

Study of Phytochemical, Anti-Microbial, Anti-Oxidant, Phytotoxic, and Immunomodulatory Activity Properties of *Bauhinia variegata*

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ABSTRACT

In this paper, we investigated the anti-microbial, anti-oxidant, phytotoxic and immunomodulatory activity of plant *Bauhinia variegata* and also established the presence of important phytoconstituents which might signify its anti-cancer property. Phytochemical screening, anti-microbial assay, phytotoxicity assay, anti-oxidant assay and immunomodulatory analysis was done according to the protocol established at Department of Biotechnology, Kathmandu University and Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Pakistan. In the phytochemical screening, we observed the presence of different phytochemicals like steroids, terpenoids, flavonoids, reducing sugars and glycosides. In case of antimicrobial assay, the plant was found to be most effective against *Bacillus cereus* with the highest zone of inhibition (ZOI) of 12 mm and against *Rhizopus* with ZOI of 11 mm. During the antioxidant assay in comparison to ascorbic acid; at highest concentration, the scavenging activity shown by the plant *B. variegata* are 43.38% as compared to ascorbic acid 89.25%. In the immunomodulatory assay at the highest concentration i.e. 250 µg/mL, the plant showed 75.1% effect, which showed that the plant has potential anti-inflammatory potential. In phytotoxicity assay, *B. variegata* showed 20% phytotoxicity. *B. variegata* has thus been proven to be an important candidate to be used as an antimicrobial, antioxidant, anti-inflammatory, phytotoxic and anticancer agent. Separation of pure compounds with bioassay guided extraction, spectrometric analysis and subsequent cytotoxicity assay of the pure bioactive compounds from *B. variegata* is highly recommended as crude extract itself showed promising phytotoxicity and other pharmaceutical potential.

Keywords: Medicinal plants, phytotoxicity, zone of inhibition, phytochemical screening

INTRODUCTION

Plants have been the single most productive source of leads for the development of drugs, and over a hundred new products are already in clinical development. Indeed, several scientific studies have described the use of various medicinal plants and their constituents for treatment of diseases. Plants have shown the presence of many valuable compounds, such as lignans, flavonoids, tannins, polyphenols, triterpenes, sterols, and alkaloids, that show a wide spectrum of pharmacological activities, including anti-inflammatory, anti-amyloidogenic, anti-cholinesterase, hypolipidemic, and antioxidant effects [1]. Considerable research on pharmacognosy, phytochemistry, pharmacology and clinical

therapeutics has been carried out on ayurvedic medicinal plants. Natural products, including plants, animals and minerals have been the basis of treatment of human diseases [2]. Many complementary and alternatives medicines are offered to patients with cancer. Among them, herbal medicines have a substantial place. These plants are mainly used to reduce adverse effects of anticancer treatments and for specific anticancer properties [3]. Plant extracts are kinds of important anticancer drugs. Anticancer drugs research has focused on several important plant extracts such as resveratrol, ricin, epigallocatechin-3-gallate, oridonin, juglone, quercetin, matrine, nordihydroguaiaretic acid.

In vitro and *in vivo* studies have shown that the an-

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titumor mechanism of plant extract includes induction of apoptosis, cytotoxicity, redifferentiation, anti-angiogenesis, anti-invasion and anti-metastasis. Plant extracts have characteristics of multi-link and multi-target. Part of plant extracts have been used in preclinical studies. In-depth study of the anti-tumor mechanisms of plant extracts on cancer treatment is important, and the full mechanism needs further study [4]. A huge reservoir of bioactive compounds exists in many species of plants of Earth, only a small percentage of which have been examined and continued to be an important source of anticancer agents. Worldwide efforts are ongoing to identify new anticancer compounds from plants. With the current decline in the number of new molecular entities from the pharmaceutical industry, novel anticancer agents are being sought from traditional medicines [5]. A number of undesired side effects sometimes occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. Currently, few plant products are being used to treat cancer. However, a myriad of many plant products exist that have shown very promising anti-cancer properties *in vitro*, but have yet to be evaluated in humans [6]. For over 40 years, natural products have served us well in combating cancer. The main sources of these successful compounds are plants from the terrestrial and marine environments. Another major contribution comes from plant alkaloids. A vast array of metabolites can be obtained from the marine world, which can be used for effective cancer treatment. The search for novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to chemotherapeutic drugs. In addition, the high toxicity usually associated with some cancer chemotherapy drugs and their undesirable side-effects increase the demand for novel anti-tumour drugs active against untreatable tumours, with fewer side-effects and/or with greater therapeutic efficiency [7]. Studies on a wide spectrum of plant secondary metabolites extractable as natural products from fruits, vegetables, teas, spices, and traditional medicinal herbs, show that these plant natural products can act as potent anti-inflammatory, antioxidant or anticancer agents. The recent advances in genomics, proteomics and metabolomics have enabled biologists to better investigate the potential use of immunomodulatory natural products for treatment or control of various cancerous diseases. The cancer preventive or protective activities of the various immunomodulatory natural products lie in their effects on cellular levels in-

