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ESTIMATION OF PHYTOCHEMICAL CHARACTERISTICS AND ANTIOXIDANT PROPERTY OF SECONDARY METABOLITES OF A MEMORY ENHANCING MEDICINAL HERB *BACCOPA MONNIERI* OF EAST KOLKATA WETLAND

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Keywords:

Bacopa monnieri, Antioxidant, Phytochemicals, Secondary metabolites

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ABSTRACT: Herbal medicine nowadays offers several options to modify the progress and symptoms of medicines. There has been a new trend in the preparation and marketing of drugs based on medicinal plants and their scientific and commercial significance. These plant-derived products are carefully standardized and their efficacy and safety for a specific application have been demonstrated. The objective of this work is to prepare different crude extracts from the leaves of a medicinal plant, *Bacopa monnieri* through the maceration method and determine their total phenolics, flavonoids and other components and also the antioxidant activity by established methods. The *Bacopa monnieri* leaves were collected from East Kolkata Wetland, India. The different crude extracts were prepared by using different solvents of different polarities. The total phenolic contents were determined by using the UV visible spectroscopy method. The content of the total phenolic of different leaves crude extracts was in the range of 17.43 mg/g-46.24 mg/g of dry crude extracts. The antioxidant activity was assessed by conventional a-diphenyl-b-s-picrylhydrazyl (DPPH) methods. The highest antioxidant activity was found in the chloroform extract of the plant selected. Also, a significant correlation between phenol and glycoside content was observed. The crude extracts of Brahmi showed significant antioxidant activity; thus, these extracts could be used as natural antioxidants for the preparation of medicines to treat different diseases.

INTRODUCTION: *Bacopa monnieri*, also known as Brahmi, is a plant commonly used in traditional Indian medicine for Alzheimer's disease, improving memory, anxiety and attention deficit-hyperactivity disorder.

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Bacopa monnieri is a non-aromatic herb. The leaves of this plant are succulent, oblong and 4-6 mm (0.16-0.24 in) thick. The flowers are small, actinomorphic and white, with four to five petals [1], [2]. The best-characterized compounds in *Bacopa monnieri* are bacosides and also other saponins called bacopasides I-XII that have been identified recently.

The constituent most studied has been bacoside A, which was found to be a blend of bacoside A3, bacopacide II, bacopasaponin C and a jujubogenin isomer of bacosaponin C. *Bacopa monnieri*

displays *in-vitro* antioxidant and cell-protective effects [3-5]. The role of free radicals in diseases can lead to cellular necrosis, which may cause some membrane path physiological conditions, including atherosclerosis, rheumatoid arthritis, also diseases like diabetes, immune-suppression, neuro-degenerative diseases, and others [6-7]. Nowadays many medicinal plants having antioxidant properties have gained special attention because they can protect the human body from free radicals [8-11]. In *Bacopa monnieri*, saponins are secondary metabolites, which show the antioxidant property.

As *B. monnieri* contains large amounts of saponins it is thought worthwhile to investigate the anti-oxidant activity of the leaves [12]. It mostly grows at the eastern part of the city Kolkata known as East Kolkata Wetland [13-15]. This wetland is the largest dumping ground and contains wastewater and many other solid and liquid waste [16-17]. Despite being rich in contaminants this wetland provides a large quantity of medicinal herbs like Brahmi and many other crops and vegetables every day [18-19].

MATERIALS AND METHODS:

Materials: Solvents like acetone, chloroform, N-hexene, butanol, ethyl acetate, and methanol were obtained from Merck, India. DPPH (2, 2-diphenyl-1-pikryl-hydrazyl was obtained from SRL, New Delhi, India. Gallic acid was obtained from Himedia, Mumbai, India. Folin-Ciocaltue and Anhydrus Sodium carbonate reagent was obtained from SRL, New Delhi, India. Filter papers were obtained from Whatman no. 1, India. UV spectroscopy (Jasco spectrophotometer, Model V-630, Japan) was used to measure the absorbance of the samples.

