



Chemotherapeutic potential of *Cayratia trifolia* L n-hexane extract on A2780 cells

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Abstract

It is of interest to report the chemotherapeutic potential of n-hexane *Cayratia trifolia* L. (*C.trifolia*) extract on A2780 cell lines. mRNA and protein expression analysis of human chemokine receptor (CXCR4) and human epidermal growth factor receptors-2 (HER2) were studied using Real time-PCR analysis and western blot analysis. The results show significant cell growth inhibition with minimal IC50 values of 46.25±0.42µg/mL against A2780 cell lines. mRNA and protein expression were considerably reduced in *C.trifolia* treated A2780 cell lines for further consideration as a chemotherapeutic agents.

Keywords: *Cayratia trifolia*; CXCR4 and HER2 protein expression, Metastatic signalling, Anti-ovarian cancer activity

Background:

The frequent use of synthetic drugs cause numerous side effects with drug resistance [1]. Natural products are effective in reducing the toxicity of allopathic drugs and therapy [2]. It is known that secondary metabolites possess strong antioxidant, cytotoxicity, antimicrobial, antidiuretic, antidiabetic, anti-inflammatory activities and also used to treat other disease and disorders, hence, play a major role in the management of human disease [3]. *Cayratia trifolia* L. (*C. trifolia*, Family: Vitaceae) commonly referred to as fox grape in English, is native to India, Australia and few Asian countries [4]. The presence of yellow waxy oil, steroids, terpenoids, alkaloids, flavonoids such as kaempferol, myricetin, quercetin, triterpenes, epifriedelanol and tannins in the whole plant of *C. trifolia* is known [5-8]. The paste of the tuber from *C. trifolia* is used in the treatment of snake bite

[9]. Therefore, It is of interest to report the cytotoxicity activity of n-hexane *Cayratia trifolia* L. (*C. trifolia*). Extract on A2780 cell lines.

Materials and methods:

Plant collection and authentication:

The whole plant of *C. trifolia* was collected from in the campus of Annai College of Arts and Science, Kovilacheri, Kumbakonam, Thanjavur District, Tamil Nadu, India and the plant was authenticated by Dr. P. Sathyanarayanan, Botanical survey of India, TNAU Campus, Coimbatore (voucher number is BSI/SRC/5/23/2010-2011/Tech.1527). The plant material was shade dried, powdered and stored in air tight container at 4°C for future analysis [10].

Extract preparation:

The dried plant material was subjected to n-hexane extraction using exhaustive extraction procedure [11]. Briefly, 200g of the plant material was soaked in a flask containing 1000 mL of n-hexane and was kept on the rotating shaker for 72 hours at 25°C (average room temperature). Finally, the collected extract was concentrated through rotary evaporator (RE-2A evaporator) set at 40°C. Further, it was stored at 4°C for future studies.

Table 1: List of primers used for Real Time PCR analysis

CXCR4	Sense-5'-ATCCCTGCCCTCCTGCTGACTATTTC-3'
	Antisense-5'-GAGGGCCTTGGCTTCTGGTG-3'
HER2	Sense-5'-CCAGGACCTGCTGAAGTGGT-3'
	Antisense-5'-TGTACGAGCCGCACATCC-3'
β-actin	Sense-5'-GCTCAGGAGGAGCAAT-3'

Antisense-5'-GGCATCCACGAAACTAC-3'

Cytotoxicity analysis

The cytotoxicity assay was determined by MTT assay [12]. Briefly, 5000 cells were seeded in each well on 96 well plates and cultured for 24 hours, then treated with different concentration (3.12, 6.25, 12.5, 25, 50, 100, 200 µg/mL) of plant extract while cyclophosphamide was used as positive control. The cells were then incubated at 37°C for 24 hours in 5% CO₂. At the end of the incubation, the medium was removed and 10 µL of MTT was added followed by 100 µL of DMSO was added to each well to solubilize the formazan crystals. It was then left in dark at room temperature. The absorbance was measured at the wavelength of 595 nm using a microtitre plate reader and the results were analysed in triplicate and the percentage was calculated.

