RESEARCH PAPER

Assessment of Antioxidant Potential and in vitro Antidiabetic Activity of *Bacopa monnieri* Leaves Extract

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Abstract
The incidence of diabetes mellitus is increasing rapidly and it is expected to increase in future. Other than currently available therapeutic options, there are a lot of herbal medicines, which have been recommended for its treatment. Herbal medicines have long been used for the treatment of diabetes because of the advantage usually having no or less side-effects. Most of these plants have antioxidant activities and hence, prevent or treat hard curable diseases, other than having the property of combating the toxicity of toxic or other drugs. The available drugs for diabetes, insulin or oral hypoglycaemic agents have one or more side effects and search for new antidiabetic drugs with minimal or no side effects from medicinal plants is a challenging for us. The present study was undertaken to investigate the antidiabetic and antioxidant activity of the present study was undertaken to investigate the antidiabetic and antioxidant activity of *Bacopa monnieri* leaves. The antioxidant activity was measured by DPPH, Nitric oxide, hydroxyl radical, superoxide radical methods were carried in the present study. In-vitro antidiabetic activity was performed using α-Amylase inhibitory assay and α-Glucosidase inhibitory assay. The results showed that ethanol extract had the lowest IC50 for the DPPH, superoxide anion scavenging, hydroxyl radical and nitric oxide inhibition ability. These results indicated that *Bacopa monnieri* leaves possess strong anti-diabetic and antioxidant potentials and support traditional medicinal use for the treatment of diabetes mellitus and good source for natural antioxidants.

Keywords: Diabetes mellitus, antioxidant activity, in vitro activity, α--Amylase inhibitory assay, α-Glucosidase inhibitory assay

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.Introduction

Diabetes mellitus is a metabolic syndrome with multiple etiology, is characterized by chronic hyperglycemia together with disturbances in carbohydrate, protein and fat metabolism results from a decrease in circulating concentration of insulin (insulin deficiency), a decrease in the response of peripheral tissues to insulin (insulin resistance) or both [1]. Diabetes mellitus is the commonest clinical disorder affecting nearly 10% of the population all over the world [2]. At present, there are an estimated 246 million people with diabetes in the world, of whom about 80% reside in developing countries [3]. Diabetes is a global disease with a huge adverse impact on the health and mortality, particularly from cardiovascular disorders [4]. To avoid the risk of serious complications from diabetes such as heart and blood vessels diseases, controlling not only blood glucose levels but also lipid levels are necessary [5]. Patients with diabetes have lipid disorders and an increased risk of coronary heart disease, peripheral vascular disease and cerebrovascular disease [6]. As the pathogenesis of diabetes involves oxidative stress, antioxidant therapies should have a potential value in its treatment. Many trials in animal models of diabetes and diabetic patients have attempted to determine the role of antioxidant therapy on prevention or treatment of diabetes complications[7].

Furthermore, significant increase in endogenous prooxidant activity and decrease in antioxidants has been shown to contribute to the oxidative stress in diabetes. Several pharmacologic agents effective in reducing diabetic mortalities have been shown to have antioxidant activities. For example, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, angiotensin-converting enzyme (ACE) inhibitors or statins have beneficial effects on diabetic patients that may involve antioxidant effects. Interestingly, ACE inhibitors, which act partially to prevent the prooxidant effects of angiotensin II, were shown to prevent the onset of type 2 diabetes.

Reactive oxygen species are highly reactive, and when produced in excess amounts due to redox imbalance in the body, they attack biomolecules and may be the root cause of various diseases. Antioxidants slow or deter oxidation when acting in small amounts on substrates being oxidized [9]. An inverse association has been reported between the dietary intake of antioxidant-rich foods and the prevalence of chronic diseases. Antioxidants are also used as food additives to prevent food oxidation and deterioration [10]. Vitamin E supplementation has been associated with a

significant decline in protein oxidation, lipid peroxidation and enhancement in the antioxidant defense system. Vitamin E may promote beneficial effects on diabetic complications through the attenuation of oxidative stress.[11] Medicinal plants with antioxidant activities have also been shown to be protective in diabetes by scavenging oxygen free radicals and decreasing the expressions of intercellular cell adhesion molecule-1 protein [12]. The present study was undertaken to investigate the antidiabetic and antioxidant activity of the present study was undertaken to investigate the antidiabetic and antioxidant activity of Bacopa monnieri leaves.