Sample Collection: The *Bacopa monnieri* leaves were collected from the East Kolkata Wetland area, India on February 10, 2018. Then the collected samples were preserved in sterile bags for further analysis. The collected plant sample was further, authenticated from Botanical Survey of India and the authentication no is CNH/Tech. II/2018/32.

Sample Preparation: The leaf samples were washed with distilled water for removing any impurities. The leaf, stem, and root were separated from each other and the leaf was air dried as per requirement. After drying, the leaf sample was ground into a powder and stored in amber colored bottle [20, 18].

Extraction Process: 100 gm of the powdered leaf was dissolved in 250 ml of methanol and kept for 3 days. After 3 days, the sample was filtered. The solvent was evaporated by using a rotary vacuum evaporator under reduced pressure at 20 °C. The part of semi-solid methanolic extract was transferred into three different tubes for antioxidant, total phenolic compound and biochemical screening test. The remaining extract was dissolved in distilled water and transferred into separating funnel for fractionation with N-hexane, chloroform, butanol, ethyl acetate, and water and kept in air for evaporation to get the crude form [20, 21, 23].

Total Phenolic Content: In separate test tubes containing 4 mg of each extract (hexane, chloroform, ethyl acetate, butanol, methanol, and distilled water) was added with 4 ml of methanol to prepare a solution. Then 200 µl of each solution was transferred to another test tube containing 1.5 ml of FCR and was kept in dark for 5 min. 1.5 ml of 6% sodium carbonate solution was added to each test tube and shaken well and kept in the dark for 2 h. The absorbance was recorded using a UV visible spectrophotometer at a wavelength of 760 nm [24].

Total Flavonoid Content: Total flavonoid content was determined by colorimetric assay [34]. The absorbance of the sample mixture was determined at 510 nm versus blank. For the standard calibration curve quercetin was used [25].

Antioxidant Activity: To each 2 mg of crude extract 10 ml of methanol was added and dissolved. From this 200, 100, 50, 25 and 12.5 mg/ml concentrations were prepared. 2.5 ml of 0.004% DPPH solution was added to all test tubes of different concentrations and kept in a dark place for one and a half h. The absorbance of all of the concentrations of crude samples was observed using UV visible spectroscopy at a wavelength of 517 nm [26-29]. Calculation formula:

$$\% \text{ inhibition } ¼ A_{standard} - A_{extract} = A_{standard} \times 100$$

Hydrogen Peroxide Scavenging Activity: The H_2O_2 scavenging ability of the extract was determined according to the described method.

A solution of H_2O_2 (40 mM) and phosphate buffer (7.4) was prepared. After the addition of different concentrations of (100, 200, 300, 400, 500 µg/ml) each extract in 3.4 ml of phosphate buffer, this solution was mixed with the prepared H_2O_2, 6 ml of 40 mM solution. The absorbance value of the reaction mixture was recorded at 230 nm.

The % of scavenging of H_2O_2 was calculated by using the following equation:

% of scavenging = Control A-Sample A) / Control A × 100

Control A = absorbance of the control reaction (containing all reagents except test compound) Sample A = absorbance of the test compound [25, 28, 29].

Nitric Oxide Scavenging Activity: A solution of 2 ml of 10 mM sodium nitroprusside and 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of different extract at various concentrations and the mixture incubated at 25 °C for 150 min. After incubation 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. 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and again incubated at room temperature for 30 min and absorbance was measured at 540 nm.

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

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

Where A_0 = absorbance of the control (blank, without extract) and A_1 = absorbance in the presence of the extract [25, 28, 29].

Phytochemical Screening Test:

Alkaloids Test: In a beaker containing 3ml of ammonia solution 1 gm of powdered samples were added and kept for 5 min. Then to the beaker 10 ml of chloroform was added and mixed. It was then filtered to remove the solid particles. The filtrate was then evaporated using a water bath, then 2 ml of Mayer's reagent was added to it. A cream-colored precipitate appeared, indicating the presence of alkaloids [26, 29, 31].

Tannin Test: In a beaker 1 gm of leaf powdered samples were taken and 1 ml of ferric chloride was added. A brownish-black color observed, indicating the presence of tannin [26, 29, 31].

