**Scholars Academic Journal of Pharmacy (SAJP)** **ISSN 2320-4206**
**Sch. Acad. J. Pharm., 2013; 2(3):190-194**

(An International Publisher for Academic and Scientific Resources)
www.saspublisher.com

## Research Article

# Phytochemical analysis and Cytotoxicity studies of *Bryophyllum calycium* in BHK-21 cells

**A. Joshi A* and Chauhan RS**
Institute of Biotechnology, G B Pant University of Agriculture & Technology, Patwadangar-263128, Distt. Nainital, Uttarakhand, India

***Corresponding author**
**Joshi A**
Email: joshi.ankita11@gmail.com

**Abstract:** *Bryophyllum calycium* is a common medicinal plant used in traditional medicine of India founded in the region of Uttarakhand near Nainital, for curing various infections, bowel diseases, healing wounds and other ailments. However, its anticancer properties are poorly defined so this was done to extract its anticancer property against BHK-21 cells. This study is a step towards evaluation of the plant against cancer. Methanolic, ethanolic and aqueous extract of the leaves of *Bryophyllum calycium* were screened for their anticancer properties. The cells were seeded with all the extracts and then allowed to grow for 24hrs, the cell growth was inhibited and rounding and clumping of cells were observed within 24hrs. Ethanolic and Aqueous extracts showed better response then that of its methanolic extract. The concentration of 10 mg/ml of ethanolic extract inhibited the cancerous growth with high affinity. The extracts inhibited the growth of the cancerous cell lines.
**Keywords**: BHK-21 cells, anticancer, cell lines..

## INTRODUCTION

*Bryophyllum pinnatum* (Lam.) Kurz (Crassulaceae) Synonym: Kalanchoe pinnata, Pers, Bryophyllum Calycinum Salisb [1]; Common names: Zakham-e-hyat, Life plant, air or maternity plant, love plant, Canterbury bells, Cathedral bells, parnabija etc. [2]. It is a perennial herb growing widely and used in folkloric medicine in tropical Africa, tropical America, India, China and Australia, classified as a weed [3]. The plant flourishes throughout the Southern part of Nigeria [4]. This is the only Kalanchoe species found in South America, however, 200 other species are found in Africa, Madagascar, China and Java. A number of species are cultivated as ornamentals and are popular tropical house plants. In Brazil, the plant goes by the common names of saiao or coirama and in Peru it is called hojadelaire (air plant) or kalanchoe [5].

The plant grows all over India in hot and moist areas, especially in Bengal and Uttarakhand . It is a succulent perennial plant that grows 1-1.5 m in height and the stem is hollow four-angled and usually branched. Leaves are opposite, decussate, succulent, 10-20 cm long. The lower leaves are simple, whereas, the upper ones 3-7 foliate and are long-petioled. They are fleshy dark green that are distinctively scalloped and trimmed in red. Leaf blade pinnately compound with 3-5 leaflets, 10-30 cm; petiolules 2-4 cm; leaflet blades oblong to elliptic, 6-8 X 3-5 cm, margin crenate with each notch bearing a dormant bud competent to develop into a healthy plantlet [6] , apex obtuse. The leaves are furnished with rooting vegetative buds. Inflorescences terminal paniculate 10-40 cm. Flowers are many bell-like pendulous. Calyx tubular, 2-4 cm; Corolla reddish to purple, 5 cm, base sparsely ciliate; lobes ovate-lanceolate; stamens inserted basally on corolla; nectar scales oblong; follicles included in calyx and corolla tube. The fruit-pod with four septa and numerous, ellipsoid, smooth striate seeds within. The plant flowers in Nov-Mar and fruits in April [7].

