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Research Article

ANTICANCER ACTIVITY OF HYDROALCOHOLIC EXTRACTS FROM *PARIS POLYPHYLLA* RHIZOMES AGAINST HUMAN A549 LUNG CANCER CELL LINES USING MTT ASSAY

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ABSTRACT
The following study is designed to determine the anticancer and cytotoxic potential of hydroalcoholic extract from rhizomes of *Paris polyphylla* to produce any cytotoxic effect on human A549 lung cancer cell lines. The hydroalcoholic analysed for spectral analysis through NMR, HPLC-DAD and LC-ESI-MS/MS methods and found to have Diosgenyl and pennogenyl saponins. The test conducted using MTT method using human lung cancer A549 cell lines as part of the *in vitro* preclinical characterization of compound and compared against Doxorubicin. More than 97 % increment in cell killing at a concentration of 500 µg/ml recorded in the cell line. The EC50 for the extract was calculated to be 52.34 ug/ml and at the concentration of 1.8559 ug/ml, doxorubicin exhibited approximately 98 % killing of the cells. The EC50 for the Doxorubicin was calculated to be 0.579 ug/ml. From the study it is concluded the hydroalcoholic rhizome extracts *Paris polyphylla* has potential to exhibit anticancer activity.
Keywords: Anticancer, A549 Lung cancer cell line, *Paris polyphylla*, rhizomes, Diosgenyl saponin, pennogenyl saponin, NMR, LC-ESI-MS/MS

INTRODUCTION

Paris polyphylla LINN-HS 703.3(herbarium number) (Herb Smith) SM.avr.yunnanensis, Chinese (chonglou) Hindi name (Doodh Buch) and in Nepalese (Satua) a perennial herb originates from the Himalayas. It usually grow about 10-100 cm tall from a rhizome 1-2.5 cm thick. The rhizomes of *Paris polyphylla* have been used as anti-helminthic and vermifuge in folk communities of Nepal[1]. The powdered roots of this plant are used as ethnopediatrics for diarrhoea in Garwal Himalaya, Uttarakhand, India[2]. It is one of the medicinally important plants in traditional Chinese medicine. Rhizomes of *Paris polyphylla* have saponin glycosides. Diosgenyl and with different sugar chains at 3-hydroxy group are the major saponin glycosides present in the rhizome. These compounds have been used as hemostatic agents and promoters for shrinkage of uterus in clinics. They also exhibit antibiotic and antitumor activity[3,4] . Medicinal plants have been in use from time immemorial and their utility has been increasing day by day in the present world. Naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is also reduced[5]. Plants represent a source of leads for many pharmaceutical compounds and the phytochemical compounds and secondary metabolites present in plants have been used in treating a number of human ailments. Drugs obtained from medicinal plants comprise 25 % of total drugs in developed countries and about 80 % in developing countries[6]. Lung cancer has been regarded as a leading cause of cancer-related mortality throughout the World. Its occurrence and development are associated with a variety of factors, including oxidative stress, apoptosis, immune factors disorders, dysfunction of lung epithelial cells, inflammation, etc. Phytochemical study showed that its main components, steroidal saponins displayed a potential cytotoxicity against various tumor cells, such as CCRF leukemia cells, ECA109 esophageal cancer cells, CaEs-17 cells, human promyelocytic leukemia HL-60 cells, human liver carcinoma HepG-2 cells, human gastric cancer BGC-823 cells, human colon adenocarcinoma LoVo cells and SW-116 cells[7,8].

MATERIALS AND METHODS

Preparation of herbal extract

The rhizomes of the plant were dried in shade for about 3 weeks and ground using a mixer to a coarse powder. Using a soxhlet extraction method, the powder of dried rhizomes were processed with petroleum ether (40-50ºC) for 18 h in order to remove fat and unwanted components. The treated powder was further processed with hydroalcoholic solution (25:75) by using same extraction process for 18 h. The extract was concentrated by evaporating the solvent using a water bath maintaining at 60-80O C at ambient conditions to get a crude hydroalcoholic extract devoid of solvents.

