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In vivo antitumor potential of extracts from different parts of *Bauhinia variegata* linn. Against b16f10 melanoma tumour model in c57bl/6 mice

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Abstract

Background: Melanoma is a metastatic type of skin cancer that is difficult to treat and the majority of efforts are directed to the design of new drugs. Medicinal Plants have been the primary source of medicines since life on earth; more than 50% of existing cancer treatments is derived from plants. *Bauhinia variegata* is well-known medicinal plant used from the ancient era to till date for their medicinal values. Scientific literatures have not documented any evidence of the antitumour potential of *Bauhinia variegata* against B16F10 melanoma tumor model in C57BL mice. The present investigation was undertaken to explore the antitumour activity of Leaf, stem bark and flower extract of *Bauhinia variegata* against B16F10 melanoma tumour model in C57BL mice.

Methods: Hydro-methanolic extract prepared from the leaf, stem bark and flower of *Bauhinia variegata* were assessed for their antitumor activity. The extracts at doses of 500 and 750 mg/kg b.wt. were given orally along with cyclophosphamide (chemotherapeutic drug) for 40 days for exploring antitumor activity against melanoma tumor (B16F10) in C57BL mice. Inhibition of tumor growth, increase in survival time of animal with treatment, histopathological studies and antioxidant parameter were determined.

Results: The Present investigation showed significant effect of the *B. variegata* L. in preventing melanoma tumor by B16F10 cell line in C57BL/6 mice. As compared with the tumour control group, the remarkable results especially in the group which received *B. variegata* extract and cyclophosphamide together were obtained for all of the measured parameters. Dose dependent response was observed in tumor volume, inhibition rate, life span time and antioxidant parameter of extracts. Combination treatment of cyclophosphamide and *B. variegata* extracts showed more pronounced effect.

Conclusions: These findings suggest that *B. variegata* hydromethanolic extract may contain bioactive compounds of potential therapeutic significance which are relatively safe from toxic effects, and can compromise the medicinal use of this plant in folk medicine.

Keywords: Chemoprevention, *Bauhinia variegata*, Melanoma, B16F10 cell line, GSH

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Background

Cancer is a growing health problem in both developing and developed countries. Melanoma is the most aggressive and deadly form of skin cancer. Patients with distant metastases have a five-year survival rate of 16% [1] and a median survival of four to six months [2]. Until very recently, melanoma has been branded by the failure of chemotherapy and other therapeutic attempts. Currently, the main treatments for cancer are chemotherapy, radiotherapy and surgery. Chemotherapy is routinely used for cancer treatment. Since cancer cells lose many of the regulatory functions present in normal cells, they continue to divide when normal cells do not. This feature makes cancer cells susceptible to chemotherapeutic drugs. Approximately five decades of systemic drug discovery and development have resulted in the establishment of a large collection of useful chemotherapeutic agents. Currently, some of these plant-derived compounds are widely used for chemotherapy of cancerous patients. However, chemotherapeutic treatments are not devoid of their own intrinsic problems. Various kinds of toxicities may occur as a result of chemotherapeutic treatments. For example, 5-fluorouracil, a common chemotherapeutic agent, is known to cause myelotoxicity [3], cardiotoxicity [4] and has even been shown to act as a vasospastic agent in rare but documented cases [5]. Another widely used chemodrug, doxorubicin causes cardiac toxicity [6–8], renal toxicity [9], and myelotoxicity. [10] Similarly, bleomycin a well-known chemotherapeutic agent, is known for its pulmonary toxicity [11–13]. In addition, bleomycin shows cutaneous toxicity [14]. Cyclophosphamide, a drug to treat many malignant conditions, has been shown to have bladder toxicity in the form of hemorrhagic cystitis, immunosuppression, alopecia and at high doses cardiotoxicity [15].

A major problem associated with cancer chemotherapy is the severe side effects resulting from normal tissue damage. Consequently, agents which protect normal tissues against chemotherapy can increase the patient's tolerance to chemotherapy. Several chemicals have been found to provide notable protection in experimental animals, but their clinical utility is limited by the drug toxicity on repeated administration [16]. Therefore, there is a need to find nontoxic and inexpensive drug/(s) for clinical chemo protection. Recent studies have indicated that some of the commonly used medicinal plants may be good source of potent but nontoxic chemoprotectors [17–20].

Natural products discovered from medicinal plants have played an important role in treatment of cancer, which is projected to become the major cause of death worldwide. A wide number of plant extracts are used against diseases in various systems of medicine such as Ayurveda, Unani and Siddha. Only a few of them have been scientifically explored. Plant derived natural products such as flavonoids, terpenes and alkaloids [21–23] and soon has received considerable attention in recent years, due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects [24]. Plant based systems continue to play an essential role in healthcare and it has been estimated by WHO that approximately 80% of the world's inhabitants rely mainly on traditional medicine for their primary health care [25].

The use of natural products in cancer therapy showed that plants are a most important source of antitumor compounds, with new structures and mechanisms of action being discovered [26]. Several plant-derived products induce apoptosis in neoplastic cells but not in normal cells [27–29].

Bauhinia variegata (Family *fabaceae*, Genus *Bauhinia*) is an herbaceous plant, found throughout India. The plant is known as Kachnara in Sanskrit and Hindi. All parts of the plant (leaves, flower buds, flower, stem, stem bark, seeds and roots) were used in traditional medicine. For using various ailments like bronchitis, leprosy, and tumors. The stem bark was used as astringent, tonic, anthelmintic and antidiabetic [30]. Infusion of the leaves was used as a laxative and for piles. Dried buds were used in the treatment of worm infestations, tumors, diarrhea, and piles. It is helpful in managing skin discoloration [31–33].

The phytochemical screening revealed that *Bauhinia variegata* contained terpenoids, flavonoids, tannins, saponins, reducing sugars, steroids and cardiac glycosides. Pharmacological studies showed that *Bauhinia variegata* exerted anticancer, antioxidant, hypolipidemic, antimicrobial, anti-inflammatory, nephroprotective, hepatoprotective, antiulcer, immunomodulating, molluscicidal and wound healing effects [34, 35]. Previous phytochemical studies on the stems [36–38], flowers [34, 39], leave and seeds [40, 41] of this species have led to the isolation of several flavonoids.

