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Evaluation of *In-Vitro* Antioxidant Activity of Biochanin A

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ABSTRACT

This study was aimed to determine the in vitro antioxidant potential of biochanin A (BCA), an isoflavone phytochemical, by using various free radical scavenging assays. The free radical scavenging activity of BCA was evaluated by various standardized assays such as 1, 1-Diphenyl-2-Picryl Hydrazyl (DPPH•), 2, 2'-Azinobis-3-Ethylbenzothiazoline-6-Sulfonic Acid ($ABTS^{•+}$), Ferric Reducing Antioxidant Power (FRAP),nitric oxide scavenging activity, reducing ability, hydroxy radical activity, superoxide anion scavenging activity, hydrogen peroxide radical, metal ion chelating activity and phosphomolybdenum assay. Four different concentrations of BCA (5, 10, 20, 40, µg/ml) were taken for evaluating the scavenging activity and which were compared with the antioxidant activity of standard ascorbic acid (AA). BCA showed good free radical scavenging activity, which was calculated as IC_{50}. IC_{50} value of BCA was also comparable to Ascorbic acid (AA).Whereas AA was used as a standard. The scavenging activity of BCA was significantly elevated in a dose dependent manner. The BCA was exhibited a highest scavenging activity than the standard. The results obtained in the present study revealed that the BCA is an excellent free radical scavenger with the activity similar to that of AA.

Keywords: Biochanin A, Antioxidant, Free radicals, Nitric oxides and Ascorbic acid

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INTRODUCTION

Oxidation is an essential biological process of energy production in many living organisms. However, excessive reactive oxygen species (ROS), are responsible for oxidative stress, it in turn damages the macromolecules and it causes the various chronic diseases such as cancer, cardiovascular disease (CVDs), and diabetes [1]. The ROS such as superoxide anion (O_2^-), hydroxyl radical (OH•), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2*) are formed as a result of normal metabolic activity and exogenous sources [2]. The production of ROS and lipid peroxidation plays a major role in the development of CVDs. Free radicals interacts with membrane lipids and causes peroxidative changes leads to the enhanced lipid peroxidation [3]. In pathological conditions, the antioxidant levels are often inadequate, as excessive levels of free radicals can be produced. In past few years, there is an upsurge in the areas associated to newer developments in the prevention of diseases by scavenging the free radicals. So it will be necessary to examine the potential role of free radicals in disease and antioxidants in its prevention. ROS are a highly reactive species that causing cell damage either directly or through acting as an intermediates in diverse cell signalling pathways [4].

Antioxidants are the important compound capable of slowing or preventing the oxidation of other molecules. The synthetic antioxidants like Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA) and propyl gallate (PG) have toxic effects of some extents. However, investigations of antioxidants are focused on naturally occurring substances, especially plant physiochemical. Antioxidant compounds in the food play an important role in health protection. Over the last decade, considerable experimental evidences have confirmed the importance of a diet, rich in antioxidants, which can protect the cells and tissues from the damage caused by the free radicals. This leads to an increasing search for improving drugs with the use of phytochemical. Traditional knowledge of medicinal plants and the compounds derived from them have always direct the search for drug discovery and development. In developing countries, a huge number of people live in extreme lower condition and some are suffering and dying for need of protected water and medicine, they have no

option for primary health care [5].Consequently; they require using medicinal plants as alternatives to traditional medicines in the provision of primary health care can be over emphasizing. Management of these conditions without any side effects are still a challenge for the medical goal. Hence, develop the drugs with low cost, more potential, and without side effects are being pursued in several laboratories around the world. Medicinal plants are cheap, easily available and affordable safe for human use and also environment friendly [6].

Isoflavones are flavonoids belonging to the phytoestrogens are having most beneficial effects. BCA (5, 7-dihydroxy-4'-methoxy isoflavone) is an o-methylated isoflavone; it is a natural organic compound in the class of phytochemicals. BCA occurs in many species of vascular plants, mostly belonging to the families such as legumes (Leguminoseae), papilonaceous plants (Papilionaceae) and many species of grasses and cereals (Graminae). The plant species which are the richest source of BCA include red clover (*Trifoliumpratense*), juniper (*Genistatinctoria*), soybean (*Sojahispida*) and plum (*Prunus spinosa*). BCA is isolated from the peg trees, fruit-trees, stone-trees, and mainly from the genus plum-tree. BCA, an flavonoid, has been shown to possess various pharmacological properties including anti-inflammatory, anti-carcinogenic and hypolipidemic effects [7]. The present in vitro study was performed to investigate the free radical scavenging activity of BCA.

