ANTI-TUMORIGENIC POTENTIAL OF SOME NATURAL PRODUCTS IN BREAST CANCER

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Degree of Doctor of Philosophy (Science)

In Biotechnology

By

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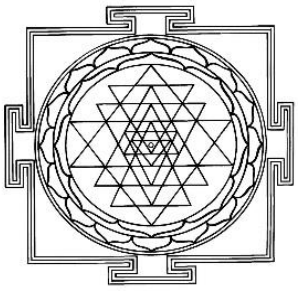

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श्रीचक्रराज-निलया श्रीमत्-त्रिपुरसुन्दरी

श्रीशिवा शिव-शक्त्यैक्य-रूपिणी ललिताम्बिका

'I dedicate my PhD thesis to the lotus feet of the Divine Feminine, the Creatrix, the Eternal Primordial Energy beyond Attributes, the fearsome protector of devotees, the benevolent Mother of Universes who has been instrumental in protecting me, loving me and guiding me as my lifeline, my strength!'

'As My Parents!'

'My everything!'

'My Ma and Baba'

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CONTENTS

LIST OF ACRONYMS

ACRONYM	FULL FORM
WHO	World Health Organization
GLOBOCAN	Global Cancer Observatory
ASR	Age Standardized Rates
HDI	Human Development Index
YLD	Years Lived with Disability
YLL	Years of Life Lost
DALYs	Disability-Adjusted Life Year
PBCRs	Population Based Cancer Registries
NCRP	National Cancer Registry Programme
TNBC	Triple Negative Breast Cancer
BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
PALB2	Partner and Localizer of BRCA2
ATM	Ataxia-telangiectasia mutated
ER	Estrogen Receptor
PR	Progesterone Receptor
HER2	Human Epidermal Growth Factor Receptor 2
FAK	Focal Adhesion Kinase
FERM	Four-Point-One, Ezrin, Radixin, Moesin
FAT	Focal Adhesion Targeting
PR	Proline Rich
NES	Nuclear Export Sequence
SH2	Src homology 2
SH3	Src homology 3
PTK2	Protein Tyrosine Kinase2
MMTV	Mouse Mammary Virus Tumor
mTORC1	Mechanistic Target of Rapamycin Complex 1

RIP	Receptor Interacting Protein
Wnt	Wingless-related integration site
FAS	Fas cell surface death receptor
MMTC	Medullary Thyroid Carcinoma
PyMT	Polyoma Middle Tumor-antigen
T3	Triiodothyronine
T47D	Thornton-47-Daughter
ERK	Extracellular Signal-Regulated Kinase
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
CAF	Cancer Associated Fibroblasts
MAPK	Mitogen Activated Protein Kinase
SRC	Proto-oncogene tyrosine-protein kinase Src
ECM	Extracellular Matrix
EMT	Epithelial to Mesenchymal Transition
RTKs	Receptor Tyrosine Kinases
GPCRs	G Protein Coupled Receptors
PI3K	Phosphatidyl-Inositol-3'Kinase
Akt /PKB	Protein Kinase B
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PDK1	Phosphoinositide-Dependent Kinase 1
PTEN	Phosphatase and Tensin homologue
4EBP1	Eukaryotic initiation factor 4E Binding Protein
S6K1	S6 kinase 1
HIF-1	Hypoxia Inducible Factor 1
Fox O	Forkhead box, class O
BCIS	Breast cancer invasion score
ERBB2	erb-b2 receptor tyrosine kinase 2
FGFR1	Fibroblast Growth Factor Receptor 1
ClpP	Caseinolytic Protease
IGF-1R	Insulin like Growth Factor 1 Receptor

IL6-STAT3	Interleukin-6 / Signal Transducer and Activator of Transcription 3
COX2	Cyclo-oxygenase 2
PDL1	Programmed Death-Ligand 1
NANOG	Homeobox protein NANOG
OCT4	Octamer-binding Transcription factor 4
PARP	Poly(ADP-ribose) Polymerase
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
TGF-α	Transforming Growth Factor–α
CR	Cysteine Rich
PTB	Phospho-Tyrosine Binding
BC-MSCs	Breast cancer associated mesenchymal stem cells
MT1-MMP	Membrane type-1 Matrix Metalloproteinase
MMP	Matrix Metalloproteinases
TIMP	Tissue Inhibitors of Metalloproteinases
GPI	Glycosylphosphatidylinositol
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
CD147	Cluster of Differentiation 147
CD44	Cluster of Differentiation 44
JNK	c-Jun N-terminal kinase
PEA3	Polyomavirus Enhancer Binding Activator -3
SP1	Specificity Protein 1
AP1	Activator Protein 1
NF-κB	Nuclear Factor-κβ
ATF2	Activating Transcription Factor 2
MKK3	Mitogen-Activated Protein Kinase Kinase 3
TGF-β	Transforming Growth Factor -β
PEX	Haemopexin
MDA-MB	M D Anderson - Metastatic Breast
MCF	Michigan Cancer Foundation
SK-BR3	Sloan-Kettering Cancer Center-Breast 3 cells
DOX	Doxycycline

EPR	Enhanced Permeability and Retention
DNA	Deoxyribonucleic Acid
ATRA	All-trans Retinoic Acid
APL	Acute Promyelocytic Leukemia
RAR	Retinoic Acid Receptor
CRABP	Cellular Retinoic Acid-Binding Protein
RXR	Retinoid X Receptor
HCC1599	Human Carcinoma1599
BT474	Breast Tumour 474 cell line
NOTCH	Neurogenic locus notch homolog
CSC	Cancer Stem Cell
JAK-STAT	Janus kinase-signal transducer and activator of transcription
AXL	'Anexelekto' receptor tyrosine kinase
IKKβ	IκB kinase β
PAM	PI3K/AKT/mTOR
MEM	Minimum Essential Media
L15	Leibovitz 15 Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
HRP	Horse Radish Peroxidase
ELISA	Enzyme-Linked Immunosorbent Assay
TMB	Tetramethylbenzidine
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
ADT	Autodock Tools
RCSB	Research Collaboratory for Structural Bioinformatics
PDB	Protein Data Bank
DMSO	Dimethyl Sulphoxide
SFCM	Serum Free Culture Medium
BSA	Bovine Serum Albumin
NP-40	Nonidet P-40
SDS	Sodium Dodecyl Sulphate
PAGE	Poly Acrylamide Gel Electrophoresis

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INTRODUCTION

1.INTRODUCTION

1.1 Statistics and Epidemiology of Breast cancer

Taking cancers in women especially, breast cancer is one of the leading cases of mortality. According to GLOBOCAN 2022, around 2,296,840 new breast cancer cases which accounts for 23.8% of total female related cancers were reported in women [Ref: WHO]. Breast cancer was reported to be the 2nd most diagnosed cancer globally. It was also the fourth leading cause of mortality with 6.9% of total cancer related deaths [*Bray et al., 2024, Ferlay et al., 2024]*.

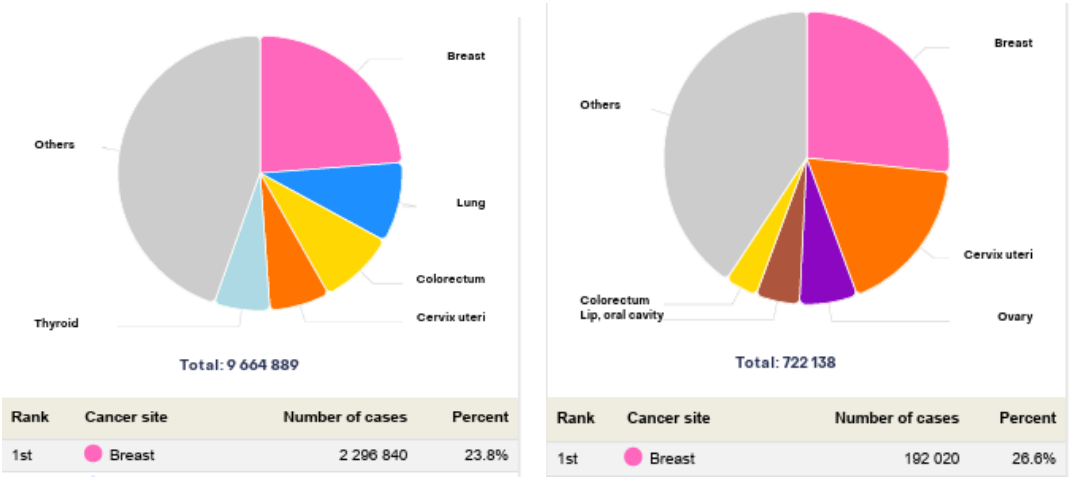

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Fig I : 'Global (A) and Indian (B) Breast cancer statistics published by GLOBOCAN 2022' (World Health Organisation).

Female breast cancer was diagnosed across 157 countries among which it was a leading cause of death in women in 112 countries. Female breast cancer also crossed all other cancers in age standardized rates of incidence and mortality (ASRs) in both transitioned countries with 54.1 per 100,000 individuals and transitioning nations with 30.8 per 100,000 individuals where the burden of mortality was more from breast, ovarian and cervical cancers in women of transitioning countries. Transitioned high income countries showed lower cases of mortality due to growing awareness, proper screening procedures and upgraded treatment systems compared to low income transitioning countries. [*Bray et al., 2024*]. Various life style issues and serious reproductive factors

like early age menarche, late age menopause, alcohol consumption, lack of healthy life style, lack of physical activity, body weight, late age pregnancy contributed to development of breast cancer in women. [*Bray et al., 2024*]

Breast cancer was also found to show a common level of incidence across all levels of HDI in India [*Bray et al., 2024*]. In India around 192,020 cases of breast cancer which accounts for 26.6% in women and 13.6% taking both sexes into consideration [GLOBOCAN 2022]. Reports have shown breast cancer to lead in areas of cancer incidence with 13.5% of newly diagnosed cases and 10.6% of total cancer related deaths in India in 2020 [WHO-Globocan 2020]. Earlier studies in 2016 also showed that patients when diagnosed at stage I have a survival rate of 93.3% compared to ones diagnosed at stage IV with 24.5 % of survival rate *[Nandkumar et al., 2016, Kulothungan et al., 2024]*. Thus, metastasis and secondary tumour formation are major causes of mortality in breast cancers.

To analyze the epidemiology of state-wise breast cancer burden in India from 2012-2016 with respect to Years Lived with Disability (YLDs), Years of Life Lost (YLLs) and DALYs per 100,000 individuals, studies based on 28 population based cancer registries (PBCRs) under National Cancer Registry Programme (NCRP) were conducted. The breast cancer burden of YLLS and YLDS were the highest in northern, southern and central regions compared to the eastern, western and northeastern regions of India and the maximum burden was from the age group of 50-69 years [*Kulothungan et al., 2024*]. The breast cancer burden of India by 2025 was projected to be 5.6 million DALYs where 5.3 million would be premature deaths (YLLs) and the rest from YLDs. Various factors which contribute in development of breast cancer in India include obesity, low literacy among women, use of tobacco, absence of physical activity, early menarche, late menopause, later pregnancy, irregular lifestyle issues, alcohol consumption, occupational hazards, stress, lesser medical awareness, low literacy, lower socio-economic status, absence of proper health screenings along with social and geographical disparities. Considering diagnosis, as age standardized incidence of breast cancer cases are on the rise over the years, prognosis among younger women diagnosed at later stages appeared to be very poor [*Malvia et al., 2017, Sathishkumar et al., 2021, Kulothungan et al., 2024*]. Thus, lesser self-awareness, inadequate screenings though self-examination and mammography, economic limitations, social stigma, fear of surgical procedures and treatment, lack of faith on treatment protocols contribute very

much in diagnosis mostly at later stages of the disease [*Malvia et al.,2017, Pal et al., 2021, D'almeida et al., 2021, Tripathi et al., 2014, Kulothungan et al., 2024*].

1.2 Anatomy and Physiology of Breast Cancer

Breast cancer study involves understanding breast anatomy and physiology which has lobules as glands producing milk; lactiferous ducts which carry milk to the nipples; areola surrounding the nipples; stroma- the connective tissue surrounding ducts and lobules along with blood and lymphatic vessels of the breast. The lymph vessels carry lymphatic fluid from the breast. This lymphatic system consists of axillary lymph nodes under the arm; internal mammary lymph nodes near breast bone and supraclavicular and infra-clavicular lymph nodes around the collar bone. Cancer cells growing within ducts and lobules metastasize at distant places via passage through this framework of lymphatic system *[Jagsi et al., 2019, Henry et al.,2020]*.

Different types of breast cancers have been classified with respect to breast physiology. As most breast cancers are carcinomas, adeno-carcinomas inside ducts and lobules of the breast stand out to be a predominant one. It includes ductal carcinomas *in situ* or inside a milk duct and invasive / infiltrating lobular or ductal carcinoma where invasion within the surrounding tissue has occurred. Invasive ductal/ lobular carcinomas include triple negative breast cancers or TNBC and inflammatory breast cancers. TNBC being highly aggressive occurs in women around age group of 40 and above with potent BRCA1 mutation resulting in poor prognosis and higher chances of disease relapse. *[Henry et al., 2020, Jagsi et al., 2019] [National Comprehensive Cancer Network (NCCN). Practice Guidelines in Oncology: Breast Cancer. Version 7.2021*]

Breast tumors have been studied and classified into various histological and molecular subtypes. These molecular subtypes are based on the presence and absence of certain important markers like Estrogen (ER), Progesterone (PR), HER2, AR (androgen receptor), EGFR etc. These include Basal like (ER-, PR-, HER2-, EGFR+), Her2/ER^{-} (ER-, PR-, HER2+, EGFR+/), normal breast like (HER2-, PR unknown, ER+/-, EGFR+), Luminal [HER2-(+), ER +(-), PR+/-], Molecular apocrine (ER-, PR-, HER2+/-, EGFR +/-, AR+), Claudin low (ER-, PR-, HER2-, EGFR+/-), interferon related (ER-/+, HER 2-, PR unknown) and TNBC (ER-, PR- and HER2-). *[Weigelt et al, 2010]*. Studies have shown genetic inheritance comprises only 10% of total breast cancer cases (1 in 10 breast cancers) compared to acquired mutations which is around

90%. Inherited BRCA1 and BRCA2 gene mutations along with PALB2, CHECK and ATM have been reported to contribute in breast tumor development *[Berger et al., 2019, Byrnes et al., 2008, Walsh et al., 2020] [National Comprehensive Cancer Network (NCCN). Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic. Version 1.2022 – August 11, 2021]*

1.3 Some Major Signalling Pathways in Breast Cancer

Extensive research over the years has showcased the underlying cross interaction of various cellular signalling pathways in breast cancer carcinogenesis. Growth factor receptor, e.g. epidermal growth factor receptor (EGFR) regulated and integrin regulated signalling pathways like phosphatidylinositol 3' kinase (PI3K)/Akt and focal adhesion kinase (FAK) pathways have been reported to play important roles in breast cancer cellular proliferation, tumour development and metastasis *[Aziz et al., 2012, Choi et al.,2016, Guan et al., 2010, Majumder et al., 2019*]

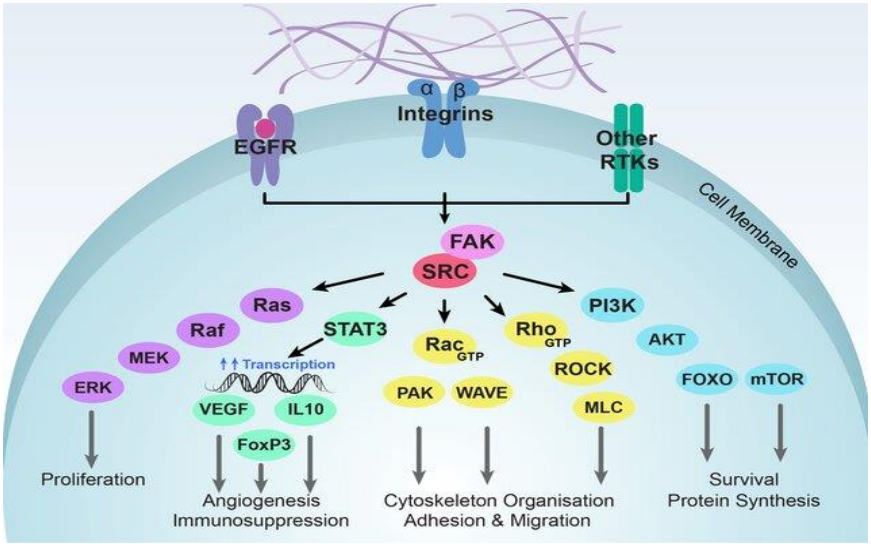

Fig II: ''Schematic of the canonical integrin/Src/FAK signalling network. Src and FAK interact with, and are activated by, numerous receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR), as well as the 'matrix receptor' integrins, which all facilitate their downstream signalling''. Figure from taken from '*Parkin, A., Man, J., Timpson, P. and Pajic, M., 2019. Targeting the complexity of Src signalling in the tumour microenvironment of pancreatic cancer: from mechanism to therapy. The FEBS journal, 286(18), pp.3510-3539)'.*

1.4. The Focal Adhesion Kinase (FAK) Pathway

Regulation of focal adhesion kinase (FAK) signalling occurs through activation of integrin receptors that are transmembrane glycoproteins possessing α and β subunits. There are eight β and eighteen α subunits expressed in humans which associate to form heterodimeric complexes and generate twenty-four different forms of integrin receptors. These subunits, via their extracellular regions, interact with various factors and components of the extracellular matrix (ECM). *[Guan et al., 2010, Hynes et al., 2002, Miranti et al., 2002]*. This interaction with components of ECM causes clustering of integrins at focal adhesions of the cell allowing interaction with cytoskeletal and signalling molecules via formation of multi protein complexes. Thus, this bi-directional signalling function of integrins between the cell and the ECM activate downstream signalling pathways regulating cellular differentiation, growth, migration and other varied functions *[Guan et al., 2010, Hynes et al., 2002, Miranti et al., 2002]*. FAK as a non-receptor tyrosine kinase gets clustered at focal adhesions of the cell. FAK signalling via integrin activation has been reported extensively to play important role in growth of cancer cells *[Guan et al., 2010]*.

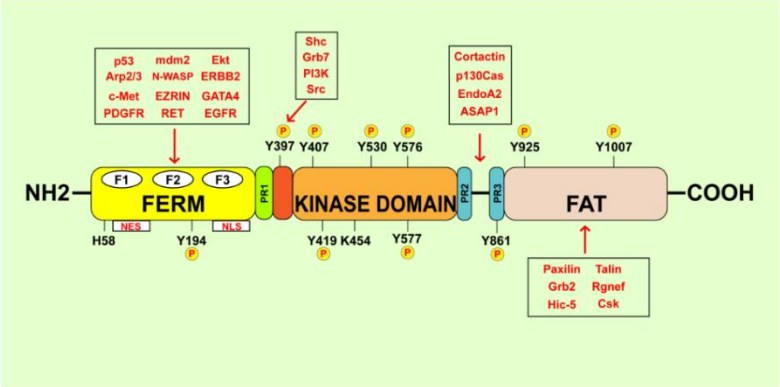

Fig III: ''Schematic representation of structural domains of FAK. FAK is composed of a central kinase domain flanked by an ezrin–radixin–moesin (FERM) homology domain on the N-terminal side and a focal adhesion targeting (FAT) domain on the C-terminal side. Both terminal domains are divided from the kinase domain by three proline-rich regions (PR1, PR2 and PR3). FAK phosphorylation sites and the main FAK binding proteins are indicated.'' **Figure taken from – '*Rigiracciolo, D.C., Cirillo, F., Talia, M., Muglia, L., Gutkind, J.S., Maggiolini, M. and Lappano, R., 2021. Focal adhesion kinase fine tunes multifaced signals toward breast cancer progression. Cancers, 13(4), p.645*'**

The FAK macromolecule is comprised of an amino terminal FERM domain; a central kinase domain, some proline rich domains followed by C-terminal focal adhesion kinase targeting (FAT) domain [*Sulzmaier et al., 2014, Rigiracciolo et al., 2021]*. The FERM domain of FAK made up of 360 amino acids specially has three domains of F1, F2 and F3 followed by a nuclear export sequence (NES) and nuclear localization sequence (NLS). This FERM domain aids in association with integrin and growth factor receptors and shares similarity with cytoskeletal proteins ezrin, radixin and moesin (ERM). This domain also helps in translocating FAK to the nucleus and epigenetic regulation of gene expression via interaction with transcription factors [*Rigiracciolo et al., 2021]*.

The central kinase domain of FAK has an ATP binding region and six tyrosine phosphorylation sites. Phosphorylation of Y576 and 577 residues gives a β-hairpin like conformation of FAK. This is followed by the C-terminal FAT domain made up of 900 amino acids with an antiparallel four helical like structure [*Rigiracciolo et al., 2021]*. The FAT domain allows localization of FAK and interaction with paxillin and talin proteins at integrin rich focal adhesions through its multi-protein binding sites. *[Rigiracciolo et al., 2021, Guan et al., 2010]*. The catalytic cleft has the Y397 site of auto-phosphorylation required for promotion of FAK activity. The C terminal FAT domain allows attachment of FAK to the cytoplasmic region of integrins where the β cytoplasmic region aids in displacing the FERM domain allowing auto-phosphorylation of Y397 residue and binding of Src kinases to FAK *[Guan et al.,2010, Sulzmaier et al., 2014]*.

Proteins like p85 and Src associate with Focal Adhesion Kinase via the phosphorylated Y397 residue through their SH2 domains. Association of FAK with the SH2 domain of Src removes the Src Y527 residue to prevent auto inhibition and causes phosphorylation of Y577 and Y576 residues in the FAK kinase domain to promote FAK activity. Phosphorylation of Y925 of the FAT domain allows adaptor proteins like Grb2 to bind to this complex through their SH2 (Src homology 2) domain and aid in downstream Ras-MAPK (mitogen activated protein kinase) signalling *[Guan et al.,2010, Rigiracciolo et al., 2021*]. FAK also plays an important role as a scaffold for phosphorylation of Src and attachment for other proteins. The C-terminal domain has two proline rich areas which has been reported to interact with SH3 domain containing p130 Cas and endophilin A2 proteins in breast cancer. The Y397 residue of FAK

interacts with PI3K which further interacts with downstream Akt molecule leading to activation of the PI3K-Akt pathway *[Guan et al.,2010]*.

FAK is an important prognostic factor in breast cancer. The chromosomal region of 8q24.3 for protein tyrosine kinase2 (PTK2) has been reported to encode for FAK. The Cancer Genome Atlas database has reported an increase in FAK mRNA levels in around 26% of breast cancers *[Sulzmaier et al., 2014]*. High PTK2 levels in TNBC subtype contribute to tumour relapse and poor prognosis. High expression of FAK was linked with high mitotic index, architectural and nuclear grade 3 tumours in breast cancers *[Rigiracciolo et al., 2021]*. FAK kinase activity in mouse mammary virus tumor (MMTV)-Wnt 1cells and regulation of Akt- mTORC1 signalling by FAK in KO-Wnt1 cells showed its importance in breast cancer cell survival. *[Rigiracciolo et al., 2021, Paul et al., 2020]*. FAK also aided in adhesion independent survival in breast cancer via interaction with receptor interacting protein (RIP) *[Kurenova et al., 2004, Rigiracciolo et al., 2021]*. Downregulation of FAK expression with loss of p125FAK in focal adhesions promoted caspase 8 mediated apoptosis along with Fas-associated death domain mediated breast cancer cell death *[Xu et al., 2000, Rigiracciolo et al., 2021]*. Thus, FAK plays an important role in survival of breast cancer cells.

FAK signalling also promotes breast cancer cellular growth. Functional enrichment studies on murine breast cancer 4T1 cells showed the importance of ECM-integrin interaction in regulation of FAK activity in cellular growth and proliferation *[Tiede et al., 2018, Rigiracciolo et al., 2021*]. Studies on mouse mammary p53R270H mutant model showed FAK expression was crucial for cellular proliferation and epithelial to mesenchymal transition (EMT) [*Rigiracciolo et al., 2021, Van Miltenburg et al., 2014]*.

FAK promotes cellular migration in breast cancers. Integin β1- mediated FAK signalling aided in cellular migration via RhoA/ROCK2/p-cofilin and RhoA/ROCK1/p-MLC signaling in breast cancers [*Rigiracciolo et al., 2021, Wilson et al., 2014]*. FAK–vinculin and phosphopaxillin link in focal adhesion regulate ECM rigidity and tissue stiffness to develop malignant nature of cells. This stiffness caused changes in cellular morphology and migration as studied in MMTC-PyMT tumors [*Plotnikov et al., 2012, Rigiracciolo et al., 2021]*. FAK mediates cytoskeletal reorganization upon ECM generated external signals and stress which allows cellular migration and invasion in breast cancer [*Rigiracciolo et al., 2021]*. Thyroid hormone T3 triiodothyronine mediated integrin αvβ3 linked FAK signalling and downstream

PI3K signalling aided in actin-cytoskeleton reorganization, rapid cellular migration and invasion in T-47D breast cancer cells *[Flamini et al., 2017]*. Integrin mediated signal transduction through FAK promotes upregulation of MMP activity in cancer cells which leads to increased invasive potential *[Banerji et al. 2008; Das et al. 2008]*.

FAK also plays an important role in angiogenesis in cancer. FAK mediated MAPK-ERK signaling was reported to cause VEGF expression in mouse 4T1 breast tumour cells *[Rigiracciolo et al., 2021]*. FAK signalling in breast cancer associated fibroblasts (CAFs) lead to regulation of cytokine signalling which maintained glycolysis of malignant cells causing enhanced cell growth. Apart from development of malignancy, CAFs are also reported to cause multidrug resistance in breast cancer cells. FAK was also reported to play important role in immune escape of cancer cells and resistance to immune therapy. [*Rigiracciolo et al., 2021]*. Thus, the role of FAK is very profound in breast cancer tumour growth, cellular proliferation, migration, angiogenesis, wound healing, immune escape and drug resistance.

FAK also plays an important role in activation of downstream PI3K signalling in breast cancers. Integrin mediated FAK activation caused PI3K and Akt phosphorylation, tumour development and EMT in breast cancers *[Chen et al., 2019, Yang et al., 2016]*. Oncogenic mutations of FAK caused FAK-Src association, $p130^{Cas}$ phosphorylation, Ras and PI3K signalling in breast cancer *[Pylayeva et al., 2009]*.

1.5. The Phosphatidylinositol 3′ Kinase (PI3K) Pathway

The phosphatidylinositol 3′ kinase (PI3K) pathway is a significant signalling pathway which mediates cellular growth, proliferation, survival, migration, angiogenesis and metabolism in breast cancer *[Miricescu et al., 2020; Ortega et al., 2020; Paplomata et al., 2014; Roy et al., 2021]*. PI3Ks are classified into three classes, class I, class II and class III. The class IA PI3Ks have been reported to play important roles in tumour development *[Ortega et al., 2020; Paplomata et al., 2014]*. The class IA PI3Ks are heterodimers comprising of catalytic p110α /p110β/p110δ subunits and regulatory p85α/p85β subunits. The regulatory subunits aid in activation of their catalytic counterparts in PI3K signalling *[Jiang et al., 2020, Miricescu et al., 2020; Ortega et al., 2020]*. G protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and certain oncogenes can directly activate the class IA PI3Ks. After activation, PI3K being a plasma membrane bound enzyme phosphorylates the inositol head group of PIP2

causing conversion of phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate i.e. PIP2 to PIP3. This process leads to recruitment of PDK1 (phosphoinositide-dependent kinase 1) and Akt leading to activation of Akt *[Miricescu et al., 2020, Ortega et al., 2020 Paplomata et al., 2014]*.

The serine/ threonine kinase protein Akt is a very important downstream protein in PI3K signalling which gets activated via phosphorylation at Thr308 and Ser473 residues. It has many downstream substrates like inducers of apoptosis, cell cycle modulators, various protein kinases, transcription factors etc *[Miricescu et al., 2020]*. The activated Akt further phosphorylates mammalian target of rapamycin 1 (mTORC1) protein complex and inactivates TSC1/2 complex. Akt phosphorylates proline-rich Akt substrate of 40 kDa (PRAS40) which activates mTORC1 to further activate the downstream proteins eukaryotic initiation factor 4E binding protein (4EBP1), S6 kinase 1 (S6K1) and hypoxia inducible factor (HIF)-1 *[Ortega et al., 2020, Roy et al., 2021]*. The activated Akt has also been reported to phosphorylate FoxO protein, inhibiting its transcriptional functions and promoting of cellular proliferation and survival *[Zhang et al., 2011]*.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylates PIP3 to PIP2 which impairs Akt phosphorylation of Ser473 and Thr308 residues. This activity of PTEN as a tumour suppressor impairs PI3K signalling which under normal conditions maintains cell homeostasis. Missense and nonsense mutations, bi-allelic and mono-allelic deletions of PTEN gene, epigenetic regulations, post translational modification and degradation of PTEN causes loss of PTEN activity and increase in Akt activity in PI3K signalling mediated cancer progression *[Miricescu et al., 2020, Ortega et al., 2020]*.

The role of PI3K pathway in breast cancer is important and extensive. Breast cancer invasion score (BCIS) model studies showed high expression levels of PI3K-Akt-mTOR which contributed in tumour aggressiveness and drug resistance *[Li et al., 2025]*. Around 70% of breast tumour samples showed mutation in PIK3CA gene encoding the p110α catalytic subunit [*Jiang et al., 2020, Ortega et al., 2020, Paplomata et al., 2014*]. Mutations with nucleotide substitutions were found in the helical and catalytic domain of p110α subunit. PI3KCA mutations were found in ER and PR positive, HER2 positive, luminal and triple negative breast cancers which makes it significant in development of aggressive breast cancer phenotype *[Miricescu et al., 2020]*. Various factors, proteins and receptors influence regulation of PI3K signalling.

Mutations in HER2 (ERBB2), FGFR1 (fibroblast growth factor receptor 1), RTKs, Akt, PDK1 and PTEN (loss of PTEN activity) can all contribute in aberrant PI3K signalling in breast cancer *[Miricescu et al., 2020]*. Role of mi-RNAs and ERα (estrogen receptor) in regulation of PI3K-Akt signalling in breast cancer has been reported *[Rahmani et al., 2020, Bocca et al., 2010]*. Expression of inner mitochondrial protein caseinolytic protease (ClpP) with high ER expression caused increased cellular growth, migration and invasion via PI3K-Akt signalling *[Luo et al., 2020]*.

