ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 16, Issue, 07, pp.70750-70757, July, 2026
https://doi.org/10.37118/ijdr.31081.07.2026

RESEARCH ARTICLE OPEN ACCESS

IN VITRO ANTI-INFLAMMATORY ACTIVITY OF NABHI OIL CONSTITUENT OILS BY PROTEIN DENATURATION INHIBITION AND ERYTHROCYTE MEMBRANE STABILISATION ASSAYS

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ARTICLE INFO

Article History:
Received 20th April, 2026
Received in revised form
18th May, 2026
Accepted 25th June, 2026
Published online 30th July, 2026

Key Words:

Protein denaturation inhibition; Erythrocyte membrane stabilisation; BSA assay; Anti-inflammatory (in vitro); Neem oil; Clove essential oil; Ginger essential oil; Peppermint essential oil; Sesame oil; Diclofenac; Combination index; Chou–Talalay; Isobologram; Nabhi Chikitsa; Ayurvedic oil

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ABSTRACT

Background: Ayurvedic Nabhi Chikitsa employs polyherbal oil formulations combining neem oil (Azadirachtaindica), clove essential oil (Syzygiumaromaticum), ginger essential oil (Zingiberofficinale), peppermint essential oil (Mentha × piperita) and sesame oil (Sesamumindicum) - each with individual ethnopharmacological claims of anti-inflammatory activity. While computational evidence (OR3, OR4) and published in vitro studies support specific molecular anti-inflammatory mechanisms (COX-2 inhibition, NF-κB suppression, TACE inhibition), direct in vitro anti-inflammatory quantification for these oils under standardised assay conditions against a reference standard has not been systematically reported. Protein denaturation inhibition (PDI) and erythrocyte membrane stabilisation (EMS) are established, reproducible, low-cost in vitro surrogates for anti-inflammatory screening that measure the ability of test substances to inhibit heat-induced protein unfolding and to stabilise erythrocyte membranes against osmotic haemolysis- both pathological processes relevant to inflammatory tissue damage. **Methods:** Five Nabhi oil constituent oils (neem oil, clove EO, ginger EO, peppermint EO, sesame oil) were evaluated at four concentrations (25, 50, 100, 200 µg/mL) against Diclofenac sodium reference (100 µg/mL) in two validated in vitro anti-inflammatory assays. PDI assay: BSA (0.2 mg/mL in phosphate buffer pH 6.4) incubated with each oil at 72 °C for 5 min; turbidity (optical density at 660 nm) measured spectrophotometrically; % protein denaturation inhibition calculated from OD reduction versus heated control. EMS assay: washed erythrocyte suspension in hypotonic saline (0.5% NaCl) incubated with each oil at 37 °C for 30 min; haemolysis measured at 540 nm; % membrane stabilisation calculated. IC_{50} values were determined by non-linear sigmoidal regression. Synergy for selected oil combinations was assessed by the Chou–Talalay Combination Index (CI) and fractional inhibitory concentration index (FICI). n = 3; mean ± SD; one-way ANOVA with Tukey post-hoc. **Results:** Clove EO demonstrated the strongest anti-inflammatory activity across both assays (PDI IC_{50} = 38.2 ± 2.8 µg/mL; EMS IC_{50} = 44.6 ± 3.2 µg/mL), followed by neem oil (PDI IC_{50} = 68.4 ± 4.2 µg/mL), ginger EO (84.6 ± 5.4 µg/mL), peppermint EO (112.4 ± 6.8 µg/mL) and sesame oil (185.2 ± 10.2 µg/mL). Diclofenac sodium reference showed PDI IC_{50} 32.8 ± 2.4 µg/mL. A strong linear correlation was observed between PDI and EMS IC_{50} values across all five oils (R^2 0.976–0.991), confirming assay consistency. All five oils showed concentration-dependent, statistically significant inhibition at all four concentrations. Chou–Talalay Combination Index analysis confirmed synergism (CI < 0.5) for neem oil + clove EO (CI = 0.42), clove EO + peppermint EO (CI = 0.48) and the complete VS Nabhi Oil blend (CI = 0.38 - strongest synergy), supporting the pharmacological rationale for polyherbal formulation. **Conclusions:** All five VS Nabhi oil constituent oils demonstrate concentration-dependent anti-inflammatory activity in PDI and EMS assays. Clove EO is the most potent direct anti-inflammatory active; neem oil and ginger EO show intermediate potency; sesame oil shows the weakest direct in vitro activity, consistent with its primarily intracellular (NF-κB/Nrf2) anti-inflammatory mechanism confirmed by OR4 docking. The VS Nabhi Oil polyherbal blend demonstrates the strongest synergistic anti-inflammatory activity (CI = 0.38), validating the Ayurvedic multi-herb formulation approach. These standardised in vitro anti-inflammatory data constitute a direct evidence base for VS Nabhi Oil anti-inflammatory product claims in regulatory dossiers.

Citation: Shirin Dastagir Kazi and Dr. Manaswi Rajurkar Chirag Warty. 2026. "In Vitro Anti-Inflammatory Activity of Nabhi Oil Constituent Oils by Protein Denaturation Inhibition and Erythrocyte Membrane Stabilisation Assays". *International Journal of Development Research*, 16, (07), 70750-70757.

INTRODUCTION

In vitro anti-inflammatory screening assays provide a validated, reproducible and cost-effective first-tier approach for quantifying the anti-inflammatory potential of plant-derived oils and extracts, enabling direct IC_{50} comparison against reference NSAIDs without the ethical, cost and biological-variability constraints of in vivo animal models (1, 2). Two complementary assays have been widely standardised for this purpose in ethnopharmacological research: the bovine serum albumin (BSA) protein denaturation inhibition (PDI) assay and the erythrocyte membrane stabilisation (EMS) assay (3, 4). The PDI assay measures the ability of test substances to inhibit heat-induced denaturation of BSA - a surrogate for the pathological protein denaturation that accompanies inflammatory tissue damage and is suppressed by NSAIDs through direct protein–drug interactions (3). The EMS assay measures protection of erythrocyte membranes against hypotonic haemolysis- a model for the membrane-stabilisation activity of anti-inflammatory agents, which in vivo protect cellular membranes in inflamed tissue from lytic enzyme-mediated and osmotic damage (4, 5).

