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Research Article

Isolation of Diterpenoid Lactones from the Leaves of *Andrographis Paniculata* and Its Anticancer Activity

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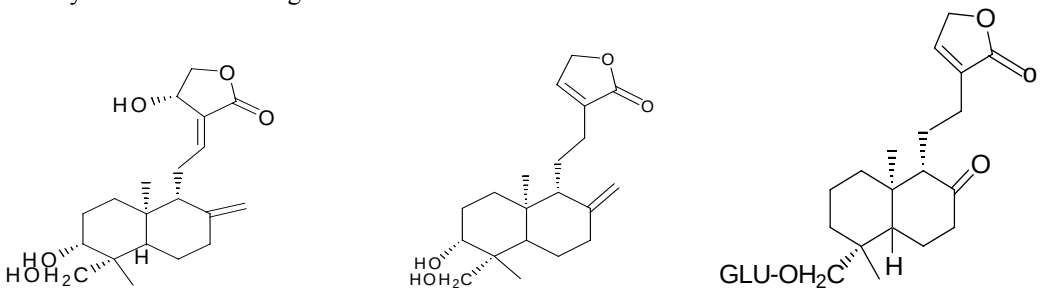
ABSTRACT

Large number of many important classes of compounds which includes flavonoids, flavones, flavone glycosides, chalcones, chalcone glycosides, xanthones, diterpenoids, dimeric diterpenes and sterols have been isolated from various parts of *Andrographis paniculata* (kalmegh) of family Acanthaceae. Though in traditional siddha and ayurvedic systems of medicine and tribal medicine, in India and some other countries, multiple clinical applications like anti-inflammatory, antiprolifiratory, antihepatic, antithrombogenic , antisnake venom, antipyretic activities, has been indicated for this plant but a very few of the isolated compounds have been tested experimentally. This gave us an impetus to isolate diterpenoid lactones from *Andrographis paniculata* and to test for anticancer activity.

Petroleum ether and chloroform extracts were prepared from the leaves of *Andrographis paniculata and* chromotographed over a column of silica gel, by gradient-elution technique and two compounds were isolated and purified by crystallization using methanol and ethyl acetate. The compounds were characterized by using IR, NMR, LC-MS spectra and compared with the authentic samples of Andrographolide (1) and 14-deoxy andrographolide (2) and neoandrographolide (3) for identification.

The identified compounds were tested on different cancer cell lines such as HepG2 (hepato cellular), Hct-116 (Human colorectal) at various concentrations using MTT-PROLIFERATION ASSAY. Further, confirmed by DAPI STAINING and ACRIDINE-ORANGE STAINING techniques. Both the compounds have shown considerable activity at micro molar ranges.



(1) **(2)** **(3)**

KEY WORDS: Andrographolide, 14-deoxy andrographolide, neoandrographolide, HepG2, Hct-116 cell lines.

INTRODUCTION

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. More than 13,000 plants have been studied during the last 5 year period.[1].These small molecules provide the source of inspiration for the majority of FDA-approved agents and continue to be one of the major sources of inspiration for drug discovery. In particular, these compounds are important in the treatment of life-threatening conditions. [2]

Andrographis paniculata an herbaceous plant, family *Acanthaceae*, Native to India and Sri Lanka which is widely cultivated in southern Asia, where it is used to treat infections and some diseases, often being used before antibiotics were created. Mostly the leaves and roots were used for medicinal purposes Although a large number of compounds have been isolated from various parts of *Andrographis paniculata*, a few of them have been studied for biological activity and very little work has been done to study the biological activity and the plausible medicinal applications of these isolated compounds and hence extensive investigation is needed to exploit the therapeutic activity of natural compounds to combat diseases. The main objective is to isolate and characterize a molecule which might show anti cancer properties or any other biological activities from the leaves of *Andrographis paniculata.*