MATERIALS AND METHODS

BCA, 2, 2-diphenyl hyrazyl (DPPH), Ascorbic acid (AA) were purchased from sigma Aldrich (St Louis, MO, USA). Sodium carbonate, Sodium phosphate, Potassium acetate, Ethylene diamine tetra acetic acid, methanol, ethyl acetate, chloroform, Sulphuric acid, trichloroacetic acid (TCA) and hydrogen peroxide reagents were obtained from Qualigens (Mumbai). All other chemicals used for performing the experiment were of high quality and analytical grade.

Antioxidant activity by DPPH$^{\bullet}$assay

The DPPH$^{\bullet}$ assay was carried out according to the method of Williams *et al*., 1995 [8]. Different concentrations of the BCA were taken in separate test tubes. The volume was adjusted to 100 µL with methanol l.5 mL of 0.1 mM methanolic solution of DPPH$^{\bullet}$ was added to these tubes and shaken vigorously. The tubes were allowed to stand for 20 min at 27°C. The control was prepared as above without BCA, and methanol. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition concentration (IC_{50}), the concentration of BCA necessary to decrease the initial concentration of DPPH$^{\bullet}$ by 50% (IC_{50}) under the specified experimental condition. Standard AA is used as a reference. The scavenging activity was calculated as follows,

DPPH scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

ABTS$^{\bullet+}$ scavenging activity

The scavenging activity of the test sample was tested using ABTS+ assay. The method was described by Re *et al.,* 1999 [9]. The ABTS$^{\bullet+}$ radical solution was prepared by mixing 14mM ABTS stock solution with 4.9 mM ammonium per sulphate and incubated 16h in the dark at room temperature until the reaction was stable. The absorbance of the ABTS+ solution was equilibrated to 0.70±0.02 by diluting with ethanol at room temperature. To 1ml of the ABTS+ solution various concentrations of the test sample of BCA were added. The antioxidant activity was estimated by calculating the percentage of the decrease in absorbance of different concentrations of BCA using the following equation.

ABTS scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample

Nitric oxide scavenging activity

Nitric oxide **(**NO) generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci *et al.,* 1994 [10]. Briefly, the reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate buffered saline (pH 7.3), with or without the BCA at different concentrations, was incubated at 25°C for 180 min in front of a visible polychromatic light source (25W tungsten lamp). The NO**.** radical thus generated interacted with oxygen to produce the nitrite ion (NO.) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the BCA was estimated using a standard curve based on sodium nitrite solutions of known concentrations.

NO**.** Scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

Ferric-reducing antioxidant power (FRAP)

The ability to reduce ferric ions was measured using the method described by Benzie and Szeto 1999 [11]. A stock solution of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, 20Mm $FeCl_3.6H_2O$ and 0.3M acetate buffer (pH 3.6) was prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution, and 25 ml acetate buffer. It was freshly prepared and warmed to 37ºC. FRAP reagent (900 ml) was mixed with 90 ml water and 30 ml BCA and standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was observed at 593 nm. An intense blue colour complex was formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex was reduced to ferrous [Fe^{2+}] form.

FRAP (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample

Hydroxyl radical scavenging activity

The scavenging activity of BCA on hydroxyl radical was measured according to the method of Klein and Cohen 1991[12]. Various concentrations of BCA were added with 1.0 mL of iron-EDTA solution, 0.5 mL of EDTA solution (0.018%), and 1.0 mL of DMSO (0.85% DMSO (v/v) in 0.1 M phosphate buffer, (pH 7.4) sequentially. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0 mL of ice-cold TCA (17.5% w/v). 3mL of Nash reagent was also added and incubated for 15min at room temperature. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated by the following formula:

HRSA (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample

Reducing power assay

The reducing power of BCA was determined by the method of Oyaizu 1986 [13]. Substances, which have reductions potential , react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+),which then reacts with ferric chloride to form ferric ferrous complex that as an absorption maximum at 700nm. Various concentrations of samples (5-40 µg) were mixed with of phosphate buffer in a test tube and 5 mL of 0.2 M phosphate buffer, pH (6.6). To this, 5 mL of 1% potassium ferricyanide solution was added. The mixture was incubated at 50°C in water bath for 20 min. After cooling, 5 mL of 10% TCA was added and the content was centrifuged at 1,000 rpm for 10 min. The upper layer of the supernatant (5 mL) was mixed with 5 mL of distilled water. To this, 1 mL of ferric chloride (0.1%) was added and vortexed. Then, the absorbance of the reaction mixture was read spectrophotometrically at 700 nm against water blank. AA used as a standard.

