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In vitro analysis of alizarin as novel therapeutic agent for murine breast cancer

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Abstract
Matrix metalloproteinases (MMPs) has been implicated as a target in cancer treatment due to their vital role in tissue remodeling, invasion and metastasis. Due to Several side effects of various synthetic drugs, now-a-days phytocompounds have taken importance in the treatment of cancer; with special significance to breast cancer by targeting MMPs. The current study is aimed at investigating the effect of Alizarin, a purified product from the Rubia plant on murine breast cancer cell line with respect to matrix metalloproteinases. The Minimum Hemolytic Concentration (MHC) and inhibitory concentration (IC50) of Alizarin were evaluated by Minimum Hemolytic Concentration assay and tetrazolium assay (MTT assay) respectively. The effect of alizarin on expression of Matrix metalloproteinase-9 (MMP-9) gene was studied on 4T1 murine breast cancer cell line by real time PCR. Cells were cultured in Roswell park memorial institute (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) and treated with different concentrations of Alizarin. Alizarin treated cells were further evaluated for apoptosis and invasion potential by Annexin binding assay and transwell chamber assay respectively. MHC and MTT assay indicate the hemolytic concentration and IC50 of the phytocompound to be 200 µM and 495 µM respectively. At high concentrations of alizarin, MMP-9 gene expression was down-regulated in a concentration dependent manner. Annexin binding assay indicates that alizarin is necrotic to the breast cancer cells. At higher drug concentrations alizarin is found to be anti-invasive in nature.

Keywords: MMPs, invasion, anti-metastatic, cytotoxicity, haemolytic, apoptosis

Introduction
Matrix metalloproteinases (MMPs), the zinc-dependent endopeptidases belong to the metzincin superfamily which is implicated in pathological conditions like cancer. MMPs play a vital role in cell-cell adhesion, cell-extracellular matrix interaction, proteolysis of ECM, cancer cell migration and provide the microenvironment for tumor growth, angiogenesis and invasion.1However, depending upon the tumor stage, tumor site, enzyme localization and substrate profile certain members of the MMPs exert contradicting roles at different stages of cancer progression. Hence MMPs are responsive to the therapeutic intervention by synthetic and natural inhibitors providing a viewpoint for future studies. Several therapeutic agents which act as matrix metalloproteinases inhibitors have been targeted to control their enzymatic activity. MMP-9, the largest member of the MMP superfamily can be correlated with breast cancer metastasis and invasion.2 Increased secretion of MMP-9 lead to increased invasiveness in MDA-MB-231 cell line *in vitro*.3 Several compounds derived from either dietary or medicinal plants having substantial chemo-preventive properties can be used effectively against cancer. In traditional Chinese medicine, Rubia plants have been used extensively for their anti-inflammatory, anti-oxidant and anti-bacterial properties. Chemically Alizarin is known as 1, 2-dihydroxyanthraquinone4 which is also commonly known as Mordant red or Turkey red. Alizarin has been effectively used as an osteotropic drug in the treatment of bone tumors.5 In the present study, we aimed to investigate the anti-metastatic property of alizarin in murine breast cancer cell line (4T1).

Materials and methods
Cell lines and cell culture
4T1 cell line (gifted by Dr T Yoshimura of National Cancer Institute, Maryland USA) was cultured in Roswell Park Memorial Institute Medium (RPMI, Hyclone), with 10% fetal bovine serum (FBS, Cell clone) and Penicillin-Streptomycin-Amphotericin B (Cellclone) in 5% CO2 incubator at 37oC. RBCs were isolated from canine blood samples collected from Veterinary Poly Clinic, IVRI.

Preparation of Drugs
Alizarin used in the current study was procured from Natural Remedies, Bangalore (India) and solubilized in methanol.

