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SCREENING AND ISOLATING AN ANTI-HUMAN EPITHELIAL COLON CANCER DLD-1 CELLS COMPOUND FROM DROSERA INDICA L. - A RARE HERBAL SPECIES IN VIETNAM

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Abstract- Species of Drosera have been proved to have important phamacognostical evaluation in treatment of ailments like: tuberculosis, cancer, HIV, cardiovascular, asthma, whooping cough, without exception of mutation related diseases [1]. Being relatively rare in nature, Drosera are such endangered species that many countries have enacted legislation to protect them [2], [3]. However, among three known species of Drosera in Vietnam [4], it is the fact that there has been no publication studying these species on the chemistry, pharmacology [5]. There is an urgent need to adopt conservation measure regionally, which may preserve these valuable species from being vanished. Moreover, studying in specific potential medicinal properties of these wild plants may suggest a source of fresh biomass and their extracted substances for interesting industrial employment. Focusing on D. indica L. collected in Vietnam, the micro propagation has been successfully established. From these in vitro materials, the screening, isolating and elucidating (both structural and anti-human epithelial colon cancer DLD-1 cells properties) plumbagin have also been carried out. Furthermore, the yield of plumbagin on in vitro culture system of Drosera indica has been reported as well.

Index Terms- Drosera, anticancer, plumbagin.

I. INTRODUCTION

Drosera, a carnivorous species, attracts attention of biologists not only for their ornamental value but also for their medicinal significance. Extract of these plants, containing quinones, are used as antispasmodic agents in the treatment of respiratory tract ailments [6]-[7], ; anti-cancer [8], [9]; cardiotonic [10]; or against tuberculosis [11]… Due to their ubiquitous medicinal properties, natural populations of these plants are becoming increasingly scarce especially in Europe where *Drosera* are included in the European Red List of Threatened Plants [12]. Several European countries have imported *Drosera* from Oriental Asia and put them as "Herba *Droserae*"on the market for sale. In Asia, exporters also have been collecting *Drosera* from the wild indiscriminately without adopting measures to propagate them [13]. *D. indica* L. is one of 2 species were found in Viet Nam. The local people also used them as a source of medicines effectively though as yet little is known of the explanation: Vinh city's hospital used them as medicinal herbs to cure whooping-cough in 1958-1959; Thanh Hoa city's inhabitant used their ethanol extract to cure bone-spavin; and the others used to cure tuberculosis, intestinal disease [5], [14]. There is an urgent need for us to adopt conservation or these plants will extinct soon. Our problem is how we can take full advantage of these medicinal plants which were listed in the Red Book of Threatened Plants in long time and actively. As a result, we do this research for screening and identifying a compound having anticancer activity from *in vitro* plantlets to not only clarify partly pharmaceutical value of this species but also affirm contributively potentiality of taking secondary metabolites from *in vitro* source.

II. EXPERIMENTAL

A. In vitro culture of Drosera indica

Material for in vitro culture: The seeds of *Drosera* are collected from the Binh Chau - Phuoc Buu Nature Reserve, Ba Ria - Vung Tau province, Viet Nam; Javel + Tween-20 (0.01% v/v) used as disinfectants; BA (6-benzylaminopurine) used as plant growth regulator.

Culture medium: MS (Murashige & Skoog), sucrose (30g /l), activated carbon (1g /l), casein hydrosylate (100mg /l), pH = 5.8 ± 0.1 (adjusted with NaOH 1N or HCl 1N). The medium is autoclaved at 1atm, 121^{o}C, for 30 minutes.

Culture conditions: light for 16 hours /day with illumination intensity of 2800 ± 200lux, room temperature of 22-25^{o}C and average humidity of 70%.

Sterilization of cultured materials: Lightly flush the fruit with alcohol, then rinse with sterile distilled water 2-3 times, then shake in 70% ethanol for 1 minute, rinse sterile water before shaking in a sterile solution of Tween-20 (0.01% v/v) and Javel (10% v/v) in 10 minutes, rinse water 5-7 times again, then remove the sterilized *Drosera* shells to obtain the seeds contained, bring the seeds soaked in 1% gibberellin solution for 24 hours before letting the seeds on the culture medium.