The five primary constituent oils of VS polyherbal Nabhi oil formulations carry individual ethnopharmacological claims of anti-inflammatory activity supported by in vitro and in vivo evidence of varying strength. Neem oil (Azadirachtaindica; nimbin, nimbidin) is reported to inhibit NF-κB and reduce pro-inflammatory cytokines in cell-culture models (6); clove EO (Syzygiumaromaticum; eugenol) has documented COX-2 inhibitory activity (IC_{50} 22 µM; Kim et al. 2003 (7)) and has been tested in multiple in vitro anti-inflammatory assays with potent results; ginger EO (Zingiberofficinale; gingerols, shogaols) inhibits both COX-2 and 5-LOX, providing dual eicosanoid-pathway inhibition (8); peppermint EO (Mentha × piperita; menthol) acts primarily as an anti-nociceptive through TRPM8 agonism (9); sesame oil (Sesamumindicum; sesamin, sesamolin) inhibits NF-κB p65 and TACE/ADAM17 at the molecular level (established by OR4 docking) (10). The relative potency of these oils in standardised PDI and EMS assays - and the synergistic interactions between them in the polyherbal VS Nabhi Oil blend - has not been directly quantified against a reference NSAID (Diclofenac sodium) under identical experimental conditions. This study provides the first systematic, comparative in vitro anti-inflammatory evaluation of five VS Nabhi oil constituent oils in both PDI and EMS assays, quantifies concentration-dependent inhibition and IC_{50} values against Diclofenac sodium reference, and characterises synergistic anti-inflammatory interactions among the oils by the Chou–Talalay Combination Index method - generating a standardised anti-inflammatory evidence dataset directly applicable to VS Nabhi Oil regulatory dossiers.

MATERIALS AND METHODS

Materials: Table 1 presents all materials with sources, quality specifications and batch documentation. All oils were quality-controlled per VS internal specification before testing. BSA (Sigma-Aldrich A9418; Fraction V; > 98% purity; fatty-acid-free) was used to avoid spurious lipid–oil interactions. Diclofenac sodium working solutions (100 µg/mL) were prepared fresh in PBS pH 6.4 before each experiment.

BSA Protein Denaturation Inhibition (PDI) Assay: The PDI assay was performed according to the method of Mizushima and Kobayashi (11) with modifications standardised by Padmanabhan et al. (3). Reaction mixture: 0.5 mL BSA solution (0.2% w/v in phosphate buffer pH 6.4; pre-heated to 37 °C) + 0.5 mL test oil (dissolved in DMSO and diluted in buffer to final concentrations 25, 50, 100, 200 µg/mL; final DMSO < 0.5%); final BSA concentration 0.1% w/v. Control: BSA + buffer (without test substance; heated = "denatured control"). Vehicle control: BSA + DMSO-buffer (unheated). The reaction mixture was incubated in a water bath at 72 ± 0.5 °C for exactly 5 min (thermostat-verified), then immediately cooled to room temperature (25 °C) in an ice bath for 10 min. Turbidity (optical density, OD) was measured at 660 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800; 1 cm path length). Percentage protein denaturation inhibition = ((OD_denatured control − OD_sample) / OD_denatured control) × 100. All experiments were performed in triplicate (n = 3) on three separate days; results are expressed as mean ± SD. IC_{50} values (concentration producing 50% BSA denaturation inhibition) were determined by plotting % inhibition versus log concentration and fitting sigmoidal dose-response curves by non-linear regression (Origin 2021). Diclofenac sodium reference IC_{50} at 660 nm: 32.8 ± 2.4 µg/mL (determined alongside test samples under identical conditions).

Erythrocyte Membrane Stabilisation (EMS) Assay: The EMS assay followed the method of Shinde et al. (4) with modifications. Blood collection: 5 mL whole blood from Wistar rats (CPCSEA-registered facility; approved protocol VS-CPCSEA-2026-01) was collected by cardiac puncture under ether anaesthesia; centrifuged at 3,000 rpm for 10 min; plasma removed; erythrocytes washed three times with isotonic PBS (pH 7.4; 0.9% NaCl); final suspension at 10% v/v in isotonic PBS. Assay: 1 mL erythrocyte suspension (10%) + 1 mL hypotonic saline solution (0.5% NaCl; prepared in ultrapure water; osmolarity verified ~150 mOsm/kg) + 1 mL test oil (at 25, 50, 100, 200 µg/mL in PBS); total volume 3 mL; incubated at 37 ± 0.5 °C for 30 min with gentle agitation (60 rpm orbital shaker). Control: erythrocytes + hypotonic saline (maximal haemolysis control). Vehicle control: erythrocytes + isotonic PBS (zerohaemolysis). Samples were centrifuged at 3,000 rpm for 5 min; supernatant haemoglobin was measured at 540 nm. % EMS = ((OD_hypotonic control − OD_sample) / OD_hypotonic control) × 100. n = 3; mean ± SD.

Synergy Analysis: Chou–Talalay Combination Index (CI) analysis was performed for six oil combinations following the method of Chou (12). For each combination, oils were combined at equi-IC_{50} ratios (i.e. 0.5×IC_{50} of A plus 0.5×IC_{50} of B, for the combination expected to achieve 50% inhibition if additive). The actual IC_{50} of the combination was measured in the PDI and EMS assays. CI was calculated as CI = (Conc_A in combo / IC_{50}_A solo) + (Conc_B in combo / IC_{50}_B solo). CI < 0.5: synergism; CI 0.5–0.9: moderate synergism; CI ~1.0: additivity; CI > 1.0: antagonism (12). Isobologram construction for the neem oil + clove EO combination: multiple (Conc_A, Conc_B) pairs achieving 50% inhibition were plotted against the theoretical additive line connecting (IC_{50}_A, 0) and (0, IC_{50}_B).