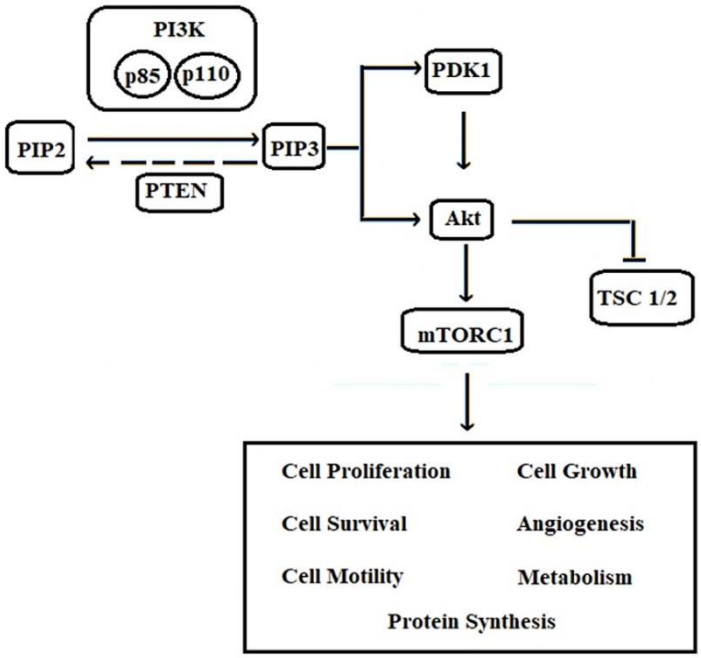

Fig IV: ‘‘Schematic Representation of PI3K Mediated Signalling Cascades’’.

Figure taken from – ‘Roy, A., Chakraborty, I. and Banerji, A., 2021. Natural Compounds as Potential Regulators of the Phosphatidylinositol 3' Kinase (PI3K) Pathway in Breast Cancer. South Asian Journal of Experimental Biology, 11(5), pp.524-538’

The PI3K pathway has been reported to play an important role in cancer stem cell development which aids in tumor progression, EMT and drug resistance *[Xia et al., 2015]*. Various factors and receptors regulate PI3K pathway to cause these processes in breast cancer. Expression of insulin like growth factor 1 receptor (IGF-1R), overexpression of IL6-STAT3, expression of cyclo-oxygenase (COX2), PDL1 mediated overexpression of NANOG and OCT4 transcription factors and Nectin 4 expression were reported in cancer stem cell development and EMT in breast cancer via regulation of PI3K pathway *[Chang et al., 2013, Yang et al., 2014, Almozyan et al., 2017, Siddharth et al., 2017]*.

PI3K/ Akt signaling also contributes in angiogenesis and neo-vascularization in breast cancer *[Zhang et al., 2017]*. Cytochrome P450 (CYP) 4Z1 caused VEGF-A linked angiogenesis, increased MMP-2 expression and metastasis through PI3K signalling in T47D and BT474 cells *[Yu et al., 2012]*. Treatment with BEZ235 on HER2 positive breast tumours showed the importance of PI3K/ Akt/ mTOR in mediating angiogenesis *[Dey et al., 2016]*.

PI3K signalling significantly mediated chemo-resistance in breast cancer. Akt signaling was reported to cause production of ROS within the breast tumour micro environment, promote drug resistance and anaerobic metabolism, prevent apoptosis and cause increased glucose uptake to defend the cells against chemotherapy *[Kaboli et al., 2021]*. The actin binding protein CapG regulated and actin binding protein Transgelin 2 caused resistance to microtubule inhibitors like paclitaxel in breast cancer through Akt activation. PI3K-Akt signalling caused resistance against letrozole (an aromatase inhibitor) and ER specific inhibitors like trastuzumab and tamoxifen. Tumour associated macrophages releasing CC-chemokine ligand 2 and HIF-1α mediated hypoxia contributed to tamoxifen and lapatinib (HER2-EGFR inhibitors) resistance via PI3K-Akt signalling *[Kaboli et al., 2021]*. PI3K-Akt signalling also has an extensive role in immune escape in breast cancer. Downregulation of PARP9 inhibited PI3K-Akt signalling mediated immune escape in breast cancer cells *[Hong et al., 2023]*. PI3K/ Akt pathway activation along with over expression of immune escape biomarker BCL2A1 was also reported in breast cancer *[Dai et al., 2022]*.

1.6 Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) or ErbB1 belongs to the ErbB family of transmembrane receptor tyrosine kinases (RTKs) and can interact with its specific ligands epidermal growth factor (EGF), amphiregulin, betacellulin and transforming growth factor–α (TGF-α). Upon such interaction, EGFR gets activated by autophosphorylation of receptor tyrosine kinase domains and subsequently activates downstream signalling cascades like PI3K and MAPK pathways to mediate cellular proliferation, differentiation and survival as well as neoplastic outcomes *[Dawson et al. 2005; Herbst 2004; Seshacharyulu et al. 2012]*.

Present on chromosome 7, the human EGFR gene codes for a single-pass transmembrane glycoprotein, EGFR, with an extracellular domain, a transmembrane

domain, a juxtamembrane domain, a kinase domain and a regulatory domain *[Alanazi et al. 2016, Herbst 2004]*. The extracellular domain has L1, L2, CR1 and CR2 subdomains. L1 and L2 regions are involved in binding to ligands like EGF whereas CR1 and CR2 are cysteine-rich regions with disulphide bonds. This is followed by the hydrophobic transmembrane domain and juxta membrane domain. The intracellular catalytic kinase domain has 20 tyrosine residues. When a ligand binds, EGFR is activated by the ligand-induced dimerization as the intracellular kinase domains come close together and tyrosine residues in the kinase domain cross phosphorylate residues in the partnering receptor and activate downstream signalling cascades [*Herbst et al. 2004; Purba et al., 2017*]. This phosphorylated region now interacts with various proteins which possess SH2 (Src-homology domain 2) and PTB (phosphotyrosine binding) domains. Thus, EGFR now interacts with signalling pathways to regulate cell signaling mediated cancer growth, invasion and metastasis *[Amelia et al., 2022, Foley et al., 2010]*. The PI3K-Akt signal transduction pathway has been reported to be activated upon interaction of EGFR with ligands [*Alanazi et al. 2016; Herbst 2004; Seshacharyulu et al. 2012*]. Several studies indicate overexpression of EGFR in a variety of tumours including breast cancers, head-and-neck cancers, renal cancers, ovarian cancers and colon cancers and association of such overexpression with poor prognosis and decreased survival. *[Guo et al. 2017; Holdman et al. 2015; Magkou et al. 2008]*.

Research has highlighted the importance of EGFR mediated signalling in breast cancer which promotes expression of matrix metalloproteinases (MMPs) and metastasis. Arachidonic acid has been reported to cause cellular migration and invasion in MDA-MB-231 cells via EGFR linked PI3K/Akt activation *[Villegas-Comonfort et al., 2014]*. Breast cancer associated mesenchymal stem cells (BC-MSCs) play a role in increasing tumour size and mammosphere formation via regulation of EGFR mediated PI3K/Akt pathway *[Yan et al., 2012]*. EGFR was reported to regulate PI3K/ Akt signaling in triple negative breast cancer cells *[Ganesan et al., 2024]*. EGFR signalling has been reported to promote MMP-2 secretion in metastatic cancers *[Periyasamy et al., 2025]*. Treatment of MDA-MB-231 breast adenocarcinoma cells with pomolic acid inhibited EGFR induced cell motility and invasion and indicated the role of EGFR signalling in FAK phosphorylation and MMP-9 expression for mediating migration and metastasis in breast cancer *[Park et al., 2016]*.

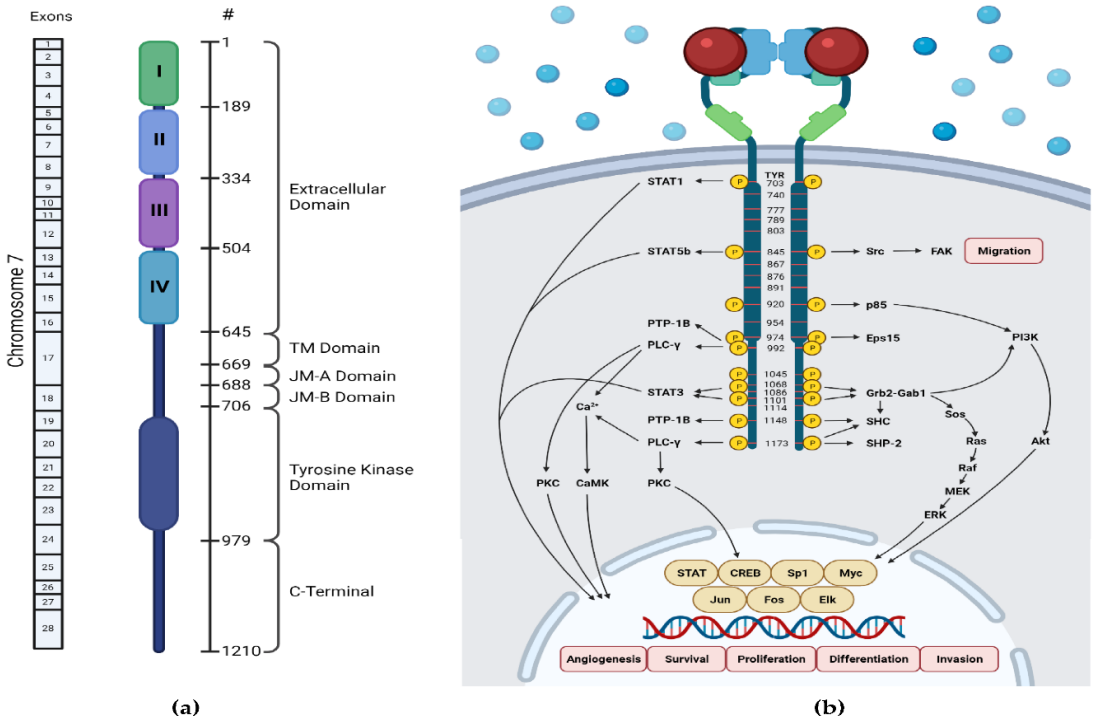

Fig V: "**Schematic diagrams of Epidermal Growth Factor Receptor (EGFR) domains.** (a) Domain structure of human EGFR and exons encoding it (b) EGFR phosphorylation sites. Blue spheres indicate the molecules present outside the cell, and red spheres indicate the EGFR-activating ligand''

Figure taken from- '*Amelia, T., Kartasasmita, R.E., Ohwada, T. and Tjahjono, D.H., 2022. Structural insight and development of EGFR tyrosine kinase inhibitors. Molecules, 27(3), p.819'*

Melittin treatment of breast cancer cells showed EGF induced downstream PI3K/ Akt signaling and MMP-9 expression *[Jeong et al.,2014]*. EGFR-EGF interactions and EGFR mediated signaling have been reported to cause increased expression of MMP-2, MMP-9 and MT1-MMP and increase of MMP-2 and MMP-9 activity via regulation of FAK and PI3K pathways in MCF-7 breast ductal carcinoma cells *[Majumder et al., 2019]*. EGFR can also cooperate with other receptors like integrins to mediate regulation of signalling pathways causing expression of matrix metalloproteinases for metastasis in cancer. Treatment of human ovarian carcinoma and epidermoid carcinoma cell line with sulphated polysaccharides from *Sepiella maindroni* inhibits EGFR mediated signalling through p38 MAPK and PI3K/Akt pathways and MMP-2 expression *[Jiang et al. 2017; Jiang et al. 2018]*.

1.7 Matrix Metalloproteinases (MMPs)

Metastasis and secondary tumour formation as a process was described through the hypothesis of 'seed and soil' proposed by English surgeon Stephen Paget in 1889. The seeds are the tumour cells and the soil is the microenvironment of the organ where secondary tumours are formed by the process of metastasis *[Langley et al., 2011]*. Metastasis is a complex multi-functional process in cancer with escape and migration of cancer cells into the lymphatic and blood vascular system via breakdown of extracellular matrix and basement membrane. After intravasation these tumour cells adhere to the endothelial wall of the blood vessels/ capillaries of the target organ. Then these tumour cells extravasate out through the endothelial layer around the basement membrane and tissue of the secondary organ causing formation of tumours *[Kozlowski et al., 2015]*. The key role players in the process of metastasis are the matrix metalloproteinases (MMPs) *[Egeblad et al. 2002; Kessenbrock et al. 2010; Nagase et al. 2006; Visse et al. 2003]*. Various cellular signalling pathways regulate MMP expression in cancer metastasis.

MMPs (also known as matrixins) are a family of zinc dependent endopeptidases which they aid in ECM degradation and remodelling, tissue reorganization, cell migration and metastasis *[Radisky et al., 2015; Egeblad et al. 2002; Kessenbrock et al. 2010; Nagase et al. 2006; Visse et al., 2003]*. Around 24 MMPs (23 are found in humans) are currently known in vertebrates. MMPs degrade components of the ECM which include laminins, osteonectin, proteoglycans, collagens and entactin. MMPs regulate cell growth and cell survival, degrade basement membrane ECM proteins, promote entry and exit of tumour cells from blood and lymphatic vessels and formation of secondary tumours *[Kwon et al., 2023; Radisky et al., 2015; Egeblad et al. 2002; Kessenbrock et al. 2010; Tauro et al., 2018]*.

On the basis of their sequence, domain and substrate specificity, MMPs have been divided into a number of groups: collagenases, gelatinases, stromelysins, matrilysins, membrane type MMPs and other MMPs *[Radisky et al., 2015; Egeblad et al. 2002; Nagase et al. 2006; Visse et al., 2003]*. A typical MMP has a pro-peptide, a catalytic domain, a hinge region and a haemopexin domain *[Egeblad et al. 2002; Nagase et al. 2006; Visse et al. 2003]*. The catalytic domain includes a conserved zinc binding motif and is similar in all MMPs.The typical MMP structure is present in many MMPs like MMP-1, MMP-3, MMP-8, MMP-10, MMP-12, MMP-13, MMP-18, MMP-

19, MMP-20 and MMP-27. Some MMPs like the matrilysins (e.g. MMP-7) do not have hinge and haemopexin domains while membrane type matrix metalloproteinases (e.g. MMP-14, MMP-15) have additional C-terminal domains *[Radisky et al., 2015; Egeblad et al. 2002; Nagase et al. 2006; Visse et al., 2003]*.

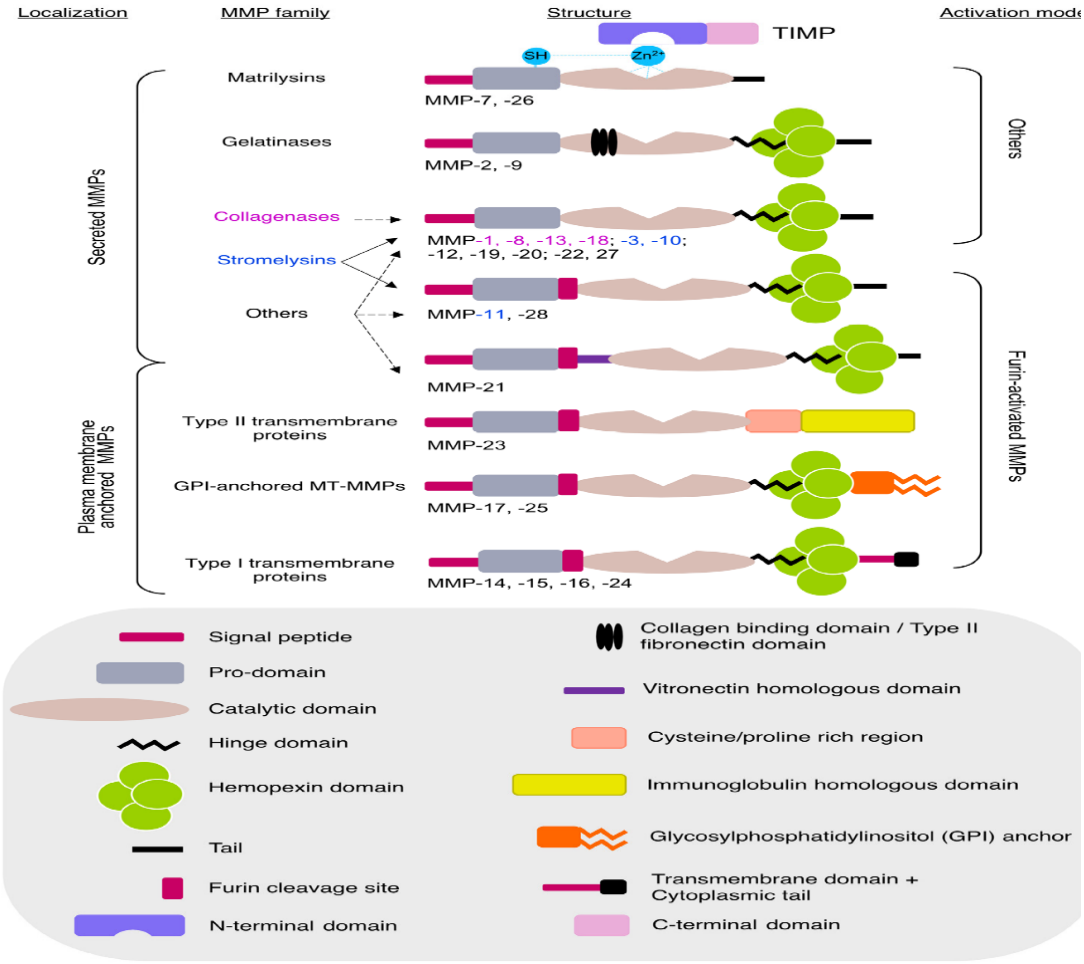

Fig VI: ''Schematic representation of MMPs and TIMPs' structural domains. MMPs are classified based on their sequence homology but also on their cellular localization. For instance, matrilysins, gelatinases, stromelysins, and collagenases are secreted MMPs whereas membrane type MMPs (MT-MMPs) and GPI-anchored proteinases are bound to the cell membrane. MMPs can be classified based on their ability to be activated by furin. The different domains of MMPs and TIMPs are indicated in the grey box''.

Figure taken from – 'Molière, S., Jaulin, A., Tomasetto, C.L. and Dali-Youcef, N., 2023. Roles of matrix metalloproteinases and their natural inhibitors in metabolism: Insights into health and disease. International Journal of Molecular Sciences, 24(13), p.10649'

Most MMPs are secreted as zymogens and are activated after cleavage of the propeptide by furin, plasmin and other specific proteases. In physiological conditions, specific inhibitors the tissue inhibitors of metalloproteinases (TIMPs), can inhibit MMP activity. Pathological effects of MMPs and loss of activity of their inhibitors, TIMPs, have been reported in arthritis, aneurysms, nephritis, ulcers, fibrosis, atherosclerosis, myocardial infarction and cancer *[Visse et al., 2003; Nagase et al. 2006]*. In such pathological conditions, imbalance between MMPs and TIMPs cause abnormal increased MMP activity. A transmembrane protein, EMMPRIN (CD147) upregulates MMP activity and MMP secretion by tumour cells and fibroblasts. Increased expression of EMMPRIN, MMP-1 and MMP-2 has been associated with tumour invasiveness and poor prognosis in breast cancers *[Gabison et al., 2005; Muramatsu, 2016]*.

MMP expression is regulated transcriptionally and post transcriptionally by various activator proteins. MMP-9 promoter region has sites for binding of activator protein 1 (AP1), specificity protein 1 (sp1), nuclear factor-κβ (NF-κβ) and multiple polyomavirus enhancer binding activator -3 (PEA3). MAPK-ERK/ JNKs aid in activation of AP1 to cause MMP-9 expression *[Augoff et al., 2022]*. Binding of AP1 protein to promoter region and binding of activating transcription factor ATF-2 to the Ap-1 binding site via signalling through MKK3/6-p38/TGF-β linked p-38-MAPK signalling caused MMP-2 overexpression *[Song et al., 2006]*. Binding of STAT3 element and STAT3 increased expression of MMP2 *[Xie et al., 2004]*. Presence of genetic polymorphisms of MMP-2 and MMP-9 genes were also reported to influence development of breast cancer phenotype.

The gelatinases include MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Gelatinases have an amino terminal signal peptide region, a pro-peptide domain with a cysteine switch PRCGXPD motif, a catalytic metalloproteinase domain with zinc binding HEXGHXXGXXH motif and fibronectin type II repeats, followed by a hinge region and C-terminal haemopexin domain *[Egeblad et al. 2002; Radisky et al., 2015; Visse et al., 2003]*. X crystallography and NMR have indicated the structure of MMP-2 and MMP-9. The pro-domain consists of connecting loops and three α helices where a bait region is present in the first loop between helix 1 and 2. After the third helix in the substrate binding region or cleft of catalytic domain lies an extended peptide region which has the cysteine switch. This cysteine switch aids in keeping the MMP in its pro-MMP inactive form. *[Visse et al., 2003]*.

The catalytic domain has superimposable polypeptide chains consisting of three α helices, connecting loops along with a 5 strand β sheet. The catalytic domain has a structural zinc, catalytic zinc along with three calcium ions. The substrate binding area is composed of a helix B, strand IV and an extended loop after the helix B. This loop has a Met-turn which aids in holding the structure around the zinc bound by three histidine residues. Water molecule being the fourth ligand of zinc along three protein residues *[Visse et al., 2003]*. In between the catalytic site helix and fifth β strand lies the fibronectin type II domain with three repeats. These domains are composed of an α helix linked with two anti-parallel β sheets and two stable disulfide bonds and are critical for collagen/gelatin binding *[Visse et al., 2003]*. Catalytic domain is followed by the haemopexin domain with a 4 bladed β propeller structure where a stable di-sulfide bond lies between blades I and IV. The haemopexin domains of pro-MMP2 interacts with C-terminal domain of TIMP-2. The β propeller structure aids in protein to protein interactions which is necessary for substrate recognition, dimerization of MT1-MMPs and activation of MMP-2 from pro-MMP-2 *[Visse et al., 2003]*.

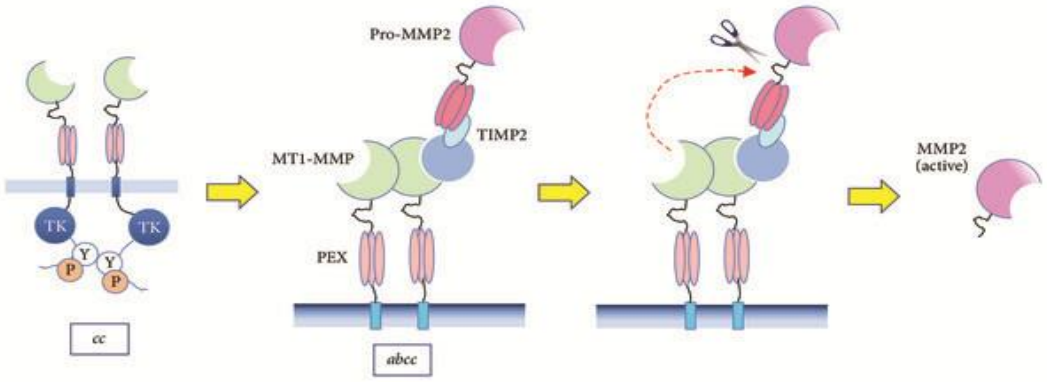

Fig VII: ‘‘Mechanism of activation of MMP-2 by TIMP-2 and MT1-MMP’’.
Figure taken from – ‘Kawasaki, S., Minerva, D., Itano, K. and Suzuki, T., 2017. Finding solvable units of variables in nonlinear ODEs of ECM degradation pathway network. Computational and mathematical methods in medicine, 2017(1), p.5924270’

Unlike most MMPs, MMP-2 is not activated by proteinases like plasmin and is activated by a cell surface “activation complex” which includes pro-MMP-2/MT1-MMP/ TIMP-2 *[Nagase et al. 2006; Seiki et al. 2003; Strongin et al. 1995; Visse et al. 2003]*. Activation of pro-MMP-2 by the complex requires MT1-MMP and catalytic

amounts of tissue inhibitor of metalloproteinases-2 (TIMP-2). The N terminal domain of TIMP-2 binds to the C terminal of a MT1-MMP in the dimeric complex of MT1-MMP molecules. The C-terminal domain of TIMP-2 interacts with the haemopexin domain of pro-MMP-2. The other free MT1-MMP of the homodimeric complex now interacts and cleaves the pro-peptide domain of pro-MMP-2 to generate active MMP-2 *[Nagase et al. 2006; Seiki et al. 2003; Strongin et al. 1995; Visse et al. 2003]*.

The membrane-type matrix metalloproteinase (MT-MMPs) have a pro-peptide domain, catalytic domain, hinge domain and haemopexin (PEX) domain followed by domains which anchor the enzyme to the cell membrane. MT1-MMP (MMP-14) has a pro-peptide domain, a catalytic metalloproteinase domain, a hinge domain, a haemopexin domain and a type I transmembrane domain with a short cytoplasmic tail *[Nagase et al. 2006; Seiki et al. 2003; Visse et al. 2003]*. MT-MMPs are initially expressed as zymogens and cleavage of their pro-peptide domains by furin and other proteases which bind to a conserved sequence site of recognition lying in between pro-peptide and catalytic domain occurs intracellularly, activating MT-MMPs *[Nagase et al. 2006; Seiki et al. 2003; Visse et al. 2003]*. MT1-MMP expression is regulated at the transcriptional level where hypoxia inducible factor 2α along with Sp1 factor and FOXA2 aid in expression of MT1-MMP gene *[Liu et al., 2025]*.

MT1-MMP causes ECM degradation through cleavage of laminin 1, laminin 5, fibronectin, vitronectin, fibrin and dermatan sulphate proteoglycans. MT1-MMP also cleaves CD44 (the major hyaluronan receptor in cells) and cause its release as a soluble fragment. As mentioned earlier, MT1-MMP also plays an important role in activation and cell surface localization of pro-MMP-2. However, truncated MT1-MMP without the transmembrane domain, with mutations in the catalytic domain or with a mutated cytosolic domain cannot activate pro-MMP-2 *[Visse et al., 2003, Nagase et al., 2006, Seiki et al.,2003, Quintero-Fabián et al., 2019, Shiomi et al., 2003]*

1.8 Role of MMP-2, MMP-9 and MT1-MMP in Breast Cancer

Increased MMP expression and activity have been reported in almost every type of cancer and such increase correlates with increased metastasis and poor prognosis *[Egeblad et al. 2002; Kessenbrock et al. 2010; Kwon et al., 2023; Radisky et al., 2015; Tauro et al., 2018; Seiki et al. 2003; Visse et al. 2003]*. The active role of MMPs has

been extensively studied in breast cancer metastasis. MMP-2 and MMP-9 overexpression was reported in high grade, node positive and basal molecular subtype of breast cancer where increased MMP-2 and MMP-9 expression was linked with metastasis to distant organs and lymph node metastasis *[Jiang et al., 2021, Lukianova et al.,2023]*. Presence of MMP-2 and MMP-9 has been reported in lymph node positive breast cancer especially the ones with high expression of Her2/neu receptors *[Stankovic et al., 2010]*. MMP-2 and MMP-9 over expression was reported in breast to brain metastasis as studied on mice model *[Mendes et al.,2005]*. MMP-9 expression in HER2 and triple negative breast cancers was associated with lymph node metastasis and relapse in breast cancer *[Yousef et al., 2014, Wu et al., 2008]*. MMP-9 overexpression was reported in advanced tumours and MMP-2 was associated with aggressive (stage III-IV), HER2 positive and triple negative breast tumours. Such increased levels of MMP-2 and MMP-9 in advanced stage and aggressive cancers indicate their importance in the metastatic process *[Sardar et al., 2025]*.

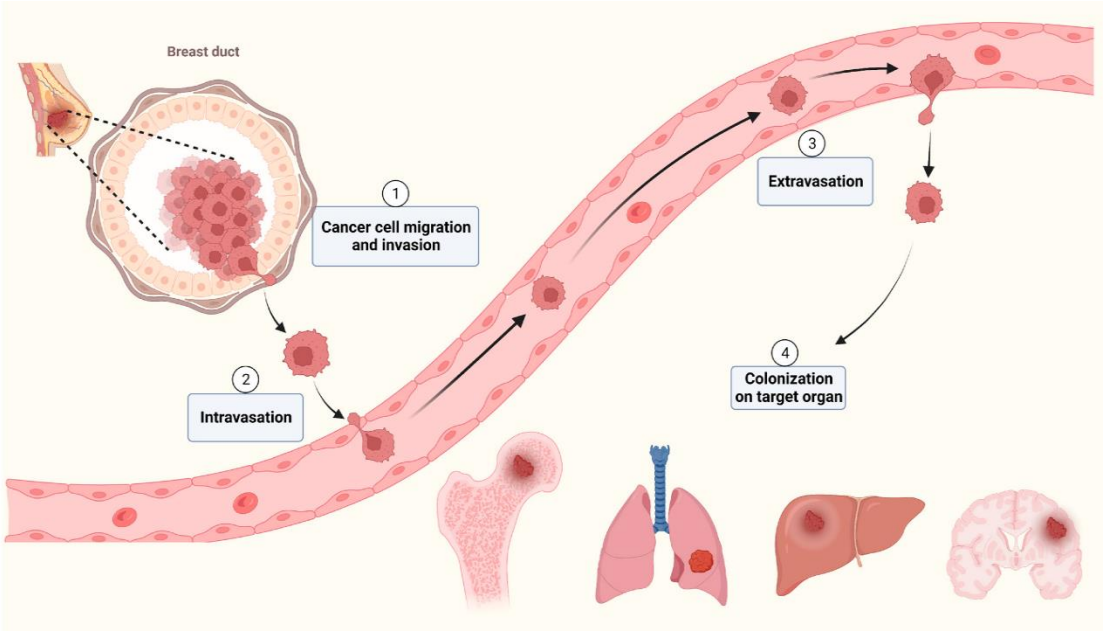

Fig VIII: 'Breast cancer metastasis to bone, lung, liver and brain'.

Figure taken from – '*Huang, S., Dong, M. and Chen, Q., 2022. Tumor-derived exosomes and their role in breast cancer metastasis. International journal of molecular sciences, 23(22), p.13993'*.

