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EVALUATION OF ANTIOXIDANT POTENTIAL OF PROPRANOLOL HCL & ASCORBIC ACID COMBINATION FORMULATION USING IN VITRO CHEMICAL METHODS

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Abstract
Amplified Oxidative stress impart a serious role in the pathophysiology of hypertension, associated cardiovascular complications, interruption in several intracellular signaling, DNA damages and genomic integrity. Various antihypertensive drugs and their metabolites reduce oxidative stress and plays a major in balancing ROS level. Combining antihypertensive agents with antioxidants may offer synergistic therapeutic benefits and increased influence on oxidative response. This study aimed to evaluate the in vitro antioxidant potential of a combination formulation containing Propranolol HCl and Ascorbic acid using established chemical methods. The antioxidant activity was assessed using (DPPH) radical scavenging assay, Superoxide Anion Scavenging Assay, Hydrogen peroxide scavenging activity, ABTS Radical Cation Decolorization Assay, Ferric Reducing Antioxidant Power (FRAP) Assay, Hydroxyl Radical Scavenging Assay. The combination formulation was compared against individual components and standard antioxidants. The combination formulation demonstrated enhanced antioxidant activity compared to individual components. The IC_{50} values for DPPH scavenging were 1052.93 µg/mL for the combination versus 1269.62 µg/mL for Propranolol HCl alone and 1473.55 µg/mL for Ascorbic acid alone. The Propranolol HCl–Ascorbic acid combination exhibited significant antioxidant potential, suggesting potential therapeutic advantages in managing hypertension-associated oxidative stress and other cellular genomic damages due to oxidative stress. These findings support further investigation in biological models.

KEYWORDS: Antioxidant activity; Antihypertensive; Ascorbic acid; DPPH; FRAP; Oxidative stress; Combination therapy; genomic integrity.

1. INTRODUCTION

Hypertension is a major global health concern affecting approximately 1.4 billion adults worldwide, representing a significant risk factor for cardiovascular disease, stroke, and renal failure based on WHO's second Global Report on Hypertension, 2025. The pathophysiology of hypertension involves multiple mechanisms, among which oxidative stress has emerged as a critical contributing factor (1,2).

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms (3). In hypertensive patients, elevated levels of superoxide anions, hydrogen peroxide, and hydroxyl radicals contribute to endothelial dysfunction, vascular remodeling, and end-organ damage (4,5). Increased ROS directly induce DNA damage and compromise genomic stability. There is a complicated association between genetic characters and oxidative stress which could be associated with the pathogenesis of various conditions (6).

Also, ROS imbalance and persistent oxidative DNA damage is a key driver of several cancer initiation and progression. This corner and relationship between oxidative stress, hypertension and pathophysiological condition related to cellular genomic damage has prompted interest in therapeutic strategies that combine antihypertensive effects with antioxidant properties.

Propranolol HCl, a drug of class non-selective Beta blocker, is widely prescribed for hypertension management. It works primarily by non-selectively blocking β-1 and β-2 adrenergic receptor, reducing heart rate, myocardial contractibility and sympathetic nervous system activity. By competitively inhibiting catecholamines (epinephrine and norepinephrine). Propranolol HCl reduces the chronotropic (heart rate), inotropic (contractility), and dromotropic (AV node conduction) responses to sympathetic stimulation. This leads to a decrease in cardiac output and myocardial oxygen demand, which is beneficial in conditions like hypertension, angina, and certain arrhythmias. Thus, making it effective for cardiovascular, neurological, and anxiety-related conditions (7,8). Previous studies have suggested that Propranolol HCl may possess intrinsic antioxidant properties attributed to its metabolite; 4-Hydroxypropranolol. The primary mechanism of action is chain-breaking activity, which neutralize free radicals and protects against lipid peroxidation and cellular damage (9).

Ascorbic acid (vitamin C) is a water-soluble antioxidant capable to scavenges free radicals, modulated enzymatic antioxidant systems and regenerates other antioxidants (10). Ascorbic Acid supplementation demonstrated improved endothelial function in hypertension (11,12). The combination of Ascorbic acid with Propranolol HCl may therefore offer synergistic benefits by addressing both hemodynamic and oxidative stress components. Even with theoretical rationale for combining antioxidants with antihypertensive molecule, a systematic assessment through established method is