Steroid Test: This test involves an addition of 1 gm of leaf powdered sample and 10 ml of chloroform in a beaker. The solid particles were removed by the filtration process. Chloroform was evaporated by using a water bath. Then 1 ml of sulphuric acid and 1 ml of acetic anhydride were added to the beaker. A green color observed, indicating positive results [26, 29, 31].

Flavonoid Test: In a test tube 1 ml of each crude extract was taken and few drops of diluted sodium hydroxide were added to it. A deep yellowish colour observed in the test tube which became colorless after adding a few drops of dilute hydrochloric acid, indicating the presence of flavonoids [26, 29, 31].

Saponin Test: To every 1 ml of crude extract 20 ml of distilled water was added. Then it was stirred for a few minutes. The formation of foam indicating the presence of saponin [26, 29, 31].

Amino Acid Test: In 1 ml of each crude extract 2-3 drops of ninhydrin reagent was added. A purple color produced, indicating positive results [26, 29, 31].

Anthraquinones Test: 1 ml of each crude extract was taken in a test tube and hydrolyzed by diluted sulphuric acid; it was then extracted with benzene. Then a dilute ammonia solution was added to the benzene layer. The formation of pink color indicates the presence of anthraquinones [26, 29, 31].

Triterpenoids Test: 5 mg of each crude extract was taken in a test tube and 5 ml of chloroform was added to it for dissolving the crude extract. 1 ml of concentration. Sulphuric acid and acetic anhydride were added to it. The appearance of reddish-violet color indicates the presence of triterpenoids [26, 29, 31].

Cardiac Glycosides (Legal's Test): To the 3 ml of extract 1 ml pyridine was added by frequent shaking followed by 1 ml sodium nitroprusside. Pink to red color appeared. It indicates the presence of cardiac glycosides [26, 29, 31].

Quantitative Phytochemical Screening of Different Extracts of *Bacopa monnieri* Leaf:

Total Alkaloid Estimation: 5 g of each plant parts sample was weighed separately into a 250 ml capacity beaker and added 200 ml of 10% solvent of acetic acid in ethanol then covered the beaker to check evaporations of solvent and allowed to stand

for 4 h. This was filtered and extracts were concentrated on a water bath to ¼ of original volume then cons. ammonium hydroxide was added dropwise into concentrated extracts until the precipitation was completed. The solution was allowed to settle the precipitate and filtered. Filtered precipitate washed with dilute ammonium hydroxide and then again filtered. This precipitate residue is an alkaloid that was dried and weighed [32, 33].

Total Flavonoids Estimation: 10 g of leaf powder sample was weighed into a 250 ml capacity beaker and added 100 ml of 80% aqueous methanol for extraction at room temperature. This was filtered through Whatman filter paper and the extract was collected into another 250 ml capacity beaker. The extraction procedure repeated in the same used sample separately and the extract was recollected. Collected extract then transferred into a crucible and evaporated till dryness on a water bath and weighed [32, 33, 34].

Total Saponin Estimation: Suspension was prepared for 10 g of a leaf powder sample in 100 ml of 20% ethanol. This sample suspension was heated over a water bath for 4 h at 55 ºC with continuous stirring. This sample was filtered and the extract was collected in 200 ml capacity of the beaker. Obtained residue re-extracted with 100 ml of 20% ethanol. Combine extracts heated over water bath at about 90 till volume was reduced to 40 ml. The concentrate was transferred into a 250 ml separating funnel and 10 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 30 ml of n-butanol was added. The combine n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven and weighted [32, 33].

Total Phenolic Compounds Estimation: 100 mg of the extract of the sample was weighed accurately and dissolved in 100 ml of triple distilled water (TDW). 1 ml of this solution was transferred to a test tube, then 0.5 ml 2 N of the folin-ciocalteu reagent and 1.5 ml 20% of Na_2CO_3 solution was added and ultimately the volume was made up to 8 ml with TDW followed by vigorous shaking and finally allowed to stand for 2 h after which the absorbance was taken at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of gallic acid [32-33].

Total Tannins Estimation: 500 mg of the sample was weighed. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.I N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min [32-33].