Gelatinases (MMP-2 and MMP-9) are crucial in promoting tumour progression by degrading ECM proteins and disrupting basement membrane integrity by digesting type IV collagen during metastasis. The gelatinases (MMP-2 and MMP-9) digest collagens,

gelatins, laminin and specially the collagen type IV component of the basement membrane. MMP-2 also digests type I, II and III collagen *[Radisky et al., 2015; Egeblad et al. 2002; Visse et al., 2003]*. Activation of pro-MMP-2 to active MMP-2 has been reported to promote increased lymph node metastasis in breast, lung, thyroid, stomach and oral carcinomas *[Brown et al. 1993; Radisky et al., 2015; Egeblad et al. 2002]*. Increased MMP-2, MMP-9 expression and activity upregulate invasive potential and cause lymph node and bone metastasis and a worse prognosis in breast cancers *[Tauro et al., 2018; Li et al., 2017]*. Altered MMP-2/ TIMP-2 ratio in invasive breast cancer subtype was correlated with lymph node metastasis *[Jinga et al., 2006]*. Overexpression of MMP-2 and MMP-9 was reported in breast-to-liver metastasis in breast cancer patients *[Golubnitschaja et al., 2016]*. Formation of lung metastases of MDA-MB-231 cells via overexpression of MMP-2 and MMP-9 was reported in mice model *[Ci et al., 2018]*. Breast-to-brain metastases in rat syngeneic model showed high MMP-2 activity *[Mendes et al., 2007]*. High expression of MMP-2 and MMP-9 promoted formation of metastatic deposits of MDA-MB-453 cells in lungs *[Stellas et al., 2010]*.

MMPs play an important role in cleaving E-cadherins resulting in loss of cell junctions, cell to cell connections and cell polarity and disruption of the epithelial structure promoting cell migration and invasion in cancer *[Egeblad et al. 2002]*. MMP-2 and MMP-9 caused E-cadherin shedding and loss of E cadherin via MMP-2 activity promoted epithelial to mesenchymal transition, loss of cell polarity and cell invasion in breast cancers *[Jezierska et al., 2009, Roy et al., 2009]*. The E-cadherin generated by cleavage by MMP-9 (known as sE-cad) in the extracellular space further localises in exosomes and interacts with various RTKs, IGF-1R, EGFR/HER receptors to activate downstream PI3K-Akt or MAPK signalling. Thus, this feedback loop aids in cellular proliferation, invasion and cell survival in cancer *[Augoff et al., 2022]*.

Significant role of MMP-9 and MMP-2 in epithelial-to-mesenchymal transition (EMT) in breast tumours has been reported *[Radisky et al., 2010]*. Signalling through TGF-β, p38 MAPK and ERK has been reported to cause MMP-2/ MMP-9 mediated epithelial to mesenchymal transition in breast cancers *[Khan et al., 2015, Kim et al., 2004, Qin et al., 2017]*. MMP-2 and MMP-9 also contribute to angiogenesis in breast cancer. Overexpression of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) along with MMP-2 and MMP-9 expression contribute to tubule formation, neovascularization and angiogenesis in

breast cancer cells *[Rao et al., 2024, Tang et al., 2013]*. Overexpression of MMP-2 and MMP-9 with downregulation of TIMP-2 and TIMP-1 along with VEGF overexpression cause neo-vascularization and angiogenesis in breast tumours *[Jobim et al., 2009]*.

The role of MT1-MMP (MMP-14) is also very important in breast cancers. Expression of MT1-MMP was reported to mediate lymphatic metastasis in breast cancer *[Yao et al., 2013]*. The proteolytic activity of MT1-MMP involves cleaving ECM and non-ECM substrates *[Seiki et al. 2003; Visse et al. 2003]*. The cytoplasmic domain of MT1-MMPs were reported to aid in cellular viability and *in vivo* tumour vascularization of breast cancer cells *[Cepeda et al., 2017]*. Overexpression of MT1-MMP and MMP-2 has been reported in invasive breast cancers compared to normal breast tissue and this increased expression promotes lymph node metastasis and increased tumour size *[Zhang et al., 2013, Seiki et al., 2003, Shiomi et al., 2003, Ueno et al., 1997]*. A study showed that MT1-MMP was localized in breast carcinoma cells and there was a strong correlation between increased MMP-2 activation and MT1-MMP expression *[Ueno et al., 1997]*. A study with 102 breast cancer biopsies showed low MT1-MMP expression by breast cancer cells reduced cell migration, metastasis and intravasation while increased MT1-MMP expression was correlated with invasion of blood vascular system in triple-negative breast cancers *[Perentes et al., 2011]*. MMP-2 and MT1-MMP were reported to be overexpressed by 64% and 60% in breast tumour samples *[Mohammad et al., 2012]*. High MMP-2 and MT1-MMP expression along with low TIMP-2 expression was reported in shorter survival and poor prognosis in breast cancer patients *[Têtu et al., 2006]*. Overexpression of MT1-MMP and VEGF-C in breast tumours were correlated with cell invasion and indicated MT1-MMP's role in angiogenesis. MT1-MMP aided in the formation of endothelial cell tubes via regulation of MMP-2 in angiogenesis *[Quintero-Fabián et al., 2019, Seiki et al., 2003]*

MMPs have also shown to play important roles in drug resistance of breast cancer. Activation of STAT3 was reported to regulate MMP-2/ MMP-9 expression and multidrug resistance in SK-BR3/EPR breast cancer cells *[Zhang et al., 2015]*. Invasive nature of MCF-7/DOX drug resistant cells was reported to be due to expression of COX-2 and MMP-2/ MMP-9 expression via EGFR-mediated PI3K/Akt signalling *[Kang et al., 2011]*. MT1-MMPs were also reported to induce radiotherapy and chemotherapy resistance through regulation of lamin B1 protein which aids in stability

of replication forks to prevent DNA damage by chemotherapy and radiotherapy *[Thakur et al., 2021]*.

Thus the role of MMP-2, MMP-9 and MT1-MMP is very extensive and profound in breast cancers. These MMPs promote increase in migratory capacity, cellular invasion and metastasis, degradation of ECM and basement membrane, loss of intercellular connections and cell polarity, angiogenesis and drug resistance.

1.9 Prevalent Methods of Treatment in Breast Cancer and Side Effects

The prevalent modes of treatment in breast cancer include chemotherapy and radiotherapy beside surgery. Extensive chemotherapy and radiotherapy often result in harmful side effects to breast cancer patients. Use chemotherapeutic drugs (like doxorubicin, taxanes, anthracyclines, trastuzumab) despite achieving some success in clinical treatment of cancers, have created toxic side effects through damage of peripheral tissues and non-cancerous healthy cells. Such treatment often leads to development of severe side effects like immunosuppression, gastrointestinal disorders, anaemia, leukopenia, neutropenia, allergies and cardiotoxicity in patients *[van den Boogaard et al., 2022; Wiranata et al., 2024, Jivani et al., 2024, Zanuso et al., 2020]*

Anthracycline-taxane combined chemotherapy has been reported to cause considerable epithelial, gastro-intestinal, and neurological damages in patients undergoing treatment. Extensive use of taxanes in breast cancer patients has been reported to induce neurological, gastrointestinal and haematological toxicity *[Wiranata et al., 2024, Jivani et al., 2024]*. Sleep disorders, fever, diarrhea, constipation, loss of appetite, allergies and ocular toxicity were also reported with taxane and anthracycline related chemotherapy in breast cancer patients *[Choulli et al., 2024]*. Cardiotoxicity from use of anthracyclines followed by cognitive neuropsychiatric disorders (affecting central nervous system) was reported from adjuvant chemotherapy *[Partridge et al., 2001, Di Nardo et al., 2022]*. Use of alkylating agents, anti-metabolites and topoisomerase inhibitors been reported to cause hepatotoxicity, nephrotoxicity, neuro toxicity and gonadal toxicity in cancer patients *[van den Boogaard et al., 2022; Katta et al., 2023; Zanuso et al., 2020]*.

To address this issue in cancer treatment, a new avenue of research with natural phytochemical compounds has gained importance over the years. In our study we have

analyzed the anti-tumorigenic potential of two important natural compounds (all-trans retinoic acid) ATRA and curcumin on breast cancer cells.

1.10 All-trans retinoic acid (ATRA)

All-trans retinoic acid (ATRA), a naturally occurring metabolite of retinol and one of the biologically most active forms of vitamin A is present in carotenoid rich fruits and vegetables. ATRA is involved in modulation of many biological processes like growth, differentiation and apoptosis via interactions mainly with the retinoic acid receptors (RARs) *[Das et al., 2013, Siddikuzzaman et al., 2013*]. ATRA has been reported to inhibit proliferation of tumour cells in vitro, to induce apoptosis in tumour cells and has been used in combination with chemotherapeutic drugs for treatment of acute promyelocytic leukemia (APL) as it is a potent inducer of myeloid differentiation *[Das et al., 2013, Siddikuzzaman et al., 2013, Lo-Coco et al., 2008*].However, the molecular effects of ATRA on many solid tumours are yet to be explored in as much detail although there are indications that it can regulate cell proliferation and induce apoptosis in breast cancer cells *[Das et al., 2013]*. ATRA has shown anti-inflammation, anti-oxidant properties along with influence on immune-stimulation according to previous experimental research [*Siddikuzzaman et al., 2013*]. ATRA can downregulate EMT by causing increase of E-cadherin expression and inhibition of expression of vimentin *[Bobal et al.,2021, Caricasulo et al., 2024]* Interaction of ATRA with RARα has been reported to regulate MAPK signalling in breast cancer cells *[Caricasulo et al., 2024]*.

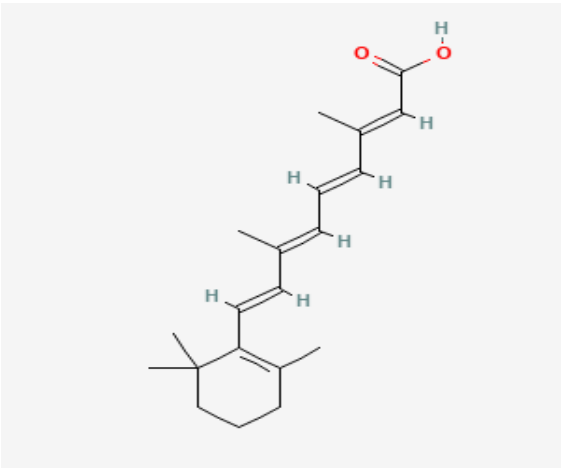

Fig IX: Structure of ATRA (PubChem Id-444795)

ATRA has shown potent effect in pro-myelocytic leukemia and has been reported to regulate downstream signaling cascades and metastasis in other cancers as well *[Das et al., 2013]*. ATRA treatment of breast cancer cells downregulated expression of FAK, NF-κB, p-ERK, MMP-2 and EMMPRIN and upregulated E-cadherin, CRABP and RAR (retinoic acid receptor) expression *[Dutta et al., 2009]*. Treatment of ATRA on MCF-7 caused formation of TR3-RXRα heterodimer receptors in nucleus and signaling which caused downstream regulation of Bcl-xl, Bcl-2 and Bax to mediate apoptosis *[Ye et al., 2004]*. Treatment of MDA-MB 231 with ATRA reduced cellular migration via downregulation of p38-MAPK signaling *[Wang et al., 2013]*.

ATRA treatment on HCC1599 and SKBR3 breast cancer cells downregulated EGF expression and heregulin-β1mediated NOTCH1 signaling and upregulated TGFβ expression to inhibit cellular migration *[Zanetti et al.,2015]*. Combinatorial treatment of ATRA with Trastuzumab on SKBR3 and BT-474 cells downregulated and reduced HER2 and FAK expression to inhibit cellular migration and invasion *[Vanderhoeven et al., 2018]*. Anti-proliferative and cardio-protective effect of trans retinoic acid was studied in trastuzumab treated rat models via regulation of signalling molecules like ERK1/2 project a future use of retinoic acid for breast cancer patients suffering due to cardiotoxicity imposed by chemotherapy *[Fan et al., 2016]*.

1.11 Curcumin

Curcumin, a phytopolylphenol, is a major component of the rhizome of turmeric (*Curcuma longa*). Curcumin is a potent phytophenol with antimicrobial, antiviral, antioxidant, anti-inflammatory, anti-diabetic and anticancer properties. It has also shown to have neuroprotective, cardio-protective properties along with effects on pulmonary fibrosis, pulmonary blockage and auto-immune disorders *[Wang et al., 2016, Barcelos et al., 2022, Fu et al., 2021]*. Studies indicate that tumour initiation, progression and metastasis can be suppressed by treatment with curcumin. Curcumin has been reported to inhibit tumour development by causing cell cycle arrest in G2/S and G2/M phase, by inhibiting estrogen receptor (ER), HER2, Wnt/β-catenin, NF-κB and MAPK mediated signaling *[Wang et al., 2016, Barcelos et al., 2022, Fu et al., 2021]*. Curcumin modulates NF-κB signalling to mediate mitochondrial-endoplasmic reticulum stress and cause cell arrest in G2/M phase, apoptosis and inhibition of cancer cell proliferation [*Liu et al., 2018].* Studies indicate curcumin can inhibit proliferation

and promote apoptosis in breast cancer cells *[Barcelos et al., 2022]*. Curcumin has been reported to reduce gemcitabine resistance and induce sensitization to paclitaxel and 5-fluro-uracil via regulation of NF-κB pathway in breast cancer cells *[Farghadani et al.,2021]*. Studies indicate that treating breast cancer cells with curcumin downregulated cancer stem cell (CSC) markers like CD44, Oct4, Nanog by inhibition of Wnt/β catenin signalling pathways *[Li et al., 2018]*

Curcumin is a hydrophobic polyphenol reported to regulate downstream signalling pathways like PI3K, MAPK, JAK-STAT, p53 and NF-κB in cancer cells *[Wang et al., 2019]*. It has also shown to have neuroprotective, cardio-protective properties along with effect on anti-pulmonary fibrosis, pulmonary blockage and auto-immune disorders *[Fu et al., 2021]*.

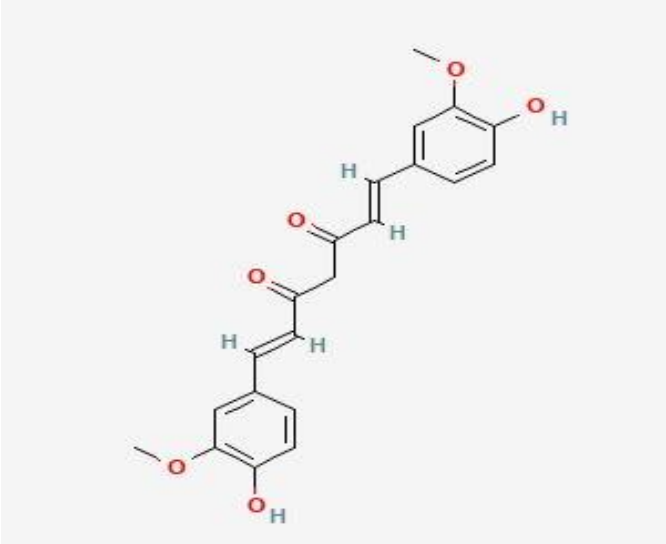

Fig X: Structure of Curcumin (PubChem Id- 969516)

Curcumin downregulated NF-κB signaling along with mediating apoptosis and inhibition of cellular proliferation and survival in breast cancer *[Nagaraju et al., 2012]*. Downregulation of PAM pathway (PI3K-Akt-mTOR), reduction in AXL mediated Akt expression, reduction in IKKβ mediated tumour development; reduction in Akt expression and apoptosis, downregulation of IL6 cytokine mediated STAT3 expression; downregulation of ERK1/2, p38-MAPK and TGF-β2 expression; downregulation of EGFR mediated ERK 1/2 and MAPK expression and downregulation p50 NF-κB gene are some of the effects of curcumin treatment on breast cancer. Curcumin reduces gemcitabine resistance along with sensitization to

paclitaxel, cyclophosphamide and 5-flouro-uracil in breast cancer cells via regulation of NF-κB pathway *[Farghadani et al.,2021]*.

Curcumin downregulated TGFβ1 mediated MMP-9 expression and p38, ERK1/2 and Smad2 e in MDA-MB 231 *[Mo et al., 2012]*. Downregulation of STAT3, regulation of tumour associated macrophage activity within the tumour microenvironment *[Golmohammadi et al.,2024]* ;downregulation of NF-κB pathway *[Nirgude et al.,2022]*; prevention of osteolysis and progression of breast to bone metastasis via regulation of TGF-β signalling pathway *[Kunihiro et al., 2022]* ; reduction in Akt expression induced AMPK mediated autophagy and ubiquitin –proteasome activity *[Guan et al., 2016]*; modulation of NF-κB signalling to mediate mitochondrial-endoplasmic reticulum stress, cell arrest in G2/M phase and apoptosis *[Liczbiński et al., 2020]* are some important after effects of curcumin treatment in breast cancer.

As both curcumin and ATRA are derived from phytochemicals, they could show lower toxicity than chemotherapeutic drugs which are of chemical origin. For example, in APL, 20% of patients treated with chemotherapeutic drug ara-C have been reported to suffer from cerebral haemorrhages and aggravated coagulopathy due to the release of pro-coagulant factors into circulation. Such side effects can be avoided by using ATRA *[Fang et al. 2002]*.

OBJECTIVES OF STUDY

2. OBJECTIVES OF STUDY

The objectives of the study are:

To investigate the effect of natural compounds with anti-tumorigenic potential, both singly and synergistically, on

1) cellular signalling cascades in breast cancer cells.

2) matrix metalloproteinases (MMPs), cellular proteases which play crucial roles in regulating tumour invasion in breast cancer cells.

3) breast cancer cell survival and invasion.

METHODOLOGY

3. METHODOLOGY

3.1 Materials, Antibodies and Reagents

Leibovitz L-15 medium, Minimal Essential Medium (MEM) and foetal bovine serum (FBS) for animal cell culture and HiPer MTT Cell Assay Teaching Kit were purchased from HiMedia (India). Anti-MMP-2, anti-MMP-9, anti-MT1-MMP, anti-p-PI3K, anti-FAK primary antibodies and secondary antibodies (HRP-coupled) were purchased from Santa Cruz Biotechnology Inc. (USA). Curcumin, all-trans retinoic acid (ATRA) and epidermal growth factor (recombinant, human and expressed in *E. coli*) was purchased from Sigma-Aldrich (USA). Tetramethylbenzidine (TMB) for ELISA was purchased from Bangalore Genei (India). For RT-PCR, BluePrint RT-PCR kit and NucleoSpinRNA XS kit was purchased from Promega (USA). Other reagents and chemicals required for experimental procedures were procured from Merck (USA), Sigma-Aldrich (USA) and Bangalore Genei (India).

3.2 Breast Cancer Cell Lines

MDA-MB-453 (metastatic human breast cancer cell line), MDA-MB-231 (invasive human breast adenocarcinoma cell line) and MCF-7 (human invasive breast ductal carcinoma cell line) were procured from National Centre for Cell Science (NCCS), Pune, India. MCF-7 cells were cultured in MEM (Minimum Essential Media) containing 10% FBS and MDA-MB-231 and MDA-MB-453 cells were cultured in L-15 (Leibovitz L-15) medium containing 10% FBS. Cells were maintained at 37℃ in CO_2 incubator.

3.3 Molecular Docking

Interactions between natural phytochemicals, synthetic inhibitors and macromolecules were studied by molecular docking technique using AutoDock Tools (ADT) 1.5.6 (Scripps Research Institute, USA, https://ccsb.scripps.edu/mgltools/1-5-6/). Crystal structures of human FAK, human PI3K p110α subunit, human MMP-1 (collagenase 1) catalytic domain, human MMP-2 (gelatinase A) catalytic domain, human MMP-9 (gelatinase B) catalytic domain, human MT1-MMP catalytic domain and human EGFR kinase domain were obtained from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (www.rcsb.org/). Structure of the

natural phytochemicals Curcumin and ATRA, synthetic inhibitors of MMPs (Rebimastat, Batimastat, Doxycycline), PI3K inhibitors (Alpelisib, LY294002) FAK inhibitor (Defactinib) and synthetic inhibitor of EGFR (Erlotinib) were obtained from PubChem, NIH, National Centre for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/).

If the macromolecules had some ligand molecules inherently bound to them, these ligand molecules were removed. The ligands and macromolecules were prepared prior to molecular docking with AutoDock Tools 1.5.6. The water molecules were removed along with addition of polar hydrogens, Kollman charges were added and AD4 subtype was assigned to prepare all the macromolecules. In case of ligand preparation, choosing and detection of torsion roots were done. This was followed by setting up grid parameters, grid box and docking parameters involving both protein and ligand. After docking, using AutoDock Tools 1.5.6, binding pattern and affinity were studied by considering the lowest ΔG value of conformations of the docked protein-ligand complexes *[Asadzadeh et al., 2015, Rauf et al., 2015, Bianco et al., 2016, Yasmeen et al., 2019]*. The pattern of interactions of the docked protein-ligand complexes was studied and analysed via PYMOL (version 2.5.4, Schrodinger, LLC), (https://www.pymol.org). For studying synergistic interactions, the pattern of interaction of one natural compound in presence of another bound to the macromolecule (e.g. docking of ATRA with MMP-2-curcumin complex and docking of curcumin with MMP-2-ATRA complex) was analysed by molecular docking and results were visualised as above.

Table 1: Molecules for molecular docking.

Molecule	PDB ID [a]
Phosphatidylinositol 3′ Kinase (PI3K)	4TUU
Focal adhesion kinase (FAK)	1MP8
Matrix metalloproteinase- 1 (MMP-1)	3SHI
Matrix metalloproteinase- 2 (MMP-2)	1QIB
Matrix metalloproteinase- 9 (MMP-9)	5I12
Membrane type-1 matrix metalloproteinase (MT1-MMP)	5H0U
Epidermal growth factor receptor (EGFR)	2GS2

a. Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank https://www.rcsb.org/

Table 2: Phytochemicals and synthetic inhibitors used for analysis

Ligand Molecules	PubChem CID
All-trans retinoic acid (ATRA)	444795 [a]
Curcumin	969516 [b]
Rebimastat	9913881 [c]
Batimastat	5362422 [d]
Doxycycline	54671203 [e]
Defactinib	25117126 [f]
LY294002	3973 [g]
Alpelisib	56649450 [h]
Erlotinib	176870 [i]

National Center for Biotechnology Information. PubChem Compound Summary for

a. CID 444795, Tretinoin. https://pubchem.ncbi.nlm.nih.gov/compound/Tretinoin

b. CID 969516, Curcumin. https://pubchem.ncbi.nlm.nih.gov/compound/Curcumin

c. CID 9913881, Rebimastat. https://pubchem.ncbi.nlm.nih.gov/compound/Rebimastat

d. CID 5362422, Batimastat. https://pubchem.ncbi.nlm.nih.gov/compound/Batimastat

e. CID 54671203, Doxycycline. https://pubchem.ncbi.nlm.nih.gov/compound/54671203

f. CID 25117126, Defactinib. https://pubchem.ncbi.nlm.nih.gov/compound/Defactinib

g. CID 3973, LY294002. https://pubchem.ncbi.nlm.nih.gov/compound/3973

h. CID 56649450, Alpelisib. https://pubchem.ncbi.nlm.nih.gov/compound/56649450

i. CID 176870, Erlotinib. https://pubchem.ncbi.nlm.nih.gov/compound/176870

3.4 Treatment of Breast Cancer Cells with ATRA and Curcumin

1 mM stock solutions of curcumin and all-trans retinoic acid (ATRA) were prepared by dissolving 3.68 mg and 3 mg respectively in 10 ml dimethyl sulphoxide (DMSO). MDA-MB 453, MDA-MB 231 and MCF-7 cells (4x 10^5) were cultured in serum free culture medium (SFCM) in 60 mm culture petri dishes. Cells were treated with 20μM/ml curcumin or 20μM/ml ATRA (singular treatments) or 20μM/ml curcumin and 20μM/ml ATRA (synergistic treatments) for 24 hours. A separate set of control cells were maintained where no treatment with curcumin or ATRA was done. In a separate set of experiments, MDA-MB-231 and MCF-7 cells were treated with two different concentrations of curcumin (20 μM/ml and 40 μM/ml). Cells were collected after 24 hours and extracted in cell extraction buffer (containing 37.5 mM Tris, 75 mM

NaCl, 0.5% Triton X-100 and 1% protease inhibitor) at 4℃. Protein concentrations of the resultant extracts were determined using modified Lowry's method [*Hartree 1972*].

Fig XI: Animal cell culture performed in Animal Tissue Culture Laboratory under Central Research Facility of St Xaviers College (Autonomous) Kolkata. Instruments used include biosafety cabinet type IIA and CO_2 incubator (Fig. A) and phase contrast inverted microscope (Fig. B). Cells were observed under inverted microscope (Fig. C) and treated with curcumin and ATRA (Fig. D).

3.5. ELISA

Samples (cell extracts, culture supernatants) were added to wells of microtitre plates in triplicate and kept at 4℃ overnight. The next day contents of the wells were discarded and 1% BSA was added to block non-specific binding sites. The wells were washed thrice with washing buffer (0.5% NP-40, 0.5% BSA in phosphate buffered saline).

Samples were incubated with anti-MMP-2/ anti-MMP-9 /anti-MT1-MMP/ anti-p-PI3K/ anti-FAK primary antibodies (1:1000 dilution) at 4℃ overnight. The wells were washed thrice with washing buffer and corresponding HRP-coupled secondary antibodies (1:1000 dilution) were added and incubated at 37℃ for 1 hour. The wells were washed several times with washing buffer and tetramethylbenzidine (TMB) substrate was added. The reaction was stopped by adding 1M H_2SO_4 and optical density was measured at 450 nm using a microtitre plate reader.

Fig XII: Preparation of cell extracts by homogenization and centrifugation of collected cells after treatment with curcumin and ATRA.

3.6. Gelatin Zymography

Samples (cell extracts, culture supernatants) were incubated with sample buffer without β-mercaptoethanol (62.5mM Tris-HCl, 2.5% SDS, 10% glycerol, 0.02% bromophenol blue; pH 6.8) at 37°C for 30 mins. Gelatin zymography was performed on 10% SDS-PAGE co-polymerized with 0.1% gelatin. Gels were washed in 2.5% Triton X-100 for 30 minutes to remove SDS, incubated for 48 hours at 37°C in reaction buffer and stained with 0.25% Coomassie Brilliant Blue following standard protocols *[Hawkes et al. 2001; Tauro et al. 2017]*. Bands were clearly visualized by destaining the gel with water.

Fig XIII: Gelatin Zymography of samples on 10% SDS-PAGE co-polymerized with 0.1% gelatin.

3.7 Wound Healing Assay

Cells were cultured in culture petri dishes (60 mm) and after the cells formed a confluent monolayer, cells were treated with curcumin and ATRA as previously described and a pipette tip was used to scratch the cell monolayer in a straight line along the centre of the petridish to create a scratch (wound). The debris was removed by washing and markings were made on the dish as reference points. The extent of cell migration into the wound area was determined by observing the culture dishes under the inverted phase contrast microscope at various time points [*Rodriguez et al., 2005*].

3.8 Cell Viability Assay using Trypan Blue

MDA-MB 453 cells ($4x\ 10^5$) were cultured in SFCM in 60 mm culture petri dishes and treated with curcumin and ATRA as previously described. Cells were collected after 24 hours, a suspension was prepared and Trypan Blue solution was added in equal proportion and thoroughly mixed. Cells were then counted on a haemocytometer for determining cell viability. Percentage of viability was determined taking viability in control cells (cultured without curcumin and ATRA treatment) as 100%.

3.9 MTT Assay

MTT assay was performed using HiPer MTT Cell Assay Teaching Kit following standard protocols. Cells were added to microtitre plate wells and treated with curcumin and ATRA as previously described. After incubation for 24 hours, MTT reagent was added to each well, incubated for 2-4 hours at 37°C and then solubilization agent was added to each well along with gentle stirring in a gyratory shaker to dissolve formazan crystals formed. Absorbance at 570 nm along with a reference wavelength of 650 nm was obtained using microtitre plate reader. Absorbance values at 650nm were subtracted from values at 570 nm and percentage of cell viability was determined.

3.10 Culture of Breast Cancer Cells on EGF and Treatment with ATRA and Curcumin

MDA-MB-453 cells were cultured in serum free culture medium (SFCM) in 1 μg/ml EGF coated 60 mm culture petri dishes. The 'Control' cells were cultured in SFCM on culture petri dishes coated with EGF and no treatment was conducted. Cells were treated with 20μM/ml curcumin or 20μM/ml ATRA. Cells were collected after 24 hours and samples were prepared as previously described. The effects of curcumin and ATRA treatment on breast cancer cells cultured on EGF were then assayed as previously described.

3.11 Treatment with PI3K Inhibitor LY294002

MDA-MB-453 cells were incubated with 50 μM LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], a specific inhibitor of PI3K [*Das et al. 2008; Vlahos et al. 1994*] for 1 hour and then cultured on 1 μg/ml EGF coated culture petri dishes. Cells were collected after 24 hours and samples were prepared as previously described. The effect of PI3K inhibition on MMPs was then assayed as previously described.

3.12 RT-PCR

RNA was extracted from MDA-MB-453 cells using NucleoSpin RNA XS kit (Takara) and RT-PCR was carried out using BluePrint RT-PCR Kit (Takara) in a thermal cycler. The forward and reverse primer sequences used for PCR were MMP-2: 5′-CAC CTA CAC CAA GAA CTT CC-3′ (forward) and 5′-AAC ACA GCC TTC TCC TCC TG-3′

(reverse); MMP-9: 5′-CGC TAC CAC CTC GAA CTT TG-3′ (forward) and 5′-GCC ATT CAC GTC GTC CTT AT-3′ (reverse). GAPDH was used as control to normalize for mRNA integrity and equal loading. After generation of cDNA with RT-PCR, 20 µl of each PCR product was run on 1.5% agarose gel and bands were visualized using an UV transilluminator.

3.13. Wound Healing Assay

MDA-MB 453 cells were cultured in culture petri dishes (60 mm) coated with 1 µg/ ml of EGF and after the cells formed a confluent monolayer, cells were treated with curcumin and ATRA as previously described and wound healing assay was performed following standard protocols [*Rodriguez et al., 2005*]. The extent of cell migration into the wound area was determined by observing the culture dishes under the inverted phase contrast microscope at various time points.

3.14 Statistical Analysis

Statistical significance of differences between results was determined using two tailed student t-test and p values were determined. Differences between groups were considered statistically significant at $p < 0.05$ and $p < 0.01$.

