ABSTRACT

Cancer is the leading cause of mortality and major hurdle of increased life expectancy worldwide. According to recent GLOBACON 2020 data Worldwide, an estimated 19.3 x 10^6 new cancer cases were reported while almost 10.0 x 10^6 cancer deaths occurred. Traditional treatments methods include chemotherapy, radio therapy, surgery, or combination of all them but they are equally toxic for the healthy cells. Natural products are best known for their safety, less toxic nature, and antioxidant characteristics making them a promising candidate to treat diseases like cancer. A vast variety of natural compounds has been reported for antitumor activities making them a potential candidates for inhibition of TGFβR signaling pathway. Transforming growth factor (TGF) beta family signaling pathway have biphasic nature having both tumor suppressive as well as tumor promoting activity. Downregµlation of transforming growth factor beta receptor (TGFβR) signaling pathway can provides better therapeutic strategy to control oncogenesis. The present study was designed to investigate the inhibitory effects of crude methanolic extracts of sixteen reported Pakistani medicinal plant species against TGFβR signaling pathway. Maceration was used to create crude methanolic extracts (CMEs) of sixteen different plant species. These CMEs were then subjected to phytochemical analyses in order to identify the main phytometabolites, notably the components that are cancer chemopreventive. *Cichorium intybus* has the highest total flavonoid concentration (0.53 mg/gQE), compared to all other plants, with a total flavonoid content that ranged from 0.02 to 0.53 mg/gQE. All plant extracts were tested for their ability to scavenge free radicals, and their IC_{50} values ranged from 2.03 to 88 µg/ml. Utilizing a colorimetric technique called 3-(4,5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay, the comparative cytotoxicity of plant extracts was assessed in HUH and MCF-7 cell lines. Based on their high antiproliferative activity > 50% inhibition against cancer cell lines, the nine active plant extracts includes *Fagonia cretica, Argemone mexicana, Rubus fruticosus, Moringa oleifera, Punica granatum, C. intybus, Xanthium strumarium, Carissa opaca* and *Cyperus rotundus* were identified. They were then subjected to relative expression studies Using GAPDH (housekeeping gene) as the reference gene, RT-PCR was used to evaluate the genetic expression of ubiquitous proteins (SMURF1

and SMURF2) and TGFβ signaling molecules (i.e. TGFβR1,2&3, SMAD3, SMAD5) in response to a subset of nine active extracts. Expression studies revealed that CMEs of five plant extracts significantly inhibit TGF beta mediated signaling cascade by downregulating the gene expression fold change <1.0 of TGFβR 1,2 & 3 and receptor associated complex protein SMAD3 compared to control. These plant extracts were further subjected to column chromatography and column fractions were obtained using various solvent combinations of n-hexane, ethyl acetate and methanol. Thin layer chromatography was performed to check the presence of compound in each fraction. Retention factor (RF) values were calculated and cytotoxic and relative expression studies were performed again on active fractions. Two plants extracts i.e. *Cichorium intybus* and *Argemone mexicana* and their most potent column fractions were selected for further *in vitro* anticancer activities, western blotting and flow cytometry. The most potent plant extract (*Argemone mexicana* CME) was subjected to *in vivo* studies using mice model for breast cancer (MTDT) and brain cancer (SB28). Dose finding studies in MTDT mice model showed significance delay in tumor growth at 250mg/kg with respect to control SHAM group. Survival time increased in mice treated with *A. mexicana* crude extract $P < 0.05$. Among tested plant extracts, the crude methanolic extract of *A. mexicana* significantly inhibited TGFβ mediated signaling cascade and increased life expectancy in MTDT mice. The active constituents of *A. mexicana* CME can be used for further studies as potent cancer therapeutic agents paving a new way to treat cancer.

CHAPTER 1

INTRODUCTION

Cancer is still leading cause of mortality and major hurdle of increased life expectancy worldwide with increasing rate day by day. According to recent GLOBACON 2020 data Worldwide, an estimated 19.3 x 10^6 new cancer cases were reported with almost 10.0 x 10^6 cancer deaths occurred (Sung et al., 2021). Traditional methods for cancer treatment include chemo and radiotherapy work through targeting the tumor cells and ultimately kill the cancer cells by exposing them with radiation but they are equally lethal to normal body tissues and often comes with lot of side effects including post treatment anemia, bleeding issues, infection, alteration in sexual system, memory changes, constipation, tiredness, extensive hair loss, loss of appetite, urination problems, itchy and sore skin, fluid retention, nausea and vomiting are most commonly observed symptoms after these treatments. Scientist are trying hard to overcome these issues by inventing something new, less expensive, versatile and more effective and specific in nature (Neu et al., 2007; Liu, Ren and Ten Dijke, 2021).

Human development is quite complex system of cells division and organization into tissues, organs and systems in order maintain homeostasis and normal physiological processes of life during both early development of embryonic level and adult stage. This process involve variety of signaling cascades working together in complex manner which involves ligand receptor interactions and downstream signaling pathways transmitting signals through the whole body in systematic fashion (Massague, 2012). Mostly human diseases occur due to dysregulation of these pathways either inappropriate activation or inhibition. One of the important key regulators of embryonic development and cellular homeostasis is the transforming growth factor β (TGFβ) superfamily of cytokines. TGFβ was first discovered in 1978 by Todaro and Larco in transformed mouse fibroblasts with murine sarcoma virus. TGFβ expressed by all cells in human body in order to regulate normal development and homeostasis. TGFβ superfamily containing 30 structurally related proteins including three isoforms of (TGFβ 1,2 &3), activins (A,

B), inhibin's also bone morphogenetic proteins (BMP 1-20), nodal lefty's, differentiation factors and Mullerian inhibiting (MI) substances showing its main role during development (Miyazono et al., 2005).

Warm-blooded animals have three isoforms of the 25 kDa disulfide-linked dimeric protein known as TGFβ, which has 112 amino acids per unit. Each TGFβ isoform has a 64–82% succession homology, and it is often stored in the extracellular matrix as a latent complex with TGFβ latency–associated peptide and dormant TGFβ associated protein. In this pathway, TGFβ is enacted by *αv* integrins, accordingly. TGFβ signaling pathway initiated by receptor ligand binding of TGFβ to TGFβ receptor 2 (TGFβR2) a serine threonine kinase receptor which in turn recruit and phosphorylate the TGFβR1 as shown in Figure 1 (Hata and Chen, 2016).

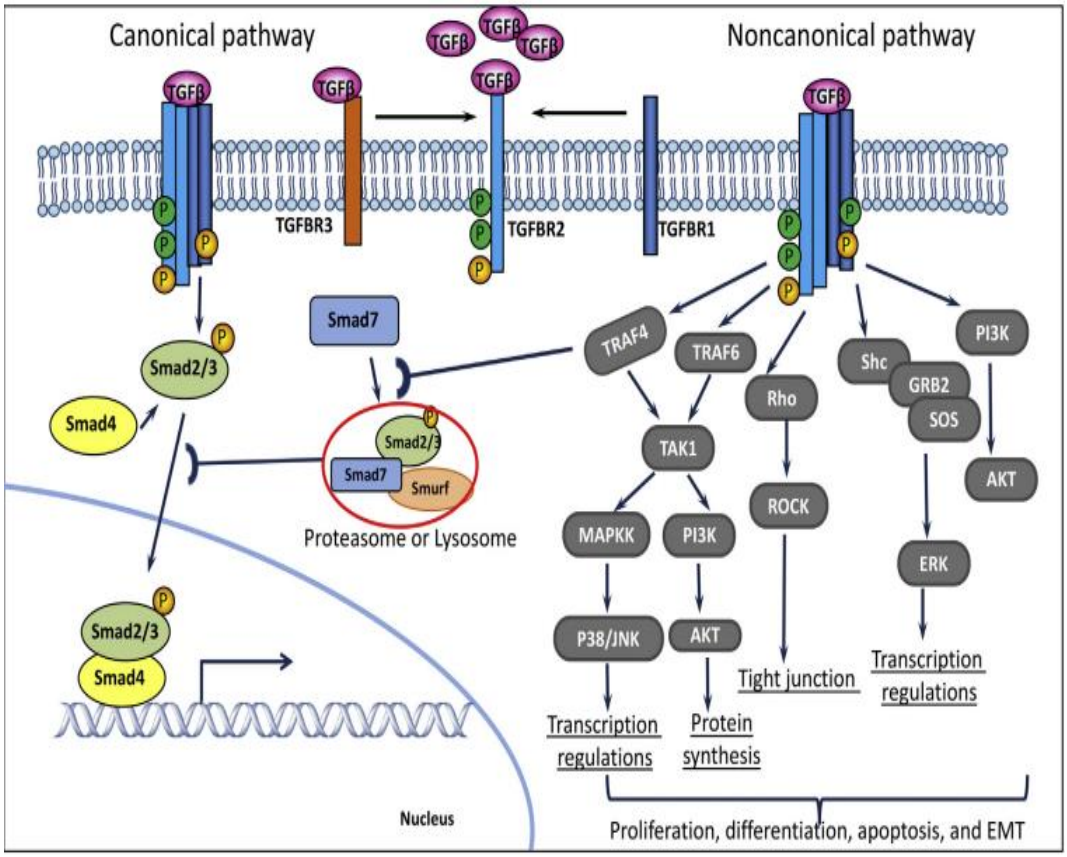

Figure 1.1: TGFβ canonical and non-canonical signaling pathway (Adopted from Vander Ark, Cao and Li, 2018)

The phosphorylated TGFβR1 also have kinase activity and initiate downstream signaling process by phosphorylation of SMAD proteins 2, 3 (mothers against decapentaplegic homolog 2 and 3) at carboxy terminus serine residue which

than binds and form complex with SMAD4 and translocate in nucleus where they activate variety of TGFβ dependent transcription factors and genes which have key role in cell apoptosis, cell proliferation and homeostasis (Vander Ark, Cao and Li, 2018).

In addition to the SMAD pathway, TGFβ promotes the activity of a number of other signaling pathways, including as TRAF6-TAK1-p38/JNK, Rho-Rock, mitogen-activated protein kinases (MAPKs), phosphoinositide 3′ kinase (PI3K), and others. TGFβ signaling mechanisms that are not SMAD (Figure 1). These optional signal transducers typically control the SMAD pathway and interfere with the signalling of many effectors. As a result, TGFβ regulates further bodily functions via the SMAD subordinate pathway as well as through additional alternative signaling pathways that provide nodal focuses for communication with additional signal transduction pathways (Zhang, 2009; Vander Ark, Cao and Li, 2018).

TGFβ regulates cell proliferation and death, acting as a tumor suppressor at the early stages of tumor development. In response to activated cytostatic genes, tumor development is inhibited by TGFβ driven G1 cell cycle arrest. For instance, TGFβ activates the expression control of the p15 or p21 genes, which represses the cyclin proteins complex (Imamura, Hikita and Inoue, 2012). Nuclear factor of activated T cells (NFAT) is a transcription factor that plays a role in tumor suppression, but it can also play a role in tumor promotion if the TGFβ signaling pathway is selectively cut off, which activates cell division and causes NFAT to be dephosphorylated and moved to the nucleus. The tumor promoting elements CDK2, Cyclin E and CDK4 upregulation occur when NFAT interacts with SMAD2 (Sengupta, Jana, and Bhattacharyya, 2014). TGFβ also involved in regulation of proapoptotic genes including cell death related protein kinases, inositol phosphatase, caspases and GADD45β (Zhao et al., 2015).

TGFβ prompted tumor suppressive effects must be circumvent by cancer cells to transform into malignant state. Cancer cells can take two alternative paths to this end, firstly selective amputation of tumor suppressive arm of this pathway or secondly incorporating receptor-inactivating mutation to decapitate the pathway (Figure 1.2). The prior path gives additional benefits to cancer cells by co-opting

TGFβ response for protumorgenic purpose.In both situation cancer cells use TGFβ signaling pathway to harmonize the tumor microenvironment to evade immune surveillance or production of protumorigenic cytokines which leads to metastatic stage and ultimately TGFβ involves in sustainability of cancer cells (Biswas et al., 2007; Huynh, Hipolito and Ten Dijke, 2019).

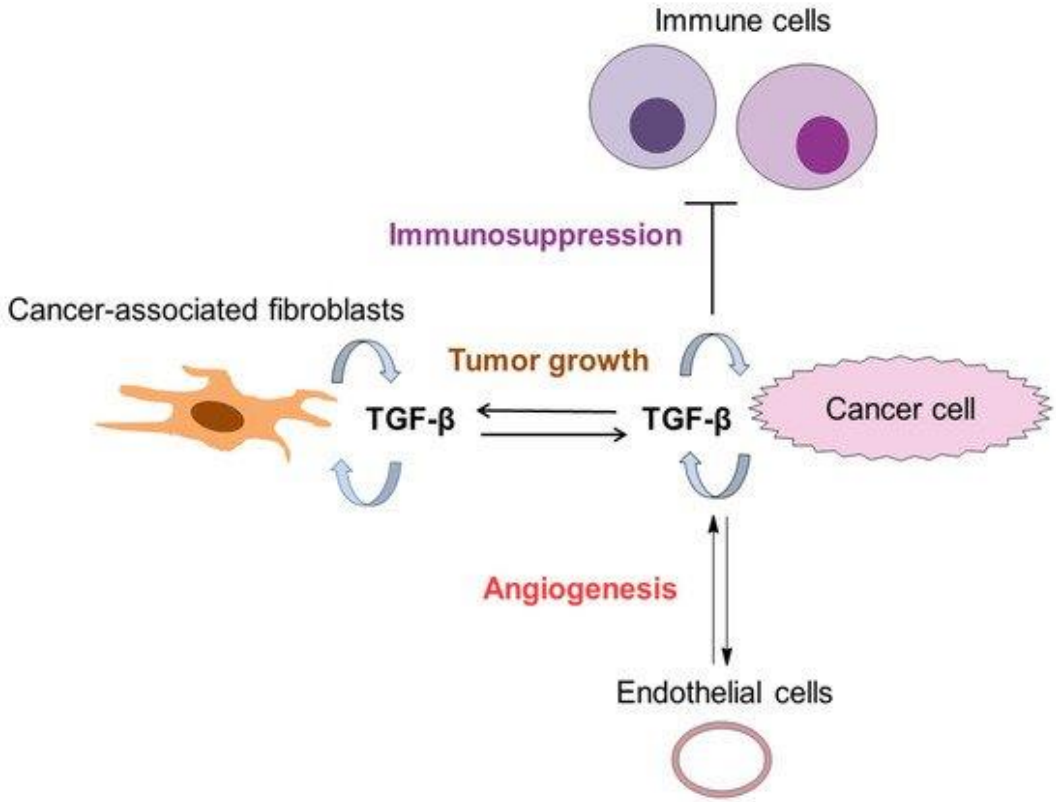

Figure 1.2: The tumor microenvironment and TGFβ signaling (Adopted from Huynh, Hipolito and Ten Dijke, 2019).

TGFβ has multifaceted role in cancer because of its dual role as tumor suppressor and tumor promoting agent. In normal cell or premalignant stage TGFβ enforces homeostasis and tumor progression can be stopped directly by cell-autonomous tumor-suppressive processes such cytostasis, which occurs mostly at the G1 phase, differentiation, and cell death, or indirectly by impacts on the stroma (suppression of inflammation and stroma-derived mitogens). However, once cancer cells bypass this pathway's tumor-suppressive component, they can produce growth factors, differentiate into invasive phenotypes, and establish metastatic colonies by

using TGFβ to their advantage (Massague, 2008; Huynh, Hipolito and Ten Dijke, 2019).

TGFβ regulates key function in immune suppression because of its dual cytokine nature regarding immunomodulation. It mainly functions by direct inhibition of CD4 helper T cells, CD8 cytotoxic T cells, macrophages, NK cells and up-regulation of immune-compromising T cells by alternating balance of Th1/Th2 pointing toward that an up-regulation of TGFβ drive primary function of immune evasion that exclude T cells and other factors in advanced colorectal cancer. So, its inhibition will be beneficial to prevent escape from immune surveillance and decrease in metastatic phenotype of tumor results in decreased tumor growth (Tauriello et al., 2018).

TGFβ restrains specific elements of CTLs, CD8+ T cells, and regular executioner cells, leading to a tumorigenic impact. TGFβ additionally improves growth intrusiveness and angiogenesis by advancing the creation and emission of lattice metalloproteases proteinases and matrix metalloproteinase and downregulating the outflow of tissue inhibitors of metalloproteases. TGFβ additionally actuates epithelial to mesenchymal transitions (EMT), which upholds invasion and growth of cancer cells in metastatic stage (Huynh, Hipolito and Ten Dijke, 2019). So when cancer become malignant than it helps to promote cancer cell growth and progression in context dependent manner.

YAP1 is an important transcription coactivator for 14-3-3σ in premalignant human mucoepidermoid carcinoma, and 14-3-3σ stabilises p53, a Smad partner for transactivating p21, the main executor of TGFβ's cytostatic and tumor suppression programme (left). Gli2 is a Smad partner for PTHrP transactivation in breast cancer cells, which is a crucial facilitator of TGFβ's programme for bone osteolysis and bone metastases (right) (Figure 1.3). TGFβ restrains the transcription of proapoptotic and lysogenic cytokines in cytotoxic T lymphocytes (CTLs) for example, perforin, Granzyme A, Granzyme B, porphyrin interferon G, and factor-associated suicide ligands (Xu et al., 2015).