The leaves of *Bryophyllum calycium* plant have been reported to possess antimicrobial [8], antifungal [9], anti-ulcer [10- 11], anti-inflammatory and analgesic [12-13], antihypertensive [14], antidiabetic [15] and antimutagenic activities [16]. A number of active compounds, including flavonoids, glycosides, steroids, bufadienolides and organic acids have been identified in *B. calycium* [17] that have been shown individually to possess variety of activities such as antibacterial, antitumor, cancer preventive and insecticidal actions. The flavonoid glycoside, Quercitrin (quercetin 3- O- alpha- L-rhamnopyranoside) and skapinnato side were isolated with anti-leishmanial activity [18]. Juice of the fresh leaves is used very effectively for the treatment of jaundice in Bundelkhand region of India. It was studied that the juice of leaves was found more effective than ethanolic extract as evidenced by *invivo* and *invitro* histopathological studies for hepatoprotective activity of plant and justifies the use of juice of plant leaves in folk medicine for jaundice. The protective effect on gentamicin-induced nephrotoxicity in rats which may involve its antioxidant and oxidative radical scavenging activities. It is also used for the treatment of kidney stones in India where is goes by the name of Pather Chat or Paan-futti . The Quercetin has neproprotective and antioxidant role [19].

The fatty acids present in *Bryophyllum calycium* may be responsible atleast inpart for its immunosuppressive effect *in-vivo*. RossiBergmann et.

al; showed the aqueous extract of leaves cause significant inhibition of cell-mediated and humoral immune responses in mice. The spleen cells of animals pre-treated with plant extract showed a decreased ability to proliferate in response to both mitogen and antigen *in-vitro*. Treatment with extract also impaired the ability of mice to mount a delayedtype hypersensitivity reaction (DTH) to ovalbumin. The *in-vitro* and topical routes of administration were the most effective by almost completely abolishing the DTH reaction. The intraperitoneal and oral routes reduced the reaction by 73% and 47% of controls, respectively. The specific antibody responses to ovalbumin were also significantly reduced by treatment.Thus, the aqueous extract of leaves possesses immunosuppressive activities. In an investigation it was also found that leaf extracts inhibited *in-vitro* lymphocyte proliferation and showed *in-vivo* immunosuppressive activity. From the ethanolic extract a purified fraction (KP12SA) found twenty-fold more potent to block murine lymphocyte proliferation than the crude extract. Thus provides evidence that saturated fatty acids present in herb plays an important role on lymphocyte proliferation, which explain its immunosuppressive effect *in-vivo* [20], Despite having broad spectrum therapeutic potential, *B.calycium* anticancer activity in BHK-21 cells have not been explored as yet.

Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments. This Wonder plant or Divine plant Leaf, Stem and Root portions and its chemicals has high index in therapeutic values. In view of absence of anti-cancer therapeutic for prevention and treatment of cancer, in the present study, we examined leaves of *Bryophyllum calycium* for presence of anti-cancer activity against BHK-21cells.

## MATERIALS AND METHODS

### Collection of Material and its Processing

Plant has been selected from high altitudearea (1600m from sea level) from the polyhouse nursery of Institute of Biotechnology,Patwadangar (Nainital), Uttarakhand and leaves of*Bryophyllum calycium*were collected and werewashed with tap water thrice. Washing wasagain repeated five times by using distilledwater. Then the leaf samples were air driedand thereafter kept in incubator at $37^0$C for24 hrs. The dried plant material was thencrushed in mechanical grinder in order tomake fine powder which was stored at roomtemperature for further use.

### Extract Preparation

### Aqueous Extract

The aqueous extract was prepared accordingto the standard method withslight modifications. Now, 5 g of leafpowder was mixed in 120 ml of water andwas kept in incubator shaker at 36˚C and 100 rpm. The extract so obtained was evaporated to drying through heating in a china dish. Dry extract was then scrapped off, weighed and reconstituted in normal saline [21].

### Solvent Extraction

Ethanol and methanol extracts were prepared in Soxhlet's apparatus. Soxhlet's extraction was carried out at room temperature. Dried Leaves of Bryophyllum calycium weighed accurately 5gm and taken in thimble and subjected to extraction in a Soxhlet's apparatus at room temperature using ethanol (150ml) and methanol (165ml) [22]. The extract obtained was first filtered using Whatman No. 1 filter paper and solvent was then removed under reduced pressure in a vacuumed rotary evaporator and dried. The dried extract was stored in air tight containers for further studies.

