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RESEARCH ARTICLE

# Response Surface Optimization for Investigating Antioxidant Potential of *Camellia Sinensis* and *Withania Somnifera* in Synergistic Manner

**Arti Devi[1] · Vagish Dwibedi[1] · Nancy George[1] · Zaved Ahmed Khan[2]**

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**Abstract** The paper highlights the synergistic potential of the novel combination of *Camellia sinensis* (Kangra green tea) and *Withania somnifera* (Ashwagandha). One variable at a time approach was used to find antioxidant potential of *C. sinensis* and *W. somnifera* alone and in combination. Optimization of antioxidant potential was done by using different plant concentrations of *C. sinensis* and *W. somnifera* using a statistical approach of central composite design (CCD) of RSM (response surface methodology). Initial antioxidant activity during optimization of the solvent system was observed in methanol for *C. sinensis* with DPPH, superoxide radical scavenging assay and hydrogen peroxide scavenging assay ($44.9 \pm 0.62$, $43.77 \pm 0.10$, $43.88 \pm 0.10$% scavenging) and for *W. somnifera* ($40.22 \pm 0.39$, $43.29 \pm 1.12$, $41.88 \pm 0.11$% scavenging), respectively. Initially, $IC_{50}$ has been calculated for *C. sinensis* ($235.26 \pm 0.012$ µg/mL) and *W. somnifera* ($256.39 \pm 0.43$ µg/mL) in methanol. Before statistical optimization, the maximum synergistic antioxidant potential of *C. sinensis* (200 µg/mL) and *W. somnifera* (150 µg/mL) with DPPH assay, superoxide radical scavenging and hydrogen peroxide scavenging assay was found to be $56.57 \pm 0.62$, $56.99 \pm 0.42$, $55.44 \pm 0.53$% scavenging, respectively. $IC_{50}$ value has been calculated for *C. sinensis* + *W. somnifera* ($IC_{50} = 215.47 \pm 0.06$ µg/mL). Optimization of plant concentration using CCD of RSM resulted in enhancement of antioxidant potential of *C. sinensis* (200.5 µg/mL) and *W. somnifera* (200.5 µg/mL) was found to be $78.01 \pm 0.01$% scavenging when compared to the initial antioxidant potential, i.e., $56.57 \pm 0.62$% scavenging shows a 1.37-fold increase from initial antioxidant potential. Research unveils that using various combination of *C. sinensis* and *W. somnifera* enhance the antioxidant potential in vitro.

**Keywords** Response surface methodology · Central composite design · Antioxidant · *Camellia sinensis* · *Withania somnifera*

**Abbreviations**

| | |
|---|---|
| DPPH 2: | 2:-Diphenyl-1-picrylhydrazyl |
| CCD | Central composite design |
| RSM | Response surface methodology |
| PMS-NADH | Phenazine methosulfate-nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide |
| NBT | Nitro-blue tetrazolium |
| EDTA | Ethylenediamine tetra-acetic acid |

**Significance statement** In this work we have optimized the synergistic antioxidant potential of *Camellia sinensis* and *Withania somnifera* through RSM which has not reported earlier. We found RSM resulted in 1.37-fold increase from initial antioxidant potential.

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✉ Zaved Ahmed Khan
deanscience.bfc@babafaridgroup.edu.in

Arti Devi
arti.uibt@cumail.in

Vagish Dwibedi
vagish.lcc@gmail.com

Nancy George
nancy.george@cumail.in

[1] University Institute of Biotechnology, Chandigarh University, Gharuan, Mohali, Punjab, India

[2] Faculty of Sciences, Baba Farid College, BFGI, Muktsar Road, Bathinda, Punjab, India

| | |
|---|---|
| ANOVA | Analysis of variance |
| SOD | Superoxide dismutase |

## Introduction

*Camellia sinensis* (L) O Kuntze (Kangra green tea) is cultivated on the gentle slopes of Western Himalayas at elevation 1290 m above mean sea level and 32°27′15.68″ N latitude, 76°31′42.26″ E longitude since the 1850s [1]. Kangra green tea was first cultivated about 150 years ago by Dr. Jameson who was the superintendent of Botanical Gardens in North–West Province. Quality-wise, the Kangra tea estate has taken a most important place in the nineteenth and twentieth centuries. Kangra tea is very much famous for its unique flavor attributed to climatic and edaphic factors of Dhauladhar Hills having soil pH below 6, altitude 900–1400 m with annual rainfall 1500–2500 mm are highly appropriate areas for tea farming. Kangra green tea has higher total phenolic content and higher antioxidant activity as compared to Darjeeling tea. The exceptional qualities of Kangra green tea are mainly attributed to the geographical conditions of the region. In 2005, Kangra green tea received Geographical Indication tag under Himachal Pradesh Patent Information Centre, Shimla by Office of Controller-General of Patents, Designs and Trademarks, Chennai, as per Geographical Indications of Goods (Registration and Protection act, 1999). In 1886 and 1895, the tea received gold and silver medals at international conventions held in London and Amsterdam [1]. EGCG which is an active phytoconstituent of green tea and green tea extract has been proved to be anti-cancerous, anti-diabetic, anti-proliferative, anti-inflammatory, anti-atherosclerosis, neuroprotective, antioxidant, estrogenic and anti-aging [1–3]. Similarly, *Withania somnifera* has also been reported for its medicinal properties, and in Ayurveda, it is known for its anti-aging, immunomodulatory, and rejuvenating properties [4]. Nowadays, *W. somnifera* has also reported its preventive effect in COVID-19 attributed to its antiviral and immunomodulatory activities [5].

The Global Anti-Aging Market was worth $250 billion in 2016 and is estimated to be growing at a CAGR (compound annual growth rate) of 5.8% and is expected to reach $331.41 billion by 2021. Of the 4000 private and 600 public biotech companies worldwide, only a few percent have shown increasing profitability [6]. Very few drug candidates obtain approval and only a third of those retrieve their R&D costs. The future growth of the anti-aging market is observed to rely on the advancement and technological development with enhanced efficacy and safety of anti-aging products [7]. In ancient times herbal remedies were extensively used to minimize the effect of aging in India, China, and Egypt. There are several pieces of shreds of evidence that show that plant and plant extracts are effective anti-aging agents [8]. A combination of different antioxidants having different modes of action is always a better alternative to increase the effectiveness, bioavailability, and decrease the toxicity. Thus, using a potent herbal remedy in its crude form will make the therapy less expensive and affordable [9]. It has been reported that the combination of vitamin E with astaxanthin can be a powerful antioxidant as astaxanthin, a natural carotenoid can scavenge free radicals and this effect was found to increase when combined with vitamin E [10]. Another study of ferulic acid in combination with resveratrol has been shown to increase apoptotic cell death in cancer cells [11]. In another study, tetrahydrocurcumin in combination with *Centella asiatica* extract helps to improve the symptoms of aging [12].

