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Evaluation of antioxidant and anti-inflammatory effect of Resveratrol and BioPerine® combination

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ABSTRACT

The objective of the present work is to evaluate and establish the potential anti-inflammatory and antioxidant action of the Resveratrol and BioPerine® combination capsule available from Healthy Hey. The product responded positively to all the tests for flavonoids and alkaloids, confirming the presence of polyphenol and alkaloids in the capsule. The combination product was subjected to *in vitro* determination of antioxidant potential in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH, reducing power using potassium ferrocyanide, hydroxy radical scavenging using Iron-EDTA and ammonium molybdate methods. The IC_{50} value of the Resveratrol and BioPerine® combination against DPPH was found to be 57.01µg/mL. The Resveratrol and BioPerine® combination was able to produce a dose and time time-dependent action potassium ferrocyanide to potassium ferricyanide. The maximum reduction occurred at 20 min post-interaction thereafter decreasing in the 30^{th} min. The IC_{50} value of the Resveratrol and BioPerine® combination against the hydroxyl radical was found to be 106.79µg/mL. The IC_{50} value of the Resveratrol and BioPerine® combination was found to be 154.88µg/mL. Resveratrol and BioPerine® combination was able to reduce the inhibition after the third hour significantly ($p<0.01$) exhibiting 50.87% reduction in edema volume.

Keywords: Resveratrol, BioPerine, antioxidant, anti-inflammatory, DPPH, Rat paw edema, carrageenan

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INTRODUCTION

Free radicals are the types of Reactive Oxygen Species (ROS) that include all highly reactive, oxygen-containing molecules. Various inflammatory stimuli such as excessive ROS produced in the process of oxidative metabolism and some natural or artificial chemicals have been reported to initiate the inflammatory process resulting in synthesis and secretion of pro inflammatory cytokines. Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants [1]. Antioxidants may be of great help in improving the quality of life as they can either prevent or postpone the onset of several degenerative diseases [2].

Nowadays, a number of herbal supplements are being used as nutraceutical products for aging, inflammation, memory boosting etc. Several flavonoids, carotenoids and pro-oxidants have been reported for antioxidant action and treatment of several diseases related to ROS [3-7]. Resveratrol is one such active ingredient obtained from plants that has been widely used in several nutraceutical supplements. Several combination products of resveratrol are available in market. Owing to the wide use of resveratrol, it was envisioned to scientifically explore the potential of one such combination containing resveratrol and BioPerine®. The objective of the present investigation was therefore to study the antioxidant effect of the combination (*in vitro*) and its anti-inflammatory action (*in vivo*).

Material and Methods

Resveratrol + BioPerine® combination was purchased from Healthy Hey Nutrition, Mumbai. DPPH was procured from Oxford Fine Chemicals, potassium ferricyanide, ferrous ammonium sulfate, ammonium acetate, sodium phosphate and other chemicals and reagents were purchased from SD Fine, Loba Chemie, Thermo Fisher and Rankem.

Determination of solubility of the Combination Product

The procured capsules of Resveratrol and BioPerine® were emptied on a butter paper and the powder was used for determining the solubility of the product. The solubility was determined qualitatively by shaking a small amount of the powder in 1 mL of solvent, in a test tube. The test tube was visually inspected for the presence or absence of any solid particles.

Test for flavonoid [8]

Preparation of Test Solution: The powder obtained from emptying the capsules was dissolved in small amount of ethanol and used for performing the tests.

- **Shinoda test:** To the test solution, few fragments of magnesium ribbon were added and conc. hydrochloric acid was mixed drop wise to it.

- **Zinc hydrochloride reduction test:** To the test solution a mixture of zinc dust and conc. hydrochloric acid was added.
- **Alkaline reagent test:** To the test solution a few drops of sodium hydroxide solution was added. Later if colour appeared, a few drops of conc. HCl were added to it.

Test for Alkaloids [8]

Preparation of Test Solution: The powder obtained from emptying the capsules was dissolved in small amount of ethanol and used for performing the tests.

- **Mayer's test:** To a few ml of test solution, two drops of Mayer's reagent was added along the sides of test tube.
- **Wagner's test:** A few drops of Wagner's reagent were added to few ml test solution along the sides of test tube.
- **Hager's test:** A few drops of Hager's reagent were added to few ml test solution along the sides of test tube.
- **Dragendorff's Test:** A few drop of Dragendorff's reagent was added to 1 ml of the test solution.

