ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Assessment of *in vitro* Antioxidative and Anti-Inflammatory Effect of *Caesalpinia Bonducella* Seed Stabilized Silver Nanoparticles

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Article History:

Received on: 01.08.2019
Revised on: 10.11.2019
Accepted on: 16.11.2019

Keywords:

CB seed AgNPs,
flavonoid,
phenol,
antioxidant,
anti-inflammatory

ABSTRACT

Membrane lipid peroxidation and DNA, protein damage is mediated by free radicals, which form the basis of chronic pathological complications.AgNPs are an important class of nanomaterials for a wide range of biomedical applications. The present study endeavors *in vitro* antioxidant and anti-inflammatory activity of green synthesized silver nanoparticles (AgNPs) using medicinal plant extract from *Caesalpinia bonducella seeds*. Total flavonoid and phenolic contents were determined. The antioxidant potential of capped AgNPs was assessed using DPPH assay, Phosphomolybdenum assay, FRAP assay, metal chelating, hydrogen peroxide, and hydroxyl radical scavenging methods. *In vitro* anti-inflammatory assay of *CB* seed, AgNPs were performed against the standard drug. *CB* seed AgNPs possessed high flavonoid and phenol compared to aqueous *CB* seed extract. The antioxidant methods confirmed that the silver nanoparticles have more antioxidant activity as compared to vitamin C. The synthesized silver nanoparticles exhibited potential anti-inflammatory activity with the IC50 71.3μg/ml. Hence, this work clearly demonstrated that the coated AgNPs with CB seeds act as a potent free radical scavenger and could be considered as a potential source for anti-inflammatory drugs.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v11i1.1879

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INTRODUCTION

Nanotechnology is a progressing field imparting wide application in biomedical science and biotechnology research area. Though a number of approaches are convenient for the synthesis of the silver nanoparticles, environment-friendly technology holds great importance (Natarajan *et al.*, 2010; Klueh *et al.*, 2000). Existing literature reports that usage of medicinal plants is valuable as a result of their medicinal properties would pass over to the nanoparticles (Santhoshkumar *et al.*, 2011; Zargar *et al.*, 2011). The active molecules in the plant extract such as flavonoid, tannins, amines, aldehyde/ketone groups, and polyols and proteins act as reducing and capping agents transfer useful properties to the green synthesized nanoparticle by controlling morphology, size and protect the surface preventing aggregation (Verma and Mehata, 2016).

Caesalpinia bonducella belongs to the Family Caesalpiniaceae found mainly in the southern amino

acids, fatty acids alkaloids, terpenoids, phytosterols, and carbohydrates (Kannur, 2011). In traditional Indian medicine, the usage of Caesalpinia bonducella seeds has created a great impact in ameliorating various diseases. It possesses various medicinal effects such as anti-hyperlipidemic, anti-hyperglycemic effect, antipyretic, anti-inflammatory, and antioxidant activity (Kannur *et al.*, 2006; Archana *et al.*, 2005; Shukla *et al.*, 2009).

Natural antioxidants, mainly the flavonoids in the plants, provide protection against harmful free radicals counteracting the oxidative damage and are firmly associated with diminished risk of chronic diseases (Krishnaiah *et al.*, 2007; Kim *et al.*, 2003) have recorded that phytocompounds of the medicinal plants provide antioxidant properties to the nanoparticles. (Zima *et al.*, 2001) have reported that in biological systems, free radicals are produced during metabolism and other physiological activities within the limits of free radical scavenging mechanism of the system. The various forms of ROS that include superoxide anion free radicals, hydroxyl free radicals, H_2O_2, less stable excited oxygen, which is generated during inflammation and its associated diseases intensifies the cellular damage and age declining process (Halliwell, 2001; Venkataraman *et al.*, 2013). These free radical production and antioxidant defenses are correlated with adversity to a broad spectrum of molecular species that include lipids, proteins, and nucleic acids. The intricate relationship between oxidative stress and inflammation were emphasized by several studies. They are involved in multiple chronic inflammatories and deteriorating disorders imposing poor health status and enhancing the probability of persistent diseases such as cancer, atherosclerosis, and Alzheimer's disease (Birben *et al.*, 2012; Mittal *et al.*, 2014). The redox properties of phenolic and flavonoid compounds in CB seeds enable them to be potent antioxidants (Sachan *et al.*, 2010).

