
3	0.46	0.42	0.15

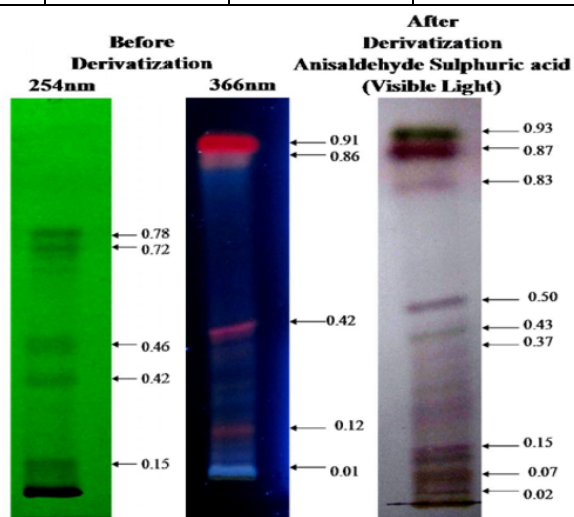
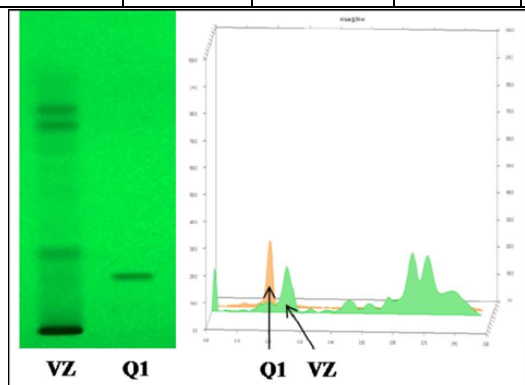


Table: 6 HPTLC co-chromatography of EEVZL against reference standards

Compound class	Standard used	Derivatizing agent	Rf (standard)	Rf (VZ extract)
Flavonoid	Quercetin	—	0.18	0.18
Steroid	Cholesterol	10% methanolic	0.35	0.34



flavonoid, and phenolic sub-classes, while cardiac glycosides and triterpenes were not detected.

Chromatographic fingerprinting

4	0.72	0.86	0.37
5	0.78	0.91	0.43
6	—	—	0.50
7	—	—	0.83
8	—	—	0.87
9	—	—	0.93

Figure: 3 TLC chromatoplate of EEVZL under 254 nm and 366 nm UV (before derivatisation) and under visible light after derivatisation with anisaldehyde-sulphuric acid reagent

Nine distinct Rf bands (0.02–0.93) were resolved after derivatisation with anisaldehyde-sulphuric acid, four more than were visible under UV alone, indicating that several secondary metabolites present in EEVZL require chemical visualisation and are not intrinsically UV-active or fluorescent.

		c H ₂ SO ₄		
Amino acid	Leucine	0.3% ninhydrin	0.56	0.56
Amino acid	Valine	0.3% ninhydrin	0.48	0.49
Amino acid	Threonine	0.3% ninhydrin	0.36	0.36

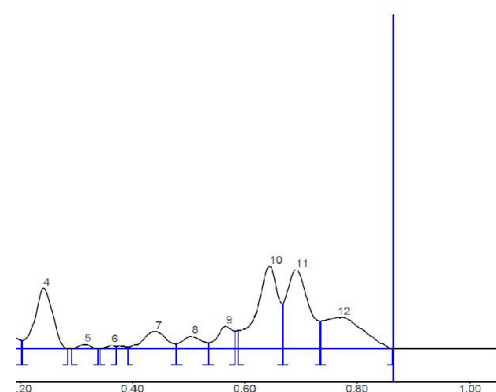


Figure: 4 HPTLC chromatogram/plate (left) and densitogram (right) of EEVZL confirming the presence

of quercetin (VZ = plant sample; Q1 = quercetin standard)

Table: 7 HPTLC profile of EEVZL for flavonoids

Peak	Start Rf	Max Rf	Max height	Max %	End Rf	Area	Area %
1	0.01	0.01	156.8	14.52	0.02	1147.2	3.57
2	0.12	0.16	27.4	2.54	0.16	488.6	1.52
3	0.17	0.18	36.6	3.39	0.20	881.5	2.74
4	0.20	0.24	165.3	15.30	0.29	4594.1	14.29
5	0.29	0.32	12.8	1.19	0.34	228.3	0.71