Identification of Chemical Components

Steroidal saponins were the main compounds of *Paris polyphylla* and they have been confirmed as contributors to the inhibition of tumor growth[9]. After being extracted with hydroalcoholic method, these main steroidal saponin compounds were analyzed by NMR and identified by high performance liquid chromatography-diode array detection (HPLC-DAD) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

MTT Assay

Cytotoxicity of the hydroalcoholic rhizome extract of *Paris polyphylla* was studied using A549 lung cancer cell line. The MTT assay was performed here as it allows assessing the viability and the proliferation of cells and thus giving an insight into cytotoxic potential of drugs. The MTT assay measures the activity of enzymes that reduce MTT to formazon dyes, giving a purple color. The amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control

cells, the effectiveness of the agent in causing death can be deduced through the production of a dose response curve. DMEM/F-12 Media and MTT were purchased from ATCC and Sigma respectively. Doxorubicin Hydrochloride was used as a reference drug along with the Paris polyphylla extract. Data Analysis was performed using GraphPad Prism software.

MTT Assay protocol

DAY1

Step 1

Plate 7000 cells in 100 ul media per well in a 96 well cell culture plate.

Step 2

Incubate (37ºC, 5 % CO_2) overnight to allow the cells to attach to the wells.

DAY2

Step 3

Prepare serial dilution of the Doxorubicin and hydroalcoholic extract in DMEM/F-12

Step 4

Add the respective concentrations of the drugs to the assigned wells as per the plate plan with cells. Place on a shaking incubator @ 150RPM for 5 minutes, to thoroughly mix the samples into the media.

Step 5

Incubate (37C, 5 % CO_2) for 72 h to allow the drug to take effect.

DAY5

Step 6

Aspirate and add 100 ul media and 20 ul MTT solutions to each well. Place on a shaking table, 150 rpm for 5 minutes, to thoroughly mix the MTT into the media.

Step 7

Incubate (37C, 5 % CO_2) for 1-5 hours to allow the MTT to be metabolized.

Step 8

Remove the media from the plate by aspiration.

Step 9

Add 100 ul DMSO to each well. Place on a shaker at 150 rpm for 5 minutes, to thoroughly mix the formazan into the solvent.

Step 10

Read optical density at 570 nM. Optical density should be directly correlated with cell quantity.

Plate plan

In a 96 well plate, the treatment scheme mentioned in Table 1.

Table 1: Plate Plan

	1	2	3	4
	DOXO	**DOXO**	**Extract**	**Extract**
A	No treatment	No treatment	No treatment	No treatment
B	0.05 uM	0.05 uM	7.81 ug/ml	7.81 ug/ml
C	0.1 uM	0.1 uM	15.62 ug/ml	15.62 ug/ml
D	0.2 uM	0.2 uM	31.25 ug/ml	31.25 ug/ml
E	0.4 uM	0.4 uM	62.5 ug/ml	62.5 ug/ml
F	0.8 uM	0.8 uM	125 ug/ml	125 ug/ml
G	1.6 uM	1.6 uM	250 ug/ml	250 ug/ml
H	3.2 uM	3.2 uM	500 ug/ml	500 ug/ml