Materials and Methods

Plant materials

The Bacopa monnieri leaves were collected from Bhopal. The plant specimen was authenticated by botanist Dr. Prabhukumar K M CSIR NBRI Lucknow. The fresh leaves were separated and cleaned to remove unwanted materials. The fresh leaves were air-dried at room temperature for about one week. The dried leaves and stems were coarsely powdered in a blender and were used for further analysis.

Preparation of plant extract

Powdered plant material were deffated with petroleum ether. Air-dried powdered drug (200g) were cold macerated with 500 ml of different solvents ranging from non-polar to polar solvents in a separate conical flask, plugged with cotton wool and the kept on a rotary shaker at 120 rpm for twenty-four hours. It was then filtered and the filtrate was evaporated to dryness at 105◦C. The percentage of the extractable matter was calculated according to the reference of the sample taken.

Phytochemical screening tests

The phytochemical tests have been performed by the standard methods

***In vitro* antioxidant activity of SA extract by DPPH free radical scavenging assay**

The scavenging effects of samples for DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical were monitored [13]. Accurately weight 0.004gm of DPPH and place it into a 100 ml of a volumetric flask. Then the volume is adjusted by methanol. Then the concentration of the solution is 0.004% of DPPH. The absorbance of this solution was taken at 517 nm against methanol as a blank and recorded as a control solution standard. Accurately weight 0.025gm of ascorbic acid and dissolved it into 5 ml of distilled water. The concentration of the solution is 5 µg/µl of ascorbic acid. This solution is called stock solution. Take 0.025gm of plant extract and dissolved it into 5 ml of methanol. The concentration of the solution is 5 µg/µl of plant extract. This solution is called stock solution. 200 µl of plant extract or standard of different concentration solution was taken in a test tube. 2 ml of reagent solution was added in test tube. Incubate the test tube for 30 mins to complete the reaction. Then the absorbance of the solution was measured at 517 nm against methanol as a blank by using UV spectrophotometer. A typical blank solution contained methanol. The mixture was mixed well and then left to stand in the dark for 30 min at room temperature, and its absorbance was read at 517 nm with a spectrophotometer against a blank. Ascorbic acid in the same concentrations was used as the positive control. All measurements were done in triplicate.The percentage (%) of scavenging of the DPPH free radical was measured by using the following equation:

$\{(A0-A1)/ A0\}\times100$

Where, A_0 = absorbance of the control

A_1 = absorbance of the extract/ standard

Then the percentage (%) of inhibition was plotted against log concentration and from the graph IC_{50} was calculated.

Determination of Nitric Oxide (NO) radical scavenging activity of Bacopa monnieri leaves extracts:

Nitric oxide (NO) was generated from sodium nitro prusside (SNP) and was measured by the griess reagent (1% w/v sulfanilamide, 2%w/v H3PO3 and 0.1% w/v N-(1-Naphthyl) ethylene diamine dihydrochloride). SNP in aqueous solution at physiological PH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO. SNP (1ml of mM) was mixed with 1ml of selected plants extracts in different concentrations in DMSO, incubated at 25 ºC for 180 minutes. To 1ml of the incubated solution, 1ml of griess reagent was added. The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with N-(1-naphtyl) ethylene diamine dihydrochloride was read at 546nm by UV-Visible spectrophotometer (Shimadzu UV-Vis1800). All the test analysis were run in triplicate and averaged. Lower absorbance of reaction mixture indicates higher free radical scavenging activity. Ascorbic was used as a positive control. All the test analysis were run in triplicate and averaged. Lower absorbance of reaction mixture indicates higher free radical scavenging activity. Ascorbic was used as a positive control. The percentage inhibition was calculated by

Percentage of Nitric oxide scavenged = [(A0–A1)/A0] X 100,

Where A0 = Absorbance of the control, and

A1 =Absorbance of the extract/ standard.

