



Characterization of phytochemicals and evaluation of anti-cancer potential of *Blumea eriantha* DC.

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Abstract The aim of the present study was to evaluate the in vitro anti-cancer and anti-oxidant potential of methanol extract of *Blumea eriantha* as well as its phytochemical characterization. The in vitro cytotoxic and antiproliferative activities of *B. eriantha* methanolic extract of leaves were evaluated using MTT assay on HeLa and B16F10 cell lines, and wound scratch and colony formation assays on B16F10 cell lines. The expressions of p53 and Bcl-2 genes were also determined by reverse transcriptase-polymerase chain reaction to establish apoptosis. Isolation and identification of chemical constituents were carried out by various chromatographic and spectroscopic analytical techniques including HPTLC and LC-MS. The methanol extract of the leaves of *B. eriantha* showed potent in vitro antioxidant and anticancer properties. Moreover, the extract showed significant loss of wound healing, thus suggesting that it could prevent a possible metastasis. Hence, *B. eriantha* could be explored as a potential anticancer plant with antimetastatic properties.

Keywords Apoptosis · *Blumea eriantha* · Cancer · Chromatography · Cytotoxicity · LC-MS

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
DPPH	1,1-diphenyl-2-picrylhydrazyl
FRAP	Ferric ion reducing antioxidant power assay
HPTLC	High performance thin layer chromatography
LC-MS	Liquid chromatography–mass spectrometry

MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
TFC	Total flavonoid content
TPC	Total phenolic content

Introduction

Phytochemicals are known to reduce the risk of chronic diseases such as cancer, cardiovascular diseases, diabetes, Alzheimer's disease, and various inflammatory disorders (Liu 2003; Shahidi et al. 2008). More than 5,000 phytochemicals have been screened and identified so far, but a large number still remain unknown and are needed to be identified before their desirable benefits are conclusively established. Human beings are constantly exposed to various oxidizing agents which generate oxidative stress and leads to redox imbalance causing damage to proteins, DNA, and lipids (Marxen et al. 2007). Researches have established that synthetic molecules are associated with toxicity and hence natural antioxidants such as polyphenolic compounds from plant origin are preferable. These molecules can donate electrons to reactive oxygen species and neutralize them (Sharma et al. 2009; Stanojević et al. 2009). Plant phenols are reported to have significant antioxidant, antitumor, antiviral and antibiotic activities (Apak et al. 2007). Several compounds extracted from plant sources such as camptothecin, vincristine, vinblastine, taxol and podophyllotoxin have been successfully identified as effective anticancer agents (Azizi et al. 2009; Pezzuto 1997; Srivastava et al. 2005).

Studies conducted on different species of *Blumea*, mainly *Blumea mollis* (Srikanth et al. 2012) and *Blumea balsamifera* (Saewan et al. 2011), have exhibited cytotoxic activity against cancer cells. The plant in the present study, *Blumea eriantha*

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DC is a small annual herb, up to 1 m in height, possessing camphor like smell and is commonly found in tropical countries. Juice of this herb is used as a 'carminative'. The essential oil extracted from the leaves and stem show potent antibacterial, antifungal and insecticidal properties (Khare 2007; Singh et al. 2011). This plant has been traditionally used as a diuretic and also for the treatment of cholera and diarrhea (Khare 2007; Singh and Parthasarathy 2012). However, there have been no studies on the in vitro anticancer properties of *Blumea eriantha* so far.

In the present study, we have investigated the antioxidant potency and cytotoxic activities of *B. eriantha* DC. leaf extract on cancer cell lines. Additionally, a preliminary screening of the chemical constituents was carried out using various chromatographic and spectroscopic techniques including HPTLC and LC-MS.

Materials and methods

Chemicals

All solvents and chemicals were of analytical grade and obtained from local suppliers. Ascorbic acid, aluminum chloride, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric chloride, Folin-Ciocalteu reagent, potassium acetate, sodium carbonate, 2,4,6-tripyridyl-s-triazine (TPTZ), trichloroacetic acid, 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Dulbecco's modified Eagle's minimum essential medium (DMEM) were purchased from Sigma Chemical Co., USA. 10 % fetal bovine serum (FBS) was procured from Gibco. Glacial acetic acid (HPLC grade) and methanol (HPLC grade) were purchased from Merck.

Plant material

B. eriantha DC. (Asteraceae) plants were collected from Kolhapur, Maharashtra, India. The identity of the plant was authenticated by Dr. Manoj Lekhak, a taxonomist from Department of Botany, Shivaji University, Kolhapur, India. A voucher specimen of the plant (MG1 and MG2) has been prepared and deposited at the herbarium in the Botany department, Shivaji University, Kolhapur, India. Leaves of *B. eriantha* are lower obovate, short petiolated, upper elliptic-ovate to oblanceolate with dense silky hairy on both the surfaces (Singh et al. 2011).

Extraction and isolation

The fresh leaves were collected, cleaned with water and air dried at the room temperature in a well ventilated and sterilized room. The dried leaves were powdered using a grinder. Leaf powder (1 g) was extracted with 25 ml of methanol,

ethanol and distilled water separately for 6 h using Soxhlet apparatus. Extracts were centrifuged at 15,000 rpm for 10 min; the supernatants were recovered and filtered using Whatman No.1 paper. The extracts were evaporated to dryness under reduced pressure by a rotatory evaporator (Rotavapor). These extracts were used for the antioxidant assays. However, the cytotoxicity assays on HeLa and B16F10 cell lines were done using the methanolic extract.

Antioxidant assays

DPPH radical scavenging activity

Antioxidant activity of the extracts was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Lee et al. 2003; Chen et al. 2005). The stock reagent solution was prepared by dissolving 24 mg of DPPH in 100 ml methanol. The working solution was obtained by mixing 10 ml of stock solution with 45 ml methanol. The different concentrations of the extracts were allowed to react with 3 ml of DPPH solution. The mixture was shaken and allowed to stand at the room temperature for 30 min in the dark. The absorbance was measured at 517 nm using a spectrophotometer. A control sample, having no extract was also included and the results were expressed as radical scavenging activity (% RSA).

$$\%RSA = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where, A = absorbance at 517 nm.

