

IN VITRO ANTI-CANCEROUS EFFECT OF THE *TAXITHELIUM NEPALENSE* (SCHWÄGR) BROTH. STUDIED BY MTT ASSAY**Athira R. K. Nair, Brijithlal N. D.*, Prakash G. Williams and Lubaina A. S.**

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ABSTRACT

The extract of *Taxithelium nepalense* (Schwägr) Broth. in various solvents are screened for phytochemical compounds and the acetone extract is screened for the anti-cancerous effect on HeLa cells (cervical cancerous cells) by MTT assay. Studies reveal that *T. nepalense* posses valuable secondary compounds like alkaloids, flavonoids, phenols etc. and the percentage of viability of cancerous cells are measured in various concentration of the extract. Studies proved that the maximum viability of cancerous cells showed in 6.25 µl and minimum viability in 100 µl. This indicates that higher concentration of the extract can kill the cancerous cells effectively. In general the results indicate that

T. nepalense shows anti-cancerous properties which is strictly based on the presence of secondary metabolites.

KEYWORDS: HeLa cells, MTT assay, *Taxithelium nepalense*, Apoptosis.

INTRODUCTION

According to the Global Cancer Report (GLOBOSCAN) in 2012 there are 14.1 million cancer cases are reported world widely, because cancer is the deadliest disease all over the world. Various anti-cancerous drugs are administrated in combination with radiation therapy to cure malignant and benign tumors. But it produce severe side effects as compared to the traditional cancer therapies like chemotherapy and radiotherapy (Cragg and Newman, 2005). Natural medicines are the plant products which contain some compounds that inhibit the growth of the cancerous cells.

Bryophytes are the primitive non-vascular amphibious cryptogams represented by approximately 960 genera and over 1500 (Gradstein et al., 2001) to 2500 species (Crum,

2001). some bryophytes are growing in shaded grounds, on moist rocks, trunks of small trees and similar moist places. They usually grew in tufts and cushions, and are responsible for providing the green colour to the floor of forests and mountains during rainy season. They used as medicines in a variety of countries for the treatment of a range of diseases, because which posses secondary compounds (Asakawa, 2007) similar to that of higher plants and has remarkable biological effects. Lal (2005) discloses that bryophytes contain several compounds which have a number of biological properties and also Zheng *et al.* (1994) discovered that the cytotoxic agents from different mosses which are directly reduce or prevent the growth of cancerous cells. *Taxithelium nepalense* is a common corticolous bryophyte mostly seen on the wet areas and also widely distributed in India, China and Philippines etc. (Gangulee's flora of Western regions of mosses). Several works have been carried out on the antioxidant property of the *T.nepalense* (Abhjith Dey and Jithendranath De, 2012). Our present study investigated that the *T. nepalense* posses secondary metabolites and shows anti-cancerous activity on HeLa (cervical cancer) cells.

MATERIALS AND METHODS

Collection of bryophytes

Collection was made as far as possible with the help of suitable instruments (chisel, knife etc). Collected bryophyte is temporarily stored in plastic bags to prevent desiccation and the protection of reproductive parts. Then the specimen is deposited in the herbarium of Bishop Moore College Mavlikkara (BMC.B-05).

Identification of bryophyte

Identification of bryophyte was made with the help of suitable literature like Gangulee's mosses of eastern India and adjacent regions. The identification was mainly based on the comparison of morphological, anatomical and reproductive characters.

Preparation of the extract of *Taxithelium nepalense*

The specimen is air dried (especially with sporophyte) and powdered with suitable instruments and then it is dissolved in suitable solvents (acetone, ethyl acetate and distilled water) for the extraction of the phytochemical compounds using Soxhlet apparatus. After 24 hours of the continuous cycle in the apparatus, we got the extract of the specimen in various solvents (William B. Jensen, 2007). Extracts are used for phytochemical analysis and the acetone extract is again used for anti-cancerous effect.

Phytochemical analysis of the bryophyte

The extracts of bryophyte were subjected to phytochemical analysis using methodology of Sofowora (1982).

