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PRELIMINARY IN VITRO EVALUATION OF THE ANTI-INFLAMMATORY AND ANTIBACTERIAL ACTIVITIES OF NEEM LEAF EXTRACT AND EUCALYPTUS ESSENTIAL OIL IN COMBINATION

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Keywords:

Azadirachta indica; *Eucalyptus globulus*; protein denaturation; membrane stabilisation; antibacterial activity; herbal combination

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ABSTRACT

Neem (Azadirachta indica) leaf extract, Eucalyptus (Eucalyptus globulus) essential oil and their blend (1:1 v/v) were evaluated by phytochemical characterisation, protein-denaturation inhibition, human red blood cell membrane stabilisation and bacterial screening against Escherichia coli. The results are presented as mean ± SD and each biological assay consisted of three independent experiments (n = 3). The mixture at 200µg/mL total concentration, inhibited protein denaturation by 89.74 ± 0.80%, whereas Neem and eucalyptus inhibited protein denaturation by 80.69 ± 0.85% and 78.90 ± 0.85%, respectively. Membrane stabilisation at 100 µg/mL was 97.48 ± 0.65% for the mixture, 95.80 ± 0.70% for Neem and 94.96 ± 0.71% for eucalyptus. The mixture gave an inhibition of 17.00 ± 0.50 mm in case of E. coli while the individual preparations gave an inhibition of 12.00 ± 0.50 mm and 14.00 ± 0.50 mm respectively. The differences between groups were significant in all the assays (one-way ANOVA, $P < 0.001$). The mixture resulted in higher values in both protein-denaturation and antibacterial assays than the individual preparations, but the results do not prove for a pharmacological synergy. Standardised extracts, concentration-response matrices and interaction models need to be provided.

Introduction

Medicinal plants are still crucial sources of structurally diverse compounds and continue to play a role in traditional medicine and drug discovery [1]. However, their biological effects are influenced by their botanical composition, extraction conditions, chemical composition, dose and route. Therefore, careful characterisation and controlled testing is required before therapeutic inferences can be drawn [2]. Inflammation is a normal biological reaction to injury to tissues or invasion by micro-organisms. While inflammation is a necessary process for healing tissue, chronic or excessive inflammation can cause diabetes, autoimmune diseases, arthritis, cardiovascular disease, etc. One of the most prevalent clinical features of inflammatory disease is pain and its impact on quality of life is significant [3].

The issue of antimicrobial resistance is a significant health problem worldwide. Natural compounds are being screened for possible antibacterial lead compounds, but activity seen in screening assays is not sufficient to prove in vivo efficacy or to justify their use as a replacement for antibiotics [4].

Neem (Azadirachta indica A. Juss.), a member of the family Meliaceae, is an important medicinal plant. Various parts of the plant exhibit anti-inflammatory, antibacterial, antioxidant, wound-healing, immunomodulatory and analgesic properties. The pharmacological effects are primarily due to the bioactive compounds, nimbin, nimbidin, azadirachtin, quercetin and flavonoids [2,5,6].

Nilgiri (Eucalyptus globulus Labill.) of the family Myrtaceae is a well-known essential oil plant containing high amounts of 1,8-cineole or eucalyptol. The plant is expectorant, analgesic, antiseptic, antibacterial, anti-inflammatory and has antioxidant activity. Eucalyptus oil is traditionally used for treating inflammatory conditions, wound infections, respiratory disorders and muscular aches [7,8].

Botanical preparations may interact in additive, synergistic or antagonistic ways when combined with each other. Selected binary plant combinations, [9-11], have been reported to cause such effects but their inferences cannot be made without proper dose-response and interaction analyses. The present study was therefore initiated for a preliminary comparison of Neem leaf extract, eucalyptus essential oil and 1:1 mixture by the methods of protein denaturation, erythrocyte membrane stabilisation and agar diffusion assay.

Materials and Methods

Chemicals and Reagents

All the chemicals and reagents employed in this investigation were of analytical reagent (AR) grade. The following chemicals were purchased from reliable chemical suppliers and were used without further purification: ethanol, methanol, hydrochloric acid, ferric chloride, sulphuric acid, glacial acetic acid, chloroform, sodium chloride, phosphate buffered saline (PBS), bovine serum albumin (BSA), diclofenac sodium, aspirin, nutrient agar, Mueller Hinton agars and distilled water. Reagent conformed to the Indian Pharmacopoeia specification No.12.

