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# ANTI-CANCEROUS EFFECT OF THE ACETONE EXTRACT OF *SEMIBARBULA ORIENTALIS* (F.WEBER) WIJK & MARGAD

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## ABSTRACT

Bryophytes are the primitive cryptogams, act as the valuable sources of secondary metabolites and posses certain biological activities. They are considered as traditional medicines and widely used to cure fever, pneumonia and lymphocytic leukemia etc. The present study aims to find out the phytochemical compounds of the moss *Semibarbula orientalis* (F. Weber) Wijk & Margad. Using acetone, ethyl acetate and distilled water and also the anti-cancerous effect of the acetone extract using MTT assay.

**KEYWORDS:** Anti-cancerous effect, SW480 cells, MTT assay, apoptosis.

## INTRODUCTION

Bryophytes are the simplest primitive non-vascular plants seen in major parts of the earth, wherever sufficient moisture is present. They include liverworts, hornworts and mosses. They are known to produce a great range of biologically active compounds, which have been reported to be possible chemical barriers against microorganisms. Troitsky *et al*. suggested that bryophytes are used as traditional medicines for thousands of years. Also certain others reported that the mosses and liverworts are medicinal plants and are said to posses certain biological activity and effects (Garnier *et al*., 1969; Suire, 1972; Ding, 1982; Wu, 1982; Ando and Matsuo, 1984). In this study the moss *Semibarbula orientals* (F. Weber) Wijk & Margad. is screened for the anti-cancerous effect by MTT assay. Here SW480 (colon cancer cells) are used for screening the anti-cancerous effect.

Apoptosis is a programmed cell death which controls the development of tissues in organisms (Kerr *et al*., 1994; Liu *et al.,* 2010). According to Hanahan, Weinberg (2000) and Abott *et al.*

(2006) dysregulation of apoptosis is usually considered as a major cancer hallmark because induction of apoptosis leads to the prevention of growths of cancerous cells. Many studies have shown that apoptosis is an important mechanism by which various anti-cancer agents exert anti-cancerous effects. The morphological characteristics of apoptosis include membrane blebbing, cellular shrinkage, chromatin condensation of apoptotic bodies etc. In this study we measure apoptotic cell (cell viability) by MTT assay.

## MATERAIALS AND METHODS

### Collection of bryophyte

Collection was made as far as possible with the help of suitable instruments (chisel, knife etc) and temporarily stored in plastic bags to prevent desiccation and the protection of reproductive parts. The specimens are deposited in the herbarium of PG Department of Botany and Biotechnology of Bishop Moore College, Mavelikara (BMC. B. 09).

### Identification of bryophyte

Collected bryophyte is carefully separated from unwanted substances and washed thoroughly with distilled water and the identification was made with the help of suitable literature like Gangulee's mosses of eastern India and adjacent regions based on morphological, anatomical and reproductive characters.

### Extract preparation of bryophyte

Identified material is subjected to air dry and the dried specimen is grinded to form fine powder for the extraction of phytochemicals. Soxhlet's extraction (hot extraction) was done for 24 hours continuously. Organic solvents like acetone (99%, Nice), ethyl acetate (99%, Nice) and distilled water were employed for the extraction of bioactive compounds. The sample is required for the extraction was weighed in a cheese cloth and kept inside the extraction chamber of soxhlet. The round bottom flask was filled with 500 ml solvents and then heated for 24 hours. The extract from the sample get dissolved into the solvent and reached the receiving flask. The flask was detached from the apparatus and the extract was kept under an evaporator to completely remove the solvent from it (William B. Jensen, 2007).

### Phytochemical analysis

The extracts of bryophytes 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 $co_2$) 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 falvonoids** - 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 flavonoids.
- **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 $H_2SO_4$ 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 $H_2SO_4$ 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 $HNO_3$ and $NH_3$ solution. Formation of reddish orange colouration 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 $H_2SO_4$ 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**- 1ml 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 $H_2SO_4$ and heated on a water bath. Formation of dark green colour indicated the presence of sugars.

