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Tissue Culture, Histochemical, Phytochemical analysis and Anti-cancer activity study on a Traditional Medicinal Plant - *Spharenthus indicus* L.

Bagath Singh C[1*], G.A.I Ebenezer[1], D. Jesubalan[2] and M.S.Sundari[3]
[1]Department of Botany, Madras Christian College, Tambaram, Chennai – 600 059.
[2]Botanical Survey of India, Western Regional Centre, Pune - 411 001, India.
[3]Department of Botany, S.P. Pune University, India.

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ABSTRACT

Spharenthus indicus L., a multipotential plant belonging to the family Asteraceae was undertaken for various investigations. Explants like leaf, stem and axillary bud were attempted for their morphogenetic potential by culturing on MS medium supplemented with different plant PGR hormones such as 2,4-D, 2 4 5-T, BAP, IBA and KIN . Leaf disc explants cultured on MS medium supplemented with 2 4-D induce callus in 15 days. The stem explants remains recalcitrant and it is interesting to observe that the axillary buds induced callus with Kinetin treatment followed by rhizogenesis with BAP. Phytochemical tests with leaf sample reveals the presence of alkaloids, terpenoids, flavonoids and phenols, as well as primary metabolites like protein and carbohydrates. Methanolic leaf extract with different concentrations viz., 7.8µg/ml, 15.6 µg/ml, 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml were tried for toxicity effect on Lung cancer cell line A549. MTT assay adopted 1000 µg/ml concentration reveals 81% anticancer activity.

Introduction

Plants have played a major part in keeping human health and refining the quality of human life and have formed a basis for traditional medicine systems that have been used for thousands of years in countries with ancient civilizations such as China (Chang and Paul, 1986), India (Kapoor, 1990) and Thailand (Subchareon, 1998a).There are many herbs, which are mainly used to treat cardiovascular problems, liver disorders, central nervous system, digestive and metabolic disorders. According to the WHO, 80% of the world population continues to rely mainly on traditional medicines for their health care (WHO, 1993; Farnsworth *et al.,* 1985). The plant *Spharenthus indicus* L. is widely distributed in Northern Australia, Indomalaya and India. The plant possesses several medicinal activities on various ailments and it has been commercially exploited in traditional medicinal system. Due to the excess usage of this species by the indigenous and pharmaceutical industries, owing to its potency to cure various illness, a reduction in its population is expected in the near future. Hence the enhancement of the population for its great demand is an impending need which is possible through the technique of tissue culture. So far there is no report on the tissueculture aspect of this species, therefore this study was undertaken to derive an efficient propagation protocol as well as to define its chemical constituents.

*Corresponding author.
Email address: rcbagath007@gmail.com
Mobile: 91- 9655411307

Materials and Methods

Source of Explant

Healthy plants of *Spharenthus indicus* L. were collected from the wild and also maintained inside the green house of botany department. Young, healthy and disease free portions of the plants were selected and used as explants.

Medium Preparation

Murashige and skoogs medium (1962) was used as the basal medium. The stock solution were prepared at a concentration of 50x and stored in refrigerator. For all PGRs stock solution of 10 M were prepared and stored at 5ÚC. The medium was supplemented with PGRs such as 2, 4-D, IBA, BAP, and KIN either alone or in combinations.The media were made up to the required volume by adding de-ionized sterile distilled water prior to the addition of 3% sucrose and 0.8% agar.The pH of the medium was adjusted to 5.8 In order to dissolve the agar, the medium was warmed up. Then liquid medium was dispersed equally in culture vials (10ml in each vial) and was closed loosely with aluminum foil immediately. Then the medium was sterilized in an autoclave at 15 lbs for 15 minutes. After that mouth of the culture vials were sealed tightly. The culture vials were labeled and kept under sterile condition.

