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

Chella Perumal Palanisamy1, Sivakumar Pethanan2, Gopinath Gurulingam Vincent2, Karthigeyan Murugesan2, Ananthavalli Ramachandran2, Ramu Sivanandum3 & Mani Panagal4*

1State Key Laboratory of Biobased Material and Green Papermaking, School of Food Science and Engineering, Qilu University of Technology, Shandong Academy of Science, Jinan 250353, China; 2Department of zoology, Arumugam Pillai Seethai Ammal College, Tiruppathur, Tamil Nadu, India; 3Department of zoology, M R Government arts college, Mannargudi, Tamil Nadu 614001, India 4Department of Biotechnology, Annnai College of Arts and Science, Kovilacheri, Tamil Nadu 612503, India; *Corresponding author - Panagal Mani - Email: mani.panagal@yahoo.com

Received June 30, 2021; Revised July 19, 2021; Accepted July 19, 2021, Published August 31, 2021

Declaration on Publication Ethics:
The author’s state that they adhere with COPE guidelines on publishing ethics as described elsewhere at https://publicationethics.org/. The authors also undertake that they are not associated with any other third party (governmental or non-governmental agencies) linking with any form of unethical issues connecting to this publication. The authors also declare that they are not withholding any information that is misleading to the publisher in regard to this article.

Author responsibility:
The authors are responsible for the content of this article. The editorial and the publisher have taken reasonable steps to check the content of the article in accordance to publishing ethics with adequate peer reviews deposited at PUBLONS.

Declaration on official E-mail:
The corresponding author declares that official e-mail from their institution is not available for all authors.

Abstract
It is of interest to report the chemotherapeutic (drug target based) potential of n-hexane Cayratia trifolia L. (C. trifolia) extract on A2780 cell lines. mRNA and protein expression analysis of the human chemokine receptor (CXCR4) and human epidermal growth factor receptors-2 (HER2) were studied using RT-PCR analysis and western blot analysis. The results show significant cell growth inhibition with minimal IC50 values of 46.25 ± 0.42 micro g/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, anti-diabetic, 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 [5–7]. The paste of the tuber from C. trifolia is used in the treatment of snake bite [9]. We have shown the antioxidant, antimicrobial and cytotoxicity potential of n-hexane extract of Cayratia trifolia L [8]. Therefore, it is of interest to explore that chemotherapeutic (drug target based) potential of Cayratia trifolia L. n-hexane extract on A2780 cells through suppression of mRNA expression.

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'-ATCCCTGCCCTCTGCTGACTATTC-3' |
|       | Antisense-5'-GAGGGCCTTGCGCTTCTGGTG-3' |
| HER2  | Sense-5'-CCAGGACCTGCTGAACCTG-3' |
|       | Antisense-5'-TGTACGAGCCGCACATCC-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. This related procedure is taken from our previous work [8].

**Figure 1:** Cell growth inhibitory assay as reproduced from elsewhere [8]

**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.
Gene expression analysis

Total RNA isolation, cDNA conversion and real-time PCR

mRNA expression levels of CXCR4 and 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 (µg). By using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2 µg 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 µl expect the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 µl of control DNA for positive control, 5 µl of water for negative control and 5 µl of template cDNA for samples were taken and reaction mixture (45 µl) 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 µg) was subjected to heat denaturation at 96 °C for 5 min with Laemmlli 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 were reprobed 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 ± 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 µg/mL against A2780 ovarian cancer cell lines. n-hexane extract showed 86% of cell growth inhibition at the highest concentration of 200 µg/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±0.42µg/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 the HER2 mRNA and protein expression which in turn down (Figure 3). Treatment with 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.

References:
[1] Mohammed SI et al. Indian J Clin Biochem. 2017 32:153 [PMID: 28428689].
[2] Priyang a Si et al. Der Pharmacia Lettre. 2015 7: 225.
[3] Sowmya S et al. Int J Pharm Pharm Sci. 2016 8: 57.
[4] Perumal PC et al. Asian Pac J Trop Dis. 2012 2: S952.
[5] Sowmya S et al. Indo Am J Pharm Res. 2015 5: 218.
[6] Perumal PC et al. Pharmacognosy Res. 2015 7: 121 [PMID: 25598646].
[7] Sowmya S et al. Int J Toxicol Pharm Res. 2015: 7: 1.
[8] Bhuvaniswari M et al. Bioinformation. 2021 17: 452.
[9] Perumal PC et al. J App Pharm Sci. 2014 4: 6.
[10] Sowmya S et al. World J Pharm Res. 2014 3: 973.5.
[11] Palanisamy CP et al. S Afr J Bot. 2019 124: 311.
[12] Ngamwongsatit P et al. J Microbiol Methods. 2008 73: 211 [PMID: 18417231].
[13] Laneve P et al. J Vis Exp. 2014 90: 51814 [PMID: 25177861].
[14] Lowry OH et al. J Biol Chem. 1951 193: 265 [PMID: 14907713].
[15] Palanisamy CP et al. J Young Pharm. 2018 10: 173.
[16] Palanisamy CP et al. J Pharm Bioallied Sci. 2019 11: 155 [PMID: 31148892].
[17] Perumal PC et al. Bangladesh J Pharmocol. 2016 11: 545.

Edited by P Kanguane

License statement: This is an Open Access article which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. This is distributed under the terms of the Creative Commons Attribution License

Articles published in BIOINFORMATION are open for relevant post publication comments and criticisms, which will be published immediately following the original article for FREE of cost without open access charges. Comments should be concise, coherent and critical in less than 1000 words.