Total Cardiac Glycosides Estimation: Cardiac glycoside content in the sample was evaluated using Buljet's reagent as described by El-Olemy. 1 g of the fine powder of samples were soaked in 10 ml of 70% alcohol for 2 h. and then filtered. The extract obtained was then purified using lead acetate and Na_2HPO_4 solution before the addition of freshly prepared Buljet's reagent (containing 95 ml aqueous picric acid +5 ml 10% aqueous NaOH). The difference between the intensity of colors of the experimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides [32, 33, 35].

Statistical Analysis: The IBM SPSS statistics 22 software was used to get the correlation matrix of quantitative phytochemicals.

RESULTS AND DISCUSSION: The Brahmi leaf samples collected from the EKW area and extracted by the maceration method with methanol and the semisolid masses were obtained. This crude extract was extracted with hexane, chloroform, ethyl acetate, butanol, methanol, and distilled water shown in **Table 1**.

TABLE 1: TOTAL AMOUNT OF N-HEXANE, ETHYL ACETATE, CHLOROFORM, BUTANOL, METHANOL AND WATER CRUDE EXTRACT FROM *BACOPA MONNIERI* LEAVES

Extract	Amount of crude leaves (gm)
N-Hexane	0.21
Ethyl acetate	0.22
Chloroform	2.43
Butanol	2.26
Methanol	10.26
Water	2.33

The crude extracts from Brahmi leaf were used for different biochemical studies. The screening was performed according to previously described methods. The results showed that alkaloids, steroids, flavonoids, and saponins were present in all polarities of crude leaf extracts. However, tannin is present in all polarities except n-hexane. Amino acids, anthraquinone, and triterpenoids were absent in all polarities shown in **Table 2**.

TABLE 2: PHYTOCHEMICAL ANALYSIS OF N-HEXANE, ETHYL ACETATE, CHLOROFORM, BUTANOL, METHANOL AND WATER CRUDE EXTRACTS FROM *BACOPA MONNIERI* LEAVES

Secondary metabolites	Inference					
	N-Hexane	Ethyl acetate	Chloroform	Butanol	Methanol	Water
Alkaloids	+	+	+	+	+	+
Steroid	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Tannins	-	+	+	+	+	+
Amino acid	-	-	-	-	-	-
Anthraquinone	-	-	-	-	-	-
Triterpenoid	-	-	-	-	-	-
Glycoside	+	+	+	+	-	-