cluding detoxifying and antioxidant enzyme systems, and the induction of anti-inflammatory and antitumor or anti-metastasis responses, often by targeting specific key transcription factors like nuclear factor kappa B (NF- κ B), activator protein (AP-1), signal transducers and activators of transcription (STAT) and other proteins [8]. Natural products have been the biggest single source of anticancer drugs and there are continued efforts to explore the bioactive chemicals. Diversity provided by nature in order to find new lead compounds, bioassay test methods have developed into high throughput screening assays using both cell-based and molecular approaches [9].

This few inventions related to a novel anticancer poly-herbal composition comprises of stem bark of *Bauhinia variegata*, *Oroxylum indicum*, *Tecomella undulata* and leaves of *Indigofera tinctoria*. Few novel composition found in plants are useful in the condition of cancer, immunosuppression, uncontrolled proliferation and angiogenesis [10, 11, 12]. Highest antioxidant potential was found in stem bark extracts of *B. variegata* [13, 14, 15]. Studies showed significant antioxidant activities of these *B. variegata* and suggested that it showed pronounced cytotoxic effect against DU-145, HOP-62, IGR-OV-1, MCF-7, and THP-1 human cancer cell lines with 90-99% cell growth inhibitory activity. Ethyl-acetate fraction of the plant also produced considerable cytotoxicity against MCF-7 and THP-1 cell lines [16, 17, 18, 19, 20, 21, 22]. Studies of ethanolic extracts of plant material showed the presence of carbohydrate, protein, glycosides, triterpenoids, saponins and steroids [23, 24]. Extracts of *B. variegata* have anti-tumor, antimicrobial, antitumorogenic, hepatoprotective and hemagglutination properties [25]. Few studies have revealed the chemopreventive role of *variegata* flower extracts against 7, 12-Dimethylbenz[a]anthracene (DMBA) induced skin carcinogenesis in mice [26]. In a search for novel anticancer compounds from medicinal plants, the isolated flavanone from *Bauhinia* was tested for cytotoxic activity against 57 human tumor lines, representing leukemia, non-small cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate and breast cancers and were found effective against most of them [27]. The inhibitory effect of bauhinione on K562 cells was correlated with its inhibition and apoptosis induction in G2-M phase [28]. A novel flavonolglycoside 5,7,3',4'-tetrahydroxy-3-methoxy-7-O- α -L-rhamnopyranosyl(1 \rightarrow 3)-O- β -D-galactopyranoside from the roots of *B. variegata* showed anti-inflammatory activity [29]. Similar research has been reported for

Withania somnifera [30, 31].

MATERIALS AND METHODS

Collection of plant materials

B. variegata whole plants were collected from Pannauti area of Kavre district, Nepal. The whole plant were then dried in shade, left over for 15 days and macerated and powdered with the help of kitchen grinder.

Preparation of plant extracts

The dry powder of *B. variegata* (100 g) was dipped in 80% aq. ethanol for three days stirring twice a day [32]. The solution at the end of third day was filtered and then concentrated in high vacuum-rotator evaporator and finally all the solvent was evaporated using freeze dryer to get the semi-solid plant extract. The same procedure was repeated three times and the freeze-dried extract was collected together. The yield of the plant powder *B. variegata* in aq. ethanol weighed after the freeze drying was 12.07 g. The extract was then kept in glass vial with airtight caps and stored at 4°C.

Phytochemical screening

Phytochemical screening was done using the methods as described [32].

