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ANTI LEUKAEMIC ACTIVITY OF *EVOLVULUS ALSINOIDES*

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ABSTRACT

*Evolvulus alsinoides (*Shankhpushpi*)* is a reputed drug of Ayurveda and reported as a brain tonic, nervine tonic, and laxative. It has also been found effective in anxiety, neurosis, and memory invigorator and used in cerebral abnormalities, epilepsy, insomnia, burning sensation, oedema, urinary disorders, and snake-bites. So the literature survey revealed that it has numerous activities and chemically it contains Alkaloids, Terpenoids, Glycosides, Flavanoids, Steroidal compounds etc. Plant derived anticancer agents are widely used for the treatment of leukemia. The main objective of present work is to isolate and characterize the compounds from the leaf extract of Shankhpushpi and to investigate antileukemic activity against different cell lines. The shade dried leaves of *Evolvolus alsinoides* were first defatted with petroleum ether and then successively extracted with Petroleum-ether and chloroform at room temperature. Both the extracts were chromatographed over a column of silica gel and Graded elution was carried out with petroleum-ether, chloroform and methanol. Repeated chromatographic purification of the fractions led to isolation of four compounds which were designated as OS-1, OS-2, OS-3and OS-4 and were identified as β-sitosterol-3-*O*-glucoside, β-sitosterol, Ursolic and Kaempferol-3, 7-di-*O*-rhamnoside by comparing the spectral (IR, ^{1}HNMR, ^{13}C NMR) and HPLC data with the authentic samples reported in the literature. These were tested for their cytotoxicity against leukemic cell lines U937, K562 and HL60 using MTT assay. The data obtained suggest that OS-4 is more cytotoxic in nature followed by OS-3 over 24 hrs of treatment. Hence the antileukemic principle was identified from the Shankhpushpi plant.

Key words: Antileukemic activity, *Evolvulus alsinoides*, MTT assay, shankapushpi**.**

INTRODUCTION

Prevention is currently an attractive and promising strategy to reduce the occurrence of cancer. Primary prevention strategies involve removing the causative agent(s) and other life-style modifications that decrease the risk of cancer. Secondary prevention (cancer chemoprevention) is the use of non-toxic natural and/or synthetic agents to decrease the risk of malignant tumor development. In the present scenario, the demand for herbal products is growing exponentially throughout the world and major pharmaceutical companies are currently conducting extensive research on plant materials (leaves) for their potential medicinal value. Cancer remains as one of the fetal diseases throughout the world and there is renewed in the discovery of novel compounds that can be used to fight cancer. Plant derived anticancer agents are widely used for the treatment of cancer/leukemia. In the present study an attempt will be undertaken to investigate the anti-cancer/antileukemic activity of *Evolvulus alsinoides.*

It is commonly known as Shankhpuspi in India. It is an important medicinal plant that grows in the open and grassy places almost throughout the India and subtropical countries of the world. Plant extracts have been used in traditional medicine for treatment of bronchitis, asthma and brain disorders like insanity, epilepsy, nervous disability, and scrofula. [1, 2]

Shankhpushpi is a reputed drug of Ayurveda and reported as a brain tonic, nervine tonic, alternative and laxative [3]. It has also been found effective in anxiety and neurosis, due to its clinical anti-anxiety effects and improved mental function highly esteemed by ancient Indian physicians as a wonderful nervine tonic & memory invigorator and used in cerebral abnormalities, epilepsy, insomnia, burning sensation, oedema, urinary disorders, snake-bites and disease caused by evil spirits. It is best tonic for brain and nerves and is also recommended for sexual & seminal debilities[4].

MATERIALS AND METHODS

General: Silica gel, 60-120 &100-200 mesh size (SRL-Mumbai, India) was used for column chromatography. TLC was carried out in Silica gel 60 F_{254} plates (Merck, Germany) and spots were visualized by spraying Libermann-Burchard reagent followed by heating. Preparative TLC was carried out on precoated Silica gel 60 F_{254} plates (Merck, Germany).The IR spectra of the compounds were recorded as KBr pellets on a JASCO 7300 FTIR spectrometer. 1H NMR and 13C NMR were carried out at 300MHz. All other chemicals and solvents were purchased from SRL- Mumbai, India.

Plant Material

Evolvulus alsinoides leaf samples were collected from the suburbs of Kolkata, India and were identified at Indian Botanic Garden, Howrah, West Bengal, Kolkata.

Extraction: The shade dried leaves of the plant (1kg) was extracted using three solvent systems – petroleum ether, chloroform and methanol successively. Extraction was done three times each with the same solvent using approx. 7 litres of solvent in each extraction. The extract was distilled and the residue was collected for further fractionation.

Isolation of Pure Compounds

Isolation of pure compound was done by using column chromatography technique.

