



Proximate analysis, Antioxidant Property and Cytotoxicity Assessment of *Pseuderanthemum reticulatum* Leaves

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ABSTRACT

The bioactive constituents derived from plants attract the attention of researchers due to their potential applications in the medicinal field. In this regard, the proximate analysis and the cytotoxicity study of the plant materials play an important role in the phytochemical research. In the present work, estimation of total ash, moisture content, fiber content, crude protein, and carbohydrate were carried out under proximate analysis and the antioxidant activity of the anthocyanin present in the plant material was evaluated by DPPH(2,2-diphenyl-1-picrylhydrazyl) method. The separation of anthocyanin pigment from the plant material was done by paper chromatography (PC) technique and they are characterized by UV-spectrum, chemical test and the R_f values obtained from paper chromatography. This study also investigated the *in vitro* cytotoxicity of *Pseuderanthemum reticulatum* leaves by means of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay using PBMC (Peripheral Blood Mononuclear Cell). The results of the proximate analysis showed that the plant material contains 7.6% of moisture content, 16.6% of total ash, 5.6% of crude protein, 23.0% of crude fiber, 3.82% of crude fat and 23.64% of carbohydrate. The free radical scavenging ability of the separated anthocyanin was found to be 72.58% at 10 $\mu\text{g/mL}$. The cytotoxicity investigation showed that the aqueous extract possess the IC_{50} value of 161.5 $\mu\text{g/mL}$. The high percentage of radical scavenging activity and low toxicity of the plant suggest that it can be extensively used for the investigation of the bioactive constituents and its applications.

Keywords: *Pseuderanthemum reticulatum*, Cytotoxicity, Peripheral blood mononuclear cell, MTT assay, DPPH, Proximate analysis.

INTRODUCTION

Natural plants are used as medicine since time immemorial. They are used as direct medicinal agent or a base for many semi-synthetic chemical compounds. The chemical structures derived from the bioactive compounds are used as models

for the synthesis of new compounds. Most of the natural plants are also used as sources for dietary supplements which are called phytopharmaceuticals and are produced from fresh or dried plants or parts of plants¹. Medicinal plants place a significant role in both humans and animals health system not only in diseased conditions but also served as a



potential material for proper maintaining of health. Qualitative and quantitative determination of the bioactive compounds from plant materials led to the discovery of new drugs and in the treatment of many diseases. The bio active compounds such as anthocyanins, phenols and flavonoids have strong anti-oxidant properties. Ajayi *et al.*, investigated The phytochemical screening, proximate analysis, mineral composition and anti-microbial activity of *Megaphrynium macrostachyum* seeds and the findings showed that the plant has good nutritional and medicinal importance². Jadid *et al.*, studied the mineral composition, and proximate analysis of the fruit *Piper retrofractum* (*P. retrofractum*) vahl and the quantitative determination of total alkaloids, phenols and flavonoids were also estimated. The plant contains 63.4% of carbohydrate, 11.4% of crude protein, 4.29% of total ash, 28.8% of fiber, 2.97% of total fat and it has high nutritional and phytochemical values³. Dastagir *et al.*, studied the proximate analysis of *Zygophyllaceae* and *Euphorbiaceae* family plants such as *Fagonia cretica* L., *Tribulus terrestris* L., *Ricinus communis* L., etc. The environment, habitat and time of harvest are the reasons for the variation in the proximate composition of these plants. The results of the investigation showed that the seeds of *R. communis* can be useful for development of biodiesel and helpful in pharmaceutical, insecticidal and food industries⁴. It is essential to investigate the host cell to examine the safety of any drug either natural or synthetic.

MTT is one of the common and the most frequently applied method to evaluate the cell viability. The cell viability is determined through colorimeter using tetrazolium dye. There is a liner relationship between metabolically active cells and the color produced by them and this will allow the quantification of the cell death rate accurately. This method is based on the capability of a mitochondrial dehydrogenase enzyme of viable cells to break the tetrazolium rings; due to which the pale yellow tetrazolium dye form purple colored crystals of formazan which are impermeable to the cell membranes. Thus the formazan crystals accumulated within healthy cells are dissolved by the addition of DMSO (Di Methyl Sulfoxide). The cell viability in MTT assay is connected with the quantification of formazan crystals at 540 nm and the number of viable cells is proportional to the formazan crystals formed⁵. Ala *et al.*, assessed the