The results were compared with a standard curve of ascorbic acid.

Ferric ion reducing antioxidant power assay (FRAP assay)

The FRAP assay was performed with some modifications (Benzie and Strain 1996). The FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10:1:1 ratio prior to use and heated to 37 °C in water bath for 10 min. The extracts of various concentrations were allowed to react with 2.7 ml of the FRAP reagent. The final volume of the reaction mixture was made up to 3 ml with distilled water. The reaction mixture was kept in the dark for 30 min. The absorbance was measured at 593 nm using a spectrophotometer (Svilaas et al. 2004).

Determination of total phenolic content

Folin-Ciocalteu reagent was used for the determination of total phenolic content. The extracts were mixed with Folin-Ciocalteu reagent and aqueous sodium carbonate. The mixtures were kept for 90 min at room temperature and

absorbance was measured at 765 nm using a spectrophotometer (Connor et al. 2002; Singleton and Rossi 1965). This was compared to the standard curve of Gallic acid concentrations and expressed as mg of Gallic acid equivalents per g (mg GAE g⁻¹) (Sengul et al. 2009).

Analysis of total flavonoids content (TFC)

The extracts were mixed with methanol, to which 10 % aluminum chloride, 1 M potassium acetate and distilled water were added. The solution was vortexed, allowed to stand for 30 min at room temperature. The absorbance of reaction mixture was measured at 415 nm using a spectrophotometer (Chang et al. 2002; Hsu 2006; Huang et al. 2005). The total flavonoids content was quantified according to the standard curve prepared for rutin and the concentration of flavonoids was reported as mg of rutin equivalents per g (mg RE g⁻¹).

In vitro anticancer assays

Cell culture

HeLa and B16F10 melanoma cell lines were obtained from National Centre for Cell Science (Pune, India). HeLa cells and B16F10 melanoma cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % heat inactivated fetal bovine serum, 100 (U) units/ml penicillin and 100 µg/ml streptomycin. The cells were incubated in an incubator with 5 % CO₂ at 37 °C.

Cytotoxicity assay of methanolic extract using MTT on HeLa and B16F10 cell lines

Ninety-six well plates were seeded with HeLa cells and B16F10 cells with a seeding density of 2×10³ cells per well and 3×10³ cells per well respectively and allowed to adhere for 24 h before treatment. After 24 h. incubation period, the plant extract was dissolved in 0.1 % DMSO and was serially diluted with DMEM supplemented with 10 % FBS and was added to the cells. Vehicle control wells contained 0.1 % DMSO + DMEM+ 10 % FBS. Negative control contained only DMEM+ 10 % FBS. Doxorubicin (10 µg/ml), a conventional cytotoxic agent, was used as standard positive control. After 48 h, 20 µl of 5 mg/ml MTT was added to all the wells. The plates were incubated for 4 h after which 100 µl of DMSO was added to all the wells. The absorbance was measured at 570 nm using a spectrophotometer (Mosmann 1983).

Wound scratch assay

B16F10 cells were seeded in 6 well plates with a seeding density of 0.4×10⁶ cells per well and allowed to adhere at

37 °C with 5 % CO₂ for 24 h. 2 sets of plates were used for the experiment. The scratch wound was introduced by scraping the monolayer in a perpendicular direction with a 200 µl pipette tip in all the plates. The cells were washed very gently with PBS. In the first set of plates, the extract was added corresponding to the IC₅₀ value i.e. 841.84 µg/ml. The second set of plates contained only medium (DMEM supplemented with 10 % FBS) and served as control. The plates were placed in incubator at 37 °C with 5 % CO₂ for 48 h. Pictures of fixed positions in the wound were taken with a digital camera mounted on an inverted microscope at the time of wounding and 48 h after addition of the extract. The wound area was then determined, after outlining the wound and measuring the area using Image J imaging software developed by NIH (Abramoff et al. 2004). The width was measured at 5 points along the scratch area and then an average was determined. The percentages of wound closure were calculated as: [(Mean width of wound at the time of wounding- mean width of wound after 48 h)/ Mean width of wound at the time of wounding] × 100 (Liang et al. 2007).

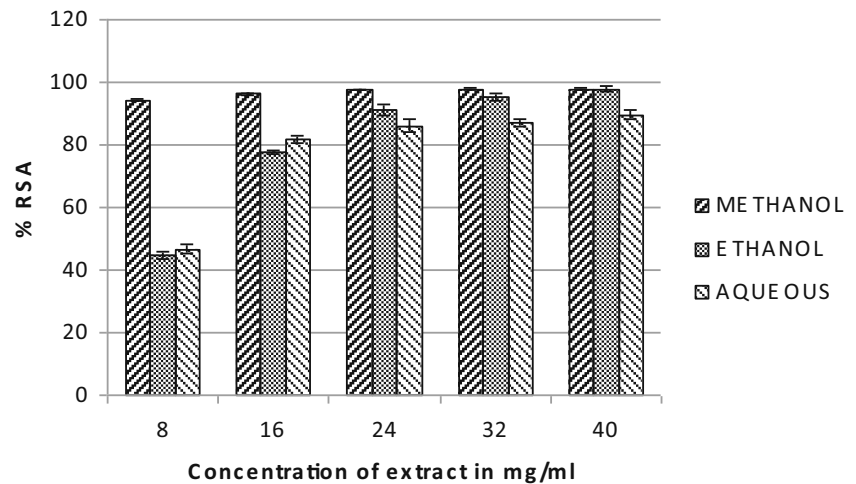
Colony formation assay

To study the effect of the extract on colony formation, subconfluent B16F10 cells were trypsinized and plated at a density of 750 cells/plate in 35 mm plates (2 sets). The plates were placed in an incubator with 5 % CO₂ at 37 °C and allowed to adhere for 24 h before treatment. After 24 h, the extract equivalent to the IC₅₀ value i.e. 841.84 µg/ml was added to 1 set of plates and the other set of plates without the extract served as a negative control. The cells were then incubated for 7 days and colonies were allowed to form. After 7 days, the plates were washed with PBS, fixed in methanol for 5 min, stained with 0.01 % crystal violet for 30 min and the number of colonies was determined by direct counting under a microscope (Franken et al. 2006).

