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In vitro Free Radical Scavenging and Antioxidant Effect of Bacoside-A

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Abstract

The aim of the present study was to evaluate the free radical scavenging activity of the compound Bacoside-A. Free radical scavenging was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS (2,2'-azinobis (3ethylbenzthiazoline-6-sulphonic acid), ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), reducing power, hydroxy radical scavenging assay, superoxide radical scavenging (SOD), hydrogen peroxide radical assay, metal chelating activity as well as phosphomolypdenum assay.The present investigation clearly indicate that the Bacoside-A possesses antioxidant properties and serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Keywords

Bacoside-A, DPPH, ABTS, FRAP, Antioxidant, Phosphomolypdenum

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INTRODUCTION

Free radicals are molecules containing one or more unpaired electrons in atomic or molecular orbitals. There is increasing evidence that abnormal production of free radicals leads to increased oxidative stress on cellular structures and causes changes in molecular pathways that underpins the pathogenesis of several important diseases, including cardiovascular diseases, neurological diseases, cancer, and in the process of physiological aging (1,2). Antioxidants are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress (3). Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidatio (4,5). Enzymes, particularly superoxide dismutase (SOD) and catalase as well as compounds like tocopherol, ascorbic acid and glutathione play a key role in protecting human cells from free radical mediated damage (6)

Bacoside-A is the dammarene type triterpenoid saponin isolated from the plant *Bacopa monniera*, which is held in high repute as a potent nerve tonic (7). *Bacopa monnera L*. is used in the indigenous systems of medicine for the treatment of various nervous system ailments such as insomnia, anxiety, epilepsy, hysteria etc (8). Preclinical and clinical studies have shown that *B. monniera* improves memory and mental function (9,10). The Bacoside-A containing plant has been shown as a potent free radical scavenger and antioxidant (11), Besides it also exhibits vasodilatory (12), calcium antagonistic (13) and muscle relaxant (14) properties. Preliminary studies indicated that bacosides, the major saponins are responsible for the facilitatory and modulatory effects of *B.monniera* (15). Hence, the present study was undertaken to assess the free radical scavenging activity of Bacoside- A.

MATERIALS AND METHOD

Chemicals

Bacoside-A, 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and ascorbic acid, were purchased from Sigma-Aldrich (St Louis, MO, USA). sodium carbonate, sodium phosphate, potassium acetate, ethylene diamine tetra acetic acid (EDTA), methanol, ethyl acetate, chloroform, sulphuric acid, trichloroacetic acid (TCA) and hydrogen peroxide reagents were obtained from Qualigens (Mumbai). All other chemicals used were of high-quality analytical grade.

DPPH radical scavenging activity

Various concentrations of Bacoside-A of the sample (4.0 mL) were mixed with 1.0 mL of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2mM. The mixture was shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517nm. Ascorbic acid was used as control (16). The percentage of DPPH decolorization of the sample was calculated according to the equation:

% decolorization = [1-(ABS sample/ABS control)] ×100

IC_{50} value (mg extract/mL) was the inhibitory concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison.

$ABTS^+$ scavenging activity

Samples were diluted to produce 0.2-1.0 mg/mL. The reaction was initiated by the addition of 1.0 mL of diluted $ABTS^+$ to 10 mL of different concentrations of Bacoside-A of the sample or 10 mL methanol as control (17). The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation

$I = A_1/A_0 \times 100$

where A_0 is the absorbance of control reaction and A_1 was the absorbance of test compound.

Ferric-reducing antioxidant power assay (FRAP)

A stock solution of 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40mM 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 37°C. FRAP reagent (900 mL) was mixed with 90 mL water and 30 mL Bacoside-A of the sample and standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+} - TPTZ) complex was reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm was recorded (18).

Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside was measured. Briefly, the reaction mixture (5.0 mL) containing sodium nitroprusside (5mM) in phosphate-buffered saline (pH 7.3), with Bacoside-A sample at different concentration was incubated at 25°C for 3 hours. The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion which was assayed at 30-minute intervals by mixing 1.0 mL of incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546 nm (19).