There are also a few reports of antitumor activity of *B. variegata* ethanolic extract against Dalton's ascetic lymphoma (DAL) in Swiss albino mice [42] and N-nitrosodiethylamine induced experimental liver tumors in rats and human cancer cell lines [43]. The present study evaluates the chemopreventive effect of the different parts and different doses of *B. variegata* on B16F10 melanoma tumor on C57BL mice.

Methods

Animals

The study was conducted on random bred, 6–7 weeks old and 24–28 g b.wt. bearing, male C57BL/6 mice. Animals were maintained under controlled conditions of temperature and light (Light: dark, 10 h: 14 h.). They were provided standard mice feed (procured from Hindustan Levers Ltd.) and water *ad libitum*.

Chemicals

Reduced glutathione, Cyclophosphamide, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), Sodium citrate and other chemicals were procured from Sigma Chemical Co (St Louis, MO).

Identification and collection of plant material

Aerial parts of *B. variegata* (Kachnar) like leaves, stem bark and floral bud were collected in the early stages of vegetation from the Bhopal, and Tah-Niwas, District Mandla (M.P.), India, during the month of October, 2007. The identification of the plant *B. variegata L.* (Kachnar) (family*: Leguminose*) was done by botanist Dr. S.S. Khan (Voucher Specimen No: SP/101/LGOB/2007), Department of Botany, Safia Science College, Bhopal, Madhya Pradesh (India).

Preparation of *B. variegata* extract

Shade dried powdered plant material such as leaves, stem bark and floral buds (50 g) were extracted by continuous mixing in 100 ml 50% methanol, and stem bark in 95% methanol, 24 h at room temperature. After filtration, methanol and water was evaporated at 60-70 °C temperature. The percentage yield of the crude extract was determined for each parts of *B. variegata* and was for leaf 12%, stem work 20% and floral buds 10%. The percentage extract yield was estimated as dry weight/dry material weight × 100 [44]. The dried powder was kept in air tight box.

Acute oral toxicity test (LD50)

Experimental animals

Acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 423 [45]. Experiments were performed using 54 male C57BL/6 mice were obtained from the Animal House of the Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal. The animals were randomly divided into nine groups of 6 animals per group.

Administration dose

Following the period of fasting, animals were weighed and extract was administered orally at a dose of 100, 200, 400, 800, 1600, 3200, 6400 and 12,800 mg/kg. After the administration of test substance, food for the mice was withheld for 2 h. Group I was given distilled water (10 ml/kg) as control [46].

Observation period

Animals were observed individually after atleast once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. All the mice were observed at least twice daily with the purpose of recording any symptoms of toxicity, survival or behavioural changes [46].

Antitumor activity of *B. variegata* in subcutaneous melanoma B16F10-bearing mice

Cell culture

B16F10 melanoma tumor cell line was purchased from the National Centre for Cell Science (NCCS, Pune, India). The cells were maintained in RPMI 1640 medium buffered with 2 g/l of HEPES and sodium bicarbonate, and supplemented with dextrose, penicillin, streptomycin and 10% of fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. When needed for experiments the cells were harvested with trypsin: EDTA (0.05:0.03 [*w*/*v*]) solution, and then washed in phosphate-buffered saline (PBS, pH 7.4). For the animal experiments, the recovered cells were adjusted to 5×10^5 cells/ml in PBS and then 200 μl of the suspension was injected subcutaneously (S.C.) into dorsal side of C57BL/6 mice. After 8–10 days of injection, the tumor was found to develop into a budding state. When the tumor was developed to a palpable level, two doses of the plant extracts (leaf, stem bark and floral bud) at 500 and 750 mg/kg b. wt. of mice were given orally and cyclophosphamide at 170 mg/kg was injected intraperitoneally (i.p.) every alternate day up to 40 days. During the treatment, the size of implanted tumor [Fig. 4a] was regularly measured at given time interval, with a digital caliper and tumor volume was calculated [47].

Experimental design

A total of 72 adult male C57BL mice aged 6–8 weeks were divided into 12 groups thus each group containing 6 animals (Fig. 1).

Group NC: receiving normal saline (10 mL/kg b. wt.) treated as Normal Control.

Group TC: (tumor-bearing mice) receiving normal saline (10 mL/kg) treated as Tumor Control.

Group CP: served as standard, which received cyclophosphamide (CP) 170 mg/kg b.wt.(tumor-bearing mice).

Group L_1 and L_2 (tumor bearing) Animals were received 500 and 750 mg/kg b.wt. *B. variegata* leaf extracts, respectively and after 30 min of *B. variegata* treatment, animals were treated with CP (170 mg/kg b.wt)

Group L_3: Animals were received orally with 500 mg/kg body weight of *B. variegata* leaf extract (tumor-bearing mice).

GroupB_1 and B_2 (tumor-bearing mice)**:** Animals were received 500 and 750 mg/kg b.wt. *B. variegata* stem bark extracts, respectively and after 30 min of *B. variegata* treatment, animals were treated with CP (170 mg/kg b.wt)

Group B_3 (tumor-bearing mice)**:** Animals were received orally with 500 mg/kg body weight of *B. variegata* stem bark extract.

Group F_1 and F_2 (tumor-bearing mice)**:** Animals were received 500 and 750 mg/kg b.wt. *B. variegata* floral bud extracts, respectively and after 30 min of *B. variegata* treatment, animals were treated with CP (170 mg/kg b.wt)

Group F_3 (tumor-bearing mice)**:** Animals were received orally with 500 mg/kg body weight of *B. variegata* floral bud extract alone.

All treatment of *B.variegata* extract (leaf, stem bark, floral bud) was administered orally through a metal oropharyngeal cannula and Cyclophosphamide and normal saline were given intraperitoneally (i.p.) by 1 ml syringe.