RNA extraction and semi quantitative RT-PCR analysis

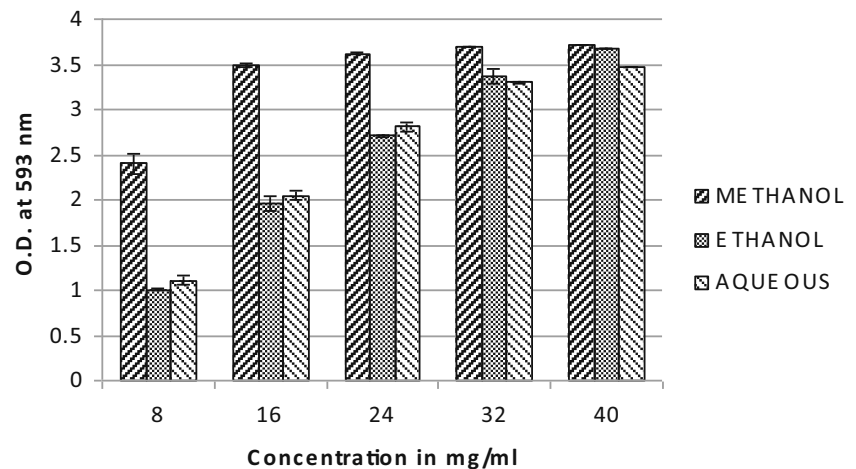
B16F10 cells were plated in a 6 well plate with a seeding density of 0.4×10⁶ cells per well. The plates were placed in an incubator with 5 % CO₂ at 37 °C for 24 h for the cells to adhere. After 24 h, each plate was treated separately with the plant extract (equivalent to the IC₅₀ value i.e. 841.84 µg/ml), doxorubicin (50 µg/ml) as a positive control and DMEM with 10 % FBS as a negative control. Then, after 48 h, RNA isolation was done from all the 3 sets of plates using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total isolated RNA by incubation for 1 h at 42 °C with 1 µl of reverse transcriptase, 2 µl oligo(dT) primer, 2 µl RNase inhibitor, 2 µl of dNTPs and 4 µl (5×) reaction buffer. Then, 2 µl of the reaction mixture was subjected to PCR for amplification of

Fig. 1 DPPH radical scavenging activity of leaf extract of *B. eriantha* in different solvent systems, data expressed in mean \pm SD, $n=3$



p53 and Bcl-2 cDNAs using gene specific primers (p53 Forward: 5'-CTGAGGTTGGCTCTGACTGTACCACCATCC-3'; p53 Reverse: 5'-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3' and Bcl-2 Forward: 5'-GTTTCGGTGGGGTCATGTGTGTGGAGA-3'; Bcl-2 Reverse: 5'-GCTGATTCGACGTTTGGCCGTAAGAC-3'). As an internal control, the house keeping gene β -Actin (β -Actin Forward: 5'-ACGGGTCAACCACACTGTGC-3'; β -Actin Reverse: 5'-CTAGAAGCATTTGCGGTGGACGATG-3') was co-amplified in each reaction. The PCR reactions was carried out in a final volume of 50 μ l containing 10 \times PCR buffer and 0.5 μ l Taq-polymerase, 10 mM of each dNTP and 10 μ M of each primer. The template was denatured for 2 min at 94 $^{\circ}$ C, followed by amplification cycles at 94 $^{\circ}$ C for 1 min, (69 $^{\circ}$ C for p53, 64 $^{\circ}$ C for Bcl-2 and 57 $^{\circ}$ C for β -actin) for 1 min and 72 $^{\circ}$ C for 1.20 min, and terminated with an additional extension step for 8 min at 72 $^{\circ}$ C. The PCR products were loaded on 1.2 % agarose gel, stained with ethidium bromide and subjected to gel electrophoresis. In negative control, DEPC water was used in place of template cDNA (Azizi et al. 2009).

Fig. 2 FRAP assay of leaf extract of *B. eriantha* in different solvent systems, data expressed in mean \pm SD, $n=3$



Separation of compounds by HPTLC

Sample preparation

The extract (1.553 g DW) was dissolved methanol (5 ml) and used as test solution for HPTLC analysis.

Sample application

Two microliter of test solution was loaded as 8 mm band length in the 10 cm \times 20 cm LiChropher Silica gel 60F254S plate using 100 μ l Hamilton syringe and CAMAG LINOMAT 5 instrument which was programmed through WIN CATS software. Several such tracks were loaded to elute the bands.

Spot development

The sample loaded plate was kept and developed in TLC twin trough developing chamber (after saturated with Solvent vapor) with mobile phase, Toluene-Chloroform-Ethanol (4: 4: 1).

Table 1 Total phenolic and flavonoid contents of the leaf extract of *B. eriantha* in different solvent systems

	Total phenol content (mg GAE/g)	Total flavonoid content (mg RE/g)
Methanol	1.345±0.083	7.729±0.037
Ethanol	1.092±0.022	5.164±0.120
Aqueous	0.935±0.104	2.029±0.005

Data shown as mean± SD, *n*=3

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in the Photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured at white light, UV 254 nm and UV 366 nm.

Scanning

The plate was fixed in a scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV light of 254 nm. The Peak table, Peak display and chromatogram were noted (Sampathkumar and Ramakrishnan 2011).