Reducing Power Assay

The reducing power was determined as described (20). Briefly, 0.13 mL of ascophyllan fractions of different concentration (10–50 µg/mL) in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.125 mL of potassium ferricyanide (1%, w/v) and incubated at 50°C for 20 min. Afterwards, 0.125 mL of TCA (10%, w/v) were added to the mixture to terminate the reaction. Then, the solution was mixed with 1.5 mL ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm.

Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) under 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 change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract (21).

Hydroxy radical activity

The reaction mixture 3.0 mL contained 1.0 mL of 1.5mM $FeSO_4$, 0.7 mL of 6mM hydrogen peroxide, 0.3 mL of 20mM sodium salicylate, and varying concentrations of Bacoside-A sample. After incubation for 1 hour at 37°C, the absence of the hydroxylated salicylate complex was measured at 562 nm (22). The percentage scavenging effect was calculated as:

Scavenging activity = $[1-(A_1 - A_2)/A_0] \times 100\%$

where A_0 was the absorbance of the control (without extract), A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without sodium salicylate.

Hydrogen peroxide radical activity

The Bacoside-A against H_2O_2 was measured according to the method (23). A solution of 40 Mm H_2O_2 was prepared in phosphate buffer (pH = 7.4). Next, 1.4 mL of different concentrations (10-1500 mg/mL) of the Bacoside-A was added to 0.6 mL of the H_2O_2 solution. The assay mixture was allowed to stand for 10 minutes at 25°C, and the absorbance measured against a blank solution at ƛ max=230 nm. The Bacoside-A on hydrogen peroxide scavenging capacity index was calculated as follows:

$$= \frac{\text{Absorbance Blank} - \text{Absorbance Test} \times 100}{\text{Absorbance Blank}}$$

Bacoside-A was expressed as IC_{50}, which is defined as the concentration (mg/mL) of the Bacoside-A required to scavenge 50 % of H_2O_2.

Chelating activity

The reaction mixture contained 1.0 mL of various concentrations of the Bacoside sample, 0.1 mL of 2mM $FeCl_2$, and 3.7 mL methanol. The control contained all the reaction reagents except the sample (24). The reaction was initiated by the addition of 2.0 mL of 5mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher iron chelating ability.The capacity to chelate the ferrous ion was calculated by

% chelation = [1 -(ABS sample/ABS control)] ×100.

Phosphomolybdenum assay

The phosphomolybdenum assay used for determining the antioxidant capacity is based on the reduction of M_0 (VI) – M_0 (V) by the antioxidants and subsequent formation of a green phosphate/M_0 (V) complex at acid P^H. 0.3 ml of Bacoside-A sample is taken in a tube and mixed with 3 ml of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95°C for 90 min. Ascorbic acid is utilised as a reference standard. The absorbance of the mixture is then measured at 695 nm with methanol blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid (25).

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean ± standard deviation. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan's multiple range test using SPSS version 16.