Preparation of Plant Extracts

Freshly collected plant materials were dried in shade and then coarsely powdered in a blender. 5g of the coarse powder was extracted successively with 150ml of various solvents in a Soxhlet apparatus for 24hours. All the extracts were filtered through Whattman No.41 filter paper individually and subjected to qualitative tests for

the identification of a range of phytochemical constituents as per the standard procedures (Brinda *et al*., 1981). The extracts were concentrated in a Soxhlet apparatus. The concentrated extracts were used for further analysis.

Preliminary Phytochemical Screening
The Phytochemical screening of leaf extracts were studied using the protocol of Khandelwal (2002).

Histochemical Studies
Standard Procedures for the histochemical localization for various components were followed: Gomori (1952); Jensen (1962); Pearse (1972); Berlyn and Miksche(1976); Horobin (1982, 1988); Gahan(1984); Krishnamurthy (1988), by using bright-field dyes and reagents to identify the storage components such as proteins, lipids, starch, nucleic acids, phytin, tannin, lignin and minerals in stems of *Spharenthus indicus* L.

***In-vitro* evaluation of anticancer activity of Methanol leaf extract of *S. indicus*L. through MTT assay**
Cell line and culture
The cells were maintain in DMEM supplemented along with 10% FBS, penicillin (100 ì/ml), and streptomycin (100 ìg/ml) in a humidified atmosphere of 50 ìg/ml CO2 at 37°C in Life Teck Research Centre Arumbakkam, Chennai. Cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS).

***In- vitro* assay for anti-cancer activity: (MTT assay) (Mosmann, 1983)**
Cells (1 × 105/well) were plated in 24-well plates and incubated in 37°C with 5% CO2 condition. After the cell attain the confluence, different concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100ìl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl—tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The following formula was used to calculated the % cell viability **% Cell viability = A570 of treated cells / A570 of control cells × 100.**

Graphs are plot using the percentage of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to contrast the full cell viability assessments.

Macro photographs were taken using Mobile digital camera. Sectioned slides were observed under Zesis-Axiostar microscope and microphotographs were taken with DSLR canon EOS 450 camera.

Results and Discussion
Medicinal plants mustremained the matters of man's curiosity since, time immemorial (Constable, 1990). Medicinal plants are globally treasured sources of new drugs (Chen *et al*., 2010).In this current investigation an important medicinal plant, the *Spharenthus indicus* L. was attempted for tissue culture, phytochemical, histochemical and anticancer studies to explore the possibility of masspropagation, determining the key chemical for anti-cancer activity. Various explants like leaf, stem and axillary bud were attempted to check their morphogenetic potential by culturing on MS medium supplemented with different plant hormones 2 4 - D, 2 4 5 - T, BAP, IBA and KIN at 10^{-5} M concentration.Leaf disc explants cultured on MS medium supplemented with 2 4-D induce callus within 15 days of inoculation (Figure 1 a). The stem explants reminds recalcitrant. A Axillary buds were dissected from the second node and cultured on MS medium supplemented with IBA, BAP and KIN. Axillary bud explants are generally used for direct caulogenesis supplementing cytokinin like BAP, 2ip, Zeatin and Kinetin to produce clones. It is interesting to observe the axillary buds induced callus in kinetin treatment (Figure 1, b-c) and callus with adventitious root in BAP (Figure 1, d).

Medicinal plants are very important due to the presence ofpotential chemicals used for healing various ailments in human being and animals. In this study simple isolation procedure and phytochemical test were adopted to test the presence of alkaloids, terpenoids, flavonoids, phenols, protein and carbohydrates from the leaf sample (Figure 2,a-f ;Table 1). Histochemical studies on stem reveals the presences of Starch, Protein, Lipids, Lignin and Tannins. (Figure 3, a-e).