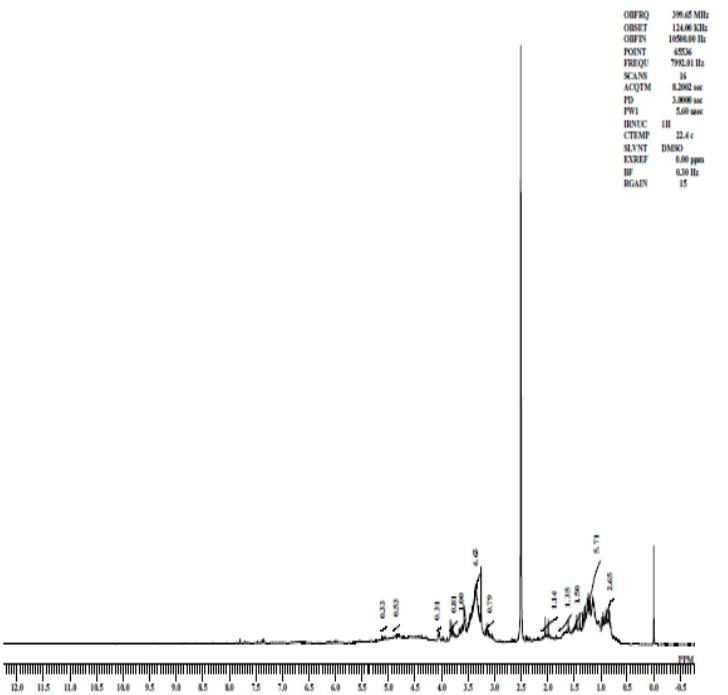
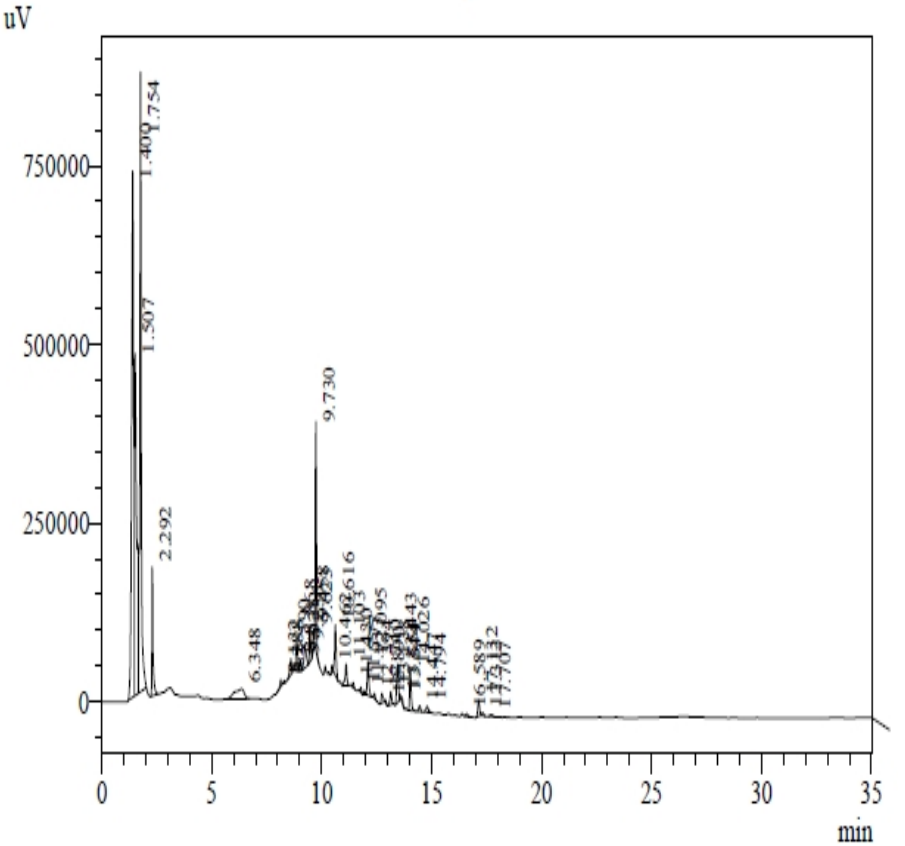


Figure 1 1H1 NMR of hydroalcoholic extract for Diosgenyl saponins



PDA Ch1 225nm 8nm

Peak#	Ret. Time	Area	Resolution	Theoretical Plate#	Area %
1	1.400	4685213	0.000	753.228	27.031
2	1.507	3592869	0.456	525.524	20.729
3	1.754	3869756	1.315	3816.212	22.327
4	2.292	594495	5.408	11522.986	3.430
5	6.348	433000	9.266	1033.208	2.498
6	8.132	50039	3.865	59688.582	0.289
7	8.288	14030	1.284	90741.184	0.081
8	8.590	116915	2.483	65968.662	0.675
9	8.868	226392	1.918	51870.465	1.306
10	9.021	60920	1.140	103332.608	0.351
11	9.236	218857	1.349	32164.488	1.263
12	9.458	212570	1.341	89731.317	1.226
13	9.623	112072	1.413	129457.811	0.647
14	9.730	1108160	0.983	126668.293	6.394
15	10.462	35860	6.781	154195.627	0.207
16	10.616	341220	1.245	89932.499	1.969
17	11.103	177471	3.266	80262.275	1.024
18	11.430	27025	2.320	133501.275	0.156

