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Phytochemical Investigation And Anti-Cancer Activity Of Glycosmis Pentaphylla.

B Edwin Jose[1]*, S Jebaseelan[1], and R Meera[2].

[1] Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai-625020.
[2] Department of Pharmaceutical Chemistry, College of Allied medical sciences, Madurai- 625020.

ABSTRACT

Evaluation of anticancer activity of methanol extract of Glycosmis pentapylla by trypan blue dye exclusion assay against Daltons ascites lymphoma cell lines. In vitro anticancer activity of methanol extract of Glycosmis pentapylla was evaluated on selected cancerous cells lines trypan blue dye exclusion assay. Trypan blue assay is based on staining of cells. Cells are then counted using hemocytometer under the microscope, non-viable cells were stained blue, viable cells remain unstained. Glycosmis pentapylla on Daltons ascites lymphoma cell lines. The medicinal plant i.e., Glycosmis pentapylla was studied by in vitro evaluation methods i.e., trypan blue exclusion assay. The Methanol extract of Glycosmis pentapylla have shown potent anticancer activity on selected cancerous cell lines. More efforts are needed to explore potent anticancer plants from the mother earth and save humans around the world from cancer.

Keywords: Anticancer activity, Glycosmis pentapylla, Trypan blue exclusion assay, Daltons ascites lymphoma cell lines.

Corresponding author

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INTRODUCTION

Cancer can be caused by any of the three ways improper diet, genetic factors, and environmental factors [1,2].Cancer is a group of diseases caused by loss of cell cycle control. Cancer is associated with abnormal uncontrolled cell growth [3]. Cancer is caused by both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). Cancer is a significant worldwide health problem generally due to the lack of widespread and comprehensive early detection methods, the associated poor prognosis of patients diagnosed in later stages of the disease and its increasing incidence on a global scale. Indeed, the struggle to combat cancer is one of the greatest challenges of mankind [4].

Glycosmis pentaphylla (Commom Name : Anam, Ban nimbu, Ashvashakota)

An erect shrub of 0.9.1.8 m. high. Twigs tomentose, terete. Leaves alternate, 3-7 foliolate; the rhachis terete, tomentose, stout, up to 18 cm long. Leaflets alternate or sub opposite, 7.5-18 by 3.8-9 cm., elliptic rhomboid or ovate, acuminate or acute, base cuneate usually acute and oblique, entire rarely obscurely toothed, pubescent on both surfaces especially along the nerves, glandular especially on the leaf margin, pellucid-punctate, thinly coriaceous aromatic when crushed, with about 7-12 pairs of lateral nerves [5-8]. Petiole 1.25-5 mm long. Flowers 5mm diameter, yellowish tetramerous, in terminal softly pubescent panicles 10-30 cm long. Juice of the leaves used in liver complaints. Root decoction given for facial inflammations. Wood used in snake-bite. Flowers and leaves used in low fever. Leaves in the form of paste used in skin affections. Leaves used in vermifuge.

Decoction of leaves is antidote for eczema, skin troubles. Roots have been used for anti inflammatory. Plant used in anemia, bechic, jaundice. The plant also having antibacterial, antifungal, antiprotozoal activity. Berry 1-1.8 cm long, ovoid, pale orange, verrucose with tufts of short hair or glabrescent when ripe. The leaves of G.pentaphylla contain a glycoside glycosmin, and alkaloids like glycosin, arborine, glycosminine, arborinine, glycolone. Root bark contains Acridone alkaloids Noracronycine, de-methylacronycine; quinazoline alkaloid ,Glycophymine, Glycosolone, glycolone; Amide, glycomide. Flowers contain alkaloids and amides like glycorine, glycosmicine, benzamide-2-methylamino, also contains carbazole alkaloid Mupamine. The presence of quinazoline bases is significant in connection with the local use of the leaves as a febrifuge [9-11].

MATERIALS AND METHODS

Plant material

The root of Glycosmis pentaphylla (1.5kg) were collected and shade dried during the month of July 2009 from Kollam hills in Kerala State, and authenticated by Dr.Chelladurai. The plant materials were taxonomically identified and the voucher specimens have been preserved in our laboratory for future reference.