For Bio-evaluation

Chemicals

RPMI 1640 medium, fetal bovine serum albumin (FBS), HEPES, streptomycine, penicillin,1-glutamine, 3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), Ara-c, DMSO and general reagents were purchased from Sigma(ST. Louis, MO, USA) and remaining chemicals and solvents were purchased from local firms and were of highest purity grade.

Cell Lines

Human leukemic cell lines U937, K562 and HL60 was obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. Cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 100U/ml penicillin and 100mg/ml streptomycin. Cultures were maintained in RPMI 164 heat 37ºC in humidified atmosphere containing 5% CO2 in air. In all the experiments untreated leukemic cells were termed as control group.

Plant material: as described before.

Extraction and Fractionation: as described before.

For pharmacological studies 1 mg of OS-3 and OS-4 were dissolved in 1ml of DMSO (0.05% DMSO in buffer) to get the stock solution of 1 mg/ml.

Statistical analyses

Statistical analyses were done by Graph Pad InStat software (La Jolla, CA, USA). Data were expressed as mean ± S.D. of three independent experiments. The differences between the

treated and control groups were analyzed by one-way ANOVA and posttests were done using Dunnett's multiple comparison tests to determine the significant levels. $p< 0.01$ (**) and $p< 0.05$ (*) were considered to be significant.

Anti-leukemic Activity

In vitro cytotoxicity assay using MTT

The MTT (method of transcriptional and translational) assay and the MTS assay are laboratory tests and standard colorimetric assays (an assay which measures changes in color) for measuring the activity of enzymes that reduce MTT or MTS + PMS to formazan , giving a purple color. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials, since those agents would result in cell toxicity and therefore metabolic dysfunction and therefore decreased performance in the assay. Cytotoxicity of OS-3 and OS-4 was assessed using the 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT), assay. Initially cells (1×10^5,100µl cell suspension per well) were seeded on 96 well tissue culture plates and incubated with OS-3 and OS-4(50, 100, 200 µg/ml) for 24 hrs at 37ºC in a humidified atmosphere containing 5% CO_2 in air. Untreated cells were taken as control. At the end of treatment, 20µl of MTT (5mg/ml in PBS) was added to each well and incubated for another 3 - 4hrs. DMSO (100 µl) was added to solublise the MTT formazan crystal and optical density (OD) was measured after 10 minutes at 492nm using micro plate manager (Reader type: Model680 XR, Bio-Rad Laboratories. Lnc.). Every sample was performed in triplicate by using all three cell lines (U937, K562 and HL60).

Percentage of OD inhibition was calculated by the following formula:

% OD inhibition = 100× (OD of Control-OD of treated)/OD of control; OD = optical density

% inhibition of OD in HL60 Cell line

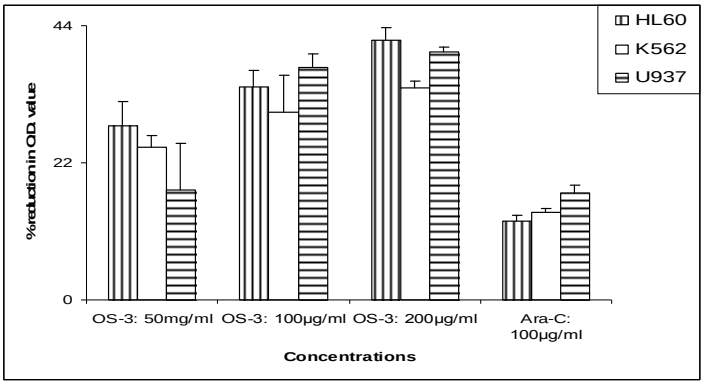
Conc.	%inhibition of OD in Cell line HL60	
	OS-3	OS-4
50 µg/ml	28.0 ± 3.8%	58.28 ± 3.5%
10 0µg/ml	34.19 ± 2.7%	71.35 ± 2%
200µg/ml	41.65 ± 2%	76.37 ± 1.8%

Inhibition of OD in K562 Cell line:

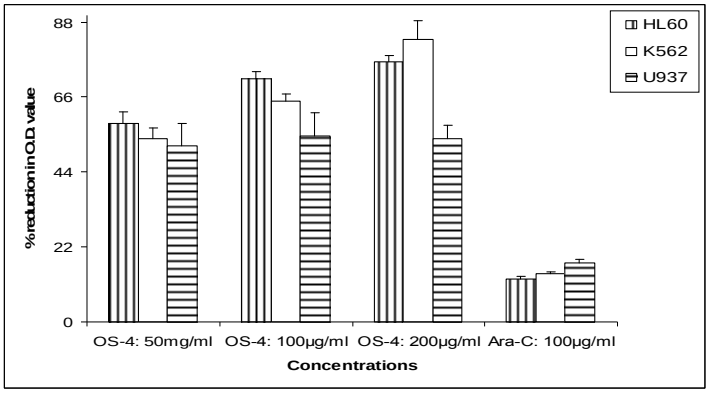
Conc.	%inhibition of OD in Cell line K562	
	OS-3	OS-4
50 µg/ml	24.63 ± 1.9%	52.68 ± 3.3%
10 0µg/ml	30.04 ± 6.0%	64.70 ± 2.1%
200µg/ml	34.03 ± 1.1%	82.87 ± 5.6%