limited. Through understanding on antioxidant behavior of Propranolol HCl in combination with Ascorbic acid is essential for formulation development and better therapeutic benefits.
This study aims to evaluate the in-vitro antioxidant potential of Propranolol HCl + Ascorbic acid combination formulation by established in-vitro chemical methods: (DPPH) radical scavenging assay, Superoxide Anion Scavenging Assay, Hydrogen peroxide scavenging activity, ABTS Radical Cation Decolorization Assay, Ferric Reducing Antioxidant Power (FRAP) Assay, Hydroxyl Radical Scavenging Assay.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Propranolol HCl (purity ≥99%) was obtained from Sigma-Aldreich, Merck. L-Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), hydrogen peroxide, sodium salicylate, and all other chemicals were of analytical grade and purchased from Sigma-Aldrich/Merck/other supplier. Methanol, ethanol, and other solvents were of HPLC grade.

2.2 Preparation of Test Solutions

2.2.1 Individual Propranolol HCl Solutions

Stock solutions of Propranolol HCl (1 mg/mL) and ascorbic acid (1 mg/mL) were prepared in methanol. Working solutions were prepared by serial dilution to obtain concentrations ranging from 62.5 to 2000 µg/mL.

2.2.2 Combination Formulation

The combination formulation was prepared by mixing Propranolol HCl and ascorbic acid in a ratio of 26:9 based on therapeutic doses. The combination was dissolved in methanol and diluted to obtain test concentrations of 62.5 to 2000 µg/mL.

2.3 DPPH Radical Scavenging Assay

The DPPH radical scavenging activity was determined according to the method of Brand-Williams et al.(13) with modifications. Briefly, 0.1 mM DPPH solution was prepared in methanol. Test samples (1 mL) at various concentrations were mixed with 2 mL of DPPH solution. The mixture was incubated in the dark at room temperature (25 ± 2°C) for 30 minutes. Absorbance was measured at 515 nm using a UV-visible spectrophotometer [2600, Shimadzu].
Color of reaction mixture before reaction: Deep purple
Color of reaction mixture after reaction: Yellow
The percentage of radical scavenging activity was calculated using the equation:

$$\text{Scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of DPPH solution without sample, and A_{sample} is the absorbance in the presence of test sample.
The IC_{50} value (concentration required to scavenge 50% of DPPH radicals) was calculated from the dose-response curve.

2.4 Superoxide Anion Scavenging Assay

The assay was performed based on Riboflavin-light-NBT scavenging system as mentioned in (14) with slightly modified form. Reaction mixture was prepared by mixing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 100 µM EDTA, 10 µM riboflavin and different concentration of test solutions. Final volume of reaction mixture was kept 3 mL. A blue color formazan was formed due to reduction of NBT by superoxide generated by reduction of riboflavin in presence of methionine and oxygen. Reaction mixture was exposed to fluorescent light for 10-15 min. The absorbance was measured at 560nm and percentage inhibition is calculated with formula:
% Inhibition=A control−A sample/A control×100
where A control = absorbance of control and A sample = absorbance of test sample.
Color of reaction mixture before reaction: Pale or light yellow
Color of reaction mixture after reaction: Dark blue or Purple

2.5 Hydrogen peroxide scavenging activity

The test was performed as detailed in method Ruch et al. (15) with slight modification. Final volume of reaction mixture (3.4mL) was prepared by adding 0.6 mL of 40 mM H_2O_2 solution in phosphate buffer, 2.4 mL of 0.1 M phosphate buffer (pH 7.4), 0.4 mL of test samples solutions at different concentration. Solution mixtures were mixed and incubated at room temperature for 10 min. and absorbance was taken at 230nm.
Color of reaction mixture before reaction: Colorless to very pale yellow
Color of reaction mixture after reaction: Solution remains colorless visually
Following formula were used for calculation the scavenging activity.
Hydrogen peroxide scavenging activity = (1- absorbance of sample / absorbance of sample) x 100.

2.6 ABTS Radical Cation Decolorization Assay

The ABTS assay was performed according to Re et al. (16). ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting 7 mM ABTS solution with 2.45 mM Potassium persulfate in a 1:1 ratio and incubating in the dark at room temperature for 12–16 hours. The $ABTS^{\bullet+}$ solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm.