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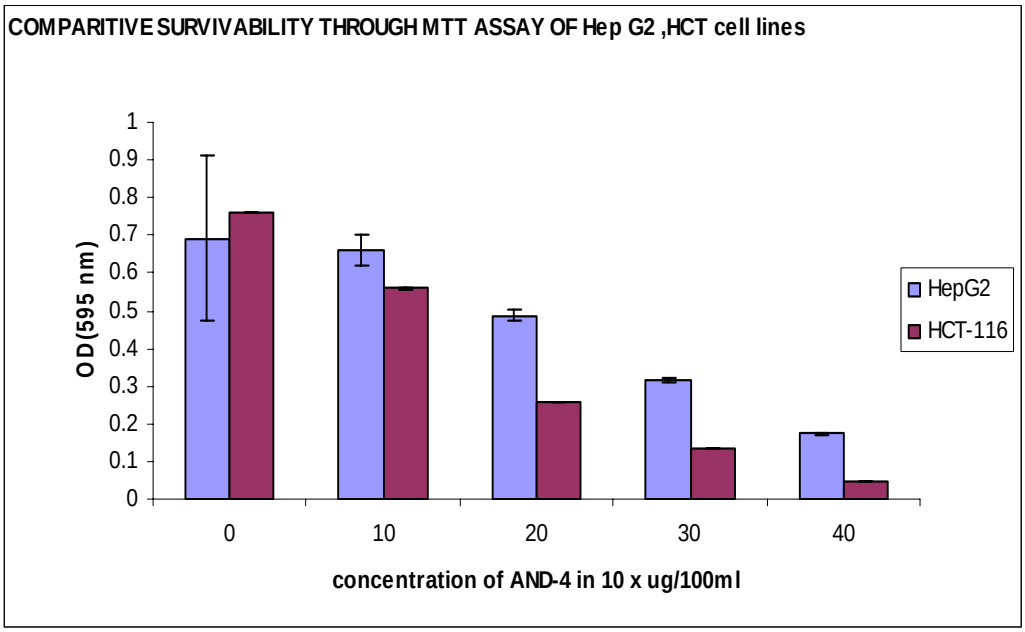

Figure:1 Comparitive survivability through MTT Assay of Hep G2, HCT cell lines

MATERIALS AND METHODS

The plant *Andrographis paniculata* has been collected from the places in Jhargham of Midnapur Dist. West Bengal. All the required materials were purchased are from sigma. Melting points were measured on yanagimoto Micro Melting point Apparatus and all uncorrected. IR spectra were determined using JASCO - 7300, FTIR spectrometer. Optical rotations were measured using JASCO DIP 370, Digital polarimeter. ^{1}H NMR recorded at 300MHz and ^{13}C NMR spectra were recorded at 74.99MHz (Brucker DPX spectrometer) in C_5D_5N with TMS as Internal standard. MALDI-MS(POSITIVE) WERE performed on a perspective biosystem voyager DE-STR Spectrometer with 2,5,dihydroxy benzoic acid as Matrix.HCT-116,Hep G2 were from National faculty for animal tissue and cell culture, pune, India.

Table: 1 Cytotoxic activity of AND-4 on HEP G2 and HCT-116 Cell lines

Blank	AVG HEP G2	AVG HCT 116
0 µg	0.6923	0.759663
10 µg	0.66233	0.56033
20 µg	0.487	0.255993
30 µg	0.31433	0.134
40 µg	0.175	0.04773

Extraction: 5oo gms of Dried leaves were weighed and taken in a percolator. Extraction was carried out using pet. ether (60-80^{0}c) to remove the fat. Then Chloroform extract was done 4 times finally extraction with methanol was carried out for 4 times. The solvents were dried in rotary evaporator for each extraction about 5lts of solvent was used and time of extraction being 16-18 hrs. Crystallization was done after the isolation of compounds by using column chromatography.

Anti Cancer Activity

Invitro proliferation assay: Cell proliferation was assessed using the 3-(4,5-dimethyl thiazol-2-yi)-2,5-diphenyl tetrazolium bromide (MTT),assay . Initially cells (1x10^5, 100 µl cell suspension per well) were seeded on 96 well tissue culture plates. The cells were then treated with samples (AND-4, AND-6, AND-11) for 48hrs at 37^0 C in a humidified atmosphere containing 5%CO_2 in air. Untreated cells were taken as control. The condition of the cell was observed under Phase contrast microscope after that that cell were treated with 50µl of MTT (5 mg/ml in PBS) was added to each cell and incubated for another 4 hrs. The supernatant in each well is replaced with DMSO (60µl) to solubilise the MTT formazan precipitate and isopropanol (200µl) was added to rupture the cells and optical density (OD) was measured immediately at 490nm using Elisa reader.

Percentage of cell/growth inhibition was calculated by the formula:

%cell inhibition =100xOD of control-OD of treated /OD of control); OD=optical density

RESULTS AND DISCUSSIONS

Chromatography: 19.7gms of chloroform extract was chromotographed over a column of silica gel by gradient elution. The compounds was identified and coded as