RESULTS

4. RESULTS

4.1 *In silico* Analysis of Interactions of Curcumin and ATRA with MMPs and Signalling Molecules in Breast Cancers

4.1.1. Interaction of curcumin, ATRA and doxycycline with the typical MMP catalytic domain: Molecular docking (Table 1) showed high binding affinity of curcumin and ATRA to the MMP-1 catalytic domain (typical catalytic domain for MMPs) with both curcumin (ΔG = -6.0 kcal/mole) and ATRA (ΔG = -6.9 kcal/mole) having binding affinity comparable to the broad spectrum MMP inhibitor doxycycline (ΔG = -6.4 kcal/mole). The binding regions of the domain with which curcumin, ATRA and doxycycline interact are as shown in Fig. 1.

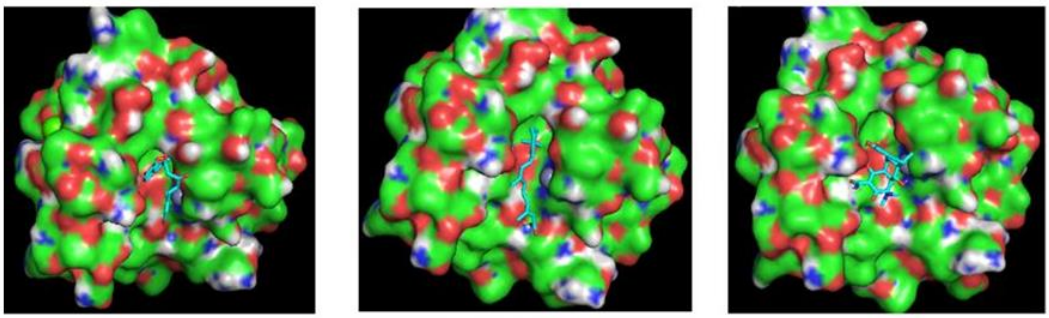

Fig 1: Interactions of catalytic domain of human MMP-1 with curcumin (Fig. 1A), ATRA (Fig. 1B) and doxycycline (Fig. 1C) using surface zoomed view.

Compound	PubChem CID	Δ G (kcal/mole)
Curcumin	969516	-6.0
ATRA	444795	-6.9
Doxycycline	54671203	-6.4

Table 1: Binding affinities of curcumin, ATRA and doxycycline with human MMP-1 catalytic domain (PDB ID: 3SHI)

4.1.2. Interaction of curcumin, ATRA and synthetic MMP inhibitors with catalytic domain of MMP-2: Molecular docking studies (Table 2) indicated that, curcumin (ΔG = -10.99 kcal/mole) and ATRA (ΔG = -10.11 kcal/mole) showed very high affinity of binding to catalytic domain of human MMP-2 which was greater than the binding

affinities of the synthetic MMP inhibitors Batimastat (ΔG = -9.62 kcal/mole) and Rebimastat (ΔG = -7.46 kcal/mole). Both curcumin and ATRA had binding affinities considerably higher than Rebimastat and Batimastat.

ATRA, curcumin, Batimastat and Rebimastat appear to preferentially interact at similar sites on the MMP-2 catalytic domain (Fig. 2A-H). Analysis with PYMOL showed ATRA formed hydrogen bonds with Leu-164 and Ala-165 (Fig. 2I) in the active site of MMP-2. Other interactive amino acids in the binding region included Gly-162, Leu-163, Leu-197, Val-198, Glu-202, His-201, His-211, Ala-220, Pro-221, Ile-222, Tyr-223, Thr-227, Pro 215, Gly-216, Ala-217, Tyr-228, Thr-229 and Phe-232. The amino acids Leu-164, Ala-165 and Tyr-223 of the MMP-2 catalytic domain formed hydrogen bonds with curcumin (Fig. 2J). Other interactive amino acids in the binding region included Gly-162, Leu-163, Tyr-193, Leu-197, Val-198, His-201, Glu-202, His- 211, Ala-217, Leu-218, Met-219, Ala-220, Pro-221, Ile-222 and Thr-227.

Analysis of MMP-2-Batimastat interactions showed amino acids Leu-164, Ala-165, Glu-202 and Pro-221 in hydrogen bond interactions with Batimastat (Fig. 2K). Other interactive amino acids in the binding region included Asp-161, Gly-162, Leu-163, His-166, Tyr-193, Leu-197, Val-198, His-201, His-205, His-211, Leu-218, Ala-220, Ile-222, Tyr-223 and Thr-227. The amino acids Gly-162, Leu-164, Pro-221 and Tyr-223 formed hydrogen bonds with Rebimastat (Fig. 2L). Other interactive amino acids in the binding region included Asp-161, Leu- 163, Ala-165, Tyr-193, His-201, Glu-202, His-211, Ala-220, Ile-222 Thr-227 and Tyr-228.

Compound	**PubChem CID**	**Δ G (kcal/mole)**	**Hydrogen Bonding to Amino Acids with Bond Lengths**
ATRA	444795	-10.11	Leu-164 (1.7Å), Ala-165 (2.0Å & 2.3Å)
Curcumin	969516	- 10.99	Leu-164 (2.0Å), Ala-165 (2.6Å & 3.0Å), Tyr-223 (2.3Ǻ)
Batimastat	5362422	- 9.62	Leu-164 (2.0Å), Ala-165 (2.3 Å, 2.3Å & 3.4Å), Glu-202 (1.8Ǻ & 2.4Ǻ), Pro-221 (1.8Ǻ & 2.1Ǻ)
Rebimastat	9913881	-7.46	Gly-162 (2.0Å & 2.9Å), Leu-164 (2.7Å), Pro-221 (2.0Ǻ & 2.1Ǻ), Tyr-223 (1.8Ǻ)

Table 2: Binding interactions of ATRA, curcumin, and synthetic MMP inhibitors Batimastat, and Rebimastat to catalytic domain of human MMP-2 (PDB ID: 1QIB).

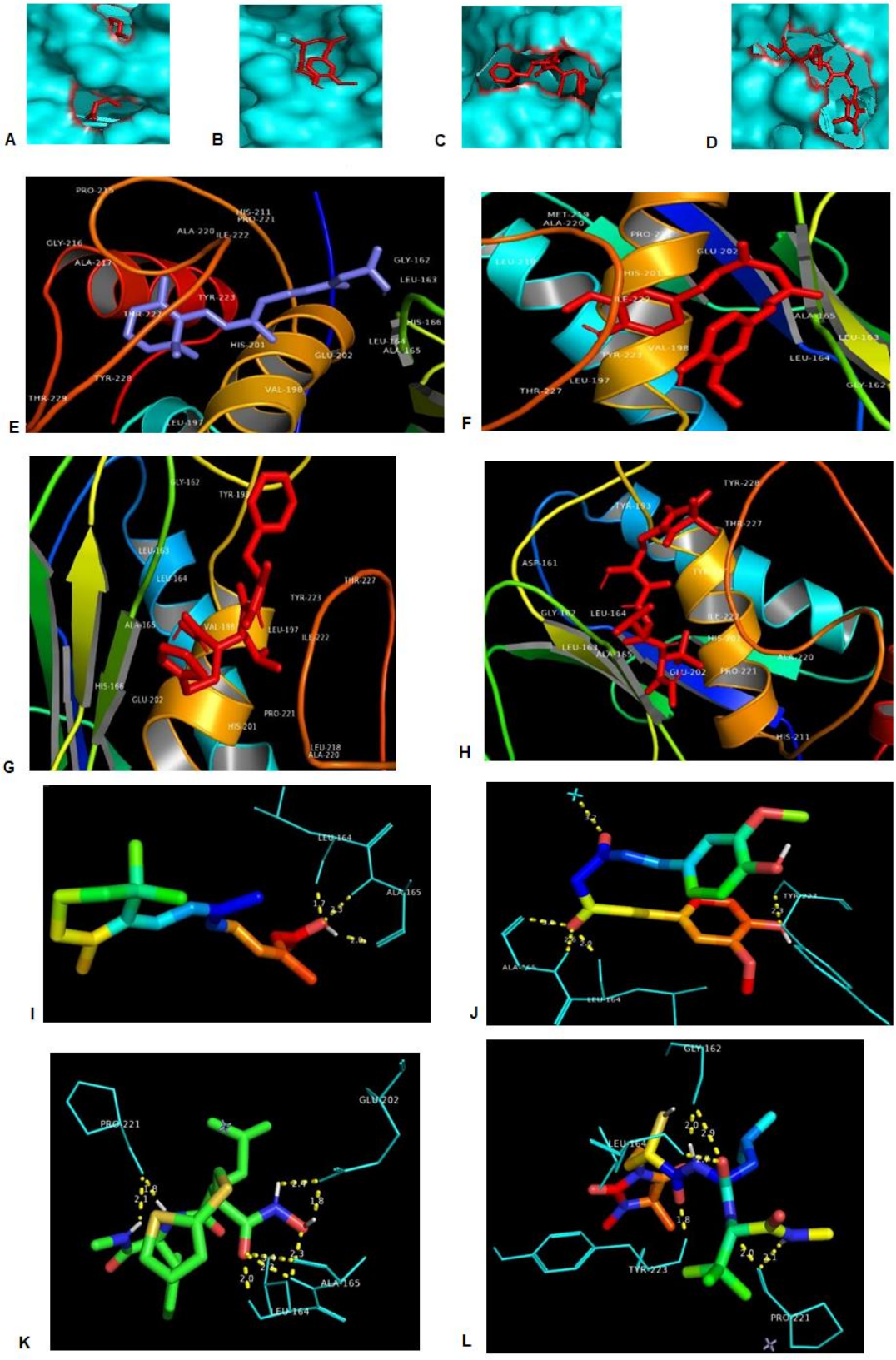

Fig. 2: Interactions of catalytic domain of human MMP-2 with ATRA, curcumin, Batimastat and Rebimastat represented using surface zoomed view (Fig. 2A, 2B, 2C and 2D respectively) and ribbon representations (Fig. 2E, 2F, 2G and 2H respectively) and hydrogen

bonding of amino acids of human MMP-2 with ATRA (Fig. 2I) curcumin (Fig. 2J), Batimastat (Fig. 2K) and Rebimastat (Fig. 2L) with estimated bond distances.

4.1.3. Interaction of curcumin, ATRA and synthetic MMP inhibitors with catalytic domain of MMP-9: Molecular docking studies (Table 3) indicated that, curcumin (ΔG = -11.23 kcal/mole) and ATRA (ΔG = -11.13 kcal/mole) showed very high affinity of binding to catalytic domain of human MMP-9 which were appreciably greater than the binding affinities of the synthetic MMP inhibitors Batimastat (ΔG = -6.42 kcal/mole) and Rebimastat (ΔG = -6.56 kcal/mole). ATRA and curcumin appear to preferentially interact at similar sites on the MMP-9 catalytic domain while both Batimastat and Rebimastat interact at a different nearby region (Fig. 3A-H).

Analysis with PYMOL showed ATRA formed hydrogen bonds with Leu-188 and Ala-189 (Fig. 3I) in the active site of MMP-9. Other interactive amino acids in the binding region included Gly-186, Leu-187, His-190, Leu-222, Val-223, His-226, His-230, His-236, Pro-240, Glu-241, Ala-242, Leu-243, Tyr-245, Pro-246, Met-247, Tyr-248, Arg-249, Phe-250, Thr-251 and Pro 255. Amino acids Leu-188, Ala-189, Leu-222, Arg-249, Pro-240 present in the catalytic site formed hydrogen bonds with curcumin (Fig. 3J). Other interactive amino acids in the binding region included Gly-186, Leu- 187, His-190, Val-223, His-226 Glu-227 Glu-241, Ala-242, Leu-243, Tyr-245, Pro- 246, Met-247, Tyr-248 and Thr 251.

Compound	PubChem CID	Δ G (kcal/mole)	Hydrogen Bonding to Amino Acids with Bond Lengths
ATRA	444795	-11.13	Leu-188 (2.0Å & 2.1Å), Ala-189 (2.1Å)
Curcumin	969516	- 11.23	Leu-188 (1.7Å & 2.0Å), Ala-189 (1.9Å), Leu-222 (2.8Å), Arg-249 (2.5Å), Pro-240 (2.3Å)
Batimastat	5362422	- 6.42	Ala-191 (2.0Å), Asp-235 (2.1Å & 1.8Å)
Rebimastat	9913881	-6.56	Asp-113 (3.3Å), Ala-191 (2.7Å & 3.6Å), Leu-234 (3.2Å), Asp-235 (2.8Å)

Table 3: Binding interactions of ATRA, curcumin, and synthetic MMP inhibitors Batimastat, and Rebimastat to catalytic domain of human MMP-9 (PDB ID: 5I12).

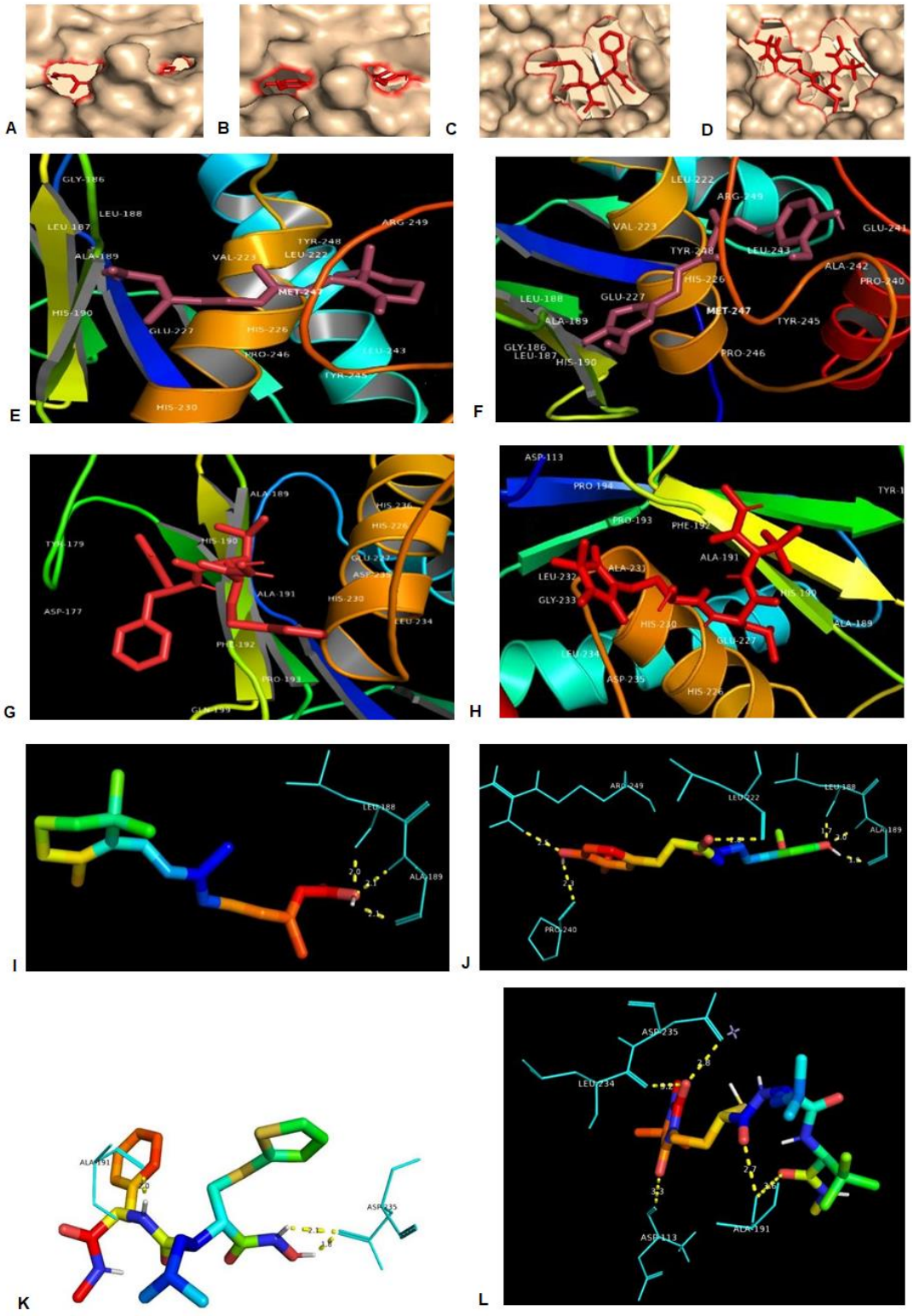

Fig. 3: Interactions of catalytic domain of human MMP-9 with ATRA, curcumin, Batimastat and Rebimastat represented using surface zoomed view (Fig. 3A, 3B, 3C and 3D respectively) and ribbon representations (Fig. 3E, 3F, 3G and 3H respectively) and hydrogen bonding of amino acids of human MMP-2 with ATRA (Fig. 3I) curcumin (Fig. 3J), Batimastat (Fig. 3K) and Rebimastat (Fig. 3L) with estimated bond distances.

Residues Ala-191 and Asp-235 were involved in hydrogen bond interactions with Batimastat (Fig. 3K). Other interactive amino acids in binding region included Asp-177, Tyr-179, Ala-189, His-190, Phe-192, Pro-193, His-226 Glu-227, His-230, Gly-233, Leu-234 and His-236. Amino acids Asp-113, Ala-191, Leu-234 and Asp-235 formed hydrogen bonds with Rebimastat (Fig. 3L). Other interactive amino acids in binding region included Leu-114, Lys-115, Tyr-179, Ala-189, His-190, Phe-192, Pro-193, Pro-194, Gln-199, His-226, Glu-227, His-230, Ala-231, Leu-232, Gly-233 and His 236.

4.1.4. Interaction of curcumin, ATRA and synthetic MMP inhibitors with catalytic domain of MT1-MMP: For MT1-MMP (MMP-14), molecular docking studies (Table 4) indicated that curcumin (ΔG = -10.78 kcal/mole) and ATRA (ΔG = - 8.28 kcal/mole) showed appreciably higher binding affinity to the catalytic domain compared to the synthetic inhibitors Batimastat (ΔG = -5.45 kcal/mole) and Rebimastat (ΔG = -6.83 kcal/mole). ATRA, curcumin and Rebimastat appear to preferentially interact at similar sites on the MT1-MMP catalytic domain while Batimastat interacts at a different site (Fig. 4A-H).

Analysis with PYMOL showed ATRA formed hydrogen bonds with His-201 (Fig. 4I) in the active site of MT1-MMP. Other interactive amino acids in the binding region included Thr-190, Pro-191, Phe-192, Phe-198, Leu-199, Ala-200, Ala-202, Leu-235, Val-236, His-239, Glu-240, His-249 Ile-256, Met-257, Pro-259, Phe-260, Tyr-261, Ala- 258 and Gln-262. Amino acids Leu-199, Ala-202, His-239, Pro-259 and Gln-262 present in the catalytic site formed hydrogen bonds with curcumin (Fig. 4J). Other interactive amino acids in the binding region included Gly-197, Phe-198, Ala 200, His 201, Phe-204, Leu-235, Val-236, Glu-240, His-243, His-249, Ala-255, Ile-256, Met-257, Ala-258, Phe-260, Tyr-261, Trp-263 and Met-264.

Residues Ser-153, Phe-269 and Leu-271 formed hydrogen bonds with Batimastat (Fig. 4K). Other interactive amino acids in binding region included Lys-146, Arg-149, Val-150, Ala-154, Phe-234, Thr-266, Glu-267, Asn-268, Val-270 and Arg-276. Amino acids Leu-199, Ala-202, Glu-240 and Pro-259 formed hydrogen bonds with Rebimastat (Fig. 4L). Other interactive amino acids in binding region included Gly-197, Phe-198, Ala-200, His-201, Asn-231 Val-236, His-239, His-243, His-249, Phe-260 and Tyr-261.

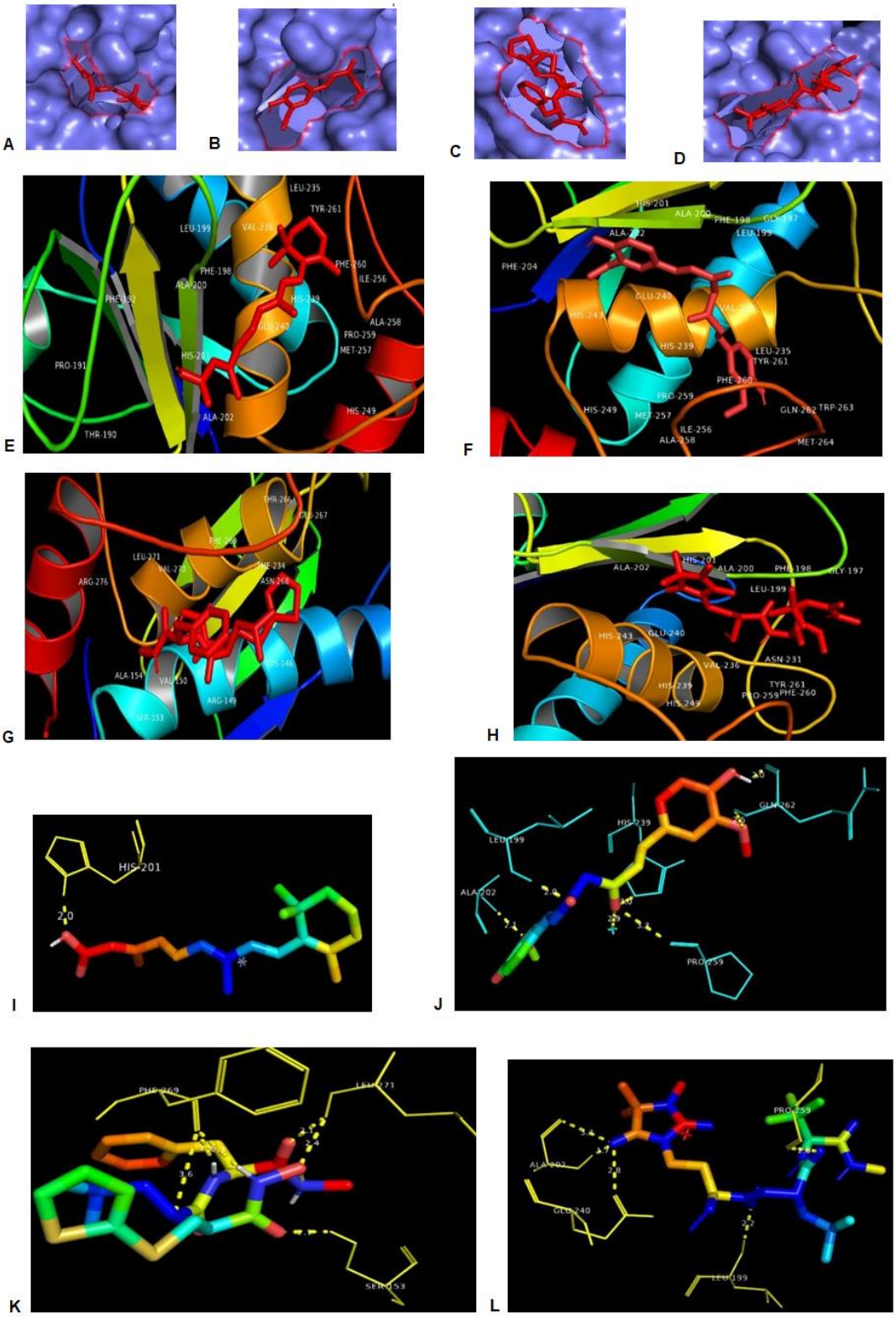

Fig. 4: Interactions of catalytic domain of human MT1-MMP with ATRA, curcumin, Batimastat and Rebimastat represented using surface zoomed view (Fig. 4A, 4B, 4C and 4D respectively) and ribbon representations (Fig. 4E, 4F, 4G and 4H respectively) and hydrogen bonding of amino acids of human MT1-MMP with ATRA (Fig. 4I) curcumin (Fig. 4J), Batimastat (Fig. 4K) and Rebimastat (Fig. 4L) with estimated bond distances.

Compound	PubChem CID	Δ G (kcal/mole)	Hydrogen Bonding to Amino Acids with Bond Lengths
ATRA	444795	-8.28	His-201 (2.0Å)
Curcumin	969516	-10.78	Leu-199 (2.0Å), Ala-202 (2.1Å), His-239 (3.0Å), Pro-259 (3.3Å), Gln-262 (2.0Å & 2.6Å)
Batimastat	5362422	-5.45	Ser-153 (2.1Å), Phe-269 (1.9Å, 2.3Å & 3.6Å), Leu-271 (2.1Å & 2.4Å)
Rebimastat	9913881	-6.83	Leu-199 (2.2Å), Ala-202 (1.7Å & 3.3Å), Glu-240 (2.8Å), Pro-259 (2.8Å)

Table 4: Interactions of ATRA, curcumin, and synthetic inhibitors Batimastat and Rebimastat to catalytic domain of human MT1-MMP (PDB ID: 5H0U).

4.1.5. Analysis of possible synergistic interactions of ATRA and curcumin with catalytic domains of MMP-2, MMP-9 and MT1-MMP: Molecular docking showed high binding affinity of curcumin with MMP-2 catalytic domain-ATRA complex (ΔG = -6.27 kcal/mole) and of ATRA with MMP-2 catalytic domain-curcumin complex (ΔG = -7.11 kcal/mol). Molecular docking also showed high binding affinity of curcumin with MMP-9 catalytic domain-ATRA complex (ΔG = -6.88 kcal/mole) and of ATRA with MMP-9 catalytic domain-curcumin complex (ΔG = -6.81 kcal/mole). For MT1-MMP, docking showed high binding affinity of curcumin with MT1-MMP catalytic domain-ATRA complex (ΔG = -6.85 kcal/mole) and of ATRA with MT1-MMP catalytic domain-curcumin complex (ΔG = -8.13 kcal/mol) (Table 5). The domains with which such interactions occur are as shown in Fig. 5.

Compound	Interactions with	Δ G (kcal/mole)
Curcumin	MMP-2 catalytic domain bound to ATRA	-6.27
ATRA	MMP-2 catalytic domain bound to curcumin	-7.11
Curcumin	MMP-9 catalytic domain bound to ATRA	-6.88
ATRA	MMP-9 catalytic domain bound to curcumin	-6.81
Curcumin	MT1-MMP catalytic domain bound to ATRA	-6.85
ATRA	MT1-MMP catalytic domain bound to curcumin	-8.13

Table 5: Analysis of possible synergistic interactions of ATRA and curcumin with catalytic domains of MMP-2 and MT1-MMP.

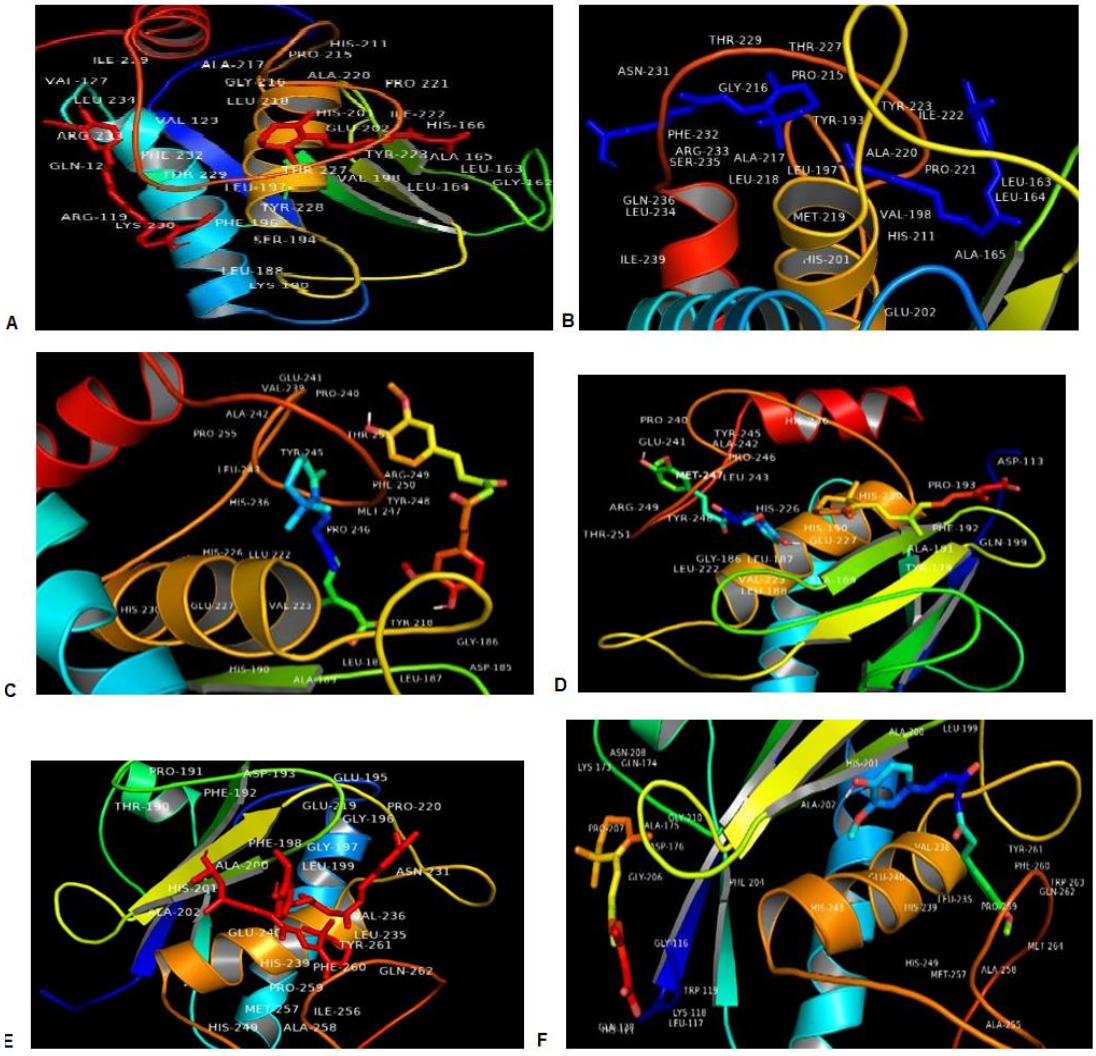

Fig. 5: Interactions of curcumin with MMP-2 catalytic domain-ATRA complex (Fig. 5A), ATRA with MMP-2 catalytic domain-curcumin complex (Fig. 5B), curcumin with MMP-9 catalytic domain-ATRA complex (Fig. 5C), ATRA with MMP-9 catalytic domain-curcumin complex (Fig. 5D), curcumin with MT1-MMP catalytic domain-ATRA complex (Fig. 5E) and ATRA with MT1-MMP catalytic domain-curcumin complex (Fig. 5F) represented using and ribbon representations.