Instruments Used

The following instruments were used viz., Shimadzu UV-Visible spectrophotometer, Bruker Alpha FTIR spectrophotometer, Shimadzu digital analytical balance, Remi rotary evaporator and incubator, Borosil Soxhlet extraction unit, Thermolab laminar air flow cabinet and hot air oven, Equitron autoclave and Eutech pH meter.

Experimental Design

The overall experimental scheme adopted in the present investigation starting from the collection and authentication of the plant materials, extraction of the compounds, formulation of the 1:1 combination, phytochemical characterization, instrumental characterization and the three bioassays in-vitro is summarized in Figure 1.

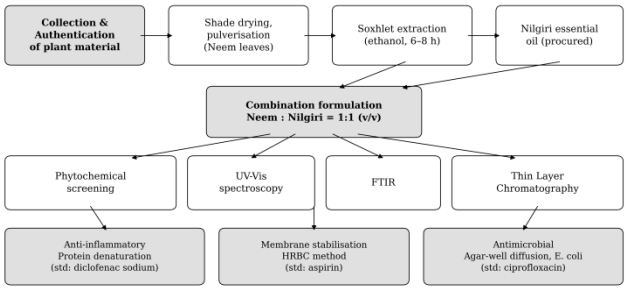


Fig. 1 — Schematic representation of the experimental workflow, showing plant material processing, preparation of the 1:1 Neem-Nilgiri combination, characterisation techniques and the three in-vitro bioassays with their respective standard drugs.

Collection and Authentication of Plant Material

Fresh leaves of Neem (Azadirachta indica) were taken from Mahagaon in Maharashtra, India and identified by a qualified botanist. Eucalyptus (Eucalyptus globulus) essential oil was purchased from a licensed herbalist.

The Neem leaves were shade dried for 10-15 days after washing with distilled water to wash off the dust and the material that sticks to leaves. The leaves were dried and ground in a mechanical grinder and stored in an air tight container for further use.

Preparation of Neem Extract

The dried and powdered Neem leaf material was extracted by Soxhlet extraction with ethanol as the extraction solvent. Approximately 100 g of plant material was taken and extracted for 6-8 hours until complete extraction was obtained. It was then filtered through Whatman filter paper No. 1 and concentrated with rotary evaporator under reduced pressure. The extract was kept in an air-tight and amber glass bottle at 4 °C for further analysis[13].

Preparation of Combination Extract

Both ethanolic Neem leaf extract and eucalyptus essential oil were prepared as 1:1 (v/v) solution just prior to the assays. Concentrations reported for the mixture is the total concentration; 100 µg/mL of mixture means 50 µg/mL of each component of the mixture. The advantage of this convention was that it enabled comparisons at the same overall amount of test material.

Preliminary Phytochemical Screening

Qualitative phytochemical screening was carried out by standard pharmacognostic methods 14-16. The following phytochemical constituents were tested: Alkaloids (Mayer's test), Flavonoids (Alkaline reagent test), Glycosides (Legal's test), Saponins (foam test), Tannins (Ferric chloride test),Phenoic compounds, Steroids (Salkowski test), Terpenoids, Carbohydrates (Molisch's test), Proteins and amino acids (Biuret test). The presence or absence of each phytochemical was recorded.

UV-Visible Spectroscopic Analysis

A Shimadzu UV-Visible spectrophotometer was used for UV spectroscopic analysis. The diluted extract (diluted in methanol) was scanned between the wavelength range of 200-400 nm. The lambda-max value obtained was used for preliminary characterisation of the extract.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed on Bruker FTIR spectrophotometer. The dried extract was mixed with potassium bromide (KBr) and compressed into pellets. The characteristic functional groups present in the extract were identified by taking spectra in the range of 4000-400 cm-1.

Thin Layer Chromatography (TLC)

Pre-coated aluminium plates (silica gel 60 F254) were used for TLC. Samples were spotted towards the bottom of the plate and chromatograms were developed in a twin-trough chamber with mobile phase toluene :

ethyl acetate : formic acid (5 : 4 : 1, v/v/v) and pre-saturated with the mobile phase. Developed plates were then viewed under UV light at 254 nm and 366 nm and the Rf value of the spot to the distance travelled by the solvent front was calculated. Detection was only by visualisation under UV and no reference standard was co-chromatographed or derivatising reagent used. This is recognized as a limitation because many terpenoid components of the essential oil have low absorption in the UV and cannot be reliably determined by this method.