Determination of antioxidant potential [9]

Determination of DPPH radicals scavenging activity

The free radical scavenging activity of the test solution was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH.

Determination of DPPH radicals scavenging activity was performed by the previously reported method. Separately, 1mM solution of DPPH and test solution (50-250 µg/mL) were prepared in ethanol. 1.5ml of the test solution was added to 1.5 ml of DPPH solution. The absorbance was measured at 517 nm against the corresponding blank solution which was prepared using 3 mL ethanol. The control sample used was 3 mL of DPPH. The assay was performed in triplicates. Percentage inhibition of free radical DPPH was calculated based on control reading by following equation.

$$\text{DPPH scavenged (\%)} = \frac{(A_{con} - A_{test})}{A_{con}} \times 100$$

A_{con} - is the absorbance of the control reaction

A_{test} - is the absorbance in the presence of the test solution.

Reducing power assay

Different concentrations of the test solution (50-250 µg/mL) in ethanol (1.0 mL) were diluted with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and mixed with 2.5 mL 1% potassium ferricyanide. After incubation at 50°C for 20 minutes, 2.5 mL of 10% trichloroacetic acid (TCA) were added to the mixture. 2.5 mL of the reaction mixture was diluted with an equal amount of distilled water and absorbance was measured at 700 nm after treatment with 0.5 mL of 0.1% ferric chloride ($FeCl_3$). Increased absorbance of the reaction mixture indicates an increase in reduction capability.

Determination of hydroxyl radical scavenging activity

Various concentrations of test solution (50, 100, 150, 200 and 250 µg) were taken and 1 mL of iron EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA.), 0.5mL of EDTA solution (0.018 g EDTA in 100 mL distilled water), 1 mL of DMSO and 0.5mL of ascorbic acid (0.22 g ascorbic acid in 100 mL distilled water) were added to it. The mixture was incubated in a boiling water bath at 80 to 90°C for 15 min. After incubation, 1 mL of ice cold TCA (17.5 g TCA in 100 mL distilled water) and 3mL of Nash reagent (7.5g ammonium acetate, 0.5mL of glacial acetic acid and 0.2 mL of acetone to 100mLdistilled water) were added and the reaction mixture was incubated at room temperature for 15 min. The absorbance was read at 412 nm. The % hydroxyl radical scavenging activity is calculated by the following formula

$$\% \, HRSA = \frac{Abs \; control - Abs \; sample}{Abs \; control} \times 100$$

Where, HRSA is the Hydroxyl Radical Scavenging Activity, Abs control is the absorbance of control and Abs sample is the absorbance of the test solution.

Phosphomolybdenum assay [10]

10mg of test solution was dissolved in 1 mL of DMSO. 100µl of the sample was taken and 1 mL of the reagent solution (0.588 mL of sulphuric acid, 0.049g ammonium molybdate and 0.036g sodium phosphate in 10 mL distilled water) was added to it. The mixture was incubated in a boiling water bath at 95°C for 90 min. After 90 min, the absorbance of the solution was read at 695 nm. Ascorbic acid (10 mg/mL in DMSO) was used as standard. The Phosphomolybdenum reduction potential (PRP) of the studied extracts were reported in percentage using the formula

% of inhibition = (control OD - sample OD/ Control OD) x 100.

Evaluation of anti-inflammatory action

Animals

Healthy Wistar rats of either sex, weighing 180-250g were used for the study. The animals were housed in cages in the animal house of SS Institute of Pharmacy, Bhopal during the course of

experimental period and maintained at 12 day and night schedule with a temperature [17-26°C] maintained at standard experimental condition. The animals were fed with standard rodent pellet feed and water *ad libitum*. The animals were fasted 12 hours before the experiment with free access to only water. The experiment was performed in accordance with the approval of protocol from the animal ethical committee of the institute.

Carrageenan induced rat paw edema method

The carrageenan induced rat paw edema method was used for evaluating the anti-inflammatory activity of Resveratol-BioPerine® combination [11].

