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(Z)-Ethylidene-4, 6-Dimethoxycoumaran-3-One Induces Apoptosis in Chronic Myelogenous Leukemia Cell Line

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ABSTRACT

The present study was performed to evaluate the antiproliferative property of (Z)-ethylidene-4, 6-dimethoxycoumaran-3-one (EDC), isolated from *Pogostemon quadrifolius* (Benth.) leaf against chronic myelogenous leukemia cell line (K-562). The purity and identity of the compound were confirmed by HPLC and ESI-MS analysis. Cell viability assay (MTT) was performed to evaluate the antiproliferative effect of EDC on K-562 cell line. The result showed that EDC inhibited the proliferation of K-562 cell line with an IC_{50} of $7.77 \pm 0.21 \mu\text{g mL}^{-1}$ ($35.3 \mu\text{M}$). Further DNA fragmentation assay was performed to identify the mode of action of EDC against K-562 cell line. The clear laddering pattern obtained after treating the cell line for 24 h with $50 \mu\text{M}$ EDC indicated that EDC induces cell death in K-562 cells by inducing apoptosis. Thus, the present study evidenced the potential antiproliferative property of EDC against chronic myelogenous leukemia cancer *in vitro*.

Key words: Antiproliferative, apoptosis, cancer, Ethylidene-4, 6-dimethoxycoumaran-3-one, K-562, leukemia

INTRODUCTION

Cancer is one of the major health issues of the present world. Leukemia is a type of cancer affecting the blood forming tissues, such as bone marrow and lymphatic system. Globocan 2012 statistics shows that, the incident number of leukemia was 351965 with a mortality number of 265471 (Ferlay *et al.*, 2013). It is estimated that, in 2015 there will be 54270 new cases of leukemia with an expected death of 24450 in United States (Siegel *et al.*, 2015). In males the chance of developing leukemia from birth to death is one in sixty and in female it is one in eighty six (Siegel *et al.*, 2014). Chronic myelogenous leukemia is a myeloproliferative tumor, which originates from hematopoietic stem cells caused by the BCR-ABL fusion gene, the oncoprotein derived from the Philadelphia chromosome 9/22 translocation (Xie *et al.*, 2014). Resistance to chemotherapy is a major problem for the successful treatment of leukemia. The researchers are trying to find new drugs which can overcome these problems.

Derivatives of many plant derived compounds like vincristine, vinblastine, podophyllotoxins etc. are in the current use for haematological malignancies (Lucas *et al.*, 2010). Recent scientific researchers are focusing to make use of plant extracts and isolated compounds to develop better drug with less side effects. *Pogostemon quadrifolius* (Benth.) is a shrub indigenous to India, Bangladesh and Myanmar. *Pogostemon* is a large genus belonging to the family Lamiaceae (Bhatti and Ingrouille, 1997; Lansdown, 2011). The plant leaf has been using in India and

Bangladesh as a herbal remedy against chicken pox, worm and also as a blood purifier (Padal and Chandrasekhar, 2013; Padal *et al.*, 2013; Padal and Raju, 2013; Raju *et al.*, 2014). The essential oil from the plant has displayed the mosquito larvicidal and antimicrobial property (Thoppil *et al.*, 2003; Trivedi, 2006). Till now the scientific community hasn't given much attention to investigate the various biological properties of the plant *P. quadrifolius* (Benth) and its constituents.

The present study was conducted to evaluate the *in vitro* antiproliferative activity of (*Z*)-ethylidene-4,6-dimethoxycoumaran-3-one (EDC), a poly phenol isolated from the *P. quadrifolius* (Benth.) leaf extracts against chronic myelogenous leukemia cell line K-562.

MATERIALS AND METHODS

Isolation of (*Z*)-ethylidene-4,6-dimethoxycoumaran-3-one: Leaves of *P. quadrifolius* (Benth.) were collected from the University of Calicut campus, India. The isolation of major cytotoxic compound, EDC was conducted as described elsewhere. The isolated EDC sample was analyzed using HPLC and ESI-MS to confirm its purity and identity.

HPLC and ESI-MS analysis of isolated EDC: One milligram EDC was dissolved in 1 mL of HPLC grade methanol and filtered through a 22 mm sterile filter. Analytical HPLC analysis was carried out using a Shimadzu HPLC system (system controller, CBM-20Alite; solvent delivery unit, LC-20AD; UV-vis detector, SPD-20A) equipped with a reverse-phase Shimadzu Shim-pack CLC-ODS (M) column. Detection was performed at 254 nm while, eluting isocratically with a flow rate of 1 mL min⁻¹ using ultrapure water-methanol (1:9) for a sample injection volume of 20 mL. Further the compound was analyzed by ESI-MS, positive ion mass spectra were generated to confirm the identity of the compound. Additionally NMR analysis was also performed to confirm the structure of the compound.