Although these binding affinities were less than for curcumin or ATRA binding separately with MMP catalytic domains, they were still appreciably high and comparable to synthetic MMP inhibitors.

4.1.6. Interaction of curcumin, ATRA and synthetic inhibitor with human FAK:

Molecular docking studies (Table 6) indicated that, curcumin (ΔG = -7.54 kcal/mole)

and ATRA (ΔG = -7.24 kcal/mole) showed very high affinity of binding to FAK which was greater than the binding affinity of the synthetic inhibitor Defactinib (ΔG = -5.82 kcal/mole). ATRA, curcumin and Defactinib appear to preferentially interact at different sites on the FAK molecule (Fig. 6A-F).

Analysis with PYMOL showed ATRA formed hydrogen bonds with Arg-541 in FAK (Fig. 6G). Other interactive amino acids in binding region included Glu538, Phe542, Val 543, Phe599, Thr600, Ser601, Ala602, Val605, Pro663, Ser664, Arg665, Arg666, Pro667, Arg668 and Phe669. Analysis of the FAK-curcumin complex with PYMOL showed amino acids Ser-653, Lys-657, Thr-678 and Glu-683 formed hydrogen bonds with curcumin (Fig. 6H). Other interactive amino acids in binding region included Leu520, Ile524, Pro648, Pro649, Thr 650, Leu 654, Gln675, Ile 679, Glu 682 and Gln686. Analysis of the FAK-Defactinib complex showed amino acids Gln-437, Glu-506, Asp-546, Arg-550 and Asn-551 formed hydrogen bonds with Defactinib (Fig. 6I). Other interactive amino acids in binding region included Glu 430, Gly 431, Glu 471 Leu507, Arg 508, Ser509, His544, Ala549, Val552 and Asp 564.

Compound	PubChem CID	Δ G (kcal/mole)	Hydrogen Bonding to Amino Acids with Bond Lengths
ATRA	444795	-7.24	Arg-541 (1.8Å, 2.3Å & 2.1Å)
Curcumin	969516	-7.54	Ser-653 (2.2Å), Lys-657 (2.2Å), Thr- 678 (2.0Å), Glu-683 (1.9Å)
Defactinib	25117126	-5.82	Gln-437 (2.2Å), Glu-506 (3.5Å), Asp-546 (2.9Å), Arg-550 (2.2Å), Asn-551 (2.4Å, 3.2Å & 3.3 Å)

Table 6: Interactions of ATRA, curcumin, and synthetic inhibitor Defactinib with human FAK (PDB ID: 1MP8).

4.1.7. Interaction of curcumin, ATRA and synthetic inhibitors with human PI3K p110α catalytic subunit: Molecular docking studies (Table 7) indicated that, curcumin (ΔG = -6.94 kcal/mole) and ATRA (ΔG = -6.46 kcal/mole) showed very high affinity of binding to human PI3K p110α catalytic subunit which was comparable with the binding affinity of synthetic PI3K inhibitor LY294002 (ΔG = -6.99 kcal/mole) and slightly lower than affinity of the synthetic inhibitor Alpelisib (ΔG = -7.13 kcal/mole).

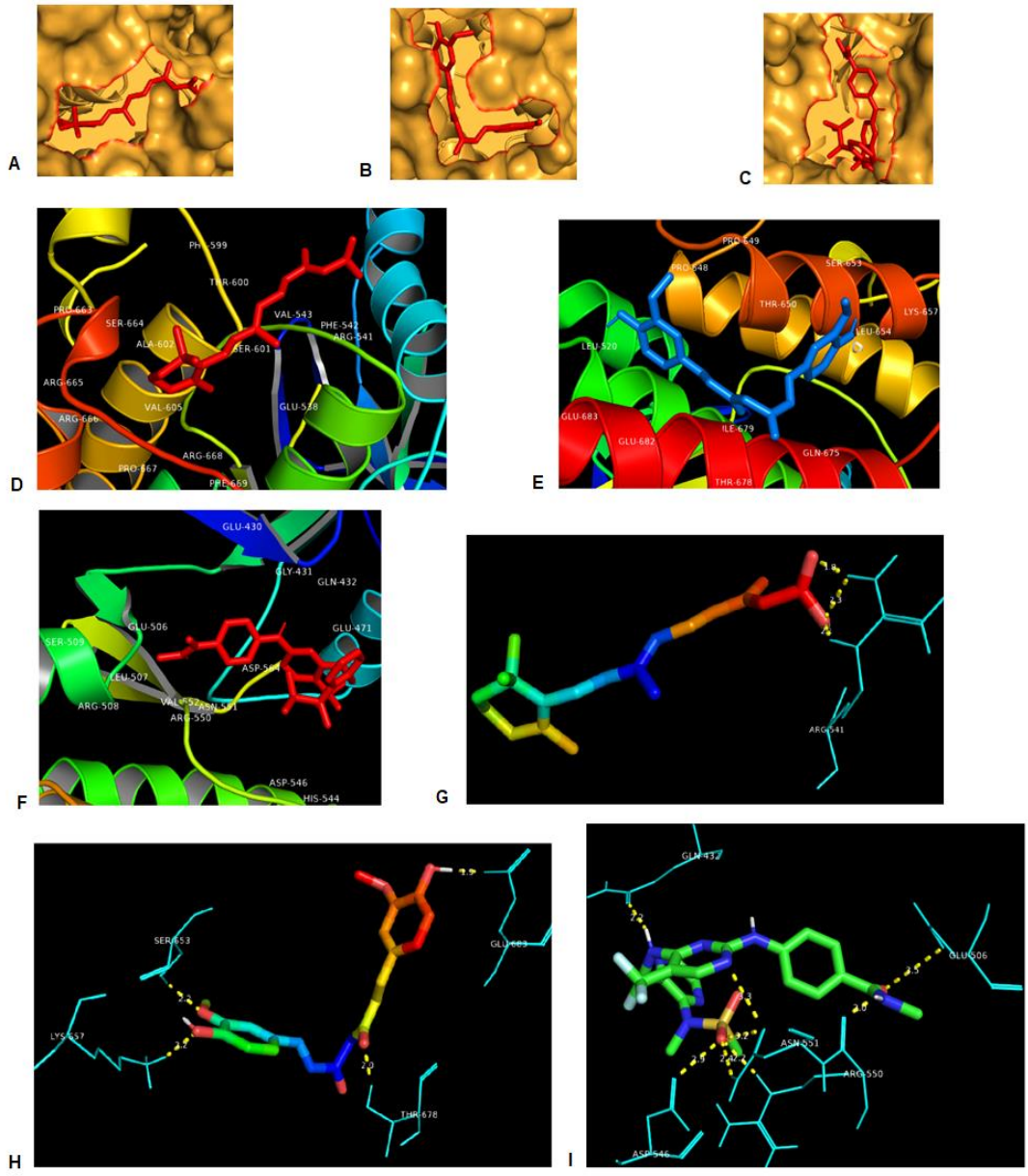

Fig. 6: Interactions of human FAK with ATRA, curcumin and Defactinib. Interactions represented using surface zoomed view for ATRA, curcumin and defactinib (Fig. 6A, 6B and 6C respectively) and ribbon representations (Fig. 6D, 6E and 6F respectively) and hydrogen bonding of amino acids of human FAK with ATRA (Fig. 6G) curcumin (Fig. 6H) and Defactinib (Fig. 6I) with estimated bond distances.

ATRA, curcumin and LY294002 appear to preferentially interact at nearby sites on p110α catalytic subunit of PI3K while Alpelisib interacts at a different site (Fig. 7A-H). Analysis of PI3K-ATRA complex with PYMOL showed ATRA formed hydrogen bonds with Asn-170, Lys-271, Ser-629 and His-670 (Fig. 7I). Other interactive amino

acids in binding region included Met 811, Gln 815, Arg 818, Ile819, Gly837, Cys838, Leu839, Gln630, Tyr 631, Leu 632, Ile 633, Arg 662 and Phe 666. Analysis of the PI3K-curcumin complex with PYMOL showed amino acids Lys-802, Glu-849 and Asn-853 formed hydrogen bonds with curcumin (Fig. 7J). Other interactive amino acids in binding region included Arg 770, Trp 780, Glu798, Ile 800, Leu 807, Asp 810, Ile 848, Val 850, Val 851 Arg 852, His 855, Gln 859, Met 922, Phe 930, Asp 933, Phe 934, Ile 939 and Ser 954.

Analysis of the PI3K-LY294002 complex showed LY294002 formed hydrogen bonds with Asn-756 (Fig. 7K). Other interactive amino acids in binding region included Ser 753, Leu 755, His 759, Leu 793, Phe 794, Asn 796, Asn 797, Glu798, Ile 799, Glu 849 Val 850 and Arg 852. For PI3K-Alpelisib complex, analysis with PYMOL showed no hydrogen bonds and Trp 424, Pro 449, His 450, Gly 451, Leu 452, Glu 453 Asp 454, Leu 456, Asn 457 Pro 458, Ile 459, Gly 460, Val 461, Lys 640, Tyr 641, Asn 677, Thr 679, Val 680, Leu 1006, Gly 1007, Gln 1014 and Ser 1015 as interactive amino acids in binding region.

Compound	**PubChem CID**	**Δ G (kcal/mole)**	**Hydrogen Bonding to Amino Acids with Bond Lengths**
ATRA	444795	-6.46	Asn-170 (1.8Å & 2.0Å), Lys-271 (1.8Å), Ser-629 (2.3Å), His-670 (2.2Å)
Curcumin	969516	-6.94	Lys-802 (2.1Å), Glu-849 (3.0Å), Asn-853 (2.4Å)
LY294002	3973	-6.99	Asn-756 (2.0Å)
Alpelisib	56649450	-7.13	None

Table 7: Interactions of ATRA, curcumin, and synthetic inhibitors LY294002 and Alpelisib with human PI3K p110α catalytic subunit (PDB ID: 4TUU).

4.1.8. Analysis of possible synergistic interactions of ATRA and curcumin with FAK and PI3K: Molecular docking showed high binding affinity of curcumin with FAK-ATRA complex (ΔG = -6.11 kcal/mole) and of ATRA with FAK-curcumin complex (ΔG = -7.02 kcal/mol). Molecular docking also showed high binding affinity of curcumin with PI3K-ATRA complex (ΔG = -7.52 kcal/mole) and of ATRA with

PI3K-curcumin complex (ΔG = -6.69 kcal/mole) (Table 8). The domains with which such interactions occur are as shown in Fig. 8.

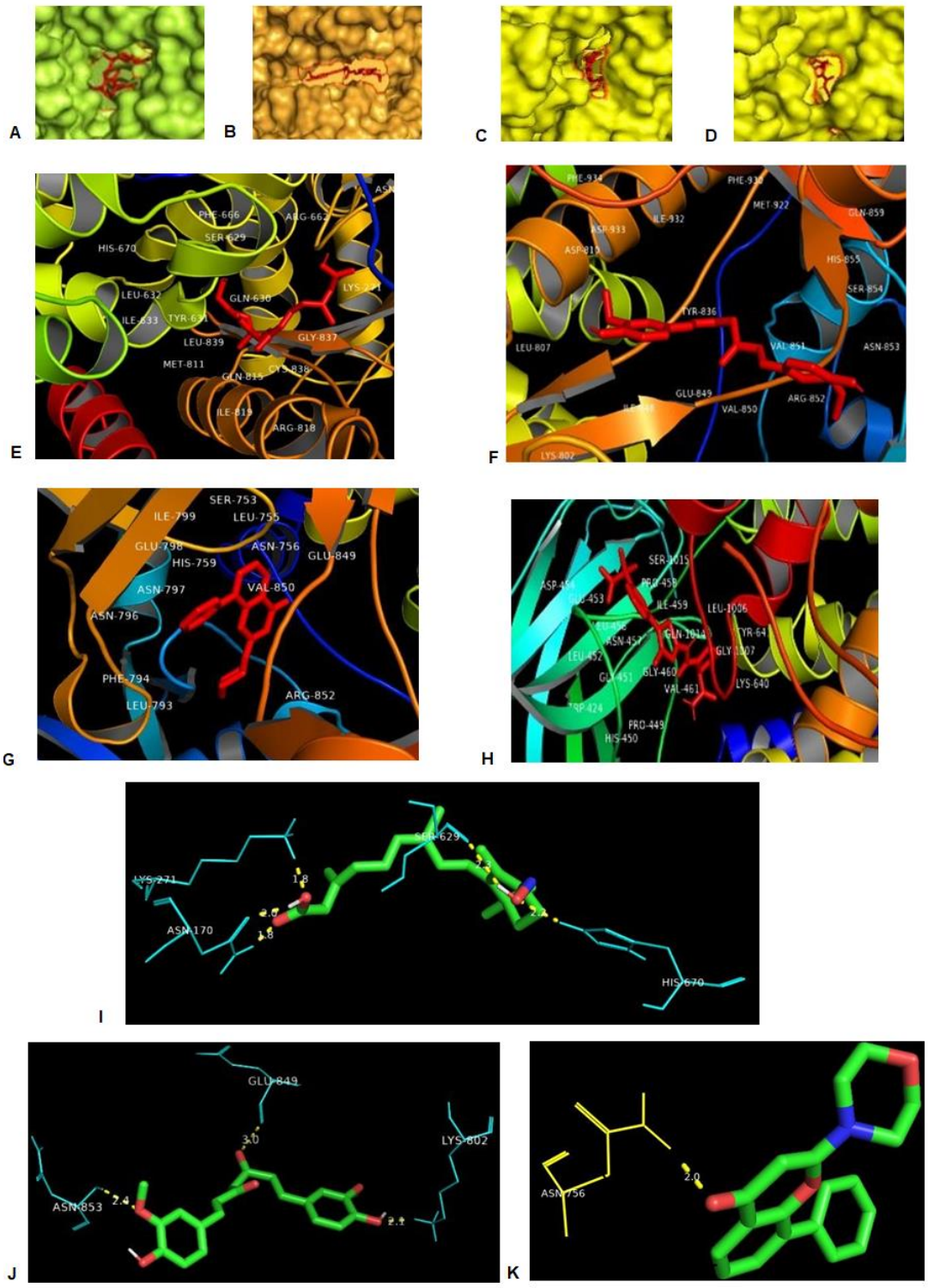

Fig. 7: Interactions of p110α catalytic subunit of human PI3K with ATRA, curcumin, LY294002 and Alpelisib. Interactions represented using surface zoomed view for ATRA, curcumin, LY294002 and alpelisib (Fig. 7A, 7B, 7C and 7D respectively) and ribbon

representations (Fig. 7E, 7F, 7G and 7H respectively) and hydrogen bonding of amino acids of human PI3K with ATRA (Fig. 7I) curcumin (Fig. 7J) and LY294002 (Fig. 7K) with estimated bond distances.

Compound	Interactions with	Δ G (kcal/mole)
Curcumin	FAK bound to ATRA	-6.11
ATRA	FAK bound to curcumin	-7.02
Curcumin	PI3K p110α subunit bound to ATRA	-7.52
ATRA	PI3K p110α subunit bound to curcumin	-6.69

Table 8: Analysis of possible synergistic interactions of ATRA and curcumin with FAK and PI3K.

Although the binding affinities were less than for curcumin or ATRA binding separately with FAK, they were still appreciably high and more than that of synthetic FAK inhibitor. For PI3K, the binding affinities were appreciably high, greater than for curcumin or ATRA binding separately with PI3K and also comparable to the synthetic PI3K inhibitors.

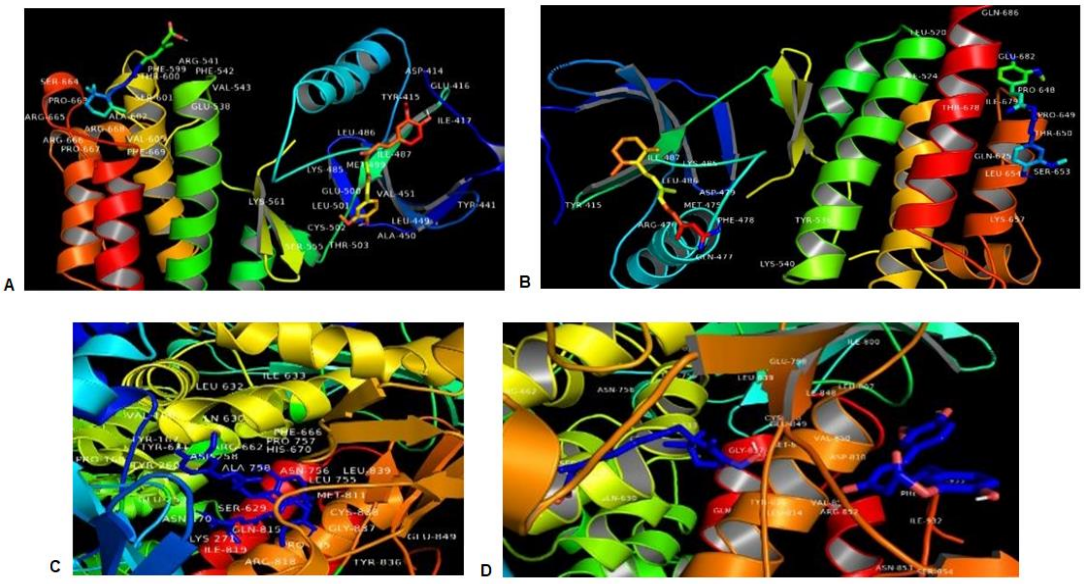

Fig. 8: Interactions of curcumin with FAK-ATRA complex (Fig. 8A), ATRA with FAK-curcumin complex (Fig. 8B), curcumin with PI3K-ATRA complex (Fig. 8C) and ATRA with PI3K-curcumin complex (Fig. 8D) represented using and ribbon representations.

4.2 Singular and Synergistic Treatment of Breast Cancer Cells with Curcumin and ATRA

4.2.1. Effect of Curcumin and ATRA Treatment on MMP-2, MMP-9 and MT1-MMP Expression in MDA-MB-453 Breast Cancer Cells: Treatment of MDA-MB-453 cells with curcumin (20 µM/ml) and ATRA (20 µM/ml) caused significant downregulation of MMP-2, MMP-9 and MT1-MMP expression ($p < 0.05$) compared to control cells. Synergistic treatment with both curcumin (20 µM/ml) and ATRA (20 µM/ml) was considerably more effective than singular treatments with curcumin or ATRA alone and caused significantly more downregulation of MMP-2, MMP-9 and MT1-MMP expression ($p < 0.01$) in comparison to control cells (Fig. 9A-C).

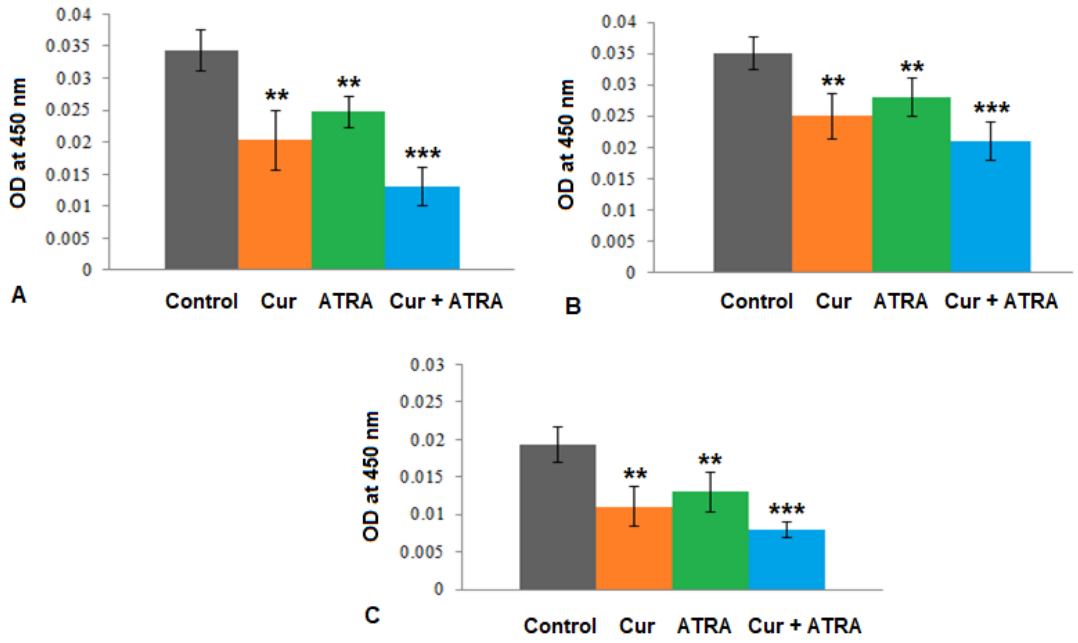

Fig 9: Effect of Curcumin and ATRA Treatment on MMP-2, MMP-9 and MT1-MMP Expression in MDA-MB-453 Breast Cancer Cells. Cell extracts of MDA-MB-453 cells treated with 20 µM/ml curcumin, 20 µM/ml ATRA and both 20 µM/ml curcumin and 20 µM/ml ATRA were subjected to ELISA and incubated with anti-MMP-2 (Fig. 9A), anti-MMP-9 (Fig. 9B) or MT1-MMP (Fig. 9C) antibodies followed by HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** $p < 0.05$; *** $p < 0.01$.

4.2.2. Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Activity in MDA-MB-453 Breast Cancer Cells: Treatment of MDA-MB-453 cells with curcumin (20 µM/ml) and ATRA (20 µM/ml) caused appreciable downregulation of MMP-2 and MMP-9 activity in cell lysates and partial downregulation of MMP-2 and MMP-9 activity in culture supernatants compared to control cells. Synergistic treatment with both curcumin (20 µM/ml) and ATRA (20 µM/ml) was more effective than singular treatments with curcumin or ATRA alone in causing appreciable downregulation of MMP-2 and MMP-9 activity in cell lysates and culture supernatants (Fig. 10A, B).

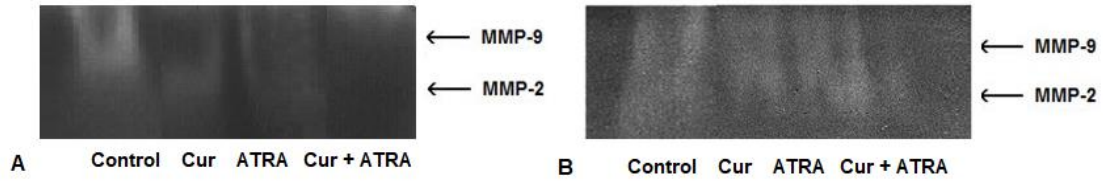

Fig. 10: Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Activity in MDA-MB-453 Breast Cancer Cells Cell extracts (Fig. 10A) and culture supernatants (Fig.10B) of MDA-MB-453 cells treated with 20 µM/ml curcumin, 20 µM/ml ATRA and both 20 µM/ml curcumin and 20 µM/ml ATRA were subjected to gelatin zymography on 10% SDS-PAGE co-polymerized with 0.1% gelatin.

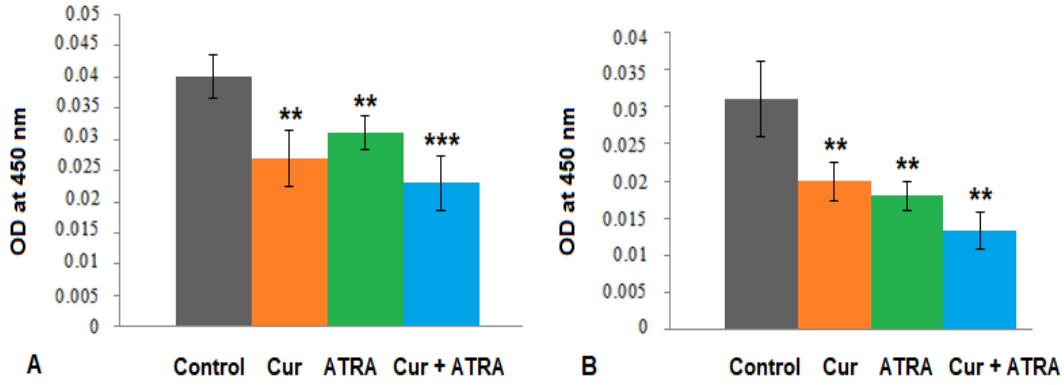

Fig 11: Effect of Curcumin and ATRA Treatment on FAK and p-PI3K in MDA-MB-453 Breast Cancer Cells. Cell extracts of MDA-MB-453 cells treated with 20 µM/ml curcumin, 20 µM/ml ATRA and both 20 µM/ml curcumin and 20 µM/ml ATRA were subjected to ELISA and incubated with anti-FAK (Fig. 11A) or anti-p-PI3K (Fig. 11B) antibodies followed by HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** $p < 0.05$; *** $p < 0.01$.

4.2.3. Effect of Curcumin and ATRA Treatment on FAK and p-PI3K in MDA-MB-453 Breast Cancer Cells: Treatment of MDA-MB-453 cells with curcumin (20 µM/ml) and ATRA (20 µM/ml) caused significant downregulation of FAK expression ($p < 0.05$) compared to control cells. Synergistic treatment with both curcumin (20 µM/ml) and ATRA (20 µM/ml) was considerably more effective than singular treatments with curcumin or ATRA alone and caused significantly more downregulation of FAK expression ($p < 0.01$) in comparison to control cells (Fig. 11A). For PI3K, treatment of cells with curcumin (20 µM/ml) and ATRA (20 µM/ml) caused significant downregulation of PI3K phosphorylation ($p < 0.05$) compared to control cells and synergistic treatment with both curcumin (20 µM/ml) and ATRA (20 µM/ml) caused more significant downregulation of PI3K phosphorylation ($p < 0.05$) and was more effective than singular treatments with curcumin or ATRA alone (Fig. 11B).

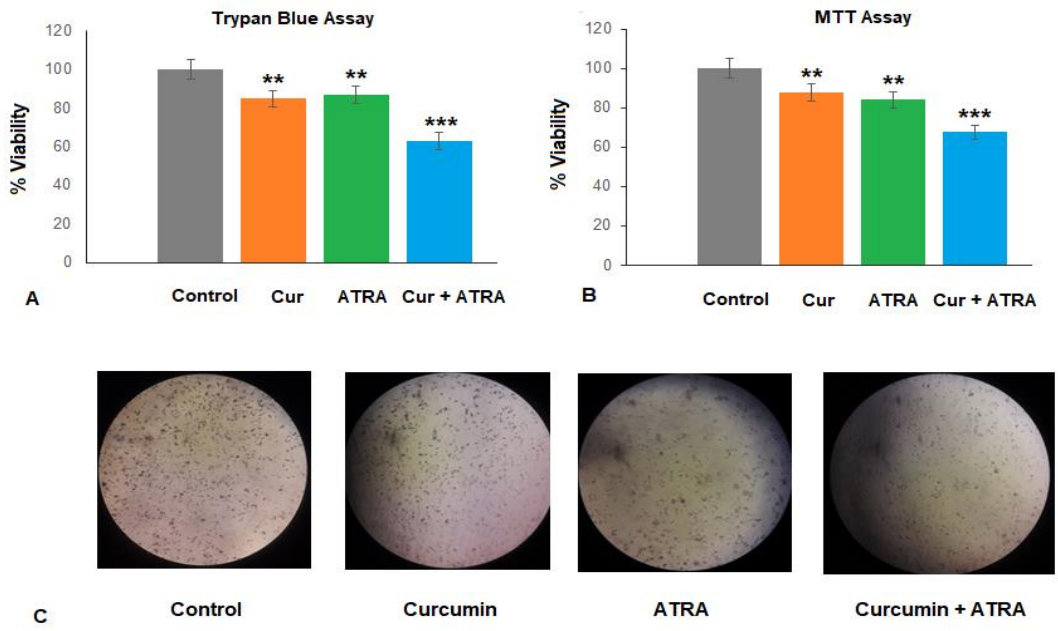

Fig. 12: Effect of Curcumin and ATRA Treatment on Cell Viability of MDA-MB-453 Breast Cancer Cells. Cell viability assays using Trypan Blue (Fig. 12A) and MTT assay (Fig. 12B) were performed with control MDA-MB-453 cells and MDA-MB-453 cells treated with curcumin (20 µM/ml), ATRA (20 µM/ml) and both curcumin and ATRA (20 µM/ml each). Viability in control cells was taken as 100%. ** $p< 0.05$, *** $p< 0.01$. Morphology of control MDA-MB-453 cells and MDA-MB-453 cells treated with curcumin, ATRA and both curcumin and ATRA as viewed under inverted phase contrast microscope (Fig. 12C).

4.2.4. Effect of Curcumin and ATRA Treatment on Cell Viability of MDA-MB-453 Breast Cancer Cells: In cell viability assays with Trypan blue, singular treatments with curcumin (20 μM/ml) and ATRA (20 μM/ml) increased the amount of non-viable cells compared to control where cell viability was stable and high. Synergistic treatment with both curcumin and ATRA (20 μM/ml each) more significantly reduced cell viability compared to singular treatments (Fig. 12A). Cell viability was also analyzed using MTT Assay. Control cells showed stable cell viability while treatment with ATRA (20 μM/ml) reduced cell viability slightly more compared to curcumin (20 μM/ml) treatment. Synergistic treatment with both ATRA and curcumin (20 μM/ml each) showed a more significant decrease in cellular viability compared to singular treatments with ATRA and curcumin alone (Fig. 12B, C).

4.2.5. Effect of Curcumin and ATRA Treatment on Cell Migration of MDA-MB-453 Breast Cancer Cells: Wound healing assay showed that treatment of MDA-MB-453 cells cultured with curcumin (20 μM/ml) or ATRA (20 μM/ml) appreciably inhibited cell migration and invasive potential in comparison to control cells cultured without such treatment. Synergistic treatment with both curcumin and ATRA (20 μM/ml each) was more effective, causing appreciably more inhibition of cell migration in comparison to treatment with curcumin or ATRA alone (Fig. 13).