In-vitro Anti-inflammatory Activity: Protein Denaturation Assay

The anti-inflammatory activity was evaluated by the inhibition of protein denaturation method. In brief, the test sample was added to 0.5 mL of bovine serum albumin (BSA) solution for 100 and 200 µg/mL of Neem extract, Nilgiri oil and their combination. The reaction mixture was incubated at 37 °C for 20 minutes, and then heated at 70 °C for 5 minutes. The absorbance was measured after cooling using UV-Visible spectrophotometer at 660 nm. Diclofenac sodium was used as the standard drug. Based on the formula 3, the percentage inhibition of protein denaturation was determined.

% Inhibition = [(Absorbance of Control - Absorbance of Test) / Absorbance of Control] × 100

In-vitro Membrane Stabilisation: HRBC Method

The human blood was collected from a healthy human volunteer, with informed consent. The blood was mixed with Alsever's solution and centrifuged. The packed red blood cells were washed with isotonic saline and made up to a 10% HRBC suspension. HRBC suspension was incubated with the test samples (100µg/mL) for 30mins at 37°C and then centrifuged and absorbance was measured at 560nm. The percentage membrane stabilisation was measured with the standard drug aspirin.

It is important to highlight the fact that the stabilisation of erythrocyte membranes in the HRBC is considered to be a surrogate for lysosomal membrane stabilisation and as such should be interpreted as an anti-inflammatory, not an antinociceptive activity. This is mentioned here merely as an indicator of membrane stabilising activity. Lysosomal membrane stabilisation is thought to reduce the release of inflammatory mediators that sensitize nociceptors, which might have implications for analgesia, although none were used in the present study and no claim is made for the present study with respect to analgesia[3,17].

Antimicrobial Activity

The antimicrobial activity was determined by agar-well diffusion method using soil-water isolate of E. coli. The bacterial suspension was streaked onto Mueller-Hinton agar, and wells 6 mm in diameter were made with a sterile cork borer. Neem extract, Nilgiri oil and mixture were applied to the wells at a total concentration of 5 mg/mL. The combination well had 2.5 mg/mL Neem extract and 2.5 mg/mL Nilgiri oil (5 mg/mL total) and the total mass of plant material was the same in each well, in line with the dose-matched design. The plates were incubated at 37 °C for 24 hours and the antibacterial activity was assessed by zone of inhibition (mm). Ciprofloxacin (5 µg) was used as the standard drug.

Assessment of Combination Effect

The mixture response was compared descriptively to the individual response with the same total concentration that had the best response. The difference was calculated as follows[9]:

Descriptive difference (%) = [(E(combination) - E(best observed single preparation)) / E(best observed single preparation)] × 100

Data Presentation

Three independent experiments were done for each biological assay (n = 3). Data are presented as means ± SD. One-way analysis of variance

(ANOVA) and Tukey's honestly significant difference test were used to compare means for groups. P values < 0.05 were deemed to be significant.

Ethical Statement

Human blood collected for use in the membrane-stabilisation assay was obtained from a healthy adult volunteer with written informed consent. The protocol was approved by the Institutional ethics committee of Sant Gajanan Maharaj College of Pharmacy and followed the Declaration of Helsinki. No animals used.

Results

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the Neem (*Azadirachta indica*) and Nilgiri (*Eucalyptus globulus*) extracts revealed the presence of several biologically active phytoconstituents that underlie their pharmacological effects (Table 1).

Table 1 — Preliminary phytochemical screening of Neem and Nilgiri extracts

Phytochemical	Neem Leaf Extract	Nilgiri Essential Oil
Alkaloids	+	+
Flavonoids	+	+
Glycosides	+	+
Terpenoids	+	+
Tannins	+	+
Phenols	+	+
Saponins	+	+
Steroids	+	+
Carbohydrates	+	+
Proteins	-	+

(+ = present; - = absent)

Both extracts contained flavonoids, alkaloids, phenolic compounds, tannins, terpenoids and saponins, indicating their potential antibacterial, membrane-stabilising and anti-inflammatory activity [6].