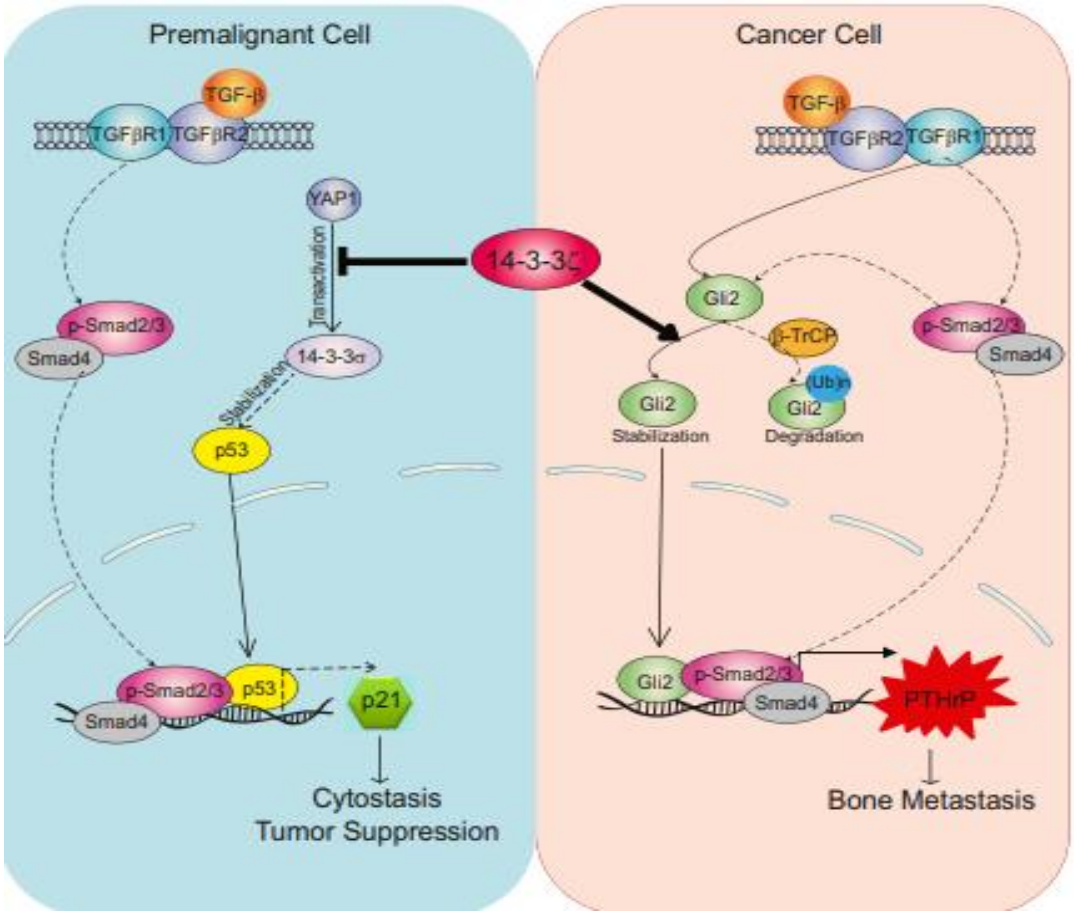

Figure 1.3: 14-3-3z-Driven contextual changes of Smad partners from p53 in premalignant cells to Gli2 in cancer cells (Adopted from Xu et al., 2015)

TGFβ mediated cytotoxic response of T cells was clearly understood by TGFβ associated inhibition in expression of Fas ligand, interferon y (Iy), perforin, and granzyme (A and B). In addition, TGFβ also induces the downregulation of human leukocyte antigen class I antigens (HLA I) in DU145 cells and inhibited the lysis of cytotoxic T cells in Prostate cancer cell line. The expression of HLA I is key element of immune response and its suppression causes an escape from immune surveillance leads to enhance in tumor progression and metastasis (Chen et al., 2015).

Epithelial to mesenchymal change (EMT) of cancer cells is an important step for change into metastatic stage (Figure 1.4).

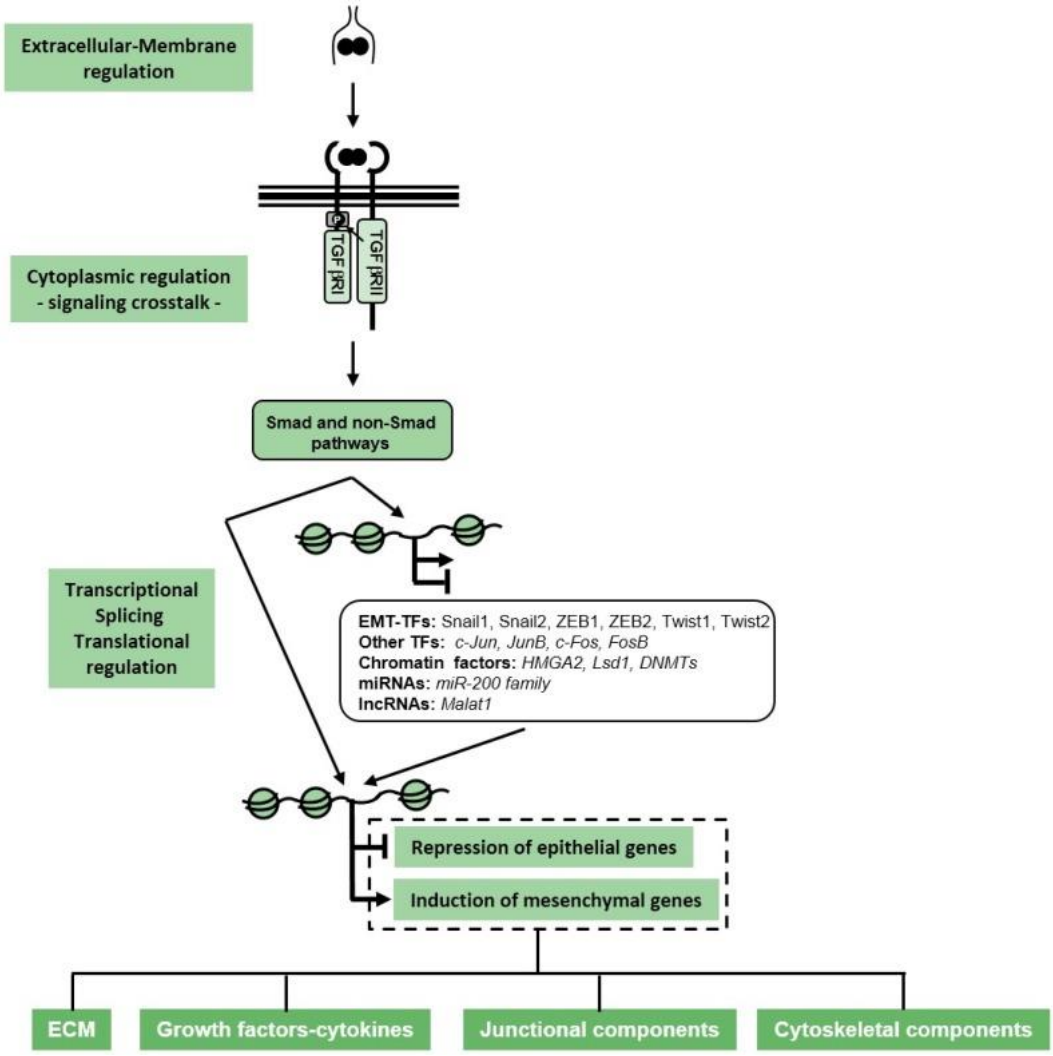

Figure 1.4: TGFβ signaling pathway role during EMT facilitated cancer cell progression and metastasis (Adopted from Moustakas and Heldin, 2016)

EMT is fundamental in injury recuperating, fibrosis, cancer growth, and normal development of embryo. TGFβ actuates EMT during normal developmental process. TGFβ driven EMT upholds cancer cells growth intrusion and spread by delivering tumor cells into the microenvironment of other cells. In numerous cancers, TGFβ incites EMT with the transcriptional guideline of cadherin (E & N), Snail and vimentin (Moustakas and Heldin, 2016). The presence of TGFβ and adhesion-dependent signaling is expected to facilitate stable articulation of myofibroblast aggregates and cytoskeletal recombination. After EMT, epithelial cells lose their extremity, tight intersections, and attachment between cells, in this way acquiring the capacity to relocate. Phenotypic change prompts diminished intercellular grip

and improved migration and invasion capacity of cancer cells, subsequently advancing cancer metastasis (Pastushenko and Blanpain, 2019).

As stated earlier, the transcription factor SOX4 may facilitate TGFβ induced activity and advance EMT, tumor growth and metastasis in breast cancer. Additionally, TGFβ can likewise activate the expression of twofold mouse minute 2 (MDM2), which makes p53 unstable, prompting EMT and cancer cells progression (Araki et al., 2010). In cancer exhibiting p53 mutated genes, TGFβ instigates the accumulation of the mutant p53, p63 protein complex, and SMADs. In this complex, the tumor suppressive capacity of P63 is inhibited, and the inactivation of P63 empowered both the mutant p53 and TGFβ to start EMT (Adorno et al., 2009).

Several experiments have shown that TGFβ is associated with the EMT of cancer cells and the progression and metastasis of a tumor cell. TGFβ initiates prostate cancer relocation by actuating stress fiber conglomeration and cytoskeletal remodeling through the cell division cycle 42 (Cdc42), Rho A and SMAD proteins (Edlund et al., 2002). TGFβ additionally actuates the expression of dedicated for cytokinesis 4 protein through the SMAD pathway, improving the exudation of cellular breakdown in the lungs carcinoma cells and expanding the motility metastasis of cancer cells (Yu et al., 2015). The actuation of the TGFβ pathway prompts a serious loss of miR-124 in small cell lung cancer, leading to EMT and metastasis. TGFβ driven EMT imitates cancer cell motility, metastasis and ancestor cell like qualities, all of which empower TGFβ role in tumor promotion and stability.

During tumor progression and metastasis, the vascular organization advancement is laid on the grounds of constant requirement of oxygen and nutrition supply to expand metastatic colonies, which in turn requires more angiogenesis. In addition to expression level of angiogenic factors likewise mirrors the intrusion capacity of the tumor (Nishida et al., 2006). Endothelial cells (EC) assume a critical part in angiogenesis. EC showed higher cell multiplication, migration and intrusion during neo-vasculature. Previous studies showed that TGFβ signaling pathway unpredictably connects with EC ability and action (Budi et al., 2019).

TGFβ likewise incites proangiogenic development factors like vascular endothelial development factor (VEGF) and connective tissue growth factor (CTGF)

through epithelial cells and fibroblast. These factors straightforwardly activate EC to start capillaries formation and assume a fundamental part in instigating and keeping up with tumor angiogenesis, subsequently speeding up cancer progression. At the same time, TGFβ can initiate endothelial migration, which is fundamental process for angiogenesis. This is further confirmed by high plasma level of TGFβ causes an increased tumor angiogenesis which leads to poor prognosis in liver, renal cell and prostate cancer respectively. Similar effect was seen in nonsmall-cell lung carcinoma that higher level of TGFβ causes tumor progression and angiogenesis (Budi et al., 2019; Darland and Damore, 2001)

In human breast cancer, the high mRNA levels of TGFβ are related with expanded micro vascular thickness and these characteristics relate to patients results in low chances of recovery. TGFβ dependent SMAD4 signaling can increase the expression of miR29a, which can target phosphatase and tensin homolog and initiate the non-canonical signaling pathway eventually leading to formation of new blood vessels. Notwithstanding TGFβ ligand activity, TGFβ receptors are likewise basic for angiogenesis (Yang et al., 2022). Numerous genetic and epigenetic changes to the TGFβ signaling pathway's components have been documented to reduce TGFβ's ability to control tumor growth (Yang et al., 2022).

Epigenetic changes are crucial for essentially all malignancies and involve changes for DNA methylation, histone alteration and microRNAs. DNA methylation assumes a basic part in tumor progression (Cedar and Bergman, 2012). Modification of DNA methylation designs prompts articulation in gene expression, even when there is no mutation in sequence of genes. All cancers that have been analyzed show changes in DNA methylation, proposing that this might address an essential component of cancer science, which bears a critical effect on growth pathology (Sandoval and Esteller, 2012). There is a significant connection between the situation with DNA hypermethylation and TGFβ motioning in cancer cells. TGFβ is a vital controller for DNA methylation through an expansion in DNA methyltransferases (DNMTs) articulation, particularly in cancer. There exists a differential impact of TGFβ induced DNMT expression among protumor genic and metastatic cells. In benign cells, TGFβ represses DNMT articulation (Cedar and Bergman, 2012).

In cancer cells, TGFβ animates DNMT expression. It should be noticed that, considering the significance of both TGFβ pathway and DNA methylation in growth movement, most of the methylated quality in cancer are pertinent to TGFβ signaling pathway. It was observed that up-regulation of TGFβ mediated methyltransferases relate to increase in metastasis and tumor progression in prostate cancer (Matsumura et al., 2011; Zhang et al., 2011). Natural products are divided in to three categories according to their response to TGFβ.

Class 1 indicates those compounds that are involved in activation of TGFβ pathway. Class 2 are natural products that have no effect on this pathway but have anticancer properties. Class 3 involves those natural products that cause inhibition of TGFβ signaling pathway (Matsumura et al., 2011). Numerous natural products have a defensive impact against carcinogenesis. Besides antioxidant role, they likewise initiate TGFβ articulation from the responsive cells. Since TGFβ has inhibitory effect on benign cells and can incite apoptosis in typical anti-tumor target cells, such property will be an appropriate for cancer supplements to avoid cancer. Models for these items incorporate seaweed and resveratrol. Different items, for example, Inchin can prompt TGFβ expression in target cells. It was studied that *Scutellaria baicalensis* and *Bupleurum scorzonerifolfium* can restrain cell multiplication by an expansion in TGFβ expression in target cells (Lee et al., 2008).

Euonymus alatus (EA) mainly present in Korea is utilized for leiomyomal cancers, showed a much lower multiplication rate than untreated cells, proposing that this plant hindered the cell division of uterine leiomyomal cells. TGFβ can accomplish a comparative impact instead of EA in combination. It was a great advantage since these products can initiate TGFβ creation in target cells, they are reasonable for cancer avoidance yet ought to be cautious in administrating these agents with the end goal of therapy of metastatic cancers (Lee et al., 2005).

The best instances of this class of natural items are flavonoids, for example, genistein, with their capacity to hinder tumor metastasis. Beside their capacity to have the antioxidant properties, they can restrain TGFβ induce signaling in the cancer cells, accordingly help to hinder cancer development and metastasis. The best illustration of this class of natural products has been discovered by Lin et al. (2011)

who have portrayed how Andrographolide suppresses hypoxia inducible factor 1β by repressing TGF-β activation in human lung cancer cell line A549. Other compounds having a place with this class incorporate green tea and black tea, *Angelica sinensis, Machilus thunbergii, Chunggan extricate*. Esculentoside A, compound from *Salvia miltiorrhiza* , *Momordica charantia* leaf extricate, and *Polypodium leucotomos* (Lee et al., 2013).

Natural compounds mostly obtained from water fractions have no effect on TGFB pathway in cancer cells like for uterine leiomyoma and citrus unshiu. Further, lycopene is a viable preventive compound for prostate cancer yet has not impact on TGFβ signaling pathway in cancer cells (Gunasekera et al., 2007). So, inhibition of TGFβ signaling could be a significance strategy to control oncogenesis. The most debatable strategy used in preclinical studies that targeting TGFβ signaling pathway having use of Monoclonal antibodies and small molecule inhibitors. However promising results could not be achieved as an anticancer drug because TGFβ signaling has a multifaceted role (Biswas et al., 2007).

Recently, natural compounds have been reported considerably in the preclinical phase of breast, cervical, ovarian, prostate and pancreatic oncogenesis. Many auspicious agents are under clinical development owing to have discriminatory activity against anti-cancer molecular targets currently being developed. Scientists are becoming more interested in using isolated phytochemicals and ethnomedicinal formulations in the disease treatment and prevention as a result of the widespread rise of cancer and development of drug resistance (Akhtar, Saleem and Saleem, 2019).

Since ancient times, Pakistan has employed medicinal plants. It was discovered that almost 108 anticancer medicinal plants were exploited in folk medicine across Pakistan to treat various neoplastic diseases. 64 of these anticancer plants were discovered to have undergone *in vivo* and *in vitro* tests for their anticancer potential. Through the use of *in vitro* and animal models, the anticancer properties of several ethnomedicinal herbs have been confirmed. These therapeutic herbs and phytochemicals prevented the development, spread, or metastasis of cancer. A few endangered species of plants are used as medicines. Through in vitro

or *in vivo* techniques, the use of fifty percent of traditional Pakistani herbs against various malignancies has been confirmed. Further pharmacological and toxicological analysis of these traditional anticancer herbs from Pakistan is required. Further promising phytochemicals must be found and isolated in order to be tested on cancer patients (Akhtar, Saleem and Saleem, 2019).

In this context present study was designed to inhibit TGFβ signaling using natural compounds. Hence the objectives of study are:

- Screening of potential TGFβ inhibitory compounds
- Selection of screened compounds for *in vitro* analysis
- Inhibition of TGFβR using selected compounds in cancer cell lines

CHAPTER 2

REVIEW OF LITERATURE

Cancer is a significant general medical issue worldwide and is the subsequent driving reason for death in the United States. In 2020, the finding and therapy of cancer was hampered by the Covid illness 2019 (COVID-19) pandemic. For instance, decreased admittance to mind in view of medical services setting terminations brought about delays in conclusion and therapy that might prompt a momentary drop-in cancer occurrence rate followed by an increase in cutting edge stage infection and eventually expanded mortality.

Notwithstanding, this optional result of the pandemic will require quite a while to measure in view of the slack in spread of populace-based surveillance information. Every year, the American Cancer Society gauges the frequency of new cancer cases and deaths in the United States and assembles the latest information on populace-based cancer incidence (Sung et al., 2021).

There were an expected 19.3 million new cases and 10 million cancer deaths worldwide in 2020. The conveyance of all-cancer frequency and mortality as indicated by world district for the two genders joined and independently for people. For the two genders consolidated, one-half of all cases and 58.3% of cancer deaths are assessed to happen in Asia in 2020, where 59.5% of the worldwide populace dwells Europe represents 22.8% of the all-out cancer cases and 19.6% of the cancer deaths, even though it addresses 9.7% of the worldwide populace, trailed by the Americas' 20.9% of frequency and 14.2% of mortality around the world.

As opposed to different locales, the portion of cancer deaths in Asia (58.3%) and Africa (7.2%) are higher than the portion of occurrence (49.3% and 5.7%, separately) considering the different dissemination of cancer types and higher case casualty rates in these areas. The exceptional variety of cancer keeps on offering pieces of information to the fundamental causes yet in addition reinforces the requirement for a worldwide heightening of endeavors to control the disease (Sung et al., 2021).

2.1 TGFB SIGNALING PATHWAY

Human body homeostasis and normal functions controlled by array of complex inter-regulated pathways and any change in normal activity of these pathway leads to abnormality. One of the key regulators of embryonic development and maintenance of cell physiological function, growth and division is TGFβ signaling pathway which belongs to superfamily of cytokines including TGFβs growth and differentiation proteins, activators, inhibitors and bone morphogenetic proteins. The alteration in these pathways leads to variety of diseases including cancer (Miyazono et al., 2005; Liu, Ren and Ten Dijke, 2021).

There are three structurally related isoform of TGFβ (1, 2 & 3) and *in vitro* studies confirmed that they have similar biological effects only difference in their potency in specific kind of cells (Chart et al., 1995). TGFβ each isoform exhibit different features as confirmed by *in vivo* mice studies while knocking down result in loss of respective function. Among tested isoforms TGFβ1 is most abundant and extensively studied isoform particularly high in bone. It was observed that mice deficient in TGFβ1 were died at postnatal phase due to autoimmune disease or at embryonic stage due to vascular defects while defective cardiac septation and valve remodeling were observed in TGFβ2 deficient mice. Mice lacking TGFβ3 isoform exhibited the defect in pulmonary system and cleft palate ((Miyazono et al., 2005; Huang et al., 2021).

Oncogenesis may be significantly controlled by inhibiting TGFβ signaling pathway. The binding of the TGFβ ligand to the TGFβR2 results in the binding of TGFβR2 to TGFβR1 and the phosphorylation of TGFβR1, while TGFβR3 aids in ligand availability. The phosphorylation of SMAD proteins by activated TGFβR1 triggered a downstream biochemical pathway. Complex creation with additional SMAD4 partners as a result. A number of genes are transcribed by TGFβ through the action of the SMAD complex, which translocated into the nucleus (Hata and Chen, 2016). Cancer cells showed activated TGFβ signaling pathway which plays a major role in gaining interest of cancer biologists because it also has a diverse role in regulating cancer cell functions including cell death, cell progression, cell to cell

interaction and differentiation to specific cell types as summarized in Figure 2.1 (Cheifetz et al., 1990, Liu, Ren and Ten Dijke, 2021; Kim et al., 2021).

