ANTIPROLIFERATIVE EFFECT OF CHLOROFORM FRACTION OF METHANOLIC EXTRACT OF SEEDS OF A*nnona muricata* IN TRIPLE NEGATIVE BREAST CANCER CELL LINES

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Abstract

The lack of expression of hormone and human epidermal growth factor receptors in breast cancer conditions has made molecular-targeted therapies unsuitable and establishing therapeutic strategies are challenging for such cancers. Screening of methanolic extract of seeds of Annona muricata against 4T1 triple negative breast cancer cells has showed evidenced cytotoxicity against cancer cells. Hence fractionation of the extract was carried out and the fraction was analysed for its phytochemical constituents and anti proliferative effect. The phytoconstituents when anlaysed using Liquid Chromatography Mass Spectroscopy revealed the presence of annonaceous acetogenins. The results of the present study reveal the anti proliferative effect of chloroform fraction of methanolic extract of seeds of A. muricata. It can be concluded that the chloroform fraction of methanolic extract of A. muricata exerts anti proliferative effect due to the presence of annonaceous acetogenins.

Keywords: *LCMS analysis, annonaceous acetogenins, A. muricata, Anti proliferative effect*

Triple negative mammary tumours are basal-like mammary tumours which lack the expression of oestrogen, progesterone and human epidermal growth factor (HER2). These tumours are highly aggressive with high lymphovascular invasion and significantly shorter survival than other types of mammary tumours. Most triple negative mammary tumours have pathobiological features in common to human triple negative breast cancers. Eventhough the protocols that are commonly followed in the treatment of human triple negative breast cancer shall be extended to animal subjects, most of the antineoplastic agents showed a very high toxicity (Sleeckx *et al.*, 2011). Moreover, tumours that lack expression of hormone receptors and HER2 are not suitable for molecular-targeted therapies and hence establishment of therapeutic strategies for triple negative breast tumours are challenging. Screening of methanolic extract of seeds of *Annona muricata* against 4T1 triple negative breast cancer cell has showed evidenced cytotoxicity against cancer cells. Hence fractionation of the extract was carried out and the fraction was analysed for its phytochemical constituents and antiproliferative effect.

1. *Assistant Professor*
2. *Professor and Head*

Materials and Methods

1) Preparation of methanolic extract of seeds of A. muricata and isolation of fractions and subfractions

The seeds of *A. muricata* were air-dried at room temperature and coarsely powdered using an electrical pulverizer. The powders obtained were extracted using a Soxhlet apparatus with methanol at 55°C. The methanolic extracts were then concentrated using rotary vacuum evaporator under reduced pressure and temperature (55°C) and kept under refrigeration for the complete evaporation of the solvent.

The fractionation was done as per the method of Chang and Wu (2001). The methanolic extract of seeds of *A. muricata* was partitioned with chloroform and water (1:1) ratio to yield chloroform fraction (CMAM). The process was repeated three times with 500 mL of the chloroform water mixture. Chloroform solvent was reduced by air drying followed by freeze drying.

2) Liquid Chromatography Mass Spectroscopy analysis of the chloroform fraction of methanolic extract of A. muricata (CMAM)

The LC-MS analysis of CMAM was performed at Sophisticated Analytical Instrument Facility, Lucknow using Waters TQD triple quadruple Mass Spectrometer (USA) equipped with Waters H-Class Acquity UPLC system and electron spray ionization source (Asthana *et al*., 2014). Mobile phase (A) acetonitrile and (B) 5 mM ammonium acetate buffer was used and isocratic elution performed at the flow rate of 0.250 mL min^{-1} for 20 min. Waters ACCUCORE C18 100 X3, 2.6µm column was used and ESI source potentials were capillary voltage 3.5 kV; cone potential at 30 V for LC-ESI-MS experiments. Nitrogen was used as the nebulizing and drying gas at flow rates of 50 and 650 L h^{-1} respectively. Mass spectral data was acquired in both positive and negative ion modes at range 150-1500 Da and Scan time 0.8 sec. Data acquisition and processing were carried out using Mass Lynx V4.1 SCN 714 software. The compound search was done using METLIN informatics platform of Scripps Centre for Metabolomics and Mass Spectrometry.

