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PHYTOCHEMICAL SCREENING AND *IN VITRO* ANTIOXIDANT ACTIVITY OF AQUEOUS AND HYDROALCOHOLIC EXTRACT OF *BACOPA MONNIERI* LINN.

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ABSTRACT

The present study was undertaken to investigate *in-vitro* antioxidant activity of aqueous and hydroalcoholic extract of whole plant of *Bacopa monnieri* Linn. Family- Scrophulariaceae. The total Phenolic content was determined using folin ciocalteau method while the total flavonoid content was determined using aluminium chloride method. *In vitro* antioxidant activity was evaluated using the Reducing power assay, Hydrogen peroxide scavenging assay, nitric oxide scavenging activity, superoxide scavenging activity and hydroxyl radical scavenging activity. The hydroalcoholic extract had more phenol concentration (116.1 mg/g of extract) when compared to aqueous extract (58 mg/g of extract). The flavonoid content was more in hydroalcoholic extract (242.6 mg/g of extract) when compared to that of aqueous extract (202.8 mg/g of extract). The reducing power and hydrogen peroxide scavenging of the extract was found to be concentration dependent. The nitric oxide scavenging activity, superoxide scavenging activity and Hydroxyl radical scavenging activity was also concentration dependent with IC_{50} value being 254.70 µg/ml , 934.06 µg/ml and 510.60 µg/ml respectively for Aqueous extract and 169.22 µg/ml, 495.83 µg/ml, 488 µg/ml respectively for hydroalcoholic extract. The order of the antioxidant potency of the whole plant extract is Hydroalcoholic >> aqueous. The results clearly indicate that aqueous and hydroalcoholic extract of *Bacopa monnieri* has anti oxidant property which may be due to the presence of phenols and flavonoids.

INTRODUCTION: Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, hypertension, arthritis, ischemia, gastritis, central nervous system injury, reperfusion injury of many tissues, cancer, Alzheimer's disease, Parkinsonism, diabetes mellitus and AIDS [1, 2]. According to the World Health Organization (WHO) "a medicinal plant is a plant which, in one or more of its organs, contains substance that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis" [3].

Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, would therefore be very important in the prevention of diseases [4]. Antioxidants have been reported to prevent oxidative damage by free radical and ROS and may prevent the occurrence of disease, cancer and aging. There is considerable evidence that antioxidants could help to prevent these diseases because they have the capacity to quench free radicals [5]. Although some synthetic antioxidants, such as butylated hydroxyl anisole (BHA) and butylated hydroxytoluene (BHT),

exhibit potent free radical scavenging effects, they have been demonstrated to exert toxicological effects as compared with natural antioxidants [6, 7].

Antioxidative compounds obtained from natural sources such as grains, oilseeds, beans, leaf waxes, bark, roots, spices, fruits and vegetables have been investigated [8]. Nowadays food scientists and nutrition specialists agree that food antioxidants, consumed daily contribute to the conservation of good health [9].

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), super oxide anion (O^{2-}) and hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2) are often generated as byproducts of biological reactions or from exogenous factors.[10] These reactive species exert oxidative damaging effects by reacting with nearly every molecules found in living cells including DNA [11], if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimer's disease and Parkinson's disease [12, 13, 14].

Recent investigations have shown that the antioxidants with free-radical scavenging properties of plant origins could have great importance as therapeutic agents in aging process and free radical mediated diseases including neuro degeneration [15, 16]. Phytoconstituents from plant extracts such as flavonoids and other polyphenolic constituents have been reported to be effective radical scavengers and inhibitors of lipid peroxidation [17, 18, 19].

Bacopa monnieri L. (Fam.Scrophulariaceae) is a creeping, glabrous, succulent herb, rooting at nodes, distributed throughout India in all plain districts, ascending to an altitude of 1320 m. The plant is reported to show sedative, antiepileptic, vaso constrictor and anti inflammatory activity [20]. The previous studies showed positive results for antioxidant activity for ethanolic and methanolic extract of the plant. It has been reported that the plant contains tetracyclic triterpenoid saponins, bacosides A and B, hersaponin, alkaloids viz. herpestine and brahmine and flavanoids [20, 21]. In the present study we reported the *in vitro* antioxidant activity of aqueous and hydro alcoholic extract of *Bacopa monnieri* using standard laboratory procedures.