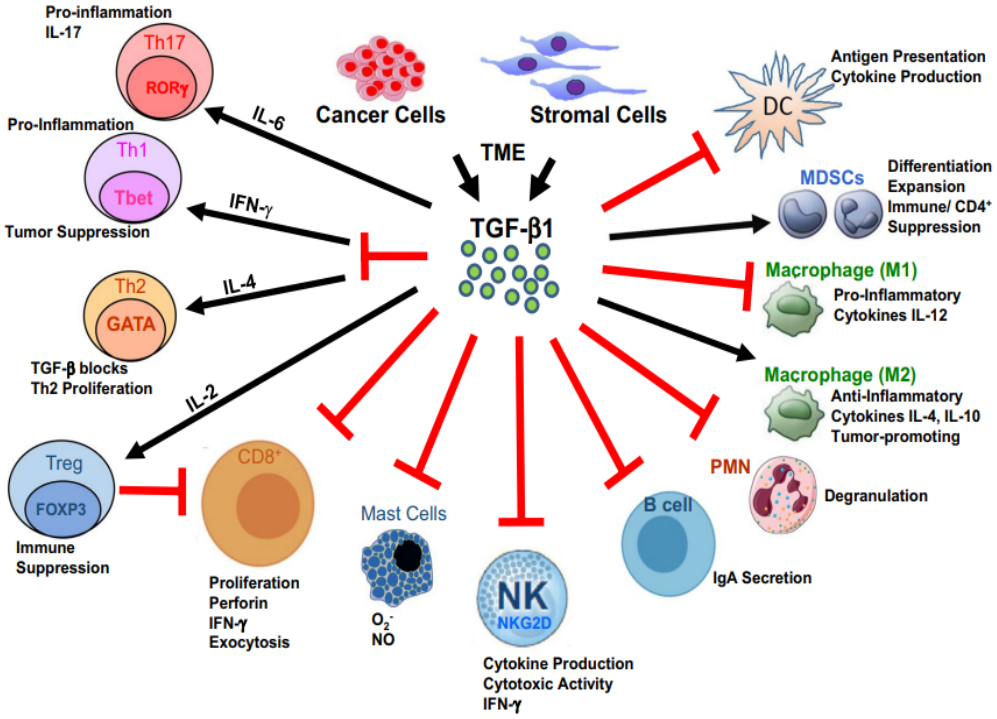

Figure 2.1: Anti-tumor immunity and role of TGFβ (Adopted from Kim et al., 2021)

2.2 TGF*B* BIPHASIC NATURE

The biphasic elements of TGFβ during malignant phase in cancer cells and other type of cells in the tumor microenvironment are summed up in Figure 2.2. In various cell types or potentially conditions, TGFβ can have unique, even inverse, effects. In typical and premalignant cells, TGFβ prevalently goes about as a growth silencer by restraining cell expansion, advancing apoptosis, and keeping up with genome stability. Notwithstanding, cancer cells can adjust to or specifically sidestep the suppressive elements of TGFβ. They use TGFβ's as promoter of development phase and go through processes, for example, the epithelial-to-mesenchymal transition (EMT), that empower their movement, intrusion, intravasation, and extravasation. Moreover, TGFβ can make a valuable TME by acting in a paracrine way to enact tumor cells related fibroblasts, initiate angiogenesis, produce

extracellular framework and help to invade immune response to promote malignant growth (Liu, Ren and Ten Dijke, 2021).

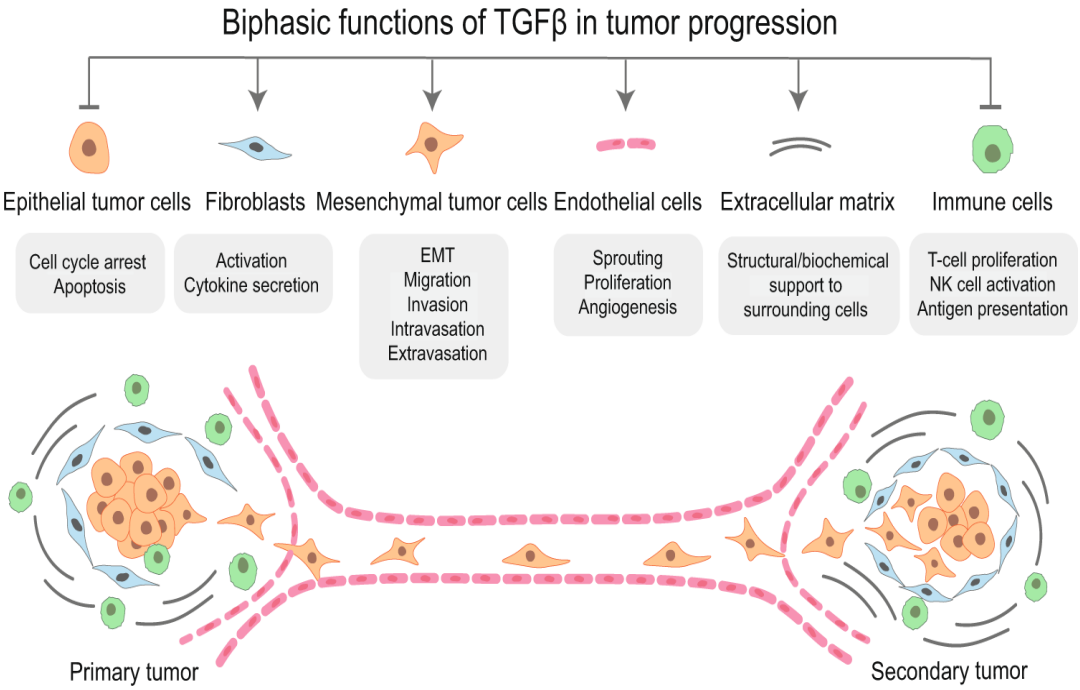

Figure 2.2: TGFβ biphasic function in tumor progression and metastasis (Adopted from Liu, Ren and Ten Dijke, 2021)

Although TGFβs can act as tumor suppressive agent (Markowitz and Roberts, 1996). But Cui et al., (1996) investigated that in peritumoral microenvironment TGFβ secretion by cancer cells can lead to tumor sustainability and invasion. This apparent conflict is resolved by introducing chemical carcinogenesis in mouse skin model and targeted expression of TGFβ1 in suprabasal keratinocytes revealed the biphasic nature of TGFβs, the development of benign mass was inhibited in skin but once tumor has been developed it increase their progression in highly invasive manner. These results showed biphasic nature of TGFβ1, early it acts as tumor repressor and later its play role as tumor promoter by initiating an EMT. It has been proved that upregulation of TGFβ3 in spindle carcinomas cause this invasive phenotype (Yang et al., 2022).

2.3 MUTATIONS IN THE TGFB RECEPTOR GENES

Levy and Hill (2006) found that mutations in the TGFβR2 alleles can result in the receptor becoming shortened or losing its ability to function as a kinase. It was commonly noticed in gliomas, head and neck carcinoma, ovarian, gastrointestinal, pancreatic, pulmonary, and colorectal cancers. They also reached the conclusion that it happens, although less frequently, in other different cancers, including those of the endometrial, pancreatic, hepatic and breast cancer. Due to modifications in mismatch repair genes, these TGFβRII inactivating mutations were more frequently found in tumors with microsatellite instability. Ovarian, breast, esophageal, and pancreatic cancer frequently include frame-shift and missense mutations in the type I TGFβR (Levy and Hill, 2006).

2.4 SMAD MUTATIONS

Similar to TGFβ receptors, the Smad protein genes are frequently altered or deleted in human malignancies. Smad mutations result from deletions, frame-shift alterations, nonsense mutations, and missense mutations as well as the loss of chromosomal regions. These mutations, which are usually prevalent in Smad2 and Smad4 and either inhibit binding interactions with the Smad partners or obstruct initiation of Smad-mediated gene transcription, were reported to be present. These genetic changes had a significant impact on the tumor suppressor Smad4, also known as dpc4 (deleted in pancreatic cancer), which was mutated or deleted in at least 50% of human pancreatic cancer, where it was first identified. (Hahn et al., 1996).

2.5 MUTATIONS IN THE NON-SMAD TGFB SIGNALING PATHWAYS

Other forms of genetic changes have also been identified to influence TGFβ signaling and tumor development in addition to the known mutations in the TGFβ receptors and canonical Smad pathway. For example, it has been observed that oncogenic activation of the Ras-Raf-MAPK pathway and the c-Jun NH2-terminal kinase in liver cancer results in the phosphorylation of the Smad3 linker domain by MAPK, further blocking the C-terminal phosphorylation of the Smad by

the TGFβRI kinase domain and limiting TGFβ cytostatic effects (Nagata et al., 2009).

2.6 TARGETING TGFB PATWHAY

Antisense oligonucleotides (ASOs), monoclonal antibodies (mAb) that block interactions between ligands and receptors, and inhibitors of TGFβ receptor I kinases against the receptor mediated signaling cascade are three strategies that have been studied to block the TGFβ pathway. Despite the fact that ASOs are very effective TGFβ inhibitors (Hau, et al., 2003), their organ and tissue penetration limits their effectiveness (Dvorchik, 2000). Despite having great route selectivity and being administered orally, small-molecule kinase inhibitors (Yingling, et al., 2004) have the potential to be hazardous, and the reason for this toxicity is being explored (Giffin, and Robinson, 2008; Gómez-Gil, 2021).

Inhibiting tumor progression in patients with metastatic cancer is anticipated by the mAbs, which are created particularly to block active TGFβ ligands and prevent their interaction with the type II receptor (Lonning, et al., 2011). Neutralization of TGFβ using mAbs has been used in animal models to explore this idea. For instance, in tumor animal models as well as in transgenic breast cancer mammary tumor virus (MMTV)-neu mouse models, systemic and transgenic administration of the soluble TGFβRII/Fc dimer that binds all three TGFβ isoforms decreased tumor burden, invasion and metastasis (Gatto, 2004).

Most patient Derived Xenografts model revealed minimal or even stimulatory tumor growth response in contrast to the reported anticancer effects of TGFβ targeting medicines in xenograft models with human cancer cell lines. In contrast, the effect is anti-metastatic or is focused on the primary tumor cells in immune-competent mice models. Stimulation of the anticancer immunological reaction in tumor bearing mice in response to TGFβ suppression is diminished, which can account for these discordant data. These trials have so far produced both favorable and unfavorable outcomes (Colak and Ten Dijke, 2017). Many potential targets are being studied to inhibit TGFβ signaling pathway as summarized in figure 2.3.

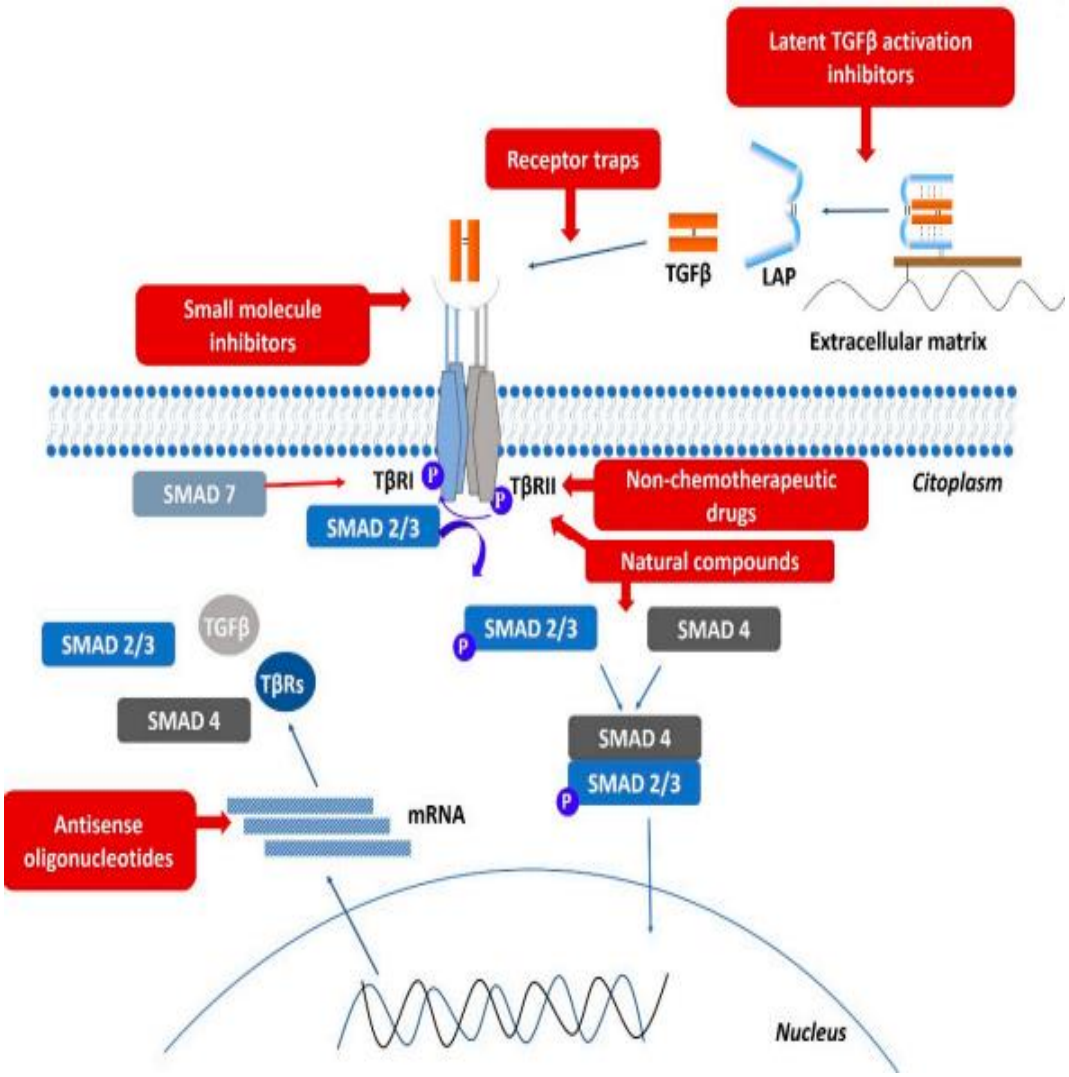

Figure 2.3: Potential therapeutic target of TGFβ signaling pathway (Adopted from Gómez-Gil, 2021)

2.6.1 Antisense Molecule

An influential method for impeding the TGFβ pathway is utilizing antisense molecules, a chemically synthesized segment of targeted RNA or DNA oligonucleotide is intended to tie target RNA or DNA to restrain its normal expected functions. For instance, the antisense molecule AP12009 known as trabedersen currently using as drug was created to invade the mRNA of the TGFβ gene and afterward repressed the development of TGFβ2 in severe stage of glioma, pancreatic,

melanoma and colorectal cancer cells. It was observed that trabedersen diminished TGFβ2 release in human pancreatic cell lines using xenograft mouse model which brought about clearly inhibiting cell growth and hindered cell metastatic ability to invade other cells. Likewise, trabedersen turned around TGFβ2 initiated immunosuppression. Previous studies showed an essential, randomized and dynamic controlled Phase II study in patients with high-grade brain tumor, intratumoral treatment with 10 μM trabedersen pave a way for additional clinical turn of events (Bogdahn et al., 2010; Yang et al., 2022).

Another antisense molecule belagenpumatucel-L is a therapeutic vaccination against TGFβ2 that has entered Phase III stage and might be useful for activating immune response. It was observed in phase III clinical trials in patients with non-small cell lung carcinoma having Stage III/IV who didn't progress after platinum-based chemotherapy. Belagenpumatucel-L was admiistered that has truly extraordinary persevered and no serious safety issues in 270 patients was observed in the belagenpumatucel-L group and 262 in untreated group. Survival time was increased upto 28.4 months in patients treated with belagenpumatucel-L as compared to control group having survival time of 16 months (Giaccone et al., 2015).

The TGFβ signaling pathway can also be targeted using antisense RNA, another tactic. Endogenous noncoding RNAs called microRNAs have the ability to control a variety of cancer-related mechanism. Studies have shown that miR-370, which can target anticipated locations in the 3′-untranslated region of the TGFβRII gene, is overexpressed in stomach tumors. To prevent TGFβ1 mediated migration, exogenous miR-370 expression reduced TGFβR II expression and Smad3 phosphorylation (Yang et al., 2022). By inhibiting TGFβRI and MMP2/9, other miRNAs, such as miR-4903p, reduced the ability of colorectal cancer to invade and metastasize. These specific compounds in the form of medications could offer a priceless insight for the development of tumor therapy strategies (Xu et al., 2015).

2.6.2 Antibodies

Neutralizing antibodies are new techniques to block the effects of TGFβ by neutralization of TGFβ activity, in addition to the use of antisense compounds to

suppress TGFβ signaling. The drug SD-208 has a promising future in the treatment of human malignant tumors. It was demonstrated that SD-208 inhibited the progression of lung metastasis in mice and the TGFβ transcriptional response mediated by Smad3/4 in osteosarcoma cell lines *in vitro*. According to literature survey SD-208 was more successful when administered as a preventive therapy and reduced osteolytic bone metastasis from prostate cancer cells in mice (Lamora et al., 2014).

TGFβ receptors in soluble form is another approach. The existence of soluble TGFβR III (glycan), which had been linked to TGFβ activation, was initially discovered to occur in several cells with the properties of mobile and cell surface proteoglycans. Glycan has been shown in some studies to be a suppressor of ovarian, prostate, kidney, and breast cancer. According to a previous study, the scaffolding protein arrestin 2 and glycan interact, activating cdc42 to stop cellular proliferation (Lee, Hempel, Lee, and Blobe, 2010; Tauriello, Sancho and Batlle, 2022).

Scientists discovered that the connection between the cytoplasmic domain of glycan and the scaffolding protein G Alpha Interacting Protein (GAIP) interaction protein C-terminus was a key factor in TGFβR III activated cell proliferation and invasion in breast cancer. The activation of the TGFβ signal transduction cascade was discovered to be inhibited by a different soluble TGFβR II in colorectal cancer, pancreatic cancer, liver cancer, Non-small cell lung cancer, and endometrial carcinoma. These soluble TGFβ- receptors are intended to target TGFβ selectively and have considerable therapeutic potential (Chen et al., 2020). A human immune globulin 4 monoclonal antibody called fesolimumab (GC1008) is capable of neutralising all TGFβ isoforms found in mammals. 29 individuals with progressed carcinoma or kidney carcinoma participated in a Phase I research that showed promising results for GC1008: The adverse reaction was curable skin keratoacanthomas/squamous-cell carcinomas and hyperkeratosis, one patient with malignant melanoma experienced a mild reaction, and six had persistent illness (Morris et al., 2014).

