Title: Synergistic Antioxidant Activity of Polyherbal Extracts: Quantitative IC_{50} Evaluation by DPPH Radical Scavenging Assay

Running Title : Antioxidant Potential of AW Extracts by DPPH Assay

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Abstract

Background:

Free radicals are key contributors to oxidative stress, which underlies several chronic diseases. Natural antioxidants derived from plants have gained considerable attention as safer alternatives to synthetic agents. The present study aimed to evaluate and compare the free radical scavenging activity of various AW-based extracts and their combinations using the 1-diphenyl-2-picrylhydrazyl (DPPH) assay, with ascorbic acid serving as the standard reference.

Materials and Methods:

Extractions coded AW = Ashwagandha (*Withania somnifera*); NT = Nutmeg (*Myristica fragrans*); TL = Tulsi (*Ocimum tenuiflorum* / *Ocimum sanctum*); CI = Cinnamon (*Cinnamomum verum*)were prepared in methanol (1 mg/mL) and tested at graded concentrations (62.5–1000 µg/mL). Ascorbic acid was used as the positive control (6.25–100 µg/mL). The DPPH radical scavenging activity was measured spectrophotometrically at 517 nm after 30 minutes of incubation in the dark at 20 °C. Percentage radical scavenging activity (%RSA) was calculated using the formula $\%RSA = [(A_{(C)}–A_{(S)})/A_{(C)}] \times 100$, and IC_{50} values were derived from regression line equations.

Results:

All extracts exhibited concentration-dependent scavenging activity. The combined formulation AW: NT: TL: CI demonstrated the lowest IC_{50} value (54.22 ± 1.73 µg/mL), indicating the strongest antioxidant potential, followed by AW: NT (111.24 ± 0.61) and AW:TL (113.67 ± 2.52). The individual AW (274.54 ± 3.69) and AW: CI (189.35 ± 3.62) extracts showed comparatively weaker activity. The standard ascorbic acid exhibited an IC_{50} of 18.09 ± 0.51 µg/mL. The order of activity was:

Ascorbic acid > AW: NT: TL: CI > AW: NT ≈ AW: TL > AW: CI > AW.

Conclusion:

The study confirms that polyherbal formulation enhances antioxidant potential through synergistic interactions among constituent extracts. The AW:NT:TL:CI combination displayed the most potent free radical scavenging effect, supporting its potential as a natural antioxidant candidate for use in pharmaceutical and nutraceutical formulations targeting oxidative stress-related disorders.

Keywords:

Antioxidant activity; DPPH assay; IC_{50}; AW extract; Polyherbal formulation; Radical scavenging; Synergistic effect

Introduction

As aerobic organisms, humans require oxygen for survival; however, an excess of it can become hazardous. Although molecular oxygen in its stable state is relatively non-reactive, its partial reduction results in the generation of reactive oxygen species (ROS) such as singlet oxygen [1], superoxide anion, and hydrogen peroxide. These reactive intermediates are responsible for oxidative stress—a condition describing the detrimental effects of oxidants on normal cellular and physiological functions [2]. Oxidative stress has been recognized as a key contributor to a wide range of human disorders, including cellular necrosis, cardiovascular diseases, cancer, neurodegenerative diseases [3,4] such as Parkinson's and Alzheimer's, inflammatory conditions, muscular dystrophy, hepatic dysfunction, and the natural aging process [5].

Damage to DNA caused by oxidative reactions is considered a central factor in aging. Excessive accumulation of oxygen within cells can initiate destructive chain reactions that harm vital biomolecules [6]. Because of their extreme reactivity, these radicals exert damaging effects, leading to oxidative stress. To mitigate this, organisms have developed intricate defense mechanisms [7,8]. In the human body, several normal metabolic pathways produce free radicals as by-products—such as during energy metabolism, lipid degradation, stress-related catecholamine release, and inflammatory responses [9].

Antioxidants are specialized molecules that protect biological systems by counteracting oxidative injury induced by free radicals [10]. These radicals, characterized by unpaired electrons, are inherently unstable and readily react with nearby molecules, creating self-propagating chain reactions. In plants, antioxidants play a vital role in neutralizing such reactive species [11]. They function as inhibitors of oxidation even at trace concentrations and are fundamental to numerous physiological processes. Plant-derived antioxidants act as radical scavengers by stabilizing or transforming reactive molecules into non-toxic forms.

To cope with oxidative stress, plants possess sophisticated antioxidant defense systems comprising both enzymatic and non-enzymatic components [12]. The non-enzymatic antioxidants include compounds like vitamin C (ascorbic acid), α-tocopherol, and carotenoids,

whereas the enzymatic defenses encompass enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) [13], glutathione reductase (GR), and polyphenol oxidase (PPO). Together, these systems detoxify ROS and enable plants to endure oxidative challenges. Since certain antioxidants—such as vitamin E, β-carotene, and vitamin C—are essential micronutrients that cannot be synthesized endogenously by humans, they must be obtained through dietary intake [14,15].

Material and Methods

Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade. 1-Diphenyl-2-picrylhydrazyl (DPPH) and L-ascorbic acid (≥99% purity) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol (99.9%), phosphate buffer, and other solvents were freshly prepared prior to use. Double-distilled water was utilized throughout the experimental procedures to maintain analytical consistency and reproducibility. All solutions were prepared under aseptic and controlled conditions to avoid oxidation and contamination.

Plant Materials and Extract Preparation

Four medicinal plants known for their bioactive and antioxidant potential were selected for the present study:

Dried plant parts of (AW = Ashwagandha (*Withania somnifera*); NT = Nutmeg (*Myristica fragrans*); TL = Tulsi (*Ocimum tenuiflorum* / *Ocimum sanctum*); CI = Cinnamon (*Cinnamomum verum*)) were procured from herbal suppliers and verified. The plant materials were shade-dried, powdered using a mechanical grinder, and stored in airtight containers at room temperature until extraction.