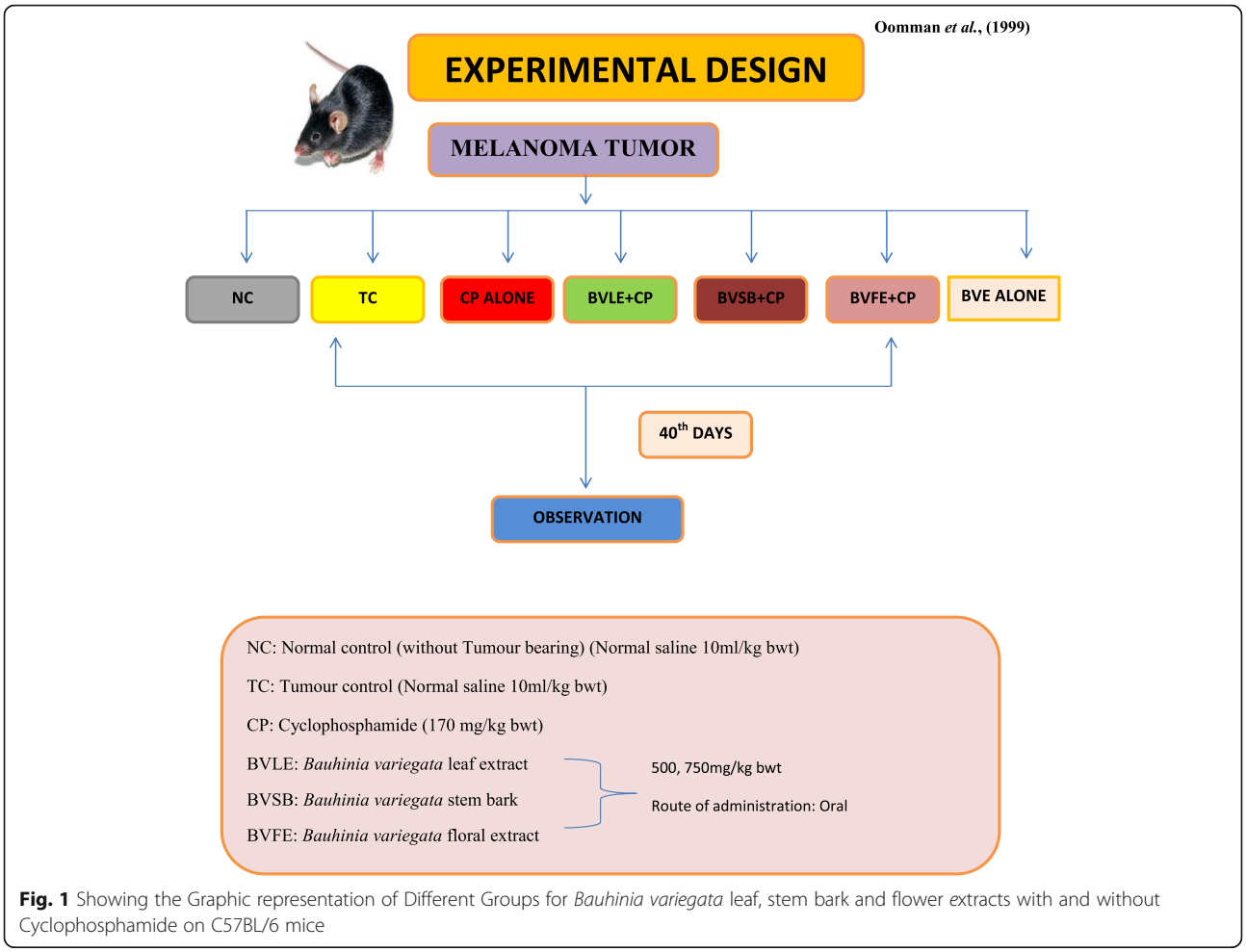

Fig. 1 Showing the Graphic representation of Different Groups for *Bauhinia variegata* leaf, stem bark and flower extracts with and without Cyclophosphamide on C57BL/6 mice

Whereas: NC: Normal control; TC: Tumour control; CP: Cyclophosphamide; L1, L2 and L3: *Bauhinia variegata* leaf extract; B1, B2 and B3: *Bauhinia variegata* Bark extract; F1, F2 and F3: *Bauhinia variegata* flower extract, i.p.: intraperitoneally

The tumour response was assessed by the tumour growth kinetics:

1. **Tumor Regression Studies** [48, 49].

Tumour Volume: During the treatment, the size of the implanted tumors was measured using Vernier calipers. Tumor volume was calculated by the following formula:

$$\textbf{Tumor volume} = \textbf{length X width}^2/2$$

Volume Doubling Time: Time required for the tumor to attain double the treatment volume.

Inhibition Rate: Tumor growth inhibition (%TGI) was determined twice weekly during the dosing period by the formula:

$$\%TGI = (1-\{T_t/T_0/C_t/C_0\}/1-\{C_0/C_t\})\ X\ 100$$

where T_t = median tumor volume of treated at time t, T_0 = median tumor volume of treated at time 0, C_t = median tumor volume of control at time t and C_0 = median tumor volume of control at time 0.

Tumor growth inhibition >50% is considered meaningful.

Study on increase in life span of melanoma tumor bearing C57BL/6 mice [50, 51]:

Mean survival time and median survival time (MST) was also calculated. The tumour response was assessed on the basis of the **percentage increase in life span (%ILS)**.

$$\%\mathbf{ILS} = \left\{\frac{\mathbf{MST\ of\ Treated\ Group}}{\mathbf{MST\ of\ Control\ Group}}\right\}-\mathbf{1} \times \mathbf{100}$$

whereas: MST = Mean survival time, ILS = Increase Life Span

An enhancement of life by 25% or more over that of control was considered as effective antitumor response [51].

Antioxidant parameter was studied in all the groups at the time of termination of the experiment (i.e., after 41 days).

Determination of glutathione (GSH) level

Glutathione was evaluated by sacrificing all the experimental mice were on day 41st by cervical dislocation and liver and Kidney was removed.

Preparation of homogenates

After collection of blood samples [52], the mice were sacrificed. Then the liver and Kidney was excised, rinsed in ice cold normal saline followed by ice cold 10% KCl solution, blotted, dried and weighed. A 10% (*w*/*v*) homogenate was prepared in ice cold KCl solution and centrifuged at 1500 rpm for 15 min at 4 °C. The supernatant thus obtained was used for the estimation of glutathione (GSH) [53, 54] level were checked using respective kits (Sigma-Aldrich Co. LLC) according to manufacturer's instruction. Reduced glutathione was used as a standard to calculate μ mole GSH/100 g tissue.

Histopathological studies

After the completion of drug treatment (40 days), on the day 41st mice were sacrificed by cervical dislocation. The tumor of three animals from each group was dissected out, fixed in 10% buffered formalin for 12 h and processed for histopathological examination. 4 μm-thick paraffin sections were cut and stained with hematoxylin and eosin and mounted in DPX (used as a synthetic resin mounting media). Sections were qualitatively assessed under the light microscope for their architecture [55].