Therefore, the present investigation was undertaken to predict the antioxidant and anti-inflammatory activities of green synthesized *C. bonducella* seed nanoparticles (CB seed AgNPs) *in vitro*. This study also attempts to explore the relationship between total phenol, flavonoid content of CB seed AgNPS, and its effective antioxidant property.

MATERIALS AND METHODS

Plant material and preparation of the extract

The synthesis *of the* silver nanoparticle is accomplished using *Caesalpinia bonducella* seeds purchased from the Madurai local market, and the nanoparticles synthesis process was carried out as described in our previous publication (Shyam *et al.*, 2018). The synthesized silver nanoparticles were used for the screening of antioxidant activity and anti-inflammatory activity.

Total phenolic contents were determined by the Folin-Ciocalteau reagent with slight modifications of (Singleton *et al.*, 1999). 1mL(1g) of CB seed AgNPs was diluted to 46 ml using distilled H_2O, added 1mL Folin–Ciocalteau reagent with intense mixing. 3 minutes later, 3mL of 2% sodium carbonate was added and left for 3 h with periodic shaking. The absorbance of the blue color that developed was read at 760 nm.

Estimation of total Flavonoids by $AlCl_3$ method

The total flavonoid was determined by the colorimetric method in which $AlCl_3$ forms complex with flavones and flavonols. To 1ml(100μg) of *Caesalpinia bonducella,* seed extract, and seed AgNPs added 0.5ml of 10% Aluminium chloride, 0.5 ml of 1M potassium acetate and 1ml of distilled H_2O. After 30 mins. Of incubation, read the absorbance at 415nm in Spectronic 20.

Antioxidant Activity Determination

DPPH radical scavenging assay

The antioxidant activity of CB seed AgNPs was measured as described by (Brand-Williams *et al.*, 1995). Absorbance was measured at 517nm after incubation for 30 minutes in the dark using a UV-Visible spectrophotometer. The following formula was applied for calculating inhibition percentage,

$$\%\ Inhibition = \left(\frac{Control - Sample}{Control}\right) \times 100$$

Phosphomolybdenum reduction assay

The assay was performed according to the procedure described by (Prieto *et al.*, 1999). Below pH 7 molybdenum is reduced by the CB seed AgNPs and results in the development of green phosphormolybdenum complex. The absorbance was measured at 695nm. Ascorbic acid was used as the standard. The antioxidant potential of CB seed AgNPs was indicated as an ascorbic acid equivalent.

Ferric (Fe^{3+}) reducing power assay

The assay using CB seed AgNPs was performed with slight modifications of the method described (Barros *et al.*, 2007). The absorbance was measured at 700nm along with ascorbic acid, which is the standard reference.

Metal chelating assay

250 μL (100μg) of CB seed AgNPs was added to a solution of 2 mmol/L FeCl2 (0.05 ml). The reaction was initiated by the addition of 5 mmol/l ferrozine (0.2 mL), and the mixture was shaken vigorously and left for 10 minutes at room temperature (Dinis *et al.*, 1994). The absorbance of the solution was then measured at 562 nm. Metal chelating activity by the nanoparticle was calculated by comparing it with standard EDTA. The results were mentioned as 1 mg EDTA proportionate to 1g CB seed AgNPs.

$$\%\ of\ inhibition = \left(\frac{Control - Sample}{Control}\right) \times 100$$

Hydroxyl Radical Scavenging Activity Assay(HRSA)