For the present study, BHK-21 cells were cultured in 24 well sterile polysterene plates using GMEM media supplemented with 5% fetal bovine serum as per standard procedure. The cells were seeded into 24 well sterile polysterene plates and were incubated for 24 hours at 37ºC. Thereafter, the medium was removed and 0.5ml of each dilution (10mg, 1mg,10µgm) of each extracts added to the assigned wells, Control well were also kept (medium without test sample) and triplicate sets of each dilution were maintained. Finally the cells were incubated for 24 hours at 37ºC and thereafter,examined under inverted microscope for their morphological studies.

### Confirmatory Tests

### MTT Assay

The MTT Assay is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) into a dark formazan product that is insoluble in water.

The amount of formazan produced is directly proportional to the cell number in a range of cell lines. It was performed as, prepared an MTT stock solution of 5 mg ml- 1 in phosphate-buffered saline (PBS), pH 7.5, and filter through a 0.22-µ filter to sterilize and the small amount of insoluble residue was removed. Add 10 µl of MTT (5mg ml-1), after 24 h of incubation and the cells were further incubated in incubator at 37°C for 3 h. Then 100 µl 0.04 M HCl in propan-2-ol to each well were added and mixed thoroughly to dissolve insoluble blue formazan crystals. The Plates were read on a micro-ELISA reader using a test wavelength of 570 nm [23].

### Neutral Red Assay

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a water soluble, weakly basic, supravital dye that accumulates in

lysosomes of viable cells. The neutral red (NR) assay is an invitro cell viability test that was developed and extensively studied for in vitro cytotoxicitydetermination. After incubation of cells withextracts, 0.33% of NR (NR in PBS) wasadded in each well and incubated for 1 h at37ºC. Dye-containing medium was removedand the well was washed twice with 150µl/well warmed PBS. The cells were thenlysed with 125 µl of 50% of v/v mixture ofethanol and 0.1M monobasic sodiumphosphate to solubilise the neutral red. Theplate was then incubated for 15 min andtake O.D at 550 nm [24].

**Cytotoxicity % = A-B/A X 100**
A = O.D of untreated well;
B = O.D of wells treated with plant extract.The data so obtained were calculated andwere presented in table-1 & 2 to find out %inhibition.

**Phytochemical analysis**

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents. Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of various infections. These are the qualitative tests performed to analyze the presence or absence of various phytochemicals such as alkaloids, tannins, flavonoids etc. in plant extract [25].

**RESULTSAND DISCUSSION**

Ethanolic extract of the leaves produced positive tests for flavonoids, steroids, terpenoids, phenolics, tannins, alkaloids and glycosides. Ethanolic extract of leaves produced positive tests for alkaloids,glycosides,phenolics and steroids. Aqueous extract showed the presence of carbohydrates, proteins, flavonoids, phenolics, tannins and glycosides**.**