Measurement of Minimum hemolytic concentration (MHC)
The MHC was determined following the protocol of Singh *et al* with modifications.6 Canine venous blood collected in the heparinized vial was centrifuged at 400g for 10mins with Histopaque (Polysucrose and Sodium diatrizoate solution) to remove serum and buffy coat. Collected RBCs were washed twice with 1X PBS and suspended in 1X PBS, followed by seeding onto a 96 well cell culture plate (100µl/well). Then RBCs were treated with alizarin in quadruplicates at final concentrations starting from 2.5µM up to 640 µM in PBS and 0.1% Triton-X-100 was used as positive control. Then the plates were incubated for 1h at 37 °C followed by centrifugation at 200g for 5min. The supernatant was transferred to another plate and absorbance was taken at 550nm. The percentage of hemolysis was calculated by plotting concentration of phytocompounds vs absorbance graph.

Cytotoxicity of alizarin by MTT assay
Cultured 4T1 cells were trypsinized from 80% confluent flask and seeded into a 96 well cell culture plate. After 24h of incubation alizarin was added to a final concentration of 0.1µM to 1000µM in quadruplicates. After 24h of incubation, each well was treated with 20µl of MTT (5mg/ml in PBS pH 7.4) and further incubation was carried out for 4h till purple formazan complex formation. The formazan complex was dissolved in 100µl DMSO and absorbance was measured at 550 nm. The IC50 was calculated using Graph pad prism software 6.0

Real-time PCR
Designing of Primers for MMP-9
For PCR based amplification of the MMP-9 gene, primers were designed (Table -1) based on the published sequence of Mus musculus matrix metallopeptidase 9 (MMP- 9), mRNA (Accession: NM_013599.4) using the software 'DNASTAR ®', Primer3 and Oligo-analyzer. The primers were supplied as desalted oligonucleotide by Eurofins (India).
Cells from the flasks were trypsinized and seeded onto 24-well tissue culture plates (5×103 cells in 100µl of growth medium/well) followed by incubation until a monolayer was formed. Alizarin was added to cells at concentrations of 400µM, 495µM, 600µM in triplicates and cells without any treatment were taken as control. Followed by drug treatment both treated and control cells were incubated for 24hr at 370C in 5% CO2 incubator. Total RNA was extracted from both alizarin treated and control cells with TRIzol (Amresco, USA), in accordance with the manufacturer's instructions. Concentrations and purity of RNA were quantified spectrophotometrically by measuring A260 and A280.The ratio A260/A280 of pure RNA was approximately 1.8. Complementary DNAs were synthesized with oligo-dT primers in a 10µl total volume reaction mixture using a Revert aid cDNA synthesis Kit (Thermo Fischer, USA) as per the instructions. The expression levels MMP-9 were detected with step one Real time PCR system (Applied Bio-systems). Real time PCR was set up in triplicates with one set of primers (MMP-9 forward and Reverse primers) as in Table 1 and conditions (as in Table 2) were used in all reactions to yield the amplification of an endogenous control gene (GAPDH) and the specific target genes of interest. Following amplification, Fold expression analysis was performed.

Table 1: Oligonucleotide sequence of primers used in this study

Gene	Sequence	%GC content	Annealing Temperature
MMP-9 (Forward)	CTCCAACCGCTGCATAAATA (20)	45	57
(Reverse)	CCCTAACGCCCAGTAGAGAG (20)	60	

Table 2: Conditions of real time PCR

Steps	Process	Temperature (^{0}C)	Duration
1	Initial Denaturation	95	5.0 min
2	Denaturation	95	30 sec
3	Annealing	57	20 sec — 35 cycles
4	Elongation	72	20 sec

Annexin and PI assay
To study the effect of alizarin on apoptosis Annexin and PI assay was carried out. 4T1 cells sub cultured in RPMI-1640 growth medium were incubated at 37 °C with 5% CO2 in an incubator for 24h. When cells reached a confluency of 80%, the cells were treated with alizarin (400µM, 495µM, 600µM) in triplicates. The plates were again incubated in the CO2 incubator for 24h. Cells treated with vincristine (positive control) to a final concentration of 10µm were used as positive control. After 48h, cells were harvested with 0.25% Trypsin 0.53 mM EDTA solution and cell pellets were incubated with 100 µl of 1x binding buffer. Then, 5µl of Annexin V-FITC and 5µl PI was added to the cells and gently vortexed followed by incubation in dark room at room temperature for 15 min. Finally, 400 µl of 1X binding buffer was added to each tube and 10,000 events per sample were analyzed by BD FACS Caliber instrument