In the present study, we have tried to combine two plant extracts, *C. sinensis* and *W. somnifera* to study their synergistic effect on free radicals in vitro. We, therefore, aimed to find out the synergistic antioxidative potential of *C. sinensis* and *W. somnifera* in vitro. To extract the maximum phytochemicals from plants, the type of solvent plays a crucial role as in the case of secondary metabolite production fermentation parameters play an important role [13].

RSM is a statistical technique practiced to augment the extraction of phytochemicals of various plants [14]. RSM is a statistical tool that is a combination of statistical methods and mathematical models that involves statistical significance, satisfactory point location, polynomial equations, to find out the interaction between various variables [14]. A statistical approach has been used in this investigation to enhance the antioxidant potential of *C. sinensis* and *W. somnifera.*

The present investigation is oriented toward the enhancement of antioxidant potential of *C. sinensis* and *W. somnifera* in a synergistic manner using Central Composite Design (CCD) based RSM using Design-Expert software version 11.

## Material and Methods

### Sample Collection

Leafy twigs of *C. sinensis* (Kangra green tea) and roots of *W. somnifera* (Ashwagandha) used in the current study were procured from Palampur tea gardens (Himachal Pradesh, India) (32°27′15.68″ N latitude, 76°31′42.26″ E) and Mohali (Punjab, India) (latitude 30.7046°N, longitude 76.7179°E), respectively, during July 2019. Plant samples were kept in a sterile bag and stored at 4 °C till further use.

## Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), riboflavin, and ascorbic acid were purchased from Sigma-Aldrich, USA, whereas hydrogen peroxide was obtained from HiMedia Lab Ltd., Mumbai, India. $DH_2O$ (distilled deionized water) was prepared by Camco Industries, Chandigarh (India). All other chemicals like methanol, sulphuric acid, Whatman filter paper, petroleum ether, and ethanol were purchased from HiMedia Lab Ltd., Mumbai, India.

## Plant Extract and Stock Solution Preparation

Petroleum ether, methanol, ethanol, water (1:1) extracts of *C. sinensis* and *W. somnifera* were prepared by the method described by [15] with slight modifications. Twenty gram powdered roots of *W. somnifera* and powdered leaves of *C. sinensis* were soaked in petroleum ether, methanol, ethanol, and water (200 mL) each for one day at room temperature. The samples were vacuum filtrated by using Whatman filter paper, and after filtration, the filtrate was subjected to evaporation with the help of a rotary evaporator. Dried crude samples were collected and stored at 4°C which were used for phytochemical analysis as well as antioxidant analysis in vitro.

## Optimization of the Solvent System for In Vitro Antioxidant Potential

To optimize the solvent system for plants *C. sinensis* and *W. somnifera*, different solvent systems were used to find out the best solvent which helps to enhance the antioxidant potential. The selection of a good solvent is very crucial for the extraction of maximum phytochemicals owing to different chemical structures as well as the polarity of phytochemicals in a specific solvent [16]. In this study, we have used different solvents (petroleum ether, methanol, ethanol, water) to evaluate antioxidant potential. After recovering the extract, it was used for different antioxidant assays (Superoxide scavenging assay, DPPH assay, and hydrogen peroxide scavenging assay).

## Antioxidant Assays

### *Superoxide Anion Scavenging*

This test was performed according to the method given by Vo et al. with minor modifications [17]. Superoxide free radical's generation takes place in PMS-NADH (phenazine methosulfate-nicotinamide adenine dinucleotide) system due to the NADH oxidation which leads to the formation of formazan which is blue during NBT (nitro-blue tetrazolium) reduction. 3 mL of final reaction volume used for the assay contains 100 µL of 20 µg of riboflavin solution + 200 µL of 12 mM EDTA solution + 100 µL of 0.1 mg NBT solution + 200 µL methanol and 50 mM phosphate buffer were used to dilute the reaction mixture and at 590 nm absorbance of the solution was taken after five minutes.

$$\% \text{ scavenging} = (\text{Absorbance of control} - \text{Absorbance of test sample} / \text{Absorbance of control}) \times 100$$

The test was performed in triplicates and data were represented as mean ± SD.

### *Hydrogen Peroxide ($H_2O_2$) Scavenging Assay*

This test was performed according to the method given by Dwibedi and Saxena with minor modifications [18]. Phosphate buffer (100 mM) pH 7.4 was prepared and 10 mM hydrogen peroxide was mixed with it. Test samples (50–200 µg/mL of *C. sinensis*, *W. somnifera,* and their combination) were added to hydrogen peroxide and after 10 min of incubation, absorbance was taken at 230 nm. Control was used for the calculation of percent inhibition. The test was performed in triplicates and data were represented as mean ± SD.

### *DPPH Assay*

This test was performed according to the method given by Emad et al. with minor modifications [19]. DPPH reaction with antioxidants leads to the formation of Diphenylpicryl hydrazine; nonradical and the purple color converted into yellow color. So, simply this is a color reduction assay that will be detected spectrophotometrically. DPPH (1 mL of 100 µM in methanol) was mixed with the test sample (50–200 µg/mL of *C. sinensis*, *W. somnifera,* and their combination) with a final reaction volume of 3 mL and after the 30 min incubation absorbance was recorded at 517 nm. Free radical scavenging activity was expressed in percent scavenging and calculated using the following formula and the inhibitory concentration ($IC_{50}$) value was recorded by linear regression analysis between concentrations of sample and inhibition percentage.

$$\text{DPPH scavenging activity } (\%) = \left[(\text{A control} - \text{A sample}) \div (\text{A control})\right] \times 100\%$$

where A control = absorbance of DPPH solution, A sample = absorbance of the sample. The test was performed in triplicates and data were represented as mean ± SD.

### Synergistic Antioxidant Potential of *C. Sinensis* and *W. Somnifera*

*C. sinensis* and *W. somnifera* were tested in conjunction to see whether there was any synergy after refining the solvent system. DPPH assay, hydrogen peroxide radical scavenging assay, and superoxide radical scavenging assay were used to determine percent scavenging when mixing *C. sinensis* and *W. somnifera* extracts (50 µg/mL + 25 µg/mL, 50 µg/mL + 50 µg/mL, 150 µg/mL + 25 µg/mL, 200 µg/mL + 150 µg/mL. The test was performed in triplicates and data were represented as mean ± SD.