The cells were observed after 24 hrs to record the changes in morphology (Fig. 1). The induction of apoptosis resulted in cell shrinkage and clumping of cells Morphological changes indicated theinhibition of cell growth, the cells in controlwells were normal but the wells treated withdifferent extracts of *Bryophyllum calycium*leavesshowed clumping of cells (Fig. 2&3). After 24 hour,apoptosis was found in most of the cells intest wells. MTT and NR assay clearlyindicated that the cells in control wells werehaving full growth upto 97% or 98% butthe other wells treated with differentconcentration like 10 mg, 1 mg and 10 µgm of*Bryophyllum calycium*leaves of differentextracts, the presence of viable cells werereduced to 52% after 24 hour of treatment.All the extracts of *Bryophyllum calycium*showedtheir anticancer effect against BHK-21 cells (Table 1).The data indicated that the ethanolic andaqueous extracts of *Bryophyllum calycium*leavesshowed more cytotoxicity then itsmethanolic extract.The aqueous and ethanolic extracts showed68% and 72% reduction in cell viability at anconcentration of 10 mg/ml in MTT Assay, while the sameconcentration of methanolic extract reduced58% as measured by MTT assay. Whileneutral red assay showed a reduction in cellviability due to ethanolic extract of *Bryophyllum calycium* as 74%. The same was 70% and56% in aqueous and methanolic extract respectively (Table. 1). The extracts of *Bryophyllum calycium* were effective against BHK-21. The cytotoxicity measured give better response with both the extracts. Morphological studies revels that the ethanolic extract of *Bryophyllum calycium* show good amount of rounding of cells and the clumping of cells were also in much amount then that of aqueous extract of *Bryophyllum calycium*. The clumping and deform cells were more in ethanolic and aqueous extract than that of methanolic extract of *Bryophyllum calycium*. In case of cytotoxicity, the ethanolic extract give better results than that of aqueous extract and methanolic extracts of *Bryophyllum calycium*. The cytotoxicity at 10 mg/ml was 72% in case of ethanolic extract of *Bryophyllum calycium*which was 68% and 58% in aqueous extract and methanolic extract of *Bryophyllum calycium*in same concentration respectively in MTT. At concentration 1 mg/ml and 10 µgm/ml in ethanolic extract , the cytotoxicity were 71% and 70% respectively whereas in aqueous and methanolic extract the cytotoxicity were found 60%, 62% and 52%, 51%,respectively at same concentration in MTT. In case of neutral red assay, the cytotoxicity at 10 mg/ml of aqueous and ethanolic extract were 70% AND 74%, respectively and it was 56% in methanolic extract of *Bryophyllum calycium*(Table. 2).Thus, the activity of aqueous extract and ethanolic extract were much better than that of its aqueous extract, these two extract were more effective and cytotoxic against BHK-21 cells then methanolic extract.

**Table 1: Percent Reduction in Cell Growth due to Extracts of *Bryophyllum calycium* leaves as Measured by MTT**

| Sl.No. | Extracts | 10mg/ml | 1mg/ml | 10µgm/ml |
|---|---|---|---|---|
| **1.** | Aqueous extract | 68.41±3.21 | 60.03±3.01 | 62.67±3.22 |
| **2.** | Methanolic extract | 58.61±3.11 | 52.74±3.26 | 51.95±3.42 |
| **3.** | Ethanolic extract | 72.91±3.56 | 71.83±3.47 | 70.63±3.62 |

**Table 2: Percent Reduction in Cell Growth due to Extracts of *Bryophyllum calycium* leaves as Measured by Neutral red Assay**

| Sl.No. | Extracts | 10mg/ml | 1mg/ml | 10µgm/ml |
|---|---|---|---|---|
| 1. | Aqueous extract | 70.24±3.41 | 69.53±3.62 | 68.36±3.52 |
| 2. | Methanolic extract | 56.71±3.34 | 55.82±3.47 | 54.75±3.51 |
| 3. | Ethanolic extract | 74.83±3.29 | 73.61±3.24 | 72.55±3.43 |