Three patients in 13 malignant pleura mesothelioma patients Phase II trials with stable illness after three months and lower expression of 2B4 and DNAM1 on natural killer cells, respectively, were observed. Additionally, five patients serum revealed higher median overall survival and novel or increasing levels of antibodies against metastatic pleura mesothelioma. The 2G7 and 1D11 antibodies are being studied in order to lessen breast cancer lung metastasis. These two antibodies mechanisms have an impact on immunological response (Stevenson et al., 2013; Tauriello, Sancho and Batlle, 2022**)**.

2.6.3 Vaccine Based Therapy

With numerous clinical assessments, tumor immunotherapy is now being studied. Cancer vaccines have recently been viewed as a successful immunotherapeutic approach. Cancer vaccines should elicit cellular and immune responses while overriding tumor induced immune suppression. Based on the utilization of tumor antigens given in the form of entire cells, nucleic acids, peptides, and proteins, several methods have been devised to create a cancer vaccine. Cancer vaccines are still in phase I/II clinical studies, despite their rapid development. For example a series of clinical trials (phase I and II) TGFβ2 or TGFβ1/2 expression was targeted by vaccines named as FANG and Belagenpumatucel-L using Vaccine-based strategy and the objective of these trials were the restoration of immune response in the tumor microenvironment. Belagenpumatucel-L, has been investigated in a phase II trial in NSCLC at different stages. Belagenpumatucel-L is a non-viral based allogeneic tumor cell vaccine, modified from TGFβ2 antisense gene, but not much encouraging results were obtained (Nemunaitis et al., 2006; Sadeghi Najafabadi, Bolhassani and Aghasadeghi, 2022)).

2.7 INHIBITION OF TGFB SIGNALING PATHWAY AND DOWNSTREAM SIGNALING PROTEINS

The TGFβ mediated receptor signaling pathway can be blocked by several small molecule tyrosine kinase antagonists of TGFβ activated receptors II and III. An example of small molecule inhibitor of TGFβR I inhibitor called galunisertib (LY2157299) hydrates has shown higher effectiveness in reducing Smad2

phosphorylation in carcinoma of the pancreas, lungs, colon, and ovaries. According to certain research, LY2157299 prevented HGSOC cells from proliferating, migrating, and invading *in vitro* (Zhang et al., 2018).

The phosphorylation of Smad2 by LY2157299 was observed to be inhibited in hepatocellular carcinoma models and in ex vivo entire tumor tissue samples from patients, and it was confirmed that this occurred in all categories. Additionally, in ex vivo samples, LY2157299 caused a reduction in proliferation biomarkers and an increase in cell death was observed (Hanafy et al., 2018). A TGFβR I kinase inhibitor called Ki26894 has been shown to bind to the ATP-binding site of TGFβR I and prevent Smad2 from being phosphorylated. According to studies, Ki26894 will inhibit motility and invasion in vitro by blocking TGFβ signaling. Additionally, X-ray radiography demonstrated that systemic Ki26894 therapy reduced bone metastasis (Tan, Alexe, & Reiss, 2009).

Additionally, Ki26894 reduced ZO2 and E-cadherin levels as well as Smad2 phosphorylation in scirrhous gastric cancer cells. On the other hand, TGFβ1 or Ki26894 therapy had no effect on non-cirrhous stomach cancer cells (Shinto et al., 2010). Another TGFβR I inhibitor, SB-431542, is being studied today. It may reduce TGFβ mediated transcription by preventing Smads nuclear translocation and activation by phosphorylation as well as by reducing the levels of plasminogen activator inhibitor-1 and vascular endothelial growth factor (VEGF) (Hjelmeland et al., 2004).

In a study conducted by Gobbi et al., (2000) correlated resistance to inhibition of tumor progression and cell proliferation mediated by TGFβ, when TGFβ type II receptor loss its expression. They used three types of cell culture including invasive mammary carcinomas (IMC), benign proliferative mass and ductal carcinoma in situ (DCIS). The detection of TGFβRII carried out by Immuno-histochemical methods and in contrast to the benign mass and normal breast cell lines, lower expression of TGFβRII has been observed in neoplastic cells. Loss of TGFβRII expression and tumor growth within both IMC ($P = 0.001$) and DCIS ($P = 0.004$) groups showed a significant inverse correlation.

2.8 ANTI-TGFB THERAPY WITH IMMUNOTHERAPY

Immune checkpoints are important immune response process regulators. Immune checkpoint signals come in two different varieties. CD (27, 28, 40, 134, and 137) and other co-stimulatory molecules facilitate the proliferation of cytotoxic effector T cells and their movement toward the tumor. Co-inhibitory signals enhance T cell depletion and impairment, and this methodology activates co-inhibitory molecules like PD-1, CTLA4, LAG3, TIM3, and SIGL. Because of this, co-stimulatory signal ligands and co-inhibitory signal inhibitors have been used in cancer immunotherapy, with CTLA4, PD-1, and its companion programmed death-ligand 1 (PD-L1) gaining the most interest and extensively studied (Chen and Flies, 2013).

2.9 OTHERS

There are various more drugs, including arsenic trioxide that can block TGFβ in addition to the three above said mechanisms. A synthetic substance known as As2O3 has been tested in several cancer treatments. According to research, As2O3 inhibits the expression of TGFβRII and Smad2/3, which controls angiogenesis. These lead us to believe that additional treatments, such as As2O3, may be effective inhibitors for blocking TGFβ signaling cascade (Zhang et al., 2017).

2.10 TARGETING TGFB IN CANCER THERAPY: CHALLENGES AND OPPORTUNITIES

2.10.1 Overcoming the Adverse Effects of Anti-TGFβ Therapies

In cases when TGFβs role in biological mechanisms is impaired, cancer patients who received treatment with TGFβ signaling inhibitors may experience adverse consequences. When used in high doses, TGFβRI kinase inhibitors can cause cardiac toxicity (hemorrhagic, debilitating, and inflammatory lesions in cardiovascular system) and dermal toxic effects (eruptive keratoacanthomas, skin rash, cutaneous squamous cell carcinomas, and basal cell carcinomas), which restricts the safe therapy window for these drugs. The practical use of many other anti-TGFβ treatments has been and remains hampered by these side effects.

TGFβ inhibitor toxicity may be reduced by giving cancer patients a careful dose. Additionally, pulsatile therapy, during which the patient takes so-called "drug holidays" during the dosage time, may help to lessen the negative effects (Fessler et al., 2016).

To lessen the (cardiac) negative effects, galunisertib has been used as a therapy regimen of two weeks on and two weeks off medication treatment. Predictive biomarkers may also make it easier to choose the patients who will benefit from anti TGFβ therapy the most. Cancer patients with mesenchymal subtype's exhibit high expression of TGFβ target genes, which is associated with a poor prognosis, according to the results of transcriptional profiling of samples from patients with a variety of malignancies, including glioblastoma, stomach cancer, breast cancer, ovarian cancer, CRC, and Non - small cell lung carcinoma. So, subsets of cancer patients with a mesenchymal phenotype may benefit more from anti TGFβ therapy (Fessler et al., 2016).

2.10.2 Perspectives on the Synergy of Combination Therapies

The resistance to several anticancer treatments, such as standard chemotherapy, targeted therapy, radiation, and immunotherapy, has been linked to increased TGFβ activity. Anti TGFβ therapy combined with these tried-and-true tactics may therefore reduce therapeutic resistance. Finding the ideal synergistic therapy regimen combination for each unique patient will be a prospective opportunity and challenge. Chemotherapeutic, laser surgery, or radiotherapy combined with anti TGFβ therapy. Chemotherapy is a common first line cancer treatment strategy made up with one or more anticancer agents that are intended to slow down and kill cancer cells that are rapidly multiplying. However, because chemotherapy is harmful to normal cells, there are significant side effects. Small compounds are used in targeted therapy to block particular signal transduction pathways that support the survival and proliferation of cancer cells. Such small molecule drugs include, for instance, selective tyrosine kinase inhibitors (Pottier et al., 2020).

Both types of therapy can result in therapy resistance and relapse because

some cells within the heterogeneous tumor population sustain and propagate after an initial valuable antitumor response, though selective small molecule antagonists tend to have fewer severe side effects than conventional chemotherapy. The mesenchymal morphology that TGFβ induced cancer cells acquire has been identified as a crucial mechanism that imparts the treatment resistant and pluripotency characteristics in cancer cells. Inhibiting metastasis and avoiding resistance to anticancer treatments are two benefits for suppressing TGFβ. For patients with Triple-Negative Breast Cancer, glioblastoma, Hepatocellular carcinoma, or Pancreatic ductal adenocarcinoma, TGFβRI kinase inhibitors, such as LY2157299, have been used with a variety of chemotherapies, such as paclitaxel, sorafenib, or gemcitabine to enhance anticancer potential (Capper et al., 2017).

Patients with solid tumors are being treated with LY3200882 in combination with gemcitabine/paclitaxel/cisplatin in a currently underway phase 1 trial (NCT02937272), or patients with resistant metastatic Colorectal cancer are being treated with LY3200882 in conjunction with capecitabine (NCT04031872). In a current phase 1/2 clinical trial, the effectiveness of vactosertib (TEW-7197) and paclitaxel combined for the therapy of advanced gastric cancer is being investigated (NCT03698825). In a phase 2 clinical trial, the pan anti TGFβ neutralizing antibody NIS793 is being evaluated in conjunction with gemcitabine/nabpaclitaxel treatment for patients with metastatic Pancreatic ductal adenocarcinoma (NCT04390763) (Dodagatta-Marri et al., 2019).

Targeted therapies have also been used in conjunction with TGFβRI kinase inhibitors to circumvent drug resistance in pre-clinical cancer models. For instance, decreased expression of mediator complex subunit 12 causes cancer cells to become resistant to a variety of tyrosine kinase inhibitors. The expression of mesenchymal markers was discovered to be induced, MEK/ERK activation was mediated, and TGFβRII protein levels were shown to rise as a result of Mediator complex depletion. Metastatic non-small cell lung tumors with downregulated complex that were treated with the TGFβRI kinase inhibitor galunisertib showed a treatment efficacy to RTK inhibitors (Huang et al., 2012).

Ionizing radiation is used in radiotherapy to destroy cancerous cells or slow

their growth. Some cancers, like glioblastoma, which generates a significant amount of TGFβ in the microenvironment and confers radio resistance on glioma-initiating cells, are especially radio resistant. The expression and release of active TGFβ can be stimulated by radiation, which can then lead to the fibrosis of normal tissues. Therefore, by increasing tumor cell radio sensitivity and safeguarding normal tissues, anti TGFβ therapy combined with radiotherapy may improve treatment (Huang et al., 2012). Fresolimumab (GC1008), a TGFβ -blocking antibody, was paired with focal irradiation in a phase 2 clinical trial for the cure of metastatic breast cancer, and it was discovered to prolong median overall life and show a positive systemic immune response. In a phase 2 clinical trial, fresolimumab and stereotactic adjuvant radiation are being used to treat early stage Carcinoma (NCT02581787). In a phase 1 clinical trial including patients with solid tumors, the combination of the TGFβRI kinase inhibitor LY3200882 with intensity modulated radiation is currently being investigated (NCT02937272) (Formenti et al., 2019).

2.11 NATURAL PRODUCTS AND TGFB PATHWAY

Natural products are best known for their safety, less toxic nature, antioxidant characteristics. So, they are promising candidate to treat different diseases like cancer (Singh et al., 2016). Some of the most well-known and efficient cancer medications on the market today have been derived from natural sources, such as plants. Therefore, it is understandable that employing alternative and complementary treatments many of which are plant-derived is considered with some trepidation or even mistrust, especially when it comes to the treatment of cancer. Clinicians should be aware that certain plant-based chemotherapy regimens are completely mainstream and backed by significant clinical research (Tewari, Priya, Bishayee and Bishayee, 2022).

A vast variety of natural compounds reported, best known for their antitumor activities could be used as potent inhibitors of Transforming Growth Factor β Receptor signaling pathway (Table 2.1). The frequency of undesirable reactions to food is generally low, despite the fact that many foods contain toxins as a naturally occurring component or as a result of handling or processing. Food allergies, which constitute for 90 % of all food allergies in the US, are another medically justified

restriction. The most prevalent food allergies are those to dairy, poultry, seafood, crustacean's crabs, tree nuts, wheat grains, peanuts and soybeans. (Dolan et al., 2010).

Table 2.1: Natural compounds targeting TGFβ pathway

Sr. No	Medicinal Plants	Bioactive Compounds	Target Genes	Reference
1	Turmeric & Parsely	Curcumin & Luteolin	TGF-beta Protein inhibitor in colon cancer an *in vitro* and *in vivo* study	Aromokeye & Si, (2022)
2	Lotus plumule	Rhoifolin	Downregulation of TGFβ2 and pSMAD2 in pancreatic cancer	Zheng et al., (2022)
3	Green tea	Epigallocatechin gallate (EGCG)	Low expression of TGFβR1 mRNA in HepG2 and PC3 cell lines	Singh et al., (2016)
4	Huang Qin	Crude extract	Inhibition of Transforming Growth Factor β (TGFβ) Signaling in Endometrial Cancer cell lines	Amber et al., (2016)
5	DanShen	Salvianolic Acid B (Sal B)	Principal target genes include TGF β1; 62Smad2/3 ;62Smad7;62MMP262	Agbarya et al., (2014)
6	Gale Of Wind	Corilagin	Inhibition of ovarian tumor growth by	Jia et al., (2013)

	blocking TGFβ signaling pathway

2.11.1 α-Tomatine

It was also claimed that the tomato plant's unripe fruit, stems, and leaves contain α-tomatine, a steroidal alkaloid made up of two molecules of D-glucose, D-xylose and D-galactose. Tomatine functions as a natural fungicide since it is harmful to a wide variety of fungus. It has been proposed that tomatine capacity to combine with membrane sterols and impair membrane function is what causes the harmful effects of tomatine on fungus. Many plant seeds' bran and germ, as well as grains, legumes, and nuts, contain phytic acid, commonly known as phytate. In addition to being an efficient chelator of divalent cations like zinc, copper, iron, magnesium and calcium, phytic acid is a simple sugar (myo-inositol) with six phosphate sidechains and serves as a dietary source of phosphorus. Phytate mineral complexes, according to studies, are soluble in the digestive system but reduce the bioavailability of minerals. (Deshpande, 2002a).

2.11.2 Satsuma Mandarin (SM)

The peel of the citrus fruit *Citrus unshiu* (also known as the Satsuma mandarin) has been used in traditional Chinese medication to cure cancer, fatigue, and the colds. Previous study investigated the ability of SM component and peel extracts to inhibit the proliferation of cancer cells. In tumor harboring mice with the renal carcinoma cell, the mechanism underlying the anti-cancer activities of SM was examined. Measurement of the tumor tissue in mice treated with varying dosages of SM content and peel extracts allowed researchers to assess how well SM suppresses growth of cancer cells. The MTT assay were used to measure the proliferation of tumor cells and splenocytes, respectively. Relevant immunological pathways were investigated using ELISA cytokine assays for TGFβ, IL-6, IFNγ, and TNFα. In tumor-bearing mice, the content and peel extracts of SM prevented the proliferation of malignant cells. In particular, the average tumor volume in the two groups treated

with 3 and 30 mg of peel extracts per kg of mice was considerably reduced to 52.32% (p 0.05) and 68.72% (p 0.01), respectively. Antitumor cytokines were evaluated in Con A-activated spleen cells from tumor bearing mice to determine the mechanism of tumor recurrence (Lee et al., 2011).

TNF alpha which had been lowered by tumor progression, was restored to the normal level in the groups treated with SM content and peel extracts, although IFN gamma was elevated in both peel extract treated groups. However, neither the SM content nor the peel extracts prevented tumor cells from proliferating and producing the tumor-promoting cytokines TGFβ and IL-6. These findings suggest that the anti-tumor effects of SM content and peel extracts in the tumor-bearing mouse model giving Strong evidence points to an immune system-mediated mechanism for how SM extracts exert their anti-tumor actions by increasing cytokines including IFN gamma and TNF alpha (Lee et al., 2011).

2.11.3 Lycopene and Lutein

Utilization of the carotenoid lycopene, which has no provitamin A activity, has been linked to a decreased risk of breast and prostate cancer. Another carotenoid, lutein, has been linked to a reduction in risk of age related retinitis pigmentosa, which is the main cause of vision loss in people 65 and older. It has been demonstrated that bioactive substances like lycopene and lutein, derived from organic plant sources, act at low substrate levels by interacting with intrinsic cytokines, matrix proteins, and their receptors in tissues, especially those belonging to the fibroblast growth factor and TGFβ families(Lee et al., 2011).

Compared to their benign parent type I melanoma epithelial cells, Dunning R3327AT3 or AT3 cells (androgen-responsive, slow-growing cancer cells with well-developed epithelium and stroma) were used to examine the consequences of citrus and commercial lycopene and lutein treatments on androgen unbiased cultured malignant type II cancer cell. Results showed that lutein and lycopene both suppressed malignant AT3 cells in a concentration and time dependent way when delivered together in cyclodextrin water soluble vehicle (Lee et al., 2011).

Examining normal DTE cells revealed no such effect, illustrating the selective suppression of particularly lethal AT3 prostate cancer cells in comparison to their benign parent. Comparable to lycopene, lutein showed a similar but slightly less pronounced reaction. No additive or synergistic effects were seen when cells were exposed to lycopene and lutein combos. These results support epidemiological research showing inverse associations between these carotenoids and prostate cancer (Gunasekera et al., 2007).

2.11.4 Chinese Herbs Crude Extract

The Chinese herbs *Scutellaria baicalensis* (SB) and *Fritillaria cirrhosa* (FC) have been demonstrated to be helpful to cancer patients in a study conducted by Amber et al., (2016), however the exact processes by which the extracts of the two herbs elicit the positive effects were unknown. In this study, the anticancer effectiveness of SB and FC on TGFβ signaling pathway members was evaluated using human endometrial cancer cells.

The expression of TGFβ isoforms, TGFβ receptors and SMADs significantly decreased after cancer cells were treated with SB and FC. Both herbs significantly reduced the growth and invasion of primitive and TGFβ1 induced cancer cells, along with the expression of Snail, Slug, matrix metalloproteinases (MMPs), avb3 integrin, focal adhesion kinase (FAK), and p-FAK. A TGFβRI inhibitor greatly reduced the anticancer effects of SB and FC and prevented TGFβ1 induced cell migration. These findings imply that downregulation of the TGFβ/SMAD signal transduction pathway by SB and FC inhibits the progression of endometrial cancer (Amber et al., 2016).

2.11.5 Corilagin

In a study conducted by Jia et al., (2013) explored the inhibition ovarian tumor growth by blocking TGF-β signaling pathway using natural medicine named as corilagin. They used *Phyllanthus niruri* L. plant best known for medicinal effect. Corilagin was extracted and treated with ovarian cell lines and analysis was done by flow cytometry and cell proliferation assay, followed by delivery of corilagin in mice

having SKOv3ip disease. Then they concluded that corilgin is a natural inhibitor of TGF-β signaling pathway, showed a potential to inhibit the ovarian cancer cell lines with IC_{50} value of less than 30 μM and having low toxicity level against normal cells made it a best choice for treatment of ovarian cancer.