Paw oedema was induced by subcutaneous injection of 0.1mL (1% solution) of Carrageenan into the plantar surface of the right hind paw of the rat. The test sample was administered in dose of 10 mg/kg in different groups of animals, 30 min prior to carrageenan injection. Ibuprofen (10 mg/kg i.p.) was used as a standard anti-inflammatory drug which was administered 30 min prior to carrageenan injection. Animals were divided into 3 groups (n = 6) as follows

Group -- I - Control - treated with vehicle (normal saline)

Group -- II - Standard drug – Ibuprofen

Group – III–Resveratol-BioPerine® combination was administered in dose of 10 mg/kg.

Paw diameters were measured immediately before the administration of the Carrageenan and thereafter at 1, 2, 4 and 6 h using vernier calliper. The results obtained were compared with control group. The percentage inhibition of paw inflammation exhibited by each group was calculated by using following formula:

$$\% \text{ inhibition} = C\text{-}T/\ C \times 100$$

C= Paw volume (mL) in vehicle-treated group (control)

T= Paw volume (mL) in drug-treated group

Results and Discussion

Solubility and phytochemical analysis

The Combination product obtained contained transparent capsule shells, filled with off white colored powder with no particular taste and odor. It was insoluble in water and soluble in methanol, ethanol, chloroform, acetone and DMSO.

As the product contained Resveratrol (polyphenolic) and BioPerine (alkaloid), it was subjected to test for flavonoids and alkaloids to confirm the presence of the secondary metabolite class.

The product responded positively to all the tests for flavonoids and alkaloids, confirming the presence of polyphenol and alkaloid in the capsule.

DPPH radicals scavenging activity

DPPH radicals react with suitable reducing agents, then losing colour stoichometrically with the number of electrons consumed, which is measured spectrophotometrically at 517 nm. The deep purple color of DPPH decreases if the compound exhibits antioxidant action. The test solution exhibited a dose depended DPPH scavenging action with a dose of 150 µg/mL producing protection against DPPH higher than the standard drug ascorbic acid (50µg/mL).

The IC_{50} value for DPPH inhibition was calculated from the plot of percent DPPH inhibition against concentration (figure 1), using the equation y=0.335x+30.90 (y=mx+c) taking the value of y as 50. The IC_{50} value of the Resveratrol and BioPerine® combination against DPPH was found to be 57.01µg/mL.

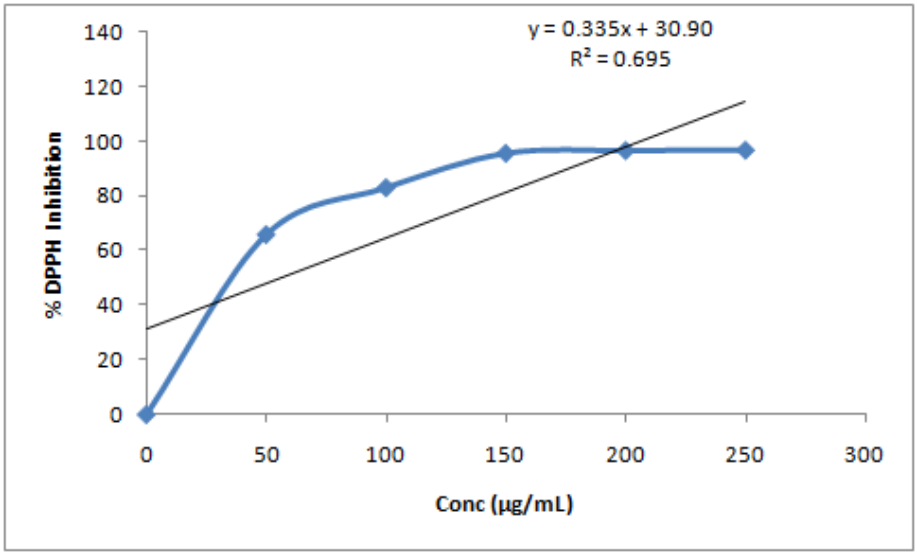

Figure 1: Percent DPPH scavenging by combination capsule

Reducing Power Assay

The capability of the extracts to reduce potassium ferrocyanide (standard) at different time periods has been depicted in table 1. Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. The pale yellow color obtained from the standard was deepened (darkened) on interaction with the contents of the capsule.