Test samples (10-20 µL) at various concentrations were mixed with 1 mL of diluted $ABTS^{\bullet+}$ solution. After 6 minutes of incubation at room temperature, absorbance was measured at 734 nm. Trolox was used as the standard, and results were expressed as Trolox equivalent antioxidant capacity (TEAC) in µmol Trolox equivalents per gram of sample. % scavenging effect is calculated as per below formula:

Scavenging effect (%) = [(control absorbance - sample absorbance)] / (control absorbance) x 100

Color of reaction mixture before reaction: Bluish green color

Color of reaction mixture after reaction: Solution discoloration

2.7 Ferric Reducing Antioxidant Power (FRAP) Assay

The reaction assay was conducted as per method (17) with slight modifications. Final volume of reaction mixture was prepared by mixing 0.5 mL sample solution (different concentrations), 0.5 mL phosphate buffer (0.2 M, pH 6.6), 0.5 mL potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 minutes further reaction was stopped by adding 0.5 mL of 10% TCA and centrifuged at 3000 rpm for 10 minutes. 0.5 mL of supernatant and 0.5 mL distilled water and 0.1 mL $FeCl_3$ (0.1%) was mixed for color development. Absorbance was taken at 700nm. Higher the absorbance of reaction mixture higher the reducing power.

Color of reaction mixture before reaction: Pale Yellow or colorless

Color of reaction mixture after reaction: Deep blue color

2.8 Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging activity was determined using Fenton reaction method with slightly modification (18,19). The reaction mixture contained 2.8mM deoxyribose, 100µM $FeCl_3$, 100 µM EDTA, 1mM H_2O_2, 100 µM Ascorbic acid, 20mM Phosphate buffer (pH 7.4) and test sample at various concentrations, the final volume of reaction mixture was kept 1mL. After incubation at 37°C for 1 Hr. 1mL of 2.8% TCA and 1%TBA were added and heated at 100°C for 15 min. Further mixture was cooled and centrifuged. Absorbance was measured at 532 nm.

Color of reaction mixture before reaction: Colorless to pale yellow

Color of reaction mixture after reaction: Yellow/brown

The hydroxyl radical scavenging activity was calculated using the equation:

$$\text{Scavenging activity (\%)} = \left[1 - \frac{A_{sample} - A_{background}}{A_{control}}\right] \times 100$$

2.9 Determination of Interaction Type

To evaluate the nature of interaction between Propranolol HCl and ascorbic acid, the combination index (CI) was calculated according to Chou and Talalay (20):

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

Where $(D)_1$ and $(D)_2$ are the concentrations of Propranolol HCl and Ascorbic Acid in combination required to achieve a 50% of activity, and $(Dx)_1$ and $(Dx)_2$ are the concentrations of each drug alone required to achieve the same effect.

CI < 1 indicates synergism, CI = 1 indicates additive effect, and CI > 1 indicates antagonism.

2.10 Statistical Analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism software (version: 10.3.x). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. IC_{50} values were calculated by nonlinear regression analysis.

3. RESULTS

3.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of Propranolol HCl, ascorbic acid, and their combination is presented in Table 1 and Figure 1. All test samples demonstrated concentration-dependent scavenging activity.

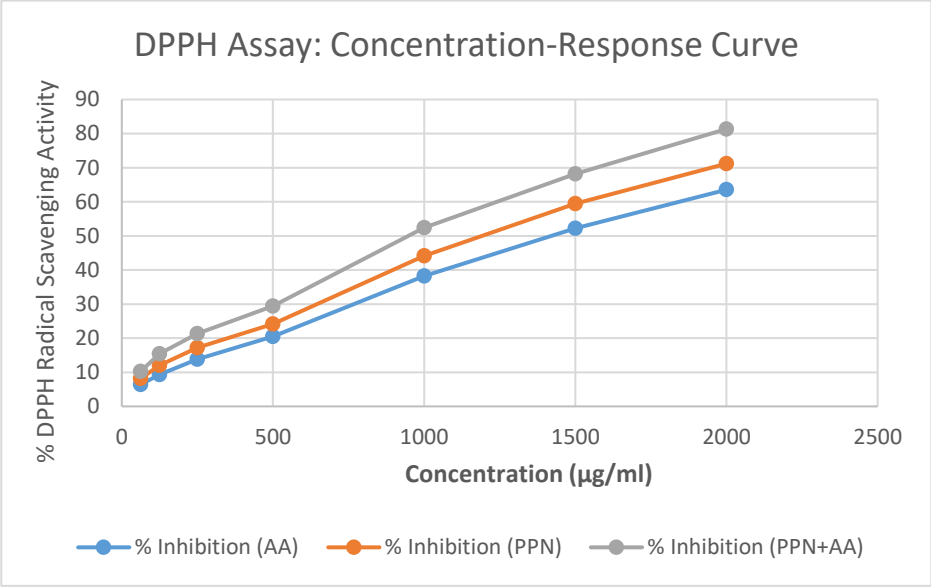
[Table 1: DPPH radical scavenging activity of test samples]