Twelve resolved peaks (Rf 0.01–0.86) were obtained, dominated by two major flavonoid components at Rf 0.65 and 0.69 that together accounted for approximately

Table: 8 HPTLC profile of EEVZL for steroids

Peak	Start Rf	Max Rf	Max height	Max %	End Rf	Area	Area %
1	–0.04	0.01	340.3	70.65	0.05	6385.7	62.0
2	0.05	0.06	41.9	8.71	0.07	584.1	5.67

Table: 9 HPTLC profile of EEVZL for amino acids

Peak	Start Rf	Max Rf	Max height	Max %	End Rf	Area	Area %
1	0.13	0.36	413.2	36.24	0.40	30109.6	49.05
2	0.40	0.43	165.9	14.55	0.46	6302.2	10.2

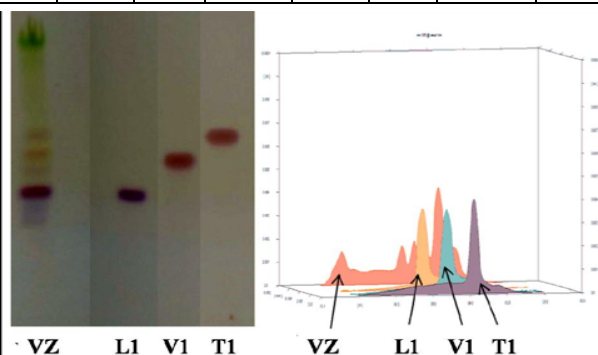


Table: 10 Total phenolic content of EEVZL by the Folin–Ciocalteu method (calibration equation $y = 0.0012x + 0.0008$, $R^2 = 0.9911$; TPC = 85.33 mg GAE/g extract)

Concentration (μg/mL)	Gallic acid – Abs ₇₆₀ (mean ± SEM)	EEVZL – Abs ₇₆₀ (mean ± SEM)
10	0.015 ± 0.0086	0.011 ± 0.0063
20	0.021 ± 0.0121	0.022 ± 0.0121
30	0.038 ± 0.0219	0.031 ± 0.0178
40	0.048 ± 0.0277	0.043 ± 0.0245
50	0.059 ± 0.0345	0.052 ± 0.0311

6	0.34	0.36	10.6	0.98	0.37	129.3	0.40
7	0.39	0.44	47.8	4.43	0.48	1731.2	5.39
8	0.48	0.51	34.1	3.16	0.54	1059.7	3.30
9	0.54	0.57	61.7	5.72	0.59	1529.2	4.76
10	0.59	0.65	225.2	20.85	0.67	7558.6	23.52
11	0.67	0.69	215.4	19.95	0.74	7555.5	23.51
12	0.74	0.77	86.2	7.98	0.86	5239.3	16.30

47% of the total peak area, indicating a flavonoid-rich, multi-component profile suitable for chemical fingerprinting and quality control.

3	0.07	0.08	22.5	4.68	0.13	474.9	4.61
4	0.30	0.35	27.3	5.67	0.39	770.5	7.48
5	0.49	0.53	26.4	5.48	0.58	929.3	9.02
6	0.61	0.67	23.2	4.81	0.72	1155.5	11.22
3	0.4	0.49	183.5	16.09	0.52	6605.5	10.76
4	0.5	0.56	164.4	14.41	0.61	7225.5	11.77
5	0.7	0.82	67.8	5.94	0.83	2466.8	4.02
6	0.8	0.88	145.5	12.76	0.9	8680.3	14.14

Figure: 5 HPTLC chromatogram and densitogram of EEVZL showing amino acid bands (VZ = plant sample; L1 = leucine; V1 = valine; T1 = threonine)

The amino-acid HPTLC profile was dominated by a single major band at Rf 0.36, contributing nearly half (49.05%) of the total peak area, consistent with a predominant free amino acid together with several minor co-migrating constituents — a fingerprint of value for standardisation of amino-acid-bearing herbal preparations.