MATERIALS AND METHODS:

Chemicals: Potassium ferric cyanide , trichloro acetic acid , ferric chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, hydrogen peroxide, Deoxyribose, EDTA, Ascorbic Acid, thiobarbituric acid, trishydrochloride, sodium dodecyl sulfate, acetic acid (glacial), butanol, pyridine, ammonium ferrous sulphate, sodium nitropursside, sulfanilic acid, N-(1-Naphthyl) ethylenediamine dihydrochloride , Dimethyl sulphoxide, NBT, sodium hydroxide, Gallic acid, sodium carbonate, Folin Ciocalteau reagent, Aluminium chloride, sodium nitrite, catechin. All the chemicals were of analytical grade and purchased from local market.

Collection and Identification of plant: The plant material (whole) i.e. *Bacopa monnieri* was collected in the month of August 2011 from a local dealer Shantanu (9437066720), Subarnarekha marketing pvt ltd. , P.O jaleswar, dst. Balasore, Pin-756032, Orissa. Around 2kgs of plant was collected. The plant material was taxonomically identified by Dr. S.K. Mahmood, Head, Department of Botany, Nizam University-Hyderabad and a specimen was deposited in their herbarium.

Preparation of the extract: The Hydro alcoholic extract of *Bacopa monnieri* was obtained from an Herbal extract manufacturing company called Amsar Goa private limited which is located in Goa, India. A sample extract (40g) was given (Batch no: L/11011 (May 2011) which was extracted using Soxhlet apparatus. The solvent used was a hydro alcoholic i.e. a mixture of Water and Alcohol (ethanol) in the ratio 60:40 % v/v. The extract was collected and stored till needed. The receipt no is AGPL/096/2011-12. The aqueous extract was prepared by Maceration process. 70g of the plant was macerated with 1000ml of water for 72 hours. The solvent was removed by evaporation and the greenish black residue was dried and stored in the dessicator. (Yield: 9.14 % w/w w.r.t to dried material).

Preliminary Phytochemical screening: The extract samples were subjected to preliminary phytochemical studies using standard procedures [22, 23] to find out the nature of the phytoconstituents present within them.

Estimation of Total Phenolic content [24]: Total Phenolic content of the extract was determined by Folin ciocalteau reagent according to Singleton and Rossi using Gallic acid as a standard. 0.1ml (100 µg) of sample solution was made up to 3ml using distilled water. About 0.5ml of Folin ciocalteau reagent was added and mixed thoroughly. Incubated for 3min at room temperature. After incubation 3ml of 20% Na_2CO_3 was added and mixed thoroughly, incubated in boiling water bath for 1 min. the absorbance was measured at 650nm. The concentration of total phenols was expressed in terms of mg of Gallic Acid equivalents per gram of extract.

Estimation of total Flavanoid content [25]: Total Flavanoid assay was measured by the aluminum chloride colorimetric assay. An Aliquot (1ml) of extracts or standard solution of catechin (20, 40, 60, 80 and 100µg/ml) was added to 10ml volumetric flask containing 4ml of distilled water. To the flask was added 0.3ml 5% $NaNO_2$. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6^{th} min, 2 ml of 1M NaOH was added and the total volume was made up to 10 ml with distilled H_2O. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg catechin equivalents (CE)/g of extract. Samples were analyzed in duplicates.

Free Radical Scavenging Assays:

Ferric Reducing Power [26]: the reducing power was determined according to the method of Oyaizu. Different concentrations of the extract (50, 100, 150, 200, 250 µg/ml) prepared in methanol were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferric cyanide { $K_3Fe(CN)_6$} (2.5ml , 1%) . The mixture was incubated at 50°C for 20 min and 2.5ml of tricholoroaceticacid (10%) was added to the mixture, which was then centrifuged at 3000rpm for 10min. the upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml , 0.1%) and the absorbance was measured at 700nm. Increased Absorbance of the reaction mixture indicated increased reducing power. Ascorbic Acid was used as Standard.

Hydrogen Peroxide Scavenging Activity: [27] The H_2O_2 scavenging ability of the extract was determined according to the method of Ruch et al. A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). 100, 200, 300, 400, 500 µg/ml concentrations of extract in 3.4ml Phosphate buffer were added to H_2O_2 solution (0.6ml, 40mM). The absorbance value of the reaction mixture was recorded at 230nm. The percent of scavenging of H_2O_2 was calculated by using the following equation.