Propagation of Drosera indica: Propagation by axillary and adventitious shoots from thin cell layers by supplemented with BA at different concentrations (0-5mg /l) on culture medium; experiment on the two-step culture process for improving shoot multiplication [15]; then creating roots and developing complete plantlets. Rate of shoot forming (%), number of buds per sample and average height of shoots (cm) were used for monitoring criteria.

B. Screening and isolating an anti human epithelial colon cancer DLD-1 cells compound from in vitro Drosera

Material: Sample was diluted with 0.25% DMSO solvent, DLD-1 human colon cancer cell line provided by American Type Culture Collections, McCoy's 5A media w. GlutaMaxTM (10% FBS), D-PBS, gentamicin and trypsin (from Gibco). Plumbagin, and resveratrol were from Sigma-Aldrich. Plumbagin Stock solution dissolved in DMSO was 120mM; 0.2% SRP, 50% TCA, 1% acetic acid, Tris-base 10mM and resveratrol (60µM) in DMSO 0.25% as positive control in screening experiment.

Extract preparation: Fresh plantlets (cultured in 8-10 weeks) were dried at 50^{o}C, and afterwards were powdered. The powdered sample was extracted consecutively with n-hexane, chloroform, ethyl acetate, acetone, and ethanol for at least 48h at room temperature and filtered later. The extracts, collected from repeated extractions (until extracts are quite transparent), was concentrated to dry by evaporating with a rotary evaporator.

Culture of colon cancer cells DLD-1: DLD-1 cultured with McCoy's 5A medium supplemented with 10% FBS; 0.1% gentamicin. The culture conditions were maintained at 37^{o}C, 95% moisture and 5% CO_2, transplanted twice weekly. Cells used in the experiment should have coverage of about 70-80% of the culture tank and initial cell density in a well of 96 wells should be 10^4cells /100µl /well. Cells were counted with the CoulterR Partikle count & size analyzer Z2 (Beckmann Coulter Inc.) supported with Multisizer AccuComp software.

Column chromatography: Chosen extract was separated into different fractions with two solvent mixture based on the results of the active extract screening.

Screening the bioactive fraction: Using Sulforhodamine B (SRB) assay [16].

SRB assay: Spreading DLD-1 cells at the bottom of each well (plate 96 wells) at a density of 10^4 cells /100µl /well in 24 hours before the cell adheres to the bottom of the well. Cells were treated with sample (100µg /ml in DMSO 0.25%) as well as the positive control (resveratrol 60µM diluted in DMSO 0.25%) and the negative control (DMSO 0.25%), a blank sample also was used to compare the color (including the culture medium with sample in each treatment but no cells), all were incubated for 48 hours. Cells were fixed after treating by TCA (50µl /well) at a cold temperature of 1-3 hours. Removing TCA and washing cells with distilled water 5 times, then let it dry at room temperature for 12-24 hours. Fixed cells after drying were stained with 0.2% SRB (100µl /well) for 20 minutes at room temperature. Subsequently, SRB staining cells were washed 4 times with 1% acetic acid (200µl /well) and let them dry for 12-24 hours. Adding 200µl Tris 10mM to each well and place the disc on the shaker for 10 minutes to completely dissolve the SRB. Record OD values at 492nm and 620nm by ELISA reader. The ability of inhibition cell proliferation is determined by the rate of inhibition - I (%), as follow:

$$I\ (\%) = (1- (OD_{(492\text{-}620)\ sample}/OD_{(492\text{-}620)\ negative\ control})) \times 100\%$$

Herein:

$$OD_{(492\text{-}620)\ Sample} = OD_{(492\text{-}620)\ Sample\ with\ cell} - OD_{(492\text{-}620)\ Blank}$$

$$OD_{(492\text{-}620)\ negative\ control} = OD_{(492\text{-}620)\ negative\ control\ with\ cell} - OD_{(492\text{-}620)\ negative\ blank}$$

Isolating pure compounds: Using silicagel chromatography and thin layer chromatography with a 25DC-Alufolien 20x20cm chromatography plate, Kieselgel 60 F254, Merck.