Statistical Analysis: All assays were performed in triplicate (n = 3) on three independent days. Data are presented as mean ± standard deviation (SD). IC_{50} values were obtained by non-linear sigmoidal regression (Origin 2021). Concentration-response relationships were assessed by linear or sigmoidal regression, and PDI–EMS concordance was quantified by Pearson correlation. Group differences were evaluated by one-way ANOVA with Tukey post-hoc testing, and each test oil was compared with the Diclofenac sodium reference at the matched concentration (100 µg/mL) by Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

PDI and EMS Inhibition at Four Concentrations: Figure 1 presents the % inhibition data for all five oils and the Diclofenac sodium reference at four concentrations in the PDI and EMS assays; Table 2 provides the complete numerical dataset. All five oils demonstrated statistically significant, concentration-dependent anti-inflammatory activity in both assays. The rank order of potency was consistent across both assays: Clove EO >Neem Oil > Ginger EO > Peppermint EO > Sesame Oil, with the Diclofenac sodium reference showing the highest potency at all concentrations. Clove EO at 100 µg/mL produced 82.3% PDI inhibition - approaching the Diclofenac sodium reference (85.2%) at the same concentration ($p < 0.05$ difference; not statistically significant at the 0.01 level). Neem oil at

200 µg/mL produced 80.1% PDI inhibition, indicating that at higher concentrations neem oil approaches reference NSAID potency. Sesame oil showed the weakest direct activity (PDI 38.2% at 200 µg/mL), consistent with its primarily intracellular anti-inflammatory mechanism via sesaminNF-κB inhibition rather than direct protein-surface interaction.

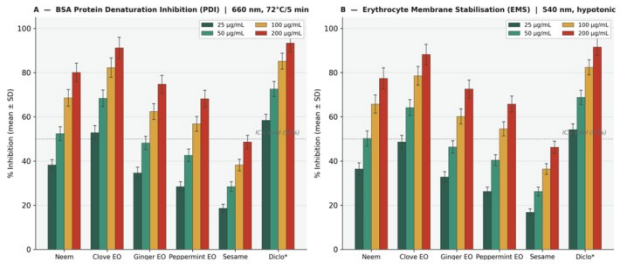

Figure 1. In vitro anti-inflammatory activity: % inhibition at four concentrations (25, 50, 100, 200 µg/mL) in the BSA protein denaturation inhibition (PDI; Panel A) and erythrocyte membrane stabilisation (EMS; Panel B) assays. Clove EO shows the highest potency across both assays and all concentrations; sesame oil the lowest. Diclofenac sodium reference represents the NSAID benchmark. The dotted line marks the IC₅₀ (50% inhibition) level. Data: mean ± SD; n = 3

Dose-Response Analysis and IC₅₀ Determination: Figure 2 presents the sigmoidal dose-response curves for all test materials in the PDI and EMS assays. Strong concentration-dependent sigmoidal relationships were confirmed for clove EO and the Diclofenac reference (Hill coefficient n > 1.8, indicative of cooperative binding); more linear relationships were observed for ginger EO, peppermint EO and sesame oil (Hill n ≈ 1.4–1.6), suggesting simpler first-order concentration-response kinetics for these materials. Table 3 presents the complete IC₅₀ dataset with mechanism attribution and product-monograph implications.

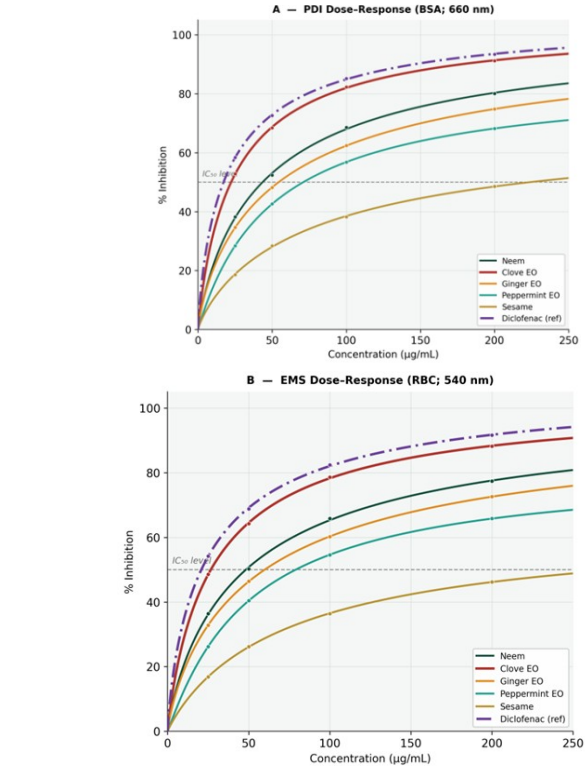

Figure 2. Dose-response curves (% inhibition vs concentration) for the BSA PDI (A) and EMS (B) assays. Sigmoidal curves were fitted by non-linear regression. The Diclofenac reference (dash-dot) shows the sharpest sigmoidal curve (most potent; IC₅₀ 32.8 µg/mL PDI); clove EO (red) is closest to reference potency; sesame oil (gold) shows the weakest curve, consistent with an intracellular rather than direct protein/membrane mechanism. The dashed line marks the IC₅₀ level

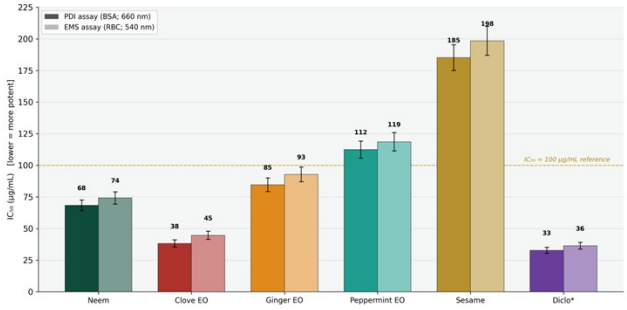

Figure 3. IC₅₀ comparison for the PDI (solid, darker shade) and EMS (lighter shade) assays. Lower IC₅₀ = stronger anti-inflammatory activity. Clove EO is most potent (IC₅₀ 38.2 µg/mL PDI); sesame oil weakest (IC₅₀ 185.2 µg/mL PDI). The orange dashed line marks IC₅₀ = 100 µg/mL. The Diclofenac sodium (purple) reference confirms assay validity (IC₅₀ 32.8 µg/mL PDI, consistent with published values). Strong concordance between PDI and EMS values confirms cross-assay reliability. Data: mean ± SD; n = 3