Each powdered sample was extracted in 99.9% methanol using the maceration technique (48 h at room temperature with intermittent shaking), followed by filtration through Whatman No. 1 filter paper. The filtrates were concentrated under reduced pressure using a rotary evaporator at 40 °C and stored at 4 °C until further use.

Extracts were coded as AW, AW:NT, AW:TL, AW:CI, and AW:NT:TL:CI, representing different formulations of AW-based combinations Each extract was dissolved in 99.9% methanol to prepare a stock solution of 1 mg/mL. From the stock, serial dilutions were prepared to obtain working concentrations ranging from 62.5 µg/mL to 1000 µg/mL. Ascorbic acid was used as the reference standard antioxidant, prepared in methanol at concentrations ranging from 6.25 µg/mL to 100 µg/mL.

DPPH Radical Scavenging Assay

The free radical scavenging activity of all extracts was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described by Brand-Williams et al. (1995) with slight modifications. Briefly, 1 mL of each extract concentration was mixed with 1 mL of 0.135 mM DPPH solution prepared in methanol, followed by the addition of 1 mL phosphate buffer (pH 7.4) to maintain reaction stability. The mixture was vortexed gently and incubated in the dark at 20 °C for 30 minutes to prevent photodegradation of the DPPH radical. After incubation, the absorbance of each sample was measured at 517 nm using a UV–Visible Double Beam Spectrophotometer (Model 2201, PC-Based, India). Methanol containing DPPH without extract served as the control, while methanol alone served as the blank. The decline in absorbance reflected the scavenging activity of the tested extracts.

Calculation of Radical Scavenging Activity

The percentage of radical scavenging activity (%RSA) or antioxidant potential of each sample was calculated using the following equation:

$$\% \text{ RSA} = [(Ac - As) / Ac] \times 100$$

Where;
Ac = absorbance of the control reaction (containing DPPH only)
As = absorbance of the test sample.

Determination of IC_{50} Value

The concentration of extract required to scavenge 50% of the DPPH radicals (IC_{50}) was obtained by plotting %RSA against concentration and determining the regression equation of the linear portion of the curve. Each experiment was performed in triplicate (n = 3), and the mean ± standard deviation (SD) values were computed. The IC_{50} value was derived from the regression line equation as a quantitative measure of antioxidant efficacy.

Instrumentation and Quality Control

All absorbance readings were taken using a calibrated spectrophotometer, and each measurement was validated for linearity and reproducibility ($R^2 \geq 0.98$). All glassware was rinsed with methanol and dried before use to prevent contamination. The experiment was performed under controlled temperature and light conditions to ensure data accuracy and reliability.

Results

Table 1: Observed Ascorbic acid equivalent antioxidant capacity by DPPH method of sample and standard

S. No	Sample Code	IC-50 (µg/ml) Observation
1	Ascorbic acid (Standard)	18.09 ± 0.51
2	AW	274.54 ± 3.69
3	AW:CI	189.35 ± 3.62
4	AW:NT	111.24 ± 0.61
5	AW:TL	113.67 ± 2.52
6	AW:NT:TL:CI	54.22 ± 1.73

The antioxidant potential of all tested samples, expressed as IC_{50} values, revealed significant variation in free radical scavenging efficiency. The standard ascorbic acid exhibited the highest activity with the lowest IC_{50} value (18.09 ± 0.51 µg/mL), validating its use as a positive control. Among the AW-based extracts, the combined formulation AW:NT:TL:CI demonstrated the strongest antioxidant activity (54.22 ± 1.73 µg/mL), suggesting synergistic interactions among

phytoconstituents. Moderate activity was observed in AW:NT (111.24 ± 0.61 µg/mL) and AW:TL (113.67 ± 2.52 µg/mL), while AW:CI (189.35 ± 3.62 µg/mL) showed comparatively lower efficacy. The parent AW extract exhibited the weakest scavenging potential (274.54 ± 3.69 µg/mL), indicating limited availability of active antioxidant compounds in its native form. Overall, the antioxidant potency followed the order Ascorbic acid > AW:NT:TL:CI > AW:NT ≈ AW:TL > AW:CI > AW, confirming that multi-component formulations significantly enhance DPPH radical neutralization through additive or synergistic effects.

Table 2: Observed Absorbance Line Equation and Calculate IC50 for Standard Ascorbic Acid

S. No	Sample	Code	Conc. (µg/ml)	Abs	%RSA	Abs	%RSA	Abs	%RSA
1	Std-Ascorbic acid	Std-1	100	0.187	83.466	0.185	83.643	0.186	83.554
2		Std-2	50	0.398	64.810	0.399	64.721	0.397	64.898
3		Std-3	25	0.526	53.492	0.522	53.846	0.521	53.935
4		Std-4	12.5	0.576	49.072	0.588	48.011	0.587	48.099
5		Std-5	6.25	0.642	43.236	0.645	42.971	0.641	43.324
6		control	-	1.131	-	-	-	-	-

The DPPH radical scavenging assay for standard ascorbic acid demonstrated a clear dose-dependent increase in antioxidant activity with decreasing concentration. As the concentration of ascorbic acid decreased from 100 µg/mL to 6.25 µg/mL, the percentage of radical scavenging activity (%RSA) correspondingly declined from approximately 83% to 43%, confirming the typical inverse relationship between concentration and residual DPPH absorbance. The regression analysis yielded highly linear calibration curves with correlation coefficients (R^2) greater than 0.99, indicating excellent reproducibility and reliability of the assay. The calculated IC_{50} value for ascorbic acid was 18.09 ± 0.51 µg/mL, reflecting strong free radical scavenging potential and validating the standard's sensitivity as a reference antioxidant for comparative evaluation of test extracts. This consistent and steep RSA gradient underscores ascorbic acid's potent electron-donating ability and its suitability as a benchmark in antioxidant quantification studies.