Test the bioactivity of the isolated compound: Investigate the effect of pure compound on the proliferation and development of DLD-1 colon cancer cells by cell counting on coulter counter.

C. Identification and quantitative analysis of isolated compound

Compound was identified with the developed methods, including ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and DEPT (perform on BRUKER AC.500 with 500MHz for ^{1}H and 125MHz for ^{13}C-NMR) and MS by Quattro Micro Tune Parameters.

The content of compound was determined by HPLC with a C18 (25x0.4cm) column with 55% Methanol + 45% CH_3COOH (0,02M), flow rate 0.4ml /min, room temperature, volume injected 25µl, and detection 254nm.

III. RESULTS

A. In vitro culture of Drosera indica

Sterilization of cultured materials

The sterilization process has an improved step than the treatment of seeds with 1% gibberellin in 24 hours, which greatly reduces the normal germination time of *Drosera* (from 21 days to 15 days), with the percentage of sterile and germinated seeds reaching 93-95%.

Axillary shoots propagation

After 5 weeks of culture, at all designed BA

concentrations (0; 0.1; 0.5; 1; 2; 3mg /l), almost shoots produced axillary buds (96-100%). Therefore, to select the appropriate BA concentration, we based only on two monitoring criteria which were average number of buds per sample (buds) and mean height of shoots (cm). Suitable concentrations of BA for axillary shoot propagation from *D. indica* were 0.5mg /l (Table 1, Fig. 1).

Table 1. Effect of BA (mg/ l) on the propagation of shoots

BA concentration (mg / l)	Number of buds/sample (buds ± SD)	Height of shoots (cm ± SD)
0	1.03 ± 0.15^{a}	3.58 ± 0.06^{e}
0.1	16.73 ± 0.25^{e}*	2.58 ± 0.02^{c}*
0.5	**18.16 ± 0.71^{f}***	**3.29 ± 0.06^{d}***
1	14.43 ± 0.15^{d}*	2.53 ± 0.10^{c}*
2	12.90 ± 1.31^{c}*	1.17 ± 0.04^{a}*
3	3.13 ± 0.21^{b}*	2.03 ± 0.26^{b}*

The mean numbers in the column with different letters differed, (*) only the difference between the treatments with the control, significant difference at p = 0.05.

Fig. 1. Effect of BA on axillary shoots propagation
A: 0mg /l; B: 0.1mg /l; C: 0.5mg /l; D: 1mg /l, E: 2mg /l, F: 3mg/l

Adventitious shoot propagation

After 5 weeks of culture, the diameter of *D. indica* stem was so small and the leaves as well as stem were so difficult to interact with the medium because of the dew drops on their surfaces, so that they could not regenerate buds directly as the flower peduncles. Thus, the effect of BA (1-5mg /l) on the adventitious shoot propagation was focused on the flower peduncles as showed in Table 2, Fig. 2. Adventitious shoot proliferation from flower peduncles plays a supporting role for axillary shoot propagation, this result show that we can get it from the thin layer of the peduncles by using BA (1mg /l).

Table 2. Effect of BA (mg /l) on adventitious shoot propagation from flower peduncle

BA concentration (mg/ l)	Shoot induction ratio (%±SD)	No of shoot / sample (shoots±SD)	Average height of shoot (cm±SD)
0	6.67±11.55^{a}	0.33±0.58^{a}	0.60±1.04^{a}
1	**86.67±11.55^{b}***	**11.55±1.78^{e}***	**1.57±0.16^{b}**
2	86.67±11.55^{b}*	5.22±0.80^{d}*	1.54±0.03^{b}
3	86.67±23.09^{b}*	3.62±0.80^{cd}*	1.22±0.17^{ab}
4	66.67±11.55^{b}*	2.28±0.67^{bc}	1.20±0.08^{ab}
5	60.00±20.00^{b}*	1.56±0.51^{ab}	0.98±0.11^{ab}