To various concentrations of silver nanoparticle of *Caesalpinia bonducella* seed AgNPs 1mL Fe - EDTA solution, 0.5mL EDTA solution, 1mL 0.85 % DMSO and 0.5ml of 0.22% Ascorbic acid were added (Klein *et al.*, 1981). The solution was placed for 15minutes in a boiling water bath of 80 to 90^{0}c. 1mL of ice-cold TCA and 3ml of Nash reagent were then added, and the reaction mixture was left at room temperature for 15 min. The absorbance was read at 412nm. The HRSA was calculated as follows,

$$\%\ HRSA = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) \times 100$$

Scavenging of Hydrogen peroxide

H_2O_2 scavenging assay by CB AgNPs was determined using the method described by (Jayaprakasha *et al.*, 2004). To different concentrations of silver nanoparticles of *Caesalpinia bonducella*, seed extract added 2ml of hydrogen peroxide solution in phosphate-buffered saline(pH 7.4). The absorbance was read at 230nm after an incubation of 10 minutes. Compared with standard Ascorbic acid.

Anti-inflammatory bioassay *in vitro*

To varying concentrations of CB seed, AgNPs extract added 0.2ml of egg albumin, 2.8ml of phosphate-buffered saline (pH6.4). After incubation at 37^{0}C in a BOD incubator for 15 minutes, the mixture was heated to 70^{o}C for 5minutes (Chandra *et al.*, 2012). Cooled and the absorbance was read 660nm against blank (Double distilled water with reagents) was used as The experiment was carried out similarly using the reference drug diclofenac

$$Percentage\ inhibition = 100 \times \left\{\frac{V_t}{V_c - 1}\right\}$$

Statistical analysis

All the experiments were performed in triplicates. The data of experimental studies were indicated as the mean $\pm$ S.d. (n=3). The statistical determination was performed using Graph Pad Prism Software version 5.01.

RESULTS AND DISCUSSION

In recent years medicinal plants have gained importance for their limited toxicity and for their efficient antioxidant property. In vitro techniques provide the tool for the detection of antioxidants present in the silver nanoparticle, which are established on the basis of the phyto compounds' ability to eliminate or scavenge free radicals (Mohanta *et al.*, 2016). Antioxidant efficiency of medicinal plants is due to the redox potential of phytochemicals, which can play an efficient role in abolishing singlet and triplet oxygen, thereby resulting in peroxide decomposition or free radicals neutralization. This antioxidant efficacy from the extract adsorbs onto the surface of the nanoparticles, enhancing its redox potential (Patil and Kumbhar, 2017). Antioxidants hold the capacity to minimize the oxidative damage precisely via counteracting with free radicals or indirectly by impeding the enzymes that produce free radicals. Free radicals may lead to inflammation, which as an early protective homeostatic immune response to tissue damage. Thus, the production of ROS plays a key role in many inflammatory diseases (Mittal *et al.*, 2014). In our study, phytoconstituents mainly flavonoid and phenol of Caesalpinia bonducella seeds silver nanoparticles were quantified and screening of their antioxidant, anti-inflammatory potential performed.

Total phenolic and flavonoid content

The total phenol components of the aqueous and CB seed AgNp extracts using Folin-Ciocalteau's reagent were detected to be 115.08 $\pm$ 0.64 and 120.15 $\pm$ 0.30 mg GAE/g, and it is the measure that corresponds to gallic acid. The total flavonoids estimation of each extract carried out using aluminum chloride reagent and quercetin as standard, and the results revealed that the CB seed AgNPs possessed 168.86$\pm$0.33 while aqueous extract contains 154.28 $\pm$0.21mg QE/g respectively, the total flavonoid contents of the extracts were calculated in terms of quercetin equivalent. Results of phytoconstituents quantification proves the fact that besides acting as good reductants and stabilizers for Ag ions they play an important role in scavenging free radicles or ROS and modulating the extent of the inflammatory response. Similar relationship was also observed by (El-Hela *et al.*, 2017) between the phytophenols and antioxidant activity in *Crataegus sinaica* leaves. Flavonoids are the classes of polyphenols that protect nucleic acids, proteins, and lipids from

oxidative destruction. There are many reports in the literature which shows the relation between flavonoids content and antioxidant activity (Firoozi *et al.*, 2016; Shukla and Mehta, 2017). The high flavonoid content in CB seed AgNPs attributes to the higher antioxidant ability Table 1.