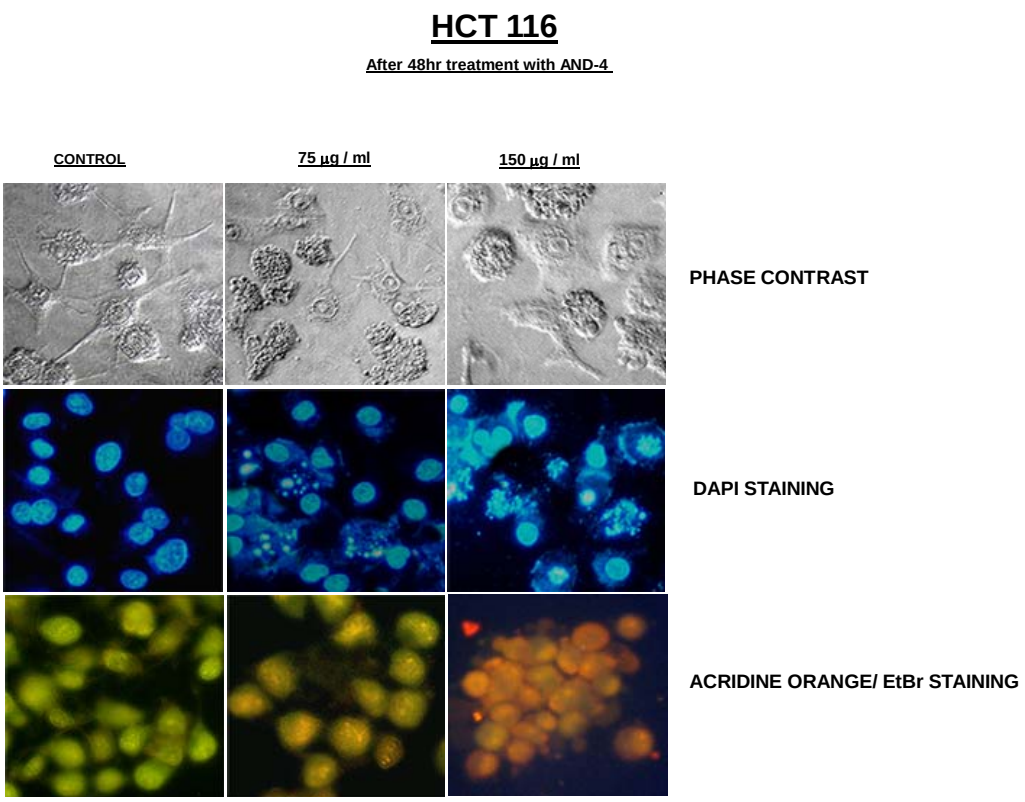

Figure: 2 Cell morphology of HCT-116

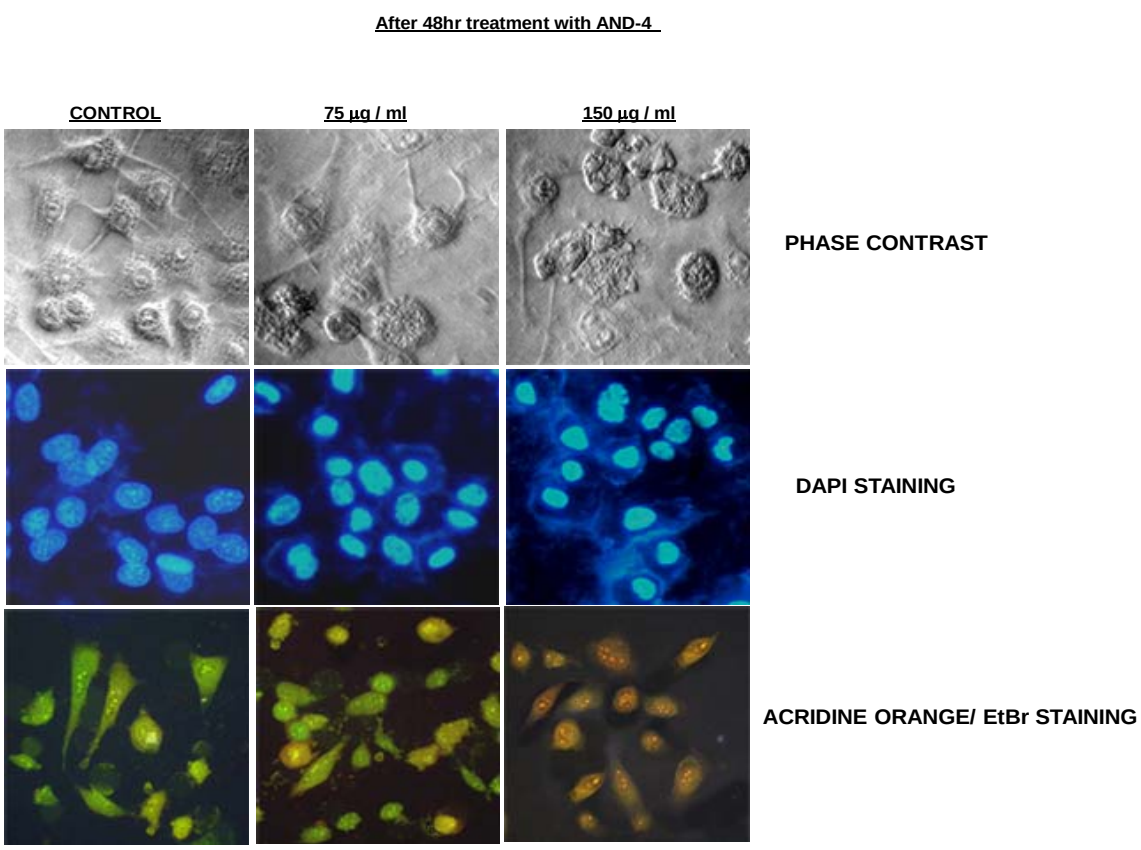

Figure: 3 Cell morphology of Hep-G2

AND-6 and conformed as Andrographolide by comparing with the authentic sample.

28.4 gms of methanolic extract were chromotographed over a column of silica gel by gradient elution. The compounds were identified and coded as AND-4, AND-11 and conformed as

14- Deoxy andrographolide, neo andrographolide respectively by comparing with the authentic sample.

Analytical IR, NMR, Mass spectral data:

AND-6 (14-deoxy Andrographolide): $C_{20}H_{30}O_4$, Mol. Wt. = 334; mp 175ºC, R_f:0.67, R_t: 23.058min, IR (KBr) v_{max}: 3283(OH stretching), 1754(γ lactone,C=O stretching), 903(exo CH_2 stretching),^{1}H NMR ($CDCl_3$, δ, ppm): 0.67 (2s, 3H, CH_3 pyridine), 1.47 (s, 2H, CH_3), 0.67 (s, 2,3H, CH_3 pyridine), (M^+): 334.

AND-4 (Andrographolide): $C_{20}H_{30}O_5$, Mol. Wt. = 350; mp 230-231ºC, R_f:0.65, R_t: 17.5min, IR (KBr) v_{max}: 3283(OH stretching), 1754(γ lactone,C=O stretching), 903(exo CH_2 stretching),^{1}H NMR ($CDCl_3$, δ, ppm): 0.65 (2s, 3H, CH_3 pyridine), 1.49 (s, 2H, CH_3), 0.67 (s, 2,3H, CH_3 pyridine), 4.8(s,2H,exo CH_2 stretching), 7.16(s, 2H,olefinic) (M^+): 350.

AND-11 (Neo Andrographolide): $C_{26}H_{40}O_8$, Mol. Wt. = 480; mp 170ºC, R_f:0.57, R_t: 15.5min, IR (KBr) v_{max}: 3449(C=C stretching), 1748(γ lactone,C=O stretching), 910(exo CH_2 stretching),^{1}H NMR ($CDCl_3$, δ, ppm): 0.65, 1.07 (s, 3H, $2CH_3$, C_{18}, C_{20}), 1.19-2.37 (m, 14H, $7CH_2$), 3.44-4.80 (m, 11H, sugar H, C_{19}), (M^+): 357.