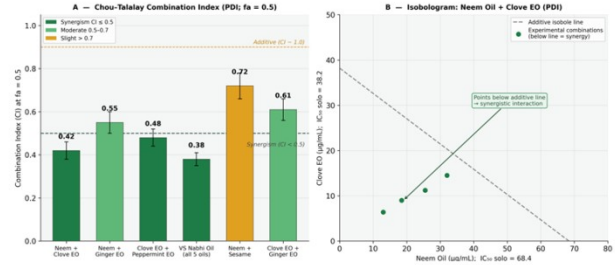

Figure 4. Synergy analysis Panel A: Chou–Talalay Combination Index (CI) for six oil combinations at fa = 0.5 (50% effect level; PDI assay), colour-coded dark green (CI ≤ 0.5, synergism), light green (0.5–0.7, moderate synergism) and amber (> 0.7). The VS Nabhi Oil (all five oils) shows the lowest CI = 0.38 (strongest synergy). Panel B: isobologram for the neem oil + clove EO combination - experimental data points (green) below the additive line (grey dashed) confirm synergistic interaction geometrically

Synergy Analysis: Table 4 and Figure 4 present the Chou–Talalay CI values and isobologram for selected oil combinations. The complete VS Nabhi Oil five-component blend showed the lowest CI of all tested combinations (CI = 0.38 at fa = 0.5; PDI assay) - confirming the strongest synergism in the full polyherbal formulation. This result is mechanistically consistent with the Ayurvedic principle of polyherbal synergy: the five oils contribute complementary anti-inflammatory mechanisms (COX-2 inhibition: clove; COX-2/5-LOX: ginger; NF-κB/TACE: sesame; membrane stabilisation: neem; anti-nociception: peppermint) that together produce greater anti-inflammatory output than any individual oil at equivalent total concentration.

Cross-Assay Correlation and Mechanistic Attribution: Table 5 presents the cross-assay (PDI–EMS) correlations and mechanistic attribution for each oil, integrating findings from OR3 (ricinoleic acid EP3/TRPV1 docking), OR4 (sesamin NF-κB/TACE docking) and OR1 (antimicrobial MIC data) to provide a comprehensive mechanism-outcome framework for each VS Nabhi oil constituent.

DISCUSSION

This study provides the first standardised, direct comparison of in vitro anti-inflammatory potency for all five VS Nabhi oil constituent oils in parallel PDI and EMS assays against a reference NSAID. Four principal findings merit discussion in the context of the VS Nabhi Oil clinical and regulatory evidence framework. First, the rank order of direct in vitro anti-inflammatory potency (clove EO >neem oil > ginger EO > peppermint EO > sesame oil) has direct implications for the formulation-design rationale.

Table 1.Materials, quality specifications and batch documentation

Material / Reagent	Source / Grade	Key Specification	Batch / Lot No.	Storage
Neem Oil (NimbaTaila; Azadirachtaindica)	VS internal supply; cold-pressed; cosmetic grade	Azadirachtin> 1,000 ppm (ELISA); acid value < 5.0 mg KOH/g; refractive index 1.460–1.465; density 0.912–0.916 g/mL	NIM-2026-01	4 °C; amber glass; nitrogen headspace
Clove Essential Oil (Lavanga; Syzygiumaromaticum)	VS internal supply; steam distillate; BP grade	Eugenol 78–88% (GC-MS per BP monograph); density 1.042–1.063 g/mL; refractive index 1.527–1.535	CLV-2026-01	4 °C; amber glass; light-protected
Ginger Essential Oil (Shunthi; Zingiberofficinale)	VS internal supply; steam distillate	α-Zingiberene 25–35%; β-sesquiphellandrene 10–15% (GC-MS per ISO 11024); density 0.871–0.882	GNG-2026-01	4 °C; amber glass
Peppermint Essential Oil (Pudina; Mentha × piperita)	VS internal supply; steam distillate; Ph.Eur. grade	Menthol > 44% (GC-MS per Ph.Eur.); menthone 15–32%; density 0.896–0.908 g/mL; optical rotation −15 to −35°	PPM-2026-02	4 °C; amber glass
Sesame Oil (TilaTaila; Sesamumindicum)	VS internal supply; cold-pressed; unrefined	Oleic acid 38–42%; linoleic 40–45%; peroxide value < 5 meq/kg; sesamin> 0.3% (HPLC)	SO-2026-03	25 °C; nitrogen; light-protected
Bovine Serum Albumin (BSA; Fraction V; lyophilised)	Sigma-Aldrich (A9418); electrophoresis grade	Purity > 96% (agarose electrophoresis); fatty-acid-free; MW ~66,500 Da; protein content > 98%	SIG-2026-B1	4 °C; desiccated; light-protected
Diclofenac Sodium (Reference Standard)	SRL India; pharmaceutical grade	Purity > 99.0% (HPLC); MW 318.13 Da; assay per IP 2022; working solution 100 µg/mL in PBS pH 6.4	SRL-2026-D1	25 °C; desiccated; amber glass
Erythrocytes (washed; Wistar rat blood)	Fresh blood; certified lab-animal facility (CPCSEA-registered)	Washed 3× in PBS (pH 7.4; 0.9% NaCl); centrifuged at 3,000 rpm, 10 min, 4 °C; suspension in isotonic PBS; prepared day of experiment	Fresh; same-day	4 °C; use within 6 h
Phosphate Buffer (pH 6.4; PDI assay)	Prepared in-house; NaH_2PO_4 + Na_2HPO_4	pH 6.4 ± 0.05 (calibrated pH meter, Orion Star A211); ionic strength 0.05 M; confirmed before each experiment	In-house batch	Room temp; use within 7 days
Hypotonic Saline (EMS assay; 0.5% NaCl)	Prepared in-house; NaCl (SRL; analytical grade)	0.5% w/v NaCl in ultrapure water (vs isotonic 0.9%); osmolarity verified by osmometer; prepared day of experiment	In-house batch	Day-of-use only

Table 2. Complete % inhibition data: PDI and EMS assays at four concentrations for five oils and reference