1. Test for Alkaloids: 5 mL of extract was concentrated to yield a residue. Residue was dissolved in 3mL of 2% (v/v) HCl, few drops of Mayer's reagent was added. Appearance of the dull white precipitate indicated the presence of basic alkaloids.
2. Test for Coumarin: 4 mL extract solution was taken; 1-2 drops of water (hot) was added. Volume was made half (UV fluorescence). 10% NH₄OH was added to another half volume (strong fluorescence). Presence of green fluorescence indicated the presence of Coumarin.
3. Test for Saponins: 2 mL extract was shaken vigorously for 30 seconds in a test tube. Persistence of thick froth even after 30 minutes indicated the presence of saponins.
4. Test for Glycosides: 2 mL of extract was dried till 1 mL. As much as 1-2mL NH₄OH was added and shaken. Appearance of cherish red colour indicated the presence of glycosides.
5. Test for Reducing Sugars: 0.5 mL of extract was taken and 1ml distilled water was added. About 5-8 drops of Fehling's solution (hot) was added. Presence of brick red precipitation indicated the presence of reducing sugar.
6. Test for steroids: 1 mL extract was dissolved in 10 mL chloroform. Equal volume of conc. H₂SO₄ was added by the side of test tube. Upper layer turned red and sulphuric acid layer turned yellow with green fluorescence. This indicated the presence of steroids.
7. Test for Quinone: 1 mL of extract was taken. As much as 1 mL of conc. H₂SO₄ was added. Formation of red colour indicated the presence of quinone.
8. Test for Terpenoids: 5 mL of extract was taken and mixed with 2 mL of chloroform. As much as 3 mL of conc. H₂SO₄ was added to form a layer. Reddish brown precipitate formation at the interface formed indicated the presence of terpenoids.

2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) free radical scavenging assay

2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) is a dark-coloured crystalline powder composed of stable free-radical molecules. DPPH has major application in laboratory research most notably in antioxidant assays. DPPH solution (95 µL, 300 µM) in Ethanol was mixed with test solution (5 µL, 500 µM). The reaction was allowed to progress for 30 min at 37°C and absorbance was monitored by microplate reader, SpectraMax340 at 517 nm. Upon reduction, the colour of the solution fades (violet to pale yellow). Percent Radical Scavenging Activity (% RSA) was determined by comparison with a Dimethyl sulfoxide (DMSO) containing control. The concentration that causes a decrease in the initial DPPH concentration by 50% is defined as IC-50 value. The IC-50 values of compounds were calculated by using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc. Amherst, MA, USA). N-acetylcysteine and ascorbic acid are used as the reference compounds [32].

Agar plate diffusion method for antimicrobial activity

Medium was dissolved and autoclaved at 121°C for 15 minutes, cooled up to 45°C and then 40-50 mL media was poured in sterile 14 cm diameter petri plate, and then allowed to solidify and kept at room temperature. Preparation of plant extract: Stock solution of 400mg/mL was prepared by weighing 200mg of plant extract in 1.5 mL eppendorf tube and the final volume of 0.5ml of DMSO was added by micropipette. Extract was completely dissolved by vortexing for 5-10 minutes. Test solution of 200 mg/mL, 100 mg/mL, 50 mg/mL, and 25 mg/mL concentrations was prepared.

Preparation of inoculums: Each culture to be tested

was streaked onto nutrient agar to obtain isolated colonies. Overnight incubation was done at 37°C. Then isolated colonies were transferred by the help of sterile loop onto Muller Hinton Broth. Overnight incubation was done at rotary shaker at 37°C.

Inoculation: For inoculation, swabbing was done with the help of cotton. Sterilized filter paper discs were dipped into the desirable concentration of the plant extracts then applied to the plates; incubated at 37°C for 24 hours. After incubation, the diameter of the Zone of inhibition was measured.

Phytotoxicity or Lemna bioassay for inhibitors and promoters of plant growth

The members of Lemnaceae family are suitable organisms to investigate physiological processes. It is a quick measure of phytotoxicity of the materials. The Lemna minor phytotoxicity assay is a useful primary screen for weedicides search. It is also observed that natural antitumor compounds can inhibit the lemna growth.