% Inhibition in of OD U937 Cell line

Conc.	%inhibition of OD in Cell line U937	
	OS-3	OS-4
50 µg/ml	17.56 ± 7.6%	51.78 ± 6.5%
10 0µg/ml	37.35 ± 2.1%	54.55 ± 6.9%
200µg/ml	39.83 ± 0.8%	53.82 ± 3.8%



Cytotoxic effect of OS-3 on human leukemic cell lines (HL60, K562 and U937) after 24h of treatment as observed by MTT assay. (Data compared with control where OS-3: 0µg/ml)



Cytotoxic effect of OS-4 on human leukemic cell lines (HL60, K562 and U937) after 24h of treatment as observed by MTT assay. (Data compared with control where OS-4: 0µg/ml).

3.8. RESULTS AND DISCUSSION

Chloroform extract was chromotographed over a column of silica gel by gradient elution. The compounds were identified and coded as OS-1 and OS-2 confirmed as β-sitosterol-3-*O*-glucoside and β-sitosterol by comparison with spectral data reported in literature. [5, 6]

OS-1:

Obtained as white powder.IR: V_{max} cm^{-1}(KBr):3413,2938,1053,953 cm^{-1}.

^{1}H NMR: δ4.44(1H, d, J=11.4Hz, H-1'), δ0.64(3H, s, H-29)

OS-2:

Obtained as white solid. IR: V_{max} cm^{-1}(KBr):3413, 2938, 1053, 955 cm^{-1}

^{1}H NMR: δ 5.35 (1H, d, J=4, H-6), δ3.56(1H, s, H-3), δ0.67(3H, s, H-29)

Pertroleum - ether extract was chromotographed over a column of silica gel by gradient elution. The compound was identified and coded as OS-3 and confirmed as Ursolic Acid, which was ascertained by comparison with its spectral data reported in literature. [7]

OS-3

Obtained as white solid. IR: V_{max} cm^{-1}(KBr): 3415, 2928, 1694, 1456 cm^{-1}

^{1}H NMR: δ5.47(1H, s, H-6), δ3.44(1H, d, J=7.2, H-3), δ 2.615(1H, d, J=11, H-18)

methanol extract was chromotographed over a column of silica gel by gradient elution. The compound was identified and coded as OS-4 and confirmed as Kaempferol-3, 7-di-*O*-rhamnoside which was ascertained by comparison with its spectral data reported in literature[8].

OS-4

Obtained as yellow solid. IR: V_{max} cm^{-1}(KBr): 3393, 3928, 1656,1064 cm^{-1}

^{1}H NMR: δ5.549(1H, s, H-1''), 5.295(1H, s, H-1'''), 6.45(1H, s, H-6), 6.79(1H, s, H-8), 6.9,(2H, d, J=7.5, H-3',5'), 7.8(2H, d, J=7.5, H-2',6'), 10.263(1H, brs,4'-OH), 12.6(1H,brs, 5-OH)

OS-3 and OS-4 isolated from petroleum ether extract and methanol extract were tested for their cytotoxicity against leukemic cell lines U937, K562 and HL60 using MTT assay (Fig. 3.1 & 3.2). The data obtained suggest that both OS-3 and OS-4 are cytotoxic in nature. The maximum cytotoxicity is shown by OS-4 followed by OS-3 over 24 hrs of treatment. Since

cell death occur by two major pathways viz: apoptosis and necrosis so detailed analysis must be carried out to determine the mode of cell death caused by drugs.

CONCLUSION

Our studies indicate that the chloroform extract contains β-sitosterol glucoside and β-sitosterol, petroleum ether extract contains Ursolic acid and methanol extract contains Kaempferol-3, 7-di-*O*-rhamnoside. The data obtained from the biological studies suggest that OS-3 and OS-4 both are cytotoxic in nature. The maximum cytotoxicity is shown by Kaempferol-3, 7-di-*O*-rhamnoside followed by ursolic acid over 24 hrs of treatment against leukemic cell line, HL60, K562 and U937. Since cell death occur by two major pathways viz.: apoptosis and necrosis so detailed analysis must be carried out to determine the mode of cell death caused by drugs.

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