DPPH radical scavenging assay

The DPPH radical scavenging potency of different concentrations of *CB* seed AgNPs is summarized in Figure 1a. Among the five different concentrations used in the study (25–125 μg/ml), test solution of 125 μg/ml exhibited the highest scavenging activity 93.25% same as ascorbic acid at the same concentration showed 93.55%, which were very close to each other (Figure 1a). The IC_{50} value of CB seed AgNPs and Ascorbic acid was 19.7 and 17.15μg. The variation in the antioxidant activity was due to the reason that AgNPs were enriched with the presence of different extent of flavonoids and phenols. The antioxidant potential, as evidenced by the DPPH assay of AgNPs (Figure 1a), could be due to the functional groups adhered to them, which were acquired from the CB seeds. Normally, the higher free radical scavenging activity and lower IC_{50} values demonstrate a higher antioxidant activity. The observed less IC_{50} value for DPPH assay supports the significance of CB seed Ag NPs as a promising source of antioxidants. Our results are in agreement with the investigation of (Shukla and Mehta, 2017), who reported similarly that aqueous extract of *CB* seeds exhibited increased DPPH radical scavenging potency in a dose-dependent manner.

Phosphomolybdenum reduction assay

Figure 1b presents the total antioxidant capacity obtained through the phosphomolybdenum assay of CB seed AgNps in comparison with that of Ascorbic acid. The *CB* seed AgNps exhibited powerful antioxidant activity with the absorbance of 0.35 at 400 μg nearing to that of the standard ascorbic acid, which showed the absorbance of 0.35 at the same concentration of AgNPs. CB seed AgNps revealed a satisfying total antioxidant activity (Figure 2a), better than that of the standard reference ascorbic acid. It has been anticipated that the bioactivity of CB seed AgNPs is due to their respective phenolics and flavonoid contents. A similar result was also observed in *Alpinia katsumadai* seed AgNPs (He *et al.*, 2017).

Ferric (Fe^{3+}) reducing power assay

From the results of FRAP (Figure 1c), the antioxidants in the CB seed AgNPs would bring about the reduction of Fe^{3+} to Fe^{2+}, which therefore correlates with the decrease in the generation of reactive hydroxyl species. CB seed AgNPs revealed a good reducing capacity with the absorbance of 0.977 at 100 μg/ml, which is bordering to the value of standard ascorbic acid. The promising antioxidant activity of a compound is evidenced by its reducing power. The reduction of Fe^{3+} was increased consistently with increasing concentration of AgNPs. Notably, the CB seed AgNPs exhibited better reducing power than the standard Ascorbic acid as the extract was enriched with phytoconstituents. Still, the phytoconstituents like phenols also possess electron-donating antioxidant capacity (Qidwai *et al.*, 2018). This result is in agreement with the attempt of (Dipankar *et al.*, 2012), who reported similarly in *Caesalpinia bonducella* leaves AgNPs.

Metal Chelating assay

The metal ion chelating efficacy of the CB seed AgNps was assayed and reported in Figure 2a. The decolorization of the color (red) relies on the reduction of ferrous ions by the CB seed nanoparticle. The results were indicated as mg EDTA eq./g. Compared to ascorbic acid, the nanoparticle showed a little lower activity of 88.36 mg EDTA/μg extract. Evidence state that ROS formation is accelerated by metals such as iron and copper. The origination of ROS associated with redox-active metal catalysis could be circumvented by chelating the metal ions (Kurutas, 2016). Antioxidant potential transferred to the silver nanoparticles by the aqueous CB seed extract forms an integrated complex with the metal ions that best describes its chelating activity and hinders the electron transfer. Thus oxidation of cellular metabolic reaction is seized, resulting in the absence of free radicals production.