2.11.6 Myricetin and Epigallocatechin Gallate

A better therapeutic approach was carried out by Singh et al., (2016) through inhibiting the tumor promoting effect of TGFβR in tumorigenesis. Selection of natural ligand molecules for inhibition of three receptors (TAK1, TGFβR1 and TGFβR2) was achieved by *in silico* approach and *in vitro* study. Inhibitors molecule having best docking energy were selected e.g. myricetin and epigallocatechin gallate (EGCG), treated with cell lines and low expression of TGFβR1 mRNA at level of 80 μM was analysed through RT-PCR.

In conclusion TGFβ plays a significant role in encouraging the spread of cancer throughout the body and can directly promote metastasis, therefore targeting its tumor-promoting activity offers considerable promise.

CHAPTER 3

MATERIALS AND METHODS

3.1 PLANT SAMPLE COLLECTION

The medicinal plants of Pakistan reported for anticancer activities or used as anticancer folk medicine were collected from various districts of Punjab and KP, Pakistan to determine their *in-vitro* anticancer and cytotoxicity and their impact on TGFβ signaling pathway. Total sixteen plants were selected for collection and collected plant samples were identified by Prof. Dr. Rahmatullah Qureshi (Table 1). The voucher sample were submitted at Department of Botany, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi (PMAS AAUR), Pakistan. Fresh plants parts (Leaves, stem, whole plant) of sixteen different plants were used for fractionation and crude methanolic extracts.

Table 3. 1: List of medicinal plants used in study

Sr. No.	Scientific Name	Voucher No.	Common Name	Location Collected	Part Used	Reference
1	*Digera muricata* (L.) Mart	PMAS-03	Anona	Murree	Leaves	antibacterial, antifungal (Vaishnav et al., 2015)
2	*Psidium guajava* L.	PMAS-08	Guava	D.G. Khan	Leaves	antispasmodic and antimicrobial (Sharma et al., 2013)
3	*Ficus carica* L.	PMAS-05	Figs	D.G. Khan	Leaves	anticancer, hepatoprotective (Gutierrez et al., 2008)
4	*Piper nigrum* L.	PMAS-07	Black pepper	D.G. Khan	Leaves and stem	antioxidant, antiasthmatic, anticarcinogenic (Mawa et al., 2013)
5	*Fagonia cretica* L.	PMAS-06	Dhamasa	Attock	Whole Plant	Antioxidant (Meghwal and Goswami, 2013)
6	*Azadirachta indica* A. Juss.	PMAS-02	Neem	Jhang	Leaves	Ailments (Satpute et al., 2012)
7	*Argemone mexicana* L.	PMAS-01	Satyanasi	Attock	Whole Plant	Antimicrobial (Kumar and Navaratnam, 2013)
8	*Rubus fruticosus* L.	PMAS-14	Blackberry	D.G. Khan	Leaves	antioxidant, anticarcinogenic,

						antiinflammatory, antimicrobial antidiabetic (Singh et al., 2010)
9	*Vitis vinifera* L.	PMAS-15	Grapes	Rawalpindi	Leaves	Antioxidant (Zia-Ul-Haq et al., 2014)
10	*Moringa oleifera* Lam.	PMAS-11	Sohanjana	Attock	Leaves	antioxidant, anticancer, cardiovascular, hepatoprotective, anti-ulcer (Apostolou et al,. 2013)
11	*Jasminum officinale* L.	PMAS-09	Jasmine	D.G. Khan	Leaves	Antiviral (Farooq et al., 2012)
12	*Punica granatum* L.	PMAS-13	Pomegranate	D.G. Khan	Leaves	Antibacterial, anti-inflammatory, anticancer, antioxidant (Zhao et al., 2009)
13	*Cichorium Intybus* L.	PMAS-12	Chicory	Islamabad	Leaves	Antibacterial (Arun and Singh, 2012)
14	*Xanthium strumarium* L.	PMAS-16	Cocklebur	Abbottabad	Leaves	Anticancer (Street et al., 2013)
15	*Carissa opaca* Stapf ex Hains.	PMAS-04	Karonda	Murree	Leaves	Antimicrobial, antitumor (Ramirez-Erosa et al., 2007)
16	*Cyperus rotundus* L.	PMAS-10	Nut Sedge	Rawalpindi	Whole Plant	diuretic, analgesic, antispasmodic (Sahreen et al., 2013)

3.2 PREPARATION OF PLANT CRUDE EXTRACTS

Fresh plant parts were shade dried and crushed to obtain fine powder. The extracts were prepared by maceration using methanol solvent and continuously shaking for 24 hours at room temperature, filtered using Whatman Filter Paper No.1 and again shaking for 72 hours followed by collection of extract in petriplates and air dried to evaporate solvent. Extractive yield of crude methanolic extracts (CMEs) was measured and extracts were stored at 4 ºC until further use (Batool et al., 2019; Tabassam et al., 2019).

3.3 TOTAL FLAVONOID CONTENT

The total flavonoid content of CMEs was determined by aluminum chloride

method using quercetin as standard (Sakanaka et al., 2005).

3.4 FREE RADICAL SCAVENGING ACTIVITY

The antioxidant activities of plants methanolic extracts were determined by DPPH method as previously reported Patel and Patel (2011). Percentage scavenging activity of DPPH free radical for each sample was calculated by using following formula:

$$Scavenging\ (\%) = \frac{Absorbance\ of\ control\ -\ Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

Antioxidant potential of the extracts were than presented as IC_{50} values as compare to control.

3.5 *IN VITRO* CYTOTOXICITY ANALYSIS

3.5.1 Cell Cultures

Hepatocellular carcinoma (HUH), human breast cancer cell line (MCF-7), Human epidermoid cancer cells (HEP2) and Human Corneal Epithelial Cells (HCEC) maintained at institute of biomedical and genetic engineering (IBGE), Islamabad were used for *in vitro* cytotoxicity studies. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) having: 10 % Fetal calf Serum (FCS) 1 % streptomycin and Penicillin. Cells were cultured at 37 °C with 5 % CO_2 in incubator (Shel lab).

An SB28-luciferase cell line (SB28), generated using the Sleeping Beauty transposon system and provided by Dr Hideho Okada University of California San Francisco, was cultured with Roswell Park Memorial Institute medium (RPMI 1640) with L-glutamine plus (gibco), supplemented with 10 % FBS (HyClone), 1 mM sodium pyruvate, and 1 % MEM Nonessential Amino Acids Solution (MEM NEAA 100X, Thermo Fisher) and cultured at 37 °C in a humidified CO_2 (5 %) environment. Cell confluency and viability was measured by using Microscope and cell counter (countess II, Life technologies) using trypan blue stain (Invitrogen) respectively.

3.5.2 Cell Viability Assay

The MTT assays were performed to evaluate the cell inhibitory effects of

crude plant extracts and their fractions on HUH, HEP2, MCF-7 and HCEC cell lines. Approximately 1×10^5 cells per well were seeded in 96-well plates. The cells were exposed to plant extracts at different concentration and incubated for 24 hours. At the end of the incubation period, media was disposed of and 10 µL of MTT (0.5 mg/ml) in PBS was added. Cultured cells were incubated at 37 ºC for three more hours and the reduced MTT dye (Invitrogen) was dissolved with 20 % DMSO or solubilization solution. Absorbance was measured at 570 nm in Microplate reader (FLUOstar Omega, BMG LABTECH). All experiments were performed in triplicate and mean values were taken for calculation of percentage cell viability. Relative cell viability was expressed as percentage difference between exposed and unexposed cells.

MTT assays were performed to evaluate the cell inhibitory effects of crude plant extracts and their selected fractions on SB28 cell line. Approximately 7.5×10^3 cells per well were seeded in 96-well plates. The cells were exposed to plants CMEs at different concentration and incubated for 24 hours. At the end of the incubation period, media was disposed of and 100 µl of media containing MTT (Biotium) 1:10 ratio was added. Cultured cells were incubated at 37 ℃ for four more hours and the reduced MTT dye formazan crystals were dissolved by adding 100 µl of 100 % DMSO (Sigma Aldrich, UK). Absorbance was measured at 570 nm in Microplate reader (BioTek). All experiments were performed in triplicate and mean values were taken for calculation of percentage cell viability. Relative cell viability was expressed as percentage difference between treated and untreated cells (Larsson 2020).

3.6 RELATIVE EXPRESSION PROFILING OF GENES

3.6.1 Isolation of Total RNA

The HUH cells were plated into the six-well culture plate with plant extracts at IC_{50} dose. The culture plates were incubated in CO_2 incubator at 37 °C for 24 hours. Total RNA was extracted as per manufacturer's instruction using Gene Jet RNA Purification Kit (Thermoscientific, USA). Quantification of RNA was done using nanodrop (Nanodrop 2000C Spectrophotometer, Thermo Scientific, USA).

3.6.2 Synthesis of cDNA

First strand cDNA was synthesized from extracted RNA using cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Thermoscientific, USA) according to manufacturer instruction. The cDNA was stored at -80 °C till further analysis.

3.6.3 Relative Quantification of Target Genes

Gene expression levels have been widely measured using quantitative real-time polymerase chain reaction (qPCR). Absolute quantification and relative quantification are the two methods used to analyze qPCR results. Based on a standard curve, absolute quantification determines the input gene amount. Relative quantification, on the other hand, quantifies alterations in gene expression in relation to a control samples. Since a standard curve does not need to be produced, relative quantification is simpler to accomplish and uses less reagents than absolute quantification. Relative expression of the genes TGFBR1, TGFBR2, TGFBR3, SMAD3, SMAD5, SMURF1 and SMURF2 was determined using GAPDH as gene of reference. RT-PCR was carried out by gene specific primers (Table 2) using Maxima SYBR Green Kit (Thermoscientific, USA) in RT-PCR machine (Slan 96P, Sansure, China). Relative gene expression levels were calculated by ΔΔCT method (Schmittgen & Livak, 2008). The level expression was presented in form of fold change over control.

Table 3. 2: Gene specific primers used in study

Sr. No.	Gene name	Forward primer	Reverse primer
1	TGFβR2	CCATGTCTCACAGCCAGCTA	CCAGGAGAAATAAGGGCAA
2	TGFβR3	CCAAGATGAATGGCACACAC	CCATCTGGCCAACCACTACT
3	TGFβR1	CATTTTTCCCAAGTGCCAGT	ACACCCCTAAGCATGTGGAG
4	SMAD3	TGCTGGTGACTGGATAGCAG	CTCCTTGGAAGGTGCTGAAG

5	SMAD5	AACCTGAGCCACAATGAACC	GTGGCATATAGGCAGGAGA
6	SMURF1	TCCGTCTCTGCTTTTCCACT	TGAGTTCTTTGGGGTGGTTC
7	SMURF2	TAGCCCTGGCAGACCTCTTA	AATACACCTGGCCTTGTTGC
8	GAPDH	ACGGATTTGGTCGTATTGGGCG	CTCCTGGAAGATGGTGATGG

3.7 COLUMN CHROMATOGRAPHY

Column chromatography is the ideal method of chromatography for purification, separation and fractination. The crude methanolic extracts (CMEs) of five plants i.e *F. cretica, A. mexicana, C. intybus, C. opaca* and *C. rotundus* was subjected to column chromatography using silica gel 60 (0.063-0.200 mm, Merck KGaA, CAS-No: 7631-86-9). The amount of 500mg of CMEs was dissolved in n-Hexane pure to make slurry. The solution was applied to a silica gel column (30 cm × 2.0 cm i.d.), which was preconditioned with n-Hexane. The column was eluted using combination of three different solvents as listed in Table 3. The eluent was collected by fraction size of 50 ml and tentative identification was carried out using TLC (Tang et al., 2007). Yield (%) was calculated for each fraction using following formula (Weight of empty bottle (g) – weight of bottle with dried fraction (g) = Net weight (g) x 1000 = weight (mg).

Table 3.3: Solvent ratios used for obtaining fractions of different polarity

Fraction No.	**Solvent Ratio (%)**		
	n-Hexane	Ethyl acetate	Methanol
F1	100	0	0
F2	100	0	0
F3	80	20	0
F4	60	40	0

F5	40	60	0
F6	20	80	0
F7	0	100	0
F8	0	80	20
F9	0	60	40
F10	0	40	60
F11	0	20	80
F12	0	0	100
F13	0	0	100

3.8 THIN LAYER CHROMATOGRAPHY

Each of the aforesaid thirteen fractions of each plant extracts was, to begin with, checked by Thin Layer Chromatography (TLC) on analytical plates TLC grade over silica gel 60 (F254, Merck Germany). For each fraction, five different solvent systems were used as developing systems. These were n-Hexane (Solvent A), n-Hexane: ethyl acetate = 1:1 (Solvent B), Ethyl acetate (Solvent C), Ethyl acetate: Methanol = 1:1 (Solvent D) and Methanol (Solvent E). 2 µl of each fraction was loaded in Silica plates followed by drying and then run-in presence of above-mentioned solvents. In each case, the spots were visualized by naked eye followed by exposure of the plates to formaldehyde solution (37 % Formaldehyde v/v in distilled water followed by mixing in concentrated H_2SO_4 to obtain 1:10 ratio with addition of small amount of 15 % Methanol to final solution to avoid polymerization). Plates were also visualized in Gel documentation system to check presence of any UV active compounds. Retention factor (Rf) values were calculated for each spot using following formula (Talukdar et al., 2010).

3.9 WESTERN BLOTTING

Western blotting also called protein blotting or immune-blotting is a robust and sensitive method for the detection and characterization of proteins.SB28 cells were

treated with CMEs of *A. mexicana* (Am CME, 30 µg/ml), *C. intybus* (Ci CME, 92 µg/ml) and their selected fractions AmF8 (62 µg/ml) and Ci F9 (185 µg/ml) according to IC_{50} obtained from MTT assay and cells treated with vehicle DMSO were taken as control. After incubation with samples at 37 ℃ and 5 % CO_2 overnight cells were trypsinized (0.05 %) and collected for protein extraction.

3.9.1 Protein Extraction Primary Preparation

Cells were centrifuged to obtain cell pellet and supernatant was discarded. RIPA buffer was added 1 ml/tube followed by incubation on ice for 1h. After this cells were centrifuged at 10000 rpm at 4 ℃ followed by collecting supernatant and stored at -80 ℃ until further process. Measure Protein concentration by using BCA kit (Thermofisher, catalog # 23227) method according to manufacturer instructions as discussed below.

3.9.1.1 Measuring concentration of treated samples

Protein standards of known concentration were prepared according to following final conc. (2000, 1500, 1000, 750, 500, 250, 125, 25, 0) µg/ml respectively. BCA working reagent (WR) was prepared by using following formula to determine the total volume of WR required: (No. of standards + No. of unknown samples) × (No. of replicates) × (volume of WR per sample) = total volume WR required. For the standard 96 well plate procedure with 6 unknowns and 2 replicates of each sample: (9 standards + 6 unknowns) × (2 replicates) × (200 µl) = 6 ml. WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). 96 well plate were taken and 20 µl of each known and unknown samples was added in respective wells in and 180 µl of WR was added in each well.

Plate was covered and incubated at 37 ℃ for 30 minutes. The wells should turn from a green color to a purple/violet color after 30 minutes and absorbance was taken at 562 nm on the Biotek plate reader. Concentration of protein was determined by drawing a regression line from known sample followed by calculating the concentration of unknown samples from standard curve (Figure 3.1). For this graph, the equation is: $y= 0.0009x + 0.2045$.

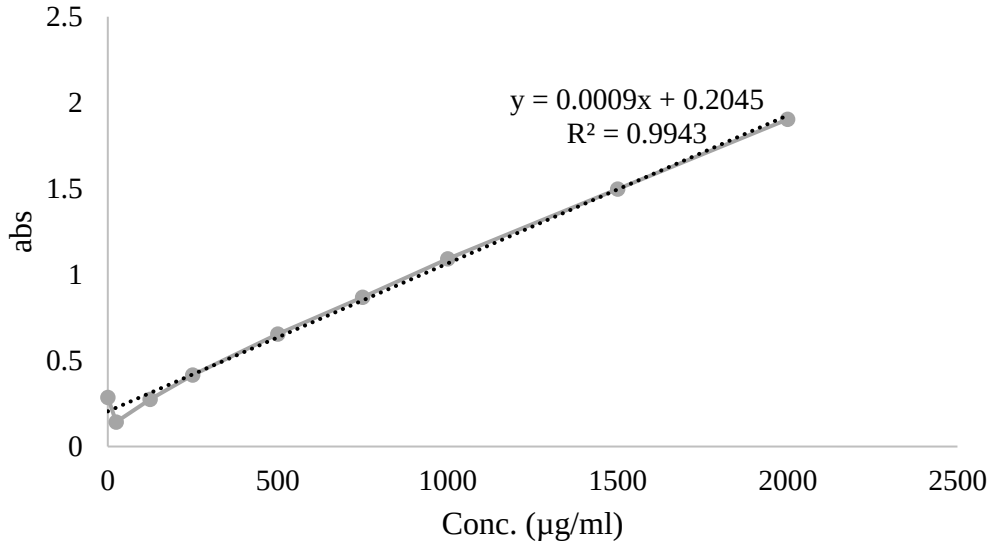

Figure 3.1: Standard curve obtained from BSA standard of known concentration

3.9.2 Gel Electrophoresis and Transfer to PVDF Membrane

Antibodies typically recognize a small portion of the protein of interest (referred to as the epitope) and this domain may reside within the 3D conformation of the protein. To enable access of the antibody to this portion it is necessary to unfold the protein, i.e. denature it. Protein concentration used for loading was 20 µg for each treated and untreated samples mixed with loading buffer with detergent sodium dodecyl sulfate (SDS) and boiled the mixture at 95-100 ℃ for 5 minutes. Based on protein of interest size gradient gel 4-15 % was used. 20 µl of each sample was loaded in lane 2 to 8 while molecular weight marker (BioRad) was loaded at lane 1 of gel and gel was run in running buffer (1X tris/glycine/SDS buffer) for 2 hours at 75 V.

Transfer Buffer 1x solution was prepared by taking 100 ml of 10 X Transfer buffer **(**10 X tris/glycine Buffer**),** 200 ml of 100 % methanol and 700 ml of deionized water. Transfer module having gel and PVDF membrane was moved to gel tank with transfer buffer and run at 75 V for 90 minutes at 4 ℃.

3.9.3 Blocking and Incubation with Antibodies of Interest

LiCor Blocking Buffer was used for blocking at Room temperature for 1h. After blocking for 1 hour membrane was probed with antibodies against following proteins: β-actin, TGFβR1, TGFβR2 and pSMAD2 according to manufacturer recommended concentrations and scanned the blots on the LiCor Odyssey.