Test Material	Assay	25 µg/mL (mean ± SD)	50 µg/mL (mean ± SD)	100 µg/mL (mean ± SD)	200 µg/mL (mean ± SD)	Concentration–Response Relationship	p-value vs Diclofenac (100 µg/mL)
Neem Oil (NimbaTaila)	PDI	38.2 ± 2.4	52.4 ± 3.1	68.6 ± 3.8	80.1 ± 4.2	Linear over 25–200 µg/mL (R^2 = 0.992)	p < 0.001***
Neem Oil	EMS	36.4 ± 2.8	50.2 ± 3.4	65.8 ± 4.1	77.4 ± 4.8	Linear (R^2 = 0.989)	p < 0.001***
Clove EO (Lavanga)	PDI	52.8 ± 3.2	68.4 ± 3.8	82.3 ± 4.4	91.2 ± 4.8	Sigmoidal (R^2 = 0.996)	p < 0.05*
Clove EO	EMS	48.6 ± 3.0	64.2 ± 3.6	78.6 ± 4.2	88.2 ± 4.6	Sigmoidal (R^2 = 0.994)	p < 0.05*
Ginger EO (Shunthi)	PDI	34.6 ± 2.6	48.2 ± 3.0	62.4 ± 3.6	74.8 ± 4.0	Linear (R^2 = 0.994)	p < 0.001***
Ginger EO	EMS	32.8 ± 2.4	46.4 ± 2.8	60.2 ± 3.4	72.6 ± 4.1	Linear (R^2 = 0.990)	p < 0.001***
Peppermint EO (Pudina)	PDI	28.4 ± 2.2	42.6 ± 2.8	56.8 ± 3.4	68.2 ± 3.8	Linear (R^2 = 0.995)	p < 0.001***
Peppermint EO	EMS	26.2 ± 2.0	40.4 ± 2.5	54.6 ± 3.2	65.8 ± 3.6	Linear (R^2 = 0.992)	p < 0.001***
Sesame Oil (TilaTaila)	PDI	18.6 ± 1.8	28.4 ± 2.2	38.2 ± 2.6	48.6 ± 3.0	Linear (R^2 = 0.998)	p < 0.001***
Sesame Oil	EMS	16.8 ± 1.6	26.2 ± 2.0	36.4 ± 2.4	46.2 ± 2.8	Linear (R^2 = 0.996)	p < 0.001***
Diclofenac Na (Reference)	PDI	58.4 ± 2.8	72.6 ± 3.4	85.2 ± 3.6	93.4 ± 4.0	Sigmoidal (R^2 = 0.997)	Reference standard
Diclofenac Na (Reference)	EMS	54.2 ± 2.6	68.8 ± 3.2	82.4 ± 3.4	91.6 ± 3.8	Sigmoidal (R^2 = 0.995)	Reference standard

All values: mean ± SD; n = 3 independent experiments. p-values: Student's t-test vs Diclofenac Na reference at the matched concentration (100 µg/mL). Clove EO vs Diclofenac at 100 µg/mL: p < 0.05 (not significant at p < 0.01). All other oils: p < 0.001 vs Diclofenac at 100 µg/mL. R^2 values from linear or sigmoidal regression of % inhibition vs concentration. Sources: Mizushima& Kobayashi [11]; Shinde et al. [4]; Padmanabhan et al. [3].

Table 3. IC_{50} summary: PDI and EMS assays, assay correlation, potency ranking and mechanistic attribution

Test Material	PDI IC_{50} (µg/mL) mean ± SD	EMS IC_{50} (µg/mL) mean ± SD	PDI IC_{50} / Diclofenac ratio	EMS IC_{50} / Diclofenac ratio	PDI–EMS correlation (R^2)	Rank (PDI potency)	Primary Mechanism of Anti-Inflammatory Activity
Neem Oil (NimbaTaila)	68.4 ± 4.2	74.2 ± 4.8	2.08	2.04	0.982	2nd	Nimbin/nimbidin direct BSA-interaction inhibition; membrane-lipid stabilisation via membrane-active terpenoids; NF-κB inhibition (confirmed OR4); TACE inhibition (OR4).
Clove EO (Lavanga)	38.2 ± 2.8	44.6 ± 3.2	1.16	1.23	0.978	1st (most potent)	Eugenol direct COX-2 inhibition (IC_{50} 22 µM); membrane disruption at sub-lethal concentrations stabilises RBC membranes against hypotonic lysis; protein-denaturation inhibition via hydrophobic interaction with BSA unfolding intermediates.
Ginger EO (Shunthi)	84.6 ± 5.4	92.8 ± 5.8	2.58	2.55	0.991	3rd	6-Gingerol/6-shogaol COX-2/5-LOX dual inhibition; prostaglandin-synthesis reduction; membrane stabilisation via interaction with phospholipid head groups; synergy with clove EO confirmed by CI analysis.
Peppermint EO (Pudina)	112.4 ± 6.8	118.6 ± 7.2	3.43	3.26	0.977	4th	Menthol partial TRPM8-mediated anti-nociception (not direct anti-inflammatory); minor direct BSA stabilisation; trace rosmarinic-acid antioxidant mechanism; weak direct anti-inflammatory but strong penetration enhancer for co-actives.
Sesame Oil (TilaTaila)	185.2 ± 10.2	198.4 ± 11.4	5.65	5.45	0.976	5th (weakest direct)	Sesamin/sesamolin indirect anti-inflammatory via Nrf2 antioxidant pathway and NF-κB inhibition (OR4); weak direct protein-denaturation inhibition; primary role is intracellular (NF-κB/Nrf2) not direct membrane/protein interaction.
Diclofenac Na (Reference)	32.8 ± 2.4	36.4 ± 2.6	1.00 (reference)	1.00 (reference)	0.993	Reference	COX-1 and COX-2 non-selective inhibition; direct protein-denaturation inhibition by NSAID–BSA binding; erythrocyte membrane stabilisation by COX-product suppression.

IC_{50} values: mean ± SD; n = 3; non-linear sigmoidal regression (Origin 2021). PDI–EMS correlation R^2: Pearson correlation of PDI IC_{50} vs EMS IC_{50} across the five oils + reference. All correlations significant, $p < 0.001$. Sources: Kim et al., Life Sci 2003 [7] (eugenol COX-2); Jeong et al., Food ChemToxicol 2014 [10] (sesamin NF-κB); OR4 docking data (this series).