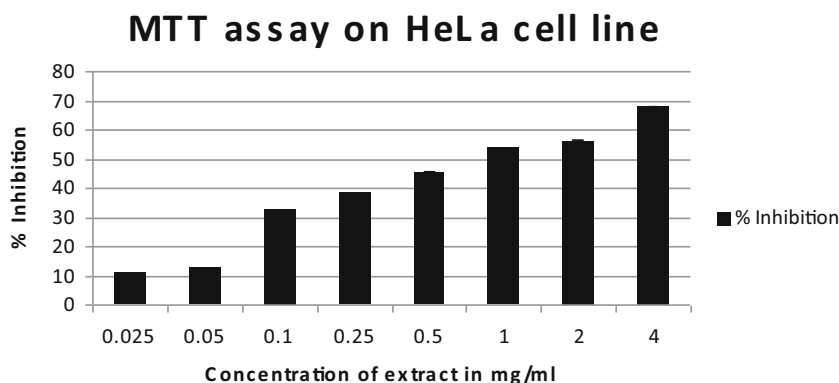
Elution

Nine bands were scraped off from the plates, eluted with methanol and used for further assays.

Cytotoxicity assay of eluted bands using MTT on B16F10 cell lines

A 96 well plate was seeded with B16F10 cells with a seeding density of 3×10^3 cells per well. After 24 h incubation, the eluted bands were serially diluted and added to the cells. MTT assay was performed as described above.

Fig. 3 MTT assay on HeLa cell line showing cytotoxicity of the serially diluted methanolic leaf extracts of *B. eriantha*. % inhibition of cells increases with increasing concentration of the extract. IC₅₀ value is 782.25 µg/ml



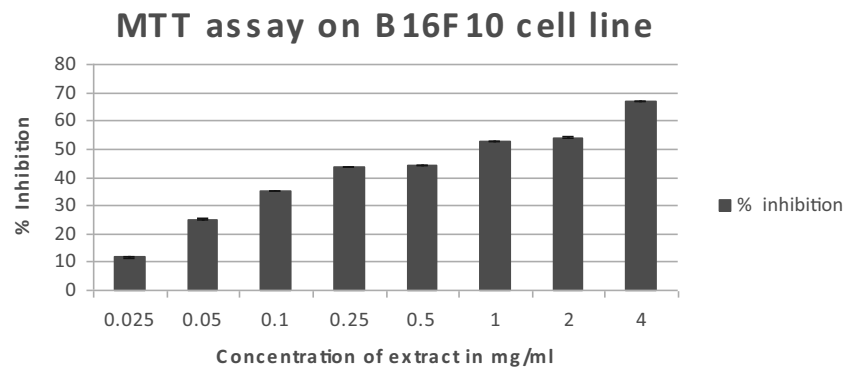
Sample analysis by LC-MS

LC-MS analysis was conducted using Agilent 6540 UHD QTOF LC-MS coupled to the 1,290 Infinity UHPLC (Agilent Technologies Inc., Bangalore, India). Zorbax SB-Aq, 3.0×100 mm; 3.5 µM column (Agilent 1,290 Infinity Binary pump, well plate autosampler, thermostatted column compartment) with a flow rate of 0.6 ml/min was used. MS with both positive and negative modes was used (Agilent 6540 UHD QTOF LC/MS/MS). Conditions for LC/MS/MS in both negative and positive ion mode included a capillary voltage of 3,500 V, nozzle voltage of 1,000 V, a nebulizing pressure of 30 psi, a drying gas flow of 10 L/min, and at 250 °C. Mass range was 50–1,700 *m/z*. MassHunter Molecular Feature Extractor (MFE) tool was used to extract the list of compounds present in the samples. The data (both positive and negative modes) was exported into Agilent Mass Profiler Professional (MPP) software to filter any background masses from system compared with blank injection. The compounds filtered through MPP which were specific for the individual sample were searched against the METLIN Personal Compound Database (PCD) with a tolerance level of 10 ppm (Ian et al. 2005).

Statistical analysis

In this study, all the observations were taken in triplicate. The mean values and standard deviations were calculated from the data obtained. Values representing the concentration of investigated extracts that cause 50 % of inhibition (IC₅₀) were determined by using the software GraFit, by Erithacus Software limited, version 7.0.2. and Microsoft excel add on ed50v10. Statistical analyses were performed using Graph pad software. Statistical analysis between treatment and control (wound scratch and colony formation assays) was determined using unpaired two-tailed Student's *t*-test. *P*-values<0.05 were considered significantly different from the control group. Statistical comparisons between the responses to doses of a test agent and control (MTT assay) were made using one way

Fig. 4 MTT assay on B16F10 cell line showing cytotoxicity of the serially diluted methanolic leaf extracts of *B. eriantha*. IC₅₀ value is 841.84 µg/ml



analysis of variance (ANOVA). When ANOVA demonstrated a significant difference between groups, a Tukey's test was performed to identify the doses that have induced a response relative to no treatment control.