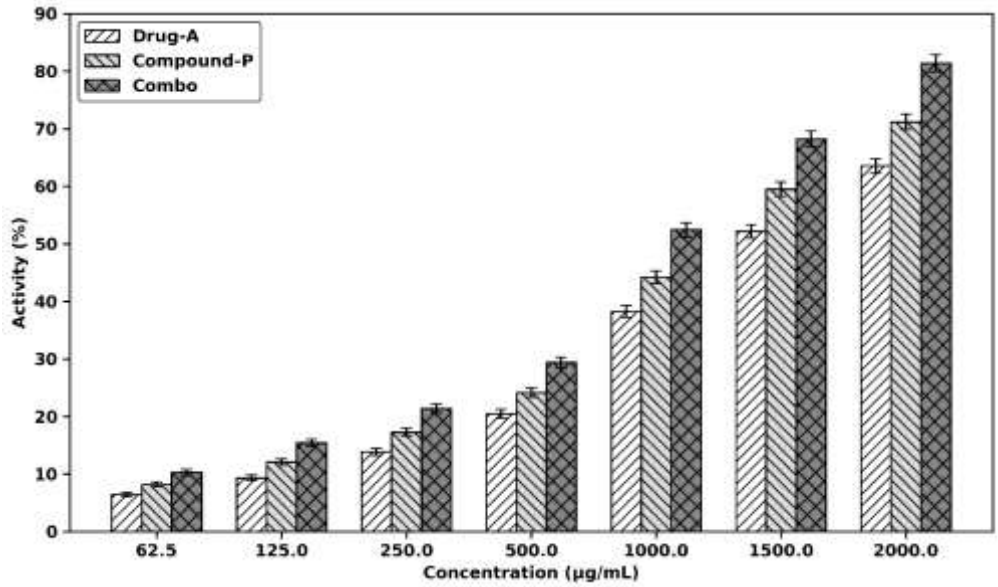
Sample	Concentration (µg/mL)	Scavenging Activity (%)	IC_{50} (µg/mL)
Ascorbic acid	62.5 - 2000	6.42 – 63.54	1473.55
Propranolol HCl	62.5 - 2000	8.16 -71.18	1269.62
Combination	62.5 - 2000	10.28 – 81.36	1052.93

Values are expressed as mean ± SD (n = 3)

The combination formulation exhibited enhanced DPPH scavenging activity compared to individual components. The IC_{50} value for the combination (1052.93 µg/mL) was significantly lower than that of Propranolol HCl alone (1269.62µg/mL) and comparable from ascorbic acid (1473.55 µg/mL).

[Figure 1: Concentration-response curves for DPPH scavenging (%)]





3.2 ABTS Radical Cation Scavenging Activity

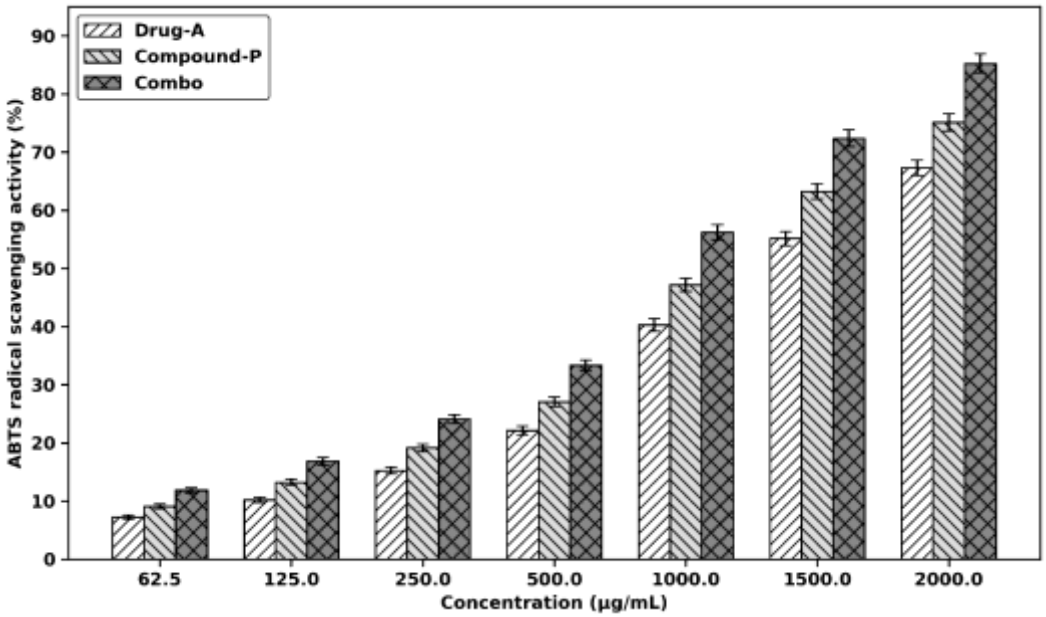
The ABTS•$^{+}$ scavenging activities are summarized in Table 2 and Figure 2.