Quantitative estimation of total phenolic and flavonoid content

10	0.015 ± 0.0086	0.011 ± 0.0063
20	0.021 ± 0.0121	0.022 ± 0.0121
30	0.038 ± 0.0219	0.031 ± 0.0178
40	0.048 ± 0.0277	0.043 ± 0.0245
50	0.059 ± 0.0345	0.052 ± 0.0311

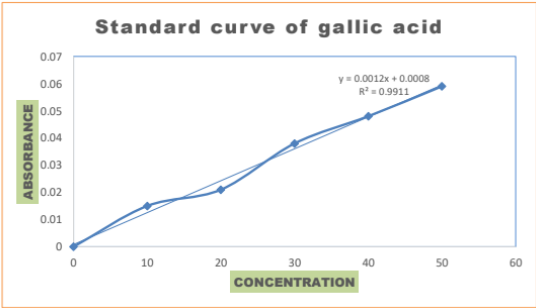


Figure: 6 Standard calibration curve of gallic acid for total phenolic content estimation

Table: 11 Total flavonoid content of EEVZL by the aluminium chloride method (calibration equation $y = 0.0055x + 0.0082$, $R^2 = 0.9901$; TFC = 68.29 mg QE/g extract)

Concentration (µg/mL)	Quercetin – Abs ₅₁₀ (mean ± SEM)	EEVZL – Abs ₅₁₀ (mean ± SEM)
10	0.061 ± 0.035	0.042 ± 0.024
20	0.136 ± 0.078	0.097 ± 0.056
30	0.167 ± 0.095	0.114 ± 0.065
40	0.231 ± 0.133	0.167 ± 0.095
50	0.275 ± 0.158	0.196 ± 0.112

	SEM)	SEM)
10	0.061 ± 0.035	0.042 ± 0.024
20	0.136 ± 0.078	0.097 ± 0.056
30	0.167 ± 0.095	0.114 ± 0.065
40	0.231 ± 0.133	0.167 ± 0.095
50	0.275 ± 0.158	0.196 ± 0.112

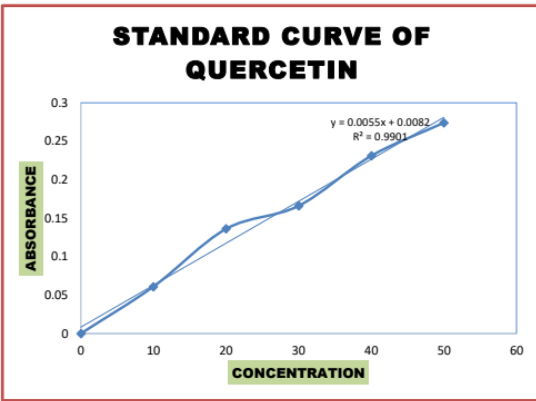


Figure: 7 Standard calibration curve of quercetin for total flavonoid content estimation

In vitro antioxidant activity

Table 12 DPPH radical-scavenging activity of EEVZL compared with ascorbic acid (IC₅₀: EEVZL = 5.39 µg/mL; ascorbic acid = 5.06 µg/mL)

Concentration (µg/mL)	VZ extract – % inhibition (mean ± SEM)	Ascorbic acid – % inhibition (mean ± SEM)
2	14.82 ± 1.24	21.1 ± 2.8
4	31.65 ± 2.11	38.5 ± 2.0
6	49.37 ± 1.95	65.6 ± 3.17
8	67.21 ± 2.36	76.2 ± 3.55
10	83.94 ± 1.68	91.1 ± 3.86

	± SD)	(mean ± SD)
2	14.82 ± 1.24	21.1 ± 2.8
4	31.65 ± 2.11	38.5 ± 2.0
6	49.37 ± 1.95	65.6 ± 3.17
8	67.21 ± 2.36	76.2 ± 3.55
10	83.94 ± 1.68	91.1 ± 3.86