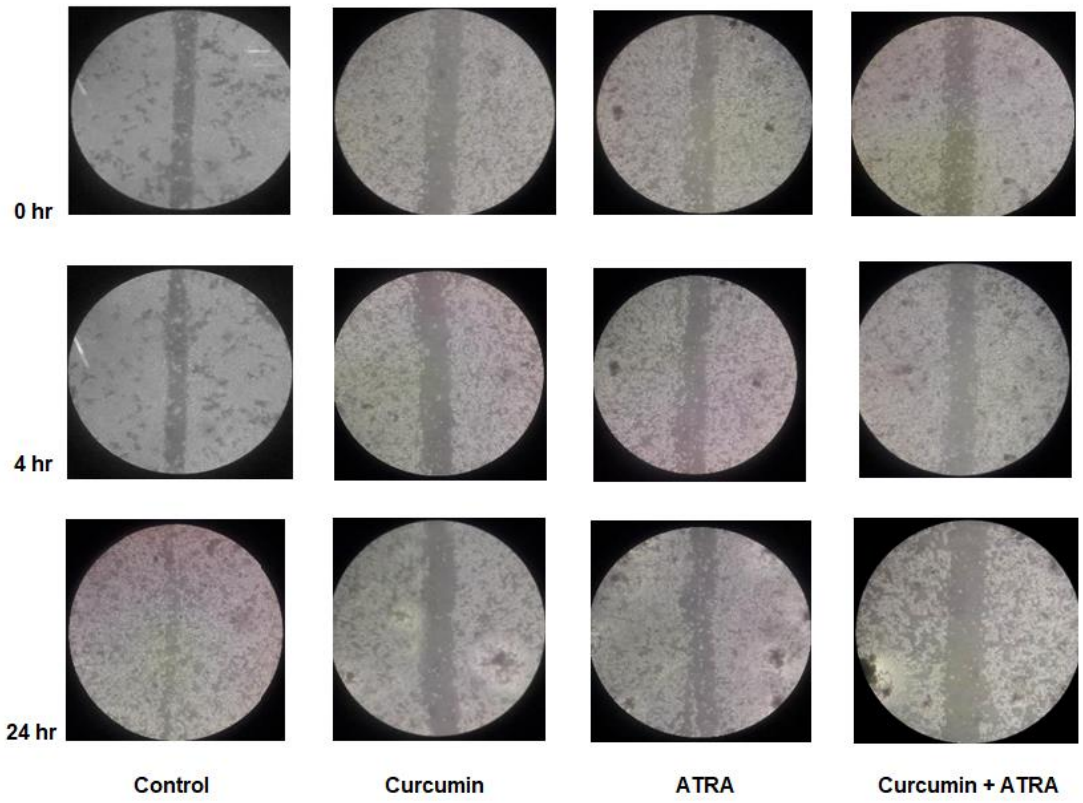

Fig. 13: Effect of Curcumin and ATRA Treatment on Cell Migration of MDA-MB-453 Breast Cancer Cells: Wound healing assay was performed with control MDA-MB-453 cells and MDA-MB-453 cells treated with curcumin (20 μM/ml), ATRA (20 μM/ml) and both curcumin and ATRA (20 μM/ml each). The extent of cell migration into the wound area was determined by viewing under inverted phase contrast microscope.

4.2.6. Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Expression in MDA-MB-231 and MCF-7 Breast Cancer Cells: Treatment of MDA-MB-231 cells with curcumin (20 μM/ml) and ATRA (20 μM/ml) caused significant downregulation of MMP-2 and MMP-9 expression ($p < 0.05$) compared to control cells. Synergistic treatment with both curcumin (20 μM/ml) and ATRA (20 μM/ml) was more effective than singular treatments with curcumin or ATRA alone and caused significantly more downregulation particularly of MMP-2 ($p < 0.01$) and MMP-9 ($p < 0.05$) expression in comparison to control cells (Fig. 14A, B). Treatment of MCF-7 cells with curcumin (20 μM/ml) and ATRA (20 μM/ml) caused significant downregulation of MMP-2 and MMP-9 expression ($p < 0.05$; $p < 0.01$ for MMP-2 for curcumin treated cells) compared to control cells. Synergistic treatment with both curcumin (20 μM/ml) and ATRA (20 μM/ml) was again more effective than singular treatments with curcumin or ATRA alone and caused significantly more

downregulation of MMP-2 and MMP-9 expression ($p < 0.01$) in comparison to control cells (Fig. 14C, D). As treatment with 20 μM/ ml of curcumin showed more appreciable reduction in MMP-2 and MMP-9 expression compared to treatment with ATRA, MDA-MB-231 and MCF-7 cells were treated with a higher dose (40 μM/ ml) of curcumin. Treatment with 20 μM/ml and 40 μM/ml of curcumin significantly reduced expression of MMP-2 and MMP-9 in MDA-MB 231 cells and MMP-2 in MCF-7 cells. In comparison to the varying doses of curcumin (20 μM/ ml and 40 μM/ ml), synergistic treatment with curcumin and ATRA more appreciably downregulated MMP-2 and MMP-9 expression in MDA-MB 231 and MCF-7 cells (Fig. 14E – G).

4.2.7. Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Activity in MDA-MB-231 and MCF-7 Breast Cancer Cells: Treatment of MDA-MB-231 cells with curcumin (20 μM/ml) and ATRA (20 μM/ml) caused appreciable downregulation of MMP-2 and MMP-9 activity in cell lysates and culture supernatants compared to control cells with curcumin being more effective in downregulating MMP (particularly MMP-2) activity. Synergistic treatment with both curcumin and ATRA (20 μM/ml each) was more effective than singular treatments with curcumin or ATRA alone in causing appreciable downregulation of MMP-2 and MMP-9 (Fig. 15A, B).

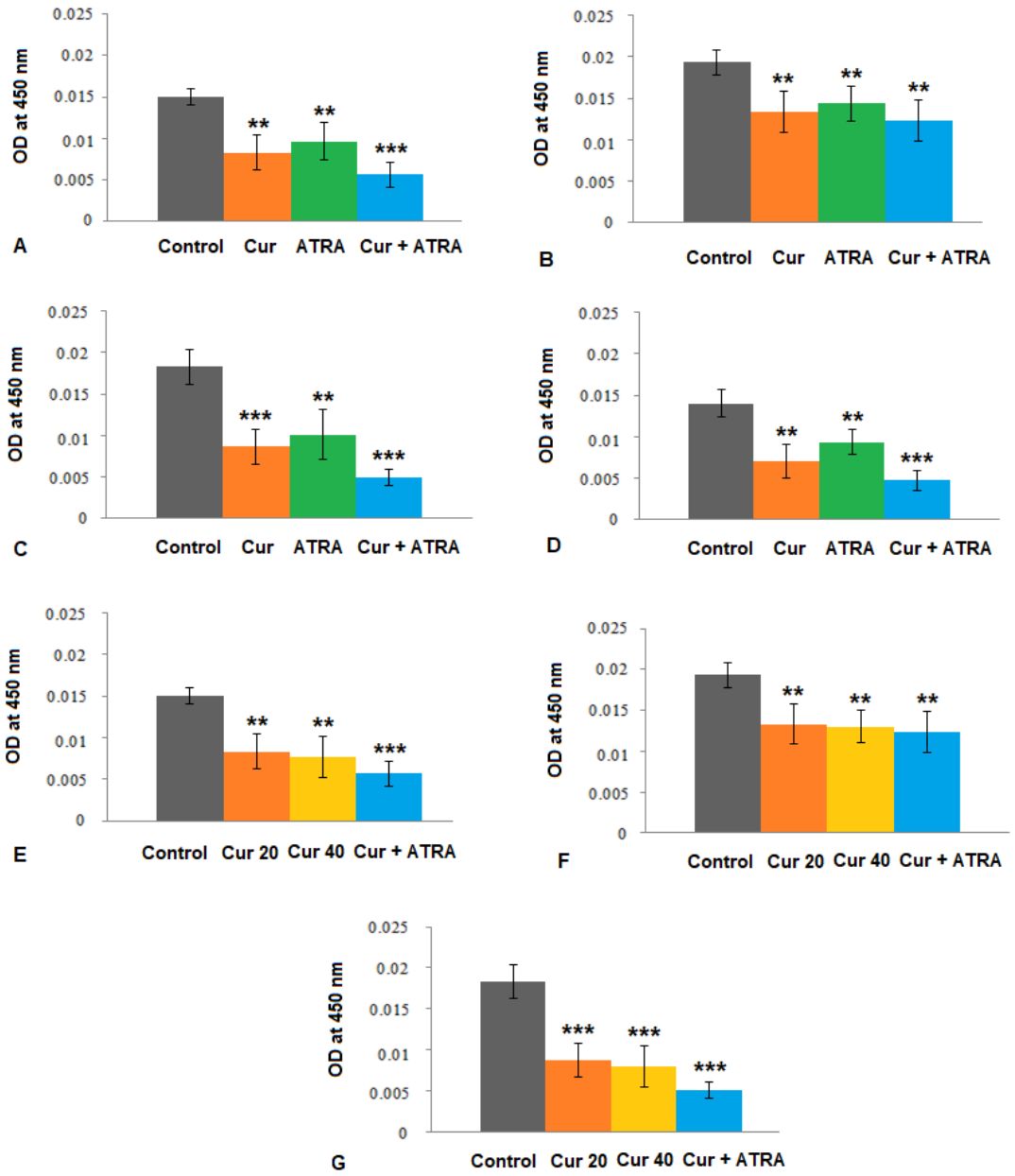

Fig 14: Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Expression in MDA-MB-231 and MCF-7 Breast Cancer Cells. Cell extracts of MDA-MB-231 cells (Fig. 14A, B) and MCF-7 cells (Fig. 14C, D) treated with 20 µM/ml curcumin, 20 µM/ml ATRA and with both 20 µM/ml curcumin and 20 µM/ml ATRA were subjected to ELISA. Cell extracts of MDA-MB-231 cells (Fig. 14E, F) and MCF-7 cells (Fig. 14G) treated with 20 µM/ml curcumin, 40 µM/ml curcumin and with both 20 µM/ml curcumin and 20 µM/ml ATRA were also subjected to ELISA. Samples were incubated with anti-MMP-2 (Fig. 14A, C, E, G) or anti-MMP-9 (Fig. 14B, D, F) antibodies followed by HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** $p < 0.05$; *** $p < 0.01$.

Similar results were obtained upon treatment of MCF-7 cells with curcumin (20 μM/ml) and ATRA (20 μM/ml) causing appreciable downregulation of MMP-2 and MMP-9 activity in cell lysates and culture supernatants compared to control cells and synergistic treatment with both curcumin and ATRA (20 μM/ml each) was more effective than singular treatments with curcumin or ATRA alone in causing appreciable downregulation of MMP-2 and MMP-9 (Fig. 15C, D).

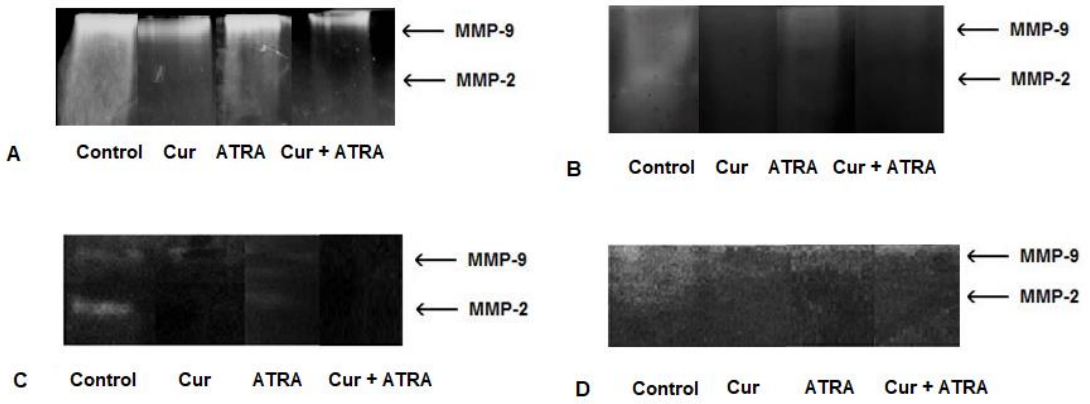

Fig. 15: Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Activity in MDA-MB-231 and MCF-7 Breast Cancer Cells. Cell extracts (Fig. 15A) and culture supernatants (Fig.15B) of MDA-MB-231 cells and cell extracts (Fig. 15C) and culture supernatants (Fig.15D) of MCF-7 cells treated with 20 μM/ml curcumin, 20 μM/ml ATRA and both 20 μM/ml curcumin and 20 μM/ml ATRA were subjected to gelatin zymography on 10% SDS-PAGE co-polymerized with 0.1% gelatin.

4.2.8. Effect of Curcumin and ATRA Treatment on FAK and p-PI3K in MDA-MB-231 and MCF-7 Breast Cancer Cells: Treatment of MDA-MB-231 (Fig. 16A) and MCF-7 cells (Fig. 16B) with curcumin (20 μM/ml) and ATRA (20 μM/ml) caused significant downregulation of FAK expression ($p < 0.05$) compared to control cells. Synergistic treatment with both curcumin (20 μM/ml) and ATRA (20 μM/ml) was considerably more effective than singular treatments with curcumin or ATRA alone and caused significantly more downregulation of FAK expression ($p < 0.01$) in comparison to control cells. Treatment of MDA-MB-231 cells with curcumin (20 μM/ml) and ATRA (20 μM/ml) caused significant downregulation of PI3K phosphorylation ($p < 0.05$) compared to control cells and synergistic treatment with both curcumin (20 μM/ml) and ATRA (20 μM/ml) caused more significant

downregulation of PI3K phosphorylation ($p < 0.01$) than singular treatments with curcumin or ATRA alone (Fig. 16C).

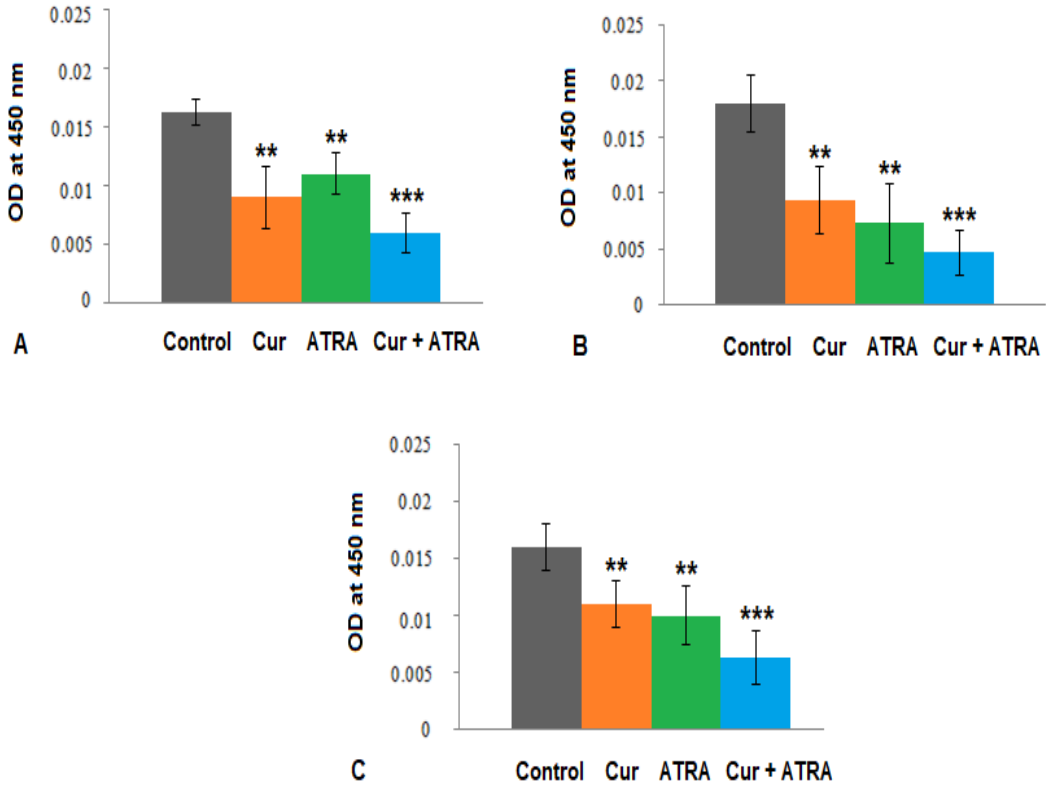

Fig 16: Effect of Curcumin and ATRA Treatment on FAK in MDA-MB 231 and MCF-7and p-PI3K in MDA-MB-231 Breast Cancer Cells. Cell extracts of MDA-MB-231 cells (Fig. 16A, C) and MCF-7 cells (Fig. 16B) treated with 20 µM/ml curcumin, 20 µM/ml ATRA and both 20 µM/ml curcumin and 20 µM/ml ATRA were subjected to ELISA and incubated with anti-FAK (Fig. 16A, B) or anti-p-PI3K (Fig. 16C) antibodies followed by HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** $p< 0.05$; *** $p< 0.01$.

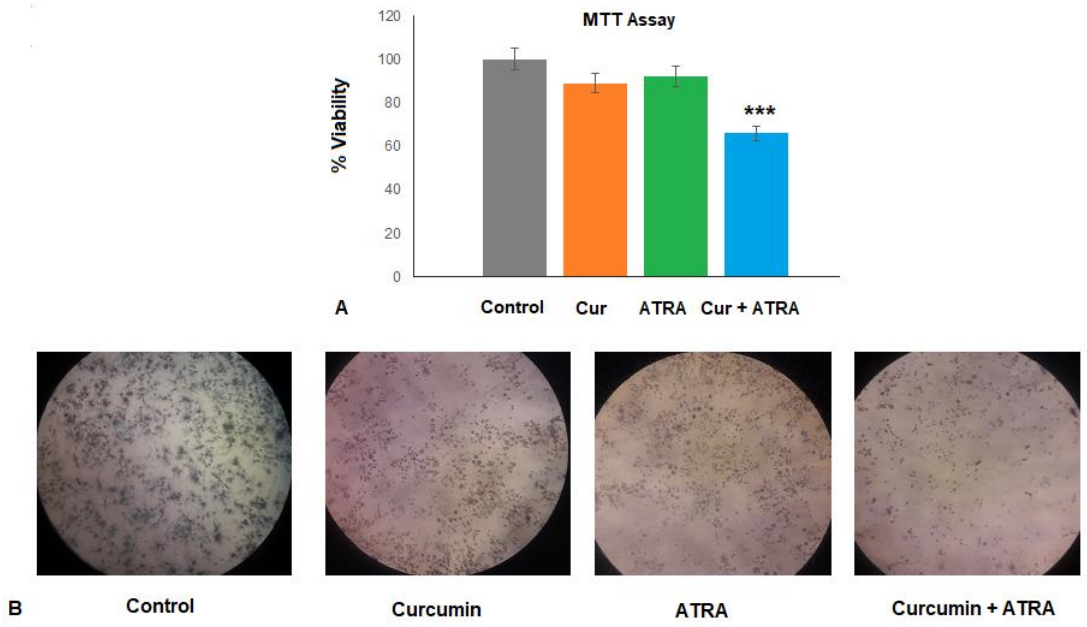

Fig. 17: Effect of Curcumin and ATRA Treatment on Cell Viability of MDA-MB-231 Breast Cancer Cells. Cell viability assays using MTT assay (Fig. 17A) were performed with control MDA-MB-231 cells and MDA-MB-231 cells treated with curcumin (20 μM/ml), ATRA (20 μM/ml) and both curcumin and ATRA (20 μM/ml each). Viability in control cells was taken as 100%. ** $p < 0.05$, *** $p < 0.01$. Morphology of control MDA-MB-231 cells and MDA-MB-231 cells treated with curcumin, ATRA and both curcumin and ATRA as viewed under inverted phase contrast microscope (Fig. 17B).

4.2.9. Effect of Curcumin and ATRA Treatment on Cell Viability of MDA-MB-231 Breast Cancer Cells: In cell viability assays with MTT assay, singular treatments with curcumin (20 μM/ml) and ATRA (20 μM/ml) showed partially loss of cellular viability compared to control where cell viability was stable and high. Synergistic treatment with both curcumin and ATRA (20 μM/ml each) significantly reduced cell viability compared to singular treatments (Fig. 17A, B).

4.2.10. Effect of Curcumin and ATRA Treatment on Cell Migration of MDA-MB-231 Breast Cancer Cells: Wound healing assay showed that treatment of MDA-MB-231 cells cultured with curcumin (20 μM/ml) or ATRA (20 μM/ml) appreciably inhibited cell migration and invasive potential in comparison to control cells cultured without such treatment. Synergistic treatment with both curcumin and ATRA (20 μM/ml each) was more effective, causing appreciably more inhibition of cell migration in comparison to treatment with curcumin or ATRA alone (Fig. 18).

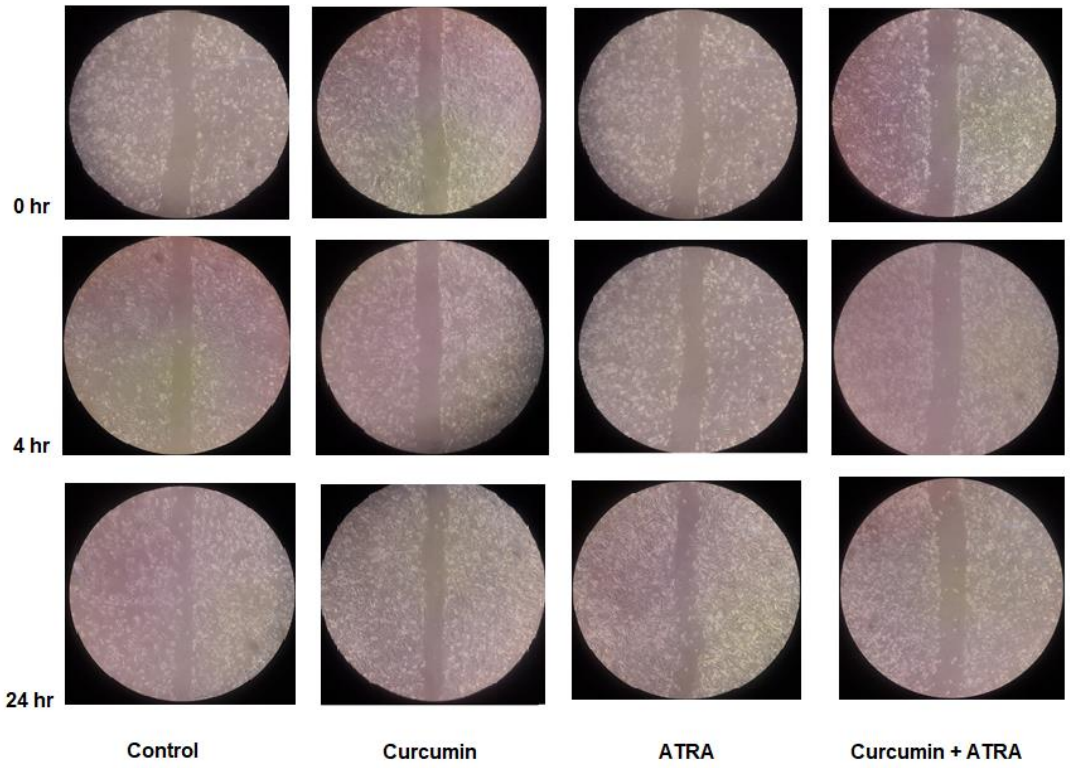

Fig. 18: Effect of Curcumin and ATRA Treatment on Cell Migration of MDA-MB-231 Breast Cancer Cells: Wound healing assay was performed with control MDA-MB-231 cells and MDA-MB-231 cells treated with curcumin (20 μM/ml), ATRA (20 μM/ml) and both curcumin and ATRA (20 μM/ml each). The extent of cell migration into the wound area was

4.3 Effect of Treatment with Curcumin and ATRA on Epidermal Growth Factor Receptor (EGFR) in Metastatic Breast Cancer Cells

4.3.1. Interaction of Curcumin, ATRA and Synthetic Inhibitor Erlotinib with kinase domain of EGFR: Molecular docking studies (Table 9) indicated that, curcumin (ΔG = -6.17 kcal/mole) and ATRA (ΔG = -8.18 kcal/mole) showed very high affinity of binding to kinase domain of human of EGFR which were appreciably greater than the binding affinity of the synthetic inhibitor Erlotinib (ΔG = -4.53 kcal/mole) (Fig. 19).

Analysis of the EGFR-ATRA docked complex with PYMOL showed ATRA formed hydrogen bonds with Lys-689, Lys-715 and Asp-982 (Fig. 19G). Other interactive amino acids in binding region included Ile 691, Lys 704, Gly 705, Leu 706, Ile 708, Pro 717, Leu 768, Pro 770 and Met 983. Analysis of the EGFR-curcumin docked complex with PYMOL revealed hydrogen bonding interactions of Gln-677, Leu-679, Arg-681 and Ile 994 with curcumin at the binding site (Fig. 19H). Other interactive amino acids included Pro 675 Asn 676, Ala 678, Trp 707, Ala 743, Ser 744, Val 745, Asp 746, Arg 752, Leu 753, Leu 754, Gly 755 Arg 807, Ala 989, Tyr 992 and Leu 993. Analysis of EGFR-Erlotinib docked complex with PYMOL revealed Gln-677 formed hydrogen bonds with Erlotinib (Fig. 19I). Other interactive amino acids included Pro675, Asn676, Ala678, Leu 679, Arg 681, Trp 707, Met 742, Ala743, Ser744, Val 745, Asp746, Arg 752, Leu 753, Leu 754, Arg807, Ala989, Tyr992, Leu 993 and Ile994.

Compound	PubChem CID	Δ G (kcal/mole)	Hydrogen Bonding to Amino Acids with Bond Lengths
ATRA	444795	-8.18	Lys-689 (2.4 Å), Lys-715 (2.0Å and 2.5 Å), Asp-982 (2.1Å and 1.6 Å)
Curcumin	969516	-6.17	Gln-677 (2.3Å), Leu-679 (2.0 Å), Arg-681 (1.9 Å), Ile-994 (2.1Å)
Erlotinib	176870	-4.53	Gln-677 (2.0Å)

Table 9: Interactions of ATRA, curcumin, and synthetic inhibitor Erlotinib with kinase domain of human EGFR (PDB ID: 2GS2).

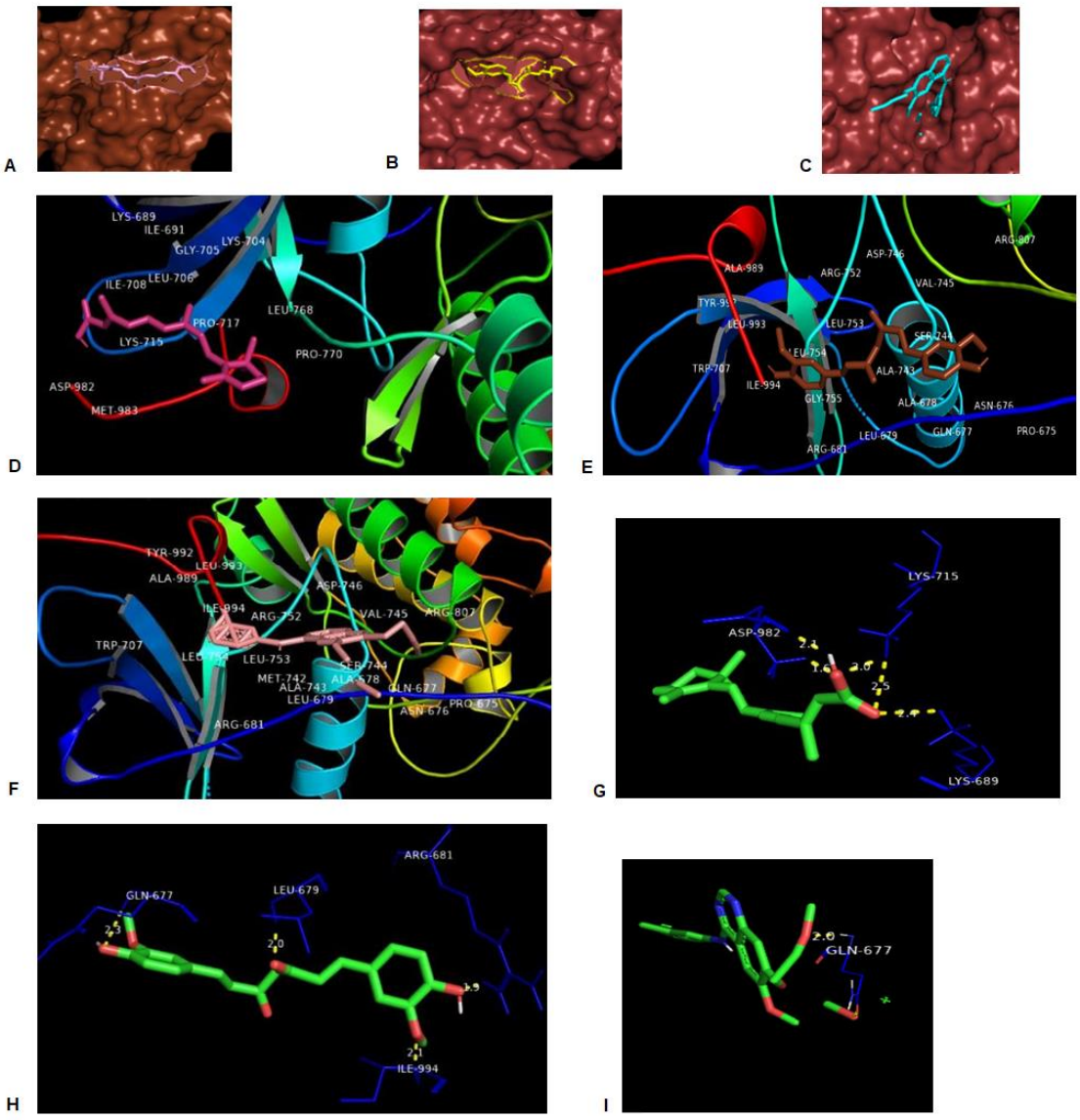

Fig. 19: Interactions of kinase domain of human EGFR with ATRA, curcumin and Erlotinib: Interactions of kinase domain of human EGFR with ATRA, curcumin and Erlotinib represented using surface zoomed view (Fig. 19A, 19B and 19C respectively) and ribbon representations (Fig. 19D, 19E and 19F respectively) and hydrogen bonding of amino acids of human EGFR with ATRA (Fig. 19G) curcumin (Fig. 19H) and Erlotinib (Fig. 19I) with estimated bond distances.