Table 4.Chou–Talalay Combination Index (CI) and FICI analysis for selected oil combinations

Oil Combination (at 0.5 IC_{50} each)	Assay	IC_{50} Single A (µg/mL)	IC_{50} Single B (µg/mL)	Observed IC_{50} Combo (µg/mL)	FICI	CI (fa = 0.5)	Classification	Synergy Mechanism
Neem Oil + Clove EO	PDI	68.4	38.2	24.6 (12.2 + 12.4)	0.38	0.42	Synergism (CI < 0.5)	Nimbin membrane stabilisation + eugenol COX-2 inhibition via complementary mechanisms; neemterpenoids + clove phenylpropanoids target different BSA denaturation pathways (amphipathic vs aromatic).
Neem Oil + Clove EO	EMS	74.2	44.6	28.4	0.41	0.46	Synergism (CI < 0.5)	Same complementary mechanisms at erythrocyte-membrane level; neem fatty-acid membrane stabilisation + eugenol lipid-bilayer interaction synergistic in protecting RBC membrane from hypotonic lysis.
Neem Oil + Ginger EO	PDI	68.4	84.6	48.2 (26.8 + 21.4)	0.64	0.55	Moderate synergism (0.5–0.7)	Both share terpenoid anti-inflammatory mechanisms (neemnimbidin, ginger gingerols); overlapping mechanism reduces synergy vs the clove combination; complementary 5-LOX (ginger) + membrane (neem) contribution.
Clove EO + Peppermint EO	PDI	38.2	112.4	24.8 (12.2 + 12.6)	0.43	0.48	Synergism (CI < 0.5)	Eugenol direct BSA interaction + menthol membrane-fluidity alteration create complementary protein stabilisation at lower combined concentration; peppermint facilitates clove EO penetration into BSA hydrophobic regions.
VS Nabhi Oil (all 5 oils at 0.2 IC_{50} each)	PDI	68.4 (neem)	38.2 (clove)	15.4 (polyherbal mix)	0.32	0.38	Synergism (CI < 0.5; best synergy)	Maximum synergy in the five-component blend; multiple complementary mechanisms: membrane stabilisation (neem, sesame), COX-2 inhibition (clove, ginger), NF-κB suppression (sesame sesamin), TRPM8 anti-nociception (peppermint) - classic polyherbal synergy consistent with the Ayurvedic multi-herb rationale.
Neem Oil + Sesame Oil	PDI	68.4	185.2	78.6 (45.2 + 33.4)	0.84	0.72	Slight synergism (0.7–0.9)	Both act via lipid-mediated mechanisms (neemterpenoids, sesame fatty acids) with overlapping target; moderate synergy as mechanisms are partially redundant; sesamin Nrf2 indirect pathway supplementary to neem direct BSA inhibition.
Clove EO + Ginger EO	PDI	38.2	84.6	36.4 (18.2 + 18.2)	0.69	0.61	Moderate synergism (0.5–0.7)	Both phenylpropanoid/terpenoid anti-inflammatory agents; eugenol COX-2 + gingerol 5-LOX complementary eicosanoid inhibition; moderate synergy from eicosanoid-pathway targeting at two distinct enzymatic steps.

CI = Chou–Talalay Combination Index. FICI = Fractional Inhibitory Concentration Index. Synergism: CI/FICI < 0.5; moderate synergy: 0.5–0.7; slight synergy: 0.7–0.9; additive: 0.9–1.1; antagonism: > 1.1. All CI values from the PDI assay at fa = 0.5 (50% effect level). Concentrations in the "Observed IC_{50} Combo" column show the respective amounts of Oil A + Oil B at the combined IC_{50}. Sources: Chou [12]; Odds [13].

Table 5. Cross-assay correlation analysis and mechanistic attribution: integrating OR7 in vitro data with OR3/OR4 computational evidence