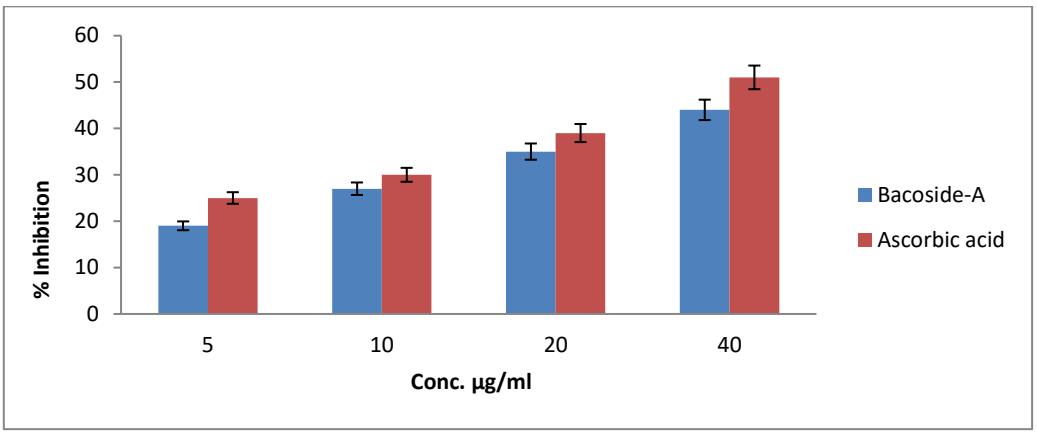
RESULT AND DISCUSSION

DPPH is a stable free radical. When antioxidant reacts with this stable radical, the electron becomes paired off and bleaching of the color stoichiometrically depends on the number of electrons taken up. From our findings, it can be postulated that Bacoside-A reduces the radical moderately to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (26, 27), Bacoside-A exhibited a significant dose dependent inhibition of DPPH activity. Which had a lesser activity than the standard of Ascorbic acid. The results are presented in **Fig.1** the IC_{50} value of Ascorbic acid and Baoside-A was 51.23 µg/ml, 44.11 µg/ml, respectively.

$ABTS^+$ assay is a decolorizing assay, which involves the direct generation of ABTS radical into monocation, which has a long wavelength absorption spectrum without the involvement of any intermediary radical. The antioxidant activity of the Bacoside-A by this assay implies that the action may be either inhibiting or scavenging radicals since both inhibition and scavenging properties of antioxidant towards this radical have been reported in earlier studies (28). The inhibition capacity of the radical $ABTS^{\bullet+}$ and the Ascorbic Acid % values, expressed in Bacoside-A, for the different concentration was presented in **Fig.2.** The results show that Bacoside-A presented the highest Ascorbic Acid % behavior, with values of 48.13µg/ml and 51.23µg/ml, respectively.

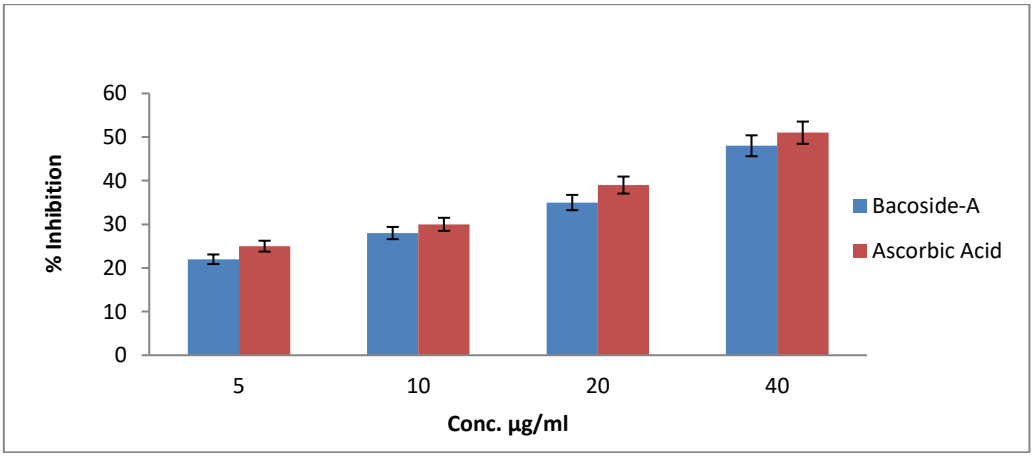
Antioxidants can be explained as reductants, and inactivators of oxidants (29). Some previous studies have also reported that the reducing power may serve as a significant indicator of potential antioxidant activity. Antioxidative activity has been proposed to be related to reducing power. In this study, we used a FRAP assay because it is quick and simple to perform, and the reaction is reproducible and linearly related to the molar concentration of the antioxidant and FRAP assay was used by several authors for the assessment of antioxidant activity of various food product samples (30,31). Suggested most of the secondary metabolites are redox-active compounds that will be picked up by the FRAP assay. **Fig.3** reveal the reductive capability Bacoside-A. The reducing power of the Bacoside-A increases with the increasing concentration was 48.21µg/ml and the IC_{50} value of Ascorbic acid was 51.23µg/ml.

