

Received: 2020-10-25

DOI: 10.2478/hepo-2021-0013

Accepted: 2021-05-20

Available online: 2021-09-01

EXPERIMENTAL PAPER

Phytochemical analysis and cytotoxicity evaluation of flowering buds of *Bauhinia variegata* L. (ahead of print)

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Summary

Introduction: *Bauhinia variegata* is used in traditional medicine in Pakistan.

Objective: The present study was designated to analyze phytochemical screening and cytotoxicity assay of crude aqueous and methanol extracts of flowering buds of *Bauhinia variegata*.

Methods: Qualitative and quantitative phytochemical screening of both extracts were performed by using standard protocols while brine shrimp lethality assay was conducted for cytotoxicity analysis of both extracts.

Results: The highest levels of phenolics, flavonoids and terpenoids were observed in methanol extract with respective standard drugs. The amino acid content was highest followed by phenol and flavonoids in crude aqueous extract of the raw materials. In cytotoxic studies (brine shrimp lethality assay) the use of methanol extract was reported as potent with LD₅₀ of 241.778 µg/ml as compared to aqueous extract with LD₅₀ of 489.7061 µg/ml.

Conclusion: This study reveals that the presence of bioactive compounds in both extracts are responsible for the treatment of various ailments and being utilized by the traditional folks as a food as well. And showed that methanol extract possess cytotoxic activity and thus needs further scientific intervention for its value addition.

Key words: *Bauhinia variegata*, phytochemical analysis, cytotoxic activity, secondary metabolites

Słowa kluczowe: *Bauhinia variegata*, analiza fitochemiczna, działanie cytotoksyczne, metabolity wtórne

INTRODUCTION

The ethnobotanical importance of plants is as old as mankind. In pre-historic societies, plant medicines were found everywhere, whereas 7,000 conventional medicines such as aspirin, digoxin, morphine, quinine, etc. have been obtained from plants and have been included in the modern pharmacopeia. A review from 1981 to 2007, showed that since 1994 majority of the drugs (35%) approved by the FDA are derived from natural sources. Researchers have made many attempts to explore many secondary metabolites (flavonoids, alkaloids, saponin, tannins, phenols, carbohydrates, phenolic glycosides, cyanogen glycosides) with pharmacological and biological activities against cancer, diabetes, ulcers, and many other diseases. Keeping in view the importance of natural drugs pharmaceutical industries are anxious about the knowledge of indigenous people regarding the uses of herbs to search for new active secondary metabolites [1, 2].

The *Fabaceae* family covers the *Bauhinia* genus. This family comprises of 630 genera and 18,000 species. The plants included in this family are deciduous trees, shrubs, vines, and are well known for ornamental purposes. *Bauhinia* is a famous plant called by various names, like Kachnar in Hindi, Raktakanchan in Marathi; English users named it as an orchid tree as well as Mountain ebony. The Kachnar word means "A glowing beautiful lady" in transcripts of Sanskrit [3]. It is widely planted in many native countries like India, South China, Pakistan, Burma, Vietnam, Cambodia, Laos, and Thailand [4]. In native countries, flowers, buds, pods, and seeds of Kachnar are cooked and used to make pickle [5]. Different plant parts are used for medicinal purposes in South Asia in Unani, Ayurveda, and homeopathic health belief models. The plant parts that are most frequently used are flowers, stem, buds, stem bark, leaves, roots, and seeds [6].

The antidiabetic activity of stem bark and leaves of *B. variegata* was discovered in rats using hydroalcohol, ethanol, and aqueous extracts. The metabolism of glucose in rats was increased with oral extract administration of various strengths that resulted in the reduction of raised glucose levels in

blood. Further experimental work established that leaves of *B. variegata* contained insulin-similar proteins that stimulate the metabolism of glucose [7, 8]. The preparation of leaves in an ethanolic extract at a dose of 300 mg/kg was related to the decrease of blood glucose levels and enhanced lipid profile. The results of *in vitro* study emphasized that *B. variegata* and roesoside leaves extracts in ethanol could upsurge the insulin release in the INS-1 β -cell line. The decrease in blood glucose levels in alloxan-induced rats was also proposed on regeneration of β -cells with the use of ethanolic bark extract, ranging from 250 to 500 mg/kg [9, 10]. The presence of flavonoid rhamnocitrin in *B. variegata* stem bark confirmed the anti-cataract potential of the plant. The anti-cancerous effect of *B. variegata* ethanolic stem extract was experimentally determined on Swiss Albino mice. The cell line growth of cancer against Dalton's ascetic lymphoma was inhibited by stem. The effect was also estimated by using the same extract against Ehrlich ascites carcinoma. The stem extract showed antimutagenic activity. The orally given ethanol extract successfully decreases N-nitrosodiethylamine enhanced enzymes [11].