in vitro cytotoxic activities of the aqueous, methanol and chloroform extracts of *Andrographis paniculata* and *Aspilia africana* leaves on murine cells. The percentage of cell viability values of the combined extracts at different concentrations were determined by the tetrazolium salt reduction assay (MTT). The results showed that extracts were cytotoxic to the murine cells at 500 µg/mL concentration⁶. Korsuwannawong *et al.*, studied the cytotoxicity of a Thai herb on Mouse fibroblast (L929) cells using tetrazolium (MTT) and sulfo rhodamine B (SRB) assays⁷. Kumar *et al.*, evaluated the *in vitro* cytotoxic activity of *Cocculus hirsutus* by MTT assay using breast cell lines (MCF-7). Antioxidant activity of the plant extracts by DPPH scavenging method was also studied. The results revealed that it shows good antioxidant property and it can act as a potential drug for breast cancer⁸. Pathogenesis of many diseases involves free radicals. Our body prevents these diseases by natural defense mechanism but when the free radicals and reactive oxygen species attack our body suddenly in a larger amount then our body needs an external antioxidant supplements. In nature many plants have natural anti-oxidants and these anti-oxidants from the natural source are highly active and preferred over the synthetic one. Anthocyanins, phenolic compounds and flavonoids derived from plant have an excellent free radical scavenging activity hence they are potent anti-oxidant⁹. Astadi *et al.*, examined the anti-oxidant activity of anthocyanins in *Malika* and *Cikuray* variety of black soybean seed coat by DPPH radical scavenging and LDL oxidation methods. The maximum radical scavenging capacity of *Malika* and *Cikuray* was 92.78% and 91.50% respectively¹⁰. The radical scavenging efficacy of anthocyanins present in a range of berries was studied by Nakajima *et al.*, The radical scavenging activities of these berry extracts were analyzed by using DPPH. The result showed that all the extracts exhibited good anti-radical activities¹¹. Bo Chu Wang *et al.*, studied the anti-oxidant properties of blueberry Anthocyanins. The blueberry Anthocyanins protect the ECV-304 cells against oxidative damage caused by H₂O₂; Hence they are used as a colorant in food products mainly of acidic in nature and as a food source to prevent diseases arising due to the oxidative processes¹². *Pseuderanthemum reticulatum* is an ornamental plant and used in traditional medicine to take care of fever, headache, cold and back pain. The native of the plant is Polynesia and belongs to

the family of Acanthaceae¹³. In the present work proximate analysis, anti-oxidant properties of Anthocyanins by DPPH method and cytotoxicity assessment of *Pseuderanthemum reticulatum* plant leaves are investigated. The proximate analysis, anti-oxidant potential and cytotoxicity assessment *Pseuderanthemum reticulatum* is an ornamental plant and used in traditional medicine to take care of fever, headache, cold and back pain was studied¹³.

EXPERIMENTAL

Materials

Sulphuric acid, Anthrone, Glucose, Methanol, Sodium hydroxide, Sulfo-phospho-Vanillin reagent, Chloroform, Fetal Bovine Serum (FBS)(Gibco USA), RPMI medium, DMSO, 3-4,5 dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT)(Sigma USA), 1X PBS (Himedia India), tissue culture 96 well plate, 0.1mM DPPH solution, Ascorbic acid, Butanol, Acetic acid of analytical grade were used for the investigation.

Plant material for proximate analysis

The fresh leaves of *Pseuderanthemum reticulatum* were shade dried for 20 days and grounded into powder using a blender and it is stored in a dry container.

Total ash

The residue remaining after incineration is called the total ash and it is expressed in terms of percentage. For the total ash determination, a clean dry crucible was weighed and the dried plant material (2g) was placed and weighed again. The crucible with the plant material was heated over an electric burner. After the material was charred; it was heated again in a muffle furnace to 600°C for 2 hours. All organic matter will be burnt at this temperature leaving behind minerals and then the crucible was taken from the furnace and cooled in a desiccator to room temperature and weighed again. The total ash percentage was determined by the following formula¹⁴.

$$\% \text{Total ash} = \frac{\text{Weight of the Ash}}{\text{Weight of the sample}} \times 100$$

Moisture content

Moisture content of the plant material was done by weighing crucible along with the sample and drying at 105°C in an oven. Heating was continued until a steady weight was obtained. Moisture content

in terms of percentage was obtained by the following formula².

$$\% \text{Moisture content} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

W1-Empty crucible weight, W2-Crucible weight with sample (before drying)

W3-Crucible weight with sample (after drying)

Estimation carbohydrate (Anthrone method)

Anthranol is an active form of the Anthrone reagent. The enol tautomer of the reagent condensed with the carbohydrate furfural derivative to form a blue colour solution which is estimated calorimetrically. The furfural derivative of carbohydrate is produced by dehydration using concentrated H₂SO₄. For the estimation of carbohydrate, standard glucose stock solution (50 mg/mL) was prepared and diluted to different concentrations. 100 µL of each standard solutions as well as the aqueous extract of the sample were mixed with 200 µL of 75% H₂SO₄ and 400 µL of Anthrone reagent. After vortexing, both the sample and standard solutions were boiled at 100°C for 15 min then the absorbance was recorded at 578 nm using micro plate reader^{15,16}.