### Synergistic Antioxidant Potential of *C. Sinensis* and *W. Somnifera* Using RSM (Response Surface Methodology)

#### *Experimental Design Using RSM*

In the current investigational study, CCD (central composite design) was used for getting different combinations of various concentrations of *C. sinensis* and *W. somnifera* extracts for enhancing antioxidant potential. RSM is used to obtain the optimized plant concentrations from all probable combinations. In the current investigation CCD (RSM) was explored to get optimized plant concentrations to get enhanced antioxidant potential from *C. sinensis* and *W. somnifera*.

#### *Central Composite Design (CCD)*

Once the independent variables having an extensive influence on the enhancement of antioxidant potential were identified, CCD was explored to augment the levels of these independent variables. The statistical software package 'Design-Expert_11.0 Stat-Ease, Inc., (Minneapolis, USA) was used to analyze the experimental design. A set of 13 experiments were generated (Table 1). All the experiments were completed in triplicates and their mean ± SD was calculated. The experimental values of antioxidant potential were obtained from the following quadratic polynomial equation analyzed by the standard ANOVA (analysis of variance), which resulted in the following equation.

$$Y = b_0 + \sum_i = 1nb_iX_i + \sum_i = 1nb_{ii}X_{2i} + \sum_i = 1n - 1\sum j = i + 1nb_{ij}X_iX_j$$

where $Y$ = response; $X_i$ and $X_j$ = independent variables; $b_0$ defines regression coefficient for the intercept, $b_i$ for linear, $b_{ij}$ for cross-product terms and $b_{ii}$ for quadratic.

**Table 1** RSM generates various combinations

| Run | Factor 1A: *C. sinensis* (µg/mL) | Factor 2B: *W. somnifera* (µg/mL) |
|---|---|---|
| 1 | 81.63 | 200.5 |
| 2 | 482.63 | 200.5 |
| 3 | 200.5 | 482.63 |
| 4 | 400 | 1 |
| 5 | 200.5 | 200.5 |
| 6 | 200.5 | 200.5 |
| 7 | 400 | 400 |
| 8 | 200.5 | 81.63 |
| 9 | 1 | 400 |
| 10 | 200.5 | 200.5 |
| 11 | 1 | 1 |
| 12 | 200.5 | 200.5 |
| 13 | 200.5 | 200.5 |

Factor 1 corresponds to the various concentrations of *C. sinensis* and factor 2 corresponds to various concentrations of *W. somnifera*. C1 to C13 run represents combination numbers

RSM *Response surface methodology*, C. sinensis *Camellia sinensis*, W. somnifera *W. somnifera*

#### *Statistical Analysis and Modeling*

To design the experiments appropriately the values on the enhancement of antioxidant potential were exposed to ANOVA (analysis of variance). A second-order polynomial equation was used to calculate the scientific relationship of the independent variables (plant concentrations) and the response (Superoxide radical scavenging and $H_2O_2$ radical scavenging). Evaluation of the linear, quadratic and interactive effects of independent variables on the response can be done with the second-order polynomial equation. Multiple nonlinear regressions help to analyze the response for each run in Design Expert which further leads to the generation of coefficients of the second-order polynomial equation included in the final models. *F*-value and *p*-value were used to determine the goodness of the model. 3D contour plots were drawn to show optimized antioxidant potential. Grooved centers in the plots indicate the highest antioxidant potential.

#### *Validation of Response Surface Model*

To determine the accuracy of the model, the concentration of two independent variables (concentration of *C. sinensis* and *W. somnifera*) which have the main impact on antioxidant potential as attained by RSM were randomly selected within the design space.

## Results and Discussions

### Optimization of a Solvent System for In Vitro Antioxidant Potential

Plant concentrations (50, 100, 150, 200 µg/mL) for optimizing the solvent system were used with different solvent systems, i.e., petroleum ether, methanol, ethanol and water. After recovering the plant extract using different solvents DPPH, superoxide radical scavenging and hydrogen peroxide free radical assay was performed and the maximum antioxidant activity (% scavenging) for *C. sinensis* was found ($44.9 \pm 0.62$, $41.99 \pm 0.11$, $38 \pm 0.54$, $20 \pm 0.45$) as shown in Fig. 1a and for *W. somnifera* ($40.22 \pm 0.39$, $36 \pm 0.58$, $37.99 \pm 0.51$, $21.67 \pm 0.21$) as shown in Fig. 1b with methanol, ethanol, water, petroleum ether, respectively, for DPPH assay. Antioxidant potential for superoxide radical scavenging assay with *C. sinensis* found was ($43.77 \pm 0.62$, $40.88 \pm 0.54$, $37 \pm 0.54$, $19.29 \pm 0.45$) as shown in Fig. 1c and with *W. somnifera* ($43.29 \pm 1.12$, $33.66 \pm 0.50$, $37.99 \pm 0.70$, $18.66 \pm 0.038$) Fig. 1d with methanol, ethanol, water, petroleum ether, respectively. Free radical scavenging activity for hydrogen peroxide assay with *C. sinensis* was found to be ($43.88 \pm 0.10$, $35.26 \pm 0.36$, $39.25 \pm 0.71$, $20.58 \pm 0.54$) as shown in Fig. 1e and with *W. somnifera* ($41.88 \pm 0.11$, $35.3 \pm 0.4$, $37.99 \pm 0.7$, $20.6 \pm 0.23$) as shown in Fig. 1f with methanol, ethanol, water, petroleum ether, respectively. $IC_{50}$ has been calculated for *C. sinensis* ($235.26 \pm 0.012$ µg/mL) and *W. somnifera* ($255.89 \pm 0.07$ µg/mL) in the optimized solvent system (methanol) as shown in Table 2.

### Synergistic Antioxidant Potential of *C. Sinensis* and *W. Somnifera*

Synergy among *C. sinensis* and *W. somnifera* has been checked using various concentrations of two plant extracts with the help of DPPH, hydrogen peroxide and superoxide free radical scavenging assay. The maximum antioxidant potential was found to be with a combination of 200 µg/mL *C. sinensis* and 150 µg/mL of *W. somnifera* $56.57 \pm 0.62$ with DPPH assay, $56.998 \pm 0.42$ with hydrogen peroxide assay and $55.44 \pm 0.53$ as shown in Fig. 2 with superoxide radical scavenging assay and $IC_{50}$ value for *Camelia sinensis* + *W. somnifera* was ($IC_{50} = 215.47 \pm 0.06$ µg/mL) for methanol was found as shown in Table 2**.** All the tests were executed in triplicates, and the data was represented in terms of mean ± standard deviation. The results were analyzed by ANOVA followed by Turkey post hoc test ($p \leq 0.05$). Statistical analysis was performed using Graph Pad Prism 7 software and $p \leq 0.05$ was considered significant.