FIG.1: DPPH RADICAL SCAVENGING ACTIVITY OF BACOSIDE-A



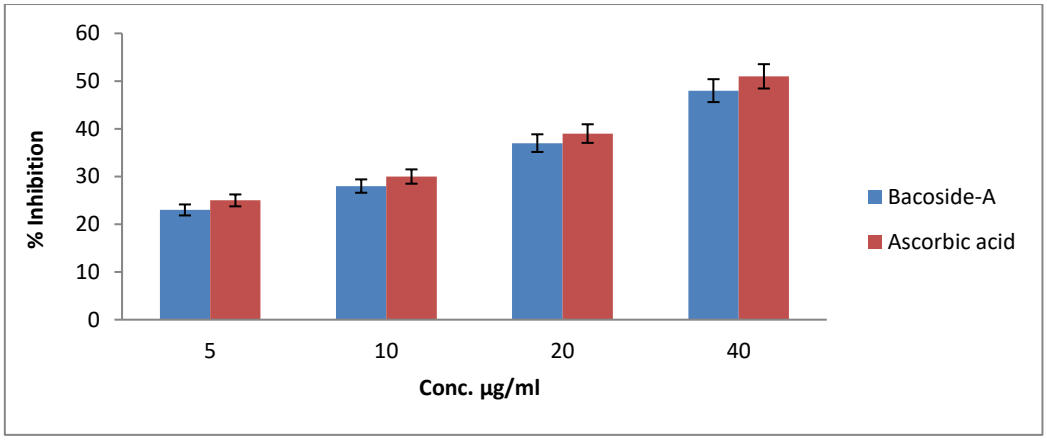
Each value is expressed as mean ± standard deviation (n=3).

FIG.2: ABTS RADICAL SCAVENGING ABILITY OF BACOSIDE-A



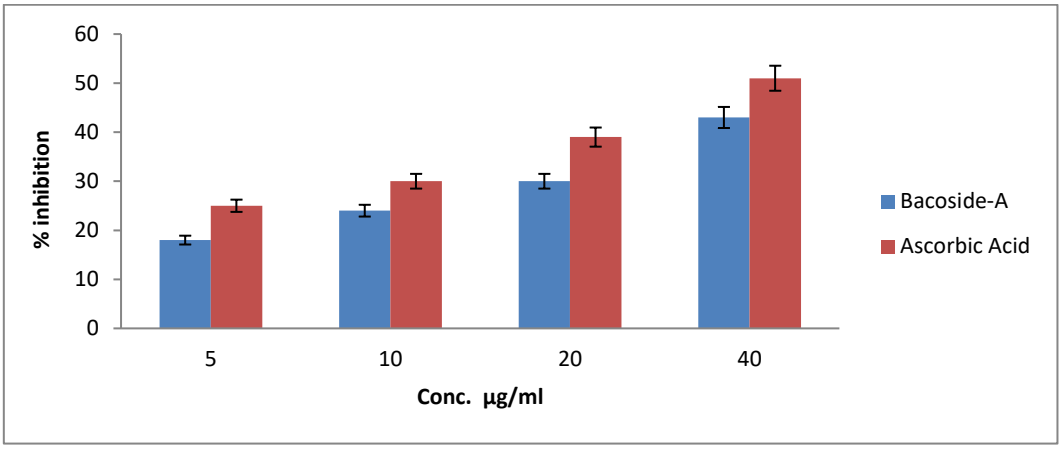
Each value is expressed as mean ± standard deviation (n=3).