Effect of Alizarin on cancer cell invasion by Transwell chamber assay
Cell invasion was performed by the Trans well chamber assay (Merck Millipore, ECM-551) according to the manufacturer's protocol by starving the 4T1 cells for 18hrs prior to assay in RPMI-1640 medium. The collagen layer of inserts was rehydrated with 300µl of serum free media. The cells were harvested with 0.25% Trypsin - 0.53mM EDTA & Trypsin was inactivated by quenching medium. The supernatant was discarded by centrifugation at 1500 RPM for 5 min and resuspended in 5ml quenching medium to have 0.6x106 cells for ml. 250µl media was removed from the inserts and 250µl of the cell suspension was added to the inserts. A final concentration of 400µM, 495µM, 600µM of Alizarin was added to different inserts respectively in triplicates. 500µl of chemoattractant medium was added to lower chamber and kept for 48h at 37oC in CO2 incubator. After treatment ~ 19 ~

The Pharma Innovation Journal invasion chambers were placed into clean wells containing 400µl of stain and incubated for 20 min at room temperature, rinsed with water several times and the non-invading cells were removed from the interior by cotton swab. The invading cells from the air dried inserts were extracted by placing in 200µl of extraction buffer for 15 min at room temperature and the absorbance was measured at 550nm.

Results

Measurement of minimal hemolytic concentration (MHC)

The minimum hemolytic concentration (MHC) was determined by treating the cells with alizarin at concentrations ranging from 2.5µM to 640µM in PBS and 0.1% triton-X-100 as a positive control and untreated RBCs as a negative control. Percentage of hemolysis in canine RBCs treated with different concentrations of alizarin with respect to the controls (shown in figure 1) were determined. 5% hemolysis of canine RBCs was observed at 200µM for alizarin.

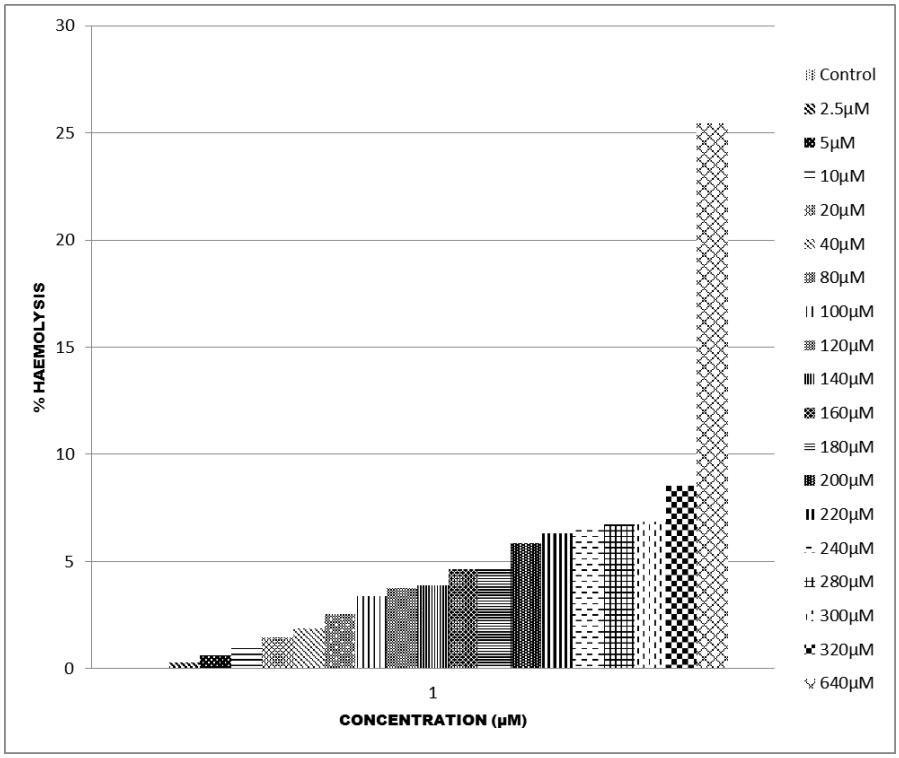

Fig 1: Minimum haemolytic concentration assay in alizarin treated and control cells.

