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Original Article

EVALUATION OF *IN VITRO*ANTICANCER ACTIVITY AND GC-MS ANALYSIS FROM LEAF *SOPHORA INTERRUPTA* BEDD

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ABSTRACT

Objective: *Sophora interrupta* Bedd (Fabaceae) is one of the well-known medicinal herb used in folk medicine for the treatment of cancer and inflammatory associated diseases. In this paper we aimed to evaluate the antioxidant and anti-cancer, properties of aqueous, methanol and n-hexane leaf extracts.

Methods: The leaf extracts were analyzed for antioxidant activity using DPPH free radical scavenging assay and anticancer activity by measuring the cell viability using MCF-7 and PC-3 cancer cell lines. GC-MS analysis was performed to identify anti-cancer compounds present in the active leaf extract.

Results: The polar solvent extracts showed good antioxidant at 500 µg/ml as related to non-polar solvents. Moreover, methanol extract exhibited highest percentage of cell death, in both MCF-7 (IC_{50} 500 µg/ml) and PC-3 cells (IC_{50} 1000 µg/ml), which is also evident from morphological observations, acridine orange and ethidium bromide dye exclusion assays.

Conclusion: Over all, it suggests that leaf methanol extract contain anticancer compounds, which are evident from our GC-MS analysis.

Keywords: *Sophora interrupta* Bedd, Leaves, Methanol, Antioxidant, GC-MS.

INTRODUCTION

Medicinal plants in a form of vegetable or in a form of food ingredients have a great importance in our daily life to maintain a balanced diet. Apart from food they also play a very important role in uptake of vitamins and minerals. For example, drinking of tulasi leaf soaked water, has shown to restore the iron absorption in anemic patients [1]. Along the lines, *Terminalia tomentosa* bark juice has shown to cure several cardiovascular diseases and therefore, it was considered as a cardiac tonic. Indeed, natural compounds and their derivatives represent more than 50% of all the drugs in clinical use for today's world. During the last 4 decades, a dozen of potent drugs have been gained from flowering plants; one example is that of Paclitaxel, a drug template derived from the bark of *Taxus brevifolia* species used to treat a number of cancers [2]. Other examples include anticancer agents derived from *Catharanthus roseus* as well as laxative agents from *Cassia* species [3]. Therefore, exploration of medicinal plants and their importance to human and animal health wellbeing is an ongoing research.

Sophora interrupta Bedd (*S. interrupta*) was commonly known as Edwariamadarasapatna and distributed in high altitude region of holy tirumala hills, Andhra Pradesh, India. Many years, this plant was employed in Ayurveda for the treatment of non-communicable diseases like cancer and cardiovascular diseases. Several active compounds, such as O-prenylated flavonol i. e; *3′, 4′-dimethoxy-7-(γ, γ-dimethylallyloxy) flavonol* a novel compound. *2′-hydroxy-3, 4-dimethoxychalcone* was a well-known natural phenol. *Biochanin A* was an isoflavone and well explored. *Kaempferol-3-O-β-D-glucopyranoside* was also a natural flavonol, were isolated from root methanol extract of *S. interrupta* [4]. In a separate experiment similar extract significantly suppressed the tumor volume and enhanced the survival time of DAL bearing mice without altering the total hemoglobin content, RBC and WBC counts [5]. In our previous study, root EtOAc significantly reported anticancer activity against cancer cells and *insilico* work explained*Kaempferol-3-O-b-D-glucopyranoside*, a secondary metabolite of *S. interrupta* root formed 6 hydrogen bond interactions with Arg 202, Gln 207, Gly 227, Gly 229, Thr 231 and Ala 232 amino acids of human DEAD box RNA helicase, DDX3 protein suggesting that the root methanol extract contain anti-neoplastic compounds [6]. Despite these reports, no studies have been made to identify the active constituents present in this plant. In this paper we report, the comparative phytochemical composition, antioxidant activity of various solvent extracts of *S. interrupta* leaf, anti-cancer activity of leaf methanol extracts (MeOH) using MCF-7 and PC-3 cancer cell lines. Of the three extracts MeOH extract was shown significant anticancer and the novelty in this article was addressed by further exploring the compounds present in it by GC-MS analysis.