Statistical analysis

Results of Statistical analysis are presented as Mean ± S.D. Statistical evaluation of data was performed by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

Results

Acute oral toxicity testing

The present study conducted as per the OECD guidelines 423 revealed that an acute toxicity test in which no death occurred in more than one of the six animals given a dose of 2000 mg /kg, the LD_{50} value can be considered greater than 2000 mg/ kg and less than 5000 mg/kg. In LD_{50} studies it were found that the different parts of *B. variegata* extract (leaf, stem bark and floral bud) did not produce any signs of toxicity or mortality up to the dose level 5000 mg/kg b.wt. Hence, the drug was considered to be safe up to the dose level of 2000 mg per kg bwt.

Effect of *B. variegata* in subcutaneous melanoma B16F10-bearing mice

The study of tumor volume of mice shown in Fig. 2a revealed that in the groups (L_3, B_3, and F_3) which received hydromethanolic extract of different parts of *B. variegata* (leaf, stem bark, floral bud) at 500 and 750 mg/kg, b. wt. a reduction in the tumor volume was observed. On the day 40th of treatment, the reduction in the tumor volume was found to be significant ($P < 0.05$) for both the doses compared to tumor control group (TC), which received normal saline, 10 mL/kg, b.wt and Cyclophosphamide alone group (CP).

When hydromethanolic leaf extract of *B. variegata* at 500 and 750 mg/kg was used as an adjuvant to chemotherapy groups (L_1 and L_2) receiving cyclophosphamide at 170 mg/kg, there was a statistically significant ($P < 0.05$) reduction in tumor volume on 40th day of tumor induction as compared to tumor control and cyclophosphamide alone group ($A_1$and A_2) as well as test drug group (L_3). Simultaneously, *B. variegata* bark extract (B_1, B_2) and flower extract (F_1 and F_2) at the dose of 500 mg/kg and 750 mg/kg with cyclophosphamide (CP) were also reduced tumor volume as compared to tumor control and cyclophosphamide group (NC and CP) as well as test drug alone group (L_3 B_3 and F_3) respectively.

The tumor doubling time (in days) Fig. 2b of Cyclophosphamide group (CP) was increased, whereas *B. variegata* leaf extract (500 mg/kg and 750 mg/kg) along with CP groups (L_1 and L_2), tumor doubling time were reduced. Simultaneously, *B. variegata* bark (B_1 and B_2) and flower extract (F_1 and F_2) (500 mg/kg and 750 mg/kg) along with CP groups were also reduced tumor doubling time respectively.

The inhibition rate Fig. 2c of *B. variegata* leaf extract alone (L_3) and cyclophosphamide group (CP) were decreased but when *B. variegata* leaf, stem bark, flower extract (L_1, L_2, B_1, B_2, F_1 and F_2) along with CP were given, the inhibition rate were increased in tumor bearing mice as compared to tumor control (TC) and cyclophosphamide group (CP) as well as test drug alone group (L_3, B_3 and C_3) respectively.

The life span Fig. 2d was also increased in *B. variegata* leaf extract (500 mg/kg and 750 mg/kg) along with CP group (L_1 and L_2), at the same time the *B. variegata* bark (B_1 and B_2) and flower extract (F_1 and F_2) along with cyclophosphamide group, life span were also increase in tumor bearing mice. The differences in the values of the results of experimental groups were statistically analyzed

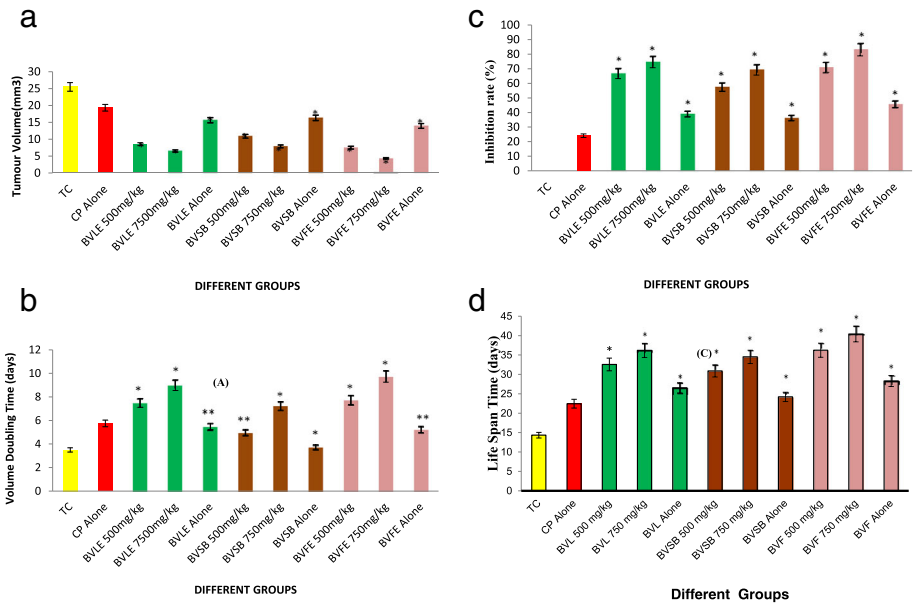

Fig. 2 Effect of *Bauhinia variegata* extract (leaf, stem bark, floral bud), individually or in combination with cyclophosphamide, on growth response of B16F10 melanoma tumour. **a** Changes in Tumour Volume of B16F10 tumour-bearing mice. The Tumour Volume was expressed in mili meter (mm^3); **b** change in volume doubling time on the growth of induced melanoma tumour. The volume doubling time was expressed in days; **c** Inhibition Rate increase as per dose dependent manner; **d** Effect of *Bauhinia variegata* alone and single (low and high) dose of *Bauhinia variegata* extract along with CP as combination therapy, on the response of B16F10 Melanoma tumour bearing mice and mean survival time of C57BL/6 mice. The data represented Means ± S.E.M. ($n = 6$ mice) from triplicate experiments. **Significantly different from Tumour control group (TC) ($P < 0.05$). * Significantly different from Cyclophosphamide group (CP) ($P < 0.05$). BVLE: *Bauhinia variegata* leaf extract; BVSB: *Bauhinia variegata* stem bark; BVF: *Bauhinia variegata* floral extract; CP: cyclophosphamide; TC: Tumour control; SEM: Standard error of mean

and found to be significant as compared to the tumor control group ($p < 0.05$).

Overall study revealed that the hydromethanolic extract of *B. variegata* floral buds exhibited stronger antitumor effect against melanoma tumor as compared to leaf extracts and stem bark extract.