Superoxide anion radical scavenging activity

Superoxide radical scavenging activity of BCA was performed according to the method of Liu *et al.,* 1997 [14]. This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotinamide adenine dinucleotide (NADH) and phenazinemethosulfate (PMS) under the aerobic condition. The 3 ml reaction mixture contained 50 mL of 1M NBT, 150 mL of 1M NADH with or without sample, and Tris buffer (0.02M, pH 8.0). The reaction was started by adding 15 mL of 1M PMS to the mixture and the absorbance was recorded at 560 nm after 2 minutes. The percentage of superoxide anion scavenging was calculated using the following formula:

Superoxide anion scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample

Hydrogen peroxide radical

BCA against H_2O_2 was measured according to the method of Ruch *et al.,* 1989 [15]. A solution of 40 mM H_2O_2 was prepared in 0.1M phosphate buffer (pH-7.4). Then, 1.4 mL of different concentrations (5-40 µg/mL) of the BCA was added to 0.6 ml of the H_2O_2 solution (40mM). The assay mixture was allowed to stand for 10 minutes at 25°C and the absorbance measured against a blank solution at 230 nm. The blank solution was contained the sodium phosphate buffer with H_2O_2. The BCA on the H_2O_2 scavenging capacity index was calculated as follows

H_2O_2 scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample

Metal ion chelating activity

The chelation of ferrous ions by extracts was estimated by method of Dinis *et al.,* 1994 [16]. The sample solution at different concentrations were added to 0.05 ml of 2mM $Fecl_2$.The reaction was initiated by the addition of 5mM ferrozine (0.2mL) and the mixture was mixed well and stands for 10 min at room temperature. The absorbance was measured at 562 nm spectrophotometrically. The Fe^{2+}chelating activity (%) was calculated from the following equation:

Metal iron chelating (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample

Phosphomolybdenum assay

The total antioxidant activity of BCA was evaluated by the phosphomolybdenum assay [17]. And this assay is based on the reduction of M_0 (VI)–M_0 (V) by the antioxidants and subsequent formation of a green phosphate/M0 (V) complex at acidic pH. 0.3 ml of BCA sample is taken in a tube and mixed with 3 ml of reagent containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and it was incubated at 95°C for 90 min. AA is utilized as a reference standard. The absorbance of the mixture is then measured at 695 nm with methanol blank. The antioxidant activity was expressed as the number of gram equivalents of AA.

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean ± standard deviation. The results were analyzed by using one-way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 17.

RESULTS AND DISCUSSION

Oxygen is essential for the survival of all living thinks on earth and its unfavourable univalent reduction generates several harm full reactive oxygen species (ROS). The most adverse effects of oxygen is due to the origination and activation of ROS and it has a tendency to donate oxygen to other molecules which results in degradation of cellular components. The ROS can be scavenged by the antioxidants. Antioxidants are the compounds which prevent the oxidation of biological molecules and protect the body from various diseases. The endogenous antioxidants are insufficient to completely remove the free radicals therefore the exogenous antioxidants are required to counteract the excess free radicals. Hence the present study was designed for the investigation of in vitro antioxidant activity of BCA.

DPPH radical scavenging ability

DPPH is a synthetic free radical and stable at room temperature commonly used to assess the antioxidant activity of various natural compounds. This assay is based on the measurement of the scavenging activity of BCA towards DPPH which reacts with a appropriate reducing agent. The electron becomes paired off and solution losses colour stoichiometrically depending upon the number of electrons gained and when this reaction takes place the colour changes from purple to yellow which shows a characteristic absorbance at 517nm [18]. The percentage of DPPH radical scavenging activity of the different concentrations of BCA is shown in Figure 1, The IC_{50} value was described as the concentration of BCA (µg/mL) that scavenges the DPPH radical by 50 %. The different concentrations such as 5, 10, 20 and 40 µg/mL of BCA showed the DPPH radical scavenging activity in a dose dependant manner. The IC_{50} values of the BCA (20 µg/mL) and AA was found to be 24.67 µg/mL, and 26.12µg/mL respectively.