FIG.3: FRAP RADICAL SCAVENGING ABILITY OF BACOSIDE-A



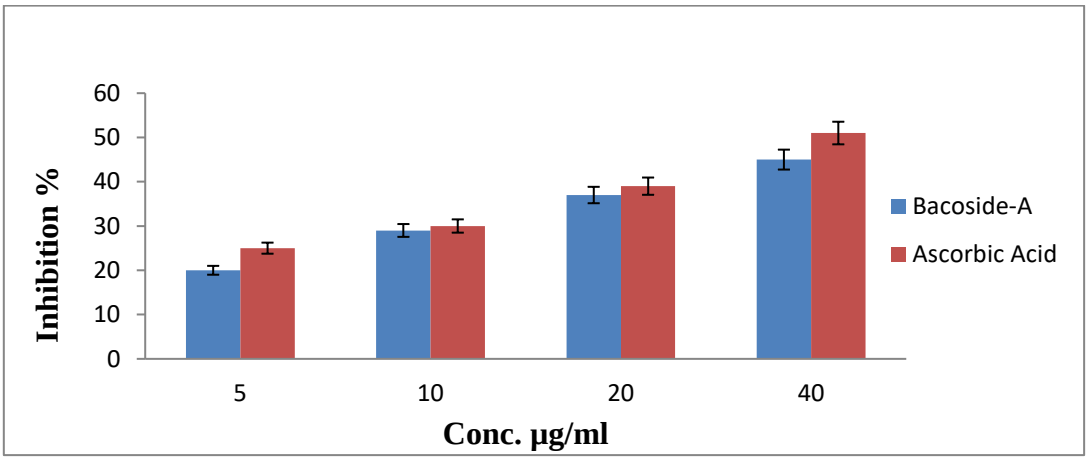
Each value is expressed as mean ± standard deviation (n=3).

FIG.4: REDUCING POWER RADICAL SCAVENGING ABILITY OF BACOSIDE-A



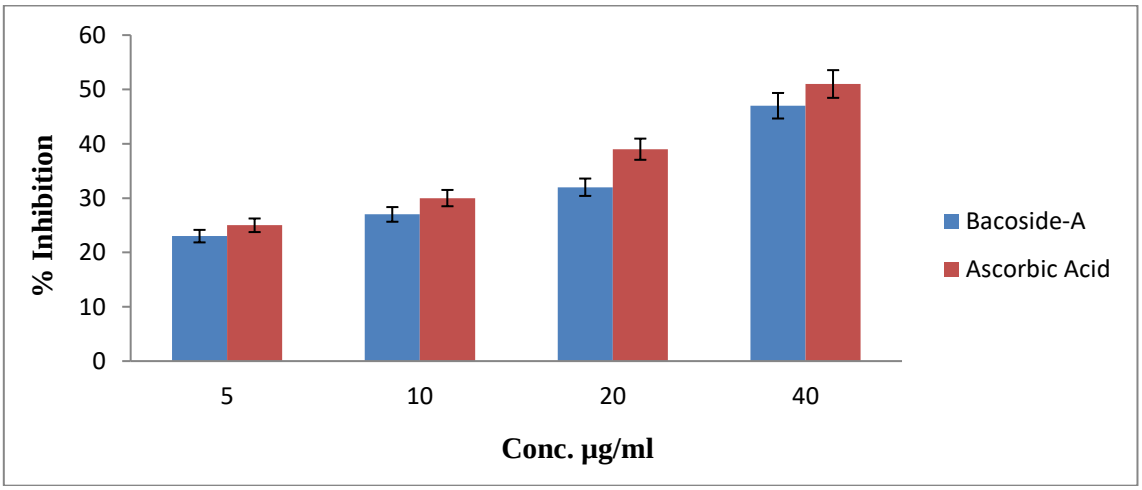
Each value is expressed as mean ± standard deviation (n=3).

FIG.5: SUPEROXIDE ANION RADICAL SCAVENGING ABILITY OF BACOSIDE-A



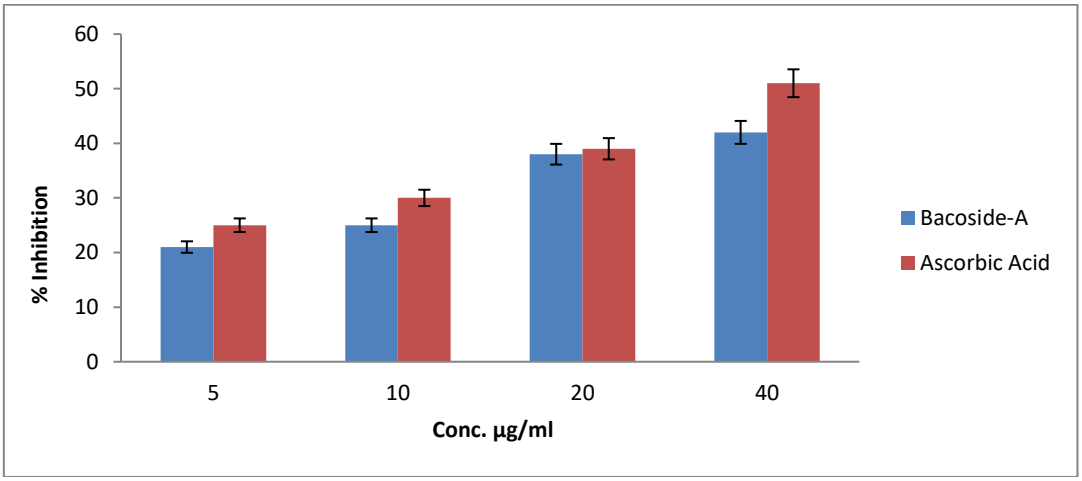
Each value is expressed as mean ± standard deviation (n=3).