Sample preparation

The chloroform fraction of methanolic extract of seeds of *A. muricata* (CMAM) was solubilized in dimethyl sulphoxide (DMSO) at concentration of 10mg/mL, further this stock solution was diluted with sterile PBS to required concentrations. The finalconcentration of DMSO in the wells was maintained less than one per cent w/v.

Cell lines

The cell lines used for the study were MDA-MB-231, an adherent epithelial human breast cancer adenocarcinoma cell line derived from metastatic site, procured from RIKEN cell bank, 4T1, an adherent epithelial Balb/cfC3H mouse mammary tumour cell line, purchased from ATCC, USA, BT-549, an adherent epithelial human breast cancer ductal carcinoma cell line, obtained as gift samples from Dr. Radha, Centre for Cellular and Molecular Biology, Hyderabad, Andhra Pradesh. All the three cell lines were Estrogen (ER) negative, Progesterone (PR) negative and Human epidermal growth factor (HER) negative. Cells were cultured in RPMI-1640 supplemented with 10 per cent fetal bovine serum, 4.5 per cent antibiotic antimycotic solution containing penicillin-streptomycin and amphotericin B. The cells were maintained in a humidified incubator at 37°C with 5 per cent CO_2. Cell lines were sub cultured by enzymatic digestion with 0.25 per cent trypsin/1mM EDTA solution when they reached approximately 70-80 per cent confluency and these trypsinized cells were used for the experiment.

3) Sulforhodamine B (SRB) assay

Sulforhadamine B assay was done as per the method of Lee *et al.* (2012). It was used to determine cellular density of compounds based on the measurement of protein content. Sulforhodamine B binds to basic amino acids of protein in electrostatic and pH dependent manner. Breast cancer cells (MDA-MB-231, BT-549 and 4T1) were plated in 96 well round bottom plates at a density of 5,000 cells/ well and allowed to attach overnight. Time zero measurement was taken at the time of drug addition by fixing of the cells with 50 µL of 30 per cent (w/v) trichloro acetic acid (TCA) in 4°C for 1 hr. Cells were treated with concentrations of 0.625, 1.25, 2.5, 5, 10, 20,

40 µg/mL of CMAM. After 48 hrs of treatment, cell monolayers were fixed with 50 µL of 30 per cent TCA. Fixed cells were washed with tap water five times and air dried. And then 50 µL of 0.4 per cent (w/v) sulforhodamine B dye was added to the plate and incubated for 20 min. The unbound dye was washed off by repeated washing with 1 per cent(v/v) acetic acid and kept for air drying. The bound stain was solubilized with 100 µL of 10 mM Trizma base for 15 min and absorbance reading was measured at 515 nm by multimode plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). Absorbance readings at time zero (T_Z), Control (C), Treatment (T_i) Growth inhibition 50 (GI_{50} - the concentration at which 50 per cent of growth was inhibited) were calculated using following formula.

Per cent of growth inhibition=100-$((T_i)-T_z)/(C-T_z))$

4) Statistical analysis

The results were statistically analyzed by One way analysis of variance (One way ANOVA) followed by Duncan's multiple comparison test.

Results and Discussion

Liquid chromatography Mass Spectroscopy analysis of the chloroform fraction of methanolic extract of A. muricata

The LC-MS analysis of CMAM showed extracted ion chromatogram (EIC) with mass at 567, 286, 224, 613, 597, 625, 581 and 561. The compound search using METLIN informatics platform of Scripps Centre for Metabolomics, Mass Spectrometry and available literatures revealed the presence of annonaceous acetogenins like Muricatacin, Muricin H, Annonacin, Muricin A, Muricin C, Isoannonacin A, Muricatetrocin B, Muricatin B, Muricatalicin, Muricatalin, Muricapentocin, Annopentocin A, Annopentocin B, Muricatocin B, Annopentocin C, Annomuricin-D-one, Annomuricin A, Muricatin A and Annomutacin.

