ISSN 2063-5346

WOUND HEALING ASSAY AND CELL VIABILITY ASSAY OF ETHANOLIC LEAF EXTRACT OF BLUMEA BALSAMIFERA AGAINST HUMAN COLORECTAL CARCINOMA CELL LINE

V. Sethumadhavan[1], K. Shakthi Sri[2], M. Saran[3], Dr.V. Jayashree[4*]

==========

Article History: Received: 19.04.2023 Revised: 02.05.2023 Accepted: 10.06.2023

Abstract

The colorectal cancer has been second leading cause of cancer that affects both men and women globally. It indicates approximately 10% of malignant growth –related mortality in Western countries The present study involves wound healing assay and cell viability assay of ethanolic leaf extract of blumea balsamifera against human colorectal carcinoma cell line.

The leaf part of Blumea balsamifera was subjected to maceration process using ethanol solvent. The collected crude extract was analysed for Qualitative and quantitative phytochemical analysis, Cell Viability Assay and Wound healing assay.

The presence of phytochemicals such as alkaloids, flavonoids, and phenols were identified by qualitative phytochemical screening. Quantitative phytochemical analysis of leaf extract revealed phenol of approximately 141 mg/g, alkaloids of approximately 105 mg/g of atropine equivalents, and flavonoids of approximately 120mg/g of quercetin equivalents. The In vitro Cytotoxicity studies of ethanolic leaf extract of Blumea balsamifera showed IC50 value was found to be 208.25 µg/ml. The in-vitro wound healing activity studies signified with the migration of cells play a vital role in wound repair. It was evidenced that the Blumea balsamifera extract showed 60.18% and 90.89% cell migration observed at 24 h and 48 h, respectively

Keyword: *Colon Cancer, Blumea Balsamifera, Maceration, MTT Assay, Wound Healing.*

[1,2,3] B pharmacy, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai.

[4*] Department of Pharmacology, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai.

[4*] mailtovjayashree@gmail.com

DOI: 10.48047/ecb/2023.12.si12.059

1. Introduction

According to the World Health Organisation, cancer accounted for 9.6 million deaths globally in 2018 and was the second largest cause of death globally. Around one in six people worldwide die from cancer, making it the most common cause of mortality. Deaths from cancer account for 70% of all deaths worldwide, mostly in low- or middle-income nations. Diagnosis of colorectal cancer weren't widespread until recent years. It is currently the fourth deadliest cancer in the world, with 900 000 fatalities per year. In addition to the aging population and the eating habits of high-income countries, unfavourable risk factors for colorectal cancer include obesity, a lack of physical exercise, and smoking. [1]

Colon cancer, commonly known as colorectal cancer (CRC), is the second-deadliest type of malignant tumour in the world and the third-most common. The most deadly cancer for both sexes is colorectal cancer, which also ranks as the third most common diagnosis. With the exception of those under 50 years old, the incidence of new cancer cases and mortality has been steadily declining over the past decade, likely due to an increase in cancer screening and enhanced treatment. [2]

Antioxidant, wound-healing, and anti-inflammatory effects in medicinal plants are supported by the structural and functional variety of phytochemicals.[3] Tumours may block the entire lumen and cause intestinal obstruction. Constipation, stomach pain, vomiting, and abdominal distension are the main symptoms of this condition. Iron deficiency anaemia may result from continuous occult bleeding brought on by a tumour; symptoms include weariness, palpitations, and a pallid aspect to the skin. Additionally, colorectal cancer may cause weight loss, typically as a result of a decreased appetite. [4]

A flowering plant in the genus *Blumea* of the family Asteraceae is called *Blumea balsamifera*. Sambong (sometimes spelt sembung) and Ngaicamphor are other names for it. For the most part, the Philippines B.[5]

Balsamifera is called sambong in Tagalog (spoken with only vowels as sahm-BOHNG), bukadkad in Visayas, and subusob, subsub, or sobsob occasionally in Ilocos. Its main applications are as a diuretic (or "water pill") and to alleviate cold symptoms. Sambong treats various cold symptoms by acting as an expectorant, an anti-diarrheal, and an antispasmodic. Sambong's primary ingredients exhibit a variety of biological properties, including anti-tumor, hepatoprotective, superoxide radical scavenging, antioxidant, antibacterial and anti-inflammation, anti-plasmodial, anti-tyrosinase, platelet aggregation, wound healing, and anti-obesity.[6]

2. Materials and Methods

Collection and Identification of Plant Material

The accumulated plant materials were cleaned with running water from the tap to remove any contaminants from the ground, and they were then allowed to air dry for two to three weeks before being ground into a coarse powder and used for additional experiments. [7]

Chemicals

Southern India Scientific Corporation in Chennai, India provided the chemicals and solvents utilised in the extraction process.