FIG.6: HYDROXY RADICAL SCAVENGING ABILITY OF BACOSIDE-A



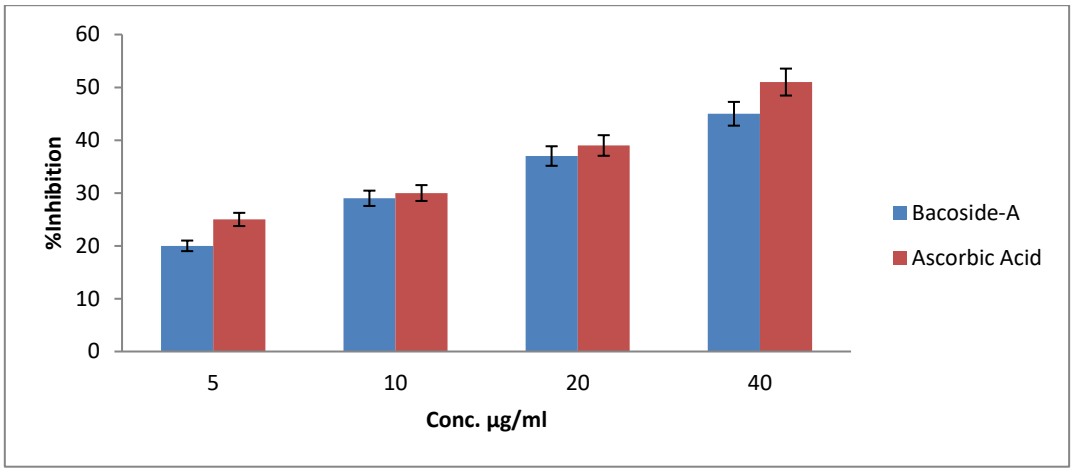
Each value is expressed as mean ± standard deviation (n=3).

FIG.7: HYDROGEN PEROXIDE RADICAL SCAVENGING OF BACOSIDE-A



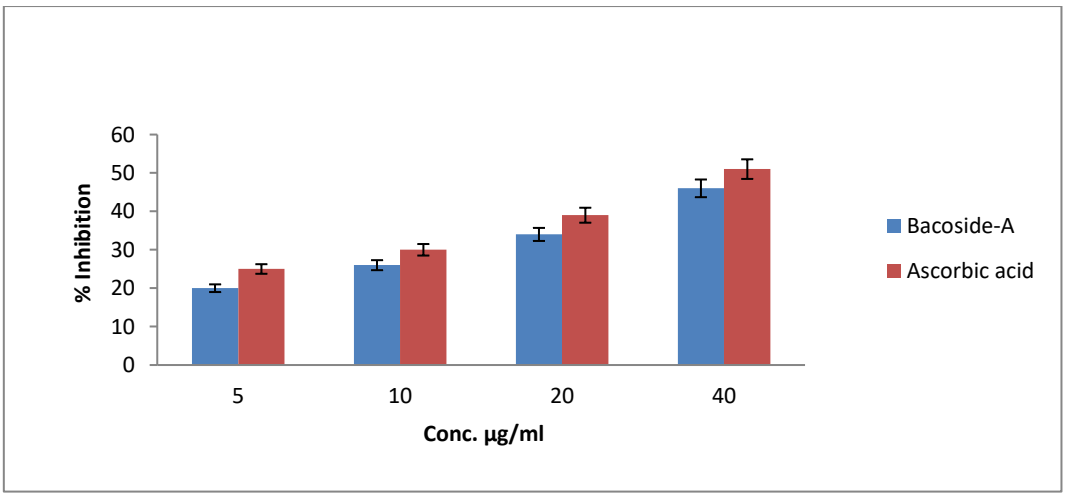
Each value is expressed as mean ± standard deviation (n=3).