MATERIALS AND METHODS

Materials

Human breast adenocarcinoma cancer cell line (MCF-7), Prostate adenocarcinoma cell line (PC-3) and Human embryonic kidney cell line (HEK293) was obtained from theNational Center for Cell Science (NCCS), Pune, India. Cell culture reagents were acquired from GIBCO (Invitrogen USA). Streptomycin, Penicillin, MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), DMSO cell culture grade purchased from Hi-Media, Mumbai, India. The shrub *S. interrupta* was collected from Tirumala hilltopin the noon time. Forest area in the location of Latitude13.667790 & Longitude 79.345880 locations, and tender leaves was collected. The plant was taxonomically pointed out by Dr. A. Prasada Rao, Senior Botanist in K LE F University, Vijayawada, Andhra Pradesh, India. Avoucher specimen has deposited, in the department (voucher number KLU 1211) for further use.

Methodologies

Phytochemical analysis

The presence of the phytoconstituents such as terpenoids, tannins, flavonoids, alkaloids, saponins, glycosides and anthraquinones was carried out based on standard protocols [7].

Measuring antioxidants by DPPH free radical

Radical scavenging potentials of n-hexane, MeOH and Aqueous(AQ) extracts were determined against free radical DPPH from standard protocol [8]. Various concentrations of extract (100, 200, 300, 400,

500 and 600 µg/ml) were added to the DPPH methanol stock solution (100 µM). The test samples were incubated for 15 min at room temperature and the optical density change from deep violet to light yellow was determined at 517 nm respectively corresponding to the blank using a UV-Vis spectrophotometer (DYNAMICA Halo DB20; Australia). Ascorbic acid was used as a standard antioxidant (5–50µg/ml). The percentage of free radical scavenging was determined by the following expression:

$$\text{Percentage (\%)Free radical scavenging} = \frac{(\text{ODof Control}) - (\text{OD of test})}{(\text{OD of Control})} \text{x } 100$$

Where, OD is the optical density. IC_{50} values were derived using linear regression analysis. IC_{50} values signify the concentration of the sample, which is appropriate in scavenging 50% of the DPPH free radicals.

Cell viability assay

To study the activity of n-hexane, MeOH and AQ extracts on (MCF-7 and PC-3) cancer cell viability, we seeded MCF-7 and PC-3 cells at a concentration of 5 x 10^3cells per well in a 96 well plate and were maintained in Dulbecco's modified eagle's medium (DMEM) and F12K medium respectively[9]. Both the cell lines were supplemented with 10% fetal bovine serum (heat inactivated) and 1% antibiotic (100U/ml of penicillin and 100µg/ml streptomycin) gently mixed and placed in a 5% CO_2-humidified incubator at 37 °C. The cells were treated with increasing concentrations (1, 10, 50, 100, 250, 300, 500 and 1000 µg/ml) of three extracts in DMSO for 24 h. Following treatment, 15µl of MTT (5 mg/ml) reagent was added to the culture media and further incubated for 4 h at 37 °C in CO_2 incubator. After an incubation period MTT containing supernatant was aspirated, 200 µl of DMSO and 25 µl of Sorenson glycine buffer (0.1 M glycine and 0.1 M NaCl, pH 10.5) were added to lyse the cells and solubilize the water insoluble formazan crystals. Absorbance values of the lysates were determined on a Fluostar optima microplate reader (BMG Labtech, Germany) at 570 nm. The percentage inhibition was calculated as:

$$= \frac{\text{Mean OD of vehicle treated cells} - \text{Mean OD of drug treated cells}}{\text{Mean OD of vehicle treated cells}} \text{ x } 100$$

The IC_{50} values were calculated using a graph pad prism, version 5.02 software (Graph Pad Software Inc., CA, USA). Negative controls were maintained with DMSO. In a separate experiment, the effects of three extracts on cells were confirmed to observe the morphological changes such as cell shape. Morphology of the cells captured using a phase contrast microscope (Zeiss, Axiovert 25, Germany).