$ABTS^{\bullet+}$ scavenging activity:

ABTS assay is a decolorizing assay and it is a simple indirect method which converts the ABTS radical into monocation, which has a broad wavelength absorption spectrum without involvement of any other intermediary radical. ABTS and

potassium persulfate reacts to form a blue coloured chromophore, and the results were compared with standard. BCA exhibited a concentration dependent increase in the free radical scavenging activity. The antioxidant activity of the BCA was assayed by this assay and it suggest that this action may be by either inhibiting or scavenging the $ABTS^{\bullet+}$ radicals. This result also coincides with the previous study [19]. Figure 2 shows the promising antioxidant potential of the BCA on ABTS radicals. The IC_{50} of the BCA and AA were found to be 26.51µg/ml, and 28.42µg/mL respectively.

FRAP assay:

FRAP is measured according to the ability of an antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+} TPTZ complex with a maximum absorption at 593 nm. The absorbance is directly proportional to the amount of antioxidant present in the sample [20]. In this assay, excess amount of reducible oxidant Fe^{III} is used and reduction of Fe^{III} is due to the antioxidant activity of BCA. Figure 3 shows that the dose responses curve of FRAP scavenging activity with IC_{50} of BCA. In the present study, we were observed that the concentration of FRAP was decreased and it might be due to the scavenging activity of BCA. The free radical scavenging ability of the BCA was significantly increased in a dose dependent manner. The IC_{50} of the BCA and AA were found to be 26.86 µg/ mL, and 28.46 µg/ mL respectively

Nitric oxide scavenging activity:

Nitric oxide (NO) is an important chemical mediator generated by the endothelial cells, neurons, macrophages etc. NO involved in the regulations of various physiological process mainly vascular homeostasis, neurotransmission, antitumor and antimicrobial activities. An excess concentration of NO is associated with tissue toxicity. Conditions such as multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis shows the involvement of its chronic level expression . It would be attractive to develop selective and potent inhibitors of NO for potential therapeutic use [21]. In this assay, sodium nitroprusside generates nitric oxide which forms nitrite and peroxy nitrite when reacts with oxygen, which act as a free radicals. In this study, BCA is act as an antioxidants and it prevents the formation of nitrite by directly competing with oxygen to react with nitric oxide and also by inhibiting the synthesis of nitric oxide. The nitric oxide scavenging ability of BCA was measured by using this assay and results were expressed as percentage inhibition. The IC_{50} of the BCA and AA were found to be 24.67µg/ml, and 26.12µg/ml respectively which were given in Figure 4.

Hydrogen Peroxide radical scavenging activity:

Hydrogen peroxide (H_2O_2) is not highly reactive but it can occasionally be toxic to the cells because it can dissociate to hydrogen (H+) and hydroxyl (OH−) radicals. Many of its toxic effects are because of H_2O_2 has the ability to rapidly cross the cell membrane and once inside the cell, it can probably react with Fe^{2+} and possible Cu^{2+} ions to form hydroxyl radicals [22]. The removal of H_2O_2 is very important for the antioxidant defence in the cell. The results of our study suggested that the BCA scavenged H_2O_2 and thus protecting living organisms by donating electrons to H_2O_2, thereby neutralizing it in to water. Figure 5 depicted that the percentage of hydrogen peroxide radical scavenging activity of BCA. IC_{50}values of the BCA was 26.12 µg/mL, this activity was comparable to the scavenging effect of AA, was found to be 28.56 µg/mL respectively.