Results and discussion

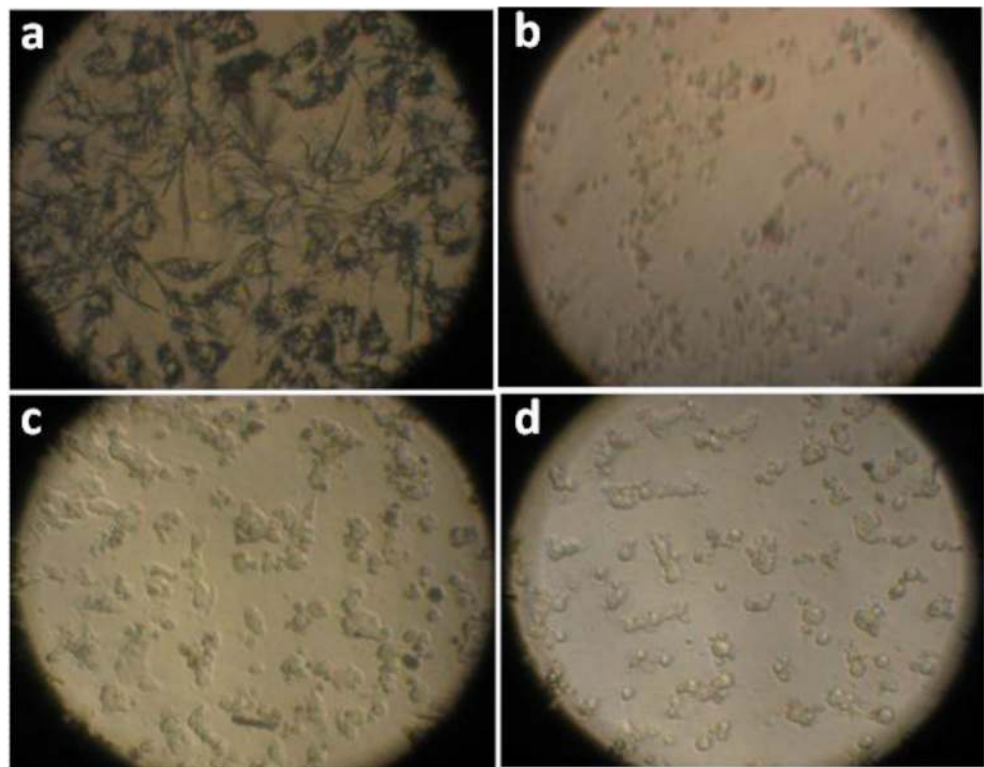
Antioxidant effect of the extract using DPPH radical scavenging activity and ferric ion reducing antioxidant power assay

The DPPH assay comprises the use of a stable free radical, 1,1-diphenyl-2-picrylhydrazyl. This is a simple, rapid and convenient method for screening of many samples for radical

scavenging activity. In its oxidized form, the DPPH radical has an absorbance maximum centered at about 520 nm and is blue in color (Marxen et al. 2007). It becomes colorless or pale yellow when neutralized which can be measured spectrophotometrically (Marxen et al. 2007). The DPPH free radical scavenging activity (RSA) of the different extracts has been depicted in Fig. 1. The % RSA was maximum in methanolic (97.90±0.24 %) followed by ethanolic (97.84±0.79 %) and aqueous (89.67093±1.45 %) extracts respectively.

FRAP is a simple, automated method for assessing the antioxidant activity of compounds. At low pH, when a ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex is reduced to the ferrous (Fe^{II}) form, by antioxidants, an intense blue color with an absorption maximum at 593 nm develops (Benzie and Strain 1996). Thus, the antioxidant activity of a sample can be

Fig. 5 MTT assay on B16F10 cells using methanolic leaf extracts of *B. eriantha*. **a** Control well with no cell death **b** well with extract concentration of 1 mg/ml showing a few crystals (viable cells) (**c** and **d**) wells with extract concentration of 2 mg/ml and 4 mg/ml respectively showing apoptotic cells



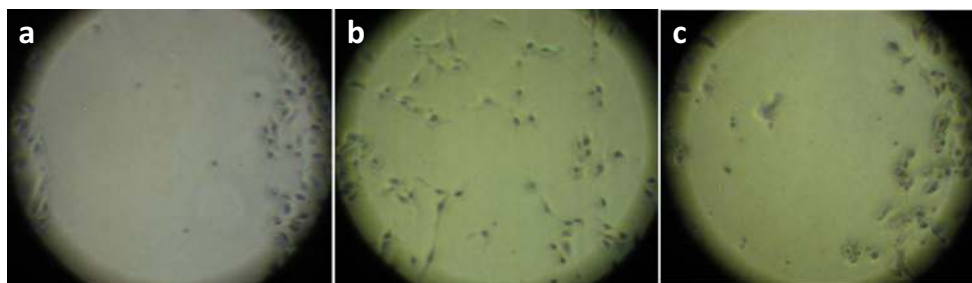


Fig. 6 Wound scratch assay on B16F10 cells using methanolic leaf extracts of *B. eriantha*. **a** scratch wound at time of scratching, $t=0$ **b** control plate 48 h after wounding, 34.41 % wound closure **c** test plate with 841.84 $\mu\text{l/ml}$ (IC_{50} value) of extract 48 h after wounding, 1.5 % wound closure

measured. The FRAP assay revealed that the methanolic extract had the maximum reducing capacity followed by the ethanolic extract and aqueous extracts (Fig. 2).

Estimation of total phenolic and total flavonoid contents

Phenols and polyphenolic compounds are widely found in plant sources, and have been shown to possess significant antioxidant activities (Ghasemi et al. 2009). The Folin-Ciocalteu reagent is sensitive to reducing compounds including polyphenols, thereby producing a blue color upon reaction, which can be measured using a spectrophotometer. Thus total phenolic content can be determined (Mongkolsilp et al. 2004). The total phenolic content in the different solvent systems is shown in Table 1. The total phenolic content was maximum in methanolic (1.345 mg GAE g^{-1}), followed by ethanolic (1.092 mg GAE g^{-1}) and aqueous (0.935 mg GAE g^{-1}) extracts respectively.

Flavonoids are a group of polyphenolic compounds naturally occurring in plants. Studies have established that flavonoids have biological properties which can lower the risk of coronary heart disease, neurodegenerative diseases, osteoporosis, and lung cancer (Lampila et al. 2009; Yao et al. 2004). The total flavonoid content in the different solvent systems is presented in Table 1. The absorbance obtained spectrophotometrically was compared to the standard curve of rutin concentrations and expressed as mg of rutin equivalents per g (mg RE g^{-1}). The total flavonoid content was maximum in methanolic (7.729 mg RE g^{-1}), followed by ethanolic (5.164 mg RE g^{-1}) and aqueous (2.029 mg RE g^{-1}) extracts respectively.

Cytotoxic effect of the extract on HeLa cells and B16F10 cells

The MTT assay is a colorimetric assay which measures changes in color to measure the activity of enzymes that reduce MTT to formazan, giving a purple color. Yellow MTT (3-(4,5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide, a tetrazole) reduces to purple formazan in living cells (Nisa et al. 2011). It is used to determine cytotoxicity of potential medicinal agents.