*The mean numbers in the column with different letters differed, * only the difference between the treatments with the control, significant difference at p = 0.05*

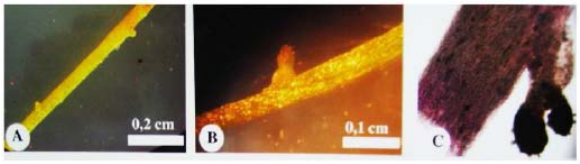

Fig. 2. Regeneration of adventitious shoot from D. indica flower peduncles after 5 days

A. sample is visible by eyes, B. sample is seen through a magnifying glass, C. sample is cut vertically, dyed in 2 colors and viewed under a 10X microscope.

Experiment on the two-step culture process for improving shoot multiplication [15]

The shoots were propagated in two steps: (1) culturing on MS medium (solid and liquid) with BA (0.5mg /l) within 2 weeks; (2) Subsequently cultured shoots were transferred to the MS medium (solid, liquid) with full complement of ingredients except BA for the next 3 weeks. After 5 weeks of culture, samples were compared with the control that did not apply liquid culture procedure 2 steps. Results showed that two-step culture according to Christoph Wawrosch's method (2009) applied were highly effective, in which process 2 (first 2 weeks induction shoots by solid MS supplemented with BA, 3 nest weeks transferring the induced sample to a non-hormone-free liquid medium) was most suitable for improving shoot multiplication with the highest shoot yield (100%) and number of shoots per simple was 1.58 times higher than control (Table 3).

Creating roots and developing complete plantlets

Shoots separated from the parents after 6-8 weeks of culture can develop into complete plantlets on liquid MS medium with sufficient parts including roots without auxin (fig. 3)

B. Screening and isolating an anti human epithelial colon cancer DLD-1 cells compound from in vitro Drosera

Extract preparation

Drosera plantlets cultured in 8-10 weeks were used as material for extract preparation, % of water occupied in the material was recorded in Table 4, and the yield of extract from dried powder are presented in the table 5.

Screening the bioactive fraction

After extraction, the *in vitro* cytotoxicity screening was performed by the SRB assay: 4 extracts of *in vitro Drosera* were mixed in 0.25% DMSO with the same concentration (100µg/ml), along with positive control (resveratrol 60µM) were induced on wells containing the same number of cells (10^4 cells /100µl /1well). The experiment was repeated six times for each treatment. Results showed that only chloroform

extracts reduced DLD-1 cell proliferation by more than 50% (Table 6).

Chloroform extract (18.45g) was separated by silica gel chromatography with the mobile phase including chloroform and petroleum ether, increasing polarization from 0% chloroform to 100% chloroform in petroleum ether. All of fractions from silica gel chromatography were screening by SRB again. Results showed that fraction which was from the mobile phase (7 petroleum ether; 3 chloroform) inhibited DLD-1 cell proliferation best.

Fig. 3. Drosera complete plantlets with all the parts, including roots on liquid MS without auxin.

Table 3. Experiment on the two-step culture process (solid and liquid)

Process	Culture process from the buds	Shoot induction ratio (% ± SD)	No. of shoots/ sample (shoots ± SD)	Morphology of plantlets
NT0	5 weeks: solid MS +BA	100.00 ± 0.00^{b}	15.60 ± 0.40^{a}	Short, no roots, less leaves
NT1	2 primary weeks: solid MS + BA 3 later weeks: solid MS	100.00 ± 0.00^{b}	17.40± 0.87^{b}*	Higher than NT0, roots and leaves enough
NT2	**2 primary weeks: solid MS + BA 3 later weeks: liquid MS**	**100.00 ± 0.00^{b}**	**25.33± 0.64^{c}***	**Highest, roots and leaves best**
NT3	2 primary weeks: liquid MS + BA 3 later weeks: liquid MS	73.33 ± 11.55^{a}*	26.03± 0.91^{c}*	Higher than NT0, roots and leaves enough

*The mean numbers in the column with different letters differed, * only the difference between the treatments with the control, significant difference at p = 0.05*