Superoxide Anion scavenging activity:

Superoxide anion is produced from molecular oxygen due to oxidative enzymes. Superoxide radicals are more toxic to the cellular components as a precursor of the ROS and it contributing to various diseases and tissue damage. In biological systems superoxide anions are removed by the enzyme superoxide dismutase (SOD). It has been found that the absence of cytosolic SOD results in muscle atrophy, liver cancer, cataracts, thymic involution and haemolytic anemia [23]. Figure 6 represents the superoxide anion radical scavenging ability of BCA. The removal of superoxide radicals were increased with the enhanced concentration of BCA. The IC_{50} value of BCA was 29.56 µg/mL which was comparatively lower than the IC_{50} (30.27µg/mL) of AA. The BCA in the concentration of 5 µg/mL showed 24.16 were as in 40 µg/mL concentrations the percentage of inhibition was recorded as 34.10 µg/mL and it clearly indicates the inhibition activity was dose dependent.

Hydroxyl radical scavenging activity:

Hydroxyl radical (OH) is a highly reactive free radical produced in the biological systems and has been considered as a more toxic radical in free radical pathology, capable of damaging all important biomolecules found in the living cells. This radical has the capacity to cause strand breakage and join nucleotides in DNA. Damaged DNA is an important mediator of various diseases including carcinogenesis, diabetes and cardiac diseases [24].

This radical also initiates lipid peroxidation by abstracting the hydrogen atoms from the unsaturated fatty acids. Hydroxyl radical scavenging activity of BCA was estimated by using ascorbic acid-iron EDTA and the hydroxyl radical is produced by the oxidation dimethyl sulfoxide (DMSO). The scavenging potential of BCA on hydroxyl radical is illustrated in Figure 7. The IC_{50} value for the hydroxyl radical scavenging activity of BCA and AA were found to be 28.46µg/mL and 29.56 µg/mL respectively. The result of the present study shows that the highly reactive hydroxyl radical was effectively scavenged by BCA thereby proved their antioxidant activity.

Reducing Ability Assay:

Antioxidant activity is associated with reducing power of the compound. Reducing power of the compounds indicates that they are electron donors and can reduce the formation of oxidized intermediates of lipid peroxidation. In this assay, the yellow colour of the reaction mixture changes to green and blue depending on the reducing ability of the test sample. Presence of reducing agents causes the conversion of Fe3+ to Fe2+ and greater absorbance at 700 nm indicated greater reducing power of the compound. In this condition, the antioxidant activity can be referred to as reducing ability [25]. From this study we found that the reducing ability of BCA was increased with increase in their concentrations and it reveals its in vitro antioxidant potential. IC50 values of BCA and AA were found to be 26.68µg/mL and 29.33µg/mL respectively, which were given in Figure 8.

Metal Chelating Assay

Irons are an extremely reactive metal, essential for the activity of various enzymes, oxygen transport and respiration and catalyse oxidative changes in proteins, lipids and other cellular components [26]. In addition, oxidative damage of protein and liposome peroxidation are induced by a Fenton reaction in which ferrous irons catalyse the conversion of hydrogen peroxide to hydroxyl radical with the formation of ferric iron. Metal Iron chelating capacity is

significant, since it reduces the concentration of the metal iron that catalyses the lipid peroxidation. Metal iron chelating activity of BCA at different concentrations were analysed and the results are shown in Figure 9. IC_{50} Value of BCA and AA were recorded as 30.35µg/ml and 32.98µg/ml respectively.

Phosphomolybdenum Assay

Phosphomolybdenum assay is a quantitative method to assess the total antioxidant capacity. This assay is based on the reduction of Phosphate Molybdenum (VI) to Phosphate-Molybdenum (V) [27]. The incubation of the BCA with the Molybdenum (VI) converts it in to a Phosphate-Molybdenum (V). Figure 10 shows the total antioxidant activity of the BCA in various concentrations and the IC_{50} value of BCA was 27.09 µg/mL and AA was 29.37µg/mL. The results of this study suggested that the BCA involved in the reduction of Phosphate Molybdenum (VI) in a dose dependent manner and confirm its antioxidant activity.