The major pharmaceutically valuable phytochemical compounds investigated in the present study were:

- ❖ Alkaloids
 - ❖ Carboxylic acids
 - ❖ Coumarins
 - ❖ Flavonoids
 - ❖ Phenols
 - ❖ Proteins and free amino acids
 - ❖ Quinones
 - ❖ Resins
 - ❖ Saponins
 - ❖ Sterols, phytosterols and triterpenoidal sapogenins
 - ❖ Tannins
 - ❖ Xanthoproteins
 - ❖ Sugars
- **Detection of alkaloids** – A few drops of dilute HCl was separately treated with 1 ml each of various extracts. Then it was filtered and the filtrates were treated with 1 ml of Dragendoff's reagent. Formation of reddish orange precipitation indicated the presence of alkaloids.
- **Detection of carboxylic acids**- 1 ml each of various extracts was separately treated with a few ml of saturated solutions of sodium bicarbonate. Observation of effervescence (due to liberation of CO₂) indicated the presence of carboxylic acids.
- **Detection of coumarins** – 1 ml each of alcoholic extracts was treated with alcoholic NaOH solution. Production of dark yellow colour indicated the presence of coumarins.
- **Detection of flavonoids**- 5 ml each of the various extracts were separately dissolved 1ml each of alcohol (stock solution) and then subjected to the following tests.

- **Ferric chloride test-** 1 ml each of stock alcoholic solution was added with a few drops of neutral FeCl_3 solution. Formation of blackish red colour indicated the presence of flavanoids.
- **Shinoda's test:** with 1ml each of alcoholic solution a small piece of Mg ribbon or Mg foil was added followed by the addition of a few drops of concentrated HCl. Change in colour showed the presence of flavanoids.
- **Detection of phenols-** 1ml of the various extracts dissolved in 5ml of alcohol was treated separately with a few drops of neutral FeCl_3 solution. Any change in colour indicated the presence of phenolic compounds.
- **Detection of protein and aminoacids-** 5 ml each of various extracts were dissolved in 5ml of water separately and were subjected to the following tests.
- **Biuret test-** 1 ml each of the various extracts was warmed gently with 10% NaOH solution and a drop of diluted CuSO_4 solution. Formation of reddish violet colour indicated the presence of proteins and amino acids.
- **Ninhydrin test-** 1 ml each of the various extracts was separately treated with a few drops of Ninhydrin solution. Change in colour showed the presence of proteins and amino acids.
- **Detection of quinones-** 1 ml of the various extracts was separately treated with alcoholic KOH solution. Quinones give colourations ranging from red to blue.
- **Detection of resins-** 1 ml of various extracts were subjected to treat with a few drops of concentrated H_2SO_4 and a few drops of acetic anhydride solution followed by 1 ml of concentrated H_2SO_4 . Resins give coloration ranging from orange to yellow.
- **Detection of saponins-** 1ml each of the various extracts was separately mixed with 20 ml of distilled water and then agitated in a graduated cylinder for 15 minutes. Foam formation indicated the presence of saponin.

- **Detection of steroids/phytosterols/triterpenoidal sapogenins-** 5 ml each of various extracts were dissolved in 5 ml each of chloroform separately (stock solution) and was subjected to the following tests.
 - **Salkowski test:** 1 ml each of concentrated H₂SO₄ was added to the stock solution and allowed to stand for 5 minutes after shaking. Turning of golden yellow colour in the lower layer indicated the presence of steroids, phytosterols, triterpenoidal and sapogenins.
 - **Libermann –Burchard test:** 1ml of each of the extract, a few drops of acetic anhydride and 1ml of concentrated H₂SO₄ were added from the sides of the test tubes and allowed to stand for 5 minutes. Formation of brown ring at the junction of the two layers and the upper layer turned green indicated the presence of steroids, phytosterols and triterpenoidal sapogenins.
- **Detection of tannins-** 5 ml each of the various extracts was dissolved in minimum amount of water separately, filtered and the filtrates were then subjected to the following tests.
 - **Ferric chloride test-** To the above filtrate a few drops of ferric chloride solution were added. The colour change indicated the presence of the tannins.
 - **Basic lead acetate test-** To the filtrate, a few drops of aqueous basic lead acetate solution was added. Formation of reddish brown precipitate indicated the presence of tannins.
- **Detection of xanthoproteins-** 1 ml of various extracts was treated separately with a few drops of concentrated HNO₃ and NH₃ solution. Formation of reddish orange indicated the presence of xanthoproteins.
- **Detection of sugars-** 5 ml each of various extracts was dissolved separately in distilled water filtered and then subjected to the following tests.
 - **Molisch's test-** To the filtrate a few drops of alcoholic α -naphthol and 2 ml of concentrated H₂SO₄ were added slowly through the sides of the test tube. Formation of reddish brown precipitate indicated the presence of sugars.