Determination of glutathione (GSH) level from non-tumor-bearing and B16-F10 melanoma-bearing mice

The down regulation level of reduced glutathione in blood, liver and kidney of experimental mice were investigated to determine the antioxidative effect of test groups against the oxidative stress induced by melanoma cells. After induction of B16F10 mice melanoma cells, the level of glutathione (GSH) of blood, liver and kidney tissues of experimental mice were recorded. The percentage change on level of glutathione (GSH) per mg protein as a function of *B. variegata* alone or in combination with cyclophosphamide has been observed.

In the groups L_1, L_2, B_1, B_2, F_1 and F_2 which received in *B. variegata* extracts at 500 and 750 mg/kg b.wt. from the day of tumor initiation, the activities of glutathione (GSH) were much closer to normal control group (NC). Decreased concentration of reduced glutathione (GSH) in tumor control group has been observed compared to normal control. The percentage change on level of glutathione (GSH) per mg protein as a function of *B. variegata* alone or in combination with cyclophosphamide has been presented in Fig. 3.

Antioxidant enzymes activities of glutathione (GSH) (units mg/protein) in the liver and kidney homogenate and blood (ml/protein) were significantly increase ($P < 0.05$) in *B. variegata* extract along with cyclophosphamide treated groups (L_1, L_2) as compared to tumor control (TC) and cyclophosphamide alone group (CP) Fig. 3.

Subcutaneous induction of B16F10 melanoma showed a significant lowering of reduced glutathione in blood, liver and kidney (characteristic of antioxidants) compared to normal control group (NC) and reduced the scavenging of reactive oxygen species. A similar results wereobserved in stem bark and floral bud extracts Fig. 3a b and c.

B. variegata floral bud extracts exhibited stronger antioxidant activity as compared to leaf and stem bark extract. Among the groups studied, optimum value of reduced GSH per mg protein is found to be in the order, floral bud > leaf > stem bark > cyclophosphamide. Based on our observation, *B. variegata* floral extract exhibited optimum antioxidant activity and rendered significant protection against oxidative stress induced by melanoma in blood, liver and kidney tissues.

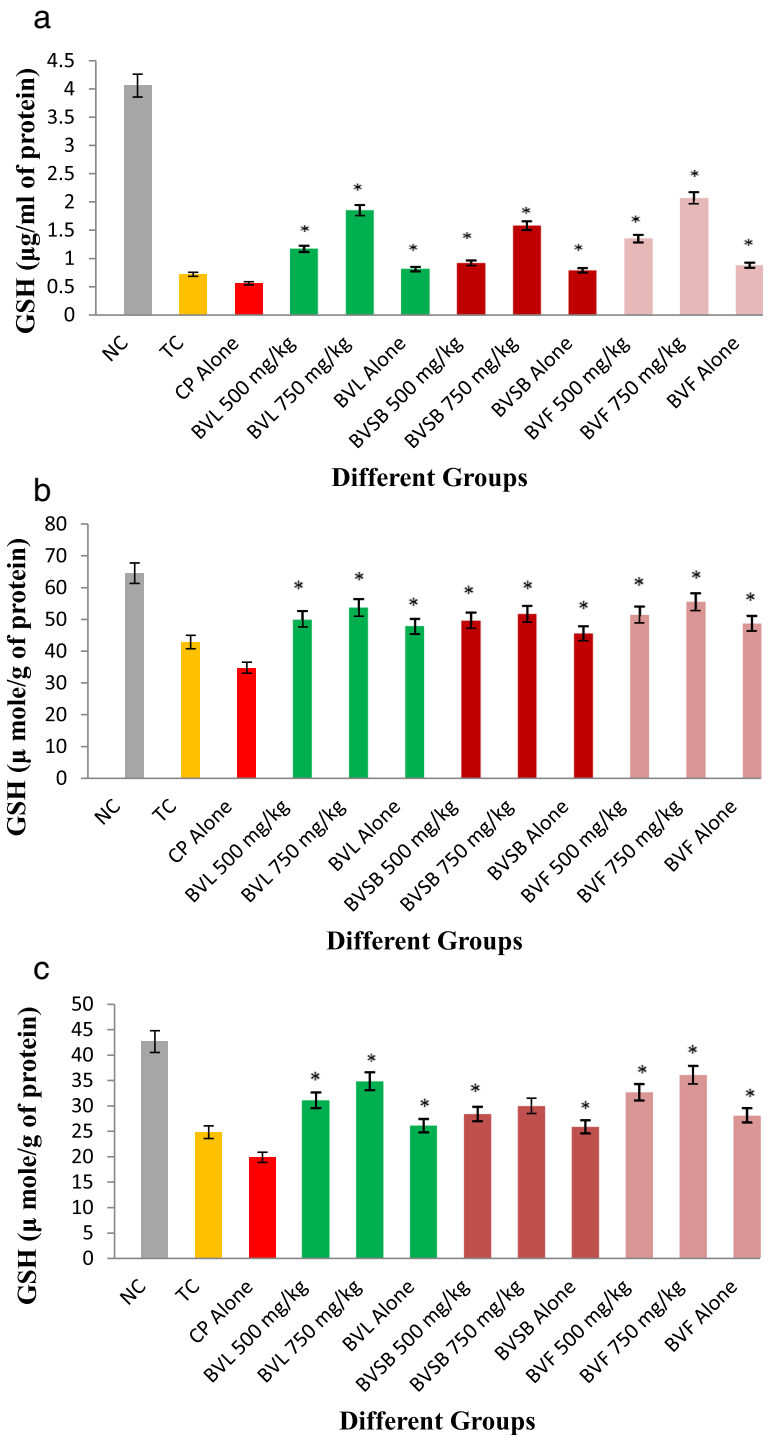

Fig. 3 Variations in the reduced glutathione (GSH) level in the liver, blood, kidney of mice during melanoma tumor with/with treatment of *B. variegata* extract. The Tumour GSH was expressed in µ mole/ml of protein in Blood (**a**); where as in Liver (**b**) and Kidney (**c**) expressed by µ mole/g of protein. The data represented Means ± S.E.M. ($N = 6$ mice) from triplicate experiments. *significantly different from cyclophosphamide group (CP) and Tumour control (TC) group ($p < 0.05$). BVLE: *Bauhinia variegata* leaf extract; BVSB: *Bauhinia variegata* stem bark; BVF: *Bauhinia variegata* floral extract; CP: cyclophosphamide; TC: Tumour control; SEM: Standard error of mean