Determination of hydroxyl radical scavenging activity of Bacopa monnieri extracts:

The ability of the plant extracts to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out [14]. Briefly, 50 mL freshly prepared different concentrations (25, 50, 75, 100

and 200 µg/mL) of the extracts was added to a reaction mixture containing 25 mL 20 mM deoxyribose, 100 mL 0.1 M phosphate buffer, 10 mL 20 mM hydrogen peroxide and 10 mL 500 mM $FeSO_4$, and the volume was made up to 200 mL with distilled water. The reaction mixture was incubated at 37 ◦C for 30 min, and the reaction was stopped by the addition of 50 mL of 2.8% TCA (trichloroacetic acid), this was followed by the addition of 50 mL of 0.6% TBA solution. The mixtures were subsequently incubated for 20 min and the absorbance was measured at 532 nm by UV-Visible spectrophotometer (Shimadzu UV-Vis1800). Ascorbic was used as a positive control. The hydroxyl radical scavenging of the extract was reported as the percentage of inhibition of the deoxyribose degradation and was calculated according to the following equation

% Inhibition = [(A0-AT)/A0]*100

Where A0 = Absorbance of the control, and

AT =Absorbance of the extract/ standard.

Determination of super oxide radical scavenging activity of Bacopa monnieri extracts:

Superoxide radical scavenging activity of the extracts was based on the method described by Liu et al. (1997). Superoxide radicals were generated in 25 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 25 mL of NBT (50 mM) solution, 25 mL NADH (78 mM) solution and different concentrations (25, 50, 75, 100 and 200 µg/mL) of extracts (50 mL). The reaction started by adding 1 mL of phenazine methosulphate (PMS) solution (10 mM) to the mixture then incubated at 25 ◦C for 5 min The absorbance measured at 560 nm. Ascorbic acid was used as a positive control. Decrease in absorbance of the reaction mixture incubated increases super oxide anion scavenging activity. All the test analysis were run in triplicate and averaged. L-ascorbic acid was used as a positive control.

The % inhibition of super oxide anion generated was calculated using the following equation

% inhibition = [(A0-AT)/A0]*100

Where A0 = Absorbance of the control, and

AT =Absorbance of test extract/ standard.

In-vitro antidiabetic activity

α-Amylase inhibitory assay: α-Amylase inhibitory assay was carried out using Bacopa monnieri extract (25, 50, 75, 100 and 200 µg/mL) (100 ml) was took in a conical flask and added 100 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing a-amylase solution. This solution was pre-incubated at 25 ◦C for 10 min, after which 100 mL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and then kept at 25 ◦C for 10 min. The reaction was terminated by adding 200 mL of dinitrosalicylic acid (DNS) reagent. The flask were then kept in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using a uv spectrophotometer. The control was prepared using the same procedure replacing the extract with distilled water while activity of the standard was tested by replacing the extract with acarbose. The α-amylase inhibitory activity was calculated as percentage inhibition, and 50% inhibition of enzyme activity (IC50) were determined. The same procedure were followed for Bacopa monnieri leaves extracts

$$\% \text{ Inhibition} = \frac{\text{A control} - \text{A extract}}{\text{A control}} \times 100$$

α-Glucosidase inhibitory assay: α-Glucosidase inhibitory assay was carried out using a modified method [15]. Rat intestinal acetone powder (100 mg) was homogenized in 3 mL of 0.9% NaCl solution. After centrifugation (30 min), the crude enzyme (100 mL) was incubated with 5 mM p-nitrophenyl glucopyranoside (pNPG), 25 mM maltose or 50 mM sucrose in 0.1 M phosphate buffer, pH 6.9 and 50 mL of the different concentrations (25, 50, 75, 100 and 200 µg/mL) of extracts at 37 ◦C for 30 min. The reaction mixture was stopped by adding 50 mL of 0.1 M Na2CO3. The enzyme activities were determined by measuring the absorbance at 405 nm (α-glucosidase). The control was prepared using the same procedure replacing the extract with distilled water while activity of the standard was tested by replacing the extract with acarbose. Percentage inhibition 50% inhibition of enzyme activity (IC50) was determined.