Anticancer activity

AND-4, AND-6, AND-11 were tested for their cytotoxicity against cancer cell lines Hep G2, HCT-116,at different concentrations using MTT assay shown in figure 1.The compounds were evaluated for cytotoxicity on normal blood cells. The highest concentration i.e., 40 µg each of the three compounds were treated on normal human bloodcells and MTT assay was done after 24 hrs of incubation. The results suggested that there is no cytotoxic effect on normal blood cells. AND-6, AND-11 were also treated against Hep G2, HCT-116 in concentrations of 30µg/ml, 60µg/ml, 120µg/ml. The results indicate that AND-4 has an anti-proliferative activity in both the cell lines further, the degree and stages of apoptosis in cells post treatment with AND-4 have been observed by imaging with fluorescent dyes (DAPI, acridine orange/ ethidium bromide) shown in figures (2&3) the maximum cytotoxicity is shown by AND-4 is shown in table 1, followed by AND-6, AND-11, over 48 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 these drugs.

CONCLUSION

Three compounds were isolated from chloroform and methanolic extract of *Andrographis paniculata* which were coded as AND-6, AND-4, AND-11. Among those AND-4 possess cytotoxic activity against cancer cell lines Hep G2,HCT-116 using MTT Assay. Since cell death may occurs by any of two major path ways *viz.* Apoptosis and necrosis so detailed analysis by DAPI and acridine orange shows DNA fragmentations which confirms that cell death occurs due to apoptosis.

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