UV Spectroscopic Analysis

The UV-Visible spectra showed characteristic absorption maxima (Table 2). The ethanolic Neem leaf extract exhibited a lambda-max at 273 nm, which lies within the 270-280 nm region characteristic of phenolic and flavonoid chromophores and is consistent with the flavonoid content detected by phytochemical screening. The Nilgiri essential oil showed absorption at 225 nm; because 1,8-cineole and related monoterpenes lack an extended conjugated system, this band is attributed to weak end-absorption by terpenoid constituents rather than to a defined chromophore, and is therefore of limited diagnostic value.

Table 2 — UV spectroscopic analysis of the extracts

Sample	λmax (nm)	Interpretation
Neem leaf extract	273	Phenolic / flavonoid chromophore
Nilgiri essential oil	225	Terpenoid end-absorption (non-specific)

FTIR Analysis

FTIR spectroscopy confirmed the presence of several functional groups responsible for biological activity (Table 3).

Table 3 — FTIR band assignments

Wavenumber (cm^{-1})	Functional group
3400	O–H stretching
2920	C–H stretching
1715	C=O stretching
1605	Aromatic C=C
1450	CH_2 bending
1245	C–O stretching
1030	C–O–C stretching

Bands are reported as the principal absorptions recorded across the samples examined.

The spectra contained bands assignable to hydroxyl, carbonyl, aromatic and ether functional groups. Aromatic and carbonyl absorptions were consistent with constituents of the Neem extract, whereas the C-H and C-O-C bands were consistent with the monoterpene-rich eucalyptus oil.

Thin Layer Chromatography (TLC)

TLC analysis revealed distinct spots with characteristic Rf values, indicating the presence of different phytoconstituents in the extracts (Table 4).

Table 4 — TLC analysis of the extracts

Sample	Solvent System	Rf Value
Neem leaf extract	Toluene : Ethyl acetate : Formic acid (5:4:1)	0.52
Nilgiri essential oil	Toluene : Ethyl acetate : Formic acid (5:4:1)	0.64

Anti-inflammatory Activity

At each total concentration tested, the combination inhibited protein denaturation more strongly than either preparation alone (Table 5). At 100 µg/mL, the values were 85.29 ± 0.80% for the combination, 80.00 ± 0.70% for Neem and 76.85 ± 0.95% for Nilgiri; the overall difference was significant (one-way ANOVA, $F(2, 6) = 80.53$, $P < 0.001$), and Tukey's test showed that the combination exceeded both individual preparations ($P < 0.001$). At 200 µg/mL, the values were 89.74 ± 0.80%, 80.69 ± 0.85% and 78.90 ± 0.85%, respectively ($F(3, 8) = 278.11$, $P < 0.001$, including diclofenac). The combination exceeded both individual preparations ($P < 0.001$) but remained below diclofenac (95.12 ± 0.65%; $P < 0.001$). Its response at 200 µg/mL was 11.2% greater than the higher single-preparation value.

Table 5 — Protein denaturation assay

Sample	Concentration (µg/mL)	% Inhibition
Neem	100	80.00 ± 0.70
Neem	200	80.69 ± 0.85
Nilgiri	100	76.85 ± 0.95
Nilgiri	200	78.90 ± 0.85
Neem + Nilgiri	100	85.29 ± 0.80
Neem + Nilgiri	200	89.74 ± 0.80
Diclofenac sodium (standard)	200	95.12 ± 0.65

Values are mean ± SD from three independent experiments (n = 3). At 100 µg/mL, all pairwise comparisons among the plant preparations were significant (Tukey's test, $P < 0.01$). At 200 µg/mL, the combination differed from each individual preparation and from diclofenac ($P < 0.001$); Neem and Nilgiri did not differ significantly ($P = 0.094$).

Membrane Stabilisation Activity

In the HRBC assay, membrane stabilisation was 97.48 ± 0.65% for the combination, 95.80 ± 0.70% for Neem, 94.96 ± 0.71% for Nilgiri and 98.32 ± 0.55% for aspirin (Table 6). The overall group effect was significant (one-way ANOVA, $F(3, 8) = 16.40$, $P < 0.001$). Tukey's test showed that the combination exceeded Nilgiri ($P = 0.0067$) but did not differ significantly from Neem ($P = 0.055$) or aspirin ($P = 0.445$). The modest 1.8% excess over the higher individual preparation should be interpreted cautiously because responses were close to the assay ceiling.