+ = presence; - = absence

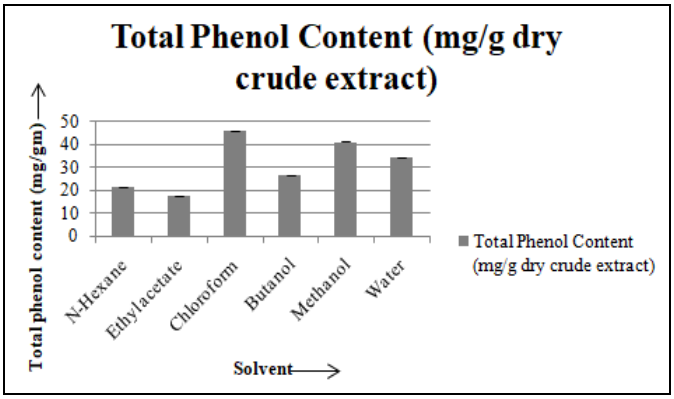

FIG. 1: TOTAL PHENOL CONTENT OF DIFFERENT CRUDE EXTRACTS FROM *BACOPA MONNIERI* LEAVES

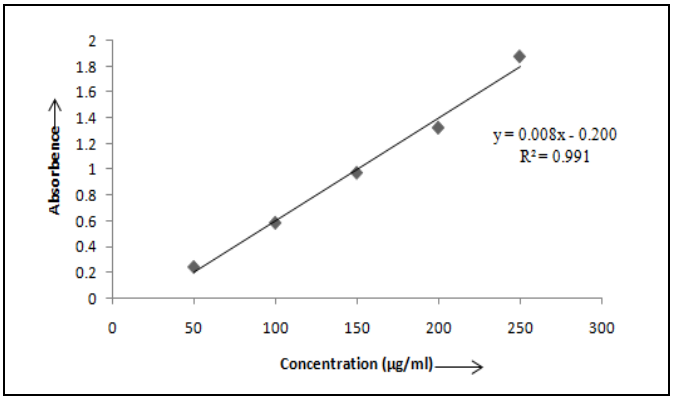

FIG. 2: GALLIC ACID STANDARD CURVE

The total phenol content of all extracts from Brahmi leaves was determined by the folin-ciocalteu method using the gallic acid curve shown in **Fig. 2**. Among the six crude extracts, the ethyl acetate crude extract contained the highest amount of phenol compound followed by methanol and the lowest was in the chloroform extract shown in **Fig. 1**.

The total flavonoid content of all leaves crude extract was determined based on quercetin standard curve shown in **Fig. 4**. The highest amount of total flavonoids was observed in methanol (38.53%) and lowest in ethyl acetate (20.37%) followed by chloroform (32.24%), water (33.55%), butanol (26.26) and n-hexane (22.45%) shown in **Fig. 3**.