Moghadamtousi *et al.* (2015) reported that most of the phytoconstituents in the seeds of *A. muricata* are annonaceous acetogenins. Reiser *et al.* (1999, 1993a, 1993b) isolated Muricatacin, Annonacin, Isoannonacin and Muricatetrocin B from the seeds of *A. muricata*. Wu *et al.* (1995) reported the isolation of Annomuricin A and Annomutacin from the leaves of *A. muricata* while Zeng *et al.* (1996) isolated Annopentocin A, Annopentocin B, Annopentocin C and Annomuricin-D-one from the leaves of *A. muricata*. Yu *et al.* (1997) isolated Muricatalicin and Muricatalin from the seeds of *A. muricata* while Muricatin A was isolated from the seeds of *A. muricata* by Huaqing *et al.* (1999). Muricin A and Muricin C were isolated from the seeds of *A. muricata* by Chang and Wu (2001). Li *et al.* (2001) isolated Muricapentocin from the leaves of *A. muricata*. Muricn H was isolated by Liaw *et al.* (2002). The present study reports the presence of Annomuricin A, Annomutacin, Annopentocin A, Annopentocin B, Annopentocin C, Annomuricin-D-one and Muricapentocin in the seeds of *A. muricata* besides the other phytoconstituents.

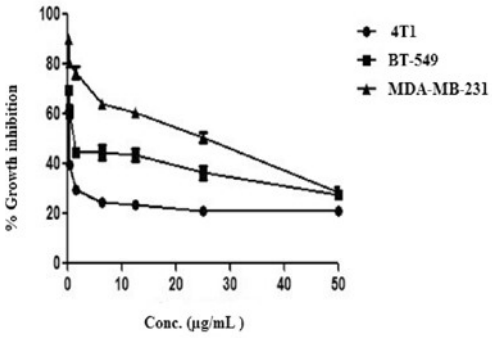

Fig. 1. Dose response curve for cell growth of breast cancer cells after treatment with chloroform fraction of methanolic extract of seeds of *A. muricata* determined by SRB assay. All values are Mean±SE, n=triplicate wells, triplicate experiments. One way ANOVA followed by Duncan's multiple comparison test, statistically significant at p<0.001

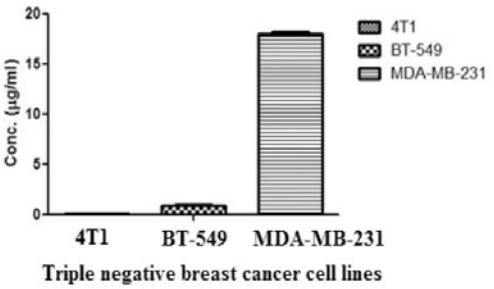

Fig. 2. GI_{50} values obtained after treatment with chloroform fraction of methanolic extract of seeds of *A. muricata* on different triple negative breast cancer cell lines determined by SRB assay. All values are Mean±SE, n=triplicate wells, triplicate experiments. One way ANOVA followed by Duncan's multiple comparison test, statistically significant at p<0.001

Anti proliferative effect

Sulphorhodamine B assay was conducted to check the potential anti proliferative effect of CMAM and the plant fraction showed dose dependent growth inhibition (Fig.1.) The GI_{50} values for 4T1, BT-549 and MDA-MB-231 were found to be 0.128+0.03, 0.95+0.04 and 18.03+0.20 µg/mL respectively as shown in Fig.2. Sulphorhodamine B (SRB) assay was conducted by Hong *et al.* (2016) to study the anti proliferative effect of hexane and ethyl acetate extracts of *Curcuma manga* on HT29 cancer cells and showed that both the extracts exhibited significant growth inhibition with GI_{50} of 15.6±0.8 and 17.9±1.2 µg/mL respectively and possessed mild toxicity on the normal colon cell line, CCD-18Co, with GI_{50} value of 46.5 ± 0.5 mg/mL and 45.7 ± 1.0 mg/mL respectively. In the present study, results of SRB buttressed the cytotoxic potential of CMAM. Hence it may be inferred that the *in vitro* cytotoxicity of CMAM could be due to decreased cell proliferation.

It can be concluded that the anti proliferative effect of chloroform fraction of methanolic extract of seeds of *A. muricata* was due to the presence of annonaceous acetogenins as identified by LC-MS analysis.

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