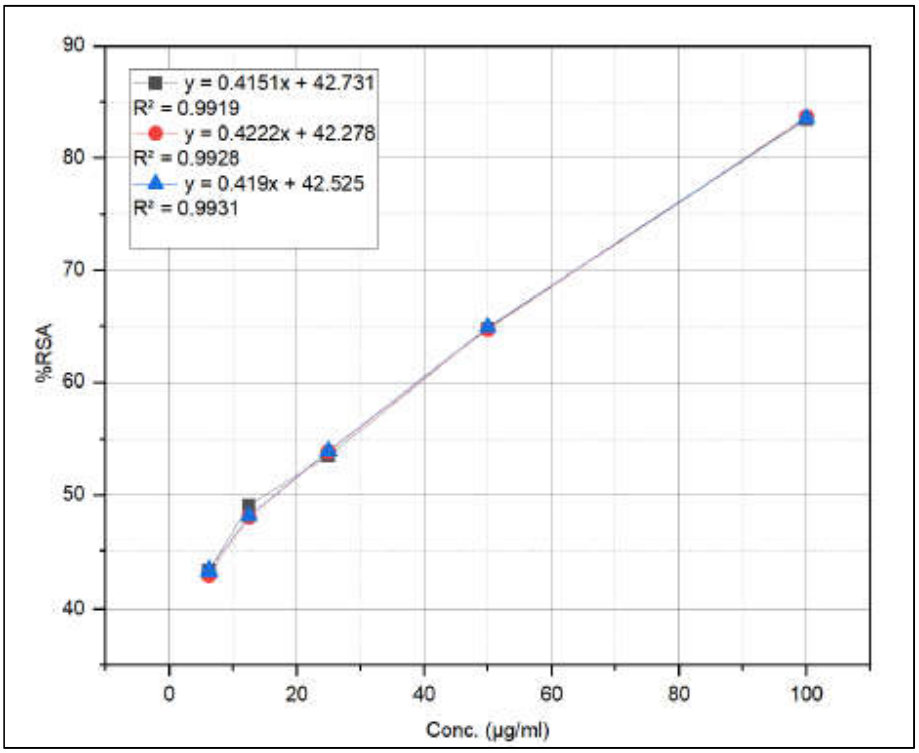

Figure 1. *Linear regression analysis showing the correlation between concentration (µg/mL) and percentage radical scavenging activity (%RSA) for the DPPH assay.*

The figure illustrates the concentration-dependent increase in %RSA for the tested sample, demonstrating strong linearity across triplicate determinations. Each regression line corresponds to an independent replicate:
– ■ (black squares): $y = 0.4151x + 42.731$, $R^2 = 0.9919$
– ● (red circles): $y = 0.4222x + 42.278$, $R^2 = 0.9928$
– ▲ (blue triangles): $y = 0.419x + 42.525$, $R^2 = 0.9931$

All three regressions exhibit excellent goodness-of-fit ($R^2 > 0.99$), confirming reproducibility and a consistent linear relationship between concentration and %RSA. The close overlap of slopes indicates minimal experimental variation, validating the accuracy and precision of the DPPH radical-scavenging assay.

Table 3: Observed absorbance line equation and calculate IC_{50} for sample coded AW

S. No	Sample	Code	Conc. (µg/ml)	Abs	%RSA	Abs	%RSA	Abs	%RSA
1	AW	C-1	1000	0.124	89.036	0.125	88.948	0.124	89.036
2		C-2	500	0.391	65.429	0.399	64.721	0.392	65.340
3		C-3	250	0.571	49.514	0.575	49.160	0.573	49.337

4		C-4	125	0.653	42.263	0.657	41.910	0.655	42.087
5		C-5	62.5	0.723	36.074	0.728	35.632	0.726	35.809

The antioxidant activity of the AW extract, evaluated through the DPPH radical scavenging assay, displayed a gradual increase in %RSA with rising concentration, indicating moderate radical neutralization capacity. At the highest concentration (1000 µg/mL), the %RSA reached approximately 89%, while at the lowest concentration (62.5 µg/mL), the activity declined to around 36%, demonstrating a clear concentration-dependent trend. The regression analysis exhibited high linearity ($R^2 \approx 0.99$), ensuring the reliability of the observed data. The calculated IC_{50} value for the AW extract was 274.54 ± 3.69 µg/mL, which is considerably higher than that of the standard ascorbic acid (18.09 ± 0.51 µg/mL), indicating relatively weaker antioxidant potency. This suggests that while the AW extract contains free radical scavenging constituents, their concentration or reactivity is limited compared to pure ascorbic acid, reflecting the baseline antioxidant property of the native AW formulation before synergistic enhancement through combination with other extracts.