Acridine orange (AO)/Ethidium bromide (EtBr) staining

To validate the changes in cell membrane, we have stained the cells using Acridine orange (AO)/Ethidium bromide (EtBr) dyes [10]. For that, we seeded 0.5 x 10^6 cells per well MCF-7 and PC-3 cells in a 6 well plates and cultured as mentioned above. Following 24 h of incubation, the media was replaced with fresh media consisting of MeOH leaf extracts and further allowed for incubation of 24 h at 37°C in a 5% CO_2 incubator. The cells were washed with Phosphate buffer saline (PBS), added 100µl of AO and EtBr (50µg/ml each) respectively to each well, and incubated for15 min in CO_2 incubator. Following incubation the medium was aspirated, and washed thrice with PBS. The intensity of fluorescent staining was observed and the images were captured with the help of fluorescent microscope (Zeiss, Axiovert 25, Germany) using appropriate color filters.

Gas chromatography-Mass spectrometer (GC-MS) analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of MeOH extract was performed using a GC-MS-QP 2010 (SHIMADZU, Kyoto, Japan) equipped with electron impact (EI) mode (ionizing potential-70eV) as well as a capillary column (VF-5 ms) (length 30 m × diameter 0.25 mm, film thickness 0.25 µm) packed with 5% phenyl dimethyl silicone. The ion source temperature was maintained at 240 °C, and helium was utilized as a carrier gas with 99.99% purity. Samples were injected at a temperature of approximately 240 °C with a split ratio of 10:1 and a flow rate of 1.51 ml/min. Mass spectra were taken at 70 eV with a scan fragments from 45 to 1000 Da and the total MS running time was 36 min.

Statistical analysis

Data generated were expressed as mean±SD Statistical analysis was performed using the statistical software Graph Pad prism v5.0. One way-ANOVA was used to analyze statistical differences between groups under different conditions. $p<0.05$ was considered as significant.

Table 1: Phytochemical analysis depicting the presence and absence of phyto constituents

S. No.	Phytoconstituents	n-hex	MeOH	AQ
1	Alkaloids	–	++	–
2	Anthroquinones	–	–	–
3	Carbohydrates	+	+	+
4	Diterpenes	+	++	+
5	Flavonoid	+	++	+
6	Glycosides	+	++	+
7	Proteins	+	+	+
8	Phenols	+	++	+
9	Saponins	+	–	+
10	Steroids	–	–	–
11	Tannins	–	++	+

++More abundance ⟶, +Moderately present ⟶, –Absent ⟶

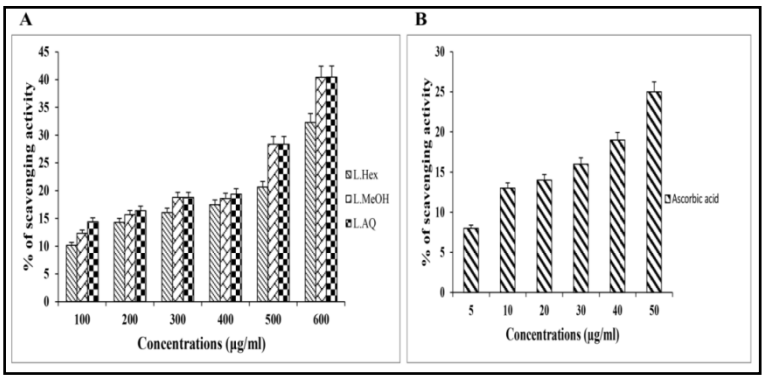

Fig. 1: Radical scavenging activity of the leaf extracts (n-hexane, MeOH & AQ) of *S. interrupta* against DPPH radical. Ascorbic acid was considered as a positive control

Table 2: List of Phytocomponents with functional activities identified using GC-MS from MeOH extract