Table 1: Reducing power of combination capsule

Solution	OD at 700 nm		
	10 min	20 min	30 min
Standard	0.033 ± 0.001	0.042 ± 0.001	0.025 ± 0.001
TS 50 µg/mL	0.056 ± 0.002	0.074 ± 0.001	0.043 ± 0.001
TS 100 µg/mL	0.097 ± 0.003	0.116 ± 0.001	0.078 ± 0.003
TS 150 µg/mL	0.139 ± 0.003	0.154 ± 0.001	0.11 ± 0.000
TS 200 µg/mL	0.176 ± 0.005	0.192 ± 0.004	0.153 ± 0.002
TS 250 µg/mL	0.221 ± 0.002	0.243 ± 0.001	0.191 ± 0.004

Results are reported as mean ± SD (n=3)

The Resveratrol and BioPerine® combination was able to produce a dose and time dependent reduction of potassium ferrocyanide to potassium ferrocyanide. The maximum reduction occurred at 20 min post interaction thereafter decreasing in the 30th min.

Hydroxy radical scavenging assay

HRS assay is used to find the scavenging activity of free hydroxyl radicals like hydrogen peroxide(which damage the body cells) in the presence of different concentrations of plant sample. A dose dependent relation was obtained for hydroxyl radical scavenging by the test solution with a solution of 200 and 250µg/mL exhibiting scavenging more than that of the standard ascorbic acid solution. The IC_{50} value for hydroxyl radical inhibition was calculated from the plot of percent hydroxyl radical inhibition against concentration (figure 2), using the equation y=0.368x+10.70 (y=mx+c) taking the value of y as 50. The IC_{50} value of the Resveratrol and BioPerine® combination against the hydroxyl radical was found to be 106.79µg/mL.

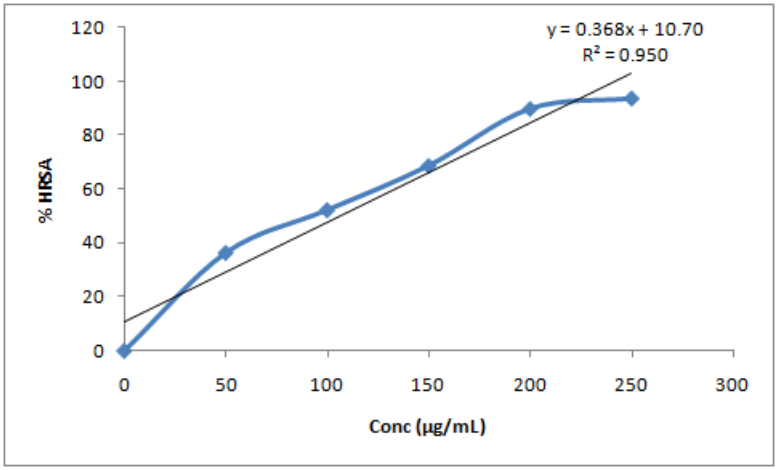

Figure 2: Plot of %HRSA against concentration

Phosphomolybdenum assay

This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The phosphomolybdenum reduction potential of the test solutions

was also found to be dose dependent with 250µg/mL solution exhibiting the highest reduction of the phosphomolybdenum complex (81.07 ± 0.710 %), comparable to ascorbic acid (92.7 ± 0.361 %). The IC_{50} value for hydroxyl radical inhibition was calculated from the plot of percent PRP against concentration (figure 3), using the equation y=0.313x+1.522 (y=mx+c) taking the value of y as 50. The IC_{50} value of the Resveratrol and BioPerine® combination was found to be 154.88µg/mL.