Extraction Method

100gm of powdered sample was taken in conical flask and 1000ml of ethanol was added to it which was subjected to maceration extraction method for about 3 to 4 days. The collected liquid was evaporated under rotary evaporator and the extract was collected. The collected crude extract was subjected to further analysis.

Qualitative Phytochemical Analysis

All the plant extracts were analysed for the phytochemical screening by using the following standard methods. [8]

Test for Alkaloids: The extract was dissolved in dilute Hydrochloric acid individually and filtered

a) **Mayer's Test:** Few drops of filtrate was added to a little amount of Mayer's Reagent (Potassium Mercuric Iodide) along the sides of the test tube. The Alkaloid was confirmed

by the formation of yellow or cream coloured precipitate.

b) **Wagner's Test:** Few ml of Wagner's reagent (Iodine in Potassium Iodide) was added to a small amount of filtrate. The presence of alkaloids was confirmed by the appearance of reddish-brown precipitate.
c) **Dragendroff's Test:** To the filtrate, Dragendroff's reagent (Potassium Bismuth Iodide solution was added. Orange brown precipitate was formed which indicates the presence of alkaloids.
d) **Hager's Test:** To the filtrate, Hager's reagent (saturated picric acid solution) was added. Presence of alkaloids was confirmed by formation of yellow coloured precipitate.

Test for Flavonoids

Shinoda Test: 2-3ml of the extract was taken in the test tube. A piece of magnesium ribbon and 1ml of Hydrochloric acid (Hcl) was added and the test tube was heated in a water bath. Formation of pink red colour or red colour was the indication for the presence of flavonoids.[9]

Test for Glycosides: To the extracts, dilute Hydrochloric acid was added and then subjected to the following tests.

Borntrager's Test: 200mg of extracts was boiled with 200ml dilute sulphuric acid and filtered. Pipette out the supernatant, cool and shake with equal volume of diethyl ether. Separate the ether layer and shake with half its volume of dilute ammonia. Formation of rose pink or red colour in the ammonical layer indicates the presence of athracene glycosides.

Keller Killiani Test: Few amounts of the extract was dissolved in 4ml of glacial acetic acid with 1 drop of 2% FeCl3 mixture and 1ml of concentrated sulphuric acid. A brown ring is formed between the layers that indicate the presence of cardiac glycosides.

Test for Terpenoids

Salkowski's Test: 1ml of chloroform was added to the 1mg of the extract followed by a few drops of concentrated sulphuric acid. Formation of reddish-brown precipitate indicates the presence of Terpenoids (triterpenes).

Copper Acetate Test: Extracts were dissolved in water and added to 3-4 drops of copper acetate solution. Formation of emerald green colour shows the presence of diterpenes.

Test for Steroids and Sterols

Liebermann Burchard's Test: 5mg of extract was treated with 2ml of chloroform and 2ml of Conc. H_2SO_4 was added along the sides of the test tubes. The lower fraction turns yellow with green fluorescence and the upper layer turns red that indicates the presence of steroids and sterols.

Test for Phenols

Ferric Chloride Test: 1-2ml of extract was treated with 3 to 4 drops of ferric chloride solution. A bluish black colour was formed which indicates the presence of phenols.

Test for Tannins

Gelatin Test: 1% gelatin solution containing sodium chloride was added to the extract. Formation of white precipitate shows the presence of tannins.[10]

3. Quantitative Analysis of Phytochemicals

Estimation of Alkaloids

To 1ml of test extract 5 ml pH 4.7 phosphate Buffer was added and 5 ml BCG solution and shake a mixture with 4 ml of chloroform. The extracts were collected in a 10-ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without extract. Atropine is used as a standard material and compared the assay with Atropine equivalents.