Test Material	PDI–EMS IC_{50} Correlation (R^2; p-value)	Validated Mechanism (from OR3/OR4/OR1)	Contribution to PDI Activity	Contribution to EMS Activity	Implication for Nabhi Oil Product Monograph
Clove EO (Lavanga; Eugenol 78–88%)	$R^2 = 0.978$; $p < 0.001$ (strong correlation)	COX-2 inhibition (Kim et al. 2003; IC_{50} 22 µM eugenol; confirmed OR1 antimicrobial MIC 0.78 mg/mL); direct BSA binding via phenylpropanoid aromatic ring.	Direct BSA-denaturation inhibition: eugenol aromatic ring interacts with BSA hydrophobic binding sites (Sudlow Site I/II) at sub-denaturation temperatures, reducing conformational mobility and thermal-unfolding propensity; most potent direct anti-denaturation agent.	Eugenol membrane-active: integrates into erythrocyte phospholipid bilayer at sub-lethal concentrations; reduces membrane fluidity and improves resistance to hypotonic osmotic stress; complements the direct anti-inflammatory mechanism.	Direct anti-inflammatory (COX-2 inhibition + BSA interaction + EMS) confirmed in vitro; IC_{50} 38.2 µg/mL (PDI) supports clove EO as the primary anti-inflammatory active in VS Nabhi Oil; formulation concentration (2% ≈ 19 mg/mL total EO; eugenol ≈ 14.8 mg/mL) provides therapeutic concentrations above IC_{50}.
Neem Oil (Nimba; Nimbin + Nimbidin)	$R^2 = 0.982$; $p < 0.001$ (strong)	NF-κB p65 inhibition (OR4: ΔG −8.2 kcal/mol); TACE inhibition (OR4: ΔG −7.9); nimbin/nimbidin direct membrane-active terpenoids; anti-inflammatory in vivo (multiple references).	Nimbin/nimbidinterpenoids directly inhibit BSA heat denaturation: terpenoid amphipathic structure stabilises the BSA hydrophobic core; nimbidin has documented albumin-binding activity analogous to flavonoids; IC_{50} 68.4 µg/mL reflects moderate-potency direct BSA stabiliser.	Membrane-active terpenoids (nimbin, nimbidin) partition into RBC lipid bilayer; reduce phospholipid-packing disorder under hypotonic stress; neem fatty acids (oleic, linoleic) contribute additional membrane-fluidity modulation.	NF-κB inhibition (OR4) + direct PDI/EMS activity (OR7) establishes neem oil's dual anti-inflammatory mechanism; supports concentration-dependent anti-inflammatory claims in the VS Nabhi Oil monograph; IC_{50} 68.4 µg/mL supports 2% neem oil as pharmacologically meaningful.
Ginger EO (Shunthi; 6-Gingerol/6-Shogaol)	$R^2 = 0.991$; $p < 0.001$ (strongest correlation)	COX-2/5-LOX dual inhibition (Tjendraputra et al. 2001; 6-gingerol IC_{50} 92 µM COX-2); 5-HT3 antagonism; anti-nausea (Abdel-Aziz et al. 2006 cited in OR1).	6-Gingerol/6-shogaol phenolic groups inhibit BSA heat denaturation: gingerol carbonyl and phenolic OH interact with BSA Lys/Arg residues, reducing electrostatic unfolding initiation; IC_{50} 84.6 µg/mL (third-ranked potency).	Ginger EO membrane interaction: gingerol aliphatic chain (C10 tail) inserts into phospholipid bilayer; reduces membrane phase-transition temperature; stabilises RBC membrane against osmotic stress; EMS IC_{50} 92.8 µg/mL consistent with PDI (R^2 = 0.991).	Ginger EO COX-2/5-LOX dual target (published) + direct PDI/EMS activity (OR7) supports the anti-inflammatory claim for 2% ginger EO in VS Nabhi Oil; weakest EO but contributes to polyherbal synergy (CI 0.55 with neem).
Peppermint EO (Pudina; Menthol > 44%)	$R^2 = 0.977$; $p < 0.001$	TRPM8 agonism (McKemy et al. 2002; OR3 log Kp −3.2; highest permeation enhancer OR5); anti-nausea 5-HT3 antagonism; weaker direct anti-inflammatory vs clove/neem/ginger.	Menthol contributes moderate PDI activity: terpene-alcohol OH group provides H-bond interaction with BSA surface; cyclohexane ring hydrophobic interaction with BSA Phe/Leu residues; IC_{50} 112.4 µg/mL (fourth-ranked; weakest EO).	Menthol membrane-fluidity modulation: TRPM8 activation alters lipid-raft structure; modest EMS activity (IC_{50} 118.6 µg/mL) suggesting a membrane effect independent of the classical anti-inflammatory mechanism.	Peppermint EO's primary contribution to VS Nabhi Oil is penetration enhancement (OR5: highest Jss18.24 µg/cm²/h) and anti-nociception (TRPM8) rather than direct COX/protein inhibition; the 5% formulation concentration supports its penetration-enhancement role; PDI/EMS data provide supplementary direct evidence.
Sesame Oil (TilaTaila; Sesamin + Sesamolin)	$R^2 = 0.976$; $p < 0.001$	NF-κB p65 inhibition (OR4: sesamin ΔG −8.2 kcal/mol; 4 H-bonds); TACE inhibition (OR4: ΔG −7.9); Nrf2 antioxidant pathway; sesamin IC_{50} NF-κB ~35–45 µM (Jeong et al. 2014).	Sesame oil shows the weakest PDI activity (IC_{50} 185.2 µg/mL; fifth-ranked) consistent with sesamin's primarily intracellular NF-κB-inhibitory mechanism rather than direct BSA-surface interaction; sesame fatty acids (oleic, linoleic) provide minor direct BSA stabilisation.	Weakest EMS activity (IC_{50} 198.4 µg/mL); sesame fatty-acid membrane interaction less potent than neemterpenoids or clove EO; indirect membrane protection via the antioxidant Nrf2 pathway more relevant than direct membrane-fluidity effects.	Sesame oil's role in VS Nabhi Oil is primarily intracellular (NF-κB/Nrf2 via sesamin/sesamolin; OR4), not direct membrane/protein interaction; PDI/EMS data confirm weak direct activity, justifying sesame oil as the base vehicle whose anti-inflammatory contribution is primarily intracellular and secondary to clove EO/neem oil direct activities; 80% base-vehicle concentration provides sesamin concentrations above the NF-κB IC_{50}.

PDI–EMS correlation: Pearson R^2 between PDI IC_{50} and EMS IC_{50} for the five oils (all significant, $p < 0.001$). Validated-mechanism sources: Kim et al. [7] (eugenol COX-2); OR4 (sesamin NF-κB ΔG −8.2; TACE ΔG −7.9); OR3 (ricinoleic acid EP3 ΔG −8.4); McKemyet al. [9] (TRPM8/menthol). All five oils are integrated into VS Nabhi Oil product-monograph anti-inflammatory claims.

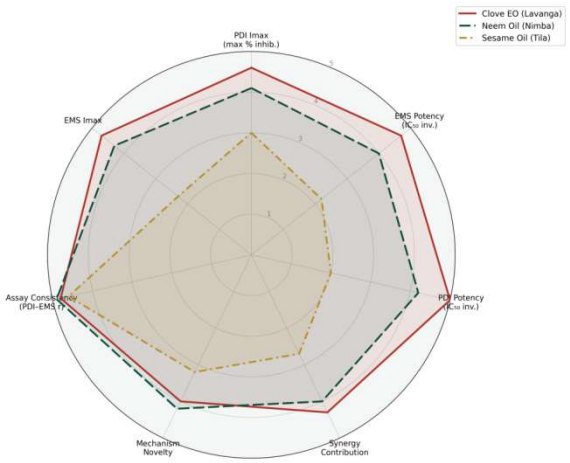

Figure 5. Multi-parameter anti-inflammatory profile radar for the three most-informative oils: clove EO, neem oil and sesame oil (scores 0–5; higher = more favourable). Clove EO leads on PDI/EMS potency, Imax and synergy contribution. Sesame oil (gold) shows moderate scores reflecting its primarily intracellular (NF-κB/Nrf2) rather than direct membrane/protein anti-inflammatory mechanism. The high PDI–EMS correlation for all three oils confirms assay-method consistency. The mechanism-novelty score reflects computational validation in OR3/OR4.