S. No.	Identified/ Similar compounds	Molecular mass (g mol⁻¹)	Nature of compound	RT	Area %	Functional activity
1	2,3-Dihydro-benzofuran	120.1	coumaran	10.551	0.68	Antiinflammatory (Closse A 1981)
2	2-Methoxy-4-vinylphenol	150.1	phenol	11.71	0.85	Antibacterial (Silici 2005)
3	3',5'-Dimethoxyacetophenone	180.2	phenol	14.951	0.34	NA
4	1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid	192.1	cyclic polyol	15.931	1.78	Antiinflammatory (Clavin M, 2007)
5	Mome Inositol	180.1	polysaccharide	17.384	80.27	Antiproliferative (Neda GD, 2013)
6	Neophytadiene	278.5	terpenoid	17.834	0.98	Antimicrobial (Stojanovic, 2010)
7	1,2-Epoxyoctadecane	268.4	Epoxide	18.279	0.42	NA
8	1-Acetyl-5-(2-Piperidinyl)-1,2,3,4-Tetrahydropyridine	208.3	Aromatic compound	18.884	1.27	NA
9	Caulophylline	204.2	Alkaloid	20.163	0.42	Anti-arthritic (Liao M, 2001)
10	Phytol	296	Diterpene alcohol	20.555	1.04	NA
11	(-)-Cytisine	190.2	Alkaloid	20.666	3.7	Mild intoxication and heightened awareness of color.
12	Lupanine	248.3	Alkaloid	21.964	0.62	Acts as weak sedative effect on CNS system
13	Cytisine, N-formyl-	218.2	Alkaloid	23.957	0.33	NA
14	d-Thermopsine	244.3	Alkaloid	24.066	2.73	Antibacterial &Allelopathic activity (N Villa-Ruano, 2012)
15	Argentamin	260.3	Alkaloid	26.346	1.17	Antihyperglycemic activity (Mardi M. Algandaby, 2010)
16	.delta.5-Ergostenol	400.6	Saponin	29.22	0.35	NA
17	Stigmasterol	412.6	Sterol	29.427	0.64	Antioxidant, hypoglycaemc, and anticancer (Panda S, 2009)
18	gamma.-Sitosterol	414.7	Sterol	29.981	0.7	Antiplatelet & anti-inflammatory (SA Saeed, 19930
19	Alpha.-Amyrin	426.7	Triterpene	30.472	0.5	Analgesic, antiinflammatory (GF Aragao, 2008)
20	Lupenone	424.7	Terpenoid	30.653	0.41	Inhibits protein tyrosine phosphatase 1B (M Na, 20090
21	Methyl Commate C	486	Triterpenes glycoside	30.975	0.58	Antibacterial, antimicrobial, insecticides, nematicides, and are highly effective in wound healing activities. (Devika 2014)