Histopathology

We have evaluated the efficacy of *B. variegata* on B16F10 mice melanoma tumors implanted subcutaneously in C57BL mice. Representative H&E-stained sections of Tumor tissues recovered from the mice are shown in Fig. 4. The observation for histopathology was made on blood vessels, tumor cells, area of tumor regression, apoptosis and reduction in tumor mass. Histology of the tumor control group (TC) revealed the presence of hypervascularization where the perimeter of blood vessels large with the presence of hyperchromic tumor cells as compared to normal control (NC) group. The apoptosis and dysplastic cells were significantly higher in tumors treated with group CP and tumor control group (TC). The groups (L, B, and F), treated with extracts of *Bauhinia variegata* at a dose of 500 and 750 mg/kg, body weight revealed the presence of less live tumor cells with apoptosis as compared of tumor control group (TC) and Cyclophosphamide group (CP).

Analysis of the melanoma tumor sections on groups (L_1 and L_2) which received extracts of *Bauhinia variegata* leaf along with cyclophosphamide at 500 and 750 mg/kg, body weight from the day of tumor induction showed a progressive increase of apoptotic cells and perimeter of blood

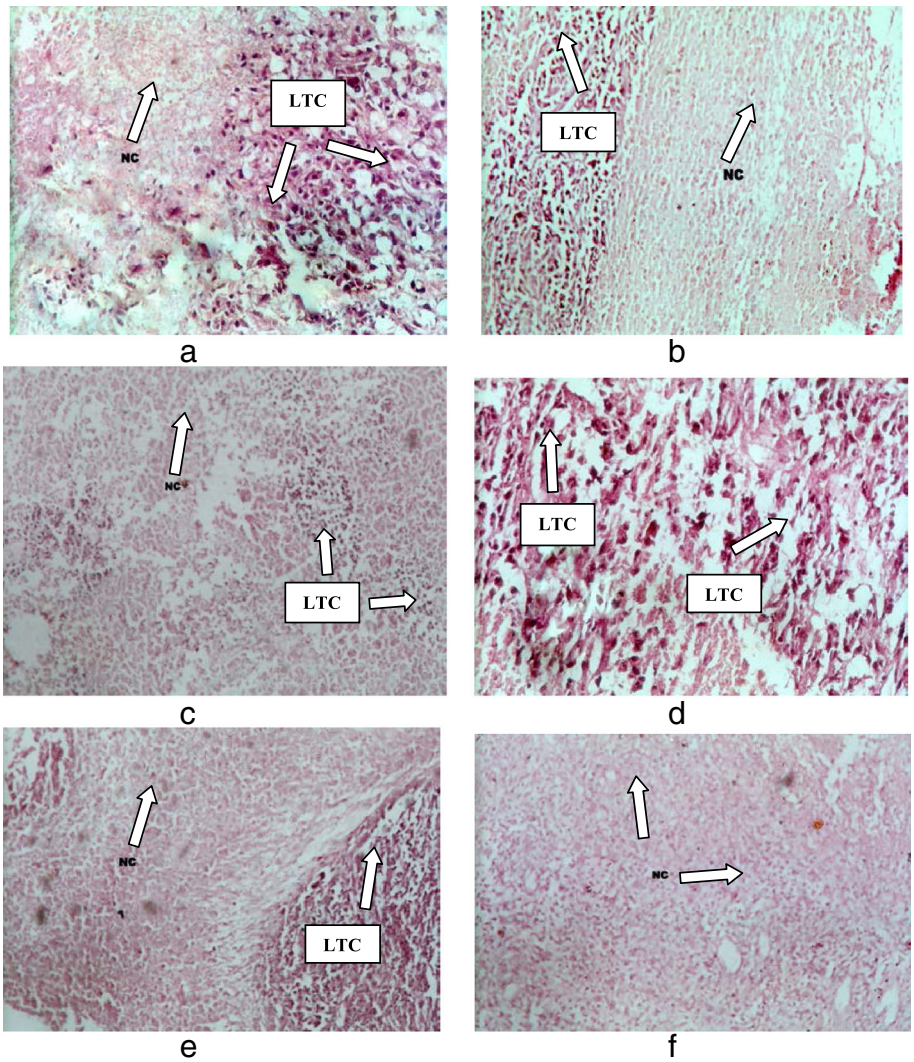

Fig. 4 Histopathology of melanoma tumor bearing C57BL/6 mice were subcutaneously transplanted with B16F10 cell line. A portion of excised tumor tissue from melanoma tumor bearing mice were fixed in 10% formalin, cut into 5-μm thickness, stained using H&E (hematoxylin and eosin) and then examined for histopathological changes. **a** The group which received cyclophosphamide (Positive control) showed equal quantity of both necrotic cells and tumor live cells but when the (**b**) Tumor bearing animals treated with *B. variegata* extract shows reduction in the live tumor cells and tumor mass; *B. variegata* (leaf, stem bark and flower) extract was given along with/without cyclophosphamide showed some histological changes such as (**c**) full necrosis with focal area of live cells, **d** Melanoma tumor suggests that total live tumor cells and (**e**) focal area of live cells with necrosis are present in the tumor control group (TC); (**f**) full necrosis and vivality with focal area of necrosis. Whereas: LTC – live Tumor cells, NC – Necrotic Cells

vessels and vascular supply was less. A similar result was observed in stem bark and floral bud extracts also. The incidence of dysplastic cells and apoptosis in the tumor almost corresponded to the effect of tumor growth inhibition, suggesting that treatment resulted in tumor regression by significant augmentation of apoptosis. However, flower extract of *B. variegata* were found more effective as compared to leaf and stem bark extract.

Discussion

Cancer is a growing health problem worldwide, with the introduction of 6 million new cases every year. Many approaches are being tried through modulation of antitumor immune response, apoptosis, and antitumor proteins for cancer treatment [56]. Cancer cells lack the growth control of normal cells, exhibiting unlimited self-sufficient replication [57, 58]. For therapeutic effectiveness, drugs are being developed that act as biological modifiers, regulating the cell cycle and promoting cell death [59]. Plant derived compounds have been reported to induce cell cycle arrest and cell death in many tumor cell lines [60–65].