- **Fehling's test-** A small portion of various filtrates were treated with 1 ml of Fehling's solution 1 and 2 then heated gently. Change in colour indicated the presence of sugars.
- **Anthrone test-** 1 ml each of the various extracts in a watch glass were separately taken and mixed thoroughly using a glass rod with an equal quantity of anthrone reagent and a few drops of concentrated H₂SO₄ and heated on a water bath. Formation of dark green colour indicated the presence of sugars.

Anti-cancerous activity of the bryophyte

Cell culture

HeLa (cervical cancer) cells were initially procured from National Centre for Cell Science (NCCS), Pune, India. and maintained in Dulbecos Modified Eagles Medium (Gibco, Invitrogen).

The cell lines were cultured in 25 cm² tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing Penicillin (100µg/ml), streptomycin (100µg/ml) and amphotericin (2.5µg/ml). Cultured cell lines were kept at 37⁰C in a humidified 5% CO₂ incubator (NBS, Germany).

Cell seeding in 96 well plates

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension (5×10⁴ cells/well) was seeded in 96 well tissue culture plates and incubated at 37⁰C in a 5% CO₂ incubator.

Cytotoxicity evaluation

After 24 hours the growth medium was removed, freshly prepared each plant extracts (in 100µl, 50µl, 25µl, 12.5µl, 6.25µl in 100µl of DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37⁰C in a humidified 5% CO₂ incubator.

Cytotoxicity assay by direct microscopic observation

Entire plates were observed at an interval of each 24 hours, up to 72 hours in an inverted microscopic observation were recorded as image. Any detectable changes in the morphology of the cells such as rounding or shrinking of cells, granulation, vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity assay by MTT method

15 mg of MTT (Sigma M-5655) was reconstructed in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period the sample content in wells were removed and 30 μ l of reconstructed MTT solution was added to all test and cell control wells the plate was gently shaken well, then incubated at 37⁰C in a humidified 5% CO₂ incubation for 4 hours. After the incubation period, the supernatant was removed 100 μ l of MTT solubilization solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the frozen crystals). The absorbance values were measured by using microplate reader at a wavelength at 570 nm (Talarico *et al.*, 2004).

% of growth of inhibition was calculated using the formula

$$\% \text{ of viability} = \frac{\text{Mean of sample}}{\text{Mean OD of the control group}} \times 100$$

RESULT AND DISCUSSION

Bryophytes are known to produce a great range of biologically active compounds. Phytochemical studies deal with the variety of organic substances that are accumulated in the plant by their biological function. Most of the compounds isolated from the bryophytes are lipophilic terpenoids and aromatic compounds of which only a few nitrogen and sulfur compounds have been found. These compounds have antibacterial, antifungal, antioxidant and anticancerous activity.

Taxithelim nepalense belongs to the family sematophyllaceae, order hypnobryales and class musci. They are corticolous plants seen in humid wet areas. Phytochemical analysis reveals that *T. nepalense* posses valuable secondary compounds like phenols, alkaloids, tannins etc. (Table: 1).

Table 1: Phytochemical analysis of *T. nepalense*.

Phytochemical Constituents	<i>Taxitheliumnepalense</i>		
	A	EA	W
Alkaloids	-	+	-
Carboxylic acids	-	-	-
Coumarins	+	+	-
Flavonoids	-	-	-
Phenol	+	+	-
Proteins & aminoacids	-	+	-
Quinones	-	-	-
Resins	-	-	-
Saponins	-	-	-
Steroids	-	-	-
Tannins	+	-	-
Xanthoproteins	-	+	-
Sugars	+	+	+

(A= Acetone, EA= Ethyl acetate, W= Water, += Presence, -= absence).