Estimation of Flavonoids

Total flavonoid content was determined by the Aluminium chloride method using catechin as a standard. 1ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 min 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added. After 6 min incubation at room temperature, 2 ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as catechin equivalents (mg catechin/g dried extract).

Estimation of Phenolic Compounds

The total phenolics content in different solvent extracts was determined with the Folin- Ciocalteu's reagent (FCR). In the procedure, different concentrations of the extracts were mixed with 0.4 ml FCR (diluted 1:10 v/v). After 5 min 4 ml of sodium carbonate solution was added. The final volume of the tubes were made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of the sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using catechol solutions as standard and total phenolic content of the extract was expressed in terms of milligrams of catechol per gram of dry weight and the standard graph.

Cell Viability

The percentage of alive, healthy cells in a sample is known as cell viability. Cell viability assays are used to assess the physical and physiological well-being of cells in response to extracellular stimuli, chemical agents, medicinal treatments, or other situations [1-3], as well as when deciding on the best conditions for cell culture growth.

Preparation of Cell Suspension

A subculture of HCT 116 cells in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To the disaggregated cells in the flask, 25 mL of DMEM with 10% FCS was added. The cells were suspended in the medium by a gentle passage with the pipette and the cells homogenized.

Seeding of Cells

One mL of the homogenized cell suspension was added to each well of a 96-welll culture plate along with the different concentrations of *blumea* extract (0 to 200 µg/mL) and incubated at 37°C in a humidified CO2 incubator with 5% CO2. After 48 hrs incubation, the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells, cytotoxic assay was carried out.

Cytotoxicity Assay

The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells,

yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48 h incubation, the wells were added with MTT and left for 3 h at room temperature. All wells have removed the content using a pipette and 100µl SDS in DMSO was added to dissolve the formazan crystals, absorbance was read in Lark LIPR-9608 microplate reader at 540 nm (Mosman et al 1983).[11]

In Vitro Wound Scratch Assay

The migration rates of HCT 116 cells were assessed by the scratch assay method (Harishkumar et al., 2013). The cell density of "2 × 105 cells" was seeded into each well of a 24-well plate and incubated with a complete medium at 37°C and 5% CO2. After 24 h of incubation, the monolayer confluent cells were scrapped horizontally with a sterile 200 µl pipette tip. The debris was removed by washing with PBS. The cell without treatment was used as the control. The scratch induced that represented the wound was photographed at 0 h using phase-contrast microscopy at × 40 magnification at 0 h, before incubation with the test and standard samples. After 24 h and 48 h of incubation, the second and third set of images was photographed. To determine the migration rate, the images were analyzed using "ImageJ" software, and the percentage of the closed area was measured and compared with the value obtained at 0 h. An increase in the percentage of the closed area indicated the migration of cells. [12]

Wound closure (%) =
(Measurement at 0 h−Measurement at 24 h or 48 h)/Measurement at 0 h × 100

4. Results and Discussion

Qualitative Phytochemical Analysis

Qualitative phytochemical screening of leaf extract of *Blumea Balsamifera* revealed the presence of major phytochemicals constituents such as Alkaloids, Flavonoids and Phenols.

Table 1: Qualitative Phytochemical Analysis of Ethanolic Leaf Extract of *Blumea Balsamifera*

Sl. No	Phytochemical Constituents	Inference
1	Alkaloids	Present
2	Flavonoids	Present
3	Glycosides	Absent
4	Terpenoids	Absent
5	Steroids	Absent
6	Phenols	Present
7	Tannins	Absent

Quantitative Phytochemical Analysis

Quantitative phytochemical analysis studies of leaf extract of *Blumea Balsamifera* showed the Alkaloids of about 105 mg/g of Atropine equivalents, Flavonoids of about 120 mg/g of Quercetin Equivalents, Phenol of about 141 mg/g.