Isolated crude plant extracts was used in anti-cancer activity in this investigation. Crude extract of leaf isolated using methanol by Soxhlet apparatus was tried with various concentrations viz., 7.8 µg/ml, 15.6 µg/ml, 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml 500 µg/ml and 1000 µg/ml on lung cancer cell line A549(Graph 1) (Figure 4). MTT assay was adopted to find out the toxicity of leaf extract on cancer cells.This study reveals 1000µg/ml leaf extract was 81% toxicity on the lung cancer A549 cell line. In comparison with other plants, *S. indicus* shows the presence of high anticancer activity.

Conclusion
Spharenthus indicus L. is one of the species which possess various phytochemical compounds and widely used in Indian traditional medicinal system for curing different types of ailments. In this present investigation the crucial tests for anti-cancer shows a significant result on the lung cancer cell line with 81 % toxicity. Clinical examination should be made to identify the chemical compound that plays a vital role against cancer cells. Furthermore studies should be undertaken on tissue culture for mass propagation and isolation of potential chemical compound through cell suspension culture for increasing the productivity of the determined cell line composite.

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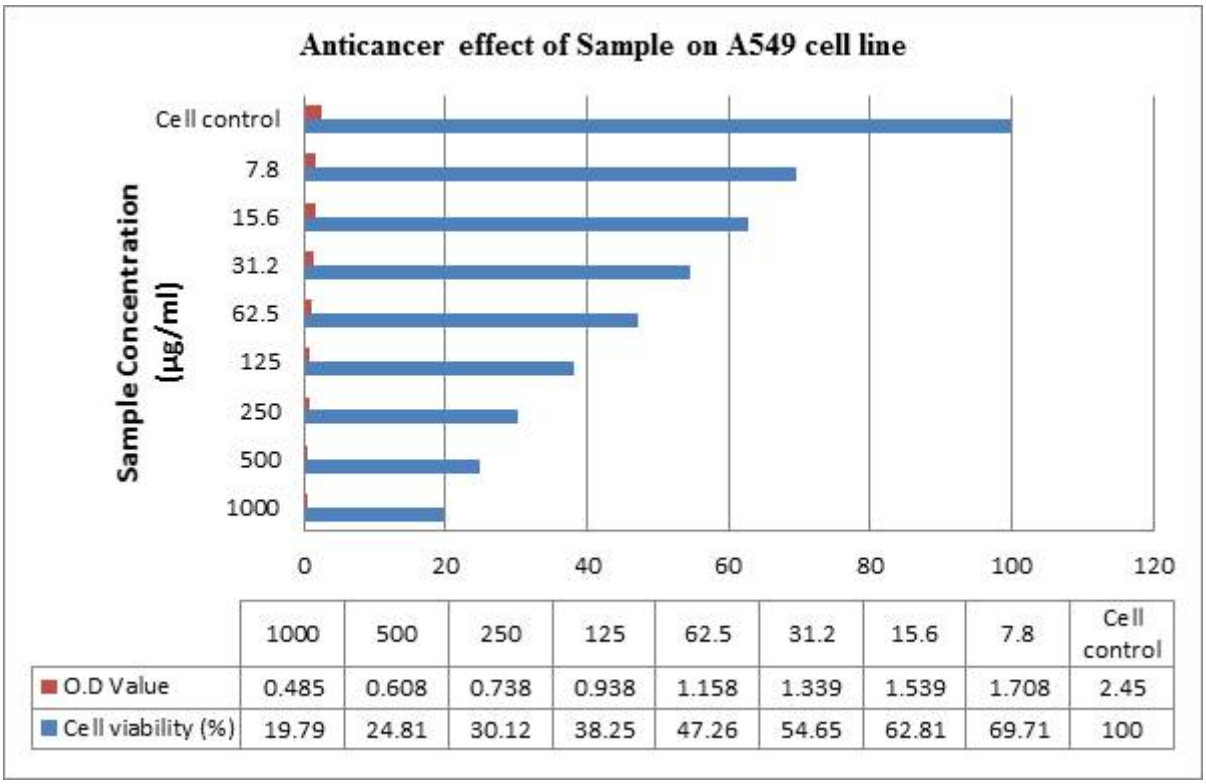
Table – 1: Qualitative analysis of phytochemicals from Leaf of *Spharenthus indicus* L.