Extraction

The root of Glycosmis pentaphylla were dried in shade and powdered to get coarse powder. About 225gms of dried powder was extracted with petroleum ether (40°-60°C) by continuous hot percolation using Soxhlet apparatus. The extraction was continued for 72 hours. The petroleum ether extract was filtered and concentrated to a dry mass by using vacuum distillation. A green waxy residue (2.6gms) was obtained. It was again extracted with chloroform for 72 hours. The chloroform extract was concentrated and a light brown residue (1.9gms) was obtained. The marc left after the chloroform extraction was dried and extracted with methanol continued up to 72 hours. The extract was filtered and concentrated by vacuum distillation. A greenish brown colour residue (3.9 gms) was obtained.

Isolation

The 80% methanol extract (15 gms) of Glycosmis Pentaphylla was chromatographed in silica gel 60 – 120 mesh, Merck, India column built in chloroform elution of column (gradient elution) with ethyl acetate of increasing the polarity. The fraction of 10 ml was collected each time. The fractions were monitored using TLC

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(silica gel G Merck, India).TLC was identified by using iodine chamber. The details of fractions are given in Table – 1.

Table 1: Fractions of the isolated compound

Fraction collected	Eluent composition	Remarks
1-30	Chloroform : ethyl acetate 30 :70 %	Light Green un separable mixture
31-60	Chloroform : ethyl acetate 40 :60 %	Light brown un separable mixture
61-100	Chloroform : ethyl acetate 50 :50 %	yellowish un separable mixture
101-150	Chloroform : ethyl acetate 90 :10 %	Light yellowish separable mixture compound
151-200	Chloroform 100 %	Light yellowish single compound

The fraction eluted from 225 yield single compound and positively for mayers test. This compound was taken up for characterization the running properties of the isolated compound are given in Table – 2. The IR, 1H NMR, 13C NMR were recorded for the isolated compound Table – 3,4,5.

Table 2: Rf (X100) Value of isolated compound

Solvent system	Rf value
Chloroform 100 %	0.46

Table 3: Ir Spectrum Of Isolated Compound

WAVE No. CM^1	GROUP ASSIGNMENT
3402.12	N-H stretching, vibration
2930.03	C-H stretching
1420.03	C-H bending
1578.07	C=O stretching bending
1498.35	C-C multiple bond stretching

Table 4: ^{1}h Nmr Spectrum Of Isolated Compound

SIGNAL ASSIGNMENT	CHEMICAL SHIFT VALUE δ H ppm
C1	14.40 (1H,s)
C2,C4	6.39(2H,m)
C3	3.81(3H,s)
C6	3.91(3H,s)
C8	7.04(1H,d)
C9	7.34(1H,d)
C10	7.46(1H,d)
C11	7.51(1H,d)

Table 5: ^{13}C NMR SPECTRUM OF ISOLATED COMPOUND

SIGNAL ASSIGNMENT	CHEMICAL SHIFT VALUE δ H ppm
C-1	161.03

C-2	120.23
C-3	163.25
C-4	120.23
C-5	129.28
C-6	130.18
C-7	140.75
C-8	121.84
C-9	122.37
C-10	123.58
C-11	121.45
C-12	138.31
C-13	176.42
C-14	130.15

PRELIMINARY PHYTOCHEMICAL INVESTIGATION

The qualitative chemical test of various extracts of Glycosmis pentapylla was carried out using standard procedure [12-15]. Carbohydrates, Tannins, Alkaloids, Cardiac glycosides, Flavanoids and Phytosterols were present in all the extracts.(Table 6)

Table 6: Preliminary Phytochemical test of Glycosmis pentaphylla

S.No	Phytochemical tests	Petroleumether extract	Chloroform extract	Methanol extract
1.	Carbohydrates	+	+	+
2.	Glycosides	+	+	+
3.	Alkaloids	+	+	+
4.	Saponins	-	-	-
5.	Tannins	+	+	+
6.	Proteins and Aminoacids	-	-	-
7.	Flavonoids	+	+	+
8.	Phytosterols	+	+	+

IN-VITRO CYTOTOXICITY ASSAY

Trypan Blue [16,17]

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It is a diazo dye. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell Trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a Dye Exclusion Method.