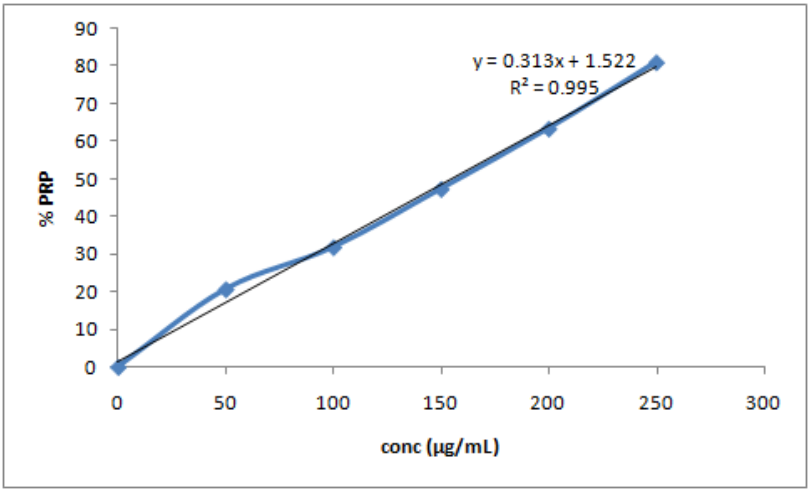

Figure 3: Plot of %PRP against concentration

Carrageenan Induced rat paw edema measurement

Carrageenan-induced acute inflammation is one of the most suitable test procedures for screening of anti-inflammatory agents [12]. As shown in table 2 and figure 4, Resveratrol and BioPerine® combination was able to reduce the inhibition after the third hour significantly ($p<0.01$).

Table 2: Rat paw edema in rats

Group	Change in Paw thickness (mm) [% inhibition of edema]			
	1h	2h	3h	4h
Normal Saline	1.476 ± 0.025	3.20 ± 0.072	3.82 ± 0.086	4.01 ± 0.047
Ibuprofen	0.48 ± 0.007** [67.48%]	0.93 ± 0.01** [70.94%]	0.96 ± 0.014** [74.87%]	0.77 ± 0.025** [80.80%]
Resveratrol and BioPerine® combination	1.24 ± 0.048 [15.99%]	2.51 ± 0.107 [21.56%]	2.61 ± 0.054* [31.66%]	1.97 ± 0.083* [50.87%]

Results are reported as mean ± SD (n=6); *p<0.01; **p<0.001

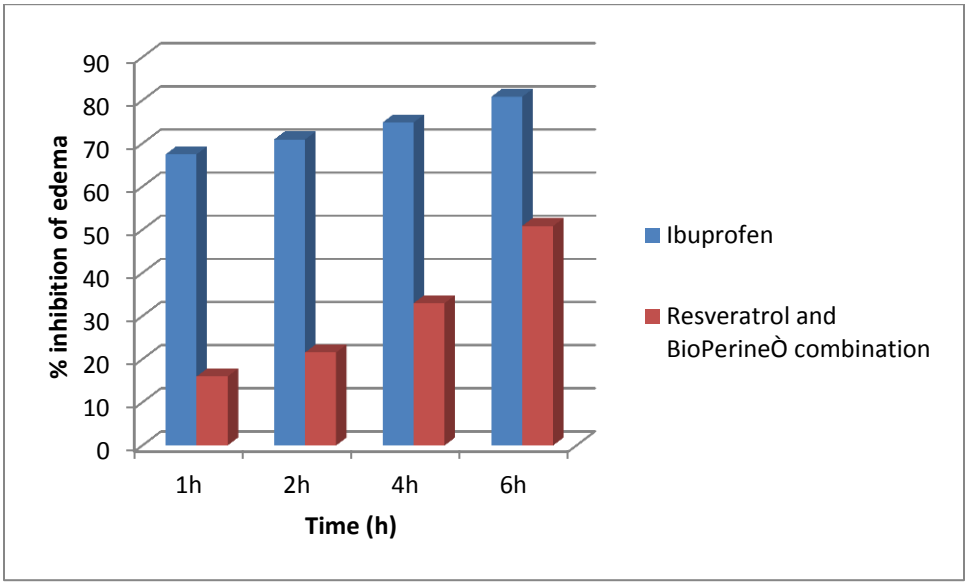

Figure 4: Comparison of anti-inflammatory effect of ibuprofen and combination capsule

CONCLUSION

The investigation resulted in establishing a scientific evidence for anti-inflammatory and antioxidant potential for the marketed supplement containing Resveratrol and BioPerine®combionation. Several of supplements claiming to contain natural antioxidant and several bioactive molecules of plant origin have flooded the nutraceutical market without proper scientific support to the claims. It is necessary to check these products for quality and pharmacological potency if any. The present study was successful in confirming the medicinal value of one such marketed product as a supplement for defying ageing and other cellular oxidation related health issues.

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