E-Medium is prepared by mixing its constituents in 1000 mL distilled water and pH is adjusted between 6.0 and 7.0 by adding KOH pellets (Stock solution). Working E-medium is prepared by mixing 100 ml of stock solution and 900 mL of distilled water. 30 mg for crude extract/ compound is dissolved in 1.5 mL of solvent (Methanol/ Ethanol etc.) serving as stock solution. Three flasks are inoculated with 10, 100 and 1000 µL of solution pipette from the stock solution for 10, 100 and 1000 µg/mL. The solvent was allowed to evaporate overnight. Add 20 mL of working E. medium and then plant of Lemna minor, each containing a rosette of two to three fronds, to each flask (total 20 fronds). Other flasks supplemented with E-medium and reference (standard drug) plant growth inhibitors and promoters serve as negative and positive controls, respectively. Place the flasks in growth cabinet for seven days. Plants should be examined daily during incubation. Count and record number of fronds per flasks on day 7. Results are analyzed as growth regulation in % age, calculated with reference to the negative control by this formula.

$$\% \text{ regulation} = \left(100 - \left(\frac{\text{Number of fronds in test}}{\text{Number of fronds in negative control}} \right) \right) \times 100$$

Immunomodulatory assay (for anti-inflammatory potential)

Immunomodulation is the regulation and modulation of immunity either by enhancing or by reducing the immune response whereas an immunomodulator

Table 1. Results of phytochemical screening of *B. variegata*

<i>Phytochemicals</i>	<i>B. variegata</i>
Alkaloids	Available
Tannins	Available
Reducing sugar	Available
Coumarin	Available
Glycosides	Available
Quinone	Not-available
Steroids	Not-available
Terpenoids	Available
Saponin	Available
Flavonoids	Available

may be defined as a biological or synthetic substance, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune responses.

Medicinal plants are a rich source of immunomodulators. Since the drugs available in the market have adverse side effects, the use of natural products as an alternative method for treatment has been on the rise in the last few decades. A large number of plants like Actinidia, Aesculus, Allium, Aloe vera, Asparagus, Centella, Emblica, Magnifera, Withania, Piper and Tinospora etc possess potent immunomodulatory activity.

Luminol-enhanced chemiluminescence assay was performed, as described by Helfand [33]. Briefly 25 µl of diluted whole blood HBSS++ (Hanks Balanced Salt Solution, containing Calcium Chloride and Magnesium Chloride) [Sigma, St. Louis, USA] was incubated with 25 µL of three different concentrations of compounds (1, 10 and 100 µg/ml), each in triplicate. Control wells received HBSS++ and cells, but no compounds. Test was performed in white half area 96 well plates [Costar, NY, USA], which was incubated at 37°C for 15 minutes in the thermostat chamber of luminometer [Labsystems, Helsinki, Finland]. After incubation, 25 µl of serum opsonized zymosan (SOZ) [Fluka, Buchs, Switzerland] and 25 µL of intracellular reactive oxygen species (ROS) detecting probe, luminol [Research Organics, Cleveland, OH, USA] were added into each well, except blank wells (containing only HBSS++). The level of the ROS was recorded in luminometer in term of relative light units (RLU). Standard used for the assay is Ibuprofen (IC-50 = 11.2 ± 1.9).

RESULTS AND DISCUSSION

In the phytochemical screening, as shown in Table 1, we observed the presence of different phytochemical