**Anti-cancerous activity of bryophytes**

**Cell culture**

SW480 (colon cancer cells) were initially procured from National Centre for Cell Science (NCCS), Pune, India, and maintained in DMEM (Gibio, Invitrogen).

The cell lines were cultured in 25 $cm^2$ tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing Penicillin (100$\mu$g/ml), streptomycin (100$\mu$g/ml) and amphothericin (2.5µg/ml). Cultured cell lines were kept at $37^0$C in a humidified 5% $CO_2$ incubator (NBS, Germany).

**Cell seeding in 96 well plates**

Two days old confluent monolayer of cells were trypsanized and the cells were suspended in 10% growth medium, 100$\mu$l cell suspension ($5\times10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at $37^0$C in a 5% $CO_2$ incubator.

**Cytotoxicity evaluation**

After 24 hours the growth medium was removed, freshly prepared each plant extracts (in 100 µl, 50 µl, 25 µl, 12.5 µl, 6.25 µl in 100 µl of DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at $37^0$C in a humidified 5% $CO_2$ incubator.

**Cytotoxicity assay by direct microscopic observation**

Entire plate was 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, and 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 µl of reconstructed MTT solution was added to all test and cell control wells the plate was gently shaken well, then incubated at $37^0$C in a humidified 5% $CO_2$ incubation for 4 hours. After the incubation period, the supernatant was removed 100 µl 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

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

**RESULT AND DISCUSSION**

The sample *Semibarbula orientalis* (F.Weber) Wijk & Margad. belongs to the family pottiaceae, order pottiales and the class musci, seen in different habitats depending upon the environmental conditions.The study shows that bryophyte extracts contain alkaloids, flavonoids, quinones, sugars, proteins and amino acids etc. but do not contain carboxylic acids, resins, steroids and saponins etc. Presence of the common compounds reveal that, bryophytes shows various biological activities as similar to that of higher plants.

Phytochemical analysis of *Semibarbula orientalis* (F. Weber) Wijk & Margad. Using acetone, ethyl acetate and distilled water.

| Phytochemical Constituents | *Semibarbulaorientalis* | | |
|---|---|---|---|
| | A | EA | W |
| Alkaloids | + | + | - |
| Carboxylic acids | - | - | - |
| Coumarins | + | + | - |
| Flavonoids | - | + | - |
| Phenol | + | + | + |
| Proteins & aminoacids | + | - | - |
| Quinones | - | - | - |
| Resins | - | - | - |
| Saponins | - | - | - |
| Steroids | - | - | - |
| Tannins | - | - | + |
| Xanthoproteins | - | - | - |
| Sugars | + | + | + |

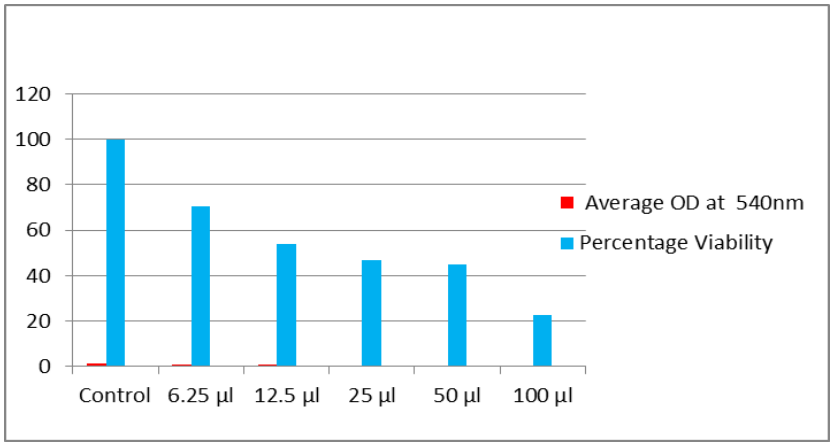
(A= Acetone, EA= Ethyl acetate, W= Water, += Presence, -= absence)

To find out the anti-proliferative effect, we can used the varying concentration of the extract of *Semibarbula orientalis* (F.Weber) Wijk & Margad. In acetone. The viability of SW480 (colon cancercells) are maximum in the control (99.99%) at 540nm. But it is varied in the variable concentration of the extract from 100 µl to 6.25 µl. The extract shows maximum viability in the concentration 6.25 µl and the percentage of viability is 70.5687. The least viability in 100 µl and the percentage of viability is 22.81398. The anti-proliferative activity of bryophyte extract in acetone of varying concentrations is given below.