[Table 2: ABTS radical cation scavenging activity (%)]

Conc (µg/mL)	Ascorbic acid (AA)	Propranolol HCl (PPN)	Combination (PPN+AA)
62.5	7.24 ± 0.36	9.12 ± 0.43	11.84 ± 0.49
125	10.18 ± 0.44	13.24 ± 0.51	16.86 ± 0.59
250	15.24 ± 0.56	19.18 ± 0.64	24.12 ± 0.72
500	22.14 ± 0.74	27.08 ± 0.82	33.36 ± 0.91
1000	40.28 ± 1.06	47.18 ± 1.14	56.24 ± 1.28
1500	55.16 ± 1.22	63.24 ± 1.33	72.38 ± 1.46
2000	67.28 ± 1.34	75.12 ± 1.48	85.24 ± 1.62
IC50	**1382.22 µg/mL**	**1174.04 µg/mL**	**965.63 µg/mL**

Combination of Propranolol HCl and Ascorbic Acid exhibited higher ABTS radical cation scavenging activity (%) as compared to individual Ascorbic acid and Propranolol HCl.

[Figure 2: ABTS Radical Cation Scavenging Activity (%)]



3.3 Ferric Reducing Antioxidant Power

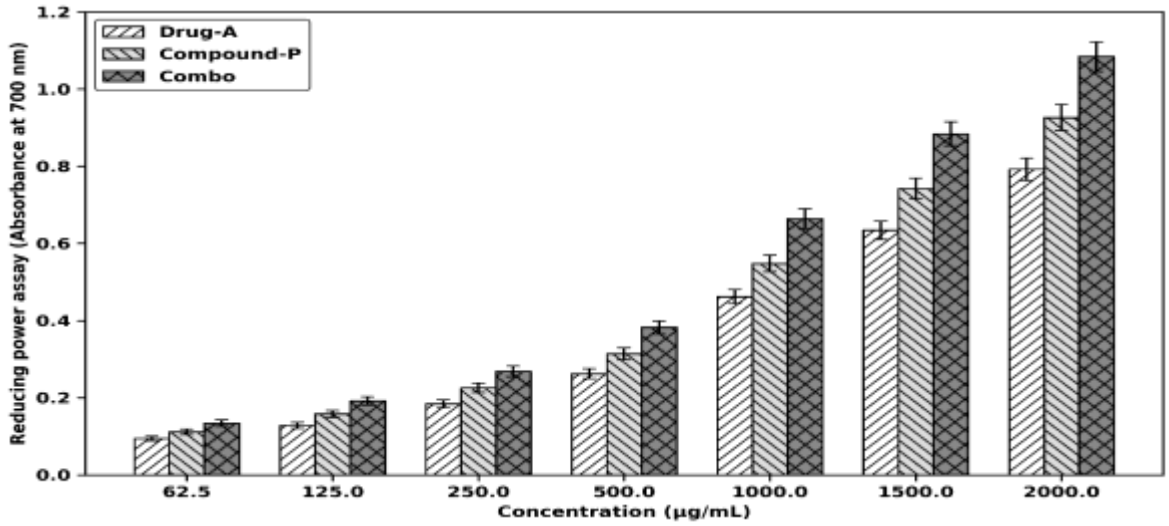
The FRAP values, expressed as Fe^{2+} equivalents, are presented in Table 3 and Figure 3.