The cytotoxicity of the extract on HeLa cells and B16F10 cells expressed as % inhibition of the cells with different concentrations of the extract is shown in Figs. 3 and 4. The IC_{50} value of the extract on HeLa cells was 782.25 $\mu\text{g/ml}$ and on B16F10 cells it was 841.84 $\mu\text{g/ml}$. The apoptotic cells can be seen in Fig. 5. Studies have been done on other species of *Blumea*, to determine their anticancer properties. A study done on methanolic extract of *Blumea mollis* (MTT assay) has reported that the IC_{50} value on SK-MEL-28 cell lines was 6.31 mg/ml and 4.45 mg/ml on NCI-H-23 cell lines (Srikanth et al. 2012). It is evident from the results, that the methanolic extract of *Blumea eriantha* has more potent cytotoxic effect as compared to *Blumea mollis*.

Inhibition of cancer cell migration

Wound scratch assay or cell migration inhibition assay is a relatively simple and economical in vitro assay which establishes arrest in the cell proliferation and migration. It also mimics to some extent the migration of the cells in vivo (Rodriguez et al. 2005). Cell migration is initiated upon a scratch wound, which is followed by growth of cells at the edges of the wound in a direction perpendicular to the scratch wound. The extract of interest was added and images were captured after 24 h or 48 h interval. The migration of the cells was compared with those in control wells. The percentage of wound closure in control plates was approximately 34.41 % and in the plates in which the extract was added it was 1.5 %. The cells at the edges of the wound in extract treated plates exhibited apoptotic changes (Fig. 6).

Table 2 Colony formation assay on B16F10 cells: Number of colonies after 7 days in control group and test group with 841.84 $\mu\text{l/ml}$ (IC_{50} value) of methanolic leaf extract of *B. eriantha*

Treatment	Number of cells seeded	Number of colonies (after 7 days)
Negative control	750	16 \pm 1.52
Test plate with extract	750	0 \pm 0

Data shown as mean \pm SD, $n=3$

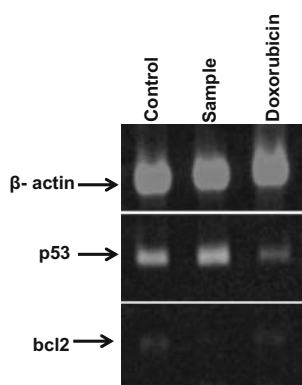


Fig. 7 Expression of housekeeping gene β actin, apoptotic gene p53 and antiapoptotic gene Bcl-2 in B16F10 cells treated with methanolic leaf extract of *B. eriantha* for 48 h. p53 level is upregulated and Bcl-2 is downregulated compared to negative control and doxorubicin. Representative data from three individual analyses have been shown here

Inhibition of colony formation

Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. Effectiveness of cytotoxic agents can be determined using this assay (Franken et al. 2006). The mean number of colonies in the treated plates were compared with the mean number of colonies in the untreated plates (colony count = $T/C \times 100$). A compound was considered active if it reduced the colony formation to less than 30 % of the control group value ($T/C \leq 30\%$) (Drees et al. 1997). The colonies in the treated plates were 0 % as compared to the control group which is less than 30 %. This suggests that the extract inhibits colony

formation. The number of colonies after 7 days have been depicted in Table 2.

Colonies in test group statistically significantly reduced as per Student's paired t test.

Confirmation of apoptosis by semiquantitative RT-PCR analysis

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a highly sensitive and specific method which can quantify specific RNA transcripts and detect expression level variation under different experimental conditions (Marone et al. 2001). The mRNA expression levels of p53 and Bcl-2 were carried out using semi-quantitative RT-PCR. The PCR products were analyzed using Image J software developed by NIH. The ratio between the sample RNA to be determined and the housekeeping gene β actin was calculated to normalize for initial variations in sample concentration and as control for reaction efficiency. The level of proapoptotic gene p53 is upregulated and antiapoptotic gene Bcl-2 is downregulated after addition of the extract as is evident from Fig. 7. The fold change ratio of sample: medium for p53 and Bcl-2 was 1.22 and 0.064 respectively.

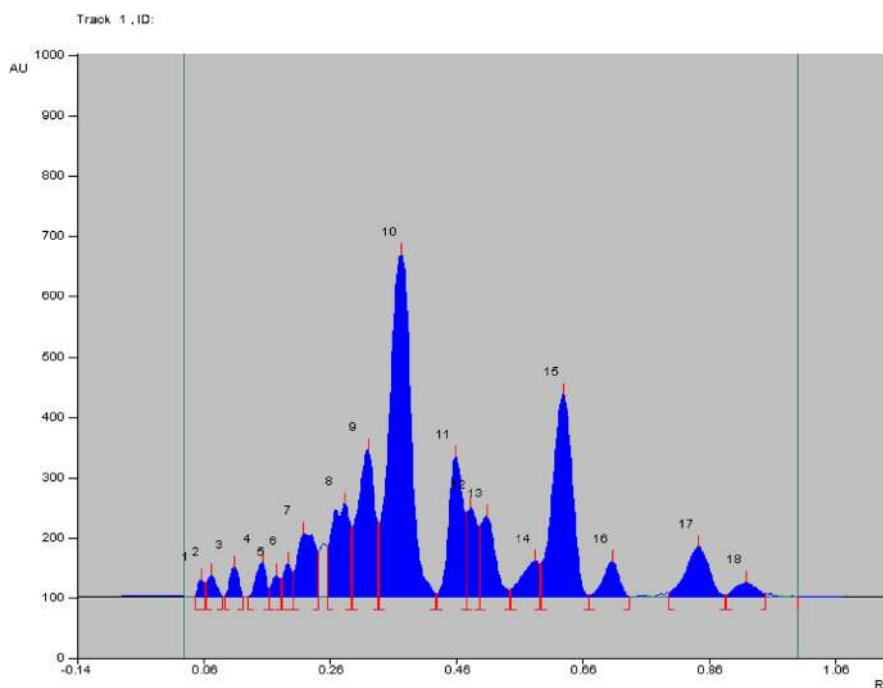
Separation and elution of compounds using HPTLC

High performance thin layer chromatography (HPTLC) is an inexpensive method for separation, qualitative identification or semi quantitative analysis of samples (Patel et al. 2012). HPTLC revealed several peaks as recorded in Table 3. The corresponding HPTLC chromatogram is presented in Fig. 8.