$$\% \text{ Inhibition} = \frac{\text{A control} - \text{A extract}}{\text{A control}} \times 100$$

(Pollock and Stevens, 1965; Trease and Evans, 1996 and Plummer, 1985)

Results

Phytochemical screening

Phytochemical analyses of the crude extract of *Semecarpus anacardium* revealed the presence of flavonoid, steroid, glycoside, saponin, tannins, and triterpinoid.

Anti-oxidant Activity: Analysis of the free radical scavenging activities of the selected Bacopa monnieri extracts revealed a concentration dependent free radical scavenging activity resulting from reduction of DPPH, NO radical to non-radical form. The scavenging activity of Ascorbic acid, a known antioxidant used as positive control, was however higher.

Sample code for *Bacopa monnieri* extract

CLBM	Chloroform extract
EABM	Ethyl acetate extract
EOBM	Ethanol extract

DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals was intiated by the lipid autoxidation. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capacity of DPPH was determined by the decrease in its absorbance at 517nm, which is induced by antioxidant. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of l-Ascorbic acid, and plant extracts increased gradually with increase in concentration.

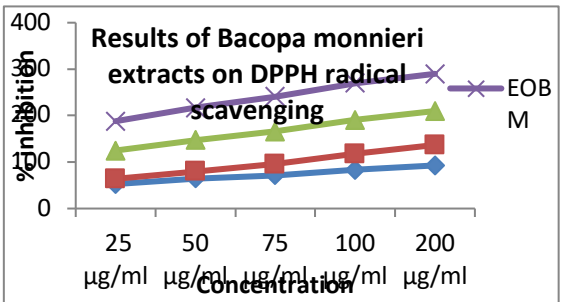

Figure 1: Results of Bacopa monnieri extracts on DPPH radical scavenging model

Determination of Nitric oxide radical scavenging model of Bacopa monnieri extracts

Nitric oxide plays an important role in various types of inflammatory processes in the body. In the present study Bacopa monnieri ethanol extracts checked for its inhibitory effect on Nitric oxide production. Nitric oxide radical generated for sodium nitroprusside at physiological pH was found to be inhibited by the extracts. The ethanol extract of Bacopa monnieri at varied concentrations showed remarkable inhibitory effect of nitric oxide radical scavenging activity compared to other extract. Results revealed that all the tested extract showed the percentage of inhibition in a dose dependent manner. The ethanol extract of Bacopa monnieri at varied concentrations showed remarkable inhibitory effect of nitric oxide radical scavenging activity.

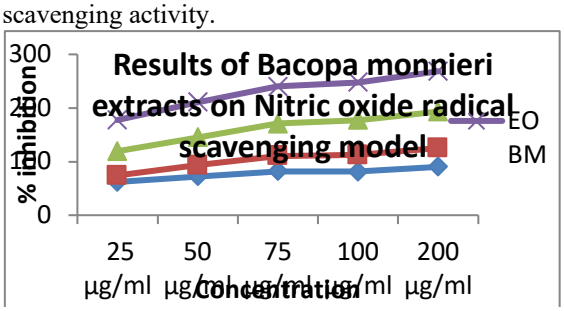

Figure 2: Results of Bacopa monnieri extracts on Nitric oxide radical scavenging model

Determination of hydroxyl radical scavenging activity of Bacopa monnieri extracts

The ethanol extract of Bacopa monnieri extracts at varied concentrations showed remarkable inhibitory effect of Hydroxyl radical scavenging activity compared to ethyl acetate and choloform extract. Superoxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases.