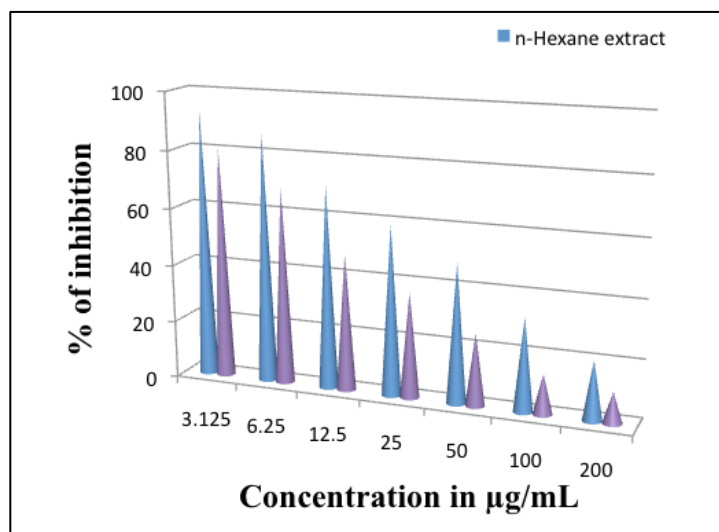


Figure 1: Cell growth inhibitory assay

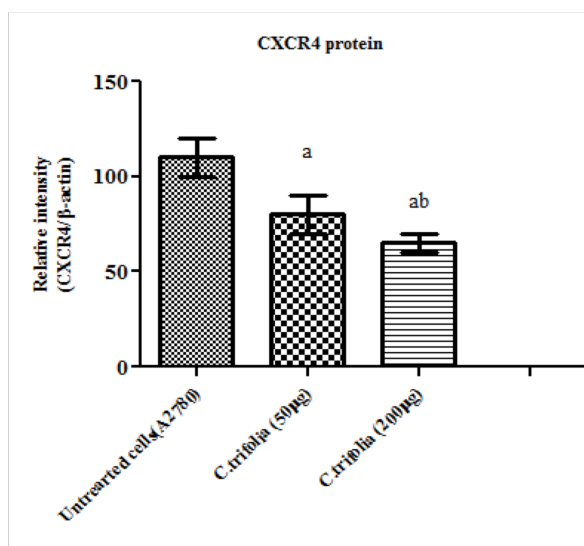
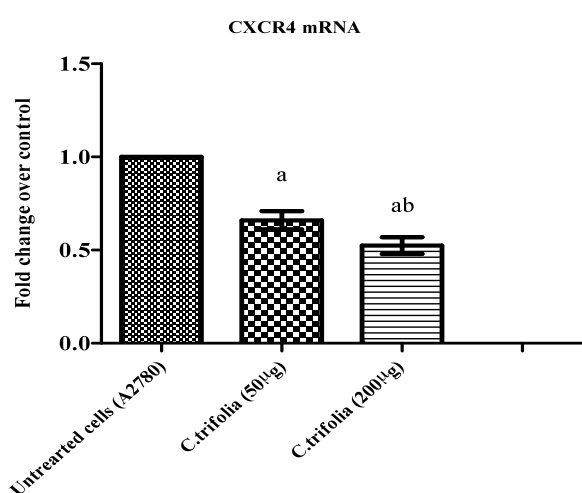
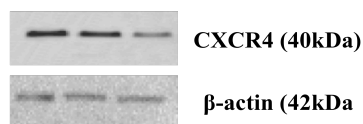


Figure 2: Effect of *C. trifolia* on CXCR4 mRNA and protein expression in A2780 human ovarian cancer cells. Cells were treated with 50 and 200µg of *C. trifolia* n-hexane extract for 24 h. The mRNA expression of CXCR4 mRNA gene was analyzed by real-time PCR using SYBR Green dye and protein expression by western blotting. Protein levels were quantified using densitometry analysis and are expressed in relative intensity. H-actin was used as an internal control. Target gene expression was normalized to H-actin mRNA expression and the results are expressed as fold change from control. Each bar represents mean ± SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cancer cells; b-compared with 50µg *C. trifolia* treated A2780 cells.