Hydroxyl Radical Scavenging Activity Assay

CB seed silver nanoparticles exhibited dynamic inhibition against hydroxyl radical (Figure 2b), which was similar to Ascorbic acid. This result proved that silver nanoparticles synthesized from seed extract of CB are highly influential in neutralizing hydroxyl radicals. The majority of the hydroxyl radicals were scavenged by the CB seed AgNPs. IC_{50} determined for Ascorbic acid and CBAgNPs were 249.92 and 261.45μg/ml. Result outcomes were found statistically satisfactory ($P < 0.05$). The hydroxyl radical is presumably the ultimate dangerous element governing the free radical-induced tissue damage. Hydroxyl radical reacts with most of the molecules found in living cells, such as phospholipids and nucleotides (Hochstein and Atallah, 1988). The hydroxyl radical scavenging activity of CB seed AgNPs predicted in comparison with that of vitamin C (Figure 2b) showed that the synthesized

Table 1: Total phenol (mg GAE/ g), flavonoid (mg QE/ g) content of aqueous CB seed, and silver nano particle extract of Caesalpinia bonducella seeds

Extract	Total phenol	Total flavonoid
CB seed	115.08 ± 0.64	154.28± 0.21
CB seed AgNPs	120.15± 0.30	168.86± 0.33