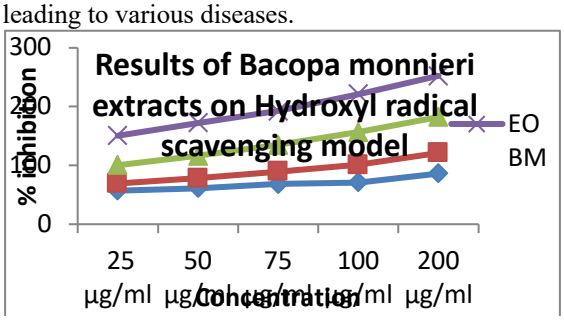

Figure 3: Results of Bacopa monnieri extracts on Hydroxyl radical scavenging model

Determination of super oxide radical scavenging activity of Bacopa monnieri extracts

The ethanol extract of Bacopa monnieri at varied concentrations showed remarkable inhibitory effect of superoxide radical activity scavenging compared to ethyl acetate and choloform extract. The ethanol extract of Bacopa monnieri at varied concentrations showed remarkable inhibitory effect of superoxide radical scavenging activity compared to ethyl acetate and choloform extract.

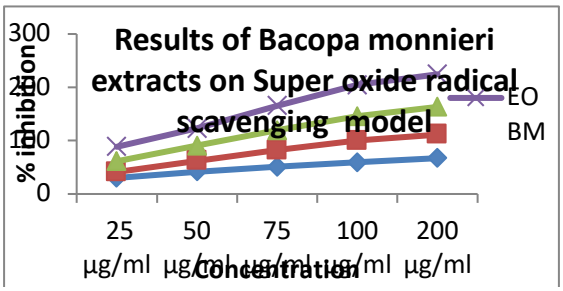

Figure 4: Results of Bacopa monnieri extracts on Super oxide radical scavenging activity

The IC50 value for the free radical scavenging abilities of the Bacopa monnieri extracts is presented.Ethanol extract had the lowest IC50 for DPPH , which is significantly lower ($p < 0.05$) than other extracts and comparable to standards (ascorbic acid). Ethanol extract possessed the lowest IC50 for hydroxyl radical scavenging ability. Aqueous extract also displayed the significant IC50 value for the inhibition of superoxide anion radicals, which is significantly different from other extracts and standard (ascorbic acid).

In-vitro antidiabetic activity

α-Amylase inhibitory assay of Bacopa monnieri extracts:

The control of postprandial plasma glucose levels is important in the treatment of diabetes mellitus and its associated complications. Inhibition of enzymes involved in the metabolism of carbohydrates such as a-amylase and α-glucosidase, is an important therapeutic approach for reducing postprandial hyperglycemia. α- Amylase found in the saliva and pancreatic juice, catalyses the hydrolysis of polysaccharides (such as starch) to disaccharides (like maltose and sucrose).

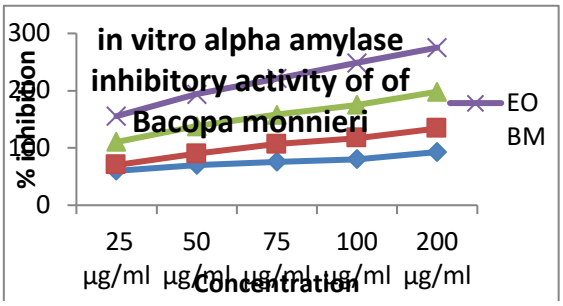

Figure 5: in vitro alpha amylase inhibitory activity of of Bacopa monnieri extracts

The in vitro alpha amylase inhibitory studies demonstrate that the ethanol extract having good alpha amylase inhibitory activity. The percentage of inhibition at various concentrations of Bacopa monnieri showed a concentration dependent reduction in the activity. The highest concentration (200µg/ml) showed maximum inhibition of nearly 94.7%.