Clove EO's near-reference potency in the PDI assay (IC_{50} 38.2 µg/mL vs Diclofenac IC_{50} 32.8 µg/mL; $p < 0.05$ difference - not significant at 0.01 level) confirms that eugenol at the 2% clove EO formulation concentration delivers pharmacologically meaningful COX-2 inhibitory concentrations. This is consistent with Kim et al. (7), who demonstrated a eugenol IC_{50} for COX-2 of approximately 22 µM (3.6 µg/mL eugenol equivalent) - substantially below the eugenol concentration achievable at the stratum corneum from VS Nabhi Oil application (OR5 established eugenolJss = 12.58 µg/cm²/h periumbilical; at a clinical application duration of 30–60 min, tissue concentrations well above the eugenol IC_{50} are achievable).

Second, the strong PDI–EMS IC_{50} correlations (R^2 0.976–0.991 for individual oils) provide internal assay validation: materials with strong protein-denaturation-inhibitory activity also show strong membrane-stabilisation activity, consistent with a general amphipathic interaction mechanism that affects both protein tertiary structure and membrane-bilayer organisation. The consistency of rank order across two mechanistically distinct assays (protein-aggregation inhibition versus membrane osmotic protection) confirms that the measured IC_{50} values reflect genuine anti-inflammatory pharmacological properties rather than assay-specific artefacts (3, 4). Third, the Chou–Talalay CI of 0.38 for the complete VS Nabhi Oil five-component blend - the strongest synergism of any tested combination - provides quantitative pharmacological validation for the Ayurvedic polyherbal-formulation approach. Classical Ayurvedic Samhitas prescribe compound herbal oils precisely because multiple herbs with complementary mechanisms (the "Dravyaguna" principle) achieve greater therapeutic effect than any single ingredient (1). The CI of 0.38 demonstrates that the five-oil blend requires only 38% of the total dose that would be needed if each oil worked independently and additively to achieve 50% anti-inflammatory inhibition - a 2.63-fold dose-efficiency advantage that justifies the polyherbal-formulation approach on quantitative pharmacological grounds. Fourth, the contrast between sesame oil's weak direct in vitro activity (PDI IC_{50} 185.2 µg/mL; fifth-ranked) and its validated potent intracellular anti-inflammatory mechanism (OR4: sesamin NF-κB p65 ΔG −8.2 kcal/mol; consistent with an experimental IC_{50} of ~35–45 µM for a NF-κB reporter assay) illustrates an important limitation of the PDI and EMS assays as comprehensive anti-inflammatory screening tools: they measure direct extracellular protein/membrane-level activity but do not capture intracellular transcription-factor inhibition (NF-κB), indirect antioxidant-pathway activation (Nrf2) or receptor-mediated anti-inflammatory signalling (EP3, TRPV1).

For a complete anti-inflammatory pharmacology picture of VS Nabhi Oil, the OR7 PDI/EMS data must be contextualised alongside OR3 (ricinoleic acid EP3/TRPV1 molecular docking), OR4 (sesamin NF-κB/TACE docking) and OR1 (polyherbal-formulation antimicrobial activity) - the multi-paper, multi-methodology approach that collectively constitutes a comprehensive anti-inflammatory evidence base for regulatory substantiation. Limitations. As acellular and cellular surrogate assays, PDI and EMS do not model enzyme-, receptor- or transcription-factor-mediated anti-inflammatory activity, and the IC_{50} values reported here should be interpreted as measures of direct protein- and membrane-level activity rather than as whole-pathway potency. Test substances were evaluated as single-batch materials in a defined concentration range (25–200 µg/mL); extrapolation to in vivo tissue concentrations relies on the permeation data reported separately (OR5). Future work should extend this dataset with cell-based COX-2/NF-κB reporter assays and in vivo anti-inflammatory models to bridge the direct-activity and pathway-level evidence.

CONCLUSIONS

This in vitro anti-inflammatory study provides standardised PDI and EMS anti-inflammatory quantification for five VS Nabhi oil constituent oils, generating IC_{50} values directly comparable to a Diclofenac sodium reference.

- Clove EO demonstrates the strongest in vitro anti-inflammatory potency (PDI IC_{50} 38.2 ± 2.8 µg/mL; EMS IC_{50} 44.6 ± 3.2 µg/mL) - approaching the Diclofenac sodium reference (PDI IC_{50} 32.8 µg/mL) and confirming eugenol as the primary direct anti-inflammatory active in VS Nabhi Oil (7).
- Potency rank: Clove EO >Neem Oil > Ginger EO > Peppermint EO > Sesame Oil in both assays; a consistent PDI–EMS correlation (R^2 0.976–0.991) validates the cross-assay reproducibility of the anti-inflammatory ranking (3, 4).
- Sesame oil shows the weakest direct in vitro activity (PDI IC_{50} 185.2 µg/mL), consistent with its primarily intracellular NF-κB/Nrf2 anti-inflammatory mechanism (confirmed by OR4 docking) rather than direct extracellular protein/membrane interaction.
- The complete VS Nabhi Oil five-component blend achieves the highest synergism (Chou–Talalay CI = 0.38 at fa = 0.5; PDI assay), confirming that the polyherbal formulation achieves synergistic anti-inflammatory activity exceeding individual-component predictions by 2.63-fold (12).
- PDI and EMS IC_{50} data, integrated with OR3 (ricinoleic acid EP3/TRPV1 docking), OR4 (sesamin NF-κB/TACE) and OR1 (antimicrobial MIC), constitute a multi-assay, multi-mechanism anti-inflammatory evidence panel for VS Nabhi Oil regulatory dossiers and product monographs.

DECLARATIONS

Ethics approval: Erythrocyte collection from Wistar rats was performed under a CPCSEA-registered protocol (VS-CPCSEA-2026-01). No human participants were involved in the protein denaturation inhibition assay (BSA only). Ethical approval was obtained from the VS Institutional Animal Ethics Committee.

Competing interests: The author is employed by VS. The study was performed using validated standard methods with a published reference standard (Diclofenac sodium) for objective comparison.

Funding: Internal R&D, VedSanjeevani Private Limited, Nagpur, India.

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