4.3.2. Effect of Curcumin and ATRA Treatment on EGFR phosphorylation in MDA-MB-453 Breast Cancer Cells cultured on EGF: Treatment of MDA-MB-453 cells cultured on 1 µg/ml EGF with curcumin (20 µM/ml) and ATRA (20 µM/ml) caused significant downregulation of EGFR phosphorylation ($p < 0.05$) compared to MDA-MB-453 cells cultured on EGF where no treatment was done (Fig. 20).

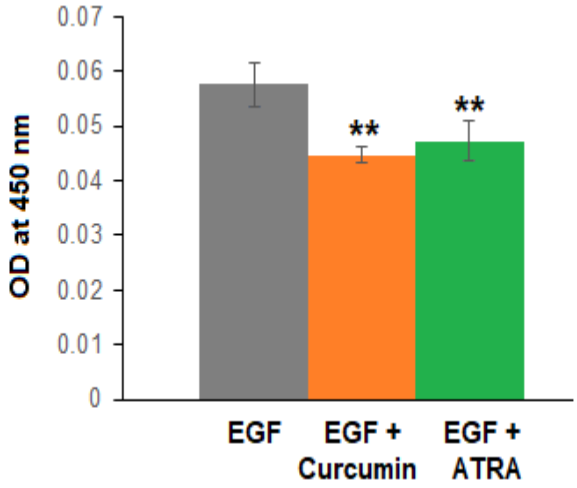

Fig 20: Effect of Curcumin and ATRA Treatment on EGFR phosphorylation in MDA-MB-453 Breast Cancer Cells. Cell extracts of MDA-MB-453 cultured on 1 µg/ml EGF and cultured on EGF with 20 µM/ml curcumin or 20 µM/ml ATRA were subjected to ELISA and incubated with anti-p-EGFR antibodies followed by HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** $p< 0.05$.

4.3.3 Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Expression in MDA-MB-453 Cells Cultured on EGF: On culturing MDA-MB-453 cells on 1 µg/ml EGF in presence of 20 µM/ml curcumin or 20 µM/ml ATRA, MMP-2 and MMP-9 expression in cell extracts and culture supernatants was significantly reduced (for MMP-2 in cell extracts, $p<0.01$; for MMP-2 in culture supernatants and MMP-9, $p<0.05$) compared to MDA-MB-453 cells cultured on 1 µg/ml EGF without such treatment. Treatment with curcumin was more effective in downregulating MMP expression than treatment with ATRA.(Fig. 21)

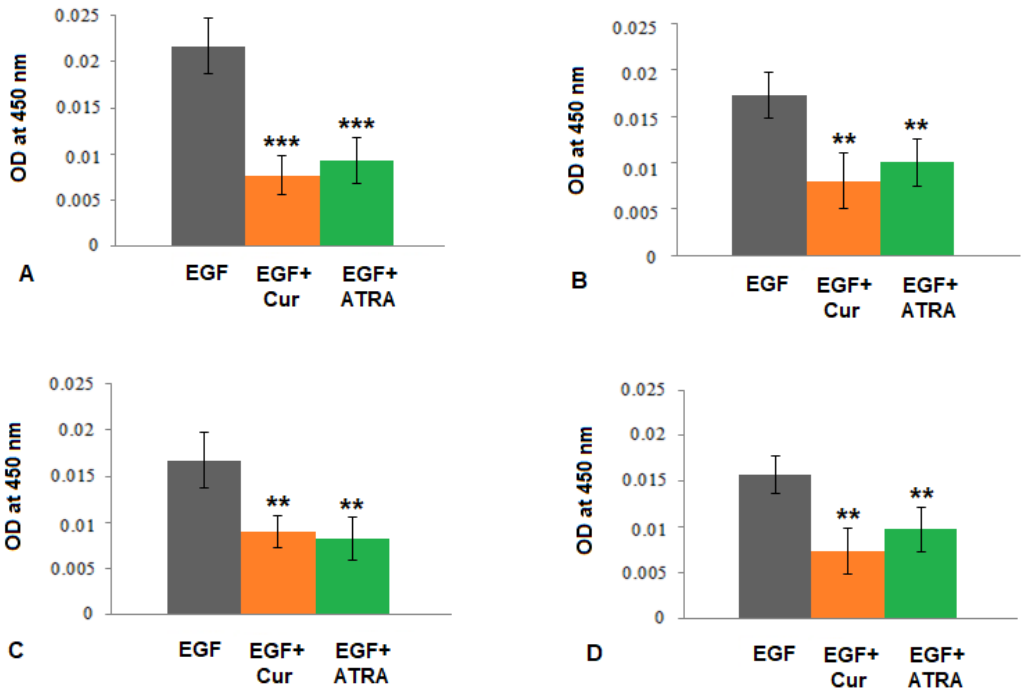

Fig. 21: Effect of Curcumin and ATRA Treatment on MMP 2 and MMP9 Expression in MDA-MB 453 Breast Cancer cells cultured on EGF: Cell extracts (Fig. 21 A and C) and culture supernatants (Fig. 21 B and D) of MDA-MB-453 cells cultured on 1 µg/ml EGF and MDA-MB-453 cells cultured on 1 µg/ml EGF with 20 µM/ml curcumin or 20 µM/ml ATRA were subjected to ELISA and incubated with anti-MMP-2 (Fig. 21 A, B) and anti-MMP-9 (Fig. 21 C, D) antibodies and HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** $p< 0.05$, *** $p<0.01$.

4.3.4. Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Activity in MDA-MB-453 Cells Cultured on EGF: Treatment of MDA-MB-453 cells cultured on 1 µg/ml EGF with curcumin and ATRA significantly downregulated MMP-2 and MMP-9 activity in comparison to MDA-MB-453 cells cultured on EGF without such treatment (Fig. 22).

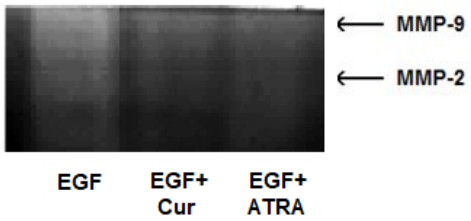

Fig. 22: Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 Activity: Cell extracts of MDA-MB-453 cells cultured on 1 µg/ml EGF and MDA-MB-453 cells cultured on

1 μg/ml EGF with 20 μM/ml curcumin or 20 μM/ml ATRA were subjected to gelatin zymography on 10% SDS-PAGE co-polymerized with 0.1% gelatin.

4.3.5 Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 mRNA Expression in MDA-MB-453 Cells Cultured on EGF: Treatment of MDA-MB-453 cells cultured in presence of 1 μg/ml of EGF with curcumin and ATRA appreciably downregulated MMP-2 and MMP-9 mRNA expression in comparison to MDA-MB-453 cells cultured on 1 μg/ml of EGF without such treatment (Fig. 23).

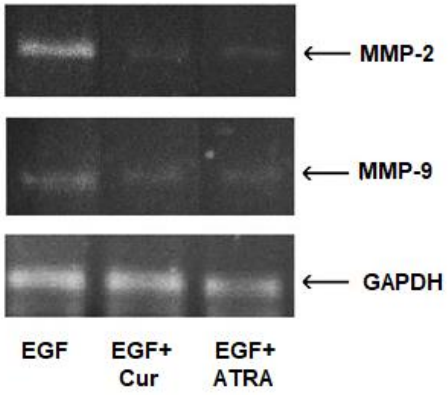

Fig.23: Effect of Curcumin and ATRA Treatment on MMP-2 and MMP-9 mRNA Expression in MDA-MB 453 cells cultured on EGF: RT-PCR was carried out with RNA from MDA-MB-453 cells cultured on 1 μg/ml EGF and MDA-MB-453 cells cultured on 1 μg/ml EGF with 20 μM/ml curcumin or 20 μM/ml ATRA using specific primers (MMP-2, MMP-9) for PCR. PCR products were run on 1.5% agarose gel. GAPDH was used as control to normalize for mRNA integrity and equal loading.

4.3.6. Effect of Curcumin, ATRA Treatment and PI3K Inhibition on MMP Expression in MDA-MB-453 Cells Cultured on EGF: Treatment of MDA-MB-453 cells cultured on 1 μg/ml EGF with PI3K inhibitor LY294002 significantly inhibited EGFR modulated expression of MMP-2 and MMP-9 in cell extracts and culture supernatants ($p<0.05$). Treatment of MDA-MB-453 cells cultured on 1 μg/ml EGF with 20 μM/ml curcumin or 20 μM/ml ATRA caused significant downregulation of MMP-2 and MMP-9 expression equivalent to or more significant (for MMP-2 in cell extracts, $p<0.01$; for MMP-2 in culture supernatants and MMP-9, $p<0.05$) compared to treatment with LY294002 (Fig. 24A-D).

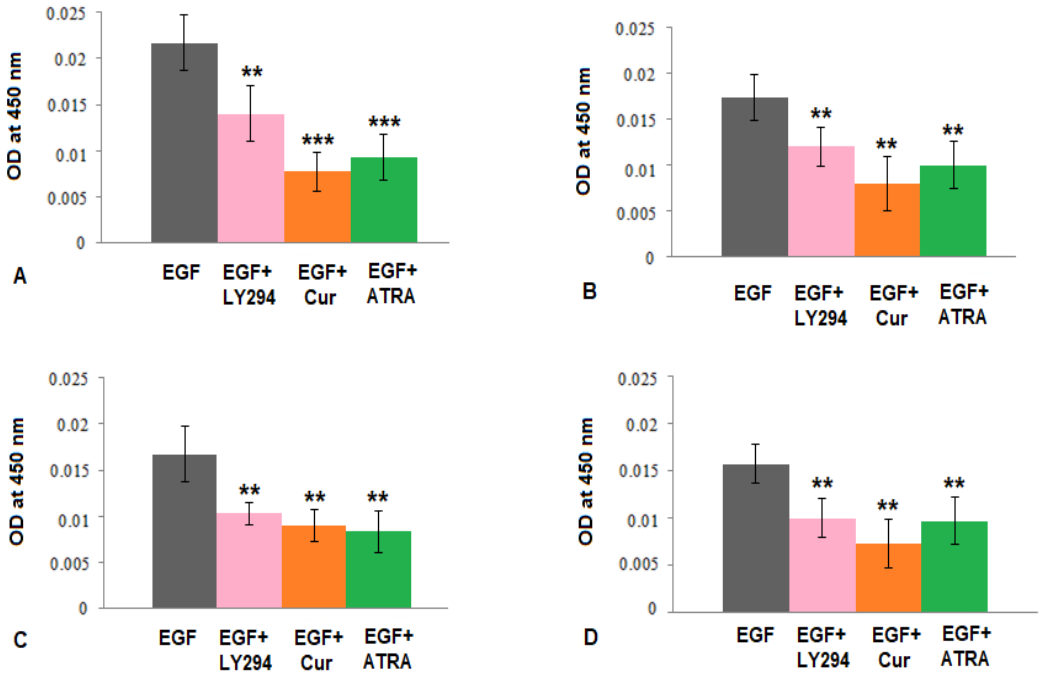

Fig. 24: Effect of Curcumin and ATRA Treatment and PI3K Inhibition on MMP Expression in MDA-MB 453 cells cultured on EGF: Cell extracts (Fig. 24 A, C) and culture supernatants (Fig. 24 B. D) of MDA-MB-453 cells cultured on 1 µg/ml EGF, MDA-MB-453 cells cultured on EGF with PI3K inhibitor LY294002 and MDA-MB-453 cells cultured on EGF with 20 µM/ml curcumin or 20 µM/ml ATRA were subjected to ELISA and incubated with anti-MMP-2 (Fig. 24 A, B) and anti-MMP-9 (Fig. 24 C, D) antibodies and HRP coupled secondary antibodies. TMB substrate was added and optical density was measured at 450 nm. ** p< 0.05, *** p,0.01.

4.3.7. Effect of Curcumin and ATRA Treatment on Cell Migration of MDA-MB 453 cells on EGF: Wound healing assay showed that treatment of MDA-MB-453 cells cultured on EGF with curcumin (20 µM/ml) or ATRA (20 µM/ml) appreciably inhibited cell migration and invasive potential in comparison to control cells cultured on EGF where no treatment was done (Fig. 25).

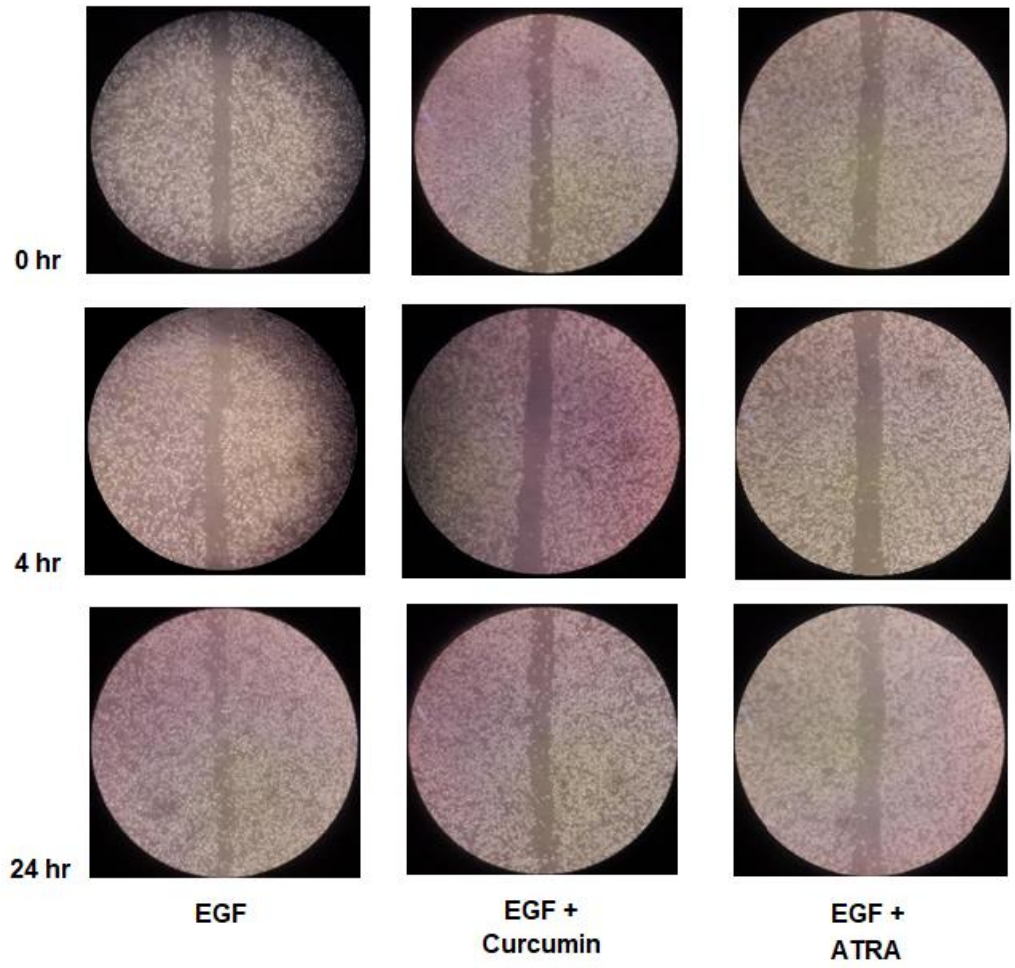

Fig. 25: Effect of Curcumin and ATRA Treatment on Cell Migration on EGF: Wound healing assay was performed with MDA-MB-453 cells cultured on EGF and MDA-MB-453 cells cultured on EGF with 20 μM/ml curcumin or 20 μM/ml ATRA. The extent of cell migration into the wound area was determined by viewing under inverted phase contrast microscope.

DISCUSSION

5. DISCUSSION

Breast cancer, is highly prevalent among women in India and worldwide and often leads to fatal outcomes due to poor prognosis and late diagnosis, making targeting of molecules which promote breast cancer progression and metastasis crucial for therapeutic purposes. Breast cancer can develop due to aberration in various signalling cascades and growth factor receptors. Advanced and invasive breast cancer can metastasize due to dysregulation of signalling cascades and increased MMP expression and activity.

MMP-2 and MMP-9, also known as gelatinase A and gelatinase B predominantly contribute in development of breast metastases in distant organs. Overexpression of MMP-2 and MMP-9 has been reported in invasive, aggressive and advanced grade breast tumours. Increase in MMP-2 and MMP-9 expression and activity has been linked with metastasis to distant organs and reported to promote lymph node, brain, bone and liver metastasis in breast cancers *[Brown et al. 1993; Radisky et al., 2015; Jiang et al., 2021; Mendes et al.,2007; Sardar et al., 2025; Golubnitschaja et al., 2016]*. Thus, high expression and activity of MMP-2 and MMP-9 has been shown to play key roles in breast cancer metastasis and contributes to poor prognosis and relapse of the disease. In our study, MDA-MB-453 (metastatic human breast carcinoma), MDA-MB-231 (invasive human breast adenocarcinoma) and MCF-7 (invasive human breast ductal carcinoma) cell lines were used as models. High expression of MMP-2 and MMP-9 has been reported in breast cancer cells lines including MDA-MB 453, MDA-MB-231 and MCF-7 *[Das et al., 2008, Choi et al., 2011, Li et al., 2017, Hui et al., 2010]*.

Our investigation was carried out with two important natural phytochemical compounds, ATRA and curcumin which have shown excellent potential as effective anti-tumorogenic agents in cancer research. ATRA induces myeloid differentiation, downregulates signalling cascades and expression of proteins and cellular factors to cause apoptosis and inhibit cellular proliferation and migration and anti-cancer effects of ATRA treatment have been reported in leukemia and various other cancers *[Das et al., 2013; Siddikuzzaman et al., 2013, Lo-Coco et al., 2008, Bobal et al., 2021, Caricasulo et al., 2024]* although the molecular effects of ATRA on many solid tumours are yet to be explored in as much detail compared to leukemic cells. Anti-tumorigenic properties of curcumin have been reported in bladder, breast, colon,

prostate, ovary, cervical, gastrointestinal and skin cancers via regulation of signal transduction, downregulation of signalling molecules, proteins and receptors, inhibition of cell proliferation and induction of apoptosis *[Sa et al., 2008; Wang et al., 2016, Barcelos et al., 2022, Fu et al., 2021, Liu et al., 2018, Farghadani et al., 2021]*.

Although varied in their mode of action, composition and chemistry, yet the increase in efficacy of action of one compound in presence of another contributes in positive outcome of synergistic treatment. ATRA in synergism with arsenic trioxide showed significant results in treatment of acute promyelocytic leukemia *[Zhou et al., 2007]*. ATRA in synergism with arsenic trioxide further showed promising results causing growth inhibition and apoptosis in human breast cancer, hepatoma and lung cancer cells *[Lin et al., 2005]*. In case of curcumin, synergistic or combinatorial treatment with drugs like doxorubicin, paclitaxel, carboplatin, metformin and docetaxel has resulted in reversal of drug resistance and increase in cytotoxicity and death of cancer cells *[Younes et al., 2022]*. Previous studies with synergistic treatment of curcumin with 5-flurouracil in HT-29 human colon cancer and fenretinide in lung cancer A549 and H1299 cells showed promising results *[Du et al., 2005; Chen et al., 2016]*. Curcumin treatment was shown to increase ATRA linked differentiation in NB4 acute promyelocytic cells *[Kini et al, 2005]*. Taking these previous studies into consideration, our investigation aimed in analysis of effects of synergistic treatment of ATRA and curcumin on signalling molecules and MMPs in breast cancers.

In our study, when MDA-MB-453 cells were treated with 20μM/ ml of ATRA and 20μM/ ml of curcumin, MMP-2 and MMP-9 expression was significantly reduced ($p<0.05$) compared to control cells which had no such treatment. However, synergistic treatment with both curcumin and ATRA (20μM/ ml each) was considerably more effective than singular treatments, causing significantly more downregulation of MMP-2 and MMP-9 expression ($p<0.01$) in cell extracts compared to control cells. Synergistic treatment with both curcumin and ATRA also caused appreciably more downregulation of MMP-2 and MMP-9 activity in comparison to treatment with curcumin or ATRA alone.

Similar results were obtained for MDA-MB-231 and MCF-7 cells. When MDA-MB-231 and MCF-7 cells were treated with 20μM/ ml of ATRA and 20μM/ ml of curcumin, MMP-2 and MMP-9 expression was significantly reduced ($p<0.05$; $p<0.01$ for MMP-2 in curcumin treated MCF-7 cells) but synergistic treatment with both curcumin and ATRA (20μM/ ml each) was considerably more effective than singular treatments

causing appreciably more downregulation of MMP-2 and MMP-9 expression in cell extracts ($p<0.01$; $p<0.05$ for MMP-9 in MDA-MB-231 cells). Synergistic treatment with both curcumin and ATRA also caused appreciably more downregulation of MMP-2 and MMP-9 activity in comparison to treatment with curcumin or ATRA alone in MDA-MB-231 and MCF-7 cells. Previous reports indicate treatment of ATRA caused reduction of MMP-2 expression in MCF-7 cells *[Dutta et al., 2009]* and curcumin treatment downregulated MMP-9 expression in MDA-MB-231 and MCF-7 cells and MMP-2 expression in MDA-MB-231 cells *[Mo et al., 2012; Kim et al., 2012; Hassan et al., 2012]*. In line with these studies, our investigations also showed effective downregulation in MMP-2 and MMP-9 expression in MCF-7 and MDA-MB-231 cells upon treatment with curcumin and ATRA and also downregulation of MMPs in metastatic MDA-MB-453 breast cancer cells. However, our investigations significantly indicated synergistic treatment with both curcumin and ATRA (20μM/ml each) was considerably more effective in appreciably reducing MMP-2 and MMP-9 activity in cell extracts and culture supernatants of MDA-MB-453, MDA-MB-231 and MCF-7 cell lines compared to singular treatments with curcumin and ATRA alone, indicating the potential of synergistic treatment with curcumin and ATRA in inhibiting MMPs in metastatic breast cancer cells.

As singular treatment with curcumin showed more appreciable reduction of MMP-2 and MMP-9 expression compared to singular treatment with ATRA, MDA-MB-231 and MCF-7 breast cancer cell lines were treated with a higher dose of curcumin (40μM/ ml) and effects were compared with synergistic treatment with both curcumin and ATRA (20 μM/ ml each). Synergistic treatment with both curcumin and ATRA downregulated MMP-2 expression in MCF-7 and both MMP-2 and MMP-9 expression in MDA-MB-231 cells more appreciably compared to the increased dose (40μM/ ml) of curcumin. These findings further indicate the efficacy of synergistic treatment of ATRA and curcumin in downregulation of MMP-2 and MMP-9 in breast cancer cells.

MT1-MMP (MMP-14) plays a crucial role in activation of MMP-2 and metastasis in breast cancers. Overexpression of MT1-MMP and MMP-2 has been correlated with development of invasive breast cancer phenotype and lymph node metastasis *[Yao et al., 2013, Zhang et al., 2013, Seiki et al., 2003, Shiomi et al., 2003, Ueno et al., 1997]*. In our study, in highly metastatic MDA-MB-453 breast cancer cells, singular treatments with curcumin (20 μM/ ml) and ATRA (20 μM/ ml) caused significant downregulation of MT1-MMP compared to control cells ($p<0.05$) and synergistic treatment with both

curcumin and ATRA (20μM/ ml each) was more significant in reduction of MT1-MMP expression ($p<0.01$) compared to singular treatments with curcumin or ATRA alone. As MT1-MMP is involved in activation of MMP-2 to promote tumour metastasis, downregulation of MT1-MMP could be a cause for observed loss of MMP-2 activity in MDA-MB-453 cells. Also, as MT1-MMP can act as a cell membrane receptor for MMP-2, its downregulation can inhibit MMP-2 localization to cell surface and breast cancer metastasis.

Thus, our study indicated treatment of breast cancer cell lines with curcumin or ATRA caused significant downregulation of MMP-2 and MMP-9 expression and activity. Synergistic treatment with both curcumin and ATRA was significantly more effective than singular treatments, causing appreciably more downregulation of MMP-2 and MMP-9 expression and activity and MT1-MMP expression in comparison to treatment with curcumin or ATRA alone.

Study of the pattern of interaction of curcumin and ATRA with MMPs was done using molecular docking to determine their affinity for MMP-2, MMP-9 and MT1-MMP catalytic domains. Affinity and interactions were compared with synthetic inhibitors of MMPs like Batimastat *[Alves et al., 2024,]*, Rebimastat *[Setti et al., 2017, Kumari et al., 2025]* and doxycycline *[Hadjimichael., 2020]*. Molecular docking studies with natural phytochemical compounds ATRA and curcumin with MMP-2 catalytic domain as target molecule showed a very high binding affinity of curcumin (-10.99 kcal/mole) and ATRA (-10.11 kcal/mole) in comparison to synthetic MMP inhibitors Doxycycline, Rebimastat and Batimastat, indicating that curcumin and ATRA have very good potential as inhibitors of MMP-2. Analysis of the docked complexes showed ATRA, curcumin, Rebimastat and Batimastat interacting with a similar region on the MMP-2 catalytic domain. There were similarities in many amino acid interactions but certain amino acids showed different kinds of interactions with the different molecules studied. In docking of MMP-2 catalytic domain with curcumin and ATRA, similarity in hydrogen bond interactions with residues Leu164 and Ala 165 were seen. Docking of MMP-2 with ATRA and curcumin, showed interactions also with Gly162, Leu163, Leu197, Val198, His201, Glu 202, His211, Ala217, Ala220, Pro221, Ile222 and Thr 227 amino acids residues. However, Tyr 223 showed hydrogen bonding with curcumin but non-polar interaction with ATRA. This similarity in amino acid interactions in the binding region indicate curcumin and ATRA interact within a similar binding region on MMP-2 catalytic domain. Batimastat-MMP-2 interactions also showed some

similarity with ATRA and curcumin where Ala 165 and Leu 164 showed hydrogen bond interactions. In batimastat–MMP-2 docking, Glu 202 and Pro221 showed hydrogen bonding whereas these two amino acids showed non-polar interactions with ATRA and curcumin. Tyr 193 showed non-polar interactions with both Batimastat and curcumin while Tyr 223 showed hydrogen bonding with curcumin and non-polar interactions with ATRA and Batimastat. For Rebimastat-MMP-2 interactions, similarity in hydrogen bond interactions with Leu164 was seen. Tyr 223 showed hydrogen bonding with curcumin and Rebimastat and non-polar interactions with ATRA. Rebimastat also showed hydrogen bonding with Pro221 which had non-polar interactions with ATRA and curcumin and non-polar interactions with Ala 165 which showed hydrogen bonding with curcumin and ATRA.

Curcumin and ATRA showed a very high binding affinity for MMP-9 catalytic domain with ΔG values of -11.23 kcal/mole and -11.13 kcal/mole respectively compared to synthetic inhibitors Rebimastat and Batimastat. In docking MMP-9 catalytic domain with curcumin and ATRA, similarities in hydrogen bond interactions were seen with Leu188 and Ala 189. Interestingly, Leu222 and Arg 249 showed hydrogen bonding with curcumin but non-polar interactions with ATRA. Docking of MMP-9 with ATRA and curcumin showed similarity in non-polar interactions like Gly186, Leu187, His190, Val223, Glu241, Ala242, Leu243, Tyr245, Pro246, Met247, Tyr248 and Thr251 which implies curcumin and ATRA interact within a similar binding region on MMP-9 catalytic domain. Analysis of the docked complexes involving MMP-9 showed curcumin and ATRA interact at similar sites on the MMP-9 catalytic domain while both Batimastat and Rebimastat bind at a different nearby region in close proximity with each other.

In our molecular docking studies, some alanine and leucine residues showed polar (hydrogen) bonding with ATRA and curcumin. Previous studies have shown Ala 189 and Leu188 residues in hydrogen bonding when cinnamic acid derivatives were docked with MMP-9 (using the same molecule used in our study, PDB ID: 5I12) *[Malekipour et al., 2023]*. When resveratrol was docked with MMP-2 for studying the importance of MMPs in cerebral ischemia, MMP-2 (the same molecule used in our study, PDB ID: 1QIB) showed polar interactions with resveratrol via Ala 165 and Leu164 residues *[Pandey et al., 2015]*. In our studies, Ala165 and Leu 164 residues of MMP-2 catalytic domain showed hydrogen bonding with both curcumin and ATRA and Ala 189 and

Leu 188 of MMP-9 catalytic domain also showed hydrogen bonding with curcumin and ATRA.

Docking of curcumin and ATRA with MT1-MMP catalytic domain showed a very high binding affinity of curcumin and ATRA with ΔG values of -10.78 kcal/mole and -8.28 kcal/mole respectively. Binding affinities of ATRA and curcumin were much higher than binding affinities of synthetic MMP inhibitors Batimastat and Rebimastat.

In docking with MT1-MMP catalytic domain, ATRA and curcumin showed similarities in amino acid interactions like Phe 198, Ala200, Leu 235, Val 236, Glu240, His 249, Ile 256, Met 257, Ala 258, Phe 260, Tyr 261 indicating similar binding sites. Leu199, Ala202, His 239, Pro259 and Gln 262 showed hydrogen bonding with curcumin and non-polar interactions with ATRA. Curcumin, ATRA and Rebimastat interact with a similar region on the MT1-MMP catalytic domain while Batimastat interacts with a different site.

Thus, the natural compounds curcumin and ATRA showed similarity with synthetic inhibitors in some amino acid interactions and binding regions on MMP-2, MMP-9 and MT1-MMP but the binding affinities of curcumin and ATRA were more compared to Batimastat and Rebimastat indicating their very good potential as inhibitors of MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MT1-MMP in breast cancers. The differences in amino acid interactions could possibly indicate different mechanisms of MMP inhibition by these compounds.

Molecular docking (double docking) showed quite high binding affinity of curcumin with MMP-2 catalytic domain-ATRA complex and ATRA with MMP-2 catalytic domain-curcumin complex with ΔG values of -6.27 kcal/mole and -7.11 kcal/mole respectively. In double docking with MMP-9 catalytic domain-curcumin complex, ATRA showed an appreciable binding affinity of ΔG = -6.81 kcal/mole and curcumin also showed an appreciable binding affinity of ΔG = -6.88 kcal/mole with MMP-9 catalytic domain in complex with ATRA. This indicated appreciable affinity for simultaneous interactions with MMP-2 and MMP-9 catalytic sites indicating good potential for synergistic inhibition of MMP-2 and MMP-9 by curcumin and ATRA as observed in our experimental studies with MDA-MB-453, MDA-MB-231 and MCF-7 cells.