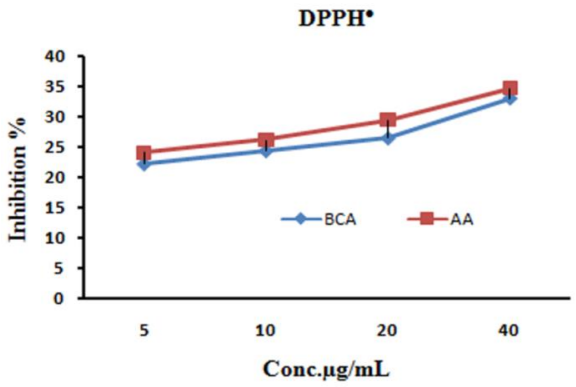

Figure 1: DPPH radical scavenging activity of BCA

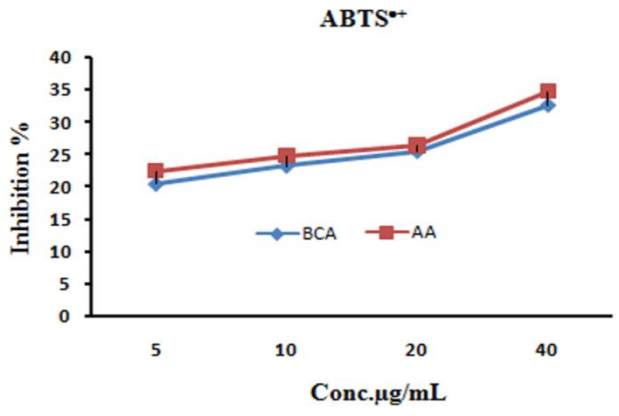

Figure 2: ABTS radical scavenging activity of BCA

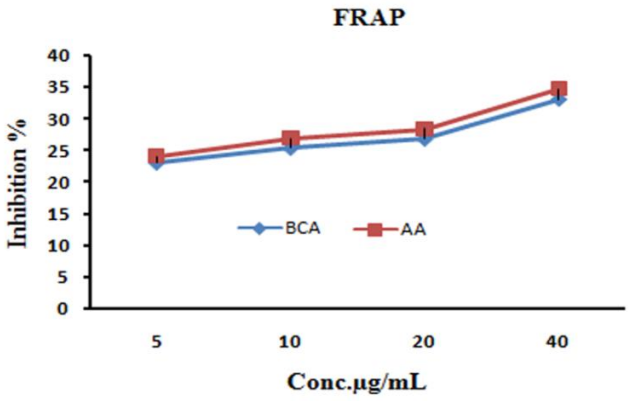

Figure 3: FRAP radical scavenging ability of BCA

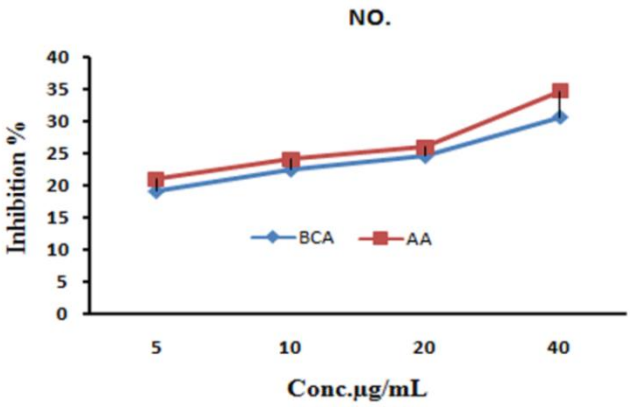

Figure 4: Nitric oxide radical scavenging ability of BCA

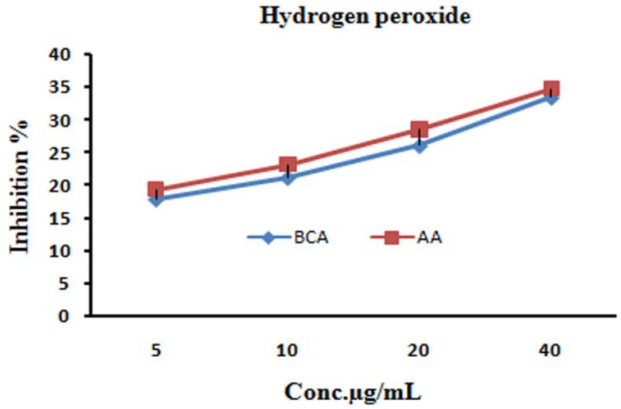

Figure 5: Hydrogen peroxide radical scavenging ability of BCA

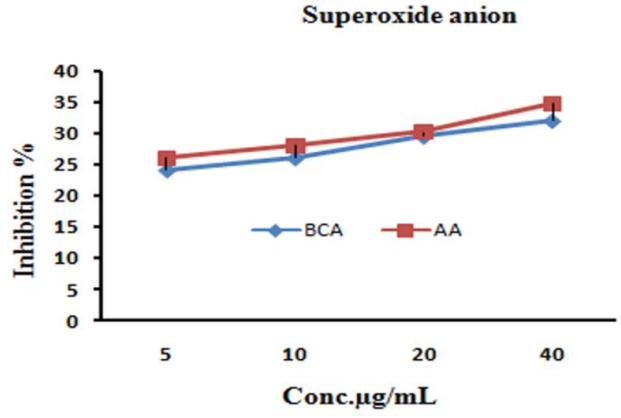

Figure 6: Super oxide anion radical scavenging ability of BCA

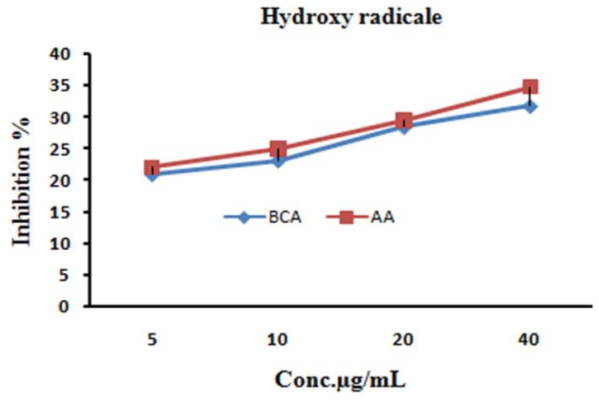

Figure 7: Hydroxyl radical scavenging ability of BCA