Table 1. % of water occupied in invitro materials

Fresh weight (g)	Dry weight (g)	Percentage of water (%)
3509.33	503.58	85.70

Table 2. The yield of extract from dried powder Drosera

Extract	Extract weight (g)	Yield of extract from dried powder (%)
n-hexane	9.71	1.93
Chloroform	**54.07**	**10.74**
Ethyl acetate	17.92	3.56
Ethanol	35.56	7.06

Table 6. Percentage (%) inhibited the proliferation of DLD-1 colon cancer cells after 48 hours treating with extracts (100µg /ml)

Extract	I (%) ± SD
Resveratrol	74.80 ± 6.78^{e}
n-hexan	12.77 ± 5.32^{a}*
chloroform	**76.16 ± 2.24^{e}***
ethyl acetate	16.17 ± 5.34^{ab}*
ethanol	44.81 ± 8.59^{d}*

Isolating pure bioactive compound

Selected fraction is repeatedly purified by thin layer chromatography prepared with a chloroform ether (8:2) solvent until the product has only one apparent round splotch with Rf = 0.48. The result allowed the isolation of a relatively pure A compound (m = 65mg) (Figure 5).

Test the bioactivity of isolated compound

The proliferation and development of DLD-1 cells after exposure to isolated compound was investigated within 48 hours. Cells were treated with A compound at different concentrations (0; 0.625; 1.25; 2.5; 3; 5µM) and spread to each well of 24 wells at a density of 3.5x10^{4} cells /well. The experiment was repeated four times, each being a well in a 24-well plate for each concentration. Cell proliferation and development after 24, 48 hours after exposure are presented in Fig.6a and Fig. 6b.

Extrapolation from the graph shows the rate of proliferation inhibition (I%) corresponding to the investigated concentrations, the 50% inhibitory concentration of IC50 of compound was determined at 24 hours and 48 hours. In addition, the effect of compound on cell morphology through the parameters of area, diameter and cell volume was also recorded in Fig. 7.

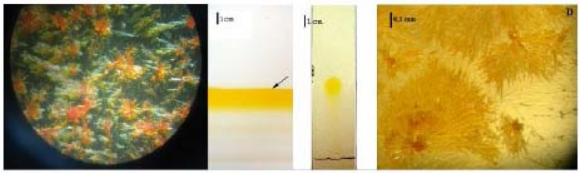

Fig. 5. Isolating A compound

Order from left to right: bottom of Erlenmeyer flask of selected fraction after solvent drying; Chromatography with orange streaks; clear round of pure compound at Rf = 0.48 when separated with petroleum ether:chloroform (8:2); Crystals of the pure compound are crystallized in chloroform

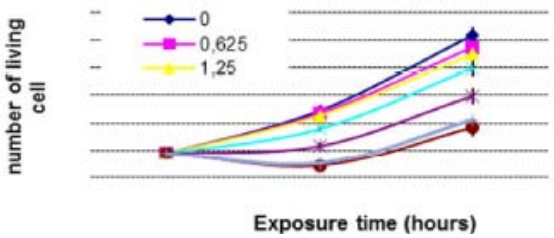

Fig. 6a. The DLD-1 cell proliferation curve over time under the influence of A compound at different concentrations

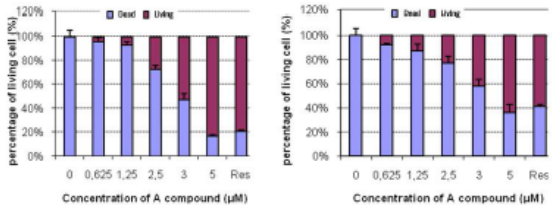

Fig. 6b. The graph shows the rate of cell proliferation of compounds at different concentrations (left: after 24 hours of exposure, right: after 48 hours of exposure)

The results show that A compound is capable of strongly inhibiting cell proliferation (IC50 = 3.53µM after 48 hours of exposure), which is more potent than resveratrol (IC50 = 27µM) [17].