Estimation of Total crude fiber

Estimation of total fiber content is one of the nutritive measurements in poultry and domestic animal feeds and it is also used to detect adulteration in the various food products both qualitatively and quantitatively. To estimate the total crude fiber, the plant material (2 g) was treated with petroleum ether at 50°C to remove the fat content. After extraction, the material was dried; then the dried material was boiled with 200 mL of sulphuric acid for 30 minutes. After boiling the material was filtered by means of muslin cloth and followed by hot water washing till the traces of all the acids were removed. The washed material was again boiled for 30 min with sodium hydroxide (200 mL) solution and washed with 25 mL of boiling water with 1.25% of H₂SO₄ followed by 25 mL of ethanol and then the residues were removed and transferred into pre-weighed ashing dish (W₁). The residue was heated for 2 h at 130°C for drying and it was cooled and weighed (W₂). Then the residue was ignited at 600°C for 30 min after that the ashing dish was weighed (W₃) again. The percentage of total fiber content in the plant material was calculated using the following formula¹⁷.

$$\% \text{Crude fiber in sample} = \frac{(W2 - W1) - (W3 - W1)}{\text{Weight of the sample}} \times 100$$

Lipid estimation by Vanillin method

The total lipid in the plant material was estimated by colorimetric Sulfo-Phospho-Vanillin method. Standard stock solution of cholesterol (5 mg/mL) was prepared using the solvent mixture of chloroform and methanol in 2:1 ratio. Standard solutions of different concentration were prepared using the stock solution. The dried plant material is also dissolved in the chloroform, methanol solvent mixture and filtered and the filtrate is used for the analysis. For colorimetric analysis Both the standards and filtrate of the plant material (500 μ L) was mixed with 1 mL of concentrated sulfuric acid and heated at 90°C for 10 min after cooling 500 μ L of Sulfo-Phosphoric-Vanillin reagent was added to each solution for the colour development and the absorbance were measured at 540 nm¹⁸.

Protein estimation by Bradford's method

The total protein content present in the plant material was determined by Bradford's method. For the estimation of protein Bovine serum albumin standard was prepared at different concentrations (0.04 to 0.5 mg/mL). 3 mL of Bradford's reagent was added to 100 μ L of each of the standard and sample solutions respectively. Then the absorbances of all the solutions were measured at a wavelength of at 595nm after incubating them in the dark for 5 minutes¹⁹.

MTT Assay

In vitro cytotoxicity of the aqueous extract of the plant material was tested via MTT assay by means of PBMC. (Peripheral Blood Mononuclear Cells). The PBMC cells were seeded into 96-well plate at a density of 1×10^5 cells/well (200 μ L) and treated with RPMI media and each sample was replicated three times and the cells were incubated in a humidified incubator at 37°C for 24 h with 5% CO₂. After the incubation period, 20 μ L of MTT (5 mg/mL) was added into each well and again the cells were incubated for 2-4 h at 37°C until purple

$$\text{DPPH radical scavenging} = \frac{(\text{absorbance of control} - \text{absorbance of reaction mixture})}{\text{absorbance of control}} \times 100 \text{ ability (\% inhibition)}$$

RESULT AND DISCUSSION

Proximate analysis

The proximate analysis result of *Pseuderanthemum reticulatum* dry leaves are

precipitate was obtained. In the end, the medium together with MTT was removed from the wells and washed by 200 μ L of 1 X PBS (Phosphate Buffered Saline) solution. The formazan crystals formed in the plate were shaken with 100 μ L of DMSO for 5 min to dissolve them. The micro plate reader (Thermo Fisher Scientific, USA) was used to measure the absorbance of the formazan crystals formed in each well at the wavelength 570nm. By using the GraphPad Prism 6.0 software (USA), the cell viability percentage and IC₅₀ value were calculated⁵.