Table 2: Quantitative Phytochemical Analysis of Ethanolic Leaf Extract of Blumea Balsamifera

Sl. No	Metabolites	Quantity (mg/g dry weight of extract)
1.	Alkaloids	105mg of Atropine Equivalents
2.	Flavonoids	120mg of Quercetin Equivalents
3.	Phenols	141 mg of Tannic acid Equivalents

Cytotoxicity Assay

The in-vitro cytotoxicity activity results of the plant extract against HCT 116 cells were inhibited marginally, however, the increasing sample concentrations showed a little increment of cytotoxicity and it was clearly observed in the results (Table 1). It was evident that the samples tested at high as 200 µg/ml showed high cytotoxicity activity as high as 56.20% against HCT 116 cells. It was proven that the high cytotoxicity effect of the test sample showed no cell disintegration after 48 h of treatment against the selected tested cell lines even at higher concentrations (Figure 1 to 4). The IC50 of the tested sample *Blumea* extract against HCT 116 cells were calculated as 208.25 µg/ml (Table 3).

Table 3: In Vitro Cytotoxicity Effect of Blumea Balsamifera Extract Against HCT 116 Cell Line

Concentrations (µg/ml)	Cell Viability (%)
0	100.00
12.5	93.48
25.0	80.16
50.0	77.20
100.0	66.08
200.0	56.20
IC50	**208.25µg/ml**

Figure 1: Cytotoxicity effect of Blumea extract against HCT 116 Cell lines

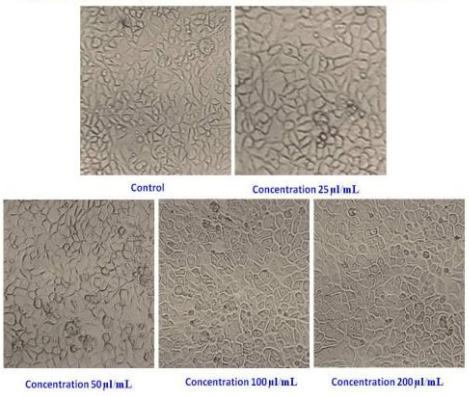


Figure 2: Cytotoxicity effect of Blumea Extract against HCT 116 Cell lines

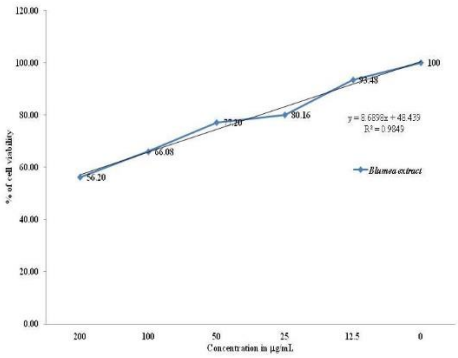


Figure 3: Effect of Blumea extract on wound healing against HCT 116 Cell lines

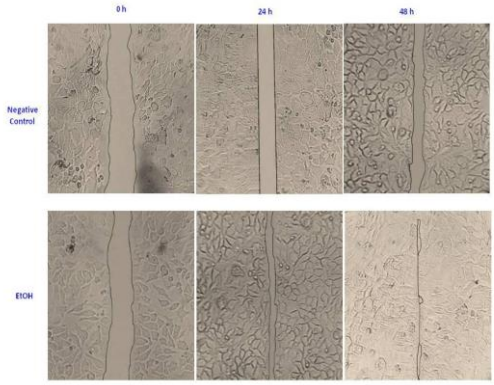
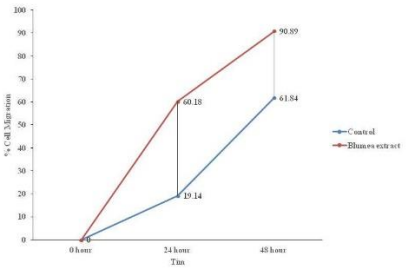


Figure 4: Effect of Blumea extract on cell migration against HCT 116 Cell lines



Wound Healing Assay

The in-vitro wound healing activity studies signified with the migration of cells play a vital role in wound repair. Cell scratch assay was performed to evaluate the wound healing potential of test samples and the minimal active concentration was chosen from the cell viability experiments. After 24 h and 48 h exposure to the test samples, it was observed that the cell migrates towards the provisional gap induced. The values given were calculated based on the scratch coverage rate in the 24 and 48 hours. It was evidenced that the *Blumea* extarct showed 60.18% and 90.89% cell migration observed at 24 h and 48 h, respectively (Table 4).