[Table 3: Ferric reducing antioxidant power (%) of test sample]

Conc (µg/mL)	Ascorbic acid (AA)	Propranolol HCl (PPN)	Combination (PPN+AA)
62.5	0.094 ± 0.006	0.112 ± 0.007	0.134 ± 0.008
125	0.128 ± 0.008	0.158 ± 0.009	0.192 ± 0.011
250	0.184 ± 0.010	0.226 ± 0.012	0.268 ± 0.014
500	0.262 ± 0.013	0.314 ± 0.015	0.382 ± 0.017
1000	0.462 ± 0.018	0.548 ± 0.022	0.664 ± 0.026
1500	0.634 ± 0.024	0.742 ± 0.027	0.884 ± 0.031
2000	0.792 ± 0.029	0.926 ± 0.034	1.084 ± 0.038

Combination of Propranolol HCl and Ascorbic Acid exhibited higher Ferric Reducing Antioxidant Power (%) as compared to individual Ascorbic acid and Propranolol HCl. The combination showed enhanced reducing power.

[Figure 3: Ferric reducing antioxidant power (%)]



3.4 Hydroxyl Radical Scavenging Activity (%)

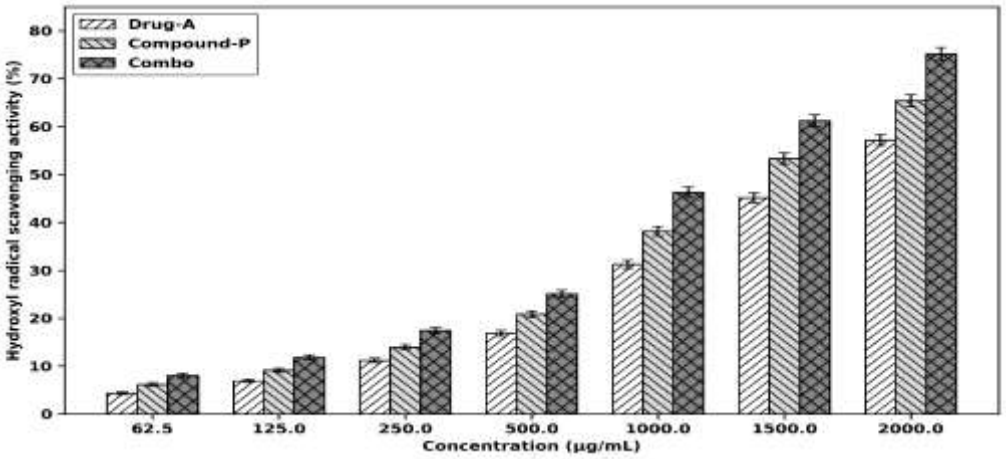
Hydroxyl radical scavenging results are shown in Table 4 and Figure 4.

[Table 4: Hydroxyl radical scavenging activity]

Conc (µg/mL)	Ascorbic acid (AA)	Propranolol HCl (PPN)	Combination (PPN+AA)
62.5	4.36 ± 0.28	6.18 ± 0.34	8.06 ± 0.39
125	6.94 ± 0.35	9.12 ± 0.42	11.84 ± 0.48
250	11.18 ± 0.47	13.92 ± 0.55	17.46 ± 0.60
500	16.86 ± 0.63	20.84 ± 0.71	25.12 ± 0.77
1000	31.24 ± 0.88	38.16 ± 1.02	46.28 ± 1.11
1500	45.12 ± 1.06	53.34 ± 1.18	61.22 ± 1.29
2000	57.18 ± 1.16	65.42 ± 1.27	75.14 ± 1.38
IC50	1707.12 µg/mL	1437.15 µg/mL	1207.50 µg/mL

Combination of Propranolol HCl and Ascorbic Acid exhibited higher Hydroxyl radical scavenging (%). The combination showed enhanced reducing power.

[Figure 4: Hydroxyl radical scavenging activity]



3.5 Superoxide anion scavenging activity (%)

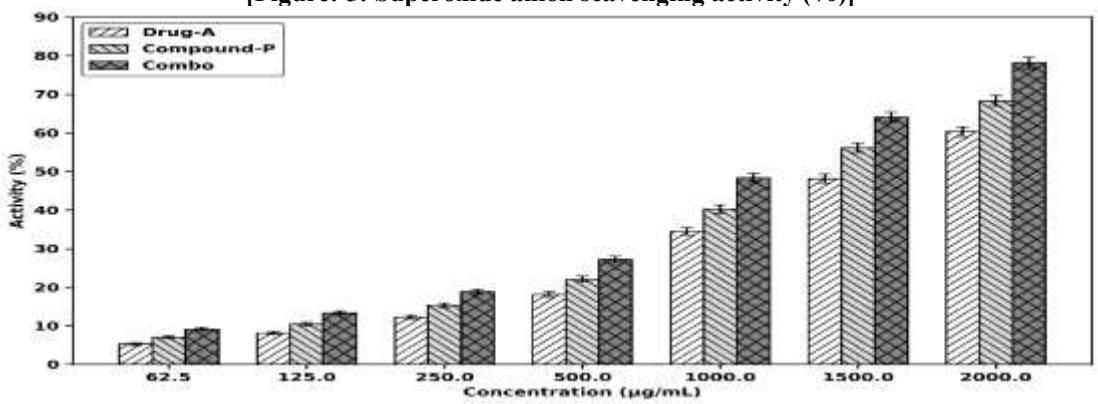
Superoxide anion scavenging activity (%) results are shown in Table 5 and Figure 5.