B. variegata flowering buds contain amino acids of four types in early stages. They are glutamic acid, aspartic acid, glycine, and α -alanine. The mature stages of buds contain α -keto glutaric acid, oxaloacetic acid, and phosphoenolpyruvic acid. In folkloric medicinal system, the flowering buds of *B. variegata* were used to treat hemorrhoids, dysentery, and diarrhoea. The flowers of *B. variegata* were used to treat oedema and dysentery and as a laxative and anthelmintic agent. The floral bud and flowers were ingested frequently for mumps and leucorrhoea treatment. *B. variegata* flowering buds in Unani medicine system were used for cough treatment, ophthalmic infections, liver complications, and hematuria menorrhagia [12, 13].

The presence of *B. variegata* in Pakistani flora with traditional usage and established pharmacological activities from investigations evoked to explore the phytochemical analysis and cytotoxicity evaluation of flowering buds of *B. variegata* [14]. This investigation was planned to estimate preliminary phytochemical analysis, with purpose to determine the

secondary metabolites that may be associated with the cytotoxic activity of flowering buds of *B. variegata* [15, 16].

MATERIAL AND METHODS

Plant collection

Bauhinia variegata was procured from Faisalabad for experimental purposes. The expert botanist Prof. Dr. Qazi Najam us Saqib from Pharmacy Department, Abbottabad University, Pakistan, identified and authenticated the plant. One portion of sample was placed in a local herbarium and served as a reference. The flowering buds were used as a plant part. The plant part was shade dried at a room temperature. After adequate drying, the plant material was then chopped to a fine powder. The powdered material was weighed properly and stored in a tight polyethylene bag for further study [17, 18].

Extract preparation

The extraction flask was used for soaking 200 g of dried powder of *B. variegata* flowering buds in 1 liter of menthol-water solution (70–30% v/v). The herbal mixture was kept in the darkroom for 14 days. Daily shaking of the mixture was done twice during this period. After 14 days, filtration was carried out with Whatman filter paper No. 1. The methanolic extract and residues were separated. The residues were added in 300 ml of methanol and the same process was adopted thrice. The rotary evaporator was used for drying filtrate at 40°C. The same procedure was performed using plant material and water as a solvent to get aqueous extract respectively [19].

Chemicals and reagent solutions

Analytical grade pure chemicals and reagent solutions procured from E. Merck Germany was used in the investigation (E. Merck, Germany).

Phytochemical analysis

Qualitative phytochemical analysis of both extracts *B. variegata* flowering buds were performed by

following standard procedures for screening secondary metabolites such as saponins, flavonoids, carbohydrates, proteins, phenols, terpenoids, glycosides, alkaloids, tannins in respective plant [3].

Alkaloid test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with dilute HCl to make acidic mixture. The reaction mixture was filtered and treated with few drops of reagents one by one and observed for any change in color. Hyoscine was used as a standard. All the reagents were freshly prepared in accordance with the reference.

1. Mayer's reagent
2. Wagner's reagent
3. Dragendroff's reagent

Carbohydrates test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with different reagents to detect the presence or absence of carbohydrates. Glucose was used as a standard.

- a) Fehling's reagent
Fehling A and Fehling B reagents were mixed in equal volume and 2 ml of this mixture was added to test sample and the changes in colour was observed.
- b) Benedict's reagent
Added Benedict's reagent to the test tube containing sample and the changes in colour was observed.

Test for flavonoids

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with different reagents to detect the presence/absence of flavonoids. Tea leaves are used as a standard.

1. 4 ml of 1% KOH was added to filtrate in a test tube and the changes were observed.
2. 4 ml of 1% already prepared AlCl_3 in methanol was added to filtrate in a test tube and the changes were observed.

Phenols test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were taken in a test tube

and few drops of 5% ferric chloride solution were added. The changes were observed. Olives were used as a reference.

Saponins test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were taken in a test tube and boiled for 3 minutes. It was filtered and cooled. The reaction mixture was treated with few drops of reagents one by one to detect the absence and presence of saponins. Glycyrrhiza was used as a standard.