In recent years, the cancer rate has increased, especially in less developed countries. Identification and development of new chemotherapy drugs has been critical for cancer treatment. Plants have been a rich source of natural drugs [66, 67]. In addition, compounds derived from plants are diverse in structure and bioactivity and exhibit low toxicity, therefore they play an important role in pharmaceutical research [68]. In cancer therapy, many plant-derived drugs such as vincristine, paclitaxel and taxol have been identified and developed. Several works on epidemiology and animal model studies demonstrated that natural compounds, which possess antioxidant or anti-inflammatory properties, could inhibit carcinogenesis [69–72].

According to the traditional recommendations and experimental studies, numerous medicinal plants have been reported to have anticancer effect. Also antiproliferative, pro-apoptotic, anti-metastatic and anti-angiogenic effects of several phytochemicals have been shown in in vitro experiments or animal studies. However, only a small number have been tested in cancerous patients and limited evidence exists for their clinical effectiveness [73].

In the current study, we have selected *B. variegata* extracts to treat the melanoma tumor. The mice were treated with *B. variegata* extracts (stem bark, leaf and floral bud) at a dose of 500 and 750 mg/kg, body weight. In the control group receiving normal saline, initially, a slow and steady growth in tumor volume was observed with a drastic increase in tumor size after few days.

Experimental tumor models are a critical pre-clinical step for the development and evaluation of chemotherapy regimens for cancer. Interestingly, we report here that the differential cytotoxic effect of these extracts was related not only to their chemical composition but may be also due to the nature of the tumor cells. In the present finding, the cancer treatment through chemotherapy with the combination of *B. variegata* was found more effective as compared to the extract alone. Earlier studies on herbs were reported that in cancer therapy many drugs when given alone give good results, however, when combined with other drugs exerting synergistic effect, the results are better. The objective of such approach is to minimize drug resistance and drug toxicity.

The *B. variegata* leaf, stem bark and flower extracts were studied on the inhibition of B16F10 melanoma tumor bearing mice. Study with the melanoma tumour model showed the effect of cyclophosphamide alone, *B. variegata* leaf, stem bark and flower extract (500 and 750 mg/kg body weight) along with Cyclophosphamide and *B. variegata* leaf, stem bark and flower extract alone groups. These were significantly reduced tumor volume and also increase survival time, inhibition rate and reduction of tumor doubling time in all *B. variegata* leaf, stem bark and flower extract as compare to tumour control (TC) and cyclophosphamide (CP) groups. Interestingly, I report here that the differential antitumor effect of these extracts was related not only to their chemical composition but may be also due to the nature of the tumor cells. Recent researches confirm the utility of herbs to both control cancer growth and to reduce side effects of chemotherapy. In addition, some herbs can reverse multidrug resistance [74].

A majority of the carcinogenic agents are regarded as powerful generators of free radicals leading to cancer. The reduction in tumor count may be due to effect in the promotional phase of tumourgenesis which prevent the reduction of free radicals [75].

Natural compounds have demonstrated strongest antioxidant and anticancer activity with multifunctional activity which also binds to and modulates activity of protein kinase involved in signal transduction cascades, show cytotoxic and cytostatic activity towards cancer cells [76]. The most quoted cancer prevention mechanism is via their activity, elicited either through direct free radical absorption or through induction od antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione via a variety of molecular mechanisms [77, 78].

Glutathione (GSH) plays an important role in a multitude of cellular processes, including cell differentiation, proliferation, and apoptosis, and disturbances in GSH homeostasis are involved in the etiology and progression of many human diseases including cancer. While GSH deficiency, or a decrease in the GSH/glutathione disulphide (GSSG) ratio, leads to an increased susceptibility to oxidative stress implicated in the progression of cancer, elevated GSH levels increase the antioxidant capacity and the

resistance to oxidative stress as observed in many cancer cells [79].

On the basis of the present study, it can be suggested that the *B. variegata* treated mice restores the changes in the activity of the antioxidant enzymes and the level of glutathione. Melanoma tumor group showed a sharp increase level of GSH in liver, kidney and Blood at *B. variegata* extract treated groups. It was observed that tumor cells produced more peroxides when they proliferate actively after inoculation of tumor. This rise in peroxides indicated the occurrence of intensification of oxygen free radical production [80]. Antioxidant molecules, such as GSH and vitamin E and C as well as antioxidant enzymes such as SOD, Catalase and glutathione peroxidase, have been long believed to have protective and anticancer activities by scavenging excess of free radicals. The liver is the main source of circulating plasma GSH (over 90% of the total GSH inflow) [81, 82]. As compared with non-tumor-bearing mice, GSH levels decrease in the brain, lung, liver, and kidney of B16-F10-bearing mice [83].

In the current study, *B. variegata* therapy restored the antioxidant levels and increases the GSH content in tumor animals which may be due to the antioxidant and free radicles scavenging ability of the plant. Many studies reported that plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells and antitumor activity in experimental animals [84].

Accumulating evidences [85–88] demonstrate that tumor growth and lethality are dependent on angiogenesis. An observation of histological slides (Fig. 4) exhibits the decrease in tumor growth in mice by the *B. variegata* extract which may be attributed to decreased host angiogenesis. *B. variegata* (leaf, stem bark and flower) extract along with/without cyclophosphamide groups showed some histological changes such as full necrosis, necrosis with focal area of live cells and vivality with focal area of necrosis, in C57BL/6 mice. A marked and dense microvasculature was observed in the tumor control group.

Preclinical animal models have been used extensively in the efficacy testing of potential chemopreventive agents. Standardized statistical methodology has been established to evaluate and compare the data from most of these animal model experiments based on the various endpoints [89]. A wide variety of naturally occurring substances have been shown to inhibit chemical carcinogenesis in animal models [90–92]. Nowadays, chemoprevention has been an important approach to control the process of cancer induction. Therefore, there is a need for exploring medicinal plants or other natural agents that can work as chemopreventive agents. The present study demonstrates the chemopreventive potential of *B. variegata* (leaf, stem bark and flower) extract on B16F10 melanoma tumour model in C57BL mice. The results of the present studies indicate that *B. variegata* (leaf, stem bark and flower) extract is effective as anticarcinogenic agent. Our observation agrees with the previous results of antitumor activity of the ethanolic extract of *B. variegata* extract against Dalton's ascetic lymphoma (DAL) in *Swiss albino* mice [93] and in N-notrosodiethylamine induced experimental liver tumour in rats and human cancer cell lines [94]. Our findings are also in agreement with the previous results of many herbal drugs such as alcoholic extract of *Thuja occidentalis* [95], aqueous-methanol (3:7) extract of *Boerhaavia diffusa* [96], methanolic extract of *Withania somnifera* roots [97], naturally occurring allyl and phenyl isothiocyanates [98], curcumin [99], sulphorafane [100], *B. variegata* leaf extract [101], *Piper longum* against A549 cell line [102], Cinnamaldehyde and eugenol [103] etc. have been reported to inhibit metastasis.