Cell culture: K-562 cell line was obtained from RGCB, India. The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and antibiotics. Cells were grown in a 5% CO₂ atmosphere in a humidified environment at 37°C in a carbon dioxide incubator.

Cell viability assays (MTT): Cell viability assay was conducted to analyze the cell viability after treatment. The MTT assay was performed as described earlier (Raghavan *et al.*, 2014). The cells were seeded in a 96-well plate at a density of 15,000 cells/well and incubated overnight. After incubation, fresh medium containing varying concentrations of EDC in DMSO was added to respective wells. Cells were incubated for a further 48 h in a CO₂ incubator. At the end of incubation, the medium was aspirated and replaced with fresh medium containing MTT (0.5 mg mL⁻¹). After 3-4 h of incubation, the formazan crystals formed, were dissolved in DMSO and the absorbance measured at 570 nm. IC₅₀ values were calculated using the ED50 plus v1.0 software program.

DNA fragmentation assay: The assay was performed as described in literature (Brooks and Harris, 2006). The cells were treated with 20 and 50 µM concentrations of EDC for 24 h. After incubation the cells were lysed, DNA was extracted and treated with RNase. Further the DNA was digested with SDS-Proteinase K together with NaCl solution. The supernatant was then extracted twice by phenol-chloroform method. The DNA was precipitated by adding an equal volume of isopropanol and incubated at -20°C for overnight. The isolated DNA was then suspended in TE buffer. After that, the DNA was separated over 2% agarose gel and visualized under UV light using ethidium bromide dye.

RESULTS AND DISCUSSION

Isolation and identification of (Z)-ethylidene-4,6-dimethoxycoumaran-3-one: Isolation of EDC was performed as described elsewhere. Structure of EDC is shown in the Fig. 1. The isolated EDC was analysed by HPLC and ESI- MS to confirm its purity and identity of the compound. Analytical HPLC profile of the isolated EDC (Fig. 2), measured at 254 nm indicated a high purity (>98%) of the sample. The HPLC spectrum displayed a prominent peak of EDC (Z form) at R_t of 3.84 min followed immediately by a minor peak (E form) at R_t of 4.11 min. ESI- MS analysis yielded a quasi-molecular ion at m/z $[M+H]^+$ 221.0 and a double $[2M+Na]^+$ sodium adduct at m/z 463.1. NMR analysis was performed in additional to confirm the compound. The results obtained were completely consistent with the data we reported earlier (Klika *et al.*, 2014).

Antiproliferative assay (MTT assay): K-562 is a human Chronic Myelogenous Leukemia (CML) cell line. Imatinib, dasatinib and nilotinib are mainly used to treat the CML patients, while the majority of patients respond to these drugs, a subset becomes resistant to these therapeutics (Gandhi *et al.*, 2014). The researchers are in progress to find the best drug which can overcome these problems. Therefore, MTT assay was conducted to evaluate the antiproliferative effect of EDC on these specific cell lines.

The cells were treated for 48 h along with various concentrations of EDC to evaluate the antiproliferative activity of the compound. The results showed an encouraging antiproliferative

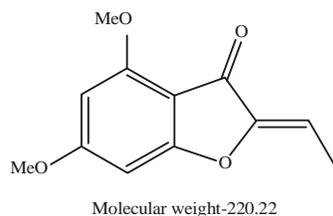


Fig. 1: Structure of (Z)-ethylidene-4,6-dimethoxycoumaran-3-one (EDC)