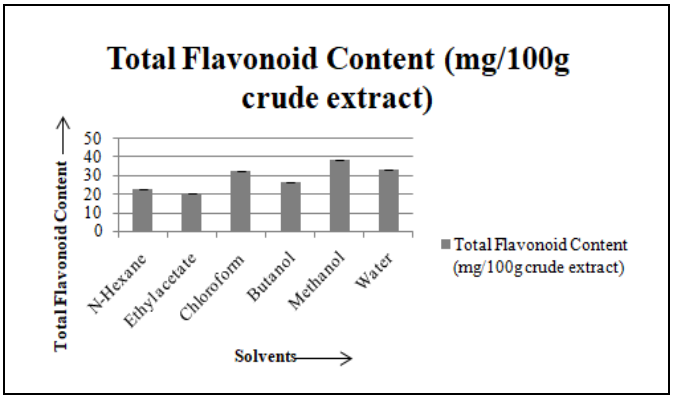

FIG. 3: TOTAL FLAVONOID CONTENT OF DIFFERENT CRUDE EXTRACT FROM *BACOPA MONNIERI* LEAVES

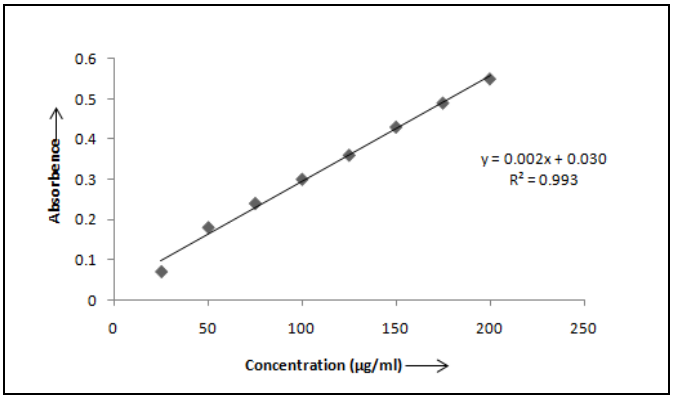

FIG. 4: QUERCETIN STANDARD CURVE

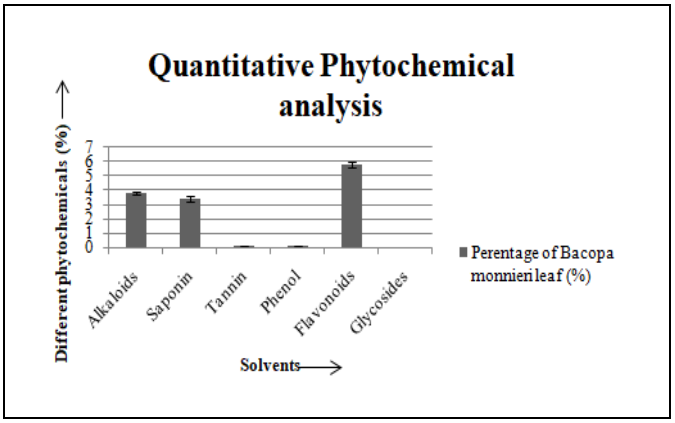

FIG. 5: QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF DIFFERENT CRUDE EXTRACT OF *BACOPA MONNIAERI* LEAVES

Quantitative Analysis: As per the positive results given in qualitative tests quantitative analysis was performed for alkaloids, saponins, tannins, phenols, flavonoids, and glycosides. In quantitative analysis flavonoids (5.8%) have shown a higher percentage followed by alkaloids (3.8%) and saponin (3.4%). The lowest percentage was observed in glycosides (0.062%) and a very low amount was observed in tannin (0.166%) and phenol (0.114%) shown in **Fig. 5**.

Antioxidant Activity: The antioxidant activity of leaf crude extracts was determined by the DPPH method. The highest antioxidant activity was found in the chloroform extract, and the lowest was in the water extract shown in **Table 3** and **Fig. 6**. Hydrogen peroxide and nitric oxide is another useful method for the determination of antioxidant activity. Both of them were checked for different solvents, and the percentage of nitric oxide was very high in butanol extract and the percentage of hydrogen peroxide was high for methanol extract. Other solvents were also observed to have a good percentage of nitric oxide and hydrogen peroxide scavenging activity shown in **Table 4** and **Fig. 7**.

TABLE 3: ANTIOXIDANT ACTIVITY OF N-HEXANE, ETHYL ACETATE, CHLOROFORM, BUTANOL, METHANOL AND WATER CRUDE EXTRACT FROM *BACOPA MONNIERI* LEAVES

Conc. (µg/ml)	N-Hexane	Ethyl acetate	Chloroform	Butanol	Methanol	Water
12.5	26.82	30.23	39.10	33.97	31.41	28.85
25	40.92	32.37	55.77	33.65	39.10	34.40
50	45.19	40.60	61.75	42.63	35.90	41.24
100	44.55	38.25	56.52	43.48	42.09	44.44
200	58.33	48.72	59.19	57.69	52.56	34.94

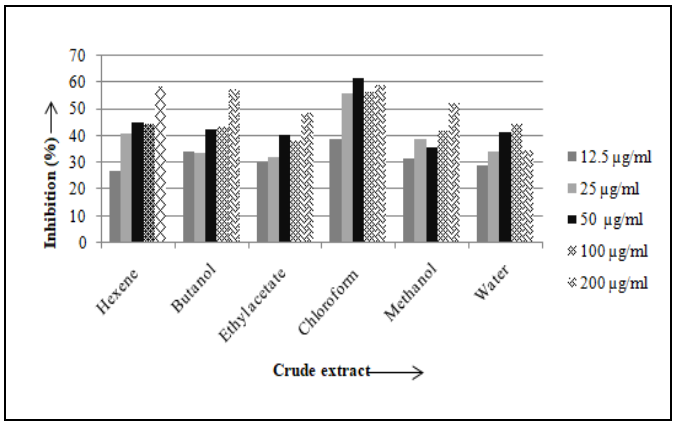

FIG. 6: ANTIOXIDANT PROPERTIES OF N-HEXANE, ETHYL ACETATE, CHLOROFORM, BUTANOL, METHANOL AND WATER CRUDE EXTRACT FROM *BACOPA MONNIERI* LEAVES

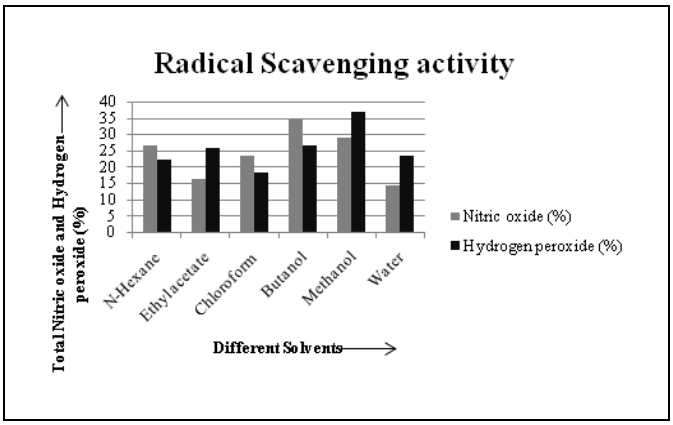

FIG. 7: NITRIC OXIDE (NO) AND HYDROGEN PEROXIDE (H_2O_2) RADICAL SCAVENGING ACTIVITY OF DIFFERENT CRUDE EXTRACT FROM *BACOPA MONNIERI* LEAVES