Separation of anthocyanin pigments using Paper Chromatography

Paper chromatography is one of the basic tools for the separation of phytoconstituents. It is mainly used to separate the colored pigments from the plant materials. To separate the anthocyanins; the plant extract of the fresh leaves was prepared using acidified methanol. The extract was placed in the paper using capillary tube. The mobile phase used in the system was a mixture of butanol, acetic acid and water in 4:1:5 ratios. Once the mobile phase reaches the solvent front, the paper is taken and the solvent front is marked and allowed to dry²⁰. The R_f values of the colored pigment spots were calculated. The colored pigment spots were cut off from the paper and dissolved in methanol with few drops of concentrated HCl. The anthocyanin pigments were confirmed by chemical test, R_f value and UV spectrum.

Antioxidant property of the anthocyanin pigments by DPPH Method

0.1 mM of DPPH solution was prepared using methanol and 100 μ L of this solution was added to the separated anthocyanin samples (300 μ L of each) at different concentrations and the solution mixture was shaken strongly and allowed to rest at room temperature for 30 minutes. Then the absorbance of each solution was measured at 517nm using a UV-Vis spectrophotometer and Ascorbic acid was used as the reference. DPPH radical scavenging ability of the separated anthocyanins was estimated by the following formula¹⁹.

shown in Table 1. Moisture content is one of the chief factors in determination of the shelf life of any plant materials. If the moisture content of the plants is higher than 15%, they can be easily contaminated by microorganisms. The moderate moisture content

(7.6%) of *Pseuderanthemum reticulatum* leaves showed that it could be stored for longer time. The availability of inorganic constituents in a plant material was given by the ash content and the plant material contains 16.6% of total ash. Fat yields more calories compared to carbohydrates and proteins. Total fat content of the plant material was found to be 3.82% by Vanillin method. Carbohydrate is one of the energy source and the estimation of carbohydrate by Anthrone method showed that the plant material contains 23.64% of carbohydrate. The protein present in the plant material was found to be 5.6% by Bradford's method. For the digestion of food materials in the food passage of animals, crude fiber is very essential and the results showed that the plant material contains 23% of crude fiber²¹.

Table 1: Proximate results of the *Pseuderanthemum reticulatum* leaves

Proximate composition	Percentage (%) of dry sample
Moisture content	07.60
Total Ash	16.60
Crude protein	05.60
Crude fiber	23.00
Crude fat	03.82
Carbohydrate	23.64

MTT Assay

MTT assay revealed the cytotoxicity of the aqueous extract of *Pseuderanthemum reticulatum* on PBMC cells at different concentrations. The OD (Optical Density) Values of the sample at different concentration and the percentage of cell viability on PBMC cells were shown in Table 2, and Fig. 1. The Cell viability of the extract at lower concentrations (10 to 40 $\mu\text{g}/\text{mL}$) was found between 83% to 99%. The cell viability percentage values for the concentrations from 60 $\mu\text{g}/\text{mL}$ to 500 $\mu\text{g}/\text{mL}$, the result were found between 109 to 215 and the values were higher than the control. The reason for the high percentage of cell viability is due to the impact of the color of the plant extract. Since the separated anthocyanin is pink in colour during the experimental observation its absorbance has also interfered the absorbance of the test solutions in MTT assay. Images of PBMC cells treated with the plant extracts at different concentrations were shown in Fig. 2 and it showed that the high concentration of the plant extract not much affect the cell viability. The overall results showed that the aqueous plant extracts have low cytotoxicity towards PBMC cells and the IC_{50} value was found to be 161.50 $\mu\text{g}/\text{mL}$ ²².

Table 2: OD Value (at 570 nm) of the control and samples at different concentrations

S. No	Tested sample concentration ($\mu\text{g}/\text{mL}$)	Mean OD values
1	Control	0.436
2	500 $\mu\text{g}/\text{mL}$	0.937
3	400 $\mu\text{g}/\text{mL}$	0.804
4	300 $\mu\text{g}/\text{mL}$	0.739
5	200 $\mu\text{g}/\text{mL}$	0.612
6	100 $\mu\text{g}/\text{mL}$	0.619
7	80 $\mu\text{g}/\text{mL}$	0.521
8	60 $\mu\text{g}/\text{mL}$	0.476
9	40 $\mu\text{g}/\text{mL}$	0.435
10	20 $\mu\text{g}/\text{mL}$	0.399
11	10 $\mu\text{g}/\text{mL}$	0.364