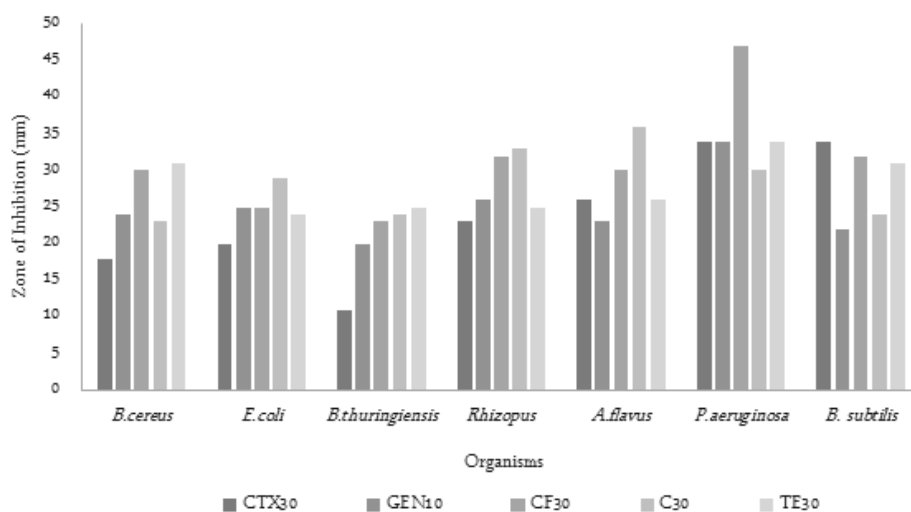
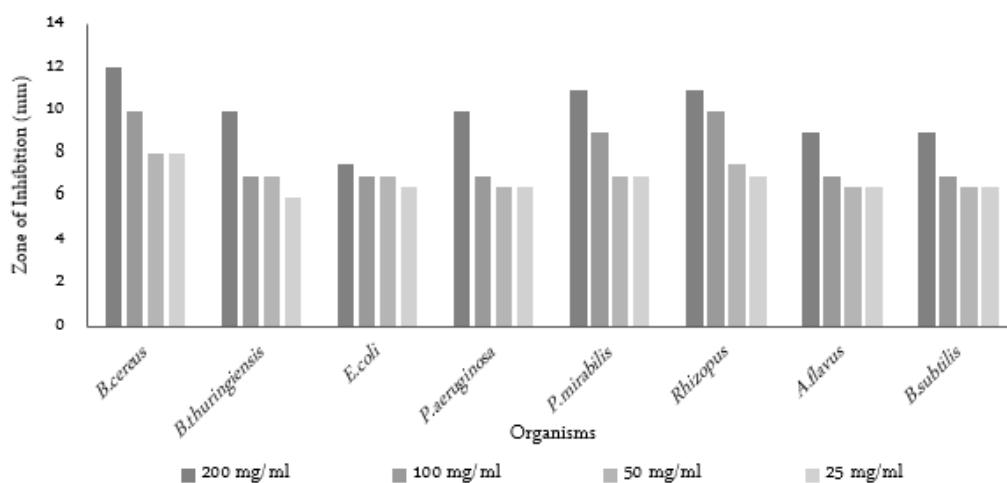


Figure 1. Zone of inhibition of standard antibiotics

Figure 2. Antimicrobial assay of *B. variegata*

als like alkaloids, tannins, reducing sugar, coumarin, glycosides, terpenoids, saponins and flavonoids.

Antimicrobial assay was performed with the plant extract of *B. variegata* by agar plate diffusion method. Five different antibiotics were used as standard drugs (Figure 1).

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Antimicrobial assay was performed with the plant extract of *B. variegata* by agar plate diffusion method. Five different antibiotics were used as standard drugs.

Results of antimicrobial assay

In case of antimicrobial assay Figure 2, *B. variegata* showed ZOI, of 12 mm, 10 mm, 8 mm and 8 mm against *Bacillus cereus* at the concentration gradient of 200 µg/mL, 100 µg/mL, 50 µg/mL and 25 µg/mL re-

spectively. Against *Bacillus thuringiensis* it was 10 mm, 7 mm, 7 mm and 6 mm; against *Escherichia coli* it was 7.5 mm, 7 mm, 7 mm and 6.5 mm; against *Pseudomonas aeruginosa* it was 10 mm, 7 mm, 6.5 mm and 6.5 mm; against *Pseudomonas mirabilis* it was 11 mm, 9 mm, 7 mm and 7 mm; against *Rhizopus* it was 11 mm, 10 mm, 7.5 mm and 7 mm; against *Aspergillus flavus* it was 9 mm, 7 mm, 6.5 mm and 6.5 mm and against *B. subtilis* it was 9 mm, 7 mm, 6.5 mm and 6.5 mm; respectively at the above mentioned concentration gradient.

Results of antioxidant assay

During the antioxidant assay Figure 3, in comparison to Ascorbic acid at 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL and 10 µg/mL, the scavenging activity shown by the plant *B. variegata* was 11.16%, 16.3%, 19.6%, 21.9%, 34.12% and 43.38% respectively and by