[Table 5: Superoxide anion scavenging activity (%)]

Conc (µg/mL)	Ascorbic acid (AA)	Propranolol HCl (PPN)	Combination (PPN+AA)
62.5	5.28 ± 0.31	7.06 ± 0.36	9.14 ± 0.42
125	8.14 ± 0.39	10.42 ± 0.45	13.36 ± 0.51
250	12.26 ± 0.52	15.34 ± 0.58	18.84 ± 0.66
500	18.12 ± 0.68	22.08 ± 0.74	27.22 ± 0.82
1000	34.46 ± 0.96	40.28 ± 1.08	48.36 ± 1.14
1500	48.18 ± 1.12	56.14 ± 1.19	64.08 ± 1.31
2000	60.34 ± 1.24	68.26 ± 1.36	78.12 ± 1.44
IC50	1595.61 µg/mL	1360.36 µg/mL	1141.17 µg/mL

Combination of Propranolol HCl and Ascorbic Acid exhibited higher Superoxide anion scavenging activity (%). The combination showed enhanced reducing power.

[Figure: 5: Superoxide anion scavenging activity (%)]



3.6 Hydrogen peroxide scavenging activity (%)

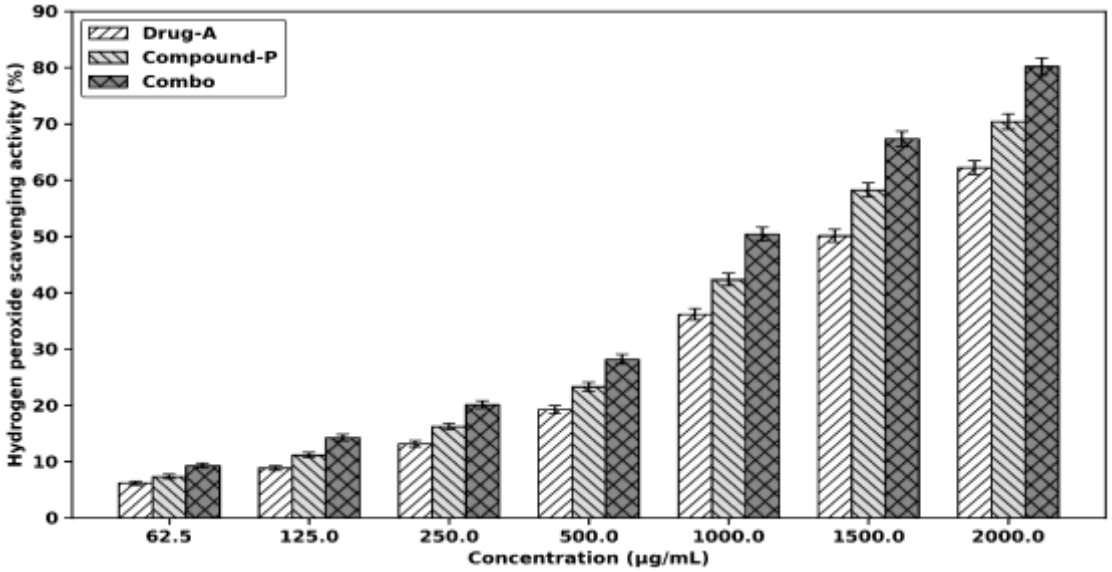
Hydrogen peroxide scavenging activity (%) results are shown in Table 6 and Figure 6.