% of scavenging = [(A of control – A of sample) / A of Control] X 100

Where A of control is the absorbance of the control reaction (containing all reagents except test compound) and a sample is the absorbance of the test compound. Test was carried out in triplicate.

Nitric Oxide Scavenging Activity: [28] Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25°C for 150 min.

From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated.

The nitric oxide radicals scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0] \times 100)$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Superoxide Radical Scavenging Activity [29]: To the reaction mixture containing 0.1 ml of NBT (1 mg/ml solution in DMSO) and 0.3 ml of the extracts, the compound and standard in dimethyl sulphoxide (DMSO), 1 ml of alkaline DMSO (1 ml DMSO containing, 5 mM NaOH in 0.1 ml water) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm.

% inhibition =

$$\frac{\text{Absorbance of Control – Absorbance of Test/ Standard}}{\text{Absorbance of Control}} \times 100$$

Hydroxyl Radical Scavenging Activity: [30] The degradation of deoxyribose generated by Fenton reaction was measured spectrophotometrically in the presence and absence of test compound. The final reaction mixture in each test tube consisted of 0.3 ml each of Deoxyribose (30 mM), ferric chloride (1mM) , EDTA (1mM), Hydrogen peroxide (20mM), in the phosphate buffer having pH 7.4 and 0.3 ml of test compound at different concentrations. The test tubes were incubated for 30 min at 37°C after incubation , trichloro acetic acid (0.5 ml , 5%) and thiobarbituric acid (0.5 ml, 1%) were added and the reaction mixture was kept in boiling water bath for 30 min. it was then cooled and the absorbance was measured at 532 nm. The results were express as a % of scavenging of hydroxyl radical.

RESULTS AND DISCUSSIONS: The participation of reactive oxygen species in the etiology and patho pysiology of human diseases such as neuro-degenerative disorders, inflammation, viral infection, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcers was already evident. To understand the role of these reactive oxygen species in several disorders and potential antioxidant, protective effect of natural compounds on affected tissues were topics of high current interest.

Initially, it was necessary to investigate *in vitro* antioxidant properties of any natural product or drug to consider it as an antioxidant substance, followed by evaluation of its antioxidant function in biological systems.

Phtyochemical analysis revealed the presence of phenols, flavanoids, glycosides, alkaloids and carbohydrates in both aqueous and hydro alcoholic extract of Bacopa monnieri.

The reducing power has been used as one of the important antioxidant capabilities for medicinal herbs. The reducing power of Aqueous and Hydro alcoholic extract of *Bacopa monnieri* was concentration dependent. The absorbance increases with increase in the concentration. From **Fig. 1,** it can be inferred that the increase in ferric reducing activity was more for hydro alcoholic extract of *Bacopa monnieri* then the aqueous extract though it was not as equal to that of standard ascorbic acid.

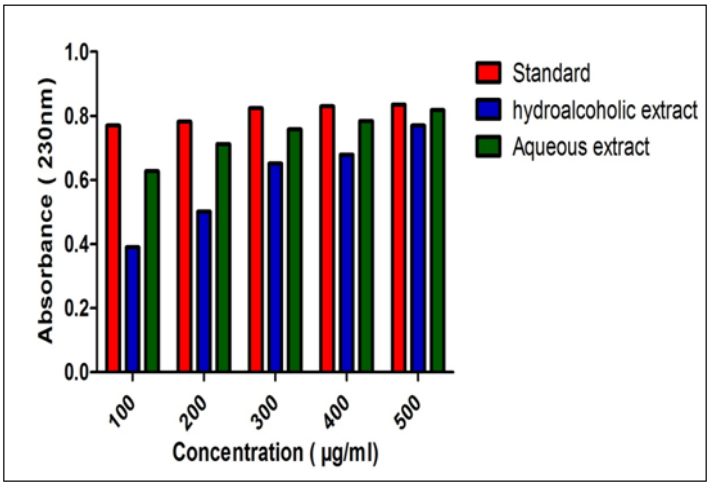

FIG 1: THE REDUCTIVE ABILITY OF AQUEOUS AND HYDRO ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* AND ASCORBIC ACID

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects it is therefore biologically advantageous for cells to control the amount of H_2O_2 that is allowed to accumulate. As shown in the **Fig. 2**, the aqueous and hydro alcoholic extract of *Bacopa monnieri* has demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner. The decomposition of H_2O_2 by the extract may at least partly result from its antioxidant and free radical scavenging activity. The activity was higher for hydro alcoholic when compared to aqueous and was comparable to that of standard i.e. ascorbic acid.