TABLE 4: NITRIC OXIDE (NO) AND HYDROGEN PEROXIDE (H_2O_2) RADICAL SCAVENGING ACTIVITY OF N-HEXANE, ETHYL ACETATE, CHLOROFORM, BUTANOL, METHANOL AND WATER CRUDE EXTRACT FROM *BACOPA MONNIERI* LEAVES

Solvent	Nitric oxide (%)	Hydrogen peroxide (%)
N-Hexane	26.56	22.36
Ethyl acetate	16.25	25.67
Chloroform	23.24	18.36
Butanol	34.63	26.53
Methanol	28.72	36.74
Water	14.32	23.42

Statistical Analysis: The IBM SPSS statistics 22 software was used to get the following correlation matrix of the data collected. In a correlation matrix, the P-value should be ≤0.05 for the test to significant and the test is not significant if the P-value is ≥0.05. In the above observed Pearson's correlation there are some values with both positive and negative significance with each other. In leaf, a negative significant correlation between phenol and glycoside was observed shown in **Table 5**.

TABLE 5: PEARSON'S CORRELATION BETWEEN QUANTITATIVE PHYTOCHEMICALS

		Saponin	Tannin	Phenol	Flavonoids	Glycosides	Alkaloids
Saponin	Pearson correlation	1	.922	.359	.280	-.359	.963
	Sig (2-tailed)		.254	.766	.819	.766	.173
	N	3	3	3	3	3	3
Tannin	Pearson correlation	.922	1	.693	-.115	-.693	.992
	Sig (2-tailed)	.254		.512	.927	.512	.081
	N	3	3	3	3	3	3
Phenol	Pearson correlation	.359	.693	1	-.795	-1.000**	.596
	Sig (2-tailed)	.766	.512		.415	.000	.593
	N	3	3	3	3	3	3
Flavonoids	Pearson correlation	.280	-.115	-.795	1	.795	.013
	Sig (2-tailed)	.819	.927	.415		.415	.992
	N	3	3	3	3	3	3
Glycosides	Pearson correlation	-.359	-.693	-1.000**	.795	1	-.596
	Sig (2-tailed)	.766	.512	.000	.415		.593
	N	3	3	3	3	3	3
Alkaloids	Pearson correlation	.963	.992	.596	.013	-.596	1
	Sig (2-tailed)	.173	.081	.593	.992	.593	
	N	3	3	3	3	3	3

CONCLUSION: Due to exposure of radiation, chemicals or other means, oxygen gets transformed to ROS. By the free radical scavenging and other mechanisms, the antioxidants provide resistance against this oxidative stress.

The phenolic compounds of plant material are mainly responsible for the antioxidant activity due to their hydroxyl groups. Due to oxidative stress, the detrimental effect of neuronal cell death and mitochondrial dysfunction has been implicated in age-related cognitive decline and neuro-degenerative disorders [36, 37]. *Bacopa monnieri* is a dietary antioxidant having *in-vitro* mode of action to protect the brain against oxidative damage. Several studies using *Baccopa monnieri* extract have shown to be a potential therapeutic antioxidant to reduce oxidative stress and can improve cognitive function in humans [38].

In the present study, the presence of saponins in the extract was confirmed through preliminary phytochemical screening. Analysis of the antioxidant activity showed that mainly the chloroform and butanol and hexane crude extracts of the selected plant are potent sources of natural antioxidants. Therefore, the selected crude extracts can be used as a natural antioxidant.

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CONFLICTS OF INTEREST: There are no conflicts of interest.

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