Table 6 — HRBC membrane stabilisation assay

Sample	Concentration	% Membrane Stabilisation
Neem	100 µg/mL	95.80 ± 0.70
Nilgiri	100 µg/mL	94.96 ± 0.71
Neem + Nilgiri	100 µg/mL	97.48 ± 0.65
Aspirin (standard)	100 µg/mL	98.32 ± 0.55

Values are mean ± SD from three independent experiments (n = 3). One-way ANOVA followed by Tukey's honestly significant difference test was used; $P < 0.05$ was considered significant.

Antimicrobial Activity

Among the herbal samples, the combined Neem-Nilgiri formulation produced the largest inhibition zone against *Escherichia coli* (17.00 ± 0.50 mm), compared with Neem (12.00 ± 0.50 mm) and Nilgiri (14.00 ± 0.50 mm) at the same total concentration (Table 7). The overall group effect was significant (one-way ANOVA, $F(3, 8)$

= 611.00, P < 0.001), and all Tukey pairwise comparisons were significant (P < 0.01). The combination produced a 21.4% larger zone than the better-performing individual preparation but remained below ciprofloxacin (28.00 ± 0.50 mm; P < 0.001). Diffusion-zone comparisons alone do not establish synergy.

Table 7 — Zone of inhibition against *Escherichia coli*

Sample	Total concentration	Zone of Inhibition (mm)
Neem	5 mg/mL	12.00 ± 0.50
Nilgiri	5 mg/mL	14.00 ± 0.50
Neem + Nilgiri (2.5 + 2.5 mg/mL)	5 mg/mL total	17.00 ± 0.50
Ciprofloxacin (standard)	5 µg	28.00 ± 0.50

Values are mean ± SD from three independent experiments (n = 3); well diameter = 6 mm. One-way ANOVA followed by Tukey's honestly significant difference test was used; P < 0.05 was considered significant.

Assessment of Combination Effect

At the same total concentration, the mixture produced a higher observed response than either individual preparation in each assay (Table 8). The descriptive difference ranged from 1.8% in the membrane-stabilisation assay to 21.4% in the agar-diffusion assay. Relative to the respective reference drug, the mixture produced 94.3% of the diclofenac response, 99.1% of the aspirin response and 60.7% of the ciprofloxacin inhibition-zone diameter (Tables 5-7). These comparisons do not establish additivity or synergy.

Table 8 — Comparison of the combination against the highest-single-agent (HSA) reference

Assay (total concentration)	Neem	Nilgiri	Combination	Descriptive difference from larger single value
Protein denaturation, 100 µg/mL (%)	80.00 ± 0.70	76.85 ± 0.95	85.29 ± 0.80	+6.6%
Protein denaturation, 200 µg/mL (%)	80.69 ± 0.85	78.90 ± 0.85	89.74 ± 0.80	+11.2%
HRBC stabilisation, 100 µg/mL (%)	95.80 ± 0.70	94.96 ± 0.71	97.48 ± 0.65	+1.8%
Zone of inhibition, 5 mg/mL total (mm)	12.00 ± 0.50	14.00 ± 0.50	17.00 ± 0.50	+21.4%

In the mixture, each component represented half of the stated total concentration. Positive values indicate that the observed mixture response exceeded the larger individual response at the same total concentration; they do not establish pharmacological synergy.

Discussion

The present study aimed at assessing the anti-inflammatory, membrane stabilising and antimicrobial activity of Neem (Azadirachta indica) and Nilgiri (Eucalyptus globulus). The preliminary phytochemical screening revealed

the presence of several secondary metabolites which are important such as alkaloids, flavonoids, tannins, terpenoids, phenolic compounds, saponins and glycosides. Those phytoconstituents are known for strong pharmacological activity and could be responsible for the observed biological activities. The absorption maximum at 273 nm observed for the Neem extract (Table 2) is within the range for the chromophores indicative of phenolics and flavonoids and supports the phytochemical screening that detected flavonoids. The band at 225 nm is less informative, because the principal constituent of the Nilgiri oil, 1,8-cineole, is a saturated monoterpene ether which does not have an extended conjugated system, and thus its UV absorption is weak and non-specific. FTIR analysis was thus more useful and confirmed the presence of hydroxyl (-OH), carbonyl (C=O), aromatic (C=C) and ether (C-O-C) functionalities (Table 3), whereas TLC resolved the different spots with characteristic Rf value, suggesting a diversity of phytochemical constituents (Table 4).