In the low concentration (0.0625-25µM), the A compound tends to pull flat, increasing the cell size slightly (10-20%) but the volume and diameter of the cell no change. However, at high concentrations (2.5-5µM), the A compound reduces the cell size gradually until the cell loses its ability to adhere and die (Fig. 8). Compared to resveratrol, a grape juice extract is being studied as a cancer treatment, they are the same in inhibiting the proliferation as well as growth and development of DLD-1 cell line, but resveratrol increased cell size in the first 48 hours of induction [17], [18]. The results show the potential for cancer treatment of the compound but a mechanism completely different from resveratrol.

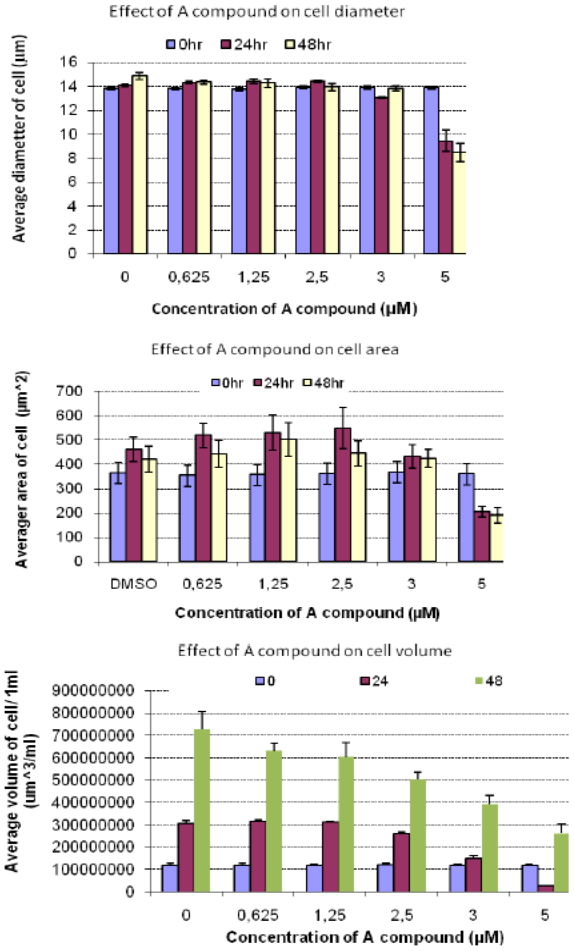

Fig. 7. The chart shows the effect of A compound on the size of DLD-1 cells

C. Identification and quantitative analysis of isolated compound

Identification

Record signals to identify the structure of A compound:

- Characteristics of the compound: long needle crystal, reddish orange; Melting temperature = 78°C; Rf = 0.47 when solubilized with chloroform: benzene (8:2).
- Mass spectra results: The peak [M-H] - 187 corresponds to the molecular weight of B of 188
- Results of ^{1}H-, ^{13}C-NMR, DEPT, COSY, HMQC and HMBC spectra: signals from the spectral results are summarized in Table 7, Fig. 9.

Basing on the signals of the characteristics and spectral results recorded from the isolated compound, compared with the characteristics and spectra of the known compounds, the A compound was identified as 2-methyl-5-hydroxy-1,4-naphthoquinone (plumbagin, plumbagone, 2-methyljuglone).

Quantitative analysis:

Fresh *D.indica* L. *in vitro* plantlets was analyzed by HPLC and compared to the standard plumbagin. The results indicated the content of plumbagin in *D.indica* L. *in vitro* plantlets was 135.1mg /kg (FW) (Fig. 10).