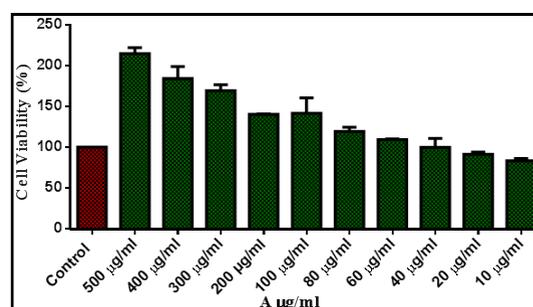


Fig. 1. Percentage of Cell Viability of PBMC cells on treatment with plant extract (A-Concentration of *Pseuderanthemum reticulatum* leaves extract at different concentration)

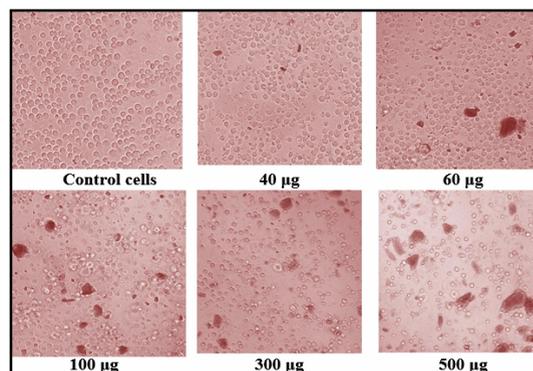


Fig. 2. Images of PBMC cells treated with plant extracts at different concentrations

Anti-oxidant activity

Anthocyanins are very reactive towards ROS (Reactive Oxygen Species), due to their structure and their electron deficient nature. Anthocyanins and anthocyanidins are natural anti-oxidants and exhibit higher scavenging activity towards free radicals²³. The coloured pigments separated from the acidified methanolic extract of *Pseuderanthemum reticulatum* showed a characteristic R_f values (0.42-0.78) and the UV absorption peak at 516nm confirmed the

presence of Anthocyanin pigments²⁴. When 2 mL of HCl was added into the extract and heated for 5 min the pink colour remains stable and the addition of 2 mL of NaOH separately to the extract gave a green colour also indicated the presence of Anthocyanin²⁰. The radical scavenging capacity of the separated Anthocyanins was done by DPPH method and the investigation result revealed that they have good anti-oxidant property. The free radical scavenging ability of extract was found to 77.54% and 72.58% at 500 µg/mL and 10 µg/mL concentrations respectively. The concentration of the sample requested to reduce the DPPH concentration by 50% is called IC₅₀ value. The IC₅₀ value for radical scavenging capacity of the extract was found to be 6.94 µg/mL and it was obtained from the linear regression analysis [log (inhibitor) Vs. normalized response] using the plot of OD value Vs. Sample concentration (Fig. 3). The free radical inhibition percentage of the separated Anthocyanin pigments at different concentrations was presented in Table 3.

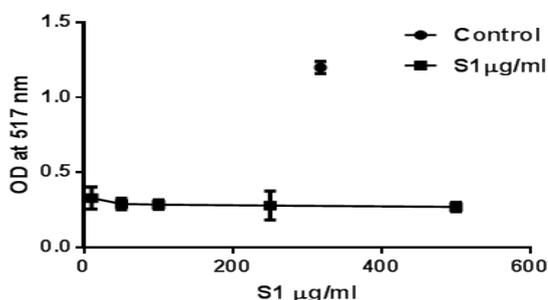


Fig. 3. OD Values (at 517 nm) of Anthocyanin extract at different concentrations after adding DPPH solution (Control Mean OD value: 1.198)

Table 3: Free radical inhibition percentage (%) of separated anthocyanin

Tested sample concentration (µg/mL)	Percentage of inhibition (%)
500	77.54
250	76.76
100	76.29
50	75.87
10	72.58
Ascorbic acid	92.97

CONCLUSION

Plants have been the source for traditional medicines and most of them are very useful for the development of new drugs in pharmacological industries. In the current work, proximate analysis, anti-oxidant potential and cytotoxicity, of *Pseuderanthemum reticulatum* leaves were studied. The results of proximate analysis showed that *Pseuderanthemum reticulatum* could be used as a feed material for animals and the free radical scavenging ability of the separated Anthocyanin by DPPH technique revealed that it has an excellent antioxidant property. The cytotoxicity studies of the aqueous extract of the plant by MTT assay on PBMC cells showed that it exhibits low toxicity towards PBMC cells. The overall results of the investigation showed that the plant can be used in pharmacological research. With the ongoing research on this plant, new bioactive principles can be derived in the future.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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