Cytotoxicity of alizarin by MTT assay

The extent of cytotoxicity caused by phytocompounds on various cell lines was determined by MTT assay which is based on the reduction of the tetrazolium salt to purple formazan product by NADH dependent succinate dehydrogenase enzyme of viable cells. The assay is a first, rapid, economical and reliable method for screening of large number of the anticancer drugs in *vitro.*7 The IC50 of alizarin on 4T1 cell line was calculated by graph pad prism V 6.0 and was found to be 495 µM as presented in Figure 2.

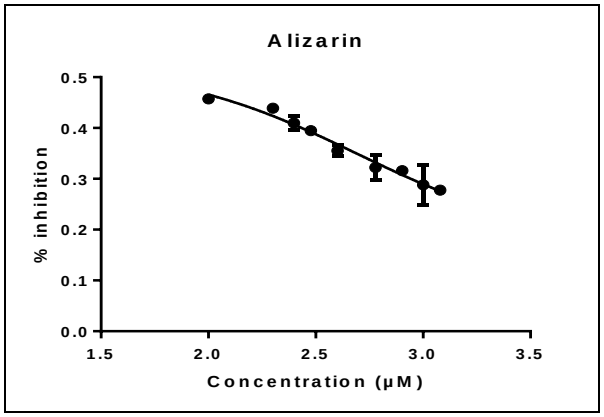

Fig 2: Effect of alizarin on cytotoxicity in 4T1 cell line by MTT assay.

Effect of alizarin on the expression of MMP-9 in 4T1 cell line by Real Time PCR:

The effect on the expression of MMP-9 gene in alizarin treated 4T1 cells as compared to non-treated cells were analyzed by qPCR. The fold change in the expression of these genes in 4T1 cells on treatment with alizarin was graphically depicted in Figure 3.

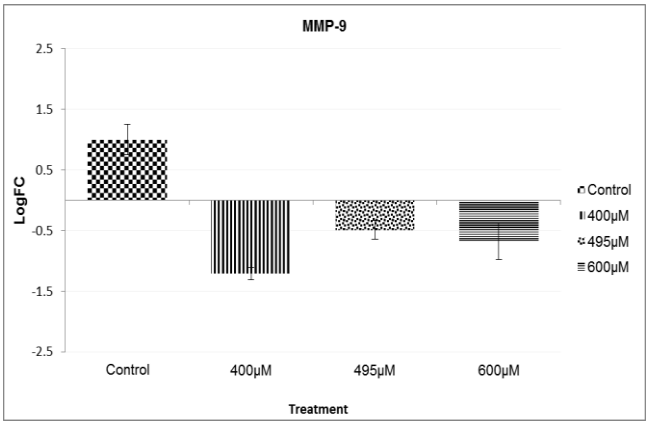

Fig 3: Change in fold expression of MMP-9 in Alizarin (400, 495 and 600 µM) treated 4T1 cells with respect to untreated cells

Effect of alizarin on apoptosis of 4T1 cell line by annexin and PI assay

Alizarin treated cells were evaluated for apoptosis by annexin binding assay and the percentage of cells obtained in necrotic, apoptosis (early and late) and live cells stage were depicted in Figure 4.