A numbers of drugs are used in cancer chemo and radiotherapy, and most of them exhibit cell toxicity and can induce genotoxic, carcinogenic, and teratogenic effects in non-tumor cells [104]. These side effects limit the use of conventional chemotherapeutic agents despite their high efficacy in treating cancerous cells. Therefore, the search for alternative drugs/molecules that are effective and non-toxic in the treatment of cancers is an important research area [105]. In fact, sincere efforts are being made to isolate bio-actives from medicinal plants for their potential in cancer treatment [106].

There is emerging scientific evidence of herbal medicines playing an important role in the supportive care of cancer therapy [107]. Herbal extract have flavonoids, anthraquinones, and saponins which might be responsible for exhibiting anticancer effect. There are some literatures which have reported biological interactions of flavonoids, polyphenols, or phenolic compounds with proteins, enzymes, and other biological processes in the cells that make them toxic to the tumour cell or serve as growth inhibitors for cancer cells [108].

Moreover, flavonoids have an anti-proliferative role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [109, 110]. Study has been reported that presence of secondary metabolites such as terpenoids, phenolics, flavonoids, anthraquinones, saponins, tannins, and alkaloids in *B. variegata* leaf, stem bark and floral bud extracts [32, 111].

Prevention and cure of diseases using phytochemicals especially flavonoids are well known. Variety of flavonoids found in the nature possesses their own physical, chemical, and physiological properties. Structure function relationship of flavonoids is epitome of major biological activities. Medicinal efficacy of many flavonoids as antibacterial, hepatoprotective, anti-inflammatory, anticancer, and antiviral agents is well established. These substances are more commonly used in the developing countries. Many flavonoids are shown to have antioxidative activity,

free radical scavenging capacity, coronary heart disease prevention, hepatoprotective, anti-inflammatory, and anticancer activities [112].

Studies have been reported that several naturally occurring compounds exhibited antitumor promoting activity in B16F10 melanoma tumour model. *Solanum lycopersicum* fruit extracts has reported to inhibit the B16F10 melanoma tumour in C57BL mice [113] and *Lawsonia inermis* leaf extract has also been reported to possess anticarcinogenic property against B16F10 melanoma tumour model [114]. *Bauhinia variegata* flower extract has been reported to show Chemopreventive activity against DMBA-induced skin Papillomagenesis in mice [115]. The *Withania somnifera*and its bioactive fraction Withanolide D were studied for their anti-metastatic activity using B16F10 melanoma cells in C57BL/6 mice. Keishi-ka-kei-to is a traditional Chinese herbal medicine which is reported to inhibit pulmonary metastasis in mice bearing B16F10 melanoma cells through the stimulation of CD8+ T cells [116].

The mechanism of tumor growth reduction in vivo induced by *B. variegata* seems to involve apoptosis induction. Presently, I have shown that the hydrometholic extract of *B. variegata* have flavonoid compounds. Finally, the isolation of the active principles of the hydromethanolic extract of *B. variegata* is currently being undertaken to investigate their cytotoxic, molecular and genetic action mechanisms, which could provide meaningful perspectives for biomedical and future drug development research.

Conclusion

Tumors employ multiple mechanisms for their uncontrolled proliferation, invasion, angiogenesis and metastasis. It is therefore logical to envision that a combination of approaches that target different mechanisms will be more effective at inhibiting tumor growth or destroying tumors than a single agent approach. As mentioned in the preceding section, few of the researches on tumor and angiogenesis have been reported using different plants in an animal model other than melanoma tumor model (study model of the present research work). The rapid increase in utilization of herbal remedies worldwide has been inspired by several factors, including the concept that herbal products are safe and effective and so investigation on medicinal plants is increasing day by day.

In conclusion, the present piece of in vivo experiments highlight the effectiveness of *B. variegata* combination among all the treatment studied and is found capable for reducing melanoma against reference drug cyclophosphamide. Oral administration of *B. variegata* extract simultaneously with tumor inoculation showed significant reduction in the tumor volume. The extract treatment also produced significant increase in the life span of tumor bearing C57BL/6 mice as compared to cyclophosphamide alone and Tumor control groups. The activity of *B. variegata* was found in following order floral bud > leaf extract > stem bark extract. Further, the antitumor activity of *B. variegata* in mice may be attributed to the presence of polar phytoconstituents such as alkaloids, flavonoids, tannins, terpenoids, and glycosides present in the crude extract of *B. variegata*. The endeavor of the present study was to travel around the potential anti-tumor activity of *B. variegata* extract on melanoma tumor. The study was not only supportive in determining the optimum dose extract employed against melanoma tumors but also in the development of a new and a potential anti-cancer drug. The hydromethanolic extract of *B. variegata* is an effective and potent antitumor agent against human malenoma so it may provide a poor man friendly and a drug of preference to the world. The use of new agents in clinical phases needs more investigations on its the molecular mechanism of action and potential usefulness of *B. variegata* as an agent for cancer therapy.

Abbreviations

b.wt.: Body weight; B16-F10: B16 melanoma F10 subline; BVFE: *Bauhinia variegata* Flower Extract; BVLE: *Bauhinia variegata* Leaf Extract; BVSE: *Bauhinia variegata* Stem bark Extract; CYP: Cyclophosphamide; GSH: Glutathione; H&E: Haemotoxylin and Eosin; I.P: Intraperitoneal; ILS: Increase in life span; IR: Inhibition rate; LTC: live Tumor cells; MST: Mean survival time; NC: Necrotic Cells; PBS: Phosphate Buffer Saline; SC: Subcutaneous; VDT: Volume doubling time

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Availability of data and materials

The data sets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

SP carried out and supervised the molecular genetic studies, interpretation of data, participated in drafting the manuscript. Author read and approved the final manuscript.

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Ethics approval and consent to participate

The study was approved by our institution internal Research Ethics Committee.

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Consent for publication

I have consent form for this publication.

Competing interests
The author declares that she has no competing interests.

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