**Fig 1: Bryophyllum calycium** **Fig 2: Normal BHK-21 cells** **Fig 3: BHK-21 Cells after Treatment with Extract.**

The aqueous and different solvent-based extraction procedure used in the present study has been routinely followed by several other studies exploring therapeutic phytochemical [26]. The procedure focused primarily on the ethanolic and aqueous-extracted fraction only and the activity. However, earlier studies show presence of cytotoxic activities such as *Bryophyllum calycium* that converges in aqueous phase [26] and could prove useful. Therefore, more refined extraction method are needed to resolve the anti-cancer activity by examining the extract without separation with chloroform as demonstrated by some of the investigators [27] and will be considered in future studies. A dose-dependent cytotoxic activity observed in leaf extract and its fraction demonstrates a potential therapeutic utility of this medicinal plant against cancer. Earlier studies by Yamagishi *et al.,* 1989 demonstrated presence of similar cytotoxic activity against KB cells in methanolic extracts of *B. pinnata*, a species of *Bryophyllum calycium* [26]. Additionally, antitumor activity of *B. pinnata* leaves owing to its antimutagenic activity has also been demonstrated [27]. Interestingly, comparative analysis of fractionated crude leaf extracts in our experiments revealed cytotoxic activity that specifically resolved in ethanolic extract:methanolic extract:70:50. Earlier studies show that antimutagenic activity can be extracted in pet ether and ethyl acetate [27]. However, the IC50 value derived from HPLC purified active principles from chloroform extracted *B. pinnata* plant in earlier studies [26] were much lower (ranged between 10ηg/ml-4 µg/ml) compared to the one isolated in current study (91 µg/ml). This may be partly due to incomplete separation, but the variability of sensitivity of the cell line used in present study, could also be a major contributor. As a general notion, HeLa cells are much more resistant than many cell lines. Therefore, further studies are warranted on a panel of cell lines to verify the anti-cancer potential of *Bryophyllum calycium*. Moreover, the inhibitory effect of both solvent extract and aqueous extract of *Bryophyllum calycium* were maximal at 24 hours. These observations suggest that active principle might get metabolized or get inactivated during culture process and is no more available to impose growth inhibitory effect. However, these assumptions need further investigation.In contrast to *Bryophyllum calycium*, methanolic extract showed a minor growth promoting activity in dose range 10gm/ml -10 µg/ml. These results, suggest accumulation of cell growth promoters in ethanolic extract that may have coexisted in the plant leaves along with cytotoxic activity. However, there is no report which specifically describes any tumor growth promoting activity associated with this plant.

## CONCLUSION

Herbalism that subsided for sometime has now made a comeback. Several plants are under investigation to discover drugs for various diseases especially the dreadful ones that still await proper medication. In this study Bryophyllum calycium was selected forearlier known for many medicinal properties. The aqueous, ethanolic and methanolic extracts were tested on BHK-21 cells, which showed cytotoxic effects on the cancer cells. Assessing its anticancer activity in our study indicates for the first time that *Bryophyllum calycium*can act as an cytotoxic and apoptosis- inducing property against BHK-21 cells. It therefore provides an important lead for development of anti-cancer therapeutics for management of cancer. Further analysis and purification of the *Bryophyllum calycium* leaf extract and *in-vivo* studies may help in discovering the full potential of *Bryophyllum calycium*as a source of an effective antiviral/anti-cancer drug.

**ACKNOWLEDGEMENT**

Authors are thankful to the Director Experiment Station and Vice-Chanceller for providing necessary facility.