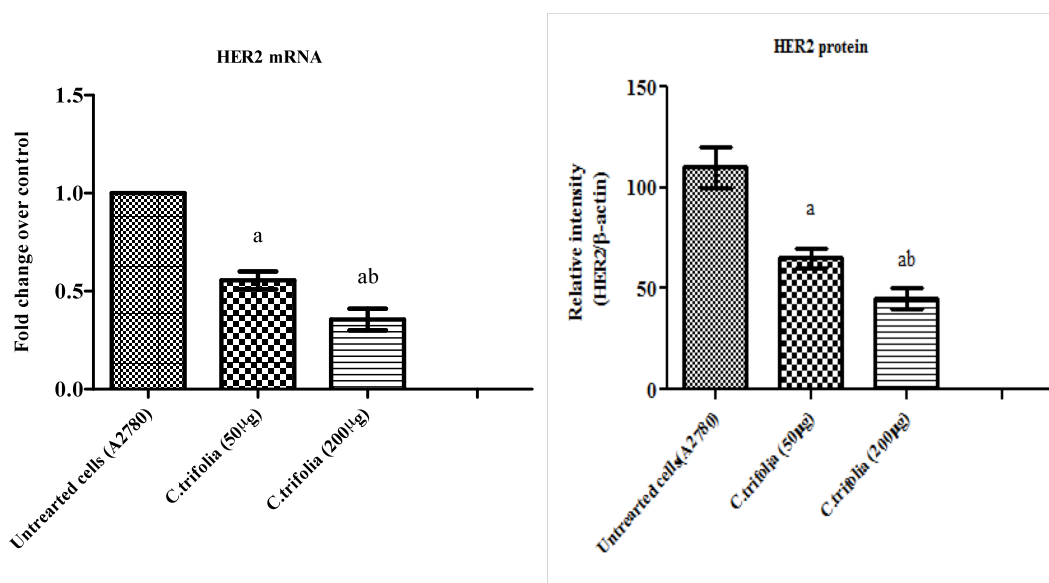
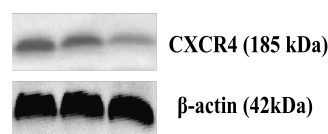


Figure 3: Effect of *C. trifolia* on HER2 mRNA expression in A2780 human ovarian cancer cells. Cells were treated with 50 and 200µg of *C. trifolia* n-hexane extract for 24 h. The mRNA expression of HER2 gene was analyzed by real-time PCR using SYBR Green dye. Target gene expression was normalized to H-actin mRNA expression and the results are expressed as fold change from control. Each bar represents mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cancer cells; b-compared with 50µg *C. trifolia* treated A2780 cells.

Gene expression analysis

Total RNA isolation, cDNA conversion and real-time PCR

mRNA expression levels of CXCR 4; HER2 were examined using real-time PCR. The total RNA was isolated by using a TRIR kit (Total RNA Isolation Reagent Invitrogen) and estimated spectrometrically by the method of Laneve et al. (2014) [13]. The RNA concentration was expressed in microgram (μ g). By using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2 μ g of total RNA as stated in the manufacturer's protocol. To perform real-time PCR, the reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), Forward and reverse primers of CXCR4 and HER2 (the primer sequences were listed in Table 1) in total volume of 45 μ l except the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 μ l of control DNA for positive control, 5 μ l of water for negative control and 5 μ l of template cDNA for samples were taken and reaction mixture (45 μ l) were added. 40 cycles (95°C for 5 min, 95°C for 5 s, 60°C for 20 s and 72°C for 40 s) was set up for the reaction and obtained results were plotted by the PCR machine (CFX96 Touch Real-Time PCR Detection System, USA) on a graph. Relative quantification was calculated from the melt and amplification curves analysis.

Protein expression analysis by western blotting

After the 24 h treatment period the cells were lysed in RIPA buffer containing 1X protease inhibitor cocktail, and protein concentrations were determined by Lowry's method [14]. Cell lysates (50 μ g) was subjected to heat denaturation at 96 °C for 5 min with Laemmli buffer. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and then transferred to PVDF membrane (Amersham Biosciences, UK). The membrane was blocked with 5% blocking buffer (Amersham Biosciences, UK) in TBS-T (Tris buffered saline and Tween 20), for 1 h at room temperature followed by incubation with primary antibody to