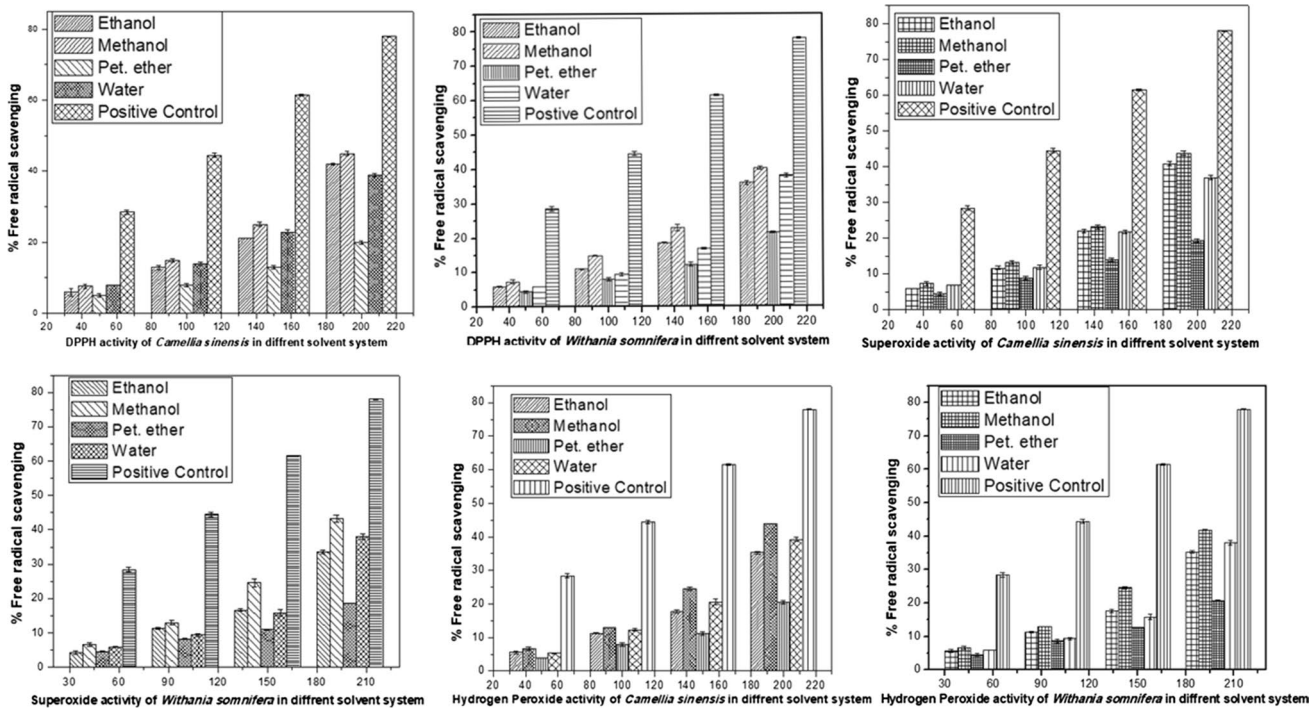


**Fig. 1** Optimization of a solvent system for **a** DPPH assay for *C. sinensis* **b** DPPH assay for *W. somnifera* **c** Superoxide radical scavenging assay for *C. sinensis* **d** Superoxide radical scavenging assay for *W. somnifera* **e** Hydrogen peroxide scavenging assay for *C. sinensis* **f** Hydrogen peroxide assay for *W. somnifera*

**Table 2** $IC_{50}$ of different extracts evaluated using DPPH, hydrogen peroxide scavenging superoxide radical scavenging

| S. No | $Sample_0$ (µg/mL)* | DPPH scavenging | Hydrogen peroxide scavenging assay | Superoxide scavenging assay |
|---|---|---|---|---|
| 1 | Standard | 115.69 ± 0.014 | 115.69 ± 0.014 | 115.69 ± 0.014 |
| 2 | Kangra green tea | 235.26 ± 0.012 | 238.44 ± 0.005 | 240.03 ± 0.12 |
| 3 | Ashwagandha | 256.39 ± 0.43 | 259.93 ± 0.16 | 255.89 ± 0.07 |
| 4 | Kangragreen tea + Ashwagandha | 215.48 ± 0.06 | 217.09 ± 0.019 | 218.06 ± 0.034 |

*Data presented are mean ± standard deviation of three replicates

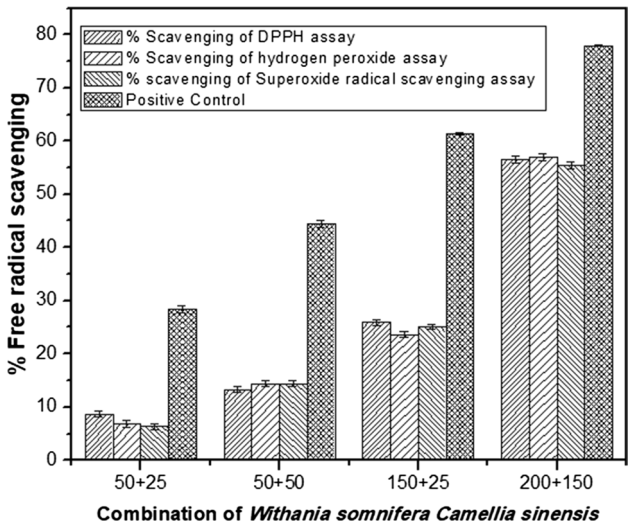


**Fig. 2** Antioxidant potential of various concentrations *Camelia sinensis* and *W. somnifera* in combination using (50 µg/mL + 25 µg/mL, 50 µg/mL + 50 µg/mL, 150 µg/mL + 25 µg/mL, 200 µg/mL + 150 µg/mL) were tested with DPPH, hydrogen peroxide and superoxide free radical scavenging assay

## Synergistic Antioxidant Potential of *C. Sinensis* and *W. Somnifera* Through RSM

Antioxidant potential in terms of percent scavenging was found to be 55.9 ± 0.62% in the presence of parameters, i.e., 200 µg/mL of *C. sinensis* and *W. somnifera,* respectively, when experiments were performed without RSM. However, in this study, optimization of antioxidant potential is carried out by using RSM, maximum antioxidant potential found was 77.08% for superoxide radical scavenging and 78.01% for hydrogen peroxide scavenging assay. At this point, the interactions between the most influencing factors obtained from OVAT (one variable at a time) approach was considered to design RSM using Central Composite Design of Design-Expert_6.0.8 Stat-Ease, Inc., (Minneapolis, USA) for getting maximum antioxidant potential.