NA = Not available

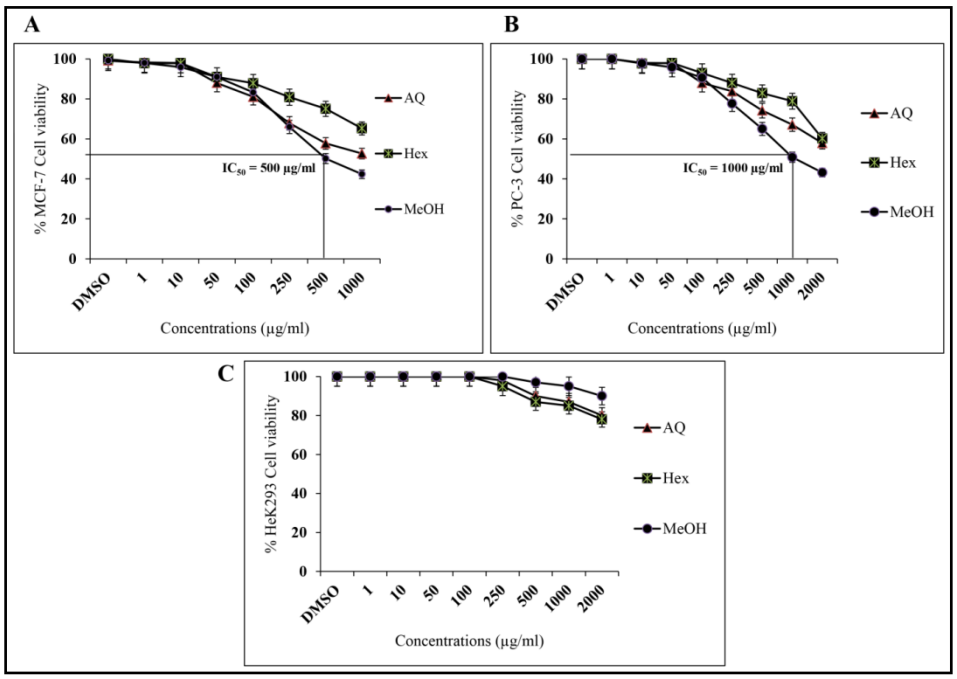

Fig. 3: Impact of *S. interrupta* leaf MeOH and AQextracts on MCF-7 and PC-3 cell morphology. A and D Control (DMSO) B) MeOH (500 µg/ml) C) AQ (1000 µg/ml)E) MeOH (1000 µg/ml) and F) AQ (2000 µg/ml).

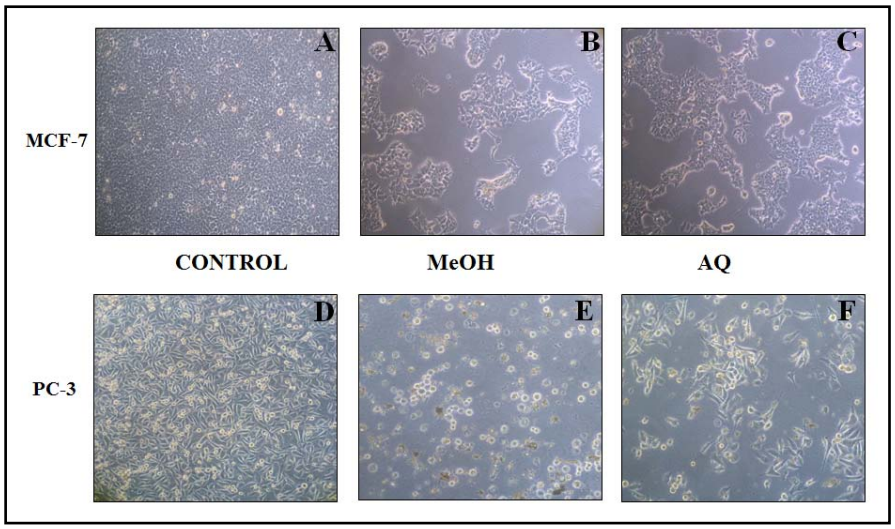

Fig. 3: Cell morphological study of *S. interrupta* leaf MeOH and AQ induced membrane blebbing and detachment were observed in both MCF-7 (500 µg/ml) and PC-3 (1000 µg/ml) cells in contrast with control (DMSO) as a vehicle