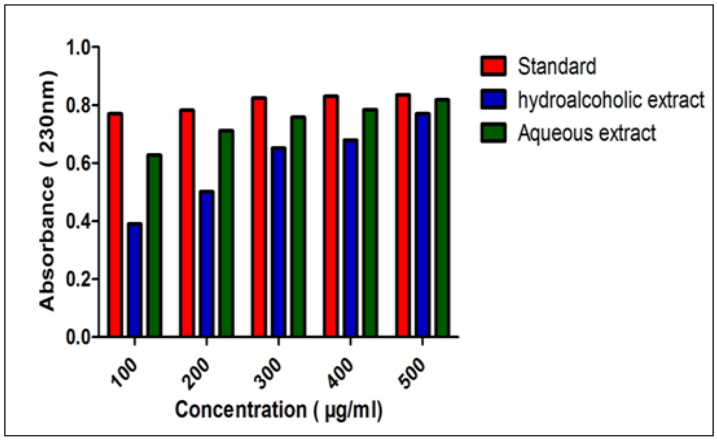

FIG 2: HYDROGEN PEROXIDE SCAVENGING OF AQUEOUS AND HYDRO ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* AND STANDARD

Active oxygen species and free radicals are involved in a variety of pathological events. In addition to ROS, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. A potential determination of oxidative damage is the oxidation of tyrosine residue of protein, peroxidation of lipids, and degradation of DNA and oligonucleosomal fragments. Nitric oxide or reactive nitrogen species formed during its reaction with oxygen or with superoxide such as NO_2, N_2O_4, N_3O_4, nitrate and nitrite are very reactive. These compounds alter the structure and function of many cellular components. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage. Aqueous and Hydro alcoholic extract of *Bacopa monnieri* shows increase in nitric oxide, as shown in the **figure 3**. The action was dose dependent. The values were comparable to that of the standard i.e. ascorbic acid.

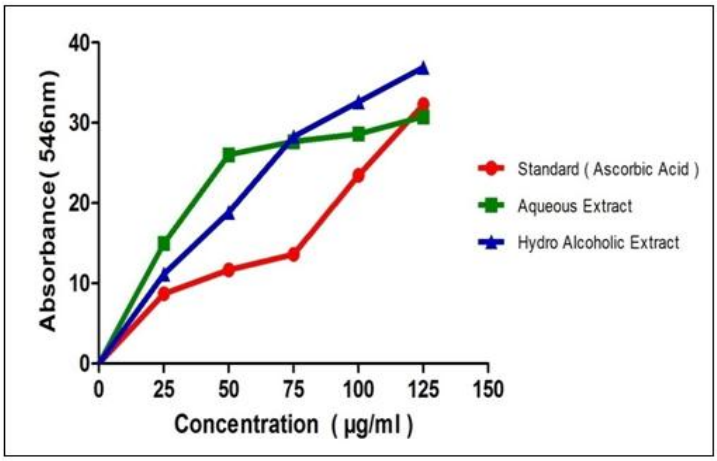

FIG 3: SCAVENGING EFFECT OF AQUEOUS AND HYDRO ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* AND STANDARD ON NITRIC OXIDE RADICAL

Superoxide radicals are known to be very harmful to the cellular component. Super oxide free radical was formed by alkaline DMSO which reacts with NBT to produce colored diformazan. The Aqueous and Hydro alcoholic extract of *Bacopa monnieri* scavenges super oxide radical and thus inhibits formazan formation. The Fig 4 illustrates increase scavenging of superoxide radicals in dose dependent manner due to the scavenging ability of the *Bacopa monnieri* extracts.IC_{50} value of ascorbic acid is 456.57 µg/ml. The IC_{50} value of Aqueous and Hydro alcoholic extract of *Bacopa monnieri* is 934.06 µg/ml and 495.83 µg/ml respectively.

From **Fig. 4,** it can be inferred that the hydro alcoholic extract has better super oxide scavenging ability when compared to aqueous and the values are comparable with that of standard Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various hydro peroxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acids in membranes and most biological molecule it contacts and is known to be capable of abstracting hydrogen atoms from membrane lipids and brings about peroxidic reaction of lipids.