S.No	Phytochemicals	Methanol	Hexane	Water
1.	Alkaloids	+++	++	++
2.	Phenols	++	++	++
3.	Carbohydrates	+	+	+
4.	Flavonoids	++	+	++
5.	Proteins	+++	++	++
6.	Terpenoids	+++	++	++

Where +++: Abundance, ++: Average, +: Poor

Graph – 1: Anti-cancer effect of Methanol leaf extract *on A549* cell line



	1000	500	250	125	62.5	31.2	15.6	7.8	Cell control
O.D Value	0.485	0.608	0.738	0.938	1.158	1.339	1.539	1.708	2.45
Cell viability (%)	19.79	24.81	30.12	38.25	47.26	54.65	62.81	69.71	100

Figure – 1: a-d, response of explants in MS medium, e- Leaf extraction in various solvents, f- crude extracts.

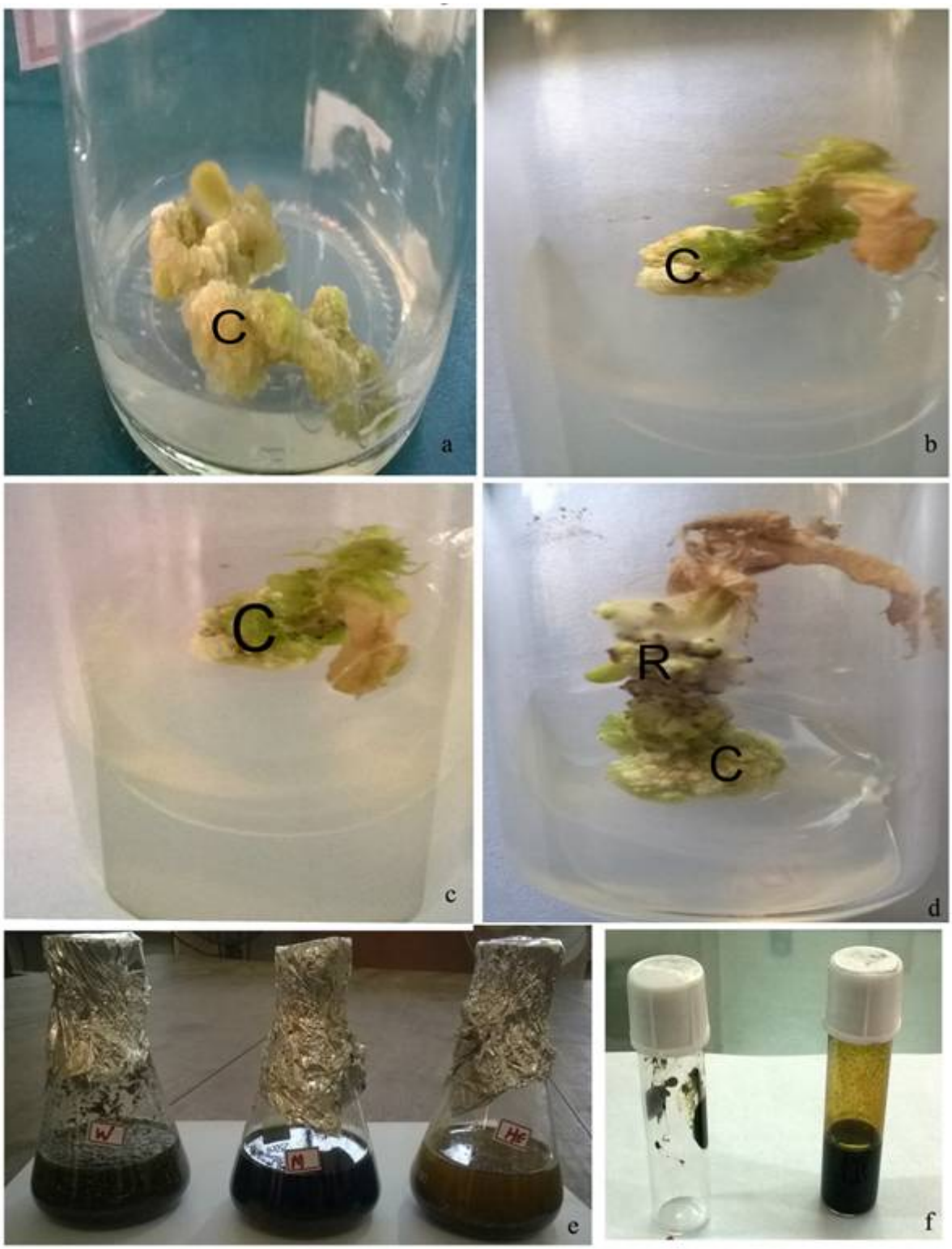


Figure – 2:. a-f, Analysis of Phytochemicals in Leaf extracts, M-Methanol, H-Hexan, W-Water