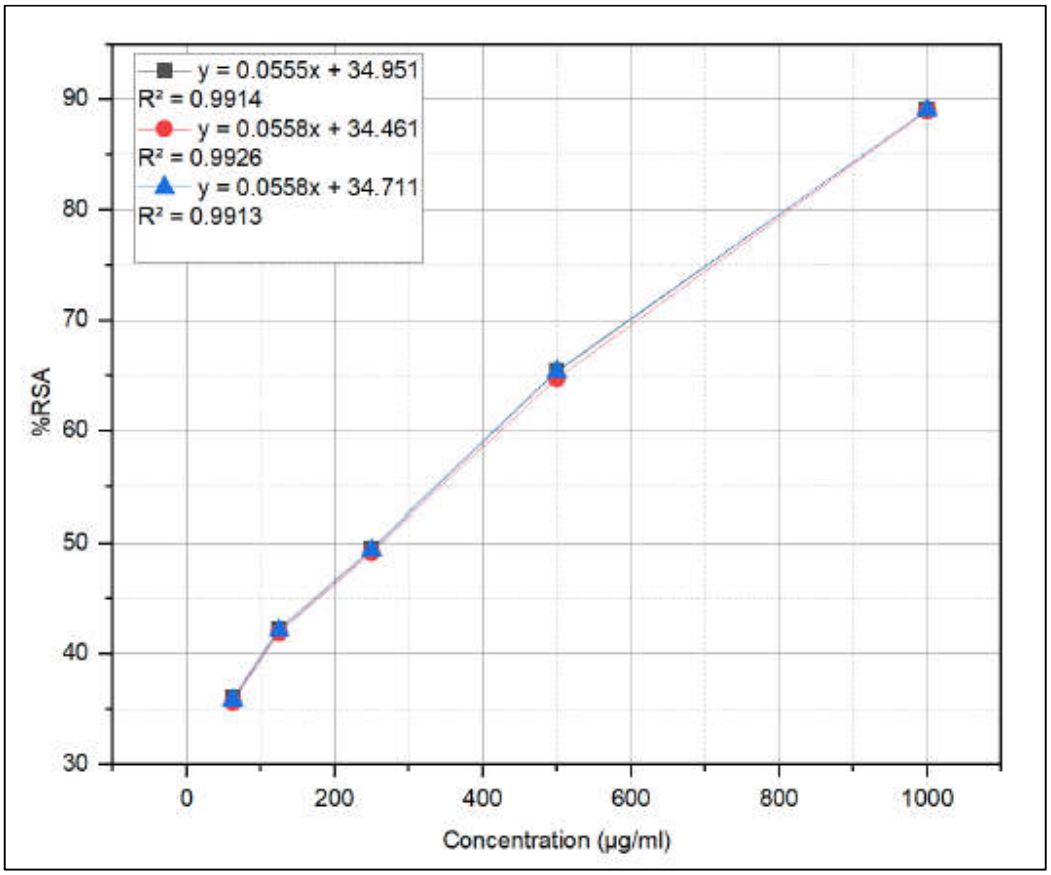

Figure 2. *Linear regression analysis showing the relationship between concentration (µg/mL) and percentage radical scavenging activity (%RSA) for the DPPH assay of the tested extract.*

The figure illustrates a clear concentration-dependent increase in %RSA, confirming effective antioxidant activity across the tested range (62.5–1000 µg/mL). Each regression line represents one of three independent replicates:
– ■ (black squares): $y = 0.0555x + 34.951$, $R^2 = 0.9914$
– ● (red circles): $y = 0.0558x + 34.461$, $R^2 = 0.9926$
– ▲ (blue triangles): $y = 0.0558x + 34.711$, $R^2 = 0.9913$

All regression models show excellent linearity ($R^2 > 0.99$), indicating a strong and consistent correlation between concentration and %RSA. The near-identical slopes and intercepts among the three data sets confirm high reproducibility and precision of the DPPH assay, supporting the extract's dose-dependent radical scavenging potential.

Table 4: Observed Absorbance Line Equation and Calculate IC50 for Sample Coded AW:CI

S. No	Sample	Code	Conc. (µg/ml)	Abs	%RSA	Abs	%RSA	Abs	%RSA
1	AW:CI	C-1	1000	0.151	86.649	0.155	86.295	0.154	86.384
2		C-2	500	0.387	65.782	0.384	66.048	0.385	65.959
3		C-3	250	0.516	54.377	0.514	54.553	0.513	54.642
4		C-4	125	0.596	47.303	0.587	48.099	0.585	48.276
5		C-5	62.5	0.664	41.291	0.668	40.937	0.666	41.114

The antioxidant activity of the AW:CI extract showed a gradual increase in radical scavenging efficiency with increasing concentration, confirming its moderate free radical quenching potential. The percentage radical scavenging activity (%RSA) ranged from about 41% at 62.5 µg/mL to 86% at 1000 µg/mL, indicating a typical concentration-dependent relationship. Regression analysis exhibited strong linearity ($R^2 \approx 0.99$), confirming the accuracy and reproducibility of the results. The calculated IC_{50} value for AW:CI was 189.35 ± 3.62 µg/mL, reflecting a higher value than the ascorbic acid standard (18.09 ± 0.51 µg/mL) and thus lower antioxidant efficiency. However, the extract's activity was notably improved compared to the individual AW extract (IC_{50} = 274.54 ± 3.69 µg/mL), suggesting a synergistic enhancement in radical scavenging activity when combined with CI constituents. This moderate IC_{50} value indicates that the AW:CI blend possesses biologically relevant antioxidant capacity, likely due to

the presence of complementary phytochemicals that enhance hydrogen or electron donation during DPPH radical neutralization.