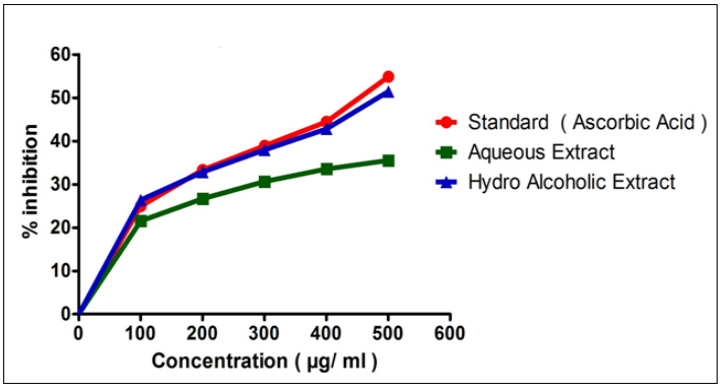

FIG 4: SCAVENGING EFFECT OF AQUEOUS AND HYDRO ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* AND STANDARD ON SUPER OXIDE RADICAL

Activity of the aqueous and hydro alcoholic extract of *Bacopa monnieri* on hydroxyl radical has been shown in above **Fig. 5** the plant extract exhibited concentration dependent scavenging activity against hydroxyl radial generated in a Fenton reaction system. Hydro alcoholic extract has better scavenging activity on hydroxyl radical when compared to aqueous extract. The IC_{50} value of ascorbic acid is 448.19 µg/ml. the IC_{50} value of aqueous and hydro alcoholic extract is 510.60 µg/ml and 488.00 µg/ml respectively.

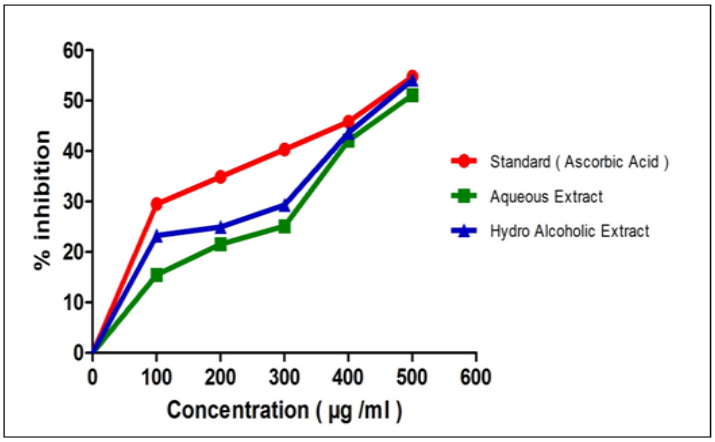

FIG. 5: SCAVENGING EFFECT OF AQUEOUS AND HYDRO ALCOHOLIC EXTRACT OF *BACOPA MONNIERI* AND STANDARD ON HYDROXYL RADICAL

CONCLUSION: Antioxidants were believed to be a panacea for many disorders in the early years of their discovery. Their importance still remain the same even several years later , when their discovery has been superseded by numerous noteworthy contributions.

The phytochemical screening of aqueous and hydro alcoholic extracts of *Bacopa monnieri* showed the presence of flavanoids and phenols which are considered to be responsible for antioxidant activity. Therefore Bacopa monnieri was considered to possess antioxidant activity.

The literature clearly suggests that *Bacopa monnieri* has been widely used as potent antioxidant as demonstrated in ethno medicine. In order to evaluate the veracity of the traditional use of *Bacopa monnieri*, in vitro antioxidant activity of aqueous and hydro alcoholic extracts of *Bacopa monnieri* were conducted.

The investigations on *Bacopa monnieri* plant extracts were found to yield substantial positive data pointing towards the evidence of antioxidant activity. The data obtained from ferric reducing power, hydrogen peroxide radical assay, nitric oxide radical scavenging , superoxide radical scavenging and microsomal lipid peroxidation assay clearly suggested that the antioxidant activity of *Bacopa monnieri* was dose dependent. It can also be noted that the extract of *Bacopa monnieri* was found to scavenge the free radicals such as peroxides, superoxides and hydroxyl radicals. From the various test it was concluded that the hydro alcoholic extract of *Bacopa monnieri* is found to possess greater antioxidant potential when compared to that of aqueous extract.

Finally, our studies concluded that *Bacopa monnieri* has antioxidant activity and therefore it can be used as an antioxidant along with the other suggested and proven therapeutic remedies such as nervine tonic, cardiotonic, cognitive enhancer and alterative.

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