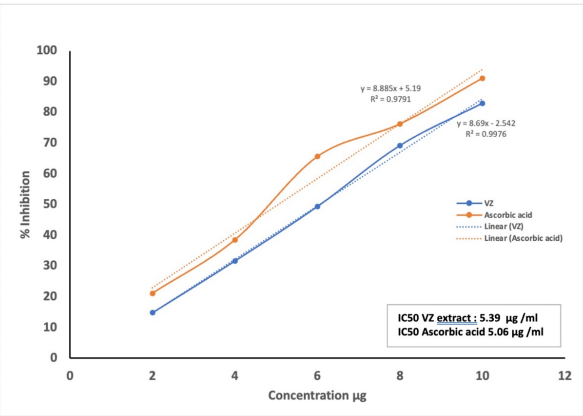


Figure: 8 Concentration-dependent DPPH radical-scavenging activity of EEVZL versus ascorbic acid
EEVZL produced a concentration-dependent increase in DPPH radical-scavenging activity with an IC₅₀ of 5.39

µg/mL, closely approaching the potency of ascorbic acid (IC₅₀ = 5.06 µg/mL), indicating strong intrinsic free-radical-scavenging capacity.

Table: 13 Reducing-power assay of EEVZL compared with ascorbic acid (IC50: EEVZL = 58.9 μ g/mL; ascorbic acid = 45.07 μ g/mL)

Concentration (μ g/mL)	VZ extract – % reducing power (mean $\pm$ SD)	Ascorbic acid – % reducing power (mean $\pm$ SD)
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10	11.5 $\pm$ 3.2	20.8 $\pm$ 1.2
20	25.7 $\pm$ 4.8	33.9 $\pm$ 1.6
40	46.8 $\pm$ 5.4	54.1 $\pm$ 1.7
80	58.4 $\pm$ 5.9	70.5 $\pm$ 1.8
100	78.2 $\pm$ 5.7	87.6 $\pm$ 1.9

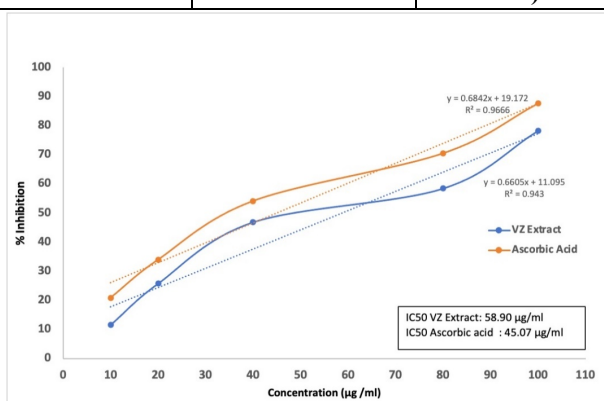


Figure: 9 Concentration-dependent ferric-reducing power of EEVZL versus ascorbic acid
In vitro immunomodulatory studies
Neutrophil locomotion and chemotaxis
Effect of VZ Extracts on Migration Activity of Neutrophils

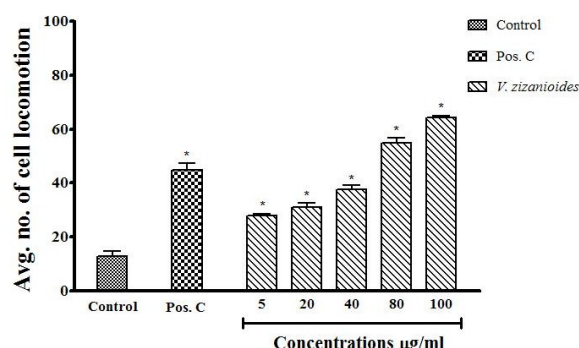


Figure: 10 Migration activity (mean number of neutrophils/field $\pm$ S.E.) of EEVZL versus negative control (PBS) and positive control (casein, 100 μ g/mL); one-way ANOVA with Tukey's post-test, * $P < 0.001$
Neutrophil migration increased in a concentration-dependent manner from 27.81 at 5 μ g/mL to 65.25 at 100 μ g/mL, compared with 12.90 for the PBS negative control and 44.25 for the 100 μ g/mL casein positive control. All extract concentrations produced a statistically significant increase in chemotactic migration relative to the negative control ($P < 0.001$), and the highest concentration exceeded the standard chemoattractant casein.