3.10 FLOW CYTOMETRY

Cells were exposed to Cells were treated with CMEs of *A. mexicana* (Am CME, 30 µg/ml), *C. intybus* (Ci CME, 92 µg/ml) and their selected fractions AmF8 (62 µg/ml) and Ci F9 (185 µg/ml) based on IC_{50} concentration obtained from MTT assay. Cells treated with vehicle having same concentration of DMSO as present in treated samples with extracts were taken as control untreated while cells treated with TGFβ were taken as positive control. SB28 cells were treated with sample for 24 hours at 37 ℃ and 5 % CO_2 and then processed for flow cytometry analysis. Cells were stained with four different fluorophores to check live dead using Zombie Aqua cell stain, cell proliferation was checked against Ki67 antigen stained with impermeable Halotag® AF647 antibody, pSMAD2 expression by impermeable Halotag® AF488 Antibody and propidium iodide (PI) was used for cell cycle analysis.

Approximately 1 x 10^6 cells/ml were taken in pre-labeled FACS tubes after counting them using Countess II machine. Cells were centrifuged and supernatant was discarded. Cells were washed with 1ml cold PBS and centrifuged at 350 g for 5min at 4 ℃. Supernatant was aspirated and cells were resuspended in 100 µl/tube of Zombie aqua solution in PBS (1:1000). Washed once with 2 ml of Cellular staining buffer (CSB) containing PBS + 10 % FBS and fixed with cold 70 % ethanol for 2-4 hours on ice. After this cells were again washed with 500 µl of Intercellular perm buffer twice and stained with AF488 and AF647 antibodies (Biolegend) according to manufacturer protocol. Cells were again washed with intercellular perm buffer and cells were resuspended in CSB 200 µl and run on CYTEK machine of flow cytometry. For cell cycle analysis incubate cells with PI solution after fixation for half hour at room temperature. Analysis was done using FCS Express 7 software

(Golden et al., 2014).

3.11 *IN VIVO* MICE STUDIES USING *A. mexicana* CME

Everything is done with the review and approval of the University of California San Francisco (UCSF) institutional animal care and use committee (IACUC) committee supervision. The Laboratory Animal Resource Center (LARC) at UCSF School of Medicine approved the animal protocol, and it was followed for all of the institute's animal experiments. Mice were raised in a conventional cage (5 mice per cage) in the animal house at the university in hygienic conditions, with air filter toppers and in a filtered air atmosphere room, with a temperature of 22 °C and a 12 hour light/dark schedule.

Experiment was designed to test whether *A. mexicana* crude methanolic extract (Am CME) can be administered to mice without gross evidence of toxicity. For this purpose 8 to 9 weeks old Balb/c mice were taken. So 5 mice (without tumor) were taken and grouped as 1 mouse receiving Single dose of 15 mg/kg, 1 mouse Single dose of 30 mg/kg, 1 mouse Single dose of 60 mg/kg, 1 mouse Single dose of 240 mg/kg and 1 mouse Single dose of 10 % DMSO + PBS vehicle. Sample injection was given on alternate days for three weeks. Primary end point was to monitor weight and any side effects occur with respect to sample injections.

Another experiment was designed to test whether Am CME administered to tumor bearing mice affects tumor growth. For this purpose 6 to 7 weeks old Xenografts model of mice for breast cancer cell line MTDT were used to study. V291LK cells (5×10^5) were subcutaneously injected using a 25-gauge needle and 200 µl of V291LK cells suspended in PBS into the right dorsal flank of the mice. Tumor volumes were computed using the formula TV = (L x W2)/2, where L stands for the longer diameter and W for the shorter diameter. Tumors were measured thrice a week. The mice were separated into six groups of five in each group and each group received an intraperitoneal injection of either DMSO (as a control vehicle) or 15, 30, 60, 120 or 240 mg/kg of Am CME once palpable tumors had developed to a diameter of $>45mm^3$. For two weeks, the mice received treatment on alternate days each week before being killed. The primary end point was when tumor volume reach up to 500

mm^3 and to monitor any side effects.

3.12 STATISTICAL ANALYSIS

All the experiments were carried out in duplicate or triplicate. Data were presented as mean ± Standard deviation. Microsoft Excel 2010 and Graph pad Prism version 9 was used for the statistical and graphical evaluations.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 TOTAL YIELD OF PLANT CRUDE EXTRACT

The CMEs percentage yield of the plants ranged from 6 to 42.67 % (Figure 4.1). *C. rotundus* has highest extraction yield 42.6 % while lowest was observed by *C. intybus* which is 6.21 %.

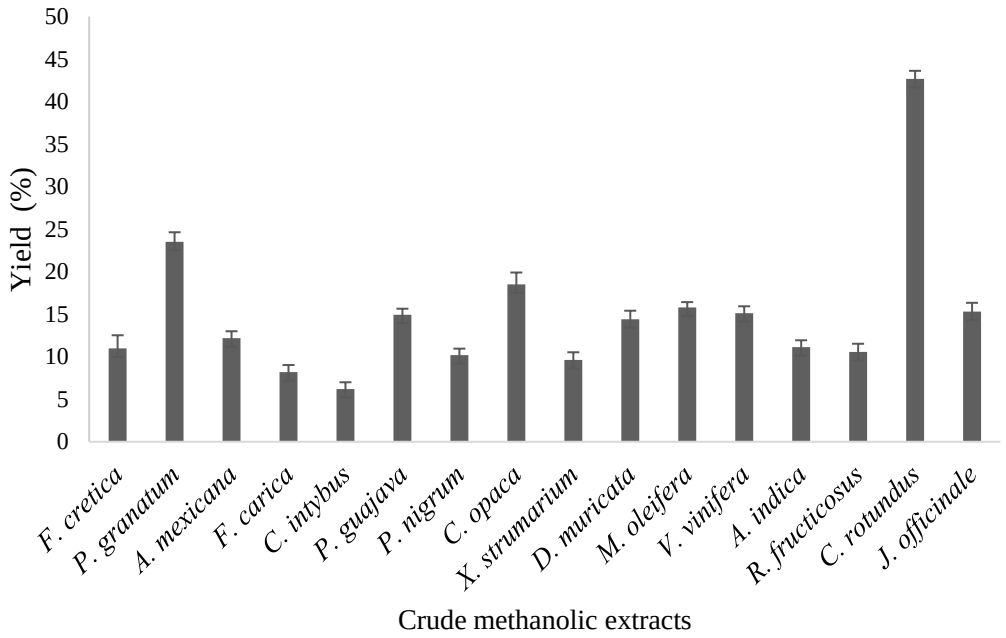

Figure 4.1: Extraction yield (%) of selected medicinal plant extracts

4.2 TOTAL FLAVONOID CONTENT (TFC)

Flavonoids play important roles for human health such as antioxidant and anti-inflammatory roles (Pourmorad, Hosseinimehr and Shahabimajd, 2006). Total flavonoid content of CMEs was determined by aluminum chloride method as presented in Figure 4.2. The analysis of figures showed that all plants have TFC <0.5 mg/gQE while *C. intybus* contain highest amount 0.45 mg/gQE and plant having lowest amount of TFC 0.02 mg/gQE was *Azadirachta indica (A. ind**ica)*.

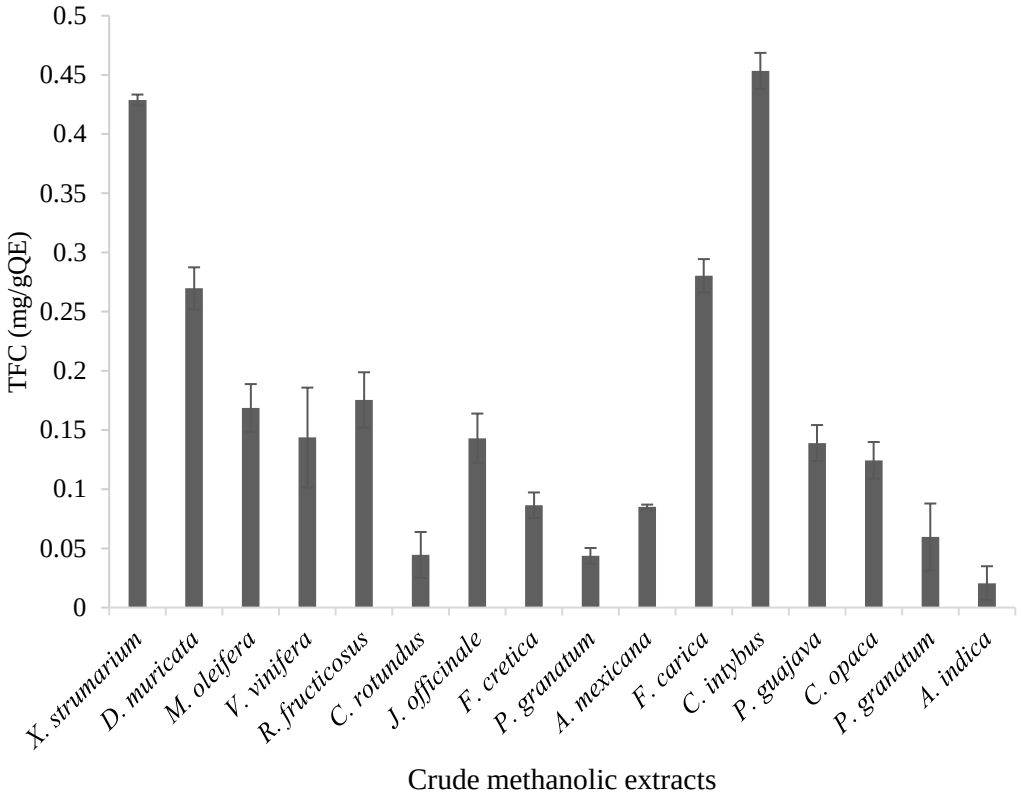

Figure 4.2: Flavonoid content (mg/gQE) of selected medicinal plant extracts

4.3 ANTIOXIDANT ACTIVITY OF CME OF SELECTED PLANTS

Free radicals lead to variety of diseases like neurological disorders and immune deficiency diseases and cancer. Scavenging activity of antioxidants is valuable for cure of those fatal diseases. DPPH assay is quick and easy method to measure antioxidant activity (Koleva et al., 2002). The DPPH free radical scavenging activity results are shown in Figure 4.3. *M. oleifera* revealed the maximum scavenging activity having IC_{50} value of 2.03 µg/ml and that of standard ascorbic acid IC_{50} was 5.67 µg/ml. Free radical scavenging activity was also remarkably good for *Digera muricata, Vitis vinifera, R. fruticosus, Psidium guajava, Piper nigrum, P. granatum, C. opaca, X. straumarium* and *F. carica* with IC_{50} values <30.00 µg/ml. While the lowest activity was observed by *C. intybus* having IC_{50} value of 87.34 µg/ml.

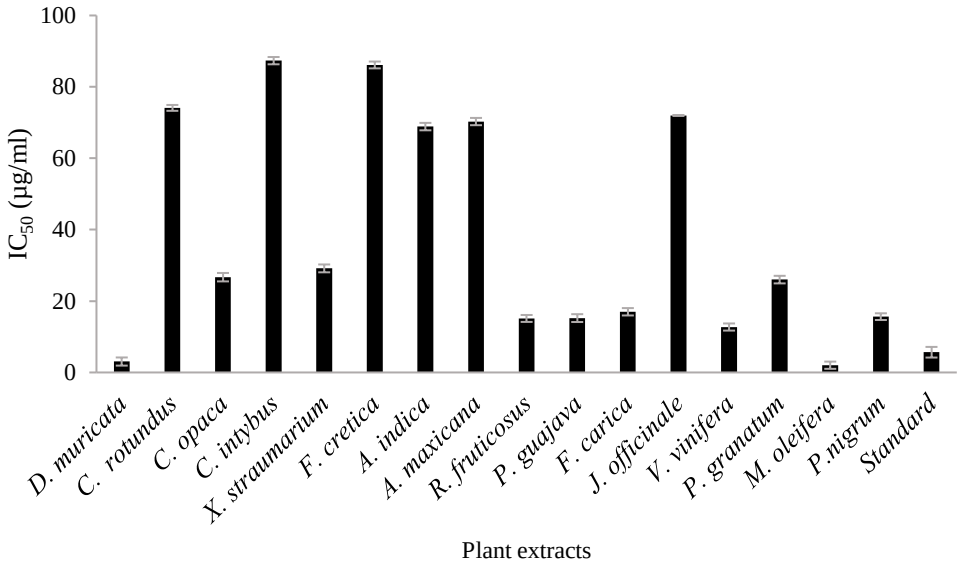

Figure 4.3: Antioxidant activity of crude methanolic extracts of selected medicinal plants

4.4 SCREENING PLANTS FOR THEIR ANTICANCER ACTIVITY

It was investigated earlier that secondary metabolites can be responsible for anticancer activities (Batra and Sharma, 2013). Cells were treated with crude methanolic extract at four different concentrations 2.5 mg/ml, 1.5 mg/ml, 0.5 mg/ml and 0.1 mg/ml. The MTT assays were performed to evaluate the cell inhibitory effects of crude plant extracts and their fractions on HUH, MCF-7 and HCEC cell lines.

The inhibition percent was calculated at different concentration of crude methanolic extracts to check the anticancer potential of plant extracts against tested cancer cell lines. The results of anticancer effect of crude methanolic extracts on HUH and MCF-7 cell lines are shown in Figure 4.4 to 4.7. All tested CMEs exhibited non-significant cytotoxicity against HCEC cells. However all plants showed significant anticancer activity against HUH cell line >50 %. *F. cretica, R. fruticosus, P. granatum, X. strumarium* and *C. rotundus* revealed significant highest activity >80 % (Figure 4.4 and 4.5).

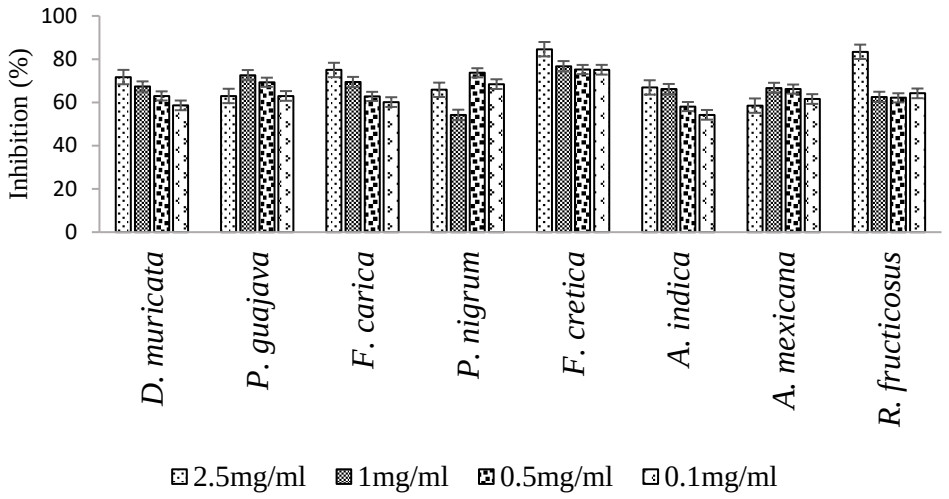

Figure 4.4: Anticancer activity of *D. muricata, P. guajava, F. carica, P. nigrum, F. cretica, A. indica, A. mexicana* and *R. fruticosus* on HUH cell line at different concentration

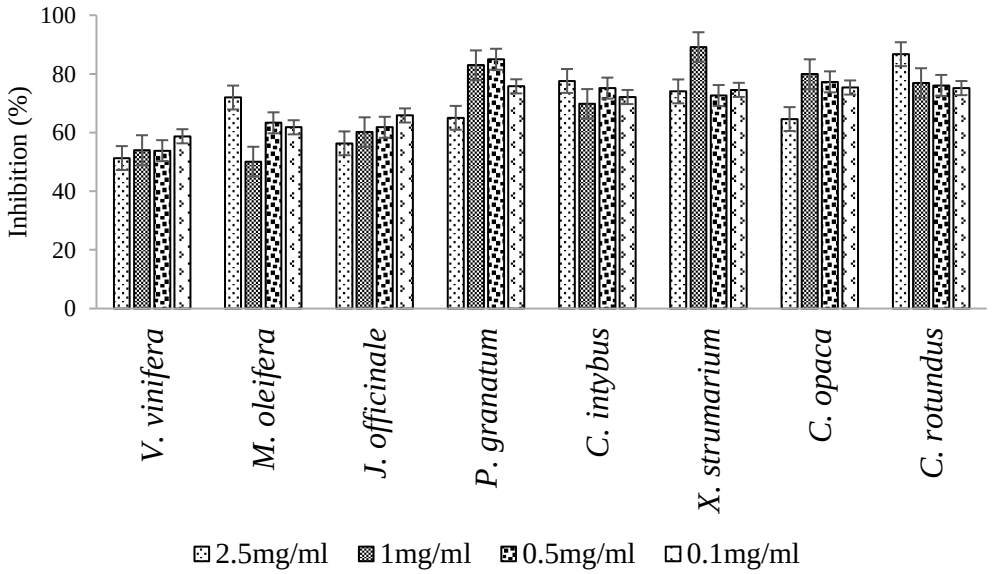

Figure 4.5: Anticancer activity of *V. vinifera, M. oleifera, J. officinale, P. granatum, C. intybus, X. strumarium, C. opaca* and *C. rotundus* on HUH cell line at different concentration

X. strumarium, C. opaca and *C. Intybus* showed high antiproliferative activity against MCF-7 cells ranging from 60 to 83 %. While *R. fruticosus, P.*

granatum and C. rotundus exhibited low anticancer activity <60 %. *D. muricate, P. guajava, F. carica, P. nigrum, F. cretica, A. indica, A. mexicana, V. vinifera, M. oleifera, Jasminum officinale* and *C. intybus* showed low anticancer activity <50 %. (Figure 4.6 and 4.7).