FIG.8: NITRIC OXIDE RADICAL SCAVENGING ABILITY OF BACOSIDE-A



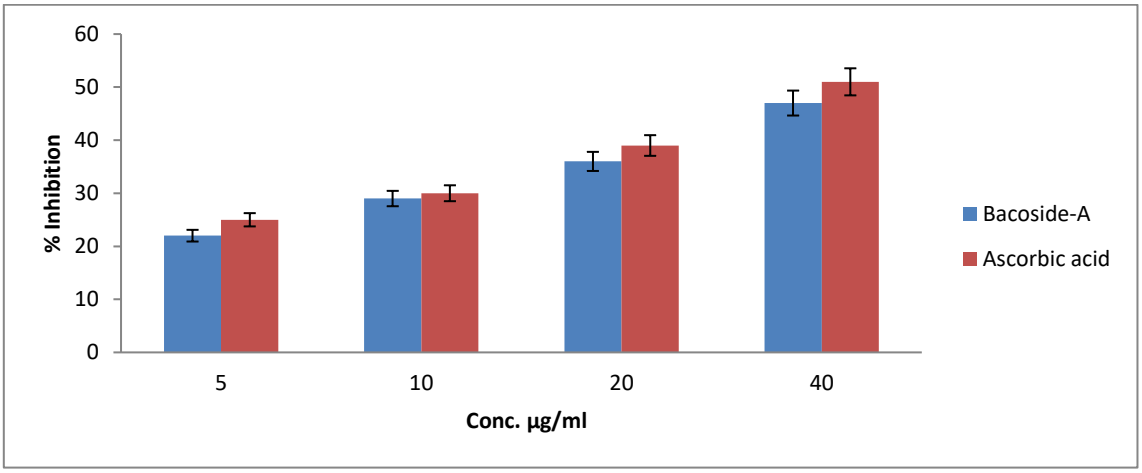
Each value is expressed as mean ± standard deviation (n=3).

FIG.9: METAL CHELATING ABILITY OF BACOSIDE-A



Each value is expressed as mean ± standard deviation (n=3).

FIG.10: PHOSPHOMOLYBDENUM RADICAL SCAVENGING ABILITY OF BACOSIDE-A



Each value is expressed as mean ± standard deviation (n=3).

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use (32). NO generated from sodium nitroprusside in aqueous solution at physiological pH reacts with oxygen to form nitrite ion. Bacoside-A inhibited nitrite formation in a concentration dependent manner (10-1500µg/ml). This may be due to the presence of antioxidant principles in the Bacoside-A, which complete with oxygen to react with nitric oxide. The scavenging of nitric oxide by Bacoside-A was increased in a dose-dependent manner as illustrated in **Fig.4** At a concentration of 45.23 µg/ml of Bacoside-A 50% of nitric oxide generated by incubation was scavenged. The IC_{50} value of Ascorbic acid was 51.23 µg/ml.

The reducing power of the Bacoside-A was evaluated by the transformation of Fe^{3+} to Fe^{2+} through electron transfer ability, which serves as a significant indicator of its antioxidant activity. The reducing properties of the Bacoside-A are generally associated with the presence of reductones, which have been shown to exert antioxidant action by donating a hydrogen atom by breaking the free radical chain. Reductions are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The presence of antioxidant substances in the compound samples causes the reduction of the Fe^{3+} ferric cyanide complex to the ferrous form. Therefore, Fe^{2+}can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (33). The **Fig.5** shows the reductive capabilities of Bacoside-A compared to Ascorbic acid. The reducing power Bacoside-A was increased with quantity of sample. The Bacoside-A could reduce the most $Fe3^{+}$ ions, which had a lesser reductive activity than the standard of Ascorbic acid. The IC50 value of Bacoside-A and Ascorbic acid was 43.18µg/ml and 51.23µg/ml respectively.