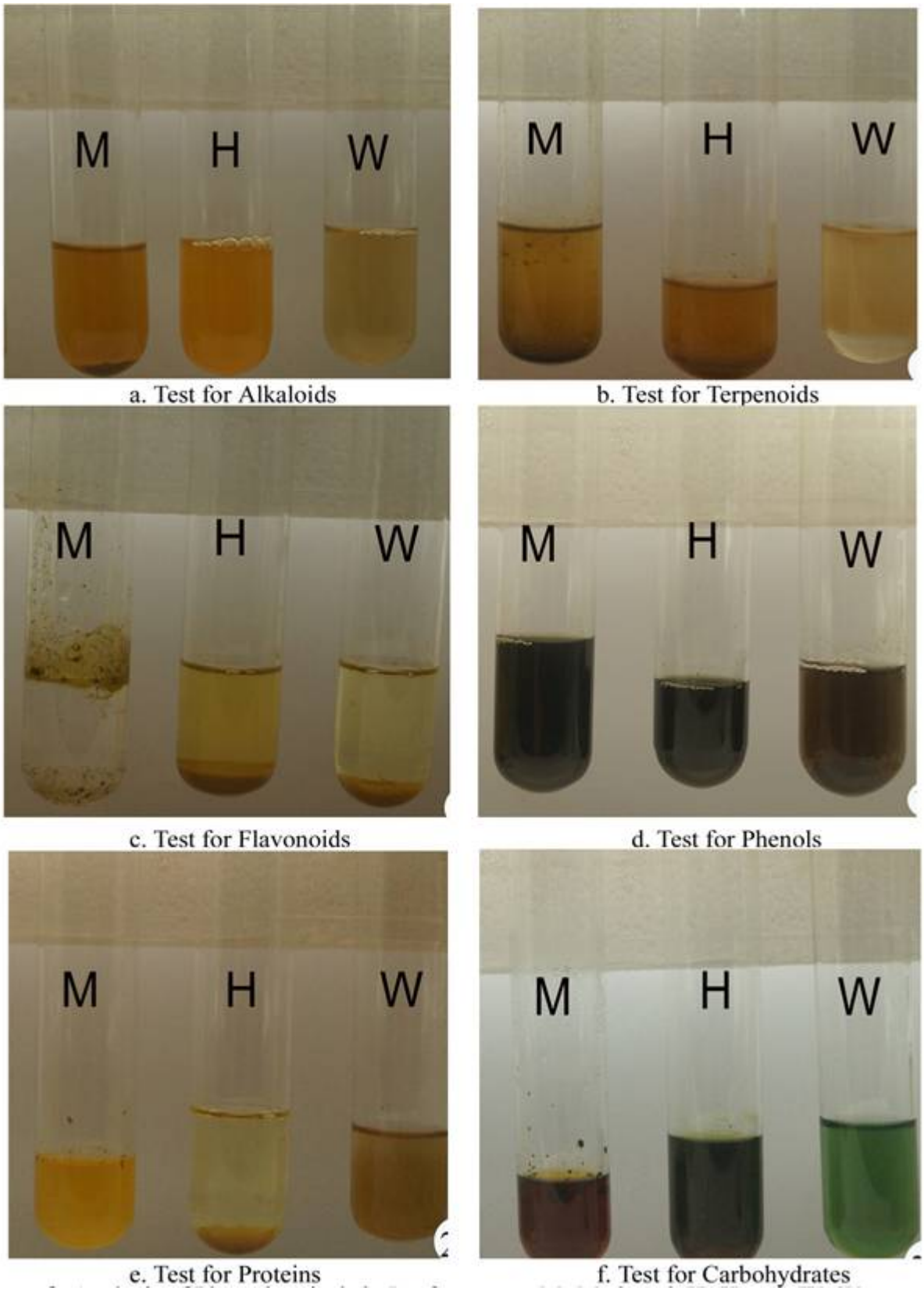


Figure 3. C.S of stem stained with different dyes. a- I2KI for the prescence of Starch, b- Fast green for protein,c- Sudan III for lipids, d- Ferric chloride for tannins, e- Safforin O for lignin