### *CCD*

The CCD was used for the enhancement of antioxidant potential which helps to analyze the behavior of the model inside the limits provided. Therefore, how the factors under investigation interact with each other can be deduced. Design-Expert_6.0.8 Stat-Ease, Inc., (Minneapolis, USA) has generated a set of 13 experiments (Table 1). A set of 13 experiments (Table 1) with plant concentrations ranging from 1 to 400 µg/mL for *C. sinensis* and 1–400 µg/mL for *W. somnifera* was generated as per Design matrix data. These concentrations were further performed and analyzed for antioxidant potential using superoxide radical scavenging assay and hydrogen peroxide scavenging assays.

Table 5 and Fig. 4a show observed and predicted percent scavenging for superoxide radical scavenging assay. ANOVA was used to analyze triplicate experimental and predicated percent scavenging values as given below in the model equation.

### *Model Equation*

An explanatory model was devised by software using Design expert. The explanatory model obtained from the data was

$$Y = +74.63 + 11.39 * A + 6.48 * B - 0.6792 * AB - 9.11 * A^2 - 8.16B^2$$

where $Y$ is the response value (% scavenging) and $A$, $B$ depicts the concentrations of *C. sinensis* and *W. somnifera,* respectively.

Table SI and Fig. 4b show the mean observed value of % scavenging and the mean predicted value of % scavenging for hydrogen peroxide scavenging assay. ANOVA was used to analyze triplicate experimental values of % scavenging and predicated values as given below.

An explanatory model was devised by software using a Design expert. The explanatory model obtained from the data was

$$Y = +75.93 + 11.45^*A + 5.77^*B - 0.6792^*AB - 9.88^*A^2 - 8.78^*B^2 \quad (5)$$

where *Y* is the response value (% scavenging) and *A*, *B* depicts the concentrations of *C. sinensis* and *W. somnifera,* respectively.

*Superoxide Radical Scavenging*

Quantification of superoxide free radical scavenging activity of 13 different combinations was attained by quantifying the scavenging of superoxide free radicals. All combinations unraveled an extensive difference in the free radical scavenging for aqueous, ethanolic, and methanolic extracts. However, the maximum response in increasing the free radical scavenging potential of *C. sinensis* and *W. somnifera* was shown by combination number 6 of methanolic extract containing (200.5 µg/mL + 200.5 µg/mL) of *C. sinensis* and *W. somnifera* concentration (77.08%), respectively, followed by combination number 3 containing (200.5 µg/mL + 482.63 µg/mL) of *C. sinensis* and *W. somnifera* concentration (67.10%), respectively, followed by combination number 4 containing (1 µg/mL + 400 µg/mL) (60.23%) of *C. sinensis* and *W. somnifera* concentration, respectively. Tables 3, 4, 5 and Figs. 3a and 4a signify the outcome of superoxide scavenging assay for methanolic extracts.

*ANOVA*

ANOVA (analysis of variance) is used to analyze the implication and authenticity of Central Composite Design (CCD) (Table 4). Variation of the data about its mean value is measured by the *F* value. The significance and authenticity of the model solely depend on the F value which should be higher and the *p*-value which should be lower. We found our model significant as the F value 67.22 suggests the model is significant enough on the other hand $p \leq 0.05$ designates the model is significant which

**Table 3** Percentage scavenging of superoxide radical scavenging assay and hydrogen peroxide free radical by phytochemicals present in the methanolic extract of *C. sinensis* (Kangra green tea) and *W. somnifera* (Ashwagandha)

| RUN | A: *C. sinensis* (µg/mL) | B: *W. somnifera* (µg/mL) | Superoxide radical scavenging assay (% Scavenging) | Hydrogen peroxide scavenging assay (% scavenging) |
|---|---|---|---|---|
| 1 | 81.63 | 200.5 | 41.08 | 40.546 |
| 2 | 482.636 | 200.5 | 74.25 | 74.09 |
| 3 | 200.5 | 482.63 | 67.10 | 65.091 |
| 4 | 400 | 1 | 60.23 | 60.23 |
| 5 | 200.5 | 200.5 | 73.2083 | 74 |
| 6 | 200.5 | 200.5 | **77.08** | **78.01** |
| 7 | 400 | 400 | 74.1 | 74.1 |
| 8 | 200.5 | 81.63 | 52.01 | 53.98 |
| 9 | 1 | 400 | 53.36 | 53.36 |
| 10 | 200.5 | 200.5 | 71.2167 | 76 |
| 11 | 1 | 1 | 36.78 | 36.78 |
| 12 | 200.5 | 200.5 | 76.7 | 76.7 |
| 13 | 200.5 | 200.5 | 74.95 | 74.95 |

The methanolic combination number 6 showed the highest percentage scavenging of superoxide radical scavenging (77.08%) and hydrogen peroxide free radical (78.01%)

**Table 4** The Analysis of Variance (quadratic model) for percentage scavenging of superoxide free radical.

| Source | Sum of squares | df | Mean square | *F*-value | *p*-value | |
|---|---|---|---|---|---|---|
| Model | 2295.31 | 5 | 459.06 | 67.22 | < 0.0001 | Significant |
| A-C. sinensis | 1037.13 | 1 | 1037.13 | 151.87 | < 0.0001 | |
| B-W. somnifera | 335.52 | 1 | 335.52 | 49.13 | 0.0002 | |
| AB | 1.85 | 1 | 1.85 | 0.2702 | 0.6192 | |
| A2 | 576.82 | 1 | 576.82 | 84.47 | < 0.0001 | |
| B2 | 463.17 | 1 | 463.17 | 67.82 | < 0.0001 | |
| Residual | 47.8 | 7 | 6.83 | | | |
| Lack of fit | 23.72 | 3 | 7.91 | 1.31 | 0.3862 | Not significant |
| Pure error | 24.08 | 4 | 6.02 | | | |
| Cor total | 2343.12 | 12 | | | | |

The lack of fit value for *p*-value was of 1.31 found as not significant, the model value of 0.0001 found significant and model is also significant