Peak#	Ret. Time	Area	Resolution	Theoretical Plate#	Area %
19	11.777	27466	2.862	162286.790	0.158
20	11.933	21533	1.323	160521.521	0.124
21	12.095	245477	1.193	100728.296	1.416
22	12.394	24421	2.267	197280.822	0.141
23	12.740	74842	2.479	92834.044	0.432
24	12.890	14465	1.108	241759.450	0.083
25	13.138	99273	1.896	112952.735	0.573
26	13.443	308379	1.872	101251.880	1.779
27	13.554	25665	0.000	0.000	0.148
28	13.644	23329	0.000	215989.331	0.135
29	14.026	281289	2.873	142678.447	1.623
30	14.447	46517	2.602	109291.770	0.268
31	14.794	65692	1.738	69082.781	0.379
32	16.589	30072	8.682	124184.812	0.174
33	17.132	125813	3.060	169131.704	0.726
34	17.313	19687	1.190	254528.826	0.114
35	17.707	21706	2.112	90086.128	0.125
Total		17332493			100.000

Figure 2 HPLC chromatogram (A) and LC-ESI-MS/MS spectra

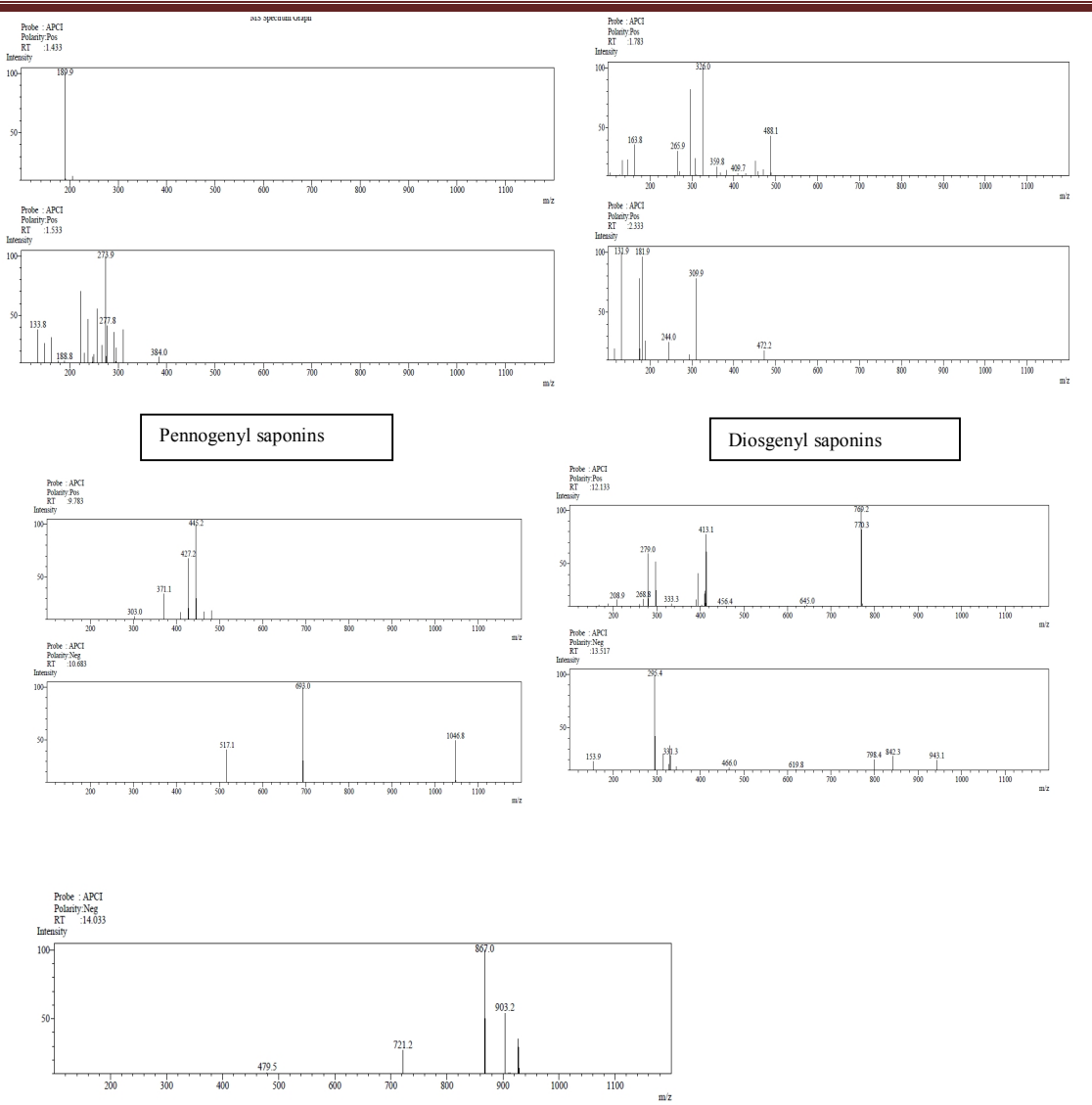


Figure 3 LCMS spectra of hydroalcoholic extract compounds

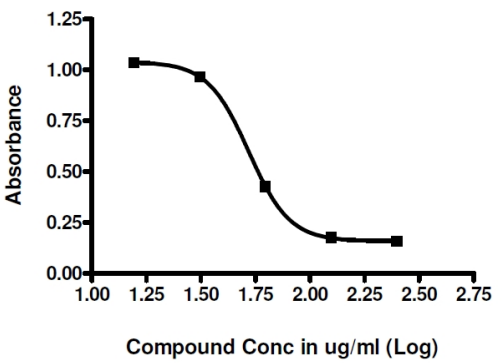


Figure 4 EC50 of extract in µg/ml

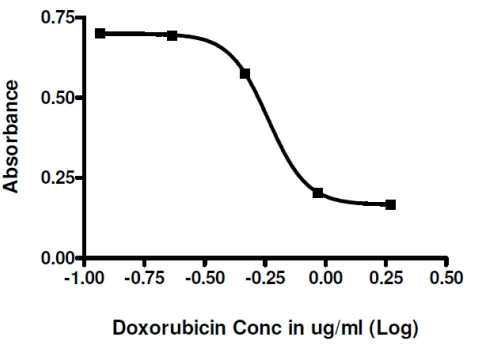


Figure 5 EC50 of Doxorubicin in µg/ml

RESULT

Spectral Analysis

NMR analysis of the hydroalcoholic extract has shown major structure f component Diosgenyl saponin as shown in Figure 1 and hydrogen shifts in HC 1H NMR DMSO-D6: 0.78–0.79 (m, 6H, CH3-18, CH3-27), 0.96–1.00 (m, 6H, CH3-21, CH3-19), 1.28 (d, 3H, J = 6.0 Hz, CH3 in rhamnose), 1.34, 1.35 (s each, 3H each, O–(CH3)2C–O), 1.52 (s, 6H, O–(CH3)2C–O), 3.37 (t, 1H, H-26a), 3.43 (t, 1H, J = 7.8; 8.4 Hz, H-4″), 3.47 (m, 1H, H-26b), 3.50 (m, 1H, H-3), 3.75 (dd, 1H, J = 4.2; 8.4 Hz, H-5′a), 3.83 (t, 1H,J = 7.2; 7.8 Hz, H-2′), 3.99 (m, 1H, H-5″), 4.05 (m, 1H, H-4′),4.07 (m, 1H, H-3″), 4.15 (m, 1H, H-3′),4.82 (m, 1H,H-1′).

Hydroalcoholic extract analysed for steroidal saponin and identified by high performance liquid chromatography-diode array detection (HPLC-DAD) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). As shown in Figure 2 HPLC Dad chromatogram has shown many components and as shown in Figure 3. Maximum ion strength has gone upto 1046 m/z for pennogenyl saponins and 796 m/z for Diosgenyl saponins

MTT assay for cell viability

More than 97 % increment in cell killing at a concentration of 500 µg/ml recorded in the cell line. The EC50 for the extract was calculated to be 52.34 ug/ml see Figure 4 and at the concentration of 1.8559 ug/ml, doxorubicin exhibited approximately 98 % killing of the cells. The EC50 for the Doxorubicin was calculated to be 0.579 ug/ml see Figure 5. From the study it is concluded the hydroalcoholic rhizome extracts *Paris polyphylla* has potential to exhibit anticancer activity.

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