1. In a test tube extracts were taken and shaken for about 1 minute and then observed the appearance of froth formation.
2. In a test tube extracts were taken and lead acetate was added and then the changes were observed.

Tannins test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with different reagents for the presence/absence of tannins. Tea leaves were used as a standard.

a) Vanillin test

Crude aqueous and methanol extract of *B. variegata* flowering buds were treated with vanillin reagent and the changes for the presence/absence of tannins were observed.

b) Gelatin test

Crude aqueous and methanol extract of *B. variegata* flowering buds were taken in a test tube and treated with 10% sodium chloride solution and 1% gelatin solution and the changes were observed.

c) Ferric chloride test

Crude aqueous and methanol extract of *B. variegata* flowering buds were treated with 5% ferric chloride solution and the changes were observed.

Glycosides test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with different reagents to detect the presence or absence of glycosides. Digitalis was used as a standard.

a) Modified Brontrager's test

Both aqueous and methanol extracts were added in a test tube along with dilute sulphuric acid. This solution was allowed to boil on a water bath for few minutes and then the mixture was

filtered. The filtrate was allowed to cool and then 2–3 drops of benzene were added to the solution, vigorously shaken to mix well and allowed to stand for complete separation of two organic layers. The organic layer was collected and equal of ammonia solution was added. The changes were observed.

b) Legal's test

To the crude aqueous and methanol extract of *B. variegata* flowering buds few ml of pyridine and 3 drops of nitroprusside and one drop of 20% sodium hydroxide solution was added and observed the changes.

Proteins and amino acids test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with different reagents to detect the absence or presence of proteins and amino acids.

a) Xanthoproteic acid test

To the test tube of crude aqueous extract and methanol extract of *B. variegata* flowering buds few drops of concentrated nitric acid were added and changes were observed.

b) Ninhydrin test

0.25% v/v ninhydrin reagent was added to the crude aqueous and methanol extract of *B. variegata* flowering buds in a test tube and boiled for a few minutes and then the changes were observed.

Phytosterols test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were treated with different reagents for the detection of phytosterols. Grapes were used as a standard.

a) Salkowski's test

The crude aqueous and methanol extract of *B. variegata* flowering buds were taken in a test tube with chloroform added. The mixture was filtered and filtrate was treated with a few drops of H_2SO_4 (conc) and allowed to stand for few minutes and then the changes were observed.

b) Libermann Burchard test

The crude aqueous and methanol extracts of *B. variegata* flowering buds were taken in a test tube with chloroform added. Filtered mixture and filtrate was treated with a few drops of H_2SO_4 (conc) was allowed to stand for a few minutes and the changes were observed. Mushroom was used as a standard.

Terpenoids test

0.2 gram of crude aqueous and methanol extract of *B. variegata* flowering buds were taken in a test tube and 10 ml of methanol was added. The mixture was shaken well and filtered. Then, 2 ml of chloroform was added in to filtrate, followed by addition of 3 ml of H₂SO₄. Then the changes in color were observed.

Estimation of amino acid

Total content of amino acid was assayed by following the ninhydrin method described by Gayathri *et al.* [20]. To 0.5 ml of both extracts, 4 ml of distilled water was added then 1 ml of ninhydrin reagent was added and mixed well. After that the mixture was boiled, the reaction mixture was left in the hot water bath for 15 minutes, and the allowed to cool. Then, ethanol in 1 ml quantity was added to the reaction mixture and mixed well. The absorbance was measured at 570 nm with the a calorimeter. The 100 µg/ml of glycine was assessed in the same way. The amounts of amino acids in flowering buds were expressed as mg/g of flowering buds.

Estimation of proteins

Plant material was investigated for total protein contents following the method described by Gayathri *et al.* [20]. To 0.4 ml of both extracts, 9 ml distilled water was added in order to increase the volume and allowed to stand for some time, before the addition of 1.0 ml of Folin-Ciocalteu reagent. The resultant mixture was shaken well before incubation at a room temperature. After five minutes, 10 ml of 7% sodium carbonates solution was added to resultant mixture. The mixture was incubated again at 23°C for 90 minutes. After incubation, the calorimeter was used to measure the absorbance at 750 nm. 100 µg/ml of gallic acid as a standard was assessed in the same way. The amounts of phenols in flowering buds were expressed as milligram of GAE/g of flowering buds.