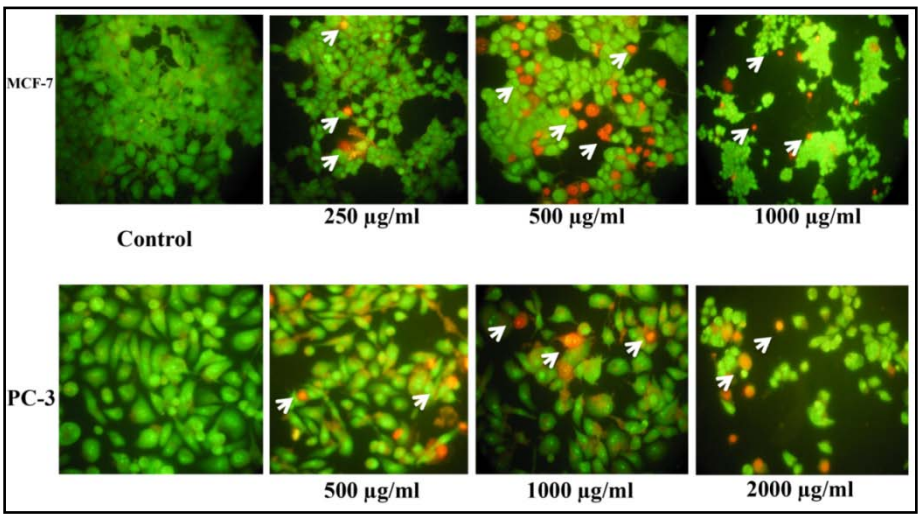

Fig. 4: Apoptotic induction of cells with *S. interrupta* leaf MeOH extract on both MCF-7 and PC-3 cells stating the early and late apoptosis depicted by orange(late death)and red colour (early death) respectivelywhich was visualized with arrow marks

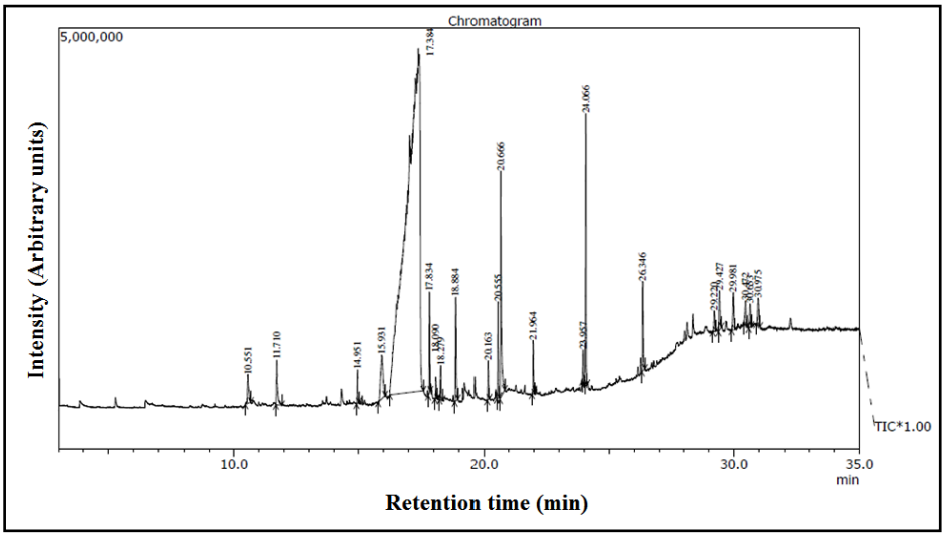

Fig. 5: Typical GC-MS chromatogram spectrum of leaf Methanol extract

RESULTS

Phytochemical analysis

In order to identify the presence and absence of phyto chemicals present in the n-hexane, MeOH and AQ extracts, we have performed several biochemical analysis. Results showed (table-1)that all three extracts were positive for terpenoids, flavonoids and Glycosides and they were documented as ++/+/based on the intensity of color change/precipitation. On the other hand, steroids and Anthraquinones are not present in either of the extracts. Along the lines, we found that leaf MeOH extract is exclusively positive for alkaloids. Our results allow choosing choice based secondary metabolites for the validation of medicinal importance of the *S. interrupta* leaf extract.

Antioxidant activity by DPPH free radical scavenging assay

Antioxidant activity is typically used to validate the medicinal importance of various plant extracts. To identify the active fraction we incubated DPPH in presence of various concentrations of *S. interrupta* leaf extracts. The percentage of free radical scavenging activity of various leaf extracts (n-Hexane, MeOH and AQ extracts) was measured and documented in fig. 1. As showed in fig. 1, all the three extracts showed dose dependent inhibition of DPPH scavenging activity at increasing concentrations ranging from 100 to 600 µg/ml. However, 5 to 10 % change in the scavenging activity was noticed between non-polar (n-hexane) to polar extracts (MeOH) at higher concentrations (500 and 600 µg/ml). In our study, we used a well-known antioxidant ascorbic acid (5–50 µg/ml) as a standard compound. Based on the standard. We found that 500 µg/ml of leaf MeOH extract is required to inhibit the 50% DPPH activity. These results suggest that this plant extract has bioactive phytochemical constituents with potential anti-oxidant activity.