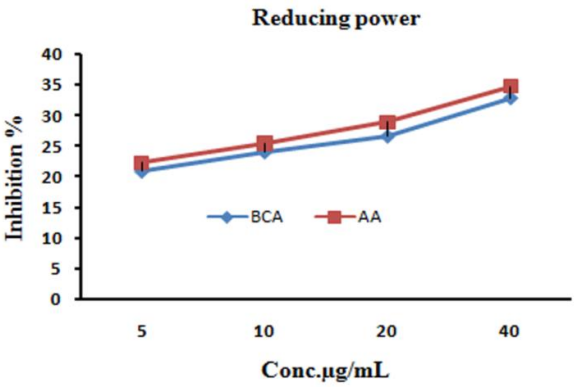

Figure 8: Reducing power radical scavenging ability of BCA

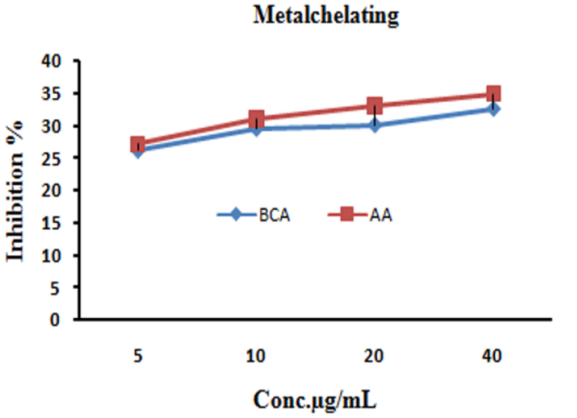

Figure 9: Metal ion chelating radical scavenging ability of BCA

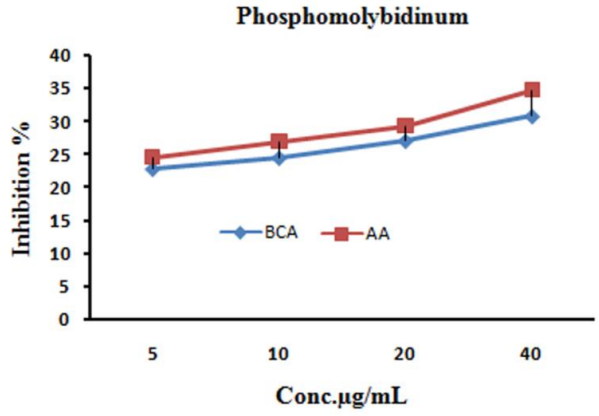

Figure 10: phosphomolybdenum radical scavenging ability of BCA

CONCLUSIONS

From the results of the present study, we can conclude that BCA exhibited potential antioxidant activity against various free radicals and this might be due to their antioxidant potential. Though, further investigations need to be done to determine the in vivo biological activity of BCA.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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