**Table 5** CCD variable validation of enhancement of antioxidant potential for superoxide radical scavenging with the help of predicted values and experimental values of *W. somnifera* and *C. sinensis* combination

| Run | Coded variables | | Response (percent scavenging) | | |
|---|---|---|---|---|---|
| | *C. sinensis* (µg/mL) | *W. Somnifera* (µg/mL) | Actual | Predicted | Difference |
| 6 | 200.5 | 200.5 | 77.08 | 76 | 1.08 |
| 3 | 200.5 | 482.63 | 67.10 | 67 | 0.10 |
| 4 | 400 | 1 | 60.23 | 61 | 1.23 |

further indicates only 0.01% chance can cause large model value could be there due to error. Significant model terms we found in our model as A, B, A2, B2. If there are values larger than 0.10 designate the model terms are not significant. In the case of several model terms which are insignificant, the model can be improved by reducing it. The significance of the model can also be analyzed by R (correlation coefficient) and $R^2$ (determination coefficient). Experimental and predicted values are said to be in better correlation if the values are close to 1. In our experiment, we found a correlation coefficient (0.97) which shows that 97% variation is mainly due to two variables and 2.04% dissimilarity in the model could not be explained by the model. Both ''Pred R-Squared'' of 0.91 and ''Adj R-Squared'' of 0.96 are in reasonable agreement with each other. All the above results showed that experimental conditions are well predicted by the model. The adequate Precision value found more than 4 is a good measure of the signal to noise ratio therefore our results show the ratio of 20.16 specifies an adequate signal. This model can be used to navigate the design space. We found 2.61, 64.01, and 47.80 values as standard deviation, mean, and predicted residual sum of squares, respectively, in the CCD model. 3D contour plots (response surface curves) have been shown to analyze how different plant concentrations interact with each other and this helps to find out the optimum value of plant concentration interaction to enhance antioxidant potential (Fig. 3a**).**

*Validation of the Model*

Three sets of experiments, with optimized antioxidant potential plant concentrations enhanced by RSM (CCD) viz-(A) concentration of *C. sinensis* (B) concentration of *W. somnifera* were carried out in triplicates to find out the authenticity of the CCD model to show that the model predicted values are reliable with experimental results. (Table 5) presents the antioxidant potential of each experiment along with the predicted response. The results verify the previous model that 200.5 µg/mL plant concentration of *C. sinensis*

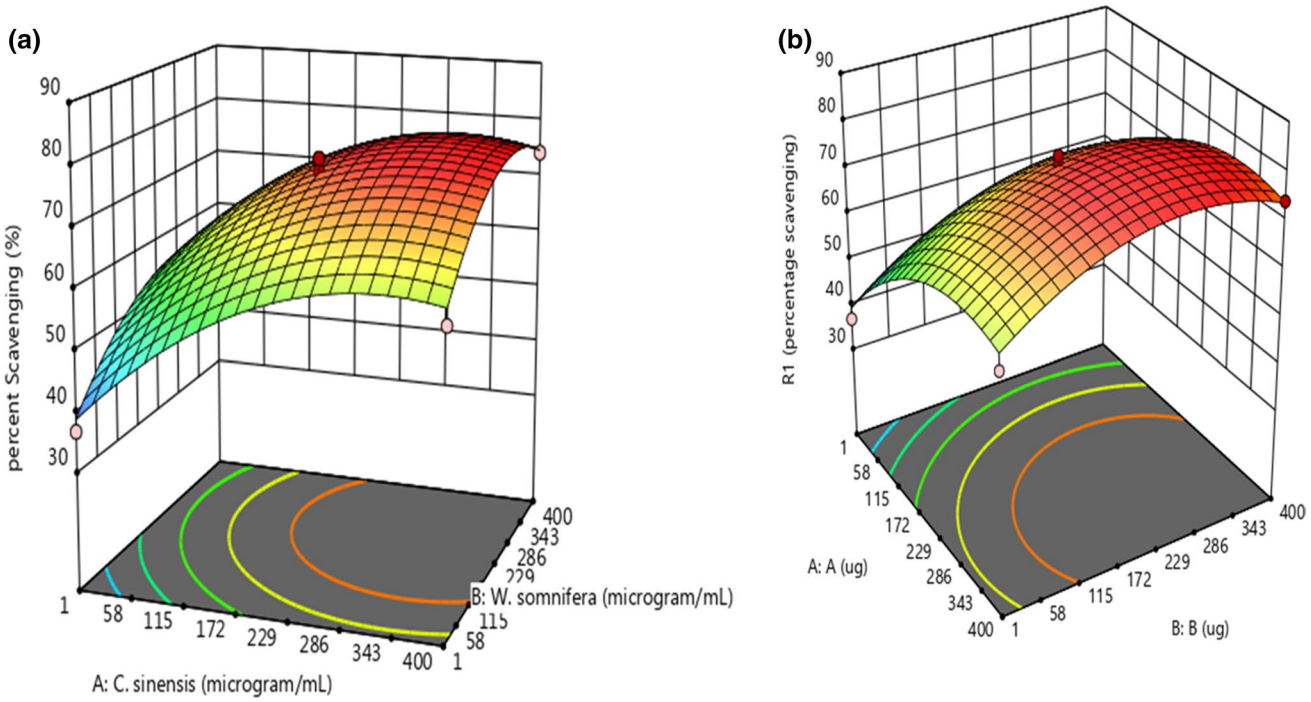


**Fig. 3** **a** 3D contour plots for the establishment of the response values between the independent variables represented by A (*C. sinensis*) and B (*W. somnifera*) and dependent variable represented by percent scavenging of superoxide free radicals through a methanolic set of extract of the mixture **b** Percentage scavenging of hydrogen peroxide free radical by phytochemicals present in the methanolic extract of A (*Camellia sinensis*) and B (*W. somnifera*). The methanolic combination number 6 showed the highest percentage scavenging of hydrogen peroxide free radical (78.01%)