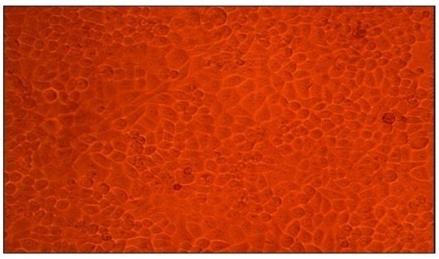
**Anti-cancerous effect of *Semibarbula orientalis* (sample-B)**

| Sample volume (µl) | Average OD at 540nm | Percentage Viability |
|---|---|---|
| Control | 1.1848 | 99.99 |
| 6.25 µl | 0.8361 | 70.56887 |
| 12.5 µl | 0.6367 | 53.73903 |
| 25 µl | 0.5534 | 46.70831 |
| 50 µl | 0.5304 | 44.76705 |
| 100 µl | 0.2703 | 22.81398 |

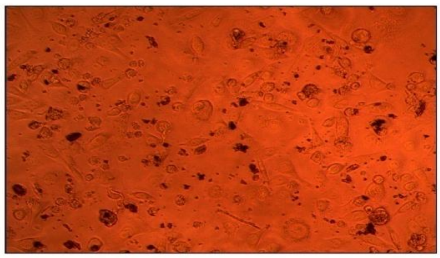
Acetone extract of *Semibarbula orientalis* shows good anti-proliferative activity against SW480 cells. The extract shows maximum viability in the concentration 6.25 µl and the least viability in 100 µl. The result indicates that the extract has the cytotoxic effect on cancerous cells that means which can induced apoptosis at higher concentrations.