α-Glucosidase inhibitory assay of of Bacopa monnieri extracts

Though ethanol extract of Bacopa monnieri displayed the more inhibition of α-amylase (IC50 62.71 µg/ml) and comparable to standard (acarbose) (IC50 55.91 µg/ml). α-Glucosidases are located in the mucosal brush border of the small intestine and catalyse the conversion of disaccharides to monosaccharide like glucose. In this study, ethanol extract of Bacopa monnieri exhibited the strongest inhibition of α-glucosidase. This is depicted by the lowest IC50 value of ethanol extract (77.65 µg/ml). The antioxidant and antidiabetic activities of different extracts of Bacopa monnieri may be due to the presence of phytochemicals such as alkaloids, tannins, saponins and cardiac glycosides.

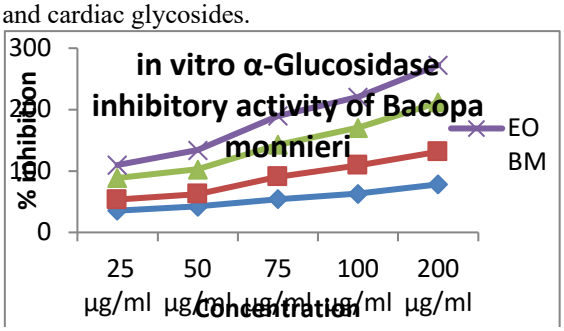

Figure 6: in vitro α-Glucosidase inhibitory activity of Bacopa monnieri extracts

Discussion

Diabetes is becoming the third "killer" of mankind after cancer and cardiovascular diseases, because of its high prevalence, morbidity and mortality. The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and failure of various organs. Hyperglycemia is an important factor in the development and progression of long-term complications of DM affecting kidney, retina, heart and nervous system. Patients with diabetes have lipid disorders (hypercholesterolemia and hypertriglyceridemia) and an increased risk of coronary heart disease, peripheral vascular disease and cerebrovascular disease. The hypoglycemic activity of many plants has been confirmed in hundreds of studies in experimental animals and several studies in diabetic patients.

The effect of antioxidants on DPPH radicals is thought to be due to their hydrogen donating ability. Radical scavenging activities are very important to prevent the deleterious role of free radical in different diseases including diabetes. DPPH free radical scavenging is an accepted mechanism by which antioxidants act to inhibit lipid peroxidation. This method has been used extensively to predict antioxidant activities because of the relatively short time required for analysis. The DPPH radical scavenging activity of extract increased with increase in concentration. In DPPH assay, the ethanol extract showed a notable radical scavenging activity in a dose-dependent manner within a certain range and was significantly different ($p < 0.05$). The phytochemical screening of the plant *Bacopa monnieri leaves* showed the presence of steroids, triterpenoids, flavonoids, glycosides, saponins and tannins. It is known that only flavonoids of a certain structure and particularly hydroxyl position in the molecule determine antioxidant properties, in general these properties depend on the ability to donate hydrogen or electron to a free radical. The present investigation established the pharmacological evidence to support that *Bacopa monnieri* leaves have antidiabetic and antioxidant activity. However, further investigations were warranted to isolate bioactive compounds from ethanol extract of *Bacopa monnieri leaves*, to observe their antidiabetic and antioxidant effects and to find out the possible mechanism action for their beneficial effects.

Conclusion

Diabetes mellitus is a global health problem and constantly increasing day by day. The number of diabetic people in world is expected to rise to 366 million in 2030. The available drugs for diabetes, insulin or oral hypoglycemic agents have one or more side effects and search for new antidiabetic drugs with minimal or no side effects from medicinal plants is a challenging for us. The present study was undertaken to investigate the antidiabetic and antioxidant activity of Bacopa monnieri leaves. Phytochemical screening showed the presence of steroids, triterpenoids, flavonoids, glycosides, saponins, and tannins that were contribute to biological activity. These results indicated that Bacopa monnieri leaves possess strong anti-diabetic and antioxidant potentials and support traditional medicinal use for the treatment of diabetes mellitus and good source for natural antioxidants.

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