Cell viability assay

Next, we validated the importance of leaf extracts by determining the mitochondrial dehydrogenase activity on two different actively proliferating cancer cell lines (MCF-7 and PC-3) of two different origins such as breast and prostate. For that we incubated the cells with increasing concentration of three leaf extracts for 24 h. Following incubation period, cells were washed with sterile PBS and measured the percentage of cell viability by measuring the formation of formazan precipitation is a measure of mitochondrial activity using MTT (*3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide*) reagent. Results showed that leaf MeOH extract delayed the cell growth as compared to aqueous and n-hexane extracts. The ability of leaf solvent extracts against cancer cell growth in MCF-7 and PC-3 as follows MeOH>AQ>n-hexane. On the other hand, the cell growth inhibitory of leaf extracts differs from MCF-7 to PC-3 cell lines. The concentration required to inhibit the 50% of the cell growth (IC_{50}) in MCF-7 was 500 µg/ml, whereas it was double the concentration (1000 µg/ml) for PC-3 cell lines (fig. 2). It suggests that cell growth inhibitory principles are present in leaf extracts and it differs from cell to cell type. In a separate experiment we have captured the phase contrast photomicrographs for both MCF-7 and PC-3cells to observe the morphological changes. The cells showed a smooth, flattenedmorphology with normal nuclei in normal culture conditions. In contrary, treated cells showed typical morphological changes with membrane blebbing and detached from the substrate in both MCF-7 and PC-3 cell lines (fig. 3)

Acridine orange (AO)/Ethidium bromide (EtBr) staining assay

To measure the apoptosis cell index, we validated the uptake of fluorescent dyes such as Acridine orange (AO) and Ethidium Bromide (EtBr) dyes in the presence and absence of leaf MeOH extract. For that we treated MCF-7 and PC-3 cancer cell lines with 500 µg/ml and 1000 µg/ml of leaf MeOH extract for 24 hours. Following time period, cells were washed with sterile PBS, incubated with fluorescent dyes (1:1 ratio i.e; 50 µg/ml of each dye) for an additional 15 min. Following incubation, cells were washed with PBS and dye uptake was captured using fluorescent microscope to measure the membrane permeability. Results showed that MeOH treated cells showed the uptake of EtBr i.e; higher membrane permeability and early or late apoptosis, which were evident from the EtBr and AO staining cells as compared to MeOH treated (Control) cells. Similarly, PC-3 cells showed similar results and data is in accordance with MCF-7 cell lines (fig. 4). Taken together. It suggests that the leaf MeOH extract contain an anti-cancerous compounds and the degree of their cell growth inhibitory action is dependent on cell type and culture condition.

GC-MS

To identify the secondary metabolites present in the leaf MeOH extract, 1 kg of leaves were collected. Shade dried, and macerated in the presence of MeOH. Following maceration, the extract was concentrated by distillation technique. Crystalline form of powder was used for GC-MS analysis using qualitative method. The number of molecules present in the leaf MeOH extract and their retention times is generated as a single spectral chromatogram. The spectrum data were compared with theNational Institute of Standards and Technology (NIST) library to identify the similar/identical compounds. Results showed three major peaks (fig. 5) and nineteen minor peaks with 21 compounds. Name of the identical/similar compounds, their retention times, % of peak area, molecular weight and their possible functional activity was described in table-2. As showed in table-2, the Mome Inositol; (-)-Cytisine and d-Thermopsine was occupied a major percentage of area with high retention times.