**References**

1. Chopra RN, Nayar SL and Chopra IL; Glossary of Indian Medicinal Plants. NISCIR (CSIR), New Dehli, 2002: 42.
2. Rastogi RP and MehrotraBN; Medicinal properties of *Bryophyllum pinnatum*. Compend. Indian Med. Plants, PID, New Dehli; 1991; 2: 112.
3. Oliver B; Medicinal plants in tropical west Africa III Antinfection therapy with higher plants. J. Ethnopharmacology; 1983; 9: 1-83.
4. Gill LS; Ethno-medical uses of plant in Nigeria. UNIBEN Press., 1992: 46.
5. Paranjpe P; Indian Medicinal Plants forgotten Healers. Chaukhamba Sanskrit Pratisthan, Delhi, 2005: 194-195.
6. Jaiswal S and Sawhney S; Correlation of epiphyllous bud differentiati on with foliar senescence in crassulacean succulent Kalanchoepinnata as revealed by thidiazuron and ethrel application. J. of Plant Physiology; 2006; 163: 717-722.
7. Varier's VPS; Indian Medicinal Plants a compendium of 500 species. Orient Longman, 2002; 3: 282-284.
8. Tan G, Gyllenhaal C, Soejarto DD; Biodiversity as a source of anticancer drugs. Curr. Drug Targets, 2006; 7(3):265-277.
9. Akinpelu DA; Antimicrobial activity of *Bryophyllum pinnatum* leaves. Fitoterapia, 2000; 71(2): 193-194.
10. Misra SN; Antifungal activity of leaf extract of some higher plants. ActaBotanicaIndica, 1979; 7:147-150.
11. Pal S, Nag CAK; Studies on the anti-ulcer activity of a *Bryophyllum pinnatum* leaf extract in experimental animals. J. Ethnopharmacol, 1991; 33(1-2): 97-102.
12. Pal S, Nag C.A.K: Preliminary studies on the anti-inflammatory and analgesic activities of *Bryophyllum pinnatum* (Lam.)., Med. Sci. Res., 1989;17: 561-562.
13. Pal S, Nag CAK; Further studies on the anti-inflammatory profile of the methanolic fraction of the fresh leaf extract of *Bryophyllum pinnatum*. Fitoterapia, 1992; 63:451-459.
14. Ojewole JAO; Antihypertensive properties of *Bryophyllum pinnatum* (Clam; oken) leaf extracts. Am. J. Hypert, 2002; 15(4): A34-A39.
15. Ojewole JAO; Antinociceptive, anti-inflammatory and antidiabetic effects of *Bryophyllum pinnatum* (Crassulaceae) leaf aqueous extract. J. Ethnopharmacol., 2005; 99(1):13-19.
16. Umbuzeiro-Valent G, Roubicek DA, Haebisch EM; Mutagenic and antimutagenic evaluation of the juice of the leaves of *Bryophyllum calycinum* (Kalanchoe pinnata), a plant with antihistamine activity. Environ. Mol. Mutagen, 1999; 33(4): 325-327.
17. Gaind KN, Gupta RL; Flavonoid glycosides from Kalanchoe pinnata. Planta Med, 1971; 20(4):368-373.
18. Marriage PB, Wilson DG; Analysis of the organic acids of Bryophyllum calycinum. Can. J. Biochem., 1971; 49(3):282-296.
19. Yadav NP and Dixit VK.; Hepatoprotective activity of leaves of Kalanchoe Pinnata Pers.. Journal of Ethnopharmacology, 2003; 86: 197-202.
20. Rossi-Bergmann B, Costa SS, Borges MBS, da Silva SA, Noleto GR, Souza MLM, and Moraes VLG; Immunosuppresive effect of the aqueous extract of Kalanchoe pinnata in mice. Phytothera. Res., 1994; 8: 399-402.
21. Marks LS, Partin AW, Epstein JI, Tyler VE, Simon I, Macairan ML, Chan TL, Dorey FJ, Garris JB, Veltri RW, Santos PB, Stonebrook KA, and deKernion JB; Effects of a saw palmetto herbal blend in men with symptomatic benign prostatic hyperplasia, J. Urol., 2000; 163 (5): 1451-1456.
22. Govindachari TR, Suresh G and Masailmani S; Antifungal activity of *Azadirachta indica* leaf hexane extract fitoterpia, 1999; 70: 427-420.
23. Mosmann T; Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immun. Methods., 1983; 65 (1-2): 55–63.
24. Flick DA and Gifford GE; Comparison of in vitro cell cytotoxic assays for tumor necrosis factor, J. Immunol. Meth., 1984; 68, 167-175.
25. Wagner H and Bladt S; Plant Drug Analysis. Second edition, Berlin, Springer, 1996: 349-354.
26. Yamagishi T, Haruna M, Yan XZ, Chang JJ, Lee KH; Antitumor agents, Bryophyllin B, a novel potent cytotoxic bufadienolide from *Bryophyllum pinnatum*. J. Nat. Prod., 52(5):1071-1079.
27. Emmanuel E, Obaseiki-Ebor KO, Hannumaiah T, Mitscher Lester A, Delbert M, Shankel: Antimutagenic activity of extracts of leaves of four common edible vegetable plants in Nigeria (West Africa). Mutat. Res. Lett., 1993; 302(2): 109-117.