Neutrophil activation and phagocytosis

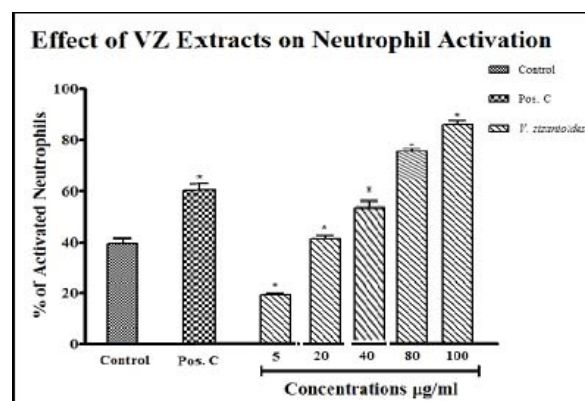


Figure: 11 Percentage of activated neutrophils following exposure to EEVZL relative to negative control (PBS) and positive control (pooled serum); * $P < 0.001$

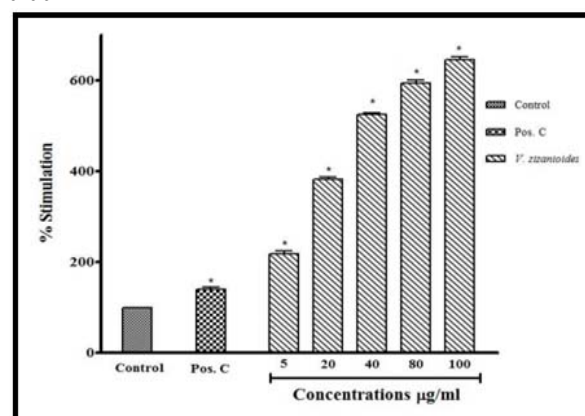


Figure: 12 Percentage stimulation of phagocytic index of EEVZL relative to negative control (PBS) and positive control (pooled serum); * $P < 0.001$

EEVZL produced a significant, concentration-dependent increase in both the percentage of activated neutrophils and the phagocytic stimulation index against *Candida albicans* ($P < 0.001$ at all tested concentrations versus control). The maximal stimulation index recorded was $140.13 \pm 8.06\%$ at the highest concentration tested, indicating pronounced enhancement of innate phagocytic function by EEVZL.

DISCUSSION

The macroscopic and quantitative microscopic characters established in this study — amphistomatic epidermis with a markedly higher abaxial stomatal index, moderate palisade ratio, and defined vein-islet/vein-termination numbers — provide reproducible diagnostic parameters for authenticating *V. zizanioides* leaves and distinguishing them from adulterant grasses,

complementing the pharmacognostic and phytochemical benchmarks previously reported for the roots of the species [29,35]. The low loss-on-drying and acid-insoluble-ash values obtained here fall within the ranges generally considered acceptable for pharmaceutical raw material under WHO guidance [40], supporting the suitability of leaf material collected from cultivated stands for standardised extract preparation.

Qualitative screening of EEVZL detected carbohydrates, amino acids, steroids, saponin glycosides, and select alkaloid, flavonoid, and tannin/phenolic sub-fractions, broadly mirroring the phytochemical classes reported for benzene and aqueous/ethanolic extracts of *V. zizanioides* in earlier work [24,26], while the absence of cardiac glycosides and triterpenes in the present leaf extract contrasts with reports of triterpenoid saponins in the root, suggesting organ-specific metabolite partitioning within the plant. The TLC and HPTLC fingerprints generated here — nine resolved bands after anisaldehyde sulphuric acid derivatisation and twelve flavonoid, six steroid, and six amino-acid HPTLC peaks extend the chromatographic characterisation reported for vetiver essential oil and root extracts [27,31,32] to the leaf, and the close R_f concordance between the extract and quercetin, cholesterol, leucine, valine, and threonine reference standards provides direct chemical confirmation of the phytoconstituent classes inferred from colour-reaction screening.

The total phenolic (85.33 mg GAE/g) and flavonoid (68.29 mg QE/g) contents recorded for EEVZL are appreciable and compare favourably with those reported for other antioxidant-rich medicinal grasses and herbs evaluated by the same Folin–Ciocalteu and aluminium-chloride methods [2,54], reinforcing the proposition that phenolic and flavonoid density is a reliable predictor of DPPH-scavenging potency across plant species. Indeed, the DPPH IC₅₀ obtained for EEVZL (5.39 µg/mL) was very close to that of ascorbic acid (5.06 µg/mL), and the reducing-power IC₅₀ (58.9 µg/mL versus 45.07 µg/mL for ascorbic acid) followed the same pattern, consistent with earlier reports that identified strong antioxidant activity in *V. zizanioides* extracts [32,33] and with the broader principle that phenolic hydroxyl groups act as efficient hydrogen donors and electron donors, simultaneously quenching free radicals and reducing Fe³⁺ to Fe²⁺ [17].