Estimation of flavonoids

Total flavonoid contents were accessed with same procedure as adopted by Gayathri *et al.* [20]. To 0.5 ml of both extracts, aluminum chloride (10%) and sodium acetate (0.1 M) were added. Distilled water in amount of 5.3 ml was added to resultant

mixture and then incubated for 30 minutes at a room temperature. Calorimeter was used to measure the absorbance at 420 nm. The 100 µg/ml of quercetin used as a standard was assessed in the same way. The amount of flavonoids in flowering buds were expressed as milligram of QE/g of flowering buds.

Cytotoxic evaluation

The Brine shrimp lethality of each extract was conducted to determine the cytotoxicity following standard protocol by Atta-ur-Rehman *et al.* [21].

Requirements

Brine shrimp's eggs (eggs of *Artemia salina*), distilled water (pH 7.4), sea salt (3.8 g/l), aluminum foil, vials, micro pipette, hatching tray and ethanol.

Sample preparation

The 20 mg of *B. variegata* flowering buds crude aqueous and methanol extracts were dissolved in 2 ml of distilled water and dimethyl sulfoxide (DMSO) which served as a stock solution. Concentrations of five diverse strengths of 500, 250, 125, 50, 25 µg/ml ppm were transported into the vials (5 vials/concentration) from the stock solution. Evaporation of vial solvents was carried out and the residue was resolubilized in 2 ml of sea water. The Pasteur pipette was used to place 10 larvae/vial. Seawater was used to makeup final volume up to 5 ml with 100 ppm, 125 ppm, 225 ppm, 500 ppm, 1000 ppm of final concentrations and incubated at 250–270°C for 24 hours under light. The negative control vials have dimethyl sulfoxide and positive control vial has reference cytotoxic drug. The magnifying glass was used to count the survivors after 24 hours.

Hatching the shrimp

The perforated partition assisted in an unequal division of the hatching tray. The sprinkling of eggs was carried out in minor quantity over the solution (3.8 g of sea salt + 1000 ml of distilled water). Aluminum foil was used to cover the solution for darkness. The tray was placed under the lamp when the eggs hatched out the larvae swam energetically and traveled to the illuminated area of the tray.

Statistical analysis

Biostat software was used to calculate LD₅₀ values by probit analysis [22].

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

Phytochemical analysis

The phytochemical analysis of both extracts of flowering buds of *B. variegata* showed the existence of a variety of significant secondary metabolites like flavonoids, proteins, amino acids, terpenoids, etc. presented in table 1. While saponins and alkaloids were absent in both extracts. The highest levels of phenolics, flavonoids, and terpenoids were observed in methanol extract with the respective standard drugs. This can be attributable to the higher solubility of these compounds in methanol than in the aqueous medium.

Table 1.

Phytochemical analysis of crude aqueous and methanol extract of flowering buds of *Bauhinia variegata*

Secondary metabolites	Extracts	
	Crude aqueous extract	Methanol extract
Alkaloids		
a) Mayer's test	--	--
b) Dragendroff's test		
Carbohydrates		
a) Fehling's test	++	++
b) Benedict's test		
Flavonoids	++	++
Phenols	++	++
Saponins	--	--
Tannins	+	+
Glycosides	+	+
Proteins		
a) Ninhydrin test	++	++
b) Xanthoproteic acid test		
Phytosterols		
a) Salkowski's test	+	+
b) Libermann Burchard test		
Triterpenoids	+	+

+ present; – absent

Quantitative analysis of phytochemicals

The quantitative analysis of both extracts of flowering buds of *B. variegata* was presented in table 2. The amino acid content was highest followed by phenol and flavonoids.

Table 2.

Quantitative analysis of phytochemicals

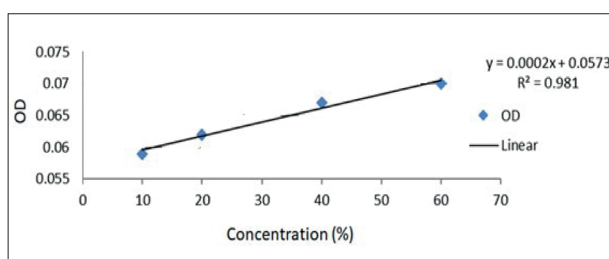
Phytochemical	BVM	BVA
Total flavonoids [mg/QE/g]	1.26±0.06	4.26±0.26
Total phenols [mg/GAE/g]	3.96±0.09	4.74±0.97
Total amino acid [mg/Gly/g]	7.71±9.8	9.47±23.74

BVM – *Bauhinia variegata* methanol extract

BVA – *Bauhinia variegata* aqueous extract

Quantitative analysis of flavonoids

The results of the quantitative analysis indicated that the content of flavonoids was higher in the aqueous extract compared with the methanol extract. The antioxidant activity and metal chelation potential of flavonoids are well recognized for herbal medicine prospective [23]. *B. variegata* is used in herbal therapy for anti-inflammatory actions and hypoglycemic effects. The probit analysis of flavonoids are presented in figure 1.