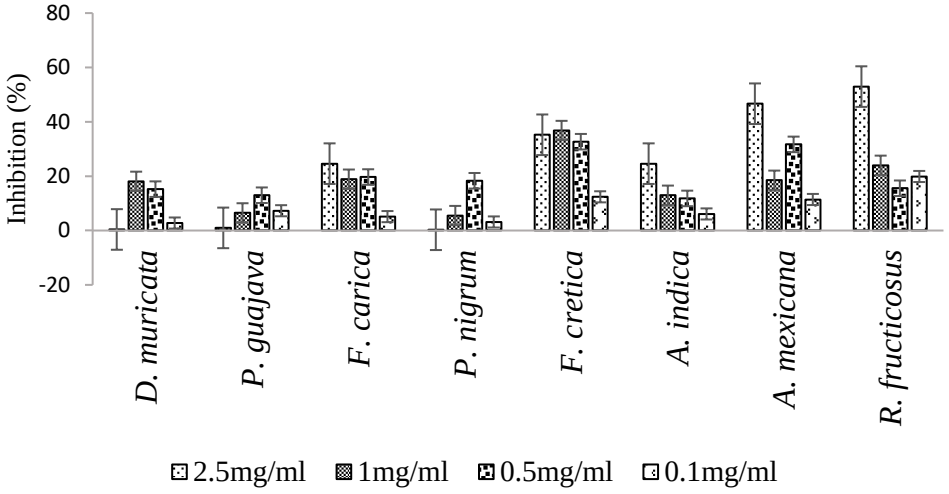

Figure 4.6: Anticancer activity of *D. muricata, P. guajava, F. carica, P. nigrum, F. cretica, A. indica, A. mexicana* and *R. fruticosus* on MCF-7 cell line at different concentration

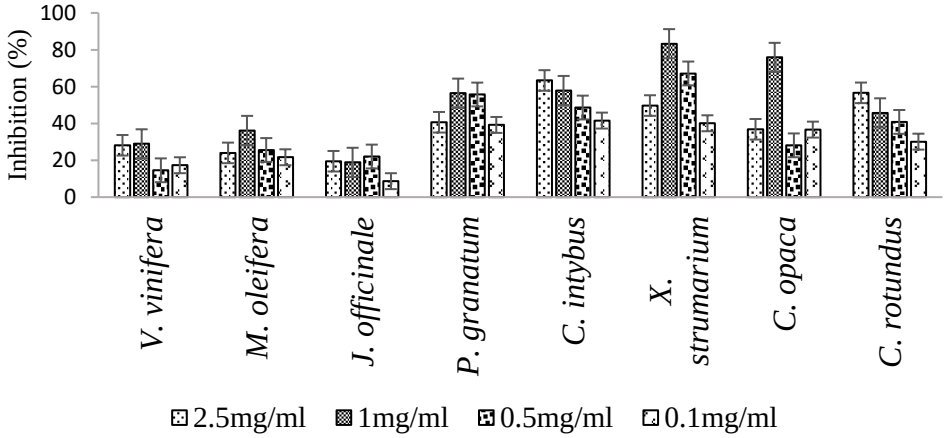

Figure 4.7: Anticancer activity of *V. vinifera, M. oleifera, J. officinale, P. granatum, C. intybus, X. strumarium, C. opaca* and *C. rotundus* on MCF-7 cell line at different concentration

Five of these plants were subjected to dose dependent cell morphological changes studies but no significant change in cell morphology was observed (Figure 4.8 to 4.12).