The immunomodulatory findings of the present study add a functional dimension that has been comparatively under-explored for the leaves of this species. Neutrophil chemotaxis and phagocytosis are archetypal innate-immune read-outs, and the concentration-dependent stimulation observed here is consistent with the general principle that saponins act as opsonising/antigen-sensitising agents that promote antigen–antibody complex binding to phagocyte Fc receptors, thereby enhancing phagocytic uptake [22]. The stimulation index recorded for EEVZL (up to 140.13 ± 8.06%) is

comparable in magnitude to the phagocytic and chemotactic enhancement reported for other saponin- and polysaccharide-rich botanicals evaluated by similar Boyden-chamber and Candida-phagocytosis protocols, including *Ricinus communis* [51], *Capparis zeylanica* [55], and *Trapa bispinosa* [56], and is consistent with the macrophage/neutrophil-activation mechanism proposed for immunomodulatory herbs more broadly [52,53]. These findings converge with, and extend, the single most directly relevant precedent in the literature: Sunitha et al. [30] demonstrated that a valencene-rich sesquiterpenoid fraction of *V. zizanioides* increased antibody titres and delayed-type hypersensitivity responses in vivo, indicating that both the sesquiterpenoid and the phenolic/flavonoid/saponin fractions of this plant may contribute, potentially synergistically, to its overall immunomodulatory profile.

Mechanistically, the amino acids identified by HPTLC (leucine, valine, threonine) are recognised substrates for lymphocyte protein synthesis and cytokine production [9], while the steroidal constituents detected may influence the balance between pro- and anti-inflammatory signalling relevant to both humoral and cell-mediated immunity [10]. Toll-like receptors and related pattern-recognition receptors on the neutrophil membrane are plausible molecular targets through which saponin- and flavonoid-rich extracts of this kind exert their chemotactic and phagocytic effects, a mechanism proposed for structurally comparable immunostimulatory botanicals [16,57,58]. Taken together, the present pharmacognostic, phytochemical, and functional bioassay data provide converging lines of evidence that support the traditional Ayurvedic use of *V. zizanioides* as a Rasayana (immune-rejuvenating) botanical, while also identifying specific chemical classes — phenolics, flavonoids, and saponin glycosides — as plausible mediators of its combined antioxidant and immunomodulatory activity.

The principal limitation of the present work is that the immunomodulatory assays were restricted to In vitro human-neutrophil read-outs; cytokine profiling, lymphocyte proliferation assays, and in vivo confirmation (e.g., delayed-type hypersensitivity or antibody-titre studies analogous to those performed for valencene [30]) were outside the present scope but are a logical next step. Bioassay-guided fractionation will also be required to attribute the observed activity to specific phenolic, flavonoid, or saponin sub-fractions rather than the crude extract as a whole.

Conclusion

This integrated pharmacognostical, phytochemical, and In vitro pharmacological investigation establishes reproducible macroscopic, microscopic, and physicochemical standards for the leaves of *Vetiveria zizanioides* and confirms a chemically rich profile of phenolics, flavonoids, steroids, saponin glycosides, and amino acids by qualitative screening and TLC/HPTLC fingerprinting. The ethanolic leaf extract (EEVZL)

demonstrated potent, concentration-dependent DPPH radical-scavenging and ferric-reducing antioxidant activity approaching that of ascorbic acid, together with significant stimulation of neutrophil chemotaxis and phagocytosis. In vitro, supporting a genuine immunomodulatory potential that complements its documented antioxidant capacity. These findings lend scientific credence to the traditional ethnomedicinal use of vetiver leaves and identify *V. zizanioides* as a promising candidate for development into a standardised natural antioxidant and immune-supportive preparation. Realising this potential will require bioassay-guided isolation of the active constituents, in vivo immunological validation, formulation development, and rigorous preclinical and clinical safety and efficacy testing before therapeutic translation can be considered.

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