MATERIALS REQUIRED

DLA (Daltons lymphoma ascites) bearing mice. Phosphate buffered saline (PBS) contains Nacl- 4gm, Na_2HPO_4- 0.72gm, KH_2PO_4- 0.1gm, KCl- 0.1gm and Distilled water- 500ml. The dye used is Trypan blue and the cell is counted by using Haemocytometer.

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PROCEDURE

Cells were aspirated from the peritoneal cavity of tumour bearing mice. The cells were washed three times using phosphate buffered saline. The viability of the cells were checked using trypan blue (cell viability should be above 98%). Different dilution of 10^{-1},10^{-2},10^{-3} were made. The numbers of cells in the 10^{-3} dilution was counted using a Haemocytometer and the cell number was adjusted to1×10^{7} cells/ml.The experiment was set up by incubating different concentration of the drug with 1×10^{6} cells. The final volume of the assay mixture was made up to 1ml using PBs and was incubated at 37°C for about 24 hour.1 ml of trypan blue was added after incubation and the number of dead cell was counted using a Haemocytometer.

$$\% \text{ Cytotoxicity} = \frac{\text{Number of dead cell}}{\text{Number of life cell + Number of dead cell}} \times 100$$

Table7: In Vitro Cytotoxic Effect

S. No	Volume of drug (ml)	Conc. Of drug µg	Volume of PBS (µl)	Volume of cell ($1X10^6$)	Volume of trypan blue	No. of viable cell	No. of dead cell	Average	% toxicity
1.	Con. I	-	900	-	-	100	0	0	0
	Con. II	-	900	-	-	100	0	0	0
2.	50	10	950	0.1ml	0.1ml	96	4	4	4
3.	50	20	950	0.1ml	0.1ml	91	9	9	9
4.	100	50	900	0.1ml	0.1ml	88	12	12	12
5.	200	100	800	0.1ml	0.1ml	70	30	30	30
6.	400	200	600	0.1ml	0.1ml	48	52	52	52

RESULTS AND DISCUSSION

The phytochemical investigation shows the presence of carbohydrates, steroids and alkaloids and phenolic compounds in the extract. The residue of the Methanol extract on column chromatography yielded the isolated compound. In column chromatography a single compound was isolated in the fraction of 151-175 (Chloroform 100%) from methanol extract. The isolated compound, slight yellowish amorphous powder (from chloroform 100% and). The melting point of isolated compound was found to be 237-240°C. The isolated compound gave positive test for alkaloids. The IR spectrum of isolated compound contained absorption band due to C=O group at 1578 cm-1. The 13C NMR shows signal at δ 176.8 ppm was assigned for C=O of ketone group. The Mass spectrum of isolated compound shows the molecular ion peak at m/z 255 and base peak at m/z186. Thus based on the IR, 1H NMR, 13C NMR spectral studies of isolated compound has been characterized as 1-hydroxy3-methoxy N-Methyl acridone.

Natural products have received increasing attention over the past 30 years for their potential as a novel cancer preventive and therapeutic agents [18,19]. In parallel, there is increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumorigenesis and associated inflammatory processes, underlining the importance of these products in cancer prevention and therapy. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products [20].

Methanol extract of Glycosmis pentapylla was tested for anticancer activity against Daltons ascites lymphoma cell lines . The number dead cells were found to increase with an increase in the concentration of drug used. The percentage toxicity was found to increase indicate that the given drug has potent cytotoxic activity in invitro condition.(Table 7). The methanolic extract exhibited maximum anticancer activity when compared with other extracts. In addition these results confirmed the evidence in previous studies which

reported that methanol is a better solvent for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents such as water, ethanol and hexane [21-23].

CONCLUSION

The plant was initially selected and tested for anticancer activity based on their historical and other traditional uses. The root extract of Glycosmis pentapylla methanol was prepared and tested for their potential as anticancer activity by in-vitro evaluation method, i.e., Trypan blue exclusion assay. This was done by closely monitoring the viability of cultured human cells exposed to the plant extracts. More efforts are needed to explore potent anticancer plants from the mother earth and save humans around the world from cancer.

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