To find out the anti-proliferative effect, we used the varying concentrations of the bryophyte extract of *Taxithelium nepalense* in acetone. The viability of HeLa cells are maximum in the control (99.99%). But it is varied in the variable concentration of the extract from 100 μ l to 6.25 μ l (**Table: 2**). The maximum viability seen in 6.25 μ l and the minimum viability seen in 100 μ l. The maximum viability of the extract of *T.nepalense* in 6.25 μ l is 58.72397 and minimum viability in 100 μ l is 22.0360.

Table 2: Anticancerous effect of *Taxithelium nepalense*.

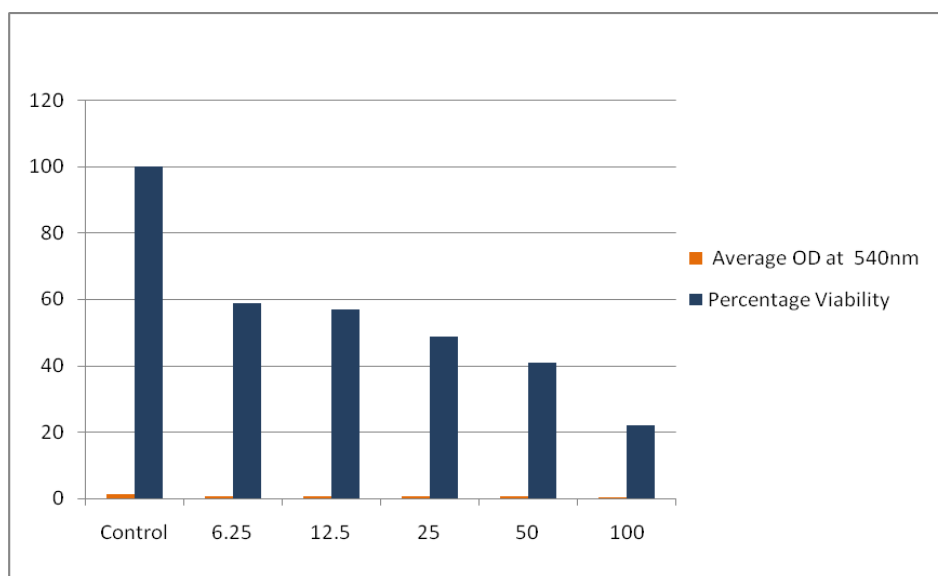
Sample volume (μ l)	Average OD at 540nm	Percentage Viability
Control	1.2053	
Sample- A		
6.25	0.7078	58.72397
12.5	0.6868	56.98166
25	0.5862	48.63519
50	0.4924	40.8529
100	0.2656	22.03601