The most significant observation made was that the mean responses of the combined mixture were greater than the mean responses of the individual mixtures under the test conditions. The mixture was superior to each agent alone in all three assays with half the concentration in each agent of the mixture (Table 8). This difference was significant with both individual preparations in the protein denaturation and antibacterial assays. In HRBC assay, the combination showed higher activity than Nilgiri and was non-significantly different from Neem. The above comparisons do not form a synergistic or supra-additive interaction due to the absence of a concentration matrix and a formal interaction model. The protein denaturation assay showed that the individual preparations were only slightly more concentrated, about 0.69 (for Neem) and 2.05 (for Nilgiri) percentage points more concentrated when the concentration of the preparation was doubled; however, the combination preparation was 4.45 percentage points more concentrated and showed an increasing descriptive difference from the higher individual preparations (6.6% to 11.2%).

The data on membrane stabilisation (Table 6) should be taken with a grain of salt. It proved to be more effective than either agent alone (no more than 1.8% above the maximum of either agent alone), but the three samples were above 94% stabilisation, at which the assay has minimal dynamic range. Erythrocyte membrane stabilization, in addition, is an indirect parameter of lysosomal membrane stabilisation and should be considered rather an anti-inflammatory activity than an antinociceptive activity. The hypothesis of reduced release of mediators sensitizing nociceptors could logically lead to the conclusion that this might result in an analgesic effect, but no such effect is claimed here, and such a model of nociception (in vivo) is required to make such a conclusion.

The combined formulation had greater zone of inhibition against Escherichia coli as compared to individual extracts (Table 7). Binary combination of Olea europaea and Ficus carica aqueous extracts has been shown to have a similar synergistic effect on antibacterial activity 9. The effect observed here could be due to the combined effect of the phytochemicals which can break the cell membrane of bacteria, inhibit essential enzymes and disrupt microbial metabolism, especially the lipophilic 1,8-cineole component in Nilgiri oil which may help the penetration of more polar constituents of Neem across the bacterial membrane 4,18.

The direction of observations are similar in the three assays, which helps support continued investigation, but there are some experimental variability issues and assay-specific effects that cannot be ruled out.

In general, the Neem-Eucalyptus mixture resulted in higher responses as compared to Neem or Eucalyptus alone under the experimental conditions. The differences between the two groups of the protein-denaturation and antibacterial assays are confirmed by the replications, but only concentration-response and formal combination studies may establish a reproducible pharmacological interaction.

Limitations of the Study

There are some significant limitations to this study. Precision and assessment of distributional assumptions were limited, as was precision of each biological assay, which contained only three independent experiments. The protein-denaturation test was performed on only two concentrations, and the other tests were performed on one concentration, so no complete concentration-response relationships or IC50 values were determined. It was not tested in a concentration matrix and it is not possible to determine if the interaction is additive, synergistic or antagonistic. Only one isolate of E. coli was screened for antibacterial activity, and the agar diffusion method was performed, which is affected by the volatility, viscosity and diffusion of essential oils. Minimum inhibitory and bactericidal concentrations were not determined. The responses to the membrane-stabilisation assays were very near the cut-off. Furthermore, the extracts were not quantified using chemical markers and batch to batch variation was not tested.

Conclusion

Neem leaf extract, eucalyptus essential oil and their 1:1 mixture showed measurable activity in the three screening assays. The mixture showed significantly higher protein-denaturation inhibition and antibacterial inhibition-zone diameters compared to each one of the preparations alone (an 11.2% difference in protein-denaturation inhibition at 200 µg/mL and a 21.4% difference in inhibition-zone diameter at 5 mg/mL). HRBC assay mixture was higher than Nilgiri but lower than Neem. The above observations do not show additivity or synergism. This combination is one that should be investigated with standardised preparations, larger numbers of independent experiments, complete concentration-response matrices, quantitative endpoints of antibacterial activity, and formal interaction models.

Abbreviations

ANOVA - Analysis of Variance; AR - Analytical Reagent; BSA - Bovine Serum Albumin; FTIR - Fourier Transform Infrared Spectroscopy; HRBC - Human Red Blood Cell; KBr - Potassium Bromide; Rf - Retardation/Retention Factor; SD - Standard Deviation; TLC - Thin Layer Chromatography; UV - Ultraviolet; WHO - World Health Organization; ZOI - Zone of Inhibition.

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Conflict of Interest

The authors declare no conflict of interest.

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