Table 3 HPTLC profile of the methanolic leaf extract of *B. eriantha*

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	Area %
1	0.05 Rf	2.3 AU	0.05 Rf	28.8 AU	1.19 %	0.06 Rf	23.7 AU	251.0 AU	0.48 %
2	0.06 Rf	24.7 AU	0.07 Rf	36.6 AU	1.52 %	0.09 Rf	1.5 AU	419.9 AU	0.81 %
3	0.09 Rf	1.3 AU	0.11 Rf	49.3 AU	2.04 %	0.12 Rf	0.8 AU	573.9 AU	1.10 %
4	0.13 Rf	0.2 AU	0.15 Rf	59.8 AU	2.48 %	0.16 Rf	12.3 AU	701.7 AU	1.35 %
5	0.16 Rf	13.0 AU	0.17 Rf	36.5 AU	1.51 %	0.18 Rf	30.2 AU	403.1 AU	0.77 %
6	0.18 Rf	31.2 AU	0.19 Rf	55.2 AU	2.29 %	0.20 Rf	40.1 AU	588.9 AU	1.13 %
7	0.20 Rf	40.1 AU	0.22 Rf	106.2 AU	4.40 %	0.24 Rf	75.0 AU	2429.9 AU	4.66 %
8	0.26 Rf	83.2 AU	0.28 Rf	154.5 AU	6.40 %	0.29 Rf	115.4 AU	3496.9 AU	6.71 %
9	0.29 Rf	115.4 AU	0.32 Rf	243.4 AU	10.09 %	0.34 Rf	121.8 AU	5304.1 AU	10.17 %
10	0.34 Rf	121.9 AU	0.37 Rf	567.1 AU	23.50 %	0.43 Rf	4.7 AU	15176.9 AU	29.10 %
11	0.43 Rf	5.1 AU	0.46 Rf	232.2 AU	9.62 %	0.47 Rf	138.7 AU	4158.6 AU	7.97 %
12	0.48 Rf	139.5 AU	0.48 Rf	148.1 AU	6.14 %	0.50 Rf	117.4 AU	2040.1 AU	3.91 %
13	0.50 Rf	118.6 AU	0.51 Rf	134.7 AU	5.58 %	0.54 Rf	13.2 AU	2437.6 AU	4.67 %
14	0.54 Rf	13.2 AU	0.58 Rf	59.7 AU	2.48 %	0.59 Rf	54.9 AU	1344.1 AU	2.58 %
15	0.59 Rf	55.6 AU	0.63 Rf	335.1 AU	13.89 %	0.67 Rf	3.4 AU	8504.4 AU	16.31 %
16	0.67 Rf	3.4 AU	0.71 Rf	58.8 AU	2.44 %	0.73 Rf	0.0 AU	1210.7 AU	2.32 %
17	0.80 Rf	7.0 AU	0.84 Rf	83.2 AU	3.45 %	0.89 Rf	3.0 AU	2449.7 AU	4.70 %
18	0.89 Rf	3.3 AU	0.92 Rf	23.9 AU	0.99 %	0.95 Rf	5.2 AU	659.0 AU	1.26 %

Fig. 8 HPTLC chromatogram of methanolic leaf extract of *Blumea eriantha* showing different peaks of phytoconstituents



The Rf values are in the range of 0.05 to 0.92. From the HPTLC plates, 9 bands were scraped, eluted with methanol and each band was tested for cytotoxicity by MTT assay. LC-MS was conducted on each band.

Cytotoxic effect of the eluted bands on B16F10 cancer cell line

MTT assay was performed on 9 bands eluted after HPTLC. All the bands exhibited positive cytotoxicity. The IC_{50} values of the bands have been explained in Table 4. Bands 5 and 6 exhibited a more potent cytotoxic effect with IC_{50} values of 87.18 $\mu\text{g/ml}$ and 76.31 $\mu\text{g/ml}$ respectively. A study done by Saewan et al., on methanolic extract of *Blumea balsamifera* in which 3 flavonoids were isolated which had cytotoxic effect on KB cells with IC_{50} value of 17.09, 47.72 and 17.83 $\mu\text{g/ml}$, however, there was no effect on MCF₇ cells (Saewan et al. 2011). In this study, bands 3 and 6 have comparable effects as that of the flavonoids isolated from *Blumea balsamifera*.

Characterization of compounds by LC-MS

In recent decade, liquid chromatography coupled with mass spectrometry (LC–MS) has become the most selective

technique for rapid screening and characterization of known and unknown constituents from plant extracts (Chen et al. 2011). Very good selectivity can be obtained due to the high power of mass separation of LC-MS (Hostettman et al. 1997). Preliminary screening of the eluted bands was done by LC-MS analysis to identify the compounds which could be responsible for the cytotoxic effect on the cancer cells. This led to the separation and identification of the constituents (Table 5), some of which have been reported to have cytotoxic activities. Bands 3 and 6 eluted from the HPTLC plate showed the most potent cytotoxic activities. Band 3 (Rf 0.29) had 7-Methylxanthine, 5-Methoxytryptophan, CAY10603, Quassamarin and band 6 (Rf 0.50) had Chryso-splenol C, Buthionine sulfoximine as its constituents which are known to have anticancer properties. The presence of these compounds could be responsible for a more potent cytotoxic effect on cancer cells.