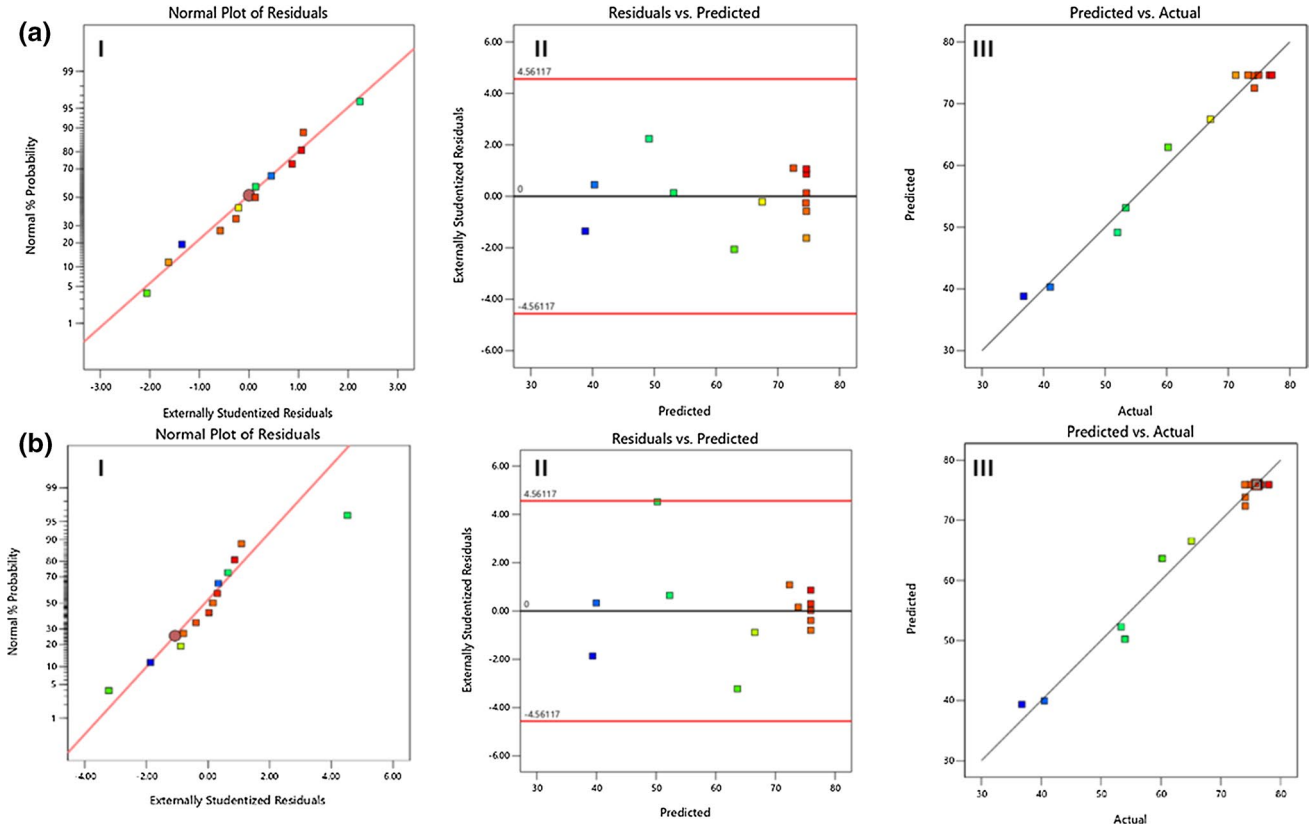


**Fig. 4** **a** ANOVA analysis of superoxide radical scavenging assay (i) normal plots for residual (ii) residual versus predicted graph (iii)The predicted vs actual graph **b** ANOVA analysis of hydrogen peroxide scavenging assay (i) normal plots for residual (ii) residual versus predicted graph (iii)The predicted vs actual graph

and 200.5 µg/mL plant concentration of *W. somnifera* was found as the most appropriate set for attaining the highest antioxidant potential. The experimental antioxidant potential of 77.08% and predicted value of 76% is very close to each other. A very minute difference between predicted and experimental values shows the validity of an experiment.

*Hydrogen Peroxide Scavenging Assay*

Determination of antioxidant potential of 13 different combinations was assessed by another chemical test, i.e., hydrogen peroxide free radical scavenging. All combinations in aqueous, ethanolic, and methanolic extracts were capable of neutralizing free radicals, whereas the maximum antioxidant potential of *C. sinensis* and *W. somnifera* was shown by combination number 6 of methanolic extract containing (200.5 µg/mL + 200.5 µg/mL) of *C. sinensis* and *W. somnifera* concentration, respectively, followed by combination number 7 of methanolic extract containing (400 µg/mL + 400 µg/mL) of *C. sinensis* and *W. somnifera* concentration, respectively, followed by combination number 2 of methanolic extract (67.12%) containing (482.63 µg/mL + 200.5 µg/mL) of *C. sinensis* and *W. somnifera* concentration, respectively. In vivo hydrogen peroxide is formed by the enzyme superoxide dismutase or SOD in a dismutation reaction (Eq. 1). Although $H_2O_2$ is not considered a free radical, it has the potential to damage the cell at very low concentrations and at high concentrations, it can deactivate glyceraldehyde-3-phosphate dehydrogenase. It can easily penetrate the biological membranes. $H_2O_2$ in the presence of transition metal ions produces hydroxyl radical which can damage DNA also [20]. Tables 3, 4, and 5 signify the outcome of the hydrogen peroxide scavenging assay of methanolic extracts.

*ANOVA*

ANOVA is used to find the implication and authenticity of Central Composite Design (CCD) (Table 6). Variation of the data about its mean value is measured by the F value. The significance and authenticity of the model solely depend on the F value which should be higher and the *p*-value which should be lower. We found our model significant as the F value 68.74 suggests the model is significant enough on the other hand $p \leq 0.05$ designates the model is significant which further indicates only 0.01% chance can cause large model

**Table 6** The Analysis of Variance (quadratic model) for percentage scavenging of hydrogen peroxide free radical. The lack of fit value for *p*-value was of 13.05 found as not significant, the model value of 0.0001 found significant and thus the model is significant

| Source | Sum of squares | df | Mean square | *F*-value | *p*-value | |
|---|---|---|---|---|---|---|
| Model | 2393.8 | 5 | 2393.8 | 68.74 | < 0.0001 | Significant |
| A-A | 1049.32 | 1 | 1049.32 | 1049.32 | < 0.0001 | |
| B-B | 266.46 | 1 | 266.46 | 266.46 | 0.0005 | |
| AB | 1.85 | 1 | 1.85 | 1.85 | 0.6226 | |
| $A^2$ | 679.59 | 1 | 679.59 | 679.59 | < 0.0001 | |
| $B^2$ | 535.67 | 1 | 535.67 | 535.67 | < 0.0001 | |
| Residual | 48.75 | 7 | 48.75 | 6.96 | | |
| Lack of fit | 39.15 | 3 | 39.15 | 13.05 | 0.0679 | Not significant |
| Pure error | 9.61 | 4 | 9.61 | 2.4 | | |
| Cor total | 2442.55 | 12 | 2442.55 | | | |

value could be there due to error. Significant model terms we found in our model as A, B, A2, B2. If there are values larger than 0.10 designate the model terms are not significant. In the case of several model terms which are insignificant, the model can be improved by reducing it. The significance of the model can also be analyzed by R (correlation coefficient) and $R^2$ (determination coefficient). Experimental and predicted values are said to be in better correlation if the values are close to 1. In our experiment, we found a correlation coefficient (0.98) which shows that 98% dissimilarity is mainly by two variables and 2% dissimilarity in the model could not be explained by the model. Both ''Pred R-Squared'' of 0.87 and ''Adj R-Squared'' of 0.96 are in reasonable agreement with each other. All the above results showed that experimental conditions are well predicted by the model. The adequate Precision value found more than 4 is good measures the signal to noise ratio therefore our results show the ratio of 20.39 specifies an adequate signal. This model can be used to navigate the design space. We found 2.64, 64.45, and 48.75 values as standard deviation, mean, and predicted residual sum of squares, respectively, in the CCD model. 3D contour plots (response surface curves) have been shown to analyze how different plant concentrations interact with each other and this helps to find out the optimum value of plant concentration interaction to enhance antioxidant potential (Fig. 3b**).**