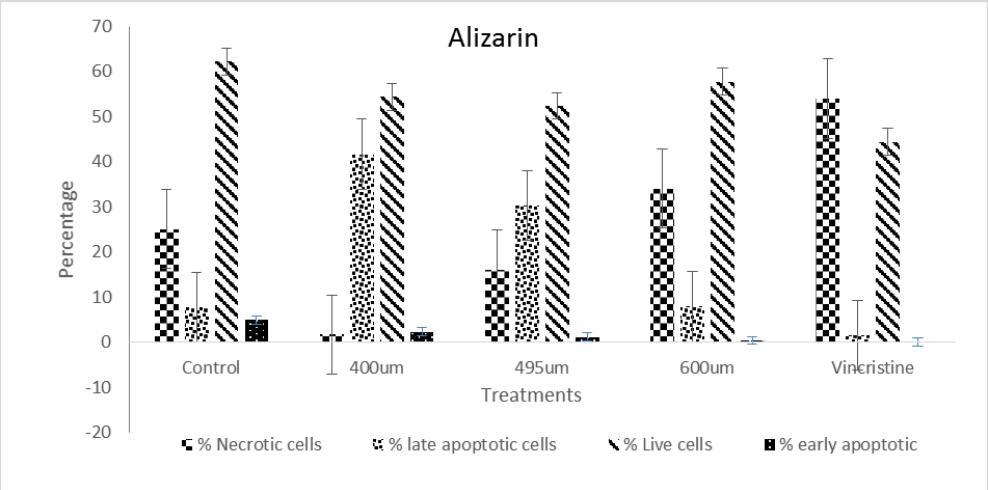

Fig 4: Percentage of cells showing necrotic, apoptotic (early and late) and live cells

Effect of alizarin on cancer cell invasion by Trans well chamber assay

Concentrations of alizarin (400 µM, 495 µM, 600 µM were tested on the invasion of 4T1 cell line in transwell chambers. Morphologically no change was observed between control and treated cells. However when statistical analysis of micro plate reading was done at significance level $p<0.05$ value at 550nm, significant difference between control (Absorbance) and treated cells was observed (Figure 5) which suggests the inhibition of cell invasion at 600µM of alizarin with an absorbance value of 0.051 at 550nm.

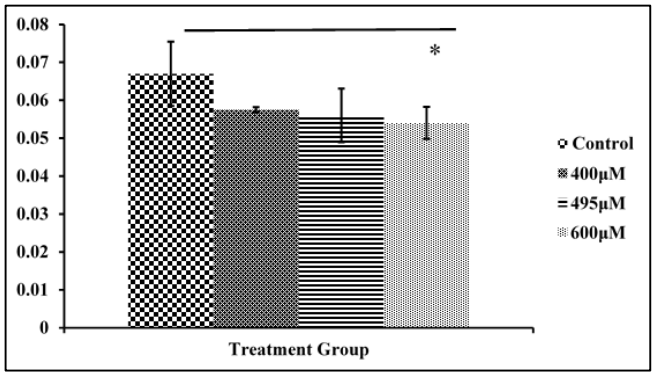

Fig 5: Concentration vs absorbance values in Transwell chamber assay.

Discussion

In the current study highly tumorigenic and invasive 4T1 (Thioguanine resistant) cell line derived from the 4th stage tumorigenic BalbC mice were used.[8]

Measurement of minimal haemolytic concentration (MHC) of alizarin

In vitro haemolysis tests have been employed by several authors for the toxicological evaluation of different plants.9The haemolysis relates to concentration and potency of plant extracts. Exposure of red blood cell (RBCs) to injurious substances such as hypotonic medium or heat results in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin. Damage to RBCs will make the cell more susceptible to secondary damage through free radical. Furthermore, haemolytic activity of each extract is related to their chemical composition. Erythrocytes were considered as the major target for the free radicals owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the oxygen transport that associated with redox active haemoglobin molecules, which were potent promoters of acid oxygen species. 5% haemolysis of RBCs is considered to be haemolytic for drugs. Alizarin was causing 5% haemolysis of blood at 200µM concentration. Certain drugs having colour may lead to false positive results, as alizarin is of yellow colour, it is showing haemolysis at low concentration.