Conclusion

The present work demonstrates presence of high phenolics and flavonoids in *B. eriantha* DC. leaf methanol extract as well as indicated free radical scavenging activity towards

Table 4 Cytotoxicity assay (MTT assay) done on B16F10 cells using 9 bands of methanolic leaf extract of *B. eriantha* eluted by HPTLC

HPTLC bands	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9
IC_{50} value ($\mu\text{g/ml}$)	539.70	168.97	817.64	125.58	87.18	76.31	138.10	201.55	336.56

IC_{50} values of the bands suggest that band 5 and 6 with IC_{50} values of 87.18 $\mu\text{g/ml}$ and 76.31 $\mu\text{g/ml}$ respectively are more potent

Table 5 Compounds present in methanolic leaf extract of *B. eriantha* identified by LS-MS reported to have cytotoxic activity against cancer cells

Compound	CAS number	R _f , min	Mass	Area	Height	Annotations	References
Liatrin	34175-79-6	11.522	418.1609	244,918	43,059	Antileukemic sesquiterpene lactone.	(Patel et al. 2010; Morris et al. 1973)
Cis-Zeatin	32771-64-5	0.839	219.1129	1,288,617	474,484	Phytohormone having cytotoxic activities.	(Voller et al. 2010)
Buthionine sulfoximine	5072-26-4	2.424	222.1031	4,275,638	853,681	Inhibitor of glutathione synthesis. Growth-inhibitory activity in different tumor cell lines. Highest cytotoxic activity against melanoma-derived cell lines and in vivo antitumor activity in B16 melanoma-bearing mice.	(Joseph et al. 1992)
12-hydroxyjasmonic acid	140631-27-2	6.974	226.1225	2,631,035	694,551	Prevents proliferation and induces death of malignant cells.	(Flescher 2005)
Chrysoptanol C - (3,7,3'-trimethoxy-5,6,4'-trihydroxyflavone)	23370-16-3	360.0864	9.874	9,025	4,543	Moderate cytotoxicity against breast cancer and human small cell lung cancer cell lines.	(Huang et al. 2005; Kanlayavattanakul et al. 2003)
CAY10621- [2,2-dimethyl-4S-(1-oxo-2-hexadecyn-1-yl)-1,1-dimethylethyl ester-3-oxazolinedicarboxylic acid]	120005-55-2	435.3354	14.427	133,838	44,229	Selective inhibitor of sphingosine kinase 1, thus inhibiting tumor growth.	(Shida et al. 2008; Wong et al. 2009)
7-Methylxanthine	552-62-5	166.0492	0.704	4,520,234	1,453,114	Tumorigenesis and teratogenesis greatly inhibited by 7-methylxanthine.	(Nomura 1983)
5-Methoxytryptophan	2504-22-5	234.1021	3.814	3,486,773	531,473	Anticancer properties by blocking cancer cell cyclooxygenase-2 overexpression.	(Cheng et al. 2012)
CAY10603-(N-[4-[3-[[[7-(hydroxyamino)-7-oxoheptylamino]carbonyl]-5-isoxazolyl]phenyl]-1,1-dimethylethyl ester])	1045792-66-2	446.2169	5.81	958,396	301,508	Suppresses A549 migration and invasion, reduced tumor growth and cancer metastasis in a murine xenograft tumor model. Potent inhibitor of several pancreatic cell lines by inhibiting histone deacetylase 6.	(Kozikowski et al. 2008; Patrick et al. 2011)
Quassimarin	59938-97-5	522.2124	6.558	2,013,565	911,869	Significantly active in the suppression of the growth of a panel of human tumor cell lines (KB, A-549, HCT-8, CAKI-1, MCF-7, and SK-MEL-2).	(Kupchan and Streehman 1976; Xu et al. 2000)
AG-494- (E)-2-Cyano-3-(3,4-dihydroxyphenyl)-N-phenyl-2-propenamide.	133550-35-3	280.0849	12.021	368,012	64,482	Antileukemic properties and antitumor activity in mice. Inhibits the growth of A549 and DU145 cancer cell lines by blocking Cdk2 (cyclin dependent kinase 2) activation, induces growth arrest of immortalized cells at G1-S and early S and is very effective in arresting the growth of EGFR (epidermal growth factor receptor) overexpressor cells. Potently inhibited the proliferation of Jurkat cells.	(Agnieszka et al. 2012; Ben-Bassat et al. 1999; Kleinberger-Doron et al. 1998; Osheroov and Levitzki 1997)
2-Naphthylalanine	58438-03-2	215.0926	1.18	21,533	8,867	Sesquiterpene having cytotoxic activities.	(Sasaki et al. 2008)
Graphinone	19683-98-8	296.1632	12.633	300,697	114,776		(Harborne et al. 1999; Martin 2010)
Etanidazole	22668-01-5	214.0712	0.599	1,468,382	682,831	Nitroimidazole drug used for its radiosensitizing properties, by decreasing glutathione concentration and inhibiting glutathione S-transferase. Administration of etanidazole causes the tissues to become more sensitive to the ionizing radiation used in cancer treatment.	(Abe et al. 2011; Stone et al. 1991; Yang et al. 2012)

DPPH and FRAP. Additionally, this plant as evident from our studies has cytotoxic activity in HeLa and B16F10 cancer cell lines along with potential anti metastatic effect and cell death by apoptosis. These favorable characteristics of this plant open further avenues for exploring the prospective of *Blumea* for effective treatment against cancer. Furthermore, remarkable progress in molecular biology and analytical instrumentation will help to extract the metabolite of interest from *Blumea eriantha* for its wider applications more precisely and accurately. The list of plants having anti cancer properties is increasing in which *Blumea* will be a plant of prime position in the near future.

Cancer is a dreaded disease and leading cause of death worldwide. Researchers are trying to establish effective treatments for the containment of this dreaded disease.

Blumea eriantha is an easily available natural material and isolation of anticancer compounds from this plant would prove to be a valuable source for the treatment of cancer.

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