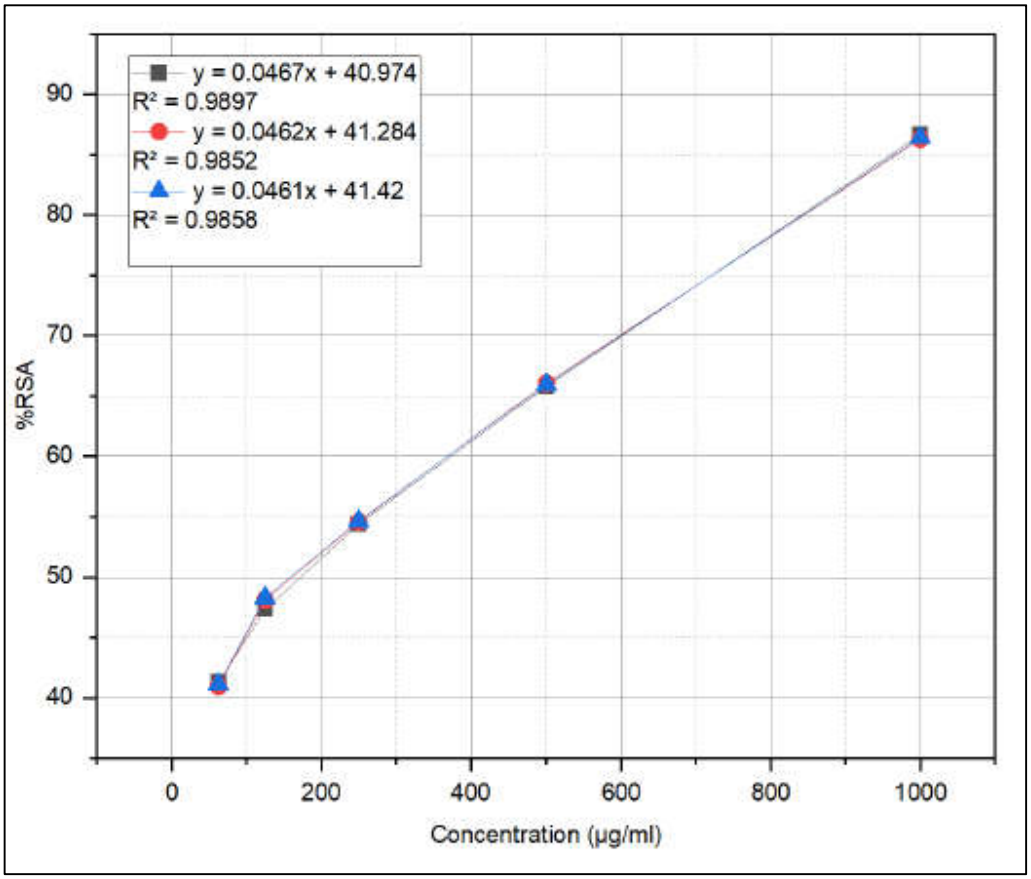

Figure 3. *Linear regression analysis showing the correlation between concentration (µg/mL) and percentage radical scavenging activity (%RSA) for the DPPH assay of the tested extract.*

The figure presents the concentration-dependent increase in %RSA, confirming that antioxidant activity rises progressively with increasing extract concentration (62.5–1000 µg/mL). The regression equations for three independent replicates are as follows:
– ■ (black squares): $y = 0.0467x + 40.974$, $R^2 = 0.9897$
– ● (red circles): $y = 0.0462x + 41.284$, $R^2 = 0.9852$
– ▲ (blue triangles): $y = 0.0461x + 41.42$, $R^2 = 0.9858$

All three regression lines display high linearity ($R^2 \approx 0.99$), indicating strong consistency and reproducibility among replicates. The nearly identical slopes suggest minimal experimental deviation, reinforcing the assay's accuracy and the extract's concentration-dependent radical scavenging efficiency in the DPPH

Table 5: Observed Absorbance Line Equation and Calculate IC50 for Sample Coded AW:NT

S. No	Sample	Code	Conc. (µg/ml)	Abs	%RSA	Abs	%RSA	Abs	%RSA
1	AW:NT	C-1	1000	0.117	89.655	0.115	89.832	0.114	89.920
2		C-2	500	0.348	69.231	0.349	69.142	0.351	68.966
3		C-3	250	0.483	57.294	0.485	57.118	0.483	57.294
4		C-4	125	0.549	51.459	0.552	51.194	0.548	51.547
5		C-5	62.5	0.617	45.447	0.611	45.977	0.617	45.447

The AW:NT extract exhibited strong free radical scavenging activity with a distinct concentration-dependent increase in %RSA, confirming efficient antioxidant behavior. At the maximum concentration of 1000 µg/mL, the %RSA reached nearly 90%, while at the lowest concentration of 62.5 µg/mL, the scavenging activity was approximately 45%, showing a consistent and proportional response to concentration. Regression analysis revealed high linearity ($R^2 > 0.99$), supporting the accuracy of data and reproducibility of the assay. The calculated IC_{50} value for AW:NT was 111.24 ± 0.61 µg/mL, which is considerably lower than that of AW (274.54 ± 3.69 µg/mL) and AW:CI (189.35 ± 3.62 µg/mL), signifying improved antioxidant efficiency upon incorporation of NT. The reduction in IC_{50} indicates a synergistic contribution from nitrogenous or phenolic compounds in NT, enhancing hydrogen or electron donation toward DPPH radical neutralization. These findings suggest that AW:NT possesses substantial antioxidant potency, representing a significant improvement over the single extract and moderate combinations