*Data represented by means ± standard error of triplicate experiments

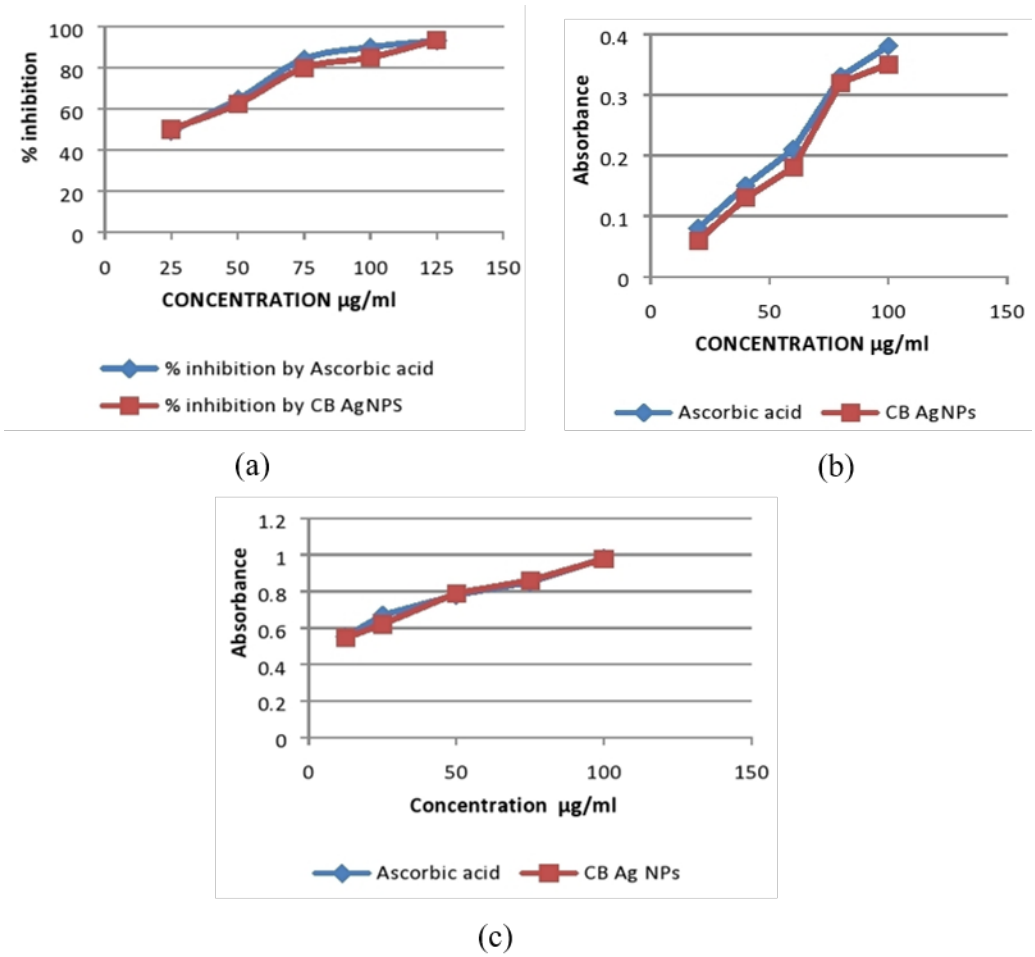

Figure 1: Antioxidant activity of CB seed AgNPs. (a) DPPH radical scavenging assay (b)Phosphomolybdenum reduction assay (c) Ferric reducing antioxidant power assay

nanoparticles are remarkably potent in neutralizing hydroxyl radicals.

Scavenging of Hydrogen peroxide

The results of H_2O_2 scavenging reported that the synthesized CB seed AgNPs possessed reliable hydrogen peroxide scavenging potential. H_2O_2 scavenging *of CB seed* AgNPs was examined to be in the range of 38% to 80%, which was closely related to that Ascorbic acid (Figure 2c). The IC_{50} values for CB seed AgNPs and Ascorbic acid were recorded to be 167.94 and 157.76. The data also suggests that the CB seeds AgNPs were efficient in scavenging H_2O_2 correlative with the standard Vitamin C. The formation of hydrogen peroxide *in vivo* occurs by the reactions catalysed by superoxide dismutase. H_2O_2 possesses the potential of crossing the membrane and can damage a number of compounds as a result of its oxidizing ability. Hydrogen peroxide targets many cellular energy-producing systems. Excess H_2O_2 leads to metabolic perturbations (Hyslop *et al.*, 1988). Thus, Dose-dependent hydrogen peroxide scavenging activity reveals that CB seed AgNPs are effectual free radical scavengers, acting probably as an essential antioxidant.

Inhibition effect of AgNPs on egg albumin denaturation

The potent anti-inflammatory strategy is the ability of any drug to prevent the denaturation of