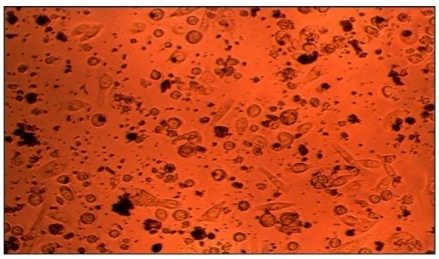

**Anti cancerous activity of *Semibarbula orientalis* in varying concentrations**



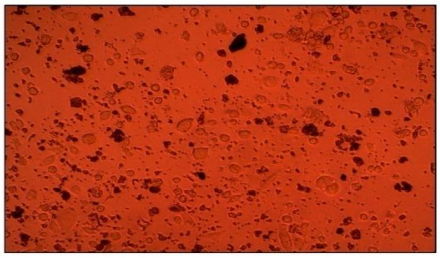
control



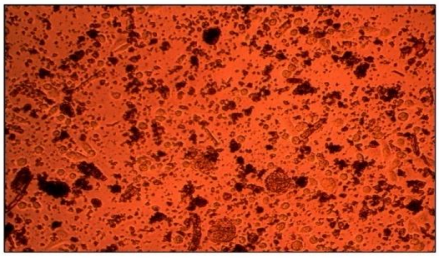
6.25 Micro litre



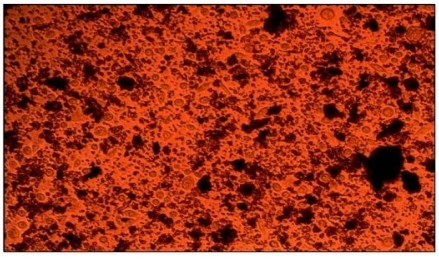
12.5 Micro litre



25 Micro litre

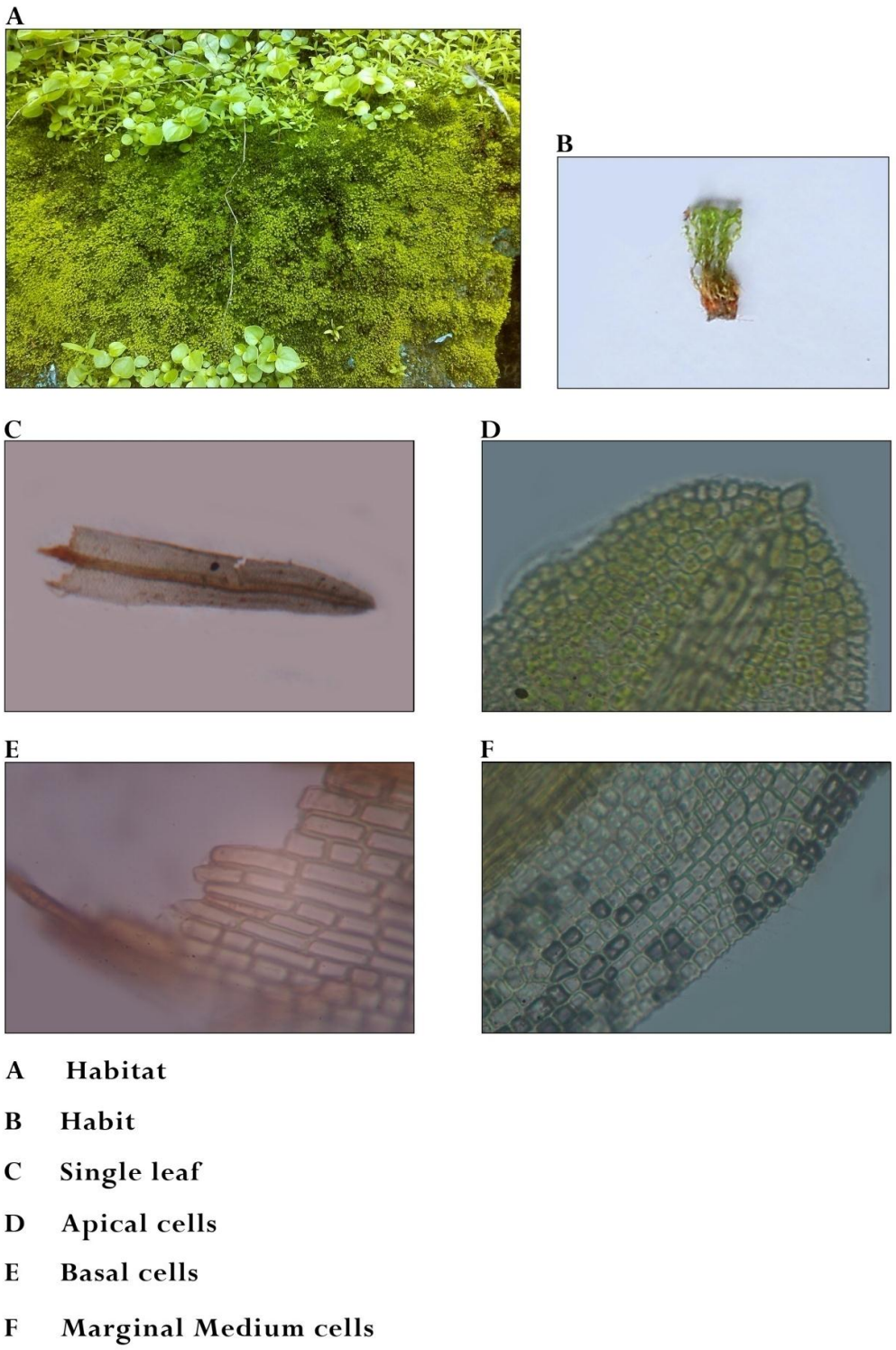


50 Micro litre



100 Micro litre

*Semibarbula orientalis (F.Weber) Wijk & Margad.*




A Habitat

B Habit

C Single leaf

D Apical cells

E Basal cells

F Marginal Medium cells

## CONCLUSION

In conclusion, we can summarised that acetone extract of *S. orientalis* can induce apoptosis and can 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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