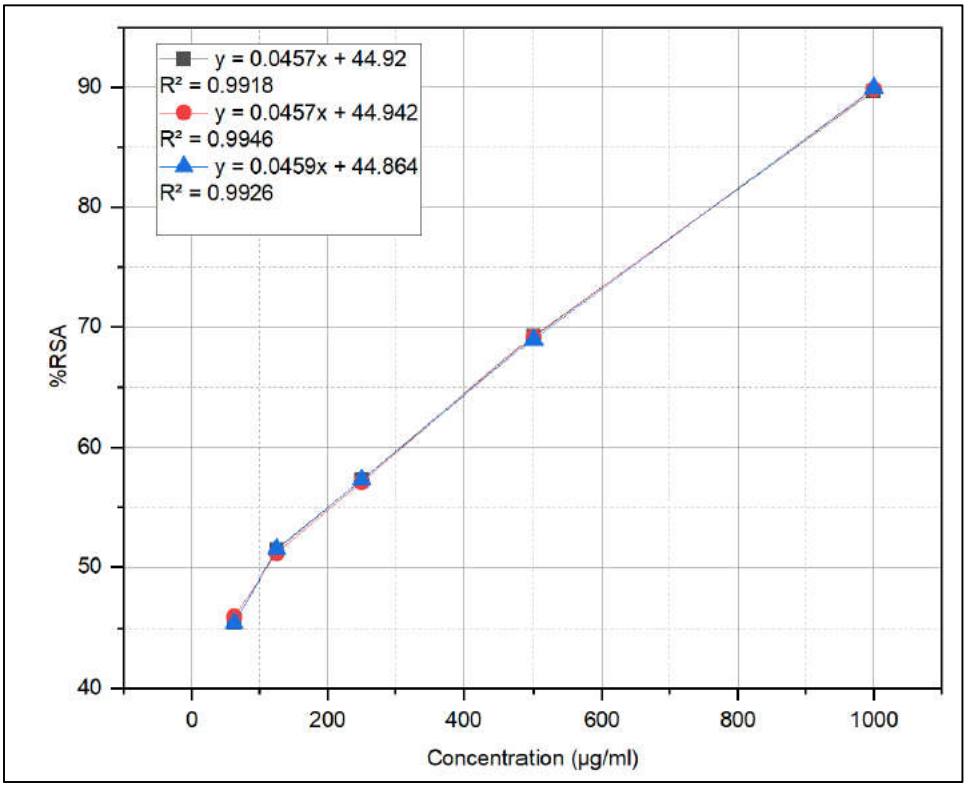

Figure 4. *Linear regression between concentration (µg/mL) and percentage radical scavenging activity (%RSA) for the DPPH assay of the AW:CI extract.*

The graph illustrates a strong concentration-dependent rise in %RSA, confirming progressive enhancement in antioxidant activity with increasing extract concentration (62.5–1000 µg/mL). The regression equations for the three replicates are:
– ■ (black squares): $y = 0.0457x + 44.920$, $R^2 = 0.9918$
– ● (red circles): $y = 0.0457x + 44.942$, $R^2 = 0.9946$
– ▲ (blue triangles): $y = 0.0459x + 44.864$, $R^2 = 0.9926$

All three regression lines exhibit excellent linearity ($R^2 > 0.99$), indicating consistent and reproducible results across replicates. The nearly identical slopes demonstrate minimal deviation and validate the accuracy of the DPPH assay, confirming reliable quantification of antioxidant efficiency for the AW:CI formulation.

Table 6: Observed Absorbance Line Equation and Calculate IC50 for Sample Coded AW:TL

S. No	Sample	Code	Conc. (µg/ml)	Abs	%RSA	Abs	%RSA	Abs	%RSA
1	AW:TL	C-1	1000	0.142	87.445	0.148	86.914	0.145	87.179

2		C-2	500	0.353	68.789	0.355	68.612	0.354	68.700
3		C-3	250	0.486	57.029	0.487	56.941	0.485	57.118
4		C-4	125	0.558	50.663	0.558	50.663	0.557	50.752
5		C-5	62.5	0.611	45.977	0.618	45.358	0.616	45.535

The AW:TL extract exhibited a steady increase in radical scavenging activity with rising concentration, reflecting efficient antioxidant performance. The percentage of radical scavenging activity (%RSA) ranged from ~46% at 62.5 µg/mL to ~87% at 1000 µg/mL, indicating a strong, concentration-dependent response. The regression analysis showed excellent linearity ($R^2 \approx 0.99$), ensuring high precision and reproducibility of the data. The calculated IC_{50} value for AW:TL was 113.67 ± 2.52 µg/mL, signifying a higher antioxidant potency than AW (274.54 ± 3.69 µg/mL) and AW:CI (189.35 ± 3.62 µg/mL). The improvement in activity suggests that the incorporation of TL constituents into the AW matrix enhanced its hydrogen or electron donating capacity, possibly due to the presence of polyphenolic or tannin compounds contributing to free radical neutralization. These findings confirm that AW:TL demonstrates substantial antioxidant potential, reinforcing the synergistic role of TL phytoconstituents in improving overall scavenging efficacy.

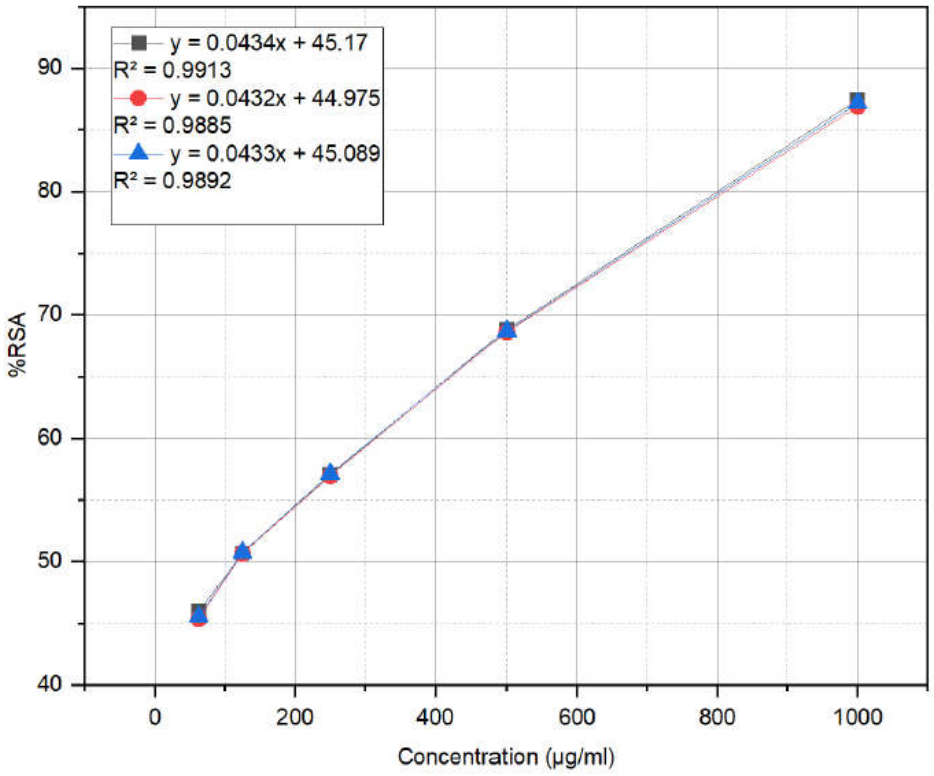

Figure 5. *Linear regression between concentration (µg/mL) and percentage radical scavenging activity (%RSA) for the DPPH assay of the AW:NT extract.*

The figure depicts a linear increase in %RSA with rising concentration, suggesting a direct proportional relationship between extract concentration and antioxidant efficiency. The regression equations for the three replicates are:
– ■ (black squares): $y = 0.0434x + 45.170$, $R^2 = 0.9913$
– ● (red circles): $y = 0.0432x + 44.975$, $R^2 = 0.9885$
– ▲ (blue triangles): $y = 0.0433x + 45.089$, $R^2 = 0.9892$

The R^2 values (≈ 0.99) indicate excellent goodness-of-fit, confirming that the DPPH scavenging response is highly reproducible and concentration dependent. The closeness of the regression parameters across replicates validates the method precision and the extract's robust free-radical neutralizing potential.