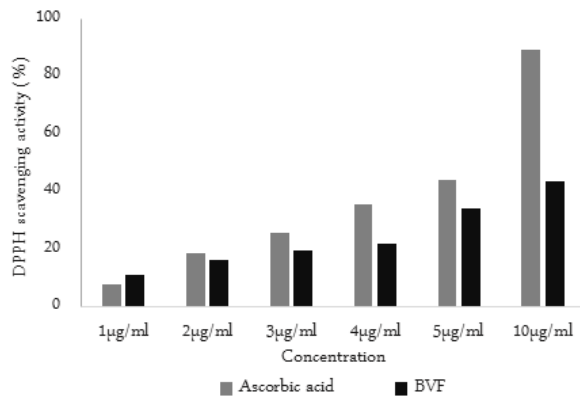


Figure 3. Results of antioxidant assay of *B. variegata*

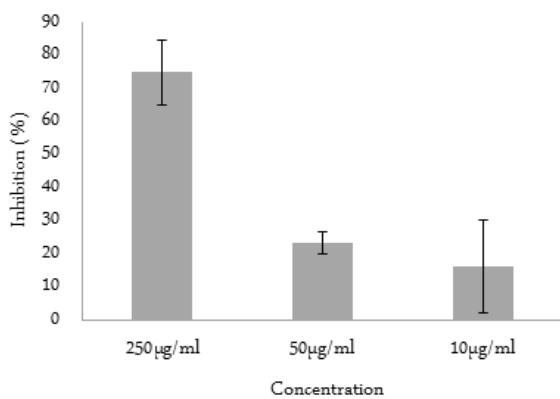


Figure 4. Immunomodulatory assay of *B. variegata*

ascorbic acid it was 8.07%, 18.45%, 25.81%, 35.46%, 44.25% and 89.25% respectively, in the same concentration gradient.

Results of immunomodulatory assay

In the immunomodulatory assay Figure 4, anti-inflammatory assay, at 250 µg/mL, 50 µg/mL and 10 µg/mL the average immunomodulatory effect are 75.1%, 23.6% and 16.4%.

Result of phytotoxicity assay

In phytotoxicity assay, at 1000 µg/mL, 100 µg/mL and 10 µg/mL the plant *B. variegata* showed 20%, 20% and 5% phytotoxicity.

Some of the reported cytotoxicity of different plant sample can be attributed to the synergic effect of all secondary metabolites that could have been present in the plants. The phytoconstituents like alkaloids, terpenoids and phenols can be attributed for the marked cytotoxicity.

The prevalence of differential anti-oxidant potential may be ascribed to their different phenolic content group and flavonoids compounds. The extract with

raised phenolic content may have contributed to the higher anti-oxidant activity. Based on the results obtained, it can be concluded that the plant contains essential phyto-constituents and possess potent anti-bacterial, anti-oxidant, phytotoxic, immunomodulatory and anti-cancer features, as has been reported earlier for *Berberis aristata* [34]. Further investigations on the isolation of the active component of the extract will throw more information on the mechanism of action.

Phenolics and flavonoids from the plants are found to possess potent antioxidants and homeostatic balance between anti-oxidant and pro-oxidant which are reported to be significant for physiological functioning as well as precluding different degenerative debilities. Conclusively, results of our study embodies the fact that the extracts are an eminent source of natural antioxidants with consequential potential in reducing the oxidative stress and preventing degenerative ailments and also cancer. Bioactivity guided purification and extraction of the pure compounds may lead to increased activity of the compounds [34].

The degree of variability in antimicrobial potential could be linked to the varying presence of different phytochemicals like flavonoids and phenols having antioxidant potential and play an important role in protection against oxidative stress. These phytochemicals exhibit restriction of generation of free radicals which damages target biomolecules. The variability in different phytochemicals contents across populations may be attributed to morphological as well as biochemical characters of the plants. This would, however, suggest source specific variation of antioxidant efficacies. The phytoconstituents like alkaloids, terpenoids and phenols can be attributed for cytotoxicity. The presence of flavonoids, and saponins in isolation or in synergy in these crude extracts used in the study might also be responsible for exhibiting anticancer efficacies. There are testaments signifying biological interactions of flavonoids, polyphenols, or phenolic compounds [35]. Figure 1, 2, 3, and 4.

CONCLUSION

B. variegata has thus been proven to be an important candidate to be used as an anti-microbial, anti-oxidant, anti-inflammatory, phytotoxic and anti-cancer agent. Separation of pure compounds with bioassay guided extraction, spectrometric analysis and subsequent cytotoxicity assay of the pure bioactive compounds from *B. variegata* is highly recommended as crude extract itself showed promising cytotoxicity.

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