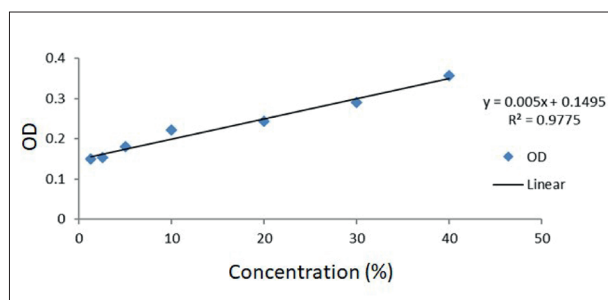
OD – optical density

Figure 1.

Total flavonoid content (mg/QE/g) of *Bauhinia variegata* flowering buds

Quantitative analysis of phenols

The results of the quantitative analysis of phenols showed that the content of phenol was higher in aqueous extract compared with methanol extract. The rich phenolic content used in food products minimizes heart disease due to antioxidant potential as well as effectiveness in chemotherapy and osteoporosis treatment [24]. The probit analysis of phenols were presented in figure 2.



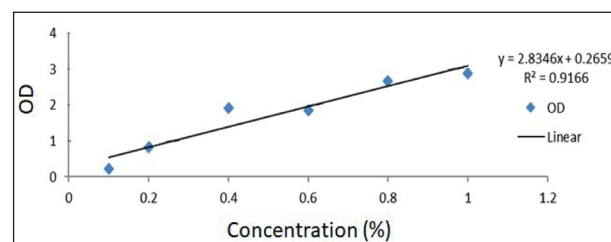
OD – optical density

Figure 2.

Total phenol content (mg/GAE/g) of *Bauhinia variegata* flowering buds

Quantitative analysis of amino acids

The results showed that aqueous extract (9.74 ± 23.74 mg/g) contains higher amount of amino acids compared to methanol extract (7.71 ± 9.8 mg/g). The human body needs protein in adequate amounts for development and as a structural constituent of body cells. Proteins act as precursors for vital molecules for lifespan. The analysis of flowering buds of *B. variegata* at early stages resulted in glutamic acid, aspartic acid, glycine, and α -alanine. The mature stages of buds contain α -ketoglutaric acid, oxaloacetic acid, and phosphoenolpyruvic acid. *B. variegata* has enriched nutrition value with amino acids and protein content and also served as a source of energy during malnourishment. The probit analysis of amino acids was presented in figure 3.



OD – optical density

Figure 3.

Total amino acid content (mg/Gly/g) of *Bauhinia variegata* flowering buds

Cytotoxic evaluation

The brine shrimp lethality assay was introduced by Michael *et al.* and further studied by Vanhaecke *et al.* and Sleet and Brendel [25-27]. The lethality of the test sample was based on the concept to kill laboratory cultured *Artemia nauplii* (brine shrimp) larvae have been used by researchers, considered as a beneficial tool for preliminary assessment of toxicity that can be backed up by more specific bioassay [28]. The probit analysis of crude aqueous extract and methanol extract are presented in figure 4.

In this study, lethality was found to be directly proportional to the concentration of extracts ranging from the lowest (100 μ g/ml) to the highest (1000 μ g/ml). This concentration-dependent increased death rate of *Artemia* showed the presence of cytotoxic active principles present in