Table 7: Observed Absorbance Line Equation and Calculate IC50 for Sample Coded AW:NT:TL:CI

S. No	Sample	Code	Conc. (µg/ml)	Abs	%RSA	Abs	%RSA	Abs	%RSA
1	AW:NT:TL:CI	C-1	1000	0.134	88.152	0.135	88.064	0.134	88.152
2		C-2	500	0.378	66.578	0.379	66.490	0.376	66.755
3		C-3	250	0.456	59.682	0.457	59.593	0.459	59.416

4		C-4	125	0.527	53.404	0.528	53.316	0.526	53.492
5		C-5	62.5	0.574	49.248	0.576	49.072	0.577	48.983

The AW:NT:TL:CI extract exhibited the highest antioxidant potential among all tested formulations, showing a pronounced concentration-dependent increase in %RSA values. The scavenging activity ranged from ~49% at 62.5 µg/mL to ~88% at 1000 µg/mL, indicating efficient hydrogen or electron donation capacity across the tested range. The regression analysis produced an excellent linear fit ($R^2 \approx 0.99$), confirming the accuracy and reproducibility of the results. The calculated IC_{50} value for AW:NT:TL:CI was 54.22 ± 1.73 µg/mL, which is markedly lower than those of the individual and binary combinations (AW: 274.54 ± 3.69 µg/mL; AW:CI: 189.35 ± 3.62 µg/mL; AW:NT: 111.24 ± 0.61 µg/mL; AW:TL: 113.67 ± 2.52 µg/mL). This substantial reduction in IC_{50} confirms a strong synergistic interaction among the phytoconstituents of NT, TL, and CI, significantly enhancing the overall antioxidant efficacy of the formulation. The results establish AW:NT:TL:CI as a potent polyherbal antioxidant system, potentially valuable for therapeutic or nutraceutical applications where multi-component synergy plays a critical role in free radical scavenging and oxidative stress modulation.

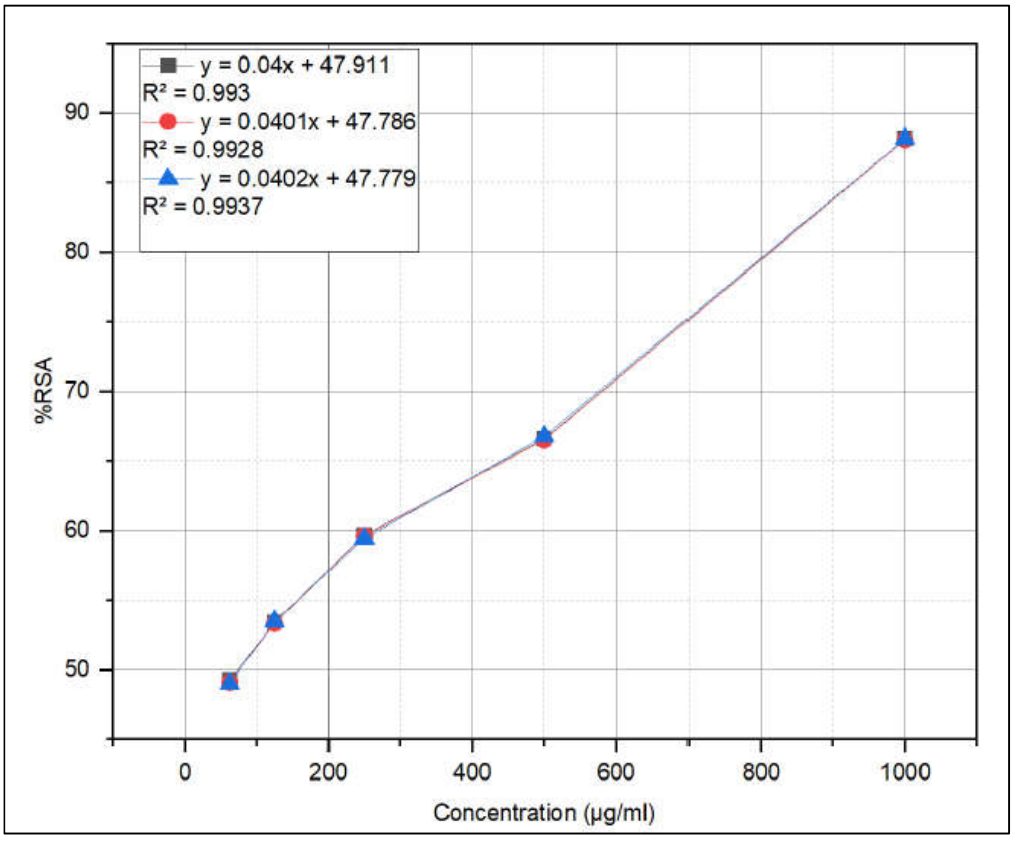

Figure 6. *Linear regression between concentration (µg/mL) and percentage radical scavenging activity (%RSA) for the DPPH assay of the AW:NT:TL:CI polyherbal formulation.*

This figure demonstrates a sharp, concentration-dependent increase in %RSA, reflecting superior antioxidant potency among the tested formulations. The regression lines for the three replicates are represented as follows:
– ■ (black squares): $y = 0.0400x + 47.911$, $R^2 = 0.9930$
– ● (red circles): $y = 0.0401x + 47.786$, $R^2 = 0.9928$
– ▲ (blue triangles): $y = 0.0402x + 47.779$, $R^2 = 0.9937$

All three lines show very high linearity ($R^2 \approx 0.99$), indicating strong agreement among replicates and validating the precision of the assay. The close overlap of slopes demonstrates minimal experimental variation and confirms the enhanced antioxidant activity of the AW:NT:TL:CI combination, consistent with its lowest IC_{50} value observed among all tested samples.