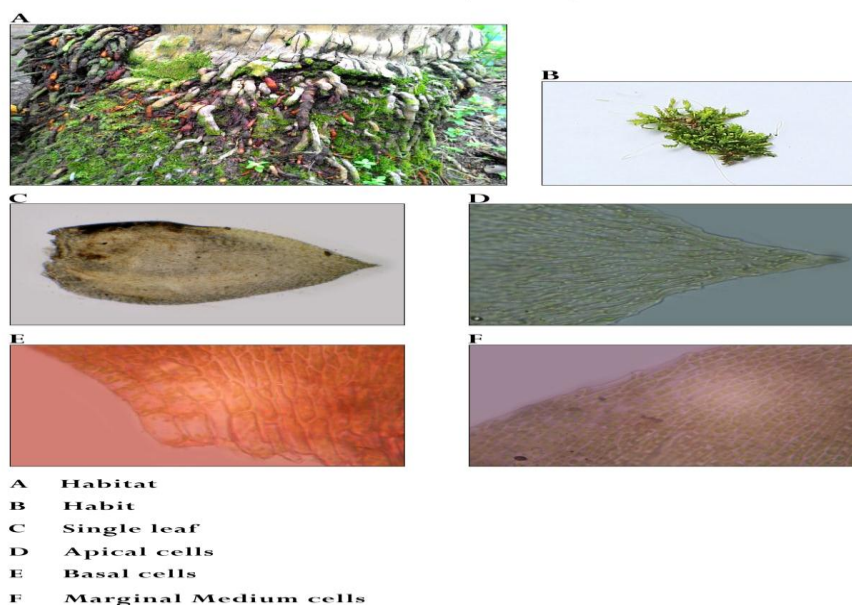
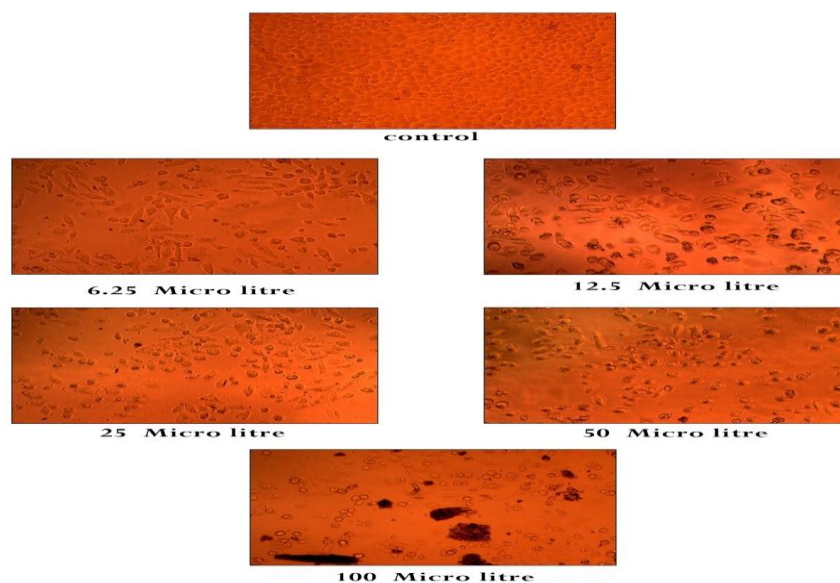
DISCUSSION

Bryophyte is a primitive plant group have no well differentiated plant body and well developed vascular system. Even though it contains several bioactive compounds such as flavonoids, phenols, alkaloids etc. for various biological properties (Asakawa *et al.*, 2007).

Several studies reveals that *Taxithelium nepalense* has an antioxidant property, hence they are used in the preparation of cosmetics (Abhjith Dey and Jithendranath De, 2012) and also it has

anti-cancerous activity. For the detection of anti-cancerous property, the extract is applied to cancerous cells and the apoptosis of cancerous cells are viewed by microscopy and measured by MTT assay. Apoptosis is a physiological process of self-destruction for maintaining cellular homeostasis between cell division and cell death (Jacobson *et al.*, Mahoney and Rosen, 2005). Induction of apoptosis can be considered as a prim factor of anti- cancer agents. Here the extract of *T.nepalense* induce apoptosis of cancerous cells HeLa (cervical cancer) caused by biochemical and physiological changes in nucleus and cytoplasm, chromatin condensation, membrane blebbing and cell shrinkage (Kerr *et al.*, 1972; Rello *et al.*, 2005). After following the MTT assay, we know that the concentration of the extract determine the amount of the viable cancerous cells. The crude extract (100 μ l) of *T.nepalense* destroy maximum number of cancerous cells. That indicates the extract has high activity against tumor cells (Figure: 1).



Taxithelium nepalense (Schwagr) Broth.Anticancerous activity of *Taxithelium nepalense* in varying concentrations**CONCLUSION**

Bryophytes rank second after the flowering plants among the major group of green land plants, which is identified based on their morphological, anatomical and reproductive characteristics. In this study anti-cancerous and phytochemical analysis of the bryophyte were checked from the acetone extract. we can summarised that the extract of the *Taxithelium*

nepalense can induce apoptosis thereby prevent the proliferation of cancerous cells. In future this bryophyte extract can be used to cure cancer in higher level organisms.

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