Table 4: Wound Healing Effect of Ethanolic Leaf Extract of Blumea Balsamifera Against HCT 116 Cell Line

Time	Control	Ethanolic leaf extract of *Blumea balsamifera*
0 hour	0	0
24 hours	19.14	60.18

5. Summary and Conclusion

In traditional medicine, the plant's primary source should be evident and certain. Thus, the Chinese Materia Medica and Flora Republicae Popularis Sinicae should be used to study the overall botanical description. The origins of the plant and medical activity of *Blumea Balsamifera* were then confirmed by additional herbal authentication work. These investigations verified the precision and originality of *B. balsamifera's* source.

The results of the current study demonstrated the nutritional value of *Blumea Balsamifera* leaf extract.It is rich in phytochemicals such as Alkaloids, Flavonoids, and Phenols according to qualitative phytochemical screening.

A quantitative examination revealed that the number of Alkaloids, Flavonoids and Phenols in the ethanolic extract is higher.

In-Vitro cytotoxicity assay (MTT assay) was performed and the IC50 value of the extract was calculated. The IC50 of the ethanolic leaf extract of Blumea *balsamifera* against HCT 116 cells were calculated as 208.25 µg/ml.

The in-vitro wound healing activity studies signified with the migration of cells play a vital role

in wound repair. It was evidenced that the *Blumea balsamifera* extract showed 60.18% and 90.89% cell migration observed at 24 h and 48 h, respectively

References

[1] Colorectal cancer. (2019). *The Lancet Journal*, *394*(10207).

[2] Lotfollahzadeh, S., Recio-Boiles, A., & Cagir, B. (2022). *Colon Cancer - StatPearls - NCBI Bookshelf*. Colon Cancer - StatPearls - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK470380/

[3] Ghuman, S., Ncube, B., Finnie, J., McGaw, L., Mfotie Njoya, E., Coopoosamy, R., & Van Staden, J. (2019). Antioxidant, anti-inflammatory and wound healing properties of medicinal plant extracts used to treat wounds and dermatological disorders. *South African Journal of Botany*, *126*, 232–240.

[4] Rathva, Bhargavi & V, Shreyas. (2020). Colorectal cancer: Etiology, pathogenesis and current treatment. 7. 20-24.

[5] Sambong listing at Stuart X Change list of Medicinal plants Accessed December 25, 2009.

[6] Pang, Y., Wang, D., Fan, Z., Chen, X., Yu, F., Hu, X., Wang, K., & Yuan, L. (2014). Blumea balsamifera—A Phytochemical and Pharmacological Review. *Molecules*, *19*(7), 9453–9477.

[7] Reddy DS. Phytochemical Analysis of Active Constituents of Alstoniascholaris and their Cytotoxicity in vitro. International Journal of Pharmaceutical Sciences and Research. 2016;7(8):32-62.

[8] Dhruti M, Bhavika P, Meonis P. Studies on phytochemical constituents and antioxidant activity of Alstoniascholaris. International Journal of Life Science. 2016;4 (4):529-38

[9] Gonzalez, R.J. and Tarloff, J.B. (2001) Evaluation of hepatic sub cellular Fractions for alamar blue and MTT reductase activity. Toxicology in vitro.15, 259-9.

[10] Crouch, S.P.M. et al. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxcity. J. Immunol. Meth.160, 81–8.

[11] Tim Mosmann. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of lmmunological Methods, 65 (1983) 55-63.

[12] M. Harishkumar, Y. Masatoshi, S. Hiroshi, et al., Revealing the mechanism of in vitro wound healing properties of citrus tamurana extract, BioMed Res. Int. (2013) 2013.

[13] Zinna Marie P Rasonabe, Jannelle D Cruz, Charlene G Tiausas, Francheska Areza. Chemical composition and antimicrobial activity of the extracts and essential oil of *Blumea balsamifera* from the Philippines. Int J Herb Med 2023;11(1):06-14.

[14] Xin Li, Mei Huang, Zhenxia Chen et al. Analysis of genetic diversity, population structure and its association with quality traits in germplasm of *Blumea balsamifera* (L.) DC. based on EST-SSR markers, 14 March 2023, PREPRINT (Version 1) available at Research Square.

[15] Zinna Marie P Rasonabe, Jannelle D Cruz, Charlene G Tiausas, Francheska Areza. Chemical composition and antimicrobial activity of the extracts and essential oil of *Blumea balsamifera* from the Philippines. Int J Herb Med 2023;11(1):06-14.