In double docking with MT1-MMP-curcumin complex, ATRA showed very good binding affinity of ΔG = -8.13 Kcal/mole and curcumin also showed an appreciable binding affinity for MT1-MMP catalytic domain in complex with ATRA with ΔG value

of ΔG = -6.85 Kcal/mole. Thus, like MMP-2 and MMP-9, these results of double ligand interaction of ATRA and curcumin with MT1-MMP catalytic domain again indicated significant potential for synergistic inhibition of MT1-MMP expression by ATRA and curcumin as shown in results of our experimental studies with MDA-MB-453.

Various intracellular signalling pathways are involved in regulation of MMP expression and activity in tumour cells including the FAK pathway and the PI3K/Akt pathway *[Aziz et al., 2012, Choi et al.,2016, Guan et al., 2010, Majumder et al., 2019*]. Aberrant and increased signalling thorough these pathways can promote tumour metastasis. PI3K has been associated with breast cancers with the catalytic subunit p110α showing aberrant increased activity due to activating mutations or gene amplifications.

In our study, when MDA-MB-453, MDA-MB-231 and MCF-7 cells were treated with 20μM/ml of ATRA and 20μM/ml of curcumin, FAK expression was significantly reduced ($p<0.05$) compared to control cells which had no such treatment. However, synergistic treatment with both curcumin and ATRA (20μM/ ml each) was considerably more effective than singular treatments, causing significantly more downregulation of FAK expression ($p<0.01$) compared to control cells. Molecular docking of FAK was done with ATRA and curcumin to study the affinity of these natural compounds for the signalling molecule in comparison to Defactinib which is a synthetic FAK inhibitor. Defactinib has been reported to downregulate FAK and inhibit downstream PI3K/Akt signalling in oesophageal cancer cells and inhibit FAK in ovarian cancers *[Zhang et al., 2021; Moore et al., 2014]*. In molecular docking, ATRA and curcumin showed a good binding affinity for FAK with ΔG values of -7.24 kcal/mole and -7.54 kcal/mole respectively which were higher compared to synthetic FAK inhibitor defactinib which indicate their very good potential as inhibitors of FAK signalling. Analysis of the docked complexes and amino acid interactions showed ATRA, curcumin and defactinib interact at different sites on the FAK molecule.

Double docking studies showed appreciable binding affinity of ATRA with FAK-curcumin complex with a ΔG value of -7.02 kcal/mole. Curcumin also showed an appreciable binding affinity to FAK-ATRA complex with ΔG value of -6.11 kcal/mole. These binding affinities were higher than that of the synthetic FAK inhibitor defactinib, indicating very good potential for synergistic inhibition of FAK by ATRA and curcumin as also shown in results of our experimental studies with MDA-MB-453, MDA-MB-231 and MCF-7 cells. Overexpression of FAK is an important prognostic

marker in breast cancer and high FAK expression levels contribute to tumour relapse and poor prognosis. FAK signalling also promotes breast cancer cellular growth and migration in breast cancers *[Sulzmaier et al., 2014; Tiede et al., 2018, Rigiracciolo et al., 2021]*. Inhibition of FAK by synergistic treatment with both curcumin and ATRA could therefore result in improved treatment outcomes for breast cancers.

Integrin receptor linked FAK activation has been reported to cause downstream PI3K activation and tumorogenesis in breast cancer *[Chen et al., 2019, Yang et al., 2016; Das et al. 2008]*. Increased phosphorylation of PI3K has been reported in the breast cancer cell lines of MDA-MB-231 and MDA-MB 453 *[Tohkayomatee et al., 2022, Elumalai et al., 2014; Guo et al., 2019]*. When MDA-MB- 453 and MDA-MB-231 breast cancer cells were treated with singular treatments of ATRA (20 μM /ml) and curcumin (20 μM/ ml), p-PI3K was significantly reduced in MDA-MB-453 and MDA-MB-231 cells compared to control cells ($p< 0.05$). Synergistic treatment with both curcumin and ATRA (20 μM/ ml each together) was again more appreciable and significant ($p< 0.01$; $p< 0.05$ for MDA-MB-453) in causing downregulation of p-PI3K in these cell lines.

In our docking studies, ATRA and curcumin showed an appreciable binding affinity with PI3K p110α catalytic subunit with ΔG values of -6.46 kcal/mole and -6.94 kcal/mole respectively which were in similar range with binding affinities of alpelisib and LY294002 (synthetic PI3K inhibitors). LY294002 is a broad spectrum PI3K inhibitor reported to bind class I PI3Ks *[Gharbi et al., 2007]* and Alpelisib is an inhibitor of p110α catalytic subunit of PI3K which has been approved for breast cancer treatment by FDA in PIK3CA mutated breast cancers *[Ortega et al., 2020; Chang et al., 2021]*. Analysis of the docked PI3K-ligand complexes indicated ATRA, curcumin and LY294002 appear to preferentially interact at nearby sites on p110α catalytic subunit of PI3K compared to Alpelisib. Curcumin showed very good binding affinity with PI3K-ATRA complex with ΔG value of -7.52 kcal/mole and ATRA when docked with PI3K-curcumin complex, showed an appreciable binding affinity with ΔG value of -6.69 kcal/mole. These binding affinities were greater than for curcumin or ATRA binding separately with PI3K and also comparable to the synthetic PI3K inhibitors which indicated significant potential for synergistic inhibition of PI3K by ATRA and curcumin.

Overexpression of class I PI3Ks has been reported in breast cancer and around 70% of breast cancers have *PIK3CA* mutations which encode for the p110α catalytic subunit

[Ortega et al., 2020; Miricescu et al., 2020]. The high affinity of ATRA and curcumin for the PI3K catalytic subunit indicates that they have very good potential as inhibitors of PI3K, indicating synergistic treatment with both curcumin and ATRA could therefore result in improved treatment outcomes for breast cancers by inhibition of PI3K signalling.

Integrin mediated signalling through FAK promotes upregulation of MMP activity in cancer cells which leads to increased invasive potential *[Banerji et al. 2008; Das et al. 2008]*. Signalling through FAK is important in regulation of MMP-2 and MMP-9 expression and activity in breast cancer *[Villegas-Comonfort et al., 2014, Das et al. 2008; Majumder et al., 2019]*. Integrin signalling and FAK activation has been correlated with overexpression of MT1-MMP in metastatic breast cancers *[Corall et al., 2014, Fan et al., 2013]*. PI3K has also been reported to play a crucial role in regulation of MMP-2 and MMP-9 expression and EMT in breast cancer cells and downregulation of PI3K mediated signalling could also cause inhibition of MMP expression and activity *[Das et al. 2008*, *Rajendran et al., 2020; Majumder et al., 2019]*. Significant downregulation of FAK and PI3K upon synergistic treatment with both curcumin and ATRA could therefore cause the observed reduction in MMP-2 and MMP-9 expression and activity and MT1-MMP expression in breast cancer cells as indicated by our experimental results.

The role of FAK in survival of breast cancer cells has been elucidated in previous studies *[Paul et al, 2020, Tan et al., 2023]*. FAK aids in adhesion independent survival in breast cancer cells and downregulation of FAK expression has been reported to promote caspase 8 mediated apoptosis and FAS-associated breast cancer cell death *[Kurenova et al., 2004*, *Rigiracciolo et al., 2021]*. FAK aids in cellular proliferation, growth, migration and survival of breast cancer cells via activation of downstream PI3K signalling pathway *[Hu et al., 2024]*. Singular treatments with ATRA (20 μM/ ml) and curcumin (20 μM/ ml) caused partial loss of cellular viability in MDA-MB-453 and MDA-MB-231 cells compared to control cells while synergistic treatment with both curcumin and ATRA (20 μM/ ml each) was effective in causing more significant loss of cell viability than singular treatments. Significant downregulation of FAK and PI3K upon synergistic treatment with curcumin and ATRA could be a possible cause for reduction in cell survival.

FAK and PI3K signalling has been reported to regulate cellular proliferation, tumour development and cellular migration in metastatic breast cancer cells. Integin mediated

FAK signalling has been reported to aid in cellular migration and FAK mediates cytoskeletal reorganization which allows cellular migration and invasion in breast cancer *[Wilson et al., 2014; Rigiracciolo et al., 2021]*. Integrin mediated signal transduction through FAK promotes upregulation of MMP activity in cancer cells which leads to increased invasive potential *[Banerji et al. 2008; Das et al. 2008]*. Activation of FAK and PI3K signalling pathways and increased expression of MMP-2 and MMP-9 contributes to increased cellular migration and invasive potential of breast cancer cells *[Das et al. 2008; Majumder et al., 2019]*. Wound healing assays with MDA-MB-231 and MDA-MB-453 cells showed treatment with curcumin and ATRA significantly inhibited cell migration in comparison to control cells with efficacy of wound healing more appreciably inhibited upon synergistic treatment, indicating the potential of synergistic treatment with curcumin and ATRA in reducing invasive potential in breast cancer cells.

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play crucial roles in regulation of invasion, metastasis, tumour progression and development in breast cancer and increase in levels of MMP-2 and MMP-9 expression and activity show significant correlation with increased invasion and metastasis. Conversely, inhibition of MMP-2 and MMP-9 expression and activity renders breast cancer cells less invasive *[Quaranta et al., 2007, Merdad et al., 2014]*. The observed inhibition of MMP-2, MMP-9 and MT1-MMP upon curcumin and ATRA treatment could therefore contribute to a loss in invasive potential of breast cancer cells. Inhibition of signalling pathways (FAK and PI3K) which regulate MMP-2 and MMP-9 expression and activity and MT1-MMP expression upon curcumin and ATRA treatment could also contribute to a loss in invasive potential of breast cancer cells. Our investigations thus indicate synergistic treatment with the phytochemicals curcumin and ATRA appears to have significantly better anti-tumorigenic potential than treatment with curcumin or ATRA alone.

Epidermal growth factor receptor (EGFR) is overexpressed in a number of breast cancers and has been associated with increased invasiveness, poor prognosis and decreased survival *[Herbst 2004; Seshacharyulu et al. 2012]*. Phosphorylated EGFR interacts with various downstream proteins having SH2 and PTB domains *[Amelia et al., 2022, Foley et al., 2010]*. Upon interaction with its ligands, EGFR modulates cellular proliferation, differentiation and survival through a number of signalling pathways including the PI3K pathway. In breast cancer cells, interaction between EGFR and its ligand EGF promote an increase in expression and activity of MMP-2

and MMP-9 leading to increased tumour invasiveness *[Herbst 2004; Seshacharyulu et al. 2012; Park et al., 2016; Villegas-Comonfort et al., 2014; Majumder et al., 2019]*. The effect of curcumin and ATRA treatment for targeting EGFR and inhibiting MMP-2 and MMP-9 expression and activity in invasive breast cancer was studied.

The EGFR kinase domain plays a very important role in receptor dimerization and activation of EGFR. Molecular docking studies showed good binding affinity of curcumin, and ATRA with EGFR kinase domain with ΔG values of -6.17 kcal/mole and -8.18 kcal/mole in comparison to the synthetic inhibitor Erlotinib indicating that curcumin and ATRA have good potential as inhibitors of EGFR. Erlotinib is an established synthetic inhibitor already used in breast cancer treatment *[Dickler et al., 2009, Catania et al., 2006]*. Although curcumin and Erlotinib showed some similarity in non-polar interactions, ATRA, curcumin and Erlotinib appeared to interact at different sites on the EGFR kinase domain and showed no similarity in hydrogen bonding interactions.

Treatment of MDA-MB-453 breast cancer cells cultured on EGF with curcumin (20 μM/ ml) or ATRA (20 μM/ ml) caused appreciable downregulation of EGFR phosphorylation compared to control cells cultured on EGF indicating the ability of curcumin and ATRA to inhibit EGFR mediated signalling. Treatment of MDA-MB-453 breast cancers cells cultured on EGF with curcumin and ATRA also caused significant reduction in MMP-2 and MMP-9 expression at both protein and mRNA levels. Treatment of MDA-MB-453 breast cancers cells cultured on EGF with curcumin and ATRA also caused appreciable reduction in MMP-2 and MMP-9 activity. These results indicate the ability of curcumin and ATRA to inhibit EGFR mediated regulation of MMP-2 and MMP-9 in breast cancer cells. Targeting EGFR regulated downstream MMP-2 and MMP-9 expression with curcumin and ATRA could be of importance in treating invasive breast cancers.

PI3K is one of the important and significant downstream signalling pathways which gets activated upon EGFR ligand interaction and EGFR linked activation of downstream PI3K/Akt pathway promotes increased MMP-2 and MMP-9 expression in breast cancer cells *[Chen et al., 2016, Hsieh et al.,2013; Ganesan et al., 2024; Majumder et al., 2019]*. When MDA-MB-453 breast cancer cells cultured on EGF were treated with the PI3K inhibitor LY294002, significant downregulation of MMP-2 and MMP-9 expression occurred in cell extracts and culture supernatants ($p<0.05$). Treatment of MDA-MB-453 breast cancer cells cultured on EGF with 20 μM/ ml of

curcumin and 20 μM/ ml of ATRA caused significant downregulation in expression of gelatinases MMP-2 and MMP-9 equivalent to/ more appreciable compared to treatment with LY294002, further indicating the potential of curcumin and ATRA as inhibitors of PI3K mediated MMP2 and MMP9 in breast cancers with overexpression of EGFR. Wound healing assays showed treatment with curcumin and ATRA significantly inhibited cell migration on EGF indicating that treatment with curcumin and ATRA can reduce invasive potential of breast cancer cells with EGFR overexpression. This could be due to inhibition of EGFR mediated signalling through FAK and downregulation in expression and activity of MMP-2 and MMP-9 which would render tumour cells less invasive. Our studies indicate curcumin and ATRA could have good therapeutic potential for treatment of breast cancers with aberrant EGFR activity.

Treatment of breast cancer patients via chemotherapy can often pose severe toxic side effects. Use of chemotherapeutic drugs like taxanes and anthracyclines has been reported to cause cardiac, gastro-intestinal and haematologic toxicity and severe allergies in breast cancer patients. Reduction or discontinuation of chemotherapy due to severity of side effects often causes poor prognosis and worse treatment outcomes *[Zanuso et al., 2020, van den Boogaard et al., 2022]*. A number of synthetic inhibitors of FAK and PI3K signalling pathways and synthetic inhibitors of MMPs are currently under study, under clinical trials or in use for treatment of cancers including breast cancers. Many of these inhibitors show good therapeutic potential *in vitro* but their efficacies *in vivo* are often reduced due to toxicity and due to effective pharmacological doses not being physiologically well tolerated. For instance, the PI3K inhibitor Alpelisib showed improved results in combination with fulvestrant but treatment had to be discontinued in around 25% of patients due to side effects like diarrhoea and hyperglycemia *[Andre et al., 2019]*. Musculoskeletal pain and hypersensitivity of skin in patients have been reported with MMP inhibitors like Rebimastat *[Winer et al., 2018]*. Using natural phytochemical compounds like curcumin and ATRA could possibly help in reducing side effects (as these compounds and their plant sources are a part of human diet) and could also lower costs of treatment, leading to the development of effective breast cancer therapeutic strategies.

CONCLUSION

6. CONCLUSION

Our study involved treatment of metastatic human breast carcinoma cells, invasive human breast adenocarcinoma cells and invasive human breast ductal carcinoma cells singly and synergistically with curcumin and ATRA. Such treatment downregulated MMP-2 and MMP-9 expression and activity, MT1-MMP expression, cellular signalling through FAK and PI3K, cell survival and migration which could render tumour cells less invasive. Synergistic treatments with both curcumin and ATRA were significantly more effective than singular treatments with curcumin or ATRA alone.

Computer based studies with molecular docking indicated the excellent potential of curcumin and ATRA as inhibitors of PI3K and FAK signalling pathways and MMPs in breast cancers as their binding affinity was, in most cases, appreciably higher than that of synthetic inhibitors. Double docking showed appreciable affinity of curcumin and ATRA for simultaneous interactions with these molecules, indicating good potential for synergistic inhibition of MMP-2, MMP-9, MT1-MMP, FAK and PI3K by treatment with curcumin and ATRA.

Targeting EGFR-EGF interactions in metastatic breast carcinoma cells by treatment with curcumin and ATRA caused downregulation of EGFR expression, MMP-2 and MMP-9 expression and activity and inhibited migration of cells on EGF, which could render tumour cells less invasive. Molecular docking studies also indicated the excellent potential of curcumin and ATRA as potential inhibitors of EGFR mediated signalling. Targeting EGFR mediated signalling through PI3K by treatment with curcumin and ATRA caused downregulation of MMP-2 and MMP-9.

Our investigations thus indicate synergistic treatment with the natural phytochemicals curcumin and ATRA appears to have significantly better anti-tumorigenic potential as inhibitors of MMPs and signalling pathways in breast cancers than treatment with curcumin or ATRA alone and such synergistic treatment could have good therapeutic potential for clinical management of metastasis in breast cancers in future.

Curcumin and ATRA also show good potential as inhibitors of EGFR mediated signalling in breast cancers and such treatment could have good therapeutic potential in breast cancers with aberrant EGFR activity.

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PRESENTATIONS AT VARIOUS CONFERENCES AND LIST OF PUBLICATIONS

8.PRESENTATIONS AT VARIOUS CONFERENCES

POSTER PRESENTATIONS

1) **A. Roy,** A. Banerji. "Regulation of Focal Adhesion Kinase and Phosphatidylinositol 3'Kinase Pathways in Breast Cancer: The Role of Natural Products" at *41st Annual International Conference of Indian Association for Cancer Research (IACR-2022)* organized by Amity University, Noida, March 2022.

2) **A. Roy**, A. Banerji. "Phytochemicals as potential Matrix Metalloproteinase (MMP) inhibitors in breast cancers" at I*nternational Conference (Online) on Biomolecules to Biome*, organized by Dept. of Life Sciences, Presidency University, Kolkata, Aug 2022.

3) **A. Roy**, A. Banerji. "Targeting EGFR-Mediated MMP-2 and MMP-9 Expression in Metastatic Breast Cancer Cells by Curcumin and ATRA" at *42nd Annual Conference of Indian Association for Cancer Research (IACR-2023)* organized by Advanced Centre for Treatment, Research and Education in Cancer, Mumbai, Jan 2023.

4)A. Roy, A. Banerji. "Curcumin and All-trans Retinoic Acid (ATRA) As Inhibitors of Matrix Metalloproteinase Expression and Activity in Breast Cancer Cells" at Bio Colloq: One Day Conference on Inter-Disciplinary Biological Sciences, organized by Ramakrishna Mission Vivekananda Centenary College (Autonomous), Rahara & Academy of Biodiversity Conservation, Jan 2024. **Awarded 1st prize for poster presentation.**

ORAL PRESENTATIONS

1**) A. Roy**, A. Banerji. "Synergistic Treatment with Curcumin and All-trans Retinoic Acid: Effects on Matrix Metalloproteinases (MMPs) in Metastatic Breast Cancer Cells" at 8th World Cancer Congress-2024, organized at JNU Convention Centre, New Delhi, March 2024. **Oral Presentation at 8th World Cancer Congress 2024 was uploaded to YouTube (30 outstanding presentations were uploaded)**

2) **A. Roy**, A. Banerji. “Synergistic Treatment with Curcumin and All-trans Retinoic Acid: Effects on Matrix Metalloproteinases (MMPs) in Metastatic Breast Cancer Cells” Abstract accepted for oral presentation at 9th World Cancer Congress, New Delhi, 2026.

9.LIST OF PUBLICATIONS

1. **A. Roy**, I. Chakraborty, A. Banerji. Natural Compounds as Potential Regulators of the Phosphatidylinositol 3′ Kinase (PI3K) Pathway in Breast Cancer. *South Asian Journal of Experimental Biology* (2021) vol. 11(5) pp. 524-538; ISSN:2230-9799.

2. **A. Roy**, I. Chakraborty, A. Banerji. Determination of Phytochemicals as Potential Inhibitors of Matrix Metalloproteinases (MMPs) with Special Reference to Breast Cancer. *Issues and Development in Health Research Vol. 5* (2021), Ed. W.M. Oo, Pub: Book Publisher International (2021) pp. 73-81. Print ISBN: 978-93-91882-30-3, eBook ISBN: 978-93-91882-32-7.

3. **A. Roy**, A. Banerji. Endogenous Regulators of Matrix Metalloproteinase Expression and Activity in Breast Cancers. *Novel Research Aspects in Medicine and Medical Science Vol. 5*, Ed. R.W. Sawadago, Pub: Book Publisher International (2023) pp. 37-49; Print ISBN: 978-8119761-32-6, eBook ISBN: 978-81-19761-68-5.

4. **A. Roy**, A. Banerji. Natural Compounds as Promising Modulators of Breast Cancer Signalling: The Significant Role of Tea Polyphenols. *Medical Science: Trends and Innovations Vol. 5.* Ed. M. Refaat, Pub: Book Publisher International (2025) pp. 88-103; Print ISBN: 978-93-49238-11-4, eBook ISBN: 978-93-49238-23-7.

5. **A. Roy**, A. Banerji. Comparative Study of Curcumin, All-Trans Retinoic Acid and Resveratrol: Therapeutic Targeting of Breast Cancer Signalling. *Annual Research & Review in Biology* (2025) vol. 40 (4) pp. 154-164; ISSN: 2347-565X.

6. I. Chakraborty, **A. Roy**, A. Banerji. Therapeutic Potential of Phosphatidylinositol 3’ Kinase (PI3K) Inhibitors in Cervical Cancer. *Science and Culture* (2021) vol. 87(1-2) pp. 57-61; ISSN: 0036-8156

Chapter 7
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Determination of Phytochemicals as Potential Inhibitors of Matrix Metalloproteinases (MMPs) with Special Reference to Breast Cancer

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ABSTRACT

Both in India as well as globally, breast cancer is the most common type of cancer in women. The major causes for mortality and poor clinical prognosis in most cancers including breast cancers is metastasis and secondary tumour formation and even with modern methods of treatment, metastasized cancers remain largely untreatable. The matrix metalloproteinases (MMPs), a family of zinc dependant endopeptidase enzymes, play critical roles in the regulation of metastasis and tumour development, making MMPs possible therapeutic targets for preventing metastasis. Treatment with phytochemical compounds has the potential to reduce side effects of treatment and make treatment more affordable. The objective of this article is to analyse the potential of four such phytochemicals resveratrol, theaflavin, curcumin and all-trans retinoic acid as MMP inhibitors with special reference to breast cancers.

Keywords: Breast cancer; matrix metalloproteinase (MMP); all-trans retinoic acid (ATRA); theaflavin; curcumin; resveratrol.

1. INTRODUCTION

Breast cancer is the most common type of cancer in women both in India and globally. In 2020, the number of new breast cancer cases reported globally was found to be 22,61,419 which accounted for 11.7% of all cancers reported in both sexes, making breast cancer the most diagnosed cancer instead of lung cancer. Globally, breast cancer is the 5th leading cause of mortality accounting for 6.9% of all cancer related deaths in 2020. In India, 1,78,361 new breast cancer cases were reported in 2020 which accounted for 26.3% of all cancers reported in women [1,2]. The age standardised incidence rates of breast cancer in India have also increased significantly over the last few decades.

Cancer research has revealed a number of cellular, molecular and biochemical events that represent the progressive transformation of normal cells into malignant cells [3]. During course of tumour progression, the cancer cells acquire a number of alterations, molecular characterizations, mutations, genetic and epigenetic changes that induce the cells to proliferate independent of any exogenous growth signals [3]. In breast cancer, metastasis is the leading cause of high morbidity and mortality. Metastasis involves multiple steps like proteolysis of basement membrane and extracellular matrix (ECM), migration through ECM, intravasation into the vascular system, reaching other organs, extravasation and multiplication at distant organs to form secondary tumours [3,4]. Several cellular signalling pathways are dysregulated in breast cancers and inappropriate signalling through these pathways can promote tumour invasion by modulating matrix metalloproteinase (MMP) expression and activity [5,6]. The MMPs, a group of zinc dependant endopeptidase enzymes, are capable of disrupting the ECM and play significant roles in tumour invasion and metastasis in breast cancers [4,7-9]. Numerous studies have shown that elevated expression of MMPs in cancers, including breast

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REVIEW ARTICLE

Natural Compounds as Potential Regulators of the Phosphatidylinositol 3' Kinase (PI3K) Pathway in Breast Cancer

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ABSTRACT

Breast cancer is one of the most prevalent forms of cancer in women both globally and in India. Although breast cancer is characterized by different molecular subtypes, a majority of breast cancers appear to have mutations in the phosphatidylinositol 3' kinase (PI3K)/ protein kinase B (Akt) pathway. Dysregulation of the PI3K/ Akt pathway in breast cancers plays important roles in promoting tumour growth, proliferation and invasion. Targeting PI3K mediated signalling cascades could be therefore of value for breast cancer treatment. Studies with synthetic inhibitors of the PI3K/ Akt pathway have yielded positive results but the efficacy shown by many of these inhibitors appear to be compromised by deleterious side effects. An alternative to synthetic inhibitors is the use of natural phytochemical compounds with antitumorigenic potential like apigenin, pomolic acid, resveratrol and its derivatives, curcumin, epigallocatechin-3 gallate and thymoquinone as potential inhibitors of PI3K/Akt signalling in breast cancer and such a strategy could lead to lesser side effects and a lower treatment cost. The current study examines the importance of the PI3K pathway in breast cancer and discusses how regulation of aberrant signalling through this pathway by natural compounds could play an important role in breast cancer therapy.

1. Introduction

Globally, breast cancer is one of the most prevalent forms of cancer in women and a major cause of cancer related mortality in the female population. According to the GLOBOCAN 2020 report, an estimated 2,261,419 new cases of breast cancer were reported in women in 2020, which accounted for around 24.5% of all female cancers reported (World Health Organisation, International Agency for Research on Cancer). In recent years, breast cancer has surpassed lung cancer as the 'most diagnosed cancer', comprising 11.7% of all cancer cases reported in both sexes in 2020. Taking both sexes into consideration, breast cancer is the 5th leading cause of cancer mortality and appears to be especially prevalent in low and middle income classes (World Health Organisation, International Agency for Research on Cancer; Sung et al., 2021). Breast cancer is a leading form of cancer affecting women in India as well. Around 134,214 cases of breast cancer were reported in 2015 (Indiastat: Revealing India statistically) and an estimated 178,361 new cases of breast cancer were reported in 2020, accounting for around 26.3% of all cancers reported in women in India during that year (World Health

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Endogenous Regulators of Matrix Metalloproteinase Expression and Activity in Breast Cancers

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ABSTRACT

Tumour metastasis and secondary tumour formation are the leading causes behind breast cancer mortality. Modulation of matrix metalloproteinase (MMP) expression and activity promotes tumour metastasis and secondary tumour formation in numerous cancers including breast cancers. Extracellular matrix metalloproteinase inducer (EMMPRIN) and membrane type-1 matrix metalloproteinase (MT1-MMP) are endogenous molecules which regulate MMP expression and activity. Our current study examines the importance of EMMPRIN (also known as Basigin or CD147) and MT1-MMP (MMP-14) with particular reference to their roles in regulating MMP expression and activity in breast cancers. MT1-MMP and EMMPRIN expression levels are increased in invasive breast cancers and correspond to an invasive phenotype and a worse prognosis by various mechanisms including by causing an increase in MMP expression and activity. MT1-MMP also plays an important role in localization of MMP-2 to the tumour cell surface, regulating proteolysis at the cell-ECM interface. Determination of MT1-MMP and EMMPRIN expression levels could act as possible biomarkers for breast cancers and may help clinicians to better diagnose the stage of the cancer. Therapies involving inhibition of MMP expression and activity to downregulate invasive potential in breast cancers could thus involve the targeting of not just MMPs but also of their endogenous inhibitors EMMPRIN and MT1-MMP for increased efficiency.

Keywords: Extracellular matrix metalloproteinase inducer (EMMPRIN); membrane type-1 matrix metalloproteinase (MT1-MMP); matrix metalloproteinases (MMPs); breast cancer; metastasis.

1. INTRODUCTION

Breast cancer is a leading cause of malignancy and mortality in women both in India and worldwide. In 2020, 178,361 breast cancer cases were reported in

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Natural Compounds as Promising Modulators of Breast Cancer Signalling: The Significant Role of Tea Polyphenols

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ABSTRACT

Breast cancer, as a disease, is highly prevalent among women in India and worldwide. Aberrant expression and signalling through various pathways like the Focal Adhesion Kinase (FAK) pathway, the Mitogen-Activated Protein Kinase (MAPK) pathway and the Extracellular Signal Regulated Kinase (ERK) pathway have been reported to cause breast cancer progression and metastasis. Treatment of breast cancer patients via chemotherapy can often pose severe toxic side effects with peripheral tissue and organ damage. Determining the anti-tumorigenic potential of various natural compounds is an interesting area of work in order to address this issue. As FAK, MAPK and ERK signalling pathways are important factors in the progression and spread of breast cancers, the potential of two tea polyphenols, epigallocatechin-3-gallate (EGCG) and theaflavin was analysed for targeting these signalling pathways. Molecular docking and analysis indicated both theaflavin and EGCG showed good binding affinity and interactions via hydrogen bonding with various amino acids on the FAK, p38MAPK and ERK2 molecules. Theaflavin and EGCG however, appear to interact with different sites on p38MAPK and ERK2 but both bind around the same site on FAK. These studies indicate the potential of the tea polyphenols, theaflavin and EGCG, as signalling pathway inhibitors for the treatment of breast cancers. Further, *in vitro* and *in vivo* studies to understand the pattern of interactions of theaflavin and EGCG with these target molecules could result in positive outcomes for breast cancer treatment.

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Comparative Study of Curcumin, All-Trans Retinoic Acid and Resveratrol: Therapeutic Targeting of Breast Cancer Signalling

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Authors' contributions

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ABSTRACT

Aims: Breast cancer is the most prevalent cancer affecting women worldwide. Aberrant signalling through the phosphatidylinositol 3' kinase (PI3K) and extracellular signal regulated kinase (ERK) pathways promotes metastatic spread of breast carcinomas. As prevalent chemotherapeutic treatments can cause toxic side effects, exploring the anti-tumorigenic potential of natural phytochemicals as inhibitors of these pathways is important.
Methodology: In our study we analyzed binding efficacy and patterns of interactions of the phytochemicals curcumin, resveratrol and all-trans retinoic acid (ATRA) with PI3K and ERK using molecular docking and computer based analysis.

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