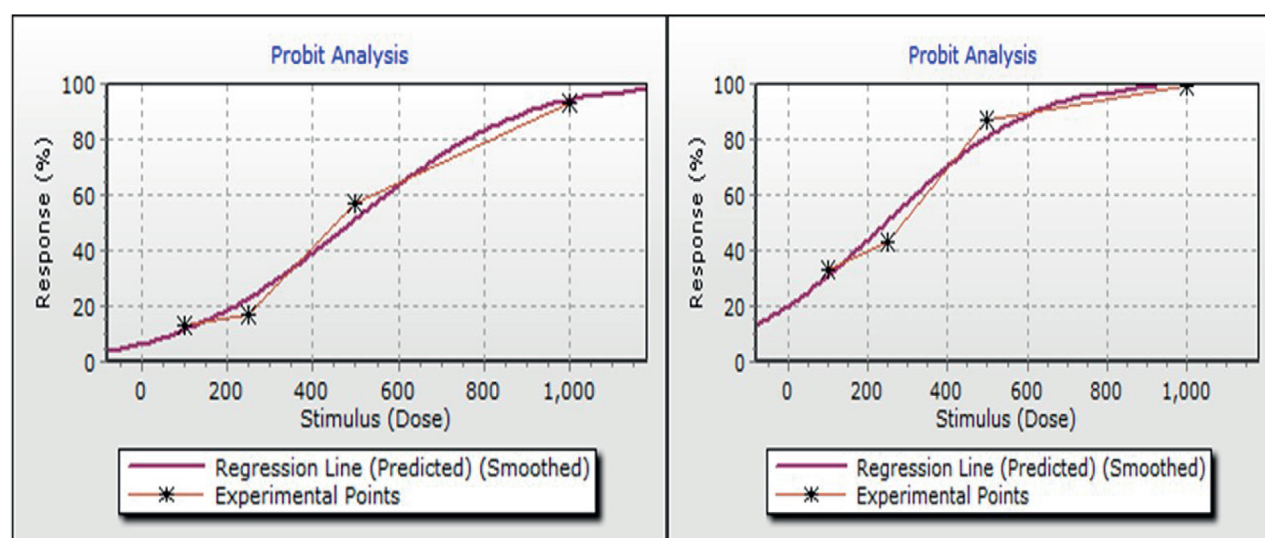


Figure 4.

Cytotoxic activity of aqueous and methanol extracts of *Bauhinia variegata* flowering buds

extracts. The results of this activity showed that methanol extract is more toxic than crude aqueous extract with LD_{50} 241.778 $\mu\text{g/ml}$ with the upper and lower confidence interval of 315.78 $\mu\text{g/ml}$, 174.98 $\mu\text{g/ml}$ and 489.7061 $\mu\text{g/ml}$ with upper and lower confidence interval of 653.5212 $\mu\text{g/ml}$ and 352.890 $\mu\text{g/ml}$, respectively. The results are presented in table 3.

Table3.

Cytotoxicity results of crude aqueous extract and methanol extract of flowering buds of *Bauhinia variegata*

Cytotoxicity results of crude aqueous extract				
Dose [$\mu\text{g/ml}$]	Log dose	Total No. of shrimps	No. of dead shrimps	Probit
1000	3.000	30	28	7.9394
500	2.699	30	17	6.1108
250	2.3979	30	5	4.8324
100	2.097	30	4	4.5697
Cytotoxicity results of methanol extract				
1000	3.000	30	30	7.9394
500	2.699	30	26	6.1108
250	2.3979	30	13	4.8324
100	2.097	30	10	4.5697

DISCUSSION

B. variegata flowering buds are used as a vegetable in most of areas of Indo-Pak but it is unfortunate that after boiling with water, water is thrown away which causes loss of some essential macro- and microelements. This genus attained great pharmaceutical and industrial interest because of the presence of polyphenols and flavonoids i.e. kaempferol and ombuin. The isolated compounds from this genus possess anti-inflammatory, anti-oxidant, anti-diabetic, immunomodulatory activity etc. Others are anthraquinones, according to the Unani system of medicine. The flowering buds of *B. variegata* enriched with anthraquinones are used in the treatment of cough, eye disease, liver complaints and as styptic in haematuria menorrhagia [16]. The brine shrimp lethality assay represents fast, cheap and simple bioassay for testing plant extracts bioactivity which in most cases correlates with the presence of flavonoids in this plant. This is an indicative of presence of potent cytotoxic compounds which warrants further studies.

CONCLUSION

From the above studies, it can be concluded that the plant *B. variegata* flowering buds possess many secondary metabolites proteins, flavonoids, terpenes, anthraquinones, terpenoids and can be incorporated for treatment of various ailments as folklore medicine such as dried buds are employed in the treatment of dysentery, diarrhea, infestation, worms, tumors, and piles. However, it provides a validation of the cytotoxic effect of the plant extracts which might indicate baseline information in understanding the possible medicinal value, and further clinical studies are needed.

Abbreviations

LD_{50} – median lethal dose
FDA – Food and Drug Administration

Conflict of interest: Authors declare no conflict of interest.

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