Effect of alizarin and on cell viability

The extent of cytotoxicity caused by Phytocompounds on cell line was determined by MTT assay. MTT assay is based on the reduction of the tetrazolium salt, to a purple Formazan product by NADH dependent mitochondrial Succinate dehydrogenase enzyme of viable cells. The formazan salt is solubilized and measured by microplate reader to determine the cytotoxicity of alizarin. The IC50 for alizarin for mice breast cancer cell line (4T1) cell line was determined by MTT assay. The IC50 is 495 µM for alizarin for 4T1 breast cancer cell line. The results indicate that alizarin was toxic to breast cancer cell line. So it will be safe to use as a drug for cancer cell line. Silvestrol was first isolated from the chloroform-soluble extract of fruits of *Aglaia foveolata* found to be cytotoxic to several human cancer cell lines.10 Betulinic acid, a secondary metabolite of plants, displayed selective cytotoxicity against human melanoma cells (MEL-2).11 Purified compounds like Curcumin, Demethoxycurcumin and bisdemethoxycurcumin, Turmerin, Turmerone, Furanodiene, Zederone from the *Curcuma longa, Curcuma zeodaria* had been shown to be cytotoxic activity in human and murine cancer cell lines.12 Galanal A and B, [8]-Gingerol and [10]-Gingerol, [6]-Paradol has been found to be cytotoxic in Jurkat, MCF`-7 and Hela, MDA-MB-231 like tumorigenic cell lines.[13]

Effect of alizarin on expression of MMP -9 by Real time PCR

The fold expression analysis of real time PCR data indicates the downstream expression of MMP- 9 for treated cells in dose dependant manner as compared to non-treated cells. Several different phytocompounds have been known to decrease the MMP-9 expressions. Vanilla bean suppresses mouse breast cancer cells and Hepatocellular carcinoma cells 14 by down regulating MMP-2 and MMP-9 expression. Ethanol extract of Ocimum extract inhibits the MMP- 2 and 9 thus showing anti-metastatic effect. 15 Apigenin, has shown anti-metastatic potential in human breast cancer MCF-7 cell line by inhibiting MMP-2 and MMP-9 16Genistin reduces the expression of the MMP 9 in HeLa cell line in a dose dependent manner 17 Quinoa (Chenopodium quinoa) crop seed extracts has potential to inhibit MMP 1 and MMP 9.[18]

Effect of alizarin on apoptosis

Apoptosis (programmed cell death); a major homeostatic mechanism of maintenance of different cell populations in tissues is marked by loss membrane integrity with the exposure of phosphatidylserine to the external cellular environment. Annexin V (35-36 kDa calcium dependent phospholipid binding protein) has affinity for the phosphatidylserine, when conjugated to a reporter molecule like FITC can be used as an indicator of apoptosis by flow cytometric analysis. Propidium iodide (PI), a fluorescent intercalating agent that binds to nucleic acids is excluded from viable cells due to intact membrane integrity when plasma membrane integrity is lost during late stages of apoptosis and necrosis, PI can enter the cell, and bind to nucleic acid and show red fluorescence. Thus combination of PI and Annexin V can be used to distinguish between viable, apoptotic and necrotic cells.19 the cytotoxic response caused The Pharma Innovation Journal by the selected concentration of alizarin (400,495,600 µM/ ml) in 4T1 cell lines is depicted in figure 4. A decrease in the population of viable cells was observed after treatment with the Phytocompound. With alizarin treated cells the necrotic cell percentage was increasing from 1% to 34% in comparison to control but there was a reduction of apoptosis of treated cells.

Effect of alizarin on cancer cell invasion by Trans well chamber assay

The invasion of 4T1 cells was not observed at a low concentration of alizarin (400 and 495 µM). However significant inhibition of cell invasion was observed 600µM concentration of alizarin (figure 5). Vanillin isolated from Vanilla bean suppresses *in vitro* invasion and in vivo metastasis of mouse breast cancer cells and Hepatocellular Carcinoma Cells.20

Conclusion

Alizarin derived from *Rubia tinctorium* showed IC50 for murine breast cancer 4T1 cell line to be 495 µM. The real time fold expression data shows down regulated expression of MMP-9 after treatment with alizarin. Alizarin is found to be necrotic and inhibits the invasion of cancer cells. Hence further studies can be carried out in using this drug as a potential drug for treatment of breast cancer.

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