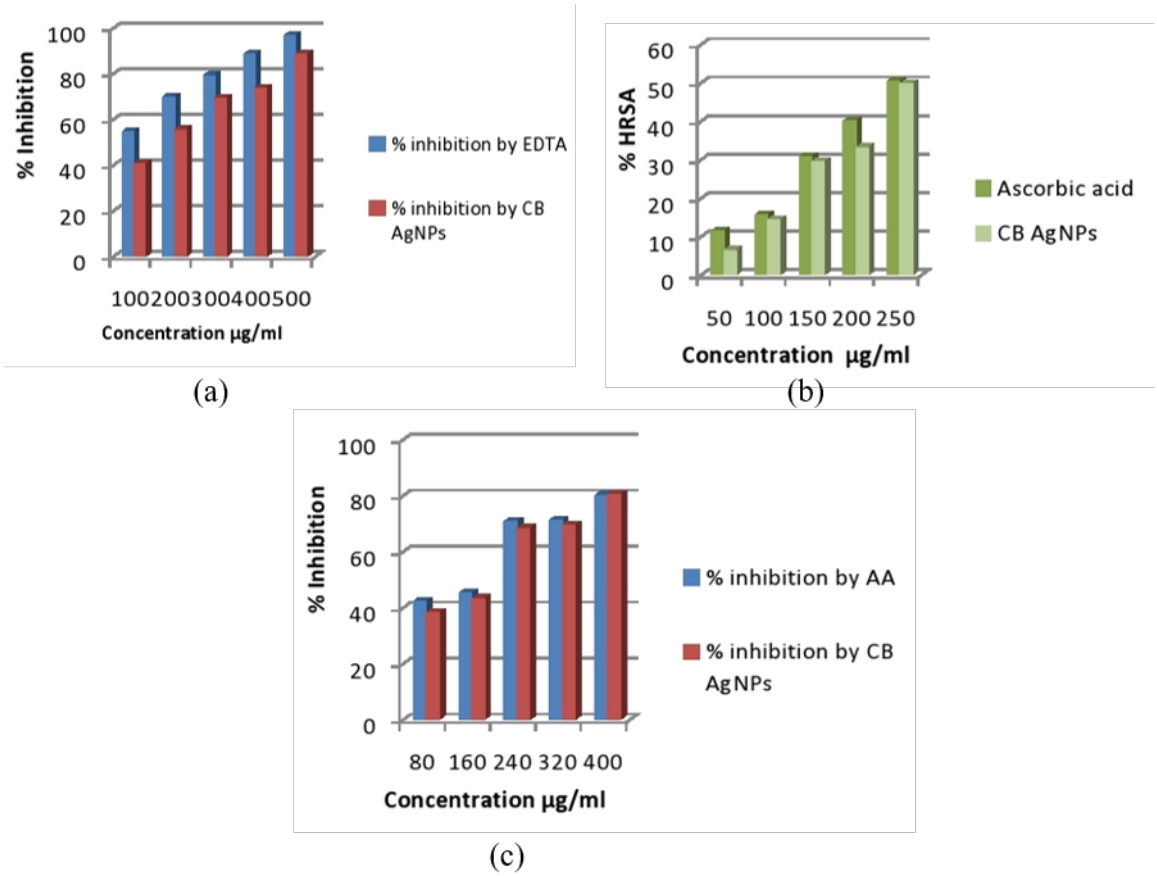

Figure 2: Free radical scavengingactivity. (a) Metal Chelating assay (b) Hydroxyl Radical Scavenging ActivityAssay (c) Scavenging of Hydrogen peroxide

Table 2: Influence of CB seed silver nano particles and Diclofenac against protein denaturation

Sample	Concentration (μg/mL)	% inhibition	IC_{50} (mg/mL)
Control	-	-	
CBS AgNps	50	49.06	71.3
CBS AgNps	100	52.09	
CBS AgNps	150	61.33	
CBS AgNps	200	68.29	
CBS AgNps	250	78.56	
DF	50	50.23	51.87
DF	100	55.21	
DF	150	68.25	
DF	200	77.26	
DF	250	80.69	

proteins during inflammatory disease conditions. The results of this assay indicated that the synthesized CB seed AgNPs retained significant anti-inflammatory potential. The highest inhibition was observed to be in the range of 78.56% and 80.69% for nanoparticle and Diclofenac, respectively Table 2. Also, the IC_{50}values for the CB AgNPs and Diclofenac were recorded to be 71.3 and 51.87. This evidence also suggests that the CB seed AgNPs were efficacious in inhibiting albumin denaturation and was much equally comparative with Diclofenac. ROS plays a crucial role in inflammatory disease progression. Inflammation is a defensive immune response against pathogens that may cause denaturation of tissue proteins, thus producing autoantigens in certain arthritic disease (Nasri and Rafieian-Kopaei, 2013). Targeting protein denaturation with the natural plant-derived nanoparticles, therefore, would serve as a potent anti-inflammatory agent. CB seed AgNps exhibited a marked *in vitro* anti-inflammatory effect by inhibiting the protein denaturation, and our results were evidenced by previous studies (Gnanasundaram and Balakrishnan, 2017; Kedi *et al.*, 2018).

CONCLUSION

Thus, the green synthesized silver nanoparticles from *C.bonducella* seed extract act as a potent free radical scavenger and exhibited excellent anti-inflammatory potential, thereby establishing their therapeutic significance. It is a promising and great finding that green bio-synthesized AgNPs capped with *CB* seed exhibited significant potential, which was much more remarkable than using this seed extract alone. This can be a promising future therapeutic agent for ROS and inflammation-mediated diseases such as cancer, Alzheimer's, ischemic injury, and rheumatoid arthritis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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