Superoxide is a highly reactive molecule that can react with many substrates produced in various metabolic processes including phagocytosis. It can cause the oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme, which catalyzes the breakdown of superoxide radical (34). The superoxide anion derived from dissolved oxygen by phenazinemethosulphate/ NADH coupling reaction reduces nitro blue tetrazolium. The decrease the absorbance at 560 nm with the Bacoside-A thus indicates the consumption of superoxide anion in the reaction mixture. As mentioned in **Fig.5**, the Bacoside-A as well as Ascorbic acid showed the scavenging activity; IC_{50} values, 40.12 µg/ml and 51.23 µg/ml, respectively.

Hydrogen peroxide itself is not very reactive, but sometimes it is toxic to cells because it may give rise to hydroxyl radical. Therefore, removing hydrogen peroxide is very important for antioxidant defense in a cell system (35). Hydroxyl radical scavenging capacity of Bacoside-A is directly related to its antioxidant activity. This method involves in vitro generation of hydroxyl radicals using Fe^{3+} /ascorbate/EDTA/ H_2O_2 system using Fenton reaction. The oxygen-derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) cause the degradation of deoxyribose into malondialdehyde, which produces a pink chromogen with thiobarbituric acid. To attack the substrate deoxyribose hydroxyl radicals were generated by

reaction of Ferric-EDTA, together with H_2O_2 and ascorbic acid. When the Bacoside-A were incubated with the above reaction mixture, it could prevent the damage against sugar. It is clear from the result that the Bacoside-A have shown a concentration dependent radical scavenging activity. The results for hydroxyl scavenging assay are shown in **Fig.6.** The concentrations for 50% inhibition were found to be 47.15 and 51.23 µg/ml for the Bacoside-A and Ascorbic acid respectively.

Hydrogen peroxide is a weak oxidizing agent that inhibits the oxidation of essential thiol (-SH) groups directed by few enzymes. Many of its toxic effects are because 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 (36). From the results, Bacoside-A was capable of scavenging H_2O_2 in a concentration dependent manner. The free radical scavenging activity of Bacoside-A was evaluated by hydrogen peroxide (H_2O_2) scavenging method. From the results, Bacoside-A showed concentration dependent activity and the H_2O_2 scavenging effect at a concentration was 42.19µg/ml. This was comparable to the scavenging effect at the concentration of Ascorbic acid was 51.23µg/ml in **Fig.7.**

Iron is an essential mineral for normal physiology, but an excess of it may result in cellular injury. If they undergo Fenton reaction, these reduced metals may form reactive hydroxyl radicals and thereby contribute to oxidative stress (37). An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyze hydroperoxide decomposition and Fenton type reactions. Therefore, it is considered important to screen the iron (II) chelating ability of the Bacoside-A. The chelating ability of ferrous ions by the Bacoside-A was estimated by the method (38). Ferrozine can quantitively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. The metal chelating activity of Bacoside-A is present in **Fig.9.**

Various concentration of Bacoside-A was also used to determine their antioxidant capacity by the formation of green phosphomolybdenum complexes. The formation of the complex was measured by the intensity of absorbance in Bacoside-A at a concentration of 5/40 µg/ml at 95^0C. The phosphomolybdenum method is based on the reduction of M_0 (VI) to M_0 (V) by the antioxidant compounds and the formation of green phosphate/M_0 (V) complex with the maximal absorption at 695 nm. In the ranking of the antioxidant capacity obtained by this method, the **Fig.10**. showed increased phosphomolybdenum reduction of Bacoside-A to the quantity of the sample. The IC50 value of Bacoside-A was 47.17µg/ml and 51.23µg/ml as a standard Ascorbic acid.

CONCLUSIONS

It is well known that free radicals are one of the causes of several diseases. The result of the present study reveals a strong antioxidant activity of the Bacoside-A. The constituents that are responsible for the antioxidant activity are unclear; hence further studies are required to evaluate the antidiabetic and antioxidant activity.

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CONFLICT OF INTEREST

There is no conflict of interest among all authors in this study.

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