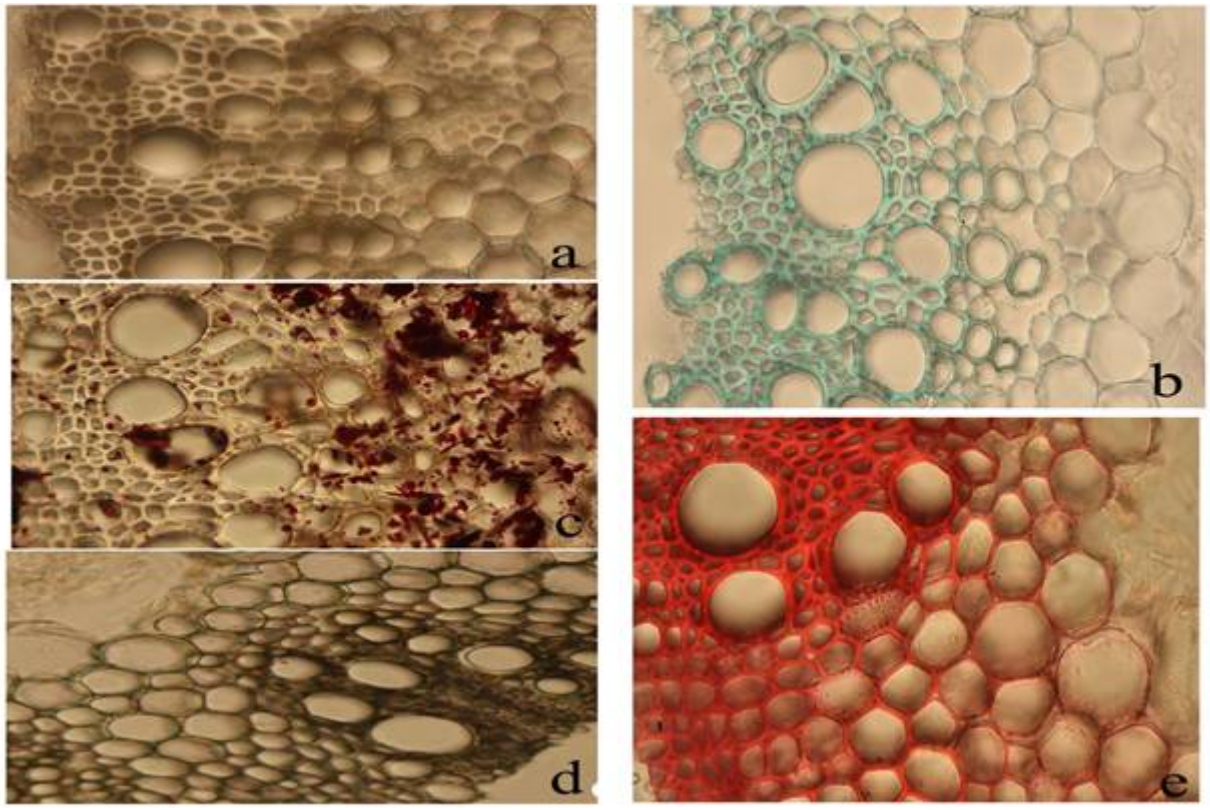
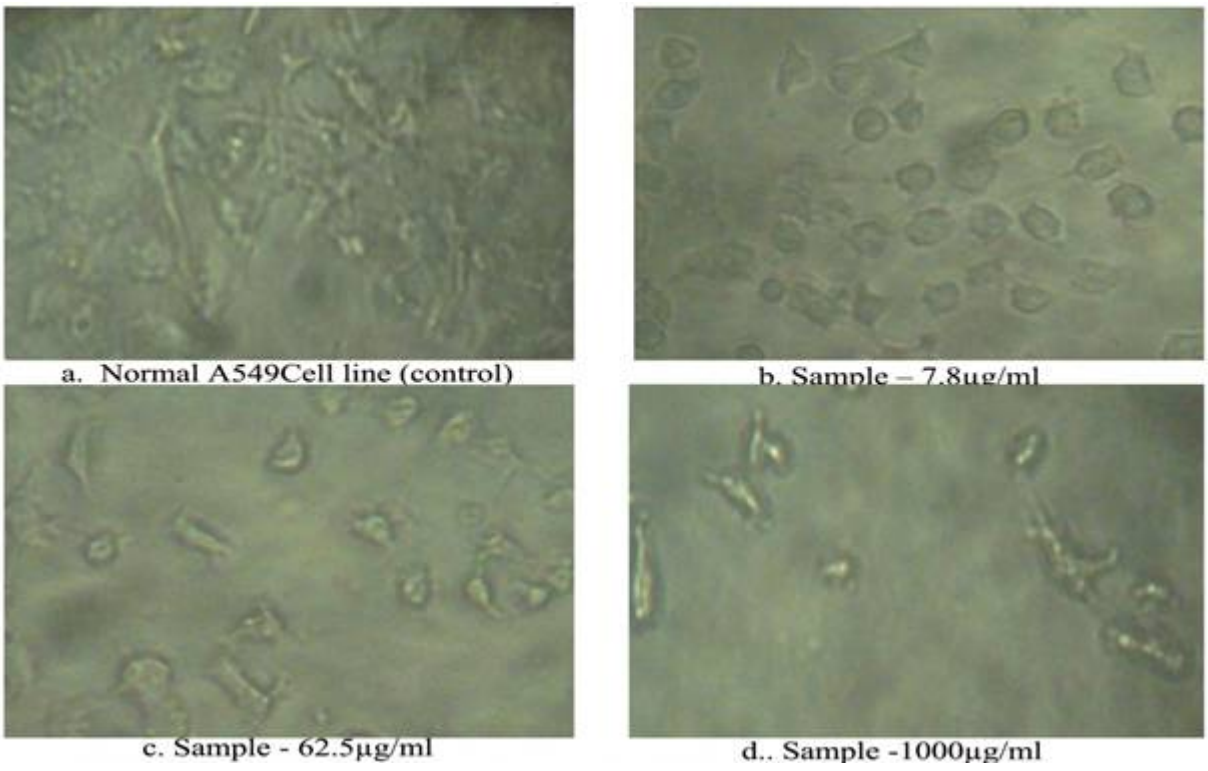


Figure – 4: a-d Effect of Methanolic leaf extract on A 570 cell line.



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