CXCR4 and HER2 at a dilution of 1:1000. The membrane was subjected to repeat wash for three times with TBS-T and then incubated for 1 h in horseradish peroxidase (HRP)-conjugated mouse/rabbit secondary antibody by 1:7500 dilutions in TBS-T. The membrane was again subjected to repeated wash for three times with TBS and TBS-T. Protein bands were visualized in chemidoc using enhanced chemiluminescence reagents (ECL; Amersham Biosciences, UK). The detected bands were quantified using the Quantity Software (Bio-Rad). Later, the membranes were incubated in stripping buffer [50 ml, containing 62.5 mM of Tris-HCl (pH 6.7) and 1 g of SDS and 0.34 ml of H-mercapto ethanol] at 55 °C for 40 min. Following this, the membranes was re probed using H-actin antibody (1:5000). In this study, H-actin was used as the loading control.

Statistical analysis

The obtained results from the assays were showed as mean \pm SD. The Statistical evaluations were measured through statistical package program (SPSS 10.0, IBM, Armonk, New York, United States).

Results and Discussion

The *in vitro* cytotoxicity activity of n-hexane extract of *C. trifolia* was investigated using different concentrations ranging from 3.12 to 200 μ g/mL against A2780 ovarian cancer cell lines. n-hexane extract showed 86% of cell growth inhibition at the highest concentration of 200 μ g/mL, where as cyclophosphamide showed 88% ie, significant cell growth inhibitory activity (IC 50 value) by n-hexane extract (Figure 1) was observed to be $46.25 \pm 0.42 \mu$ g/mL, when compared to the standard, cyclophosphamide. The present study has confirmed that the induction of cell death occurred at a very low concentration like any other potential cytotoxicity drug. Thus, it may be considered to be a good candidate for therapeutic agent.

A part of the chemokine superfamily, chemokine receptor 4 (CXCR4) is a particular stromal cell factor-1 (SDF-1, CXCL12) receptor, which is a strongly conserved G protein-coupled 7-transmembrane receptor. The only chemokine receptor expressed in about 80% of ovarian cancer tissues, although not in the usual ovarian epithelium, is CXCR4, rendering it a potential candidate for targeted ovarian cancer therapy [15]. HER2 was, on the other hand, a significant predictor of oncogenic genes in ovarian cancer. Breast cancer studies have shown that HER2 controls CXCR4 expression, which is essential for HER2-enhanced lung invasion, migration, adhesion and metastasis [16]. The X-ray structure of the bioactive compound epifriedelanol, isolated from the ethanol extract *C.trifolia* and its binding affinities to a few proteins identified to be overexpressed under ovarian cancer (HER2, EGFR and CXCR4) have been extensively investigated using molecular docking techniques. It is very impressive that the compound binds to carboplatin, an FDA-approved treatment for ovarian cancer, in order to interact with the protein targets [17]. Therefore in the present study, we attempted to investigate the underlying signalling mechanism of *C.trifolia* in A2780 ovarian cancer cells. Results of this study showed that CXCR4 ($p<0.036$) mRNA and CXCR 4 protein ($p<0.015$) expression were significantly reduced in *C.trifolia* treated A2780 cells (Figure 2). Similar to that of the CXCR4, HER2 mRNA ($p<0.022$) and protein ($p<0.007$) were also found to be down regulated in extract treated cells. Our findings showed that CXCR4 and protein expression was substantially up regulated by HER2 in untreated A2780 cells (Figure 3). Treatment with *C.trifolia* n-hexane extract controlled the HER2 mRNA and protein expression which in turn down regulated the CXCR4 expression. These data shows that natural compounds selected from the GC-MS study of *C.trifolia* ethanolic extract, such as ethyl oleate, 4,8,12,16-tetramethylheptadecan-4-olide and heptacosanol, have strong HER2 molecule docking and have an appropriate score and complex energy compared to the FDA-approved cyclophosphamide drug [16] and this may be the

reason behind the down regulation of HER2 and CXCR4 by n-hexane *C.trifolia* extract that regulates ovarian cancer.

Conclusion:

The n-hexane extract of *C.trifolia* significantly reduced CXCR4 and HER2 mRNA and protein expression A2780 cell lines. However, role of *C.trifolia* n-hexane extract on further downstream signalling molecules need to be studied to validate the data.

Conflict of author interest:

The authors declare no conflict of interest.

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