DISCUSSION

Plants and animals co-exist in existence of the nature. Plants are more adaptable species on the earth than animals. Unlike, humans plants can adapt to cold, heat and drought tolerable conditions. Due to the lack of adaptability, humans are vulnerable to many diseases in the developing world[11]. There are several methods and treatments available to treat various diseases. However, lack of adaptability, resistance developed against contagious and communicable diseases is a major threat to human existence. Therefore, identification, isolation, and characterization of natural compounds from plant or other biological sources is an ongoing research. In our continuous efforts, we have identified *S. interrupta* plant as a natural reservoir for several effective medicinal compounds. Our group and others have documented the importance of *S. interrupta* root EtOAc extract for anticancer and anti-inflammatory activities. In this paper, we describe the importance of bioactive compounds from leaf part of *S. interrupta* plant. To identify the biological activity of the compounds present in the leaf extract, we used three different solvents varying between non-polar (n-Hexane) to Polar (MeOH and AQ). The biological activity of each extract was estimated by validating the free radical scavenging activity using DPPH as a substrate. All the extracts showed scavenging activity, it suggests that the antioxidant nature of the compounds present in the leaf as a whole. However, antioxidant activity alone cannot be considered as a therapeutic index for treating emerging diseases due to the changes in environment and lifestyle factors. Based on the literature, we have tested all these extracts for anticancer activity by measuring the growth of actively proliferating cancer cells using MCF-7 and PC-3 cell lines. Both MeOH and AQ extracts declined the cell growth in both the cell lines.

The magnitude of the inhibition of cell growth is varied for both the extracts and was higher for MeOH extract as comparison to AQ. Along the lines; we also noticed that the concentration required inhibiting the 50% of cell growth varied from MCF-7 to PC-3 cells line. It may be caused by genetic heterogeneity between two different cell lines. From this experiment, it was clear that antioxidant nature alone cannot circumvent the medicinal value; in addition the compounds must produce certain biological activity. From this, we rule out the importance of n-hexane and AQ extracts for further studies. In support of the cell growth inhibitory activity, we measured the membrane permeable study using leaf MeOH extract. Influx and efflux of fluorescent dyes such as acridine orange (AO) and Ethidium Bromide (EtBr) were considered as one of the hallmark for determining the apoptotic index of the viable cells [12]. Apoptosis is a programmed cell death, during cell death; the membrane rigidity is inversely proportional to the living cells, therefore the permeability of small molecules diffuse easily and can interact with nuclear ingredients like DNA and RNA. From our

experiments, it was clear that leaf MeOH extract induced cell death in both the cancer cell lines, which was evident from the more number of EtBr stained red fluorescent cells in the field of view at the concentration of IC_{50} value for both MCF-7 (500 µg/ml) and PC-3 (1000 µg/ml) cells. Altogether, it suggests that, Leaf MeOH extract contain biologically active principles, found to have 21 phytoconstituents, which was evident from GC-MS spectral chromatogram. Among all, Mome Inositol is one of the major components with a highest percentage area of 80.27 and six other compounds have 1-5% range of abundance in this extract. The extraction of crude extracts was a successive extraction procedure from non-polar to polar solvents, in this case we have performed GC-MS but the Inositol was present only in MeOH extract and absent in other extracts. Mome Inositol with a molecular weight of 180.16g mol^{-1}shown to exhibit anti-proliferative, anti-alopecic, anti-cirrhotic and anti-neuropathic activities [13]. Among all, Mome Inositol has shown anti-proliferative activity against MCF-7, MDA-MB-231, HepG2 and Hs27 cell lines. Mome Inositol is a six hydroxyl group polysaccharide chains and was highly abundant in other medicinal plants such as *Macrotyloma uniflorum* (Lam.), *Clitoria ternatea* and *Ormocarpum cochinchinense* [14].

CONCLUSION

Sophora interrupta Bedd belong to the family of Fabaceae. The species in this family is indigenous to India. The leaf methanol extract contain several antioxidant ingredients and they exhibited anti cancerous activity in both MCF-7 and PC-3 cell lines. Mome Inositol was one of the highly abundant medicinal compounds present in this extract, confirmed by GC-MS analysis. However, studies were indeed needed to validate the importance of Mome Inositol alone or in combination with other abundant compounds against cancer using bioactive principles from *S. interrupta* leaf extract.

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CONFLICT OF INTERESTS

Declared None

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