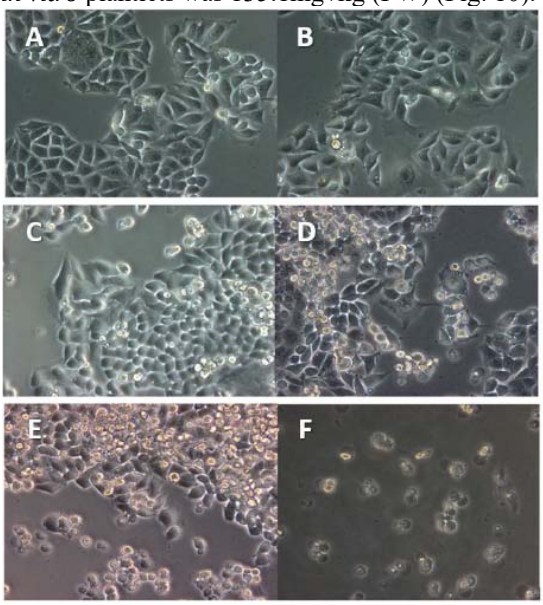

Fig. 8. DLD-1 cells under the influence of A compound after 48 hours exposure was observed under contrast microscope, 10x magnification.

A: negative control (DMSO); B: 0,625µM; C: 1,25µM; D: 2,5µM; E: 3µM; F: 5µM

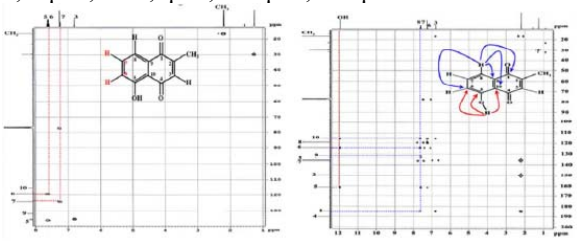

Fig. 9. Results of HMQC (left), HMBC (right) ($CDCl_3$)

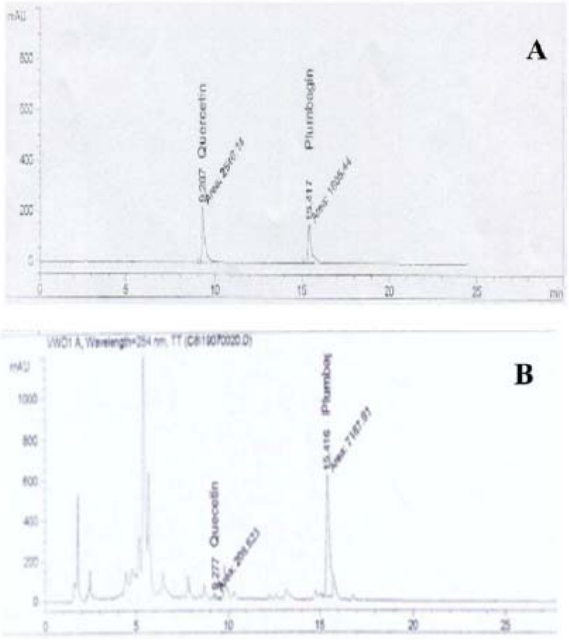

Fig. 10. HPLC chromatograms. A: Standard plumbagin – B: D. indica L. in vitro plantlets.

	Isolated compound		Standard plumbagin [19]	
	^{1}H-NMR (CDCl3, δ ppm)	*^{13}C-NMR (CDCl3, δ ppm)*	*^{1}H-NMR (CDCl3, δ ppm)*	*^{13}C-NMR (CDCl3, δ ppm)*
1		184.79		184.70
2		149.64		149.60
3	6.81 (1H, s)	135.48	6.81 (3H, q, J=1.56)	135.50
4		190.39		190.30
5		161.24		161.20
6	7.26 (1H, dd, J=8.0 & 1.5Hz)	124.18	7.25 (1H, m)	124.20
7	7.60 (1H, t, J=8.0Hz)	136.10	7.62 (2H, m)	136.10
8	7.64 (1H, dd, J=8.0 & 1.5Hz)	119.29	7.62 (2H, m)	119.30
9		132.13		132.10
10		115.19		115.20
CH3	2.19 (1H, s)	16.49	2.20 (3H, d, J=1.56)	16.50
OH	11.95		11.95	

Table 7. ^{1}H-NMR, ^{13}C-NMR of the isolated compound and the standard quercetin

CONCLUSION

Drosera indica L. *in vitro* plantlets contained a compound having strong anti-human epithelial colon cancer DLD-1 cell line activity which was isolated from chloroform extract by column chromatography. *In vitro* culture of *Drosera* for plumbagin has great potential for practical application.

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