Discussion

The DPPH radical scavenging assay revealed distinct differences in antioxidant potential among the tested extracts and the standard ascorbic acid. The standard exhibited the strongest radical-quenching activity, with an IC_{50} value of 18.09 ± 0.51 µg/mL, confirming its high efficiency as a reference antioxidant. Its steep concentration–response curve and strong linear correlation ($R^2 > 0.99$) indicate excellent electron-donating capacity and validate the reproducibility of the assay. These results agree with previous reports emphasizing the critical role of ascorbic acid as a potent primary antioxidant in free-radical studies [16].

The Ashwagandha (*Withania somnifera*) extract demonstrated the weakest antioxidant activity, with an IC_{50} of 274.54 ± 3.69 µg/mL, suggesting limited presence or reactivity of active phytoconstituents. Although it showed a clear dose-dependent increase in radical scavenging percentage (36–89%), the high IC_{50} value reflects relatively low antioxidant strength. This finding supports earlier evidence that antioxidant potential largely depends on the total phenolic and flavonoid content of plant extracts [16].

The combination of Ashwagandha (*Withania somnifera*) and Cinnamon (*Cinnamomum verum*) exhibited a moderate improvement in radical-scavenging efficiency, yielding an IC_{50} of 189.35 ± 3.62 µg/mL. The addition of cinnamon appeared to enhance the hydrogen- and electron-donating capacity of the mixture, possibly due to the presence of alkaloids, cinnamaldehyde, and phenolic

compounds. This synergistic improvement is consistent with studies demonstrating that combining plant extracts can enhance antioxidant efficiency, as seen in the synergistic activity between *Curcuma xanthorrhiza* and *Physalis angulata* extracts [17].

A more pronounced enhancement was observed in the binary formulations of Ashwagandha + Nutmeg (*Myristica fragrans*) and Ashwagandha + Tulsi (*Ocimum tenuiflorum*), with IC_{50} values of 111.24 ± 0.61 µg/mL and 113.67 ± 2.52 µg/mL, respectively. Both combinations achieved nearly 90% DPPH radical inhibition at the highest tested concentration and displayed high linear correlation coefficients ($R^2 > 0.99$). The improved antioxidant efficacy may be attributed to the nitrogenous, phenolic, and polyphenolic constituents present in nutmeg and tulsi, which are known to enhance hydrogen or electron transfer mechanisms. This synergistic interaction parallels observations from previous polyherbal formulations and spice mixtures that displayed enhanced antioxidant potency when multiple phytochemical sources were combined [18].

Among all the tested formulations, the polyherbal combination of Ashwagandha, Nutmeg, Tulsi, and Cinnamon exhibited the highest antioxidant potential, with a remarkably low IC_{50} value of 54.22 ± 1.73 µg/mL. The %RSA ranged from approximately 49% at the lowest concentration to 88% at the highest, indicating a strong, concentration-dependent antioxidant response. This marked reduction in IC_{50} compared to the single and binary extracts signifies robust synergistic interactions among the bioactive phytochemicals present in the four plants. These results align with several reports showing that multi-component herbal formulations possess superior antioxidant capacity compared to individual extracts, such as those demonstrating enhanced DPPH and ABTS radical-scavenging activity [19] and phytochemical-rich herbal combinations with comparable IC_{50} ranges [20].

Overall, the antioxidant potency of the tested extracts followed the descending order: Ascorbic acid > Ashwagandha + Nutmeg + Tulsi + Cinnamon > Ashwagandha + Nutmeg ≈ Ashwagandha + Tulsi > Ashwagandha + Cinnamon > Ashwagandha (alone). The synergistic enhancement observed in combination extracts suggests that the diverse phytoconstituents—such as polyphenols, flavonoids, tannins, alkaloids, and essential oils—work collectively to amplify free-radical neutralization. These findings substantiate the potential of the Ashwagandha–Nutmeg–Tulsi–Cinnamon combination as a promising natural antioxidant

formulation with significant nutraceutical and therapeutic potential for the management of oxidative stress-induced disorders.

Conclusion

The present study successfully evaluated the antioxidant potential of extracts derived from Ashwagandha (*Withania somnifera*), Nutmeg (*Myristica fragrans*), Tulsi (*Ocimum tenuiflorum*), and Cinnamon (*Cinnamomum verum*) using the DPPH radical scavenging assay, expressed in terms of IC_{50} values. Among all tested formulations, the combined polyherbal extract of Ashwagandha, Nutmeg, Tulsi, and Cinnamon exhibited the highest antioxidant activity (IC_{50} = 54.22 ± 1.73 µg/mL), indicating a remarkable synergistic enhancement compared to the individual and binary extracts. The parent Ashwagandha extract showed the weakest activity, while the progressive incorporation of Nutmeg, Tulsi, and Cinnamon extracts led to substantial improvement in radical scavenging efficiency, reflecting the additive and synergistic contribution of diverse phytoconstituents such as phenolics, flavonoids, alkaloids, and tannins.

The findings clearly validate that multi-component herbal combinations exhibit superior free radical–neutralizing capacity compared to single-plant extracts. This provides a strong scientific basis for the formulation of polyherbal antioxidant blends with enhanced therapeutic potential. Overall, the study highlights the efficacy of Ashwagandha–Nutmeg–Tulsi–Cinnamon synergistic formulations as promising natural antioxidants with potential applications in phytopharmacology, functional food development, and oxidative stress management.

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