*Validation of the Model*

Three sets of experiments for optimum antioxidant potential of plant concentrations enhanced by RSM (CCD) viz-(A) concentration of *C. sinensis* (B) concentration of *W. somnifera* were carried out in triplicates to find out the authenticity of the CCD model to show that the model predicted values are reliable with experimental results. (Table SI) presents the antioxidant potential of each experiment along with the predicted response. The results verify the previous model that 200.5 µg/mL plant concentration of *C. sinensis* and 200.5 µg/mL plant concentration of *W. somnifera* was found as the best combination for obtaining the maximum antioxidant potential. The experimental antioxidant potential of 78.01% and predicted value of 77.01% are very close to each other. A very minute difference between predicted and experimental shows the validity of the experiment.

## Discussion

Exploration of synergistic antioxidant potential of *C. sinensis* (Kangra green tea) and *W. somnifera* (Ashwagandha) through RSM is relatively new, although these two plant phytochemicals were known for their therapeutic potential but still work is not done on their synergistic behavior. *C. sinensis* has been linked with numerous health benefits like antioxidant activity, anti-inflammatory, anti-diabetic, anti-atherosclerosis, anti-cancer, and an anti-aging moiety. Similarly, *W. somnifera* has also been reported for various therapeutic benefits like anti-inflammatory, antioxidant, anticancer, antidiabetic, immunomodulatory, anti-atherosclerosis, antiaging and antiviral [4, 5]. However, there is no data available on enhancing the antioxidant potential of *C. sinensis* and *W. somnifera* synergistically by using response surface methodology.

The history of drinking green tea is quite ancient but the chemical component investigation has started recently. In 1827, caffeine was isolated from tea [21] and the presence of flavonoids like epicatechin, epicatechin-gallate, and epigallocatechin was confirmed in 1927 [22]. Several reports published show that green tea polyphenols help to cure age-related disorders like cardiovascular diseases, skin anti-aging [23, 24]. In Ayurveda, roots of *W. somnifera* have been used as a medicine to treat rheumatoid arthritis, in treating infertility [4]. Thus, both these plants have excellent anti-oxidant and anti-aging properties. Therefore, the current investigation was focused on the enhancement of plant concentrations of *C. sinensis* and *W. somnifera* with the help of RSM for the enhancement of antioxidant potential in a synergistic way.

The selection of appropriate solvent plays an important function in the extraction of phytochemicals to maximize the extraction as well as the antioxidant potential of plants and in this investigation, they found maximum antioxidant activity in methanol as various medicinal phytochemicals have different affinities for different solvents [16]. In our case, we have used various solvents to extract phytochemicals and methanol was optimized as the best solvent for extraction which is in agreement with the previous study.

RSM is the best method to optimize the production of industrially important enzymes, food products, pharmaceutical drugs. Statistically designed experiments can help to set up the relation between linear and quadratic terms [25]. RSM has been used to optimize polyphenol content in green tea with the help of the ultrasonic extraction method [26]. In the current study, plant concentrations of *C. sinensis* and *W. somnifera* have been optimized using RSM. Two independent variables *C. sinensis* and *W. somnifera* were considered to find out the optimum combination of plant concentrations to enhance the antioxidant potential. The predicted values given by RSM were again performed experimentally to validate the results. The superoxide radical scavenging method is popularly used to find out the antioxidant capacity of various foods [17]. The contour plot diagram of superoxide radical scavenging has been found to show better scavenging potential with concentrations (200.5 + 200.5 µg/mL) of *C. sinensis* and *W. somnifera,* respectively. In one of the similar studies, *Hibiscus sabdariffa* extract and *Theobroma cacao* combination have been found to show high antioxidant activity which is good for cardiovascular diseases [27]. The actual superoxide radical scavenging activity of combined formulation of *C. sinensis* and *W. somnifera* was found as 77.08% for superoxide anion scavenging assay and 77.01% for hydrogen peroxide scavenging assay which is significantly higher as compared to the scavenging effects of the formulated beverage (pineapple, orange, carrot and *Hibiscus sabdariffa*) ranging from 33 to 73% as reported by Ogundele et al. [28]. In another study tea and ginger blend in 2:1 ratio has been reported to show 78.7% scavenging of peroxide-free radicals [29].

Three-dimensional contour plots generated in CCD in superoxide anion scavenging and hydrogen peroxide scavenging assay were able to describe (97.96% and 98%, respectively) effect of variation on a combined formulation which is in agreement with the previous investigation reported by Ogundele et al. in 2016. From ANOVA, the model's F-value of 67.22 (superoxide anion scavenging assay) and 68.74 (hydrogen peroxide scavenging assay) implies that there was a significant effect of the herbal extract mixture on the antioxidant property at $P \leq 0.05$ which is in agreement with the previous report [27].

After RSM studies, we were able to achieve a 1.37-fold increase in the antioxidant potential of the herbal mixture. Thus, the present study has shown that RSM is the best method to optimize antioxidant potential. We also conclude that plant's antioxidant potential can be enhanced synergistically. Therefore, in future clinical studies, *C. sinensis* and *W. somnifera* can be used in combination to analyze their efficacy on animal models which will be helpful to release its combinatorial effect to delay the onset of aging diseases in humans.

## Conclusion

A combination of strong antioxidant factors which are specific in their action, are nontoxic, and show anti-stress properties from plants will be a better solution for anti-aging. We expect to make a suitable formulation containing *C. sinensis* and *W. somnifera* to control ROS-mediated pathogenesis, and its control by suitable antioxidant therapy/therapies. Development of a formulation in the form of nutritional supplement that will not have any side-effects, thus the product can have market value in near future.

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**Authors' Contribution** Zaved Ahmed Khan conceived the idea and designed the experiments. Arti Devi carried out the experiments and wrote the manuscript. Zaved Ahmed Khan, Vagish Dwibedi and Nancy George supervised the work and edited the paper.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflicts of interest.

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