[Table 6: Hydrogen peroxide scavenging activity (%)]

Conc (µg/mL)	Ascorbic acid (AA)	Propranolol HCl (PPN)	Combination (PPN+AA)
62.5	6.18 ± 0.33	7.34 ± 0.39	9.28 ± 0.44
125	8.84 ± 0.41	11.16 ± 0.48	14.24 ± 0.56
250	13.18 ± 0.53	16.24 ± 0.60	20.12 ± 0.69
500	19.24 ± 0.71	23.28 ± 0.79	28.16 ± 0.86
1000	36.18 ± 0.98	42.34 ± 1.10	50.42 ± 1.22
1500	50.12 ± 1.11	58.26 ± 1.23	67.34 ± 1.34
2000	62.18 ± 1.24	70.42 ± 1.36	80.18 ± 1.49
IC50	1529.63 µg/mL	1304.02 µg/mL	1089.27 µg/mL

Combination of Propranolol HCl and Ascorbic Acid exhibited higher Hydrogen peroxide scavenging activity (%). The combination showed enhanced reducing power.

[Figure 6: Hydrogen peroxide scavenging activity (%)]



3.7 Combination Index Analysis

CI=(D1/Dx1) + (D2/Dx2)
D1 & D2 = Doses of drug in combination required to achieve a 50% of activity
Dx1 & Dx2 = Doses of drug alone the would achieve the same effect (50%)
CI<1 indicates synergy

The combination index values for different assays are presented in Table 7.

[Table 7: Combination index values for Propranolol HCl –ascorbic acid combination]

Assay Method	CI Value	Interaction Type
DPPH	0.279936	CI<1: Synergism
ABTS	0.276721	CI<1: Synergism
FRAP	0.279816	CI<1: Synergism
Hydroxyl radical	0.282111	CI<1: Synergism
Hydrogen Peroxide	0.281273	CI<1: Synergism
Superoxide	0.282475	CI<1: Synergism

From all the test the CI values obtained are lower than 1, indicating synergistic effect of combination as compared to individual effect at 50% reducing activity and antioxidant potential.

4. DISCUSSION

The present study evaluated the in vitro antioxidant potential of a combination formulation containing Propranolol HCl and Ascorbic acid using multiple complementary chemical methods. The use of multiple assays is essential because different methods measure distinct aspects of antioxidant activity, including electron transfer, hydrogen atom transfer, and metal chelation mechanisms (21) etc.

4.1 Antioxidant Activity of Individual Components

Propranolol HCl demonstrated potent antioxidant activity across all assays. This activity is attributed to its active metabolite, 4-hydroxypropranolol capable to effectively neutralize free radicals and protects against lipid peroxidation causing cellular damage. Previous studies have reported similar findings.

Ascorbic acid, as expected, exhibited potent antioxidant activity due to its ability to donate electrons and hydrogen atoms from its enediol structure (22,23). The lactone ring and enediol system enable ascorbic acid to effectively scavenge various reactive oxygen species.

4.2 Combination Effects

The combination formulation showed synergistic antioxidant effects compared to individual components. This can be explained by several potential mechanisms:

1. Complementary mechanisms: Propranolol HCl and Ascorbic acid may scavenge radicals through different mechanisms, providing broader antioxidant coverage.
2. Regeneration effects: Ascorbic acid is known to regenerate other antioxidants from their oxidized forms. It may regenerate the oxidized form of Propranolol HCl, thereby enhancing overall antioxidant capacity (24,25).
3. Synergistic electron transfers: The combination may facilitate more efficient electron transfer to neutralize free radicals.

4.3 Relevance to Hypertension Therapy and Other Pathophysiological conditions

The enhanced antioxidant activity of the combination formulation has potential clinical implications. A formulation that combines blood pressure-lowering effects with antioxidant activity may provide more comprehensive cardiovascular protection than antihypertensive therapy alone. This therapeutic strategy may be useful for other pathophysiological conditions related to oxidative stress, genomic damages and cancers.

4.5 Limitations

The study employed in vitro chemical assays, which may not directly translate to in vivo antioxidant effects. Further studies using biological models and clinical trials are needed to confirm therapeutic benefits.

5. CONCLUSION

This study demonstrated that the combination of Propranolol HCl and Ascorbic acid exhibits synergistic antioxidant activity compared to individual components, as evidenced by DPPH, ABTS, FRAP, hydroxyl radical scavenging assays, Hydrogen peroxide scavenging activity and superoxide scavenging activity. The combination showed synergistic interaction with CI values CI<1: Synergism across multiple assay methods.

These findings suggest that the Propranolol HCl –Ascorbic acid combination may offer therapeutic advantages in managing hypertension-associated oxidative stress and various pathophysiological conditions linked to cellular signaling interruption and DNA damages. The results support the rationale for developing fixed-dose combination formulations and warrant further investigation in biological models and clinical settings to confirm the potential cardiovascular benefits of this combination.

Declarations

Conflicts of Interest

The authors declare no conflicts of interest.

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