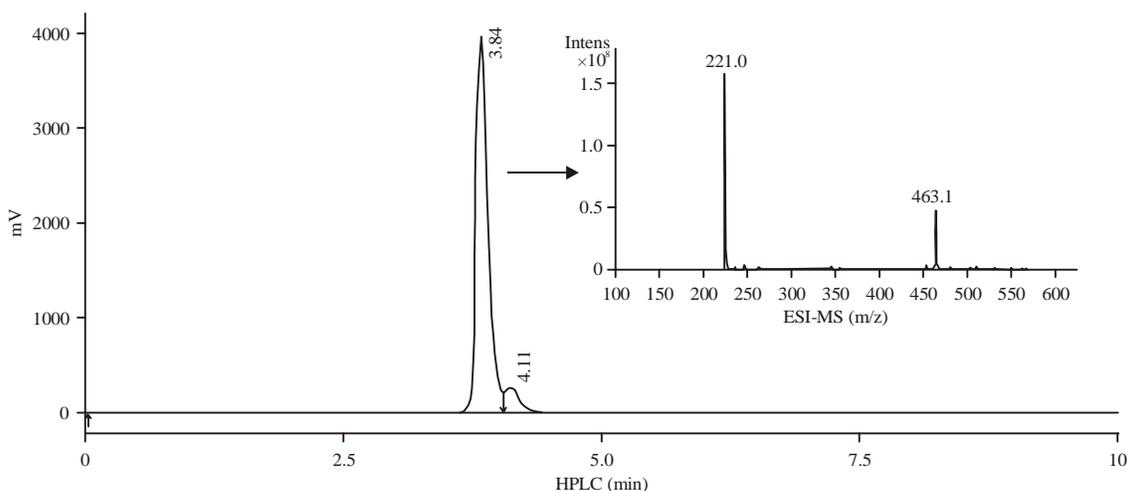


Fig. 2: HPLC spectrum of isolated (Z)-ethylidene-4,6-dimethoxycoumaran-3-one (EDC) analyzed at 254 nm. The ESI-MS spectra of EDC obtained also shown in the figure

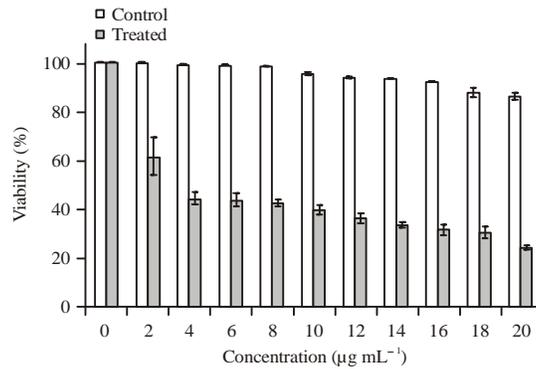


Fig. 3: MTT assay: The graph showing percentage of viability after treatment of K-562 with EDC for 48 h treatment is represented in comparison with DMSO. The results are indicated in Mean±SD of three independent experiments

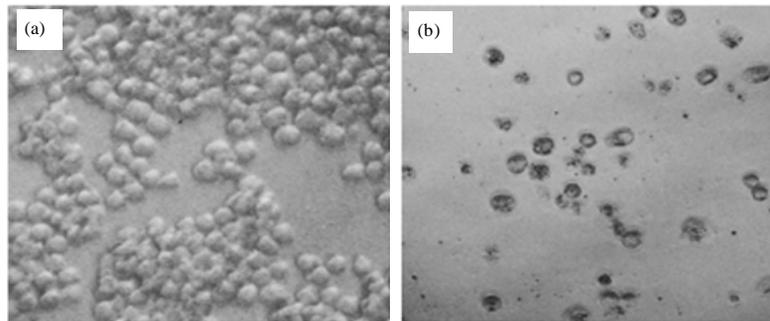


Fig. 4(a-b): Effect of EDC on cancer cell morphology after 48 h treatment of K-562 with IC₅₀ (a) Control and (b) Treated

activity of EDC against K-562 with an IC₅₀ of 7.77±0.21 (35.3 µM). The graph (Fig. 3) is showing the percentage of viability after treatment with EDC for 48 h. After treatment the cells were shrunk in size. Figure 4 shows the effect of EDC on the cancer cell morphology after 48 h treatment at IC₅₀.

DNA fragmentation assay: Apoptosis is the programmed cell death, which is a normal physiological process, occurs in cells during development and normal cellular processes. The damaged cells, which can't work normally, will die during apoptotic process. It was considered that the defect in the normal apoptosis mechanism is the main reason for the development of cancer. The major number of chemotherapy drugs clinically used for the treatments function by initiation of apoptosis (Hassan *et al.*, 2014). DNA fragmentation assay was performed to analyze the apoptotic inducing property of EDC on K-562 cell line. Two different concentrations, one which is less (20 µM) and one which is more (50 µM) to IC₅₀ obtained, were selected for this study to understand the effect of EDC on K-562. Cells were treated with 20 and 50 µM EDC for 24 h (Fig. 5). The result showed a considerable formation of DNA ladder pattern after treatment with 50 µM EDC within 24 h treatment. The results also indicated the concentration dependent induction of cell death.



Fig. 5: DNA fragmentation assay: Formation of DNA ladder after EDC treatment of K-562 cells for 24 h

DNA fragmentation is an important marker to detect the apoptosis induction, wherein the cells activated endonuclease cleaves DNA strands between nucleosome units, resulting in the formation of DNA/histone fragments (Brooks and Harris, 2006). The results confirm that EDC causes the cell death in K-562 by inducing apoptosis.

CONCLUSION

(*Z*)-ethylidene-4,6-dimethoxycoumaran-3-one (EDC) is a phenolic compound isolated from the plant leaves of *Pogostemon quadrifolius* (Benth.). The present study helped to identify the antiproliferative property of EDC against K-562 cell line. It also revealed that EDC induces apoptosis in K-562 cells. Thus, the study was facilitated to identify the potential effect of EDC against the chronic myelogenous leukemia cancer cells *in vitro*.

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