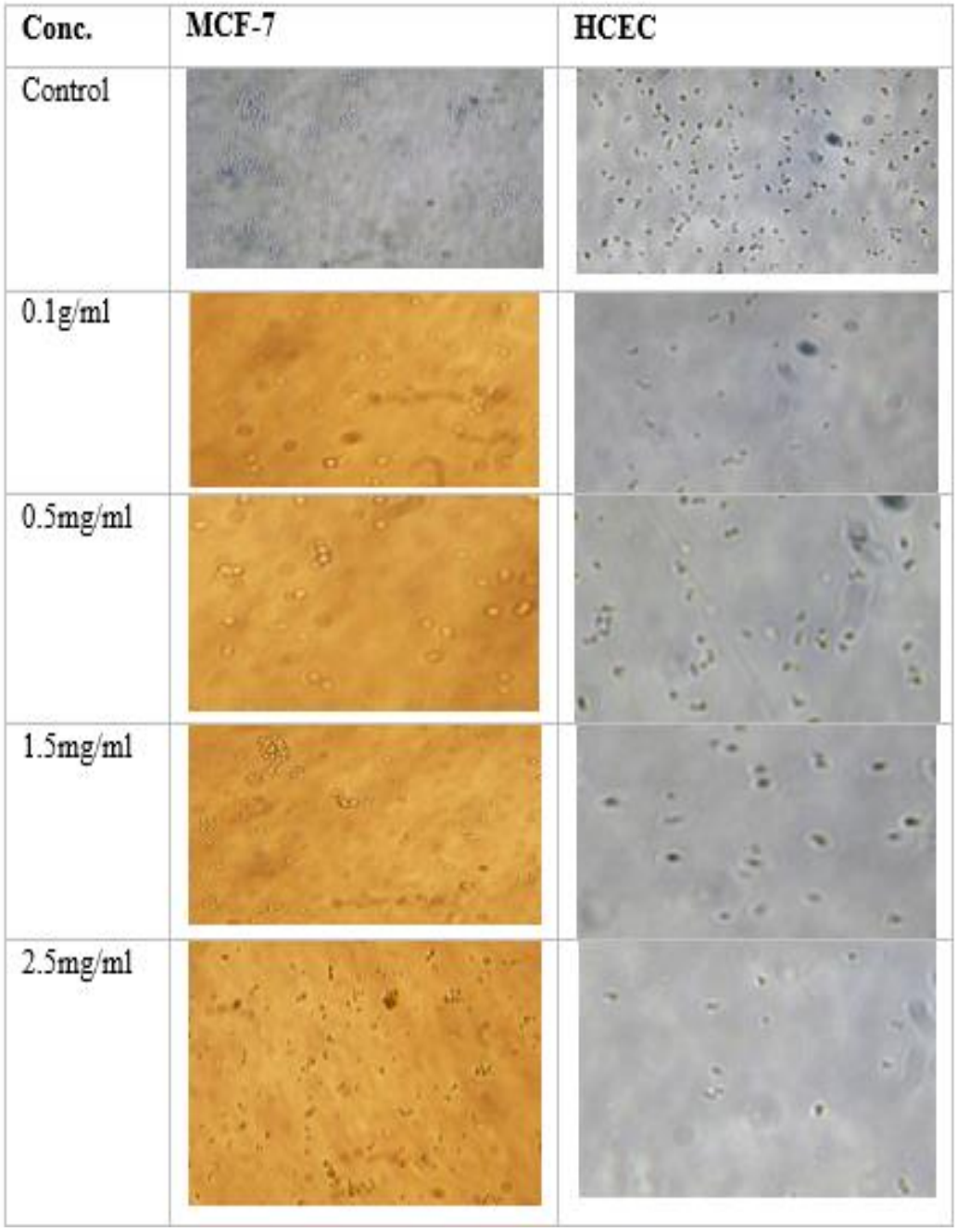

Figure 4.8: *C. opaca* dose dependent effect of cell morphological changes

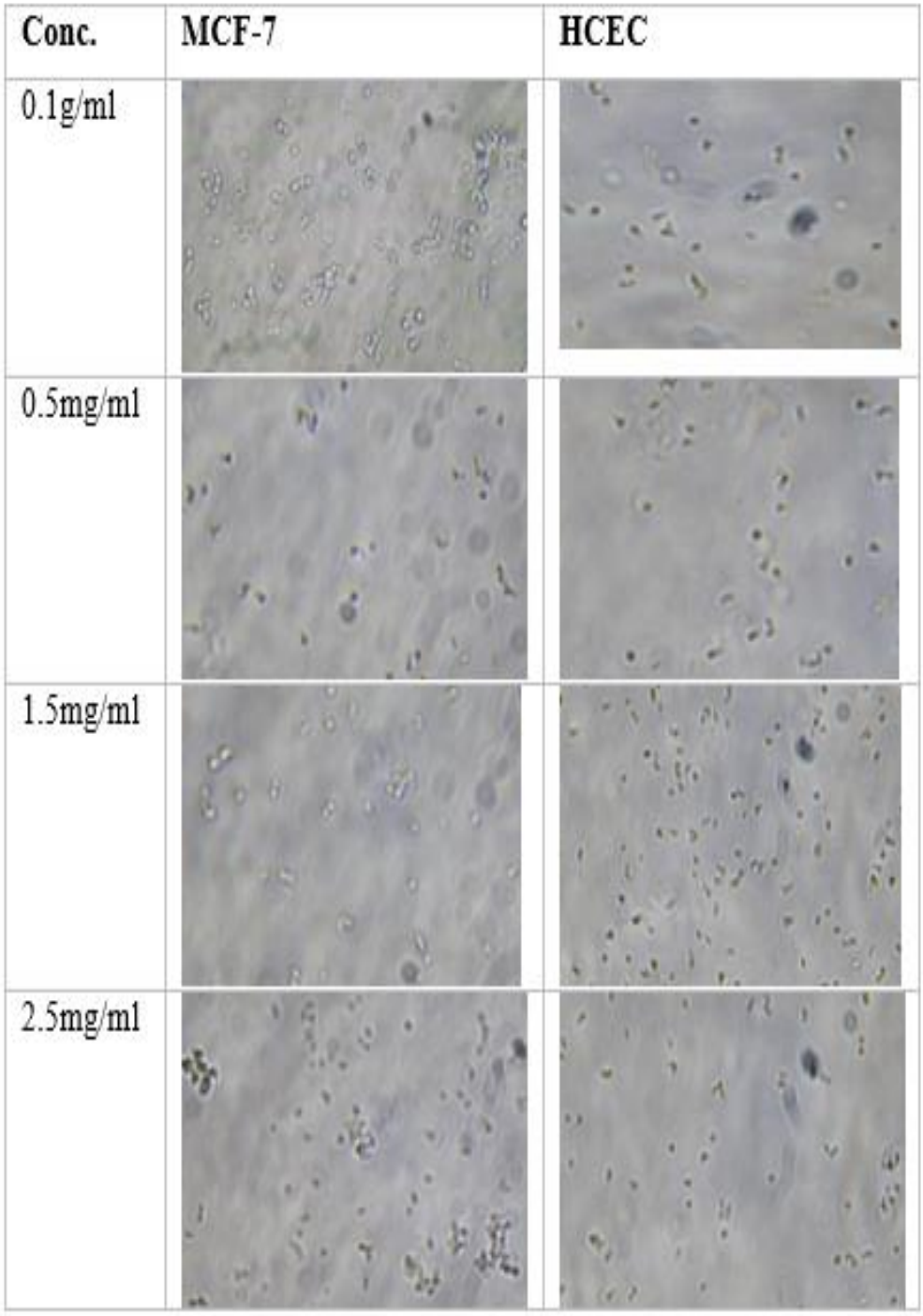

Figure 4.9: *C. intybus* dose dependent effect of cell morphological changes

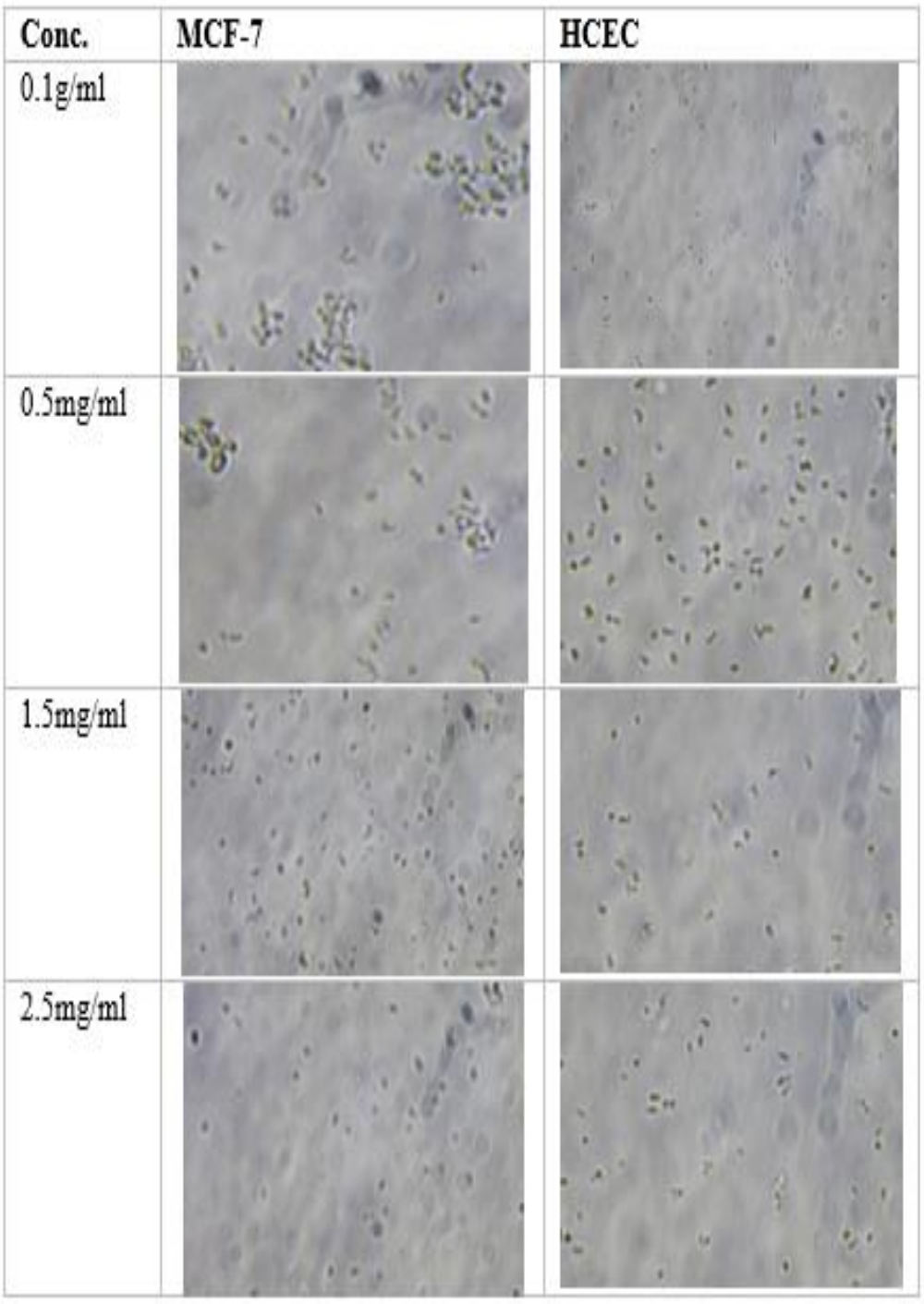

Figure 4.10: *C. rotundus* dose dependent effect of cell morphological changes

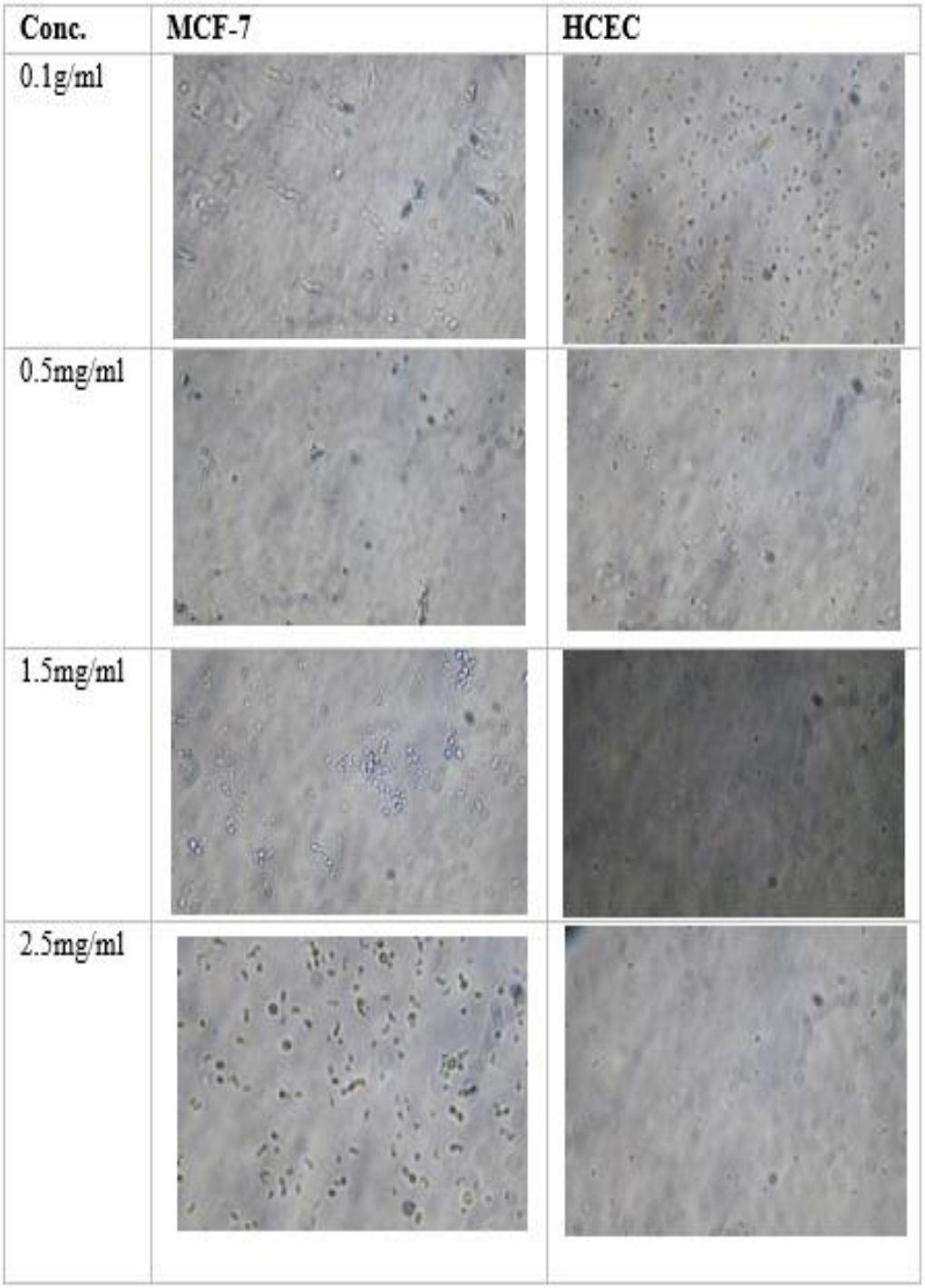

Figure 4.11: *F. cretica* dose dependent effect of cell morphological changes

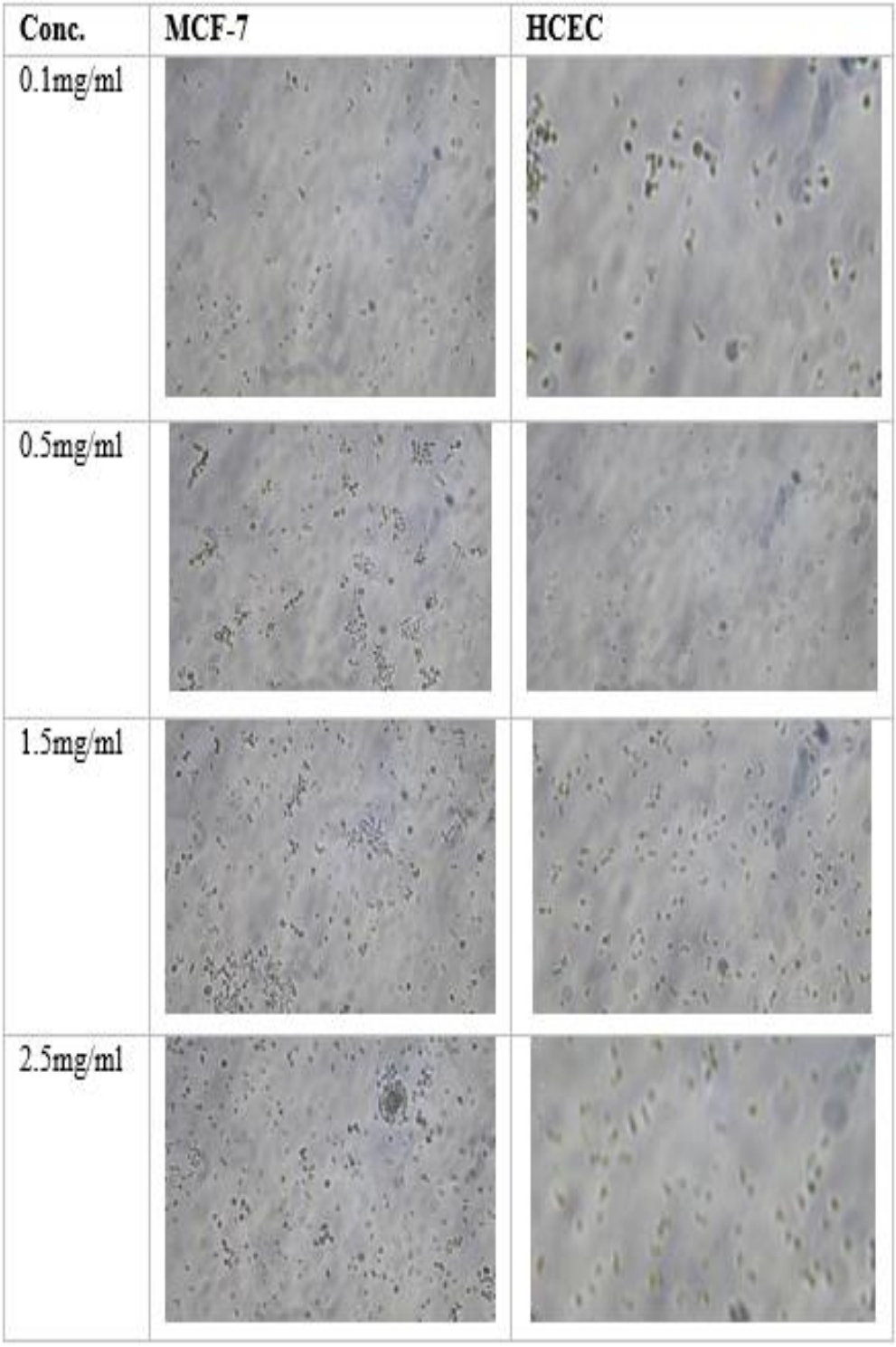

Figure 4.12: *A. mexicana* dose dependent effect of cell morphological changes

4.5 RT PCR RESULTS OF CRUDE METHANOLIC EXTRACTS

TGFβ activated signaling pathway increases the tumor invasion, epithelial to mesenchymal transition and metastasis. Cancer cells exhibit activated TGFβ signaling pathway and its inhibition is an attractive strategy for cancer treatment. The first step of TGFβ induce signaling pathway is the binding of TGFβ ligand to its receptor TGFβR2 that's result in activation of TGFβR1 triggered by TGFβR2 and phosphorylation of TGFβR1 while TGFβR3 help in ligand availability. In present study Relative expression studies were carried out for TGFβ receptors expressed in HUH cell line by nine plant extracts (Figure 4.13 and 4.14). Significant *in vitro* downregulation of all three receptors of TGFβ was observed by two plant extracts i.e. *F. cretica, A. mexicana,* reported for the first time.

Downstream signaling process initiated by activated TGFβR1 induced phosphorylation of SMAD proteins. Result in complex formation with other SMAD4 partner. SMAD complex translocated into nucleus and regulate TGFβ mediated transcription of several genes (Massagué, 2012). Remarkably attenuated expression of SMAD3 gene was observed by exposure of *F. cretica, A. mexicana, M. oleifera, R. fruticosus and P. granatum* plant extracts in this study (Figure 4.13 and 4.14). These results are consistent with studies indicating inhibition of TGFβ1-induced tumorigenesis potential of colon, breast, and lung cancer cells (Mo et al., 2012: Wang et al., 2013; Wang et al., 2014).

Our key findings identified two most potent plants extracts *F. cretica* and *A. mexicana*. Both of the plants exhibited inhibitory effects against TGFβR 1, 2 and 3 and SMAD3 and over expression of one of ubiquitin gene (SMURF) in case of *A. mexicana* was observed. No previous studies were found on relative expression of TGFβR signaling pathway by these two plants an *in vitro* approach. TGFβR signaling pathway is also tightly regulated by ubiquitin mediated degradation pathway an evolutionary preserved cascade. SMURF 1 and 2 play important role in SMAD degradation via E3 ligases (Izzi and Attisano, 2004). Our findings reveal first time that *A. mexicana, R. fruticosus* and *P. granatum* have significantly increase the expression of SMURFs genes.

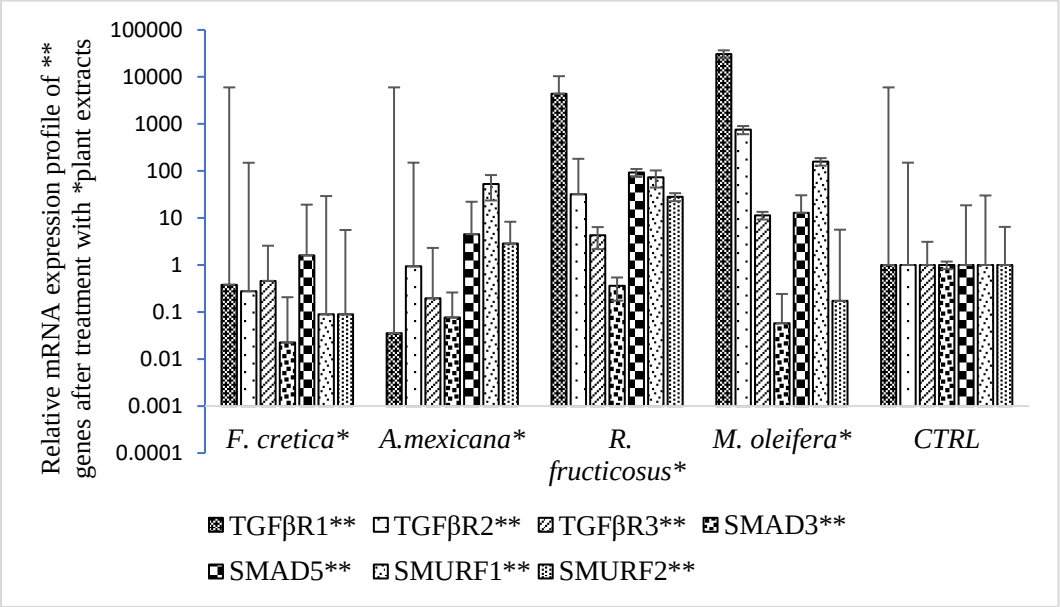

Figure 4.13: Relative mRNA expression showing relative mRNA expression profile of TGFβR (1,2 and 3) and their downstream signaling protein SMAD 3,5 and Ubiquitin proteins SMURF 1,2 mentioned as** after treatment with CMEs of *F. cretica, A. mexicana, R. fructicosus* and *M. oleifera* denoted as * presented as fold change with respect to control

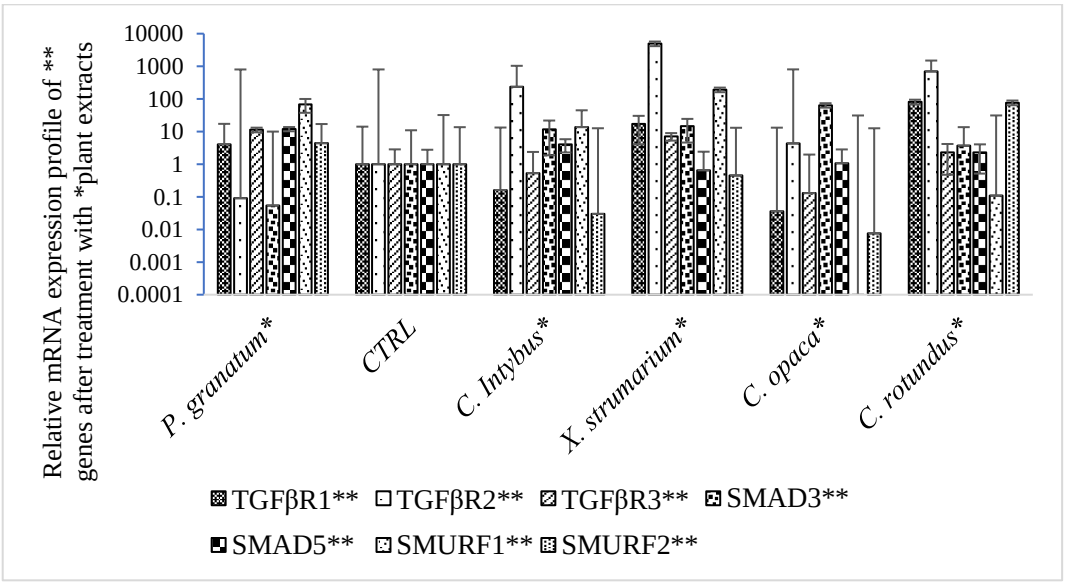

Figure 4.14: Relative mRNA expression profile of TGFβR (1,2 and 3) and their downstream signaling protein SMAD 3,5 and Ubiquitin proteins SMURF 1,2 mentioned as** after treatment with CMEs of *P. granatum, C. intybus, X.*

strumarium, C. opaca and *C. rotundus* extracts denoted as * presented as fold change with respect to control

4.6 COLUMN CHROMATOGRAPHY

Column chromatography is common method used for compound isolation and purification from a complex mixture (Tang et al., 2007). Crude methanolic extracts of five selected plants were subjected to column chromatography using solvents of different polarity from non-polar to polar as mentioned earlier (Figure 4.15).

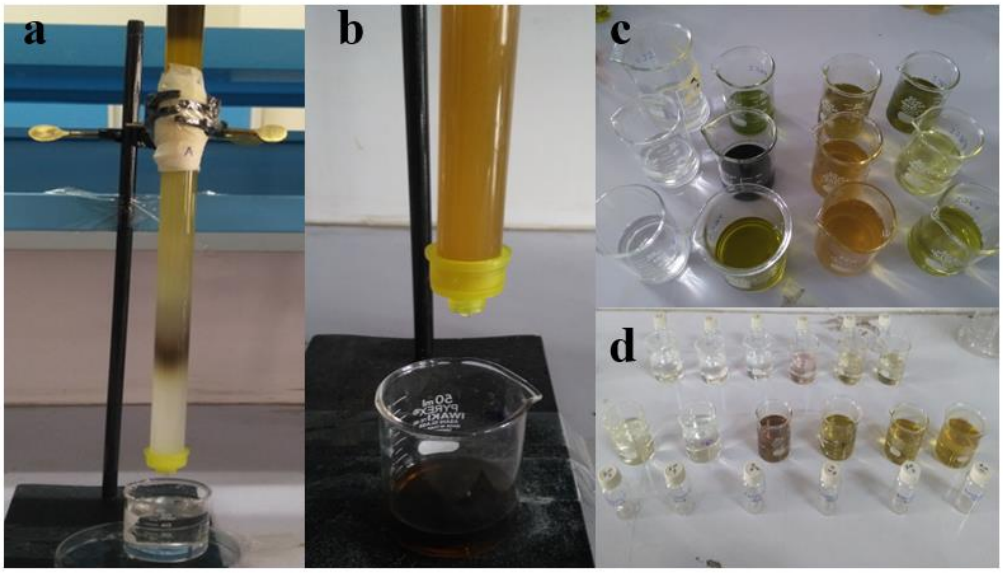

Figure 4.15: Column chromatography, a) and b) are showing collection of fraction, c) is showing 13 different types of fractions obtained after use of combination of solvent system while d) is collection and preservation of fraction in fraction tubes.

Fractions were collected in separate vials, air dried at room temperature and yield of fractions (mg/500 mg) was calculated (Table 4.1). It was observed that polar fractions which have combination of methanol and ethyl acetate like F9, F10 and F11 have the highest yield of compounds eluted. *F. cretica* fraction 9 eluted most of the compounds almost 166 mg while lowest was observed in slightly polar fraction 3 which was only 3.31 mg.

While in case of *A. mexicana* and *C. intybus* lowest compounds were collected in completely non-polar fraction which are 1.9 mg and 1.22 mg respectively. Both plants showed highest yield in F10 which is 110 mg and 154 mg respectively. Other two plants i.e., *C. opaca* and *C. rotundus* highest yields was observed in fractions 10 and 11, which is 238 mg and 102 mg respectively. So, it was concluded that all these plants are good source of polar secondary metabolites like flavonoids, alkaloids etc.

Table 4.1: Amount of compounds in mg obtained from each fraction

Fraction No.	**Plant Fractions obtained in (mg /500 mg)**				
	F. cretica	***A. mexicana***	***C. intybus***	***C. opaca***	***C. rotundus***
F1	6.21	6.04	1.22	1.91	4.51
F2	3.97	1.9	1.99	3.61	3.8
F3	3.31	3.18	2.31	5	2.29
F4	24.62	60.08	19.26	12.63	8.05
F5	7.44	80.96	16.5	21.22	4.84
F6	7.56	37.8	17.71	13.61	7.27
F7	4.08	24.7	9.59	1.13	7.99
F8	18.79	2.28	2.83	1	0.97
F9	166.42	43.94	32.83	25.2	17.23
F10	142.65	109.55	153.85	238.18	99.83
F11	55.33	49.97	108.1	39.3	102.37
F12	17.6	20.73	126.3	37.21	27.98
F13	16.99	33.69	87.11	33.89	43.55

4.7 THIN LAYER CHROMATOGRAPHY

TLC is a common method used for purification of specific compound from mixture of compounds and also to check the number of compounds present in mixture. RF values of unknown compounds can be compared with already known compounds to identify the nature of compound of interest. So, this method can be used for identification of compounds (Talukdar et al., 2010). The fractions obtained from each plants were subjected to TLC to check the nature and number of compounds in each fraction and check for presence of any UV active compounds. Rf value for each of compound visible through naked eye or after treatment with formaldehyde was calculated as shown in Tables 4.2 to 4.6.

It was observed that most of the mixture of compounds are present in polar fractions which are again combination of methanol and ethyl acetate or n-hexane and ethyl acetate based separation. No UV active compounds were seen in any fraction obtained from crude extract fractions of *F. cretica, C. intybus* and *C. rotundus*. While fraction number 5 of *A. mexicana* showed presence of UV active compound(s), (Figure 4.16a). The UV active compounds were also observed in *C. opaca* polar fractions; fraction number 8, 9 and 10 (Figure 4.16b). No UV active compound was reported in these plant extracts in recently reviewed literature.

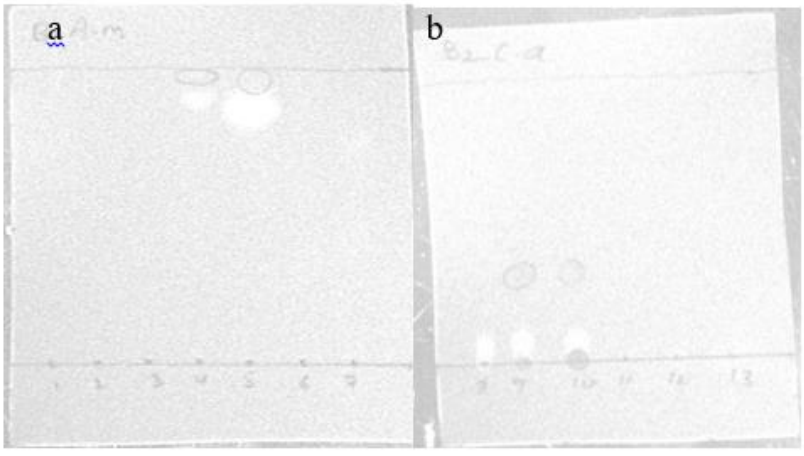

Figure 4.16: Part a) is showing UV active compound in fraction 4 and 5 of *A. mexicana*, b) is showing UV active compounds in fraction 8, 9 and 10 of *Carissa opaca*

Table 4.2: The retention factor (Rf) for each of the thirteen fractions of *Fagonia cretica* in different solvent system

Fraction No.	**RF Value (*Fagonia cretica* Fractions)**					**Remarks**
	Solvent A	**Solvent B**	**Solvent C**	**Solvent D**	**Solvent E**	
F1	-	-	-	-	-	
F2	-	-	-	-	-	
F3	-	-	-	-	-	
F4	-	0.8,0.96	0.98	0.64,0.98	0.75	
F5	-	0.87	-	0.98	-	
F6	-	0.12,0.53, 0.62,0.84	0.68,0.98	0.77,0.96	0.69,0.77	
F7	-	0.62	0.68,0.9	0.96	-	**No UV active compound was seen**
F8	-	-	0.11,0.62, 0.8,0.96	0.53,0.7	0.6,0.8	
F9	0.12	-	0.11	0.85,0.96	0.44,0.98	
F10	-	-	-	0.53	0.68	
F11	-	-	-	0.62	0.68	
F12	-	-	-	0.62	-	
F13	-	-	-	-	-	

Table 4.3: The retention factor (Rf) for each of the thirteen fractions of *Argemone mexicana* in different solvent system

	RF Value (*Argemone mexicana* fractions)	**Remarks**

Fraction No.	Solvent A	Solvent B	Solvent C	Solvent D	Solvent E	
F1	-	-	-	-	-	
F2	-	-	-	-	-	
F3	-	-	-	-	-	
F4	-	0.9,0.97	0.93	0.77,0.93	0.42,0.64	
F5	-	0.81,0.9,0.97	0.93	0.95	0.57,0.75	
F6	-	0.46,0.76,0.93	0.93	0.9,0.97	0.86	**UV active compound was seen in Fraction 5**
F7	-	0.25,0.74	0.66,0.91	0.9,0.97	0.93	
F8	-	-	0.77,0.83	0.97	0.93	
F9	-	0.47,0.58	0.1,0.6,0.7,0.77	0.73,0.95	0.66,0.8	
F10	-	0.1	-	0.73,0.95	0.64,0.8	
F11	-	0.1	-	0.93	0.77,0.88	
F12	-	-	-	0.93	0.68,0.88	
F13	-	-	-	0.93	-	

Table 4.4: The retention factor (Rf) for each of the thirteen fractions of *Cichorium intybus* in different solvent system

Fraction No.	RF Value (*Cichorium intybus* fractions)					Remarks
	Solvent A	Solvent B	Solvent C	Solvent D	Solvent E	
F1	-	-	-	-	-	

F2	-	-	-	-	-	
F3	-	-	-	-	-	
F4	-	0.86,0.97	0.97	0.71,0.93	0.54,0.7	
F5	-	0.67,0.91, 0.97	0.68,0.91, 0.97	0.71,0.93	0.6,0.74,0.8	**No UV active compound was seen**
F6	-	0.93	1	0.78,0.93	0.7	
F7	-	0.21	0.64,0.84	0.71,0.78,0.93	0.78	
F8	-	-	-	0.95	-	
F9	-	0.27	0.15,0.24,0.77	0.9,1	0.84,0.88	
F10	-	-	0.11	0.61,1	0.57,0.71`	
F11	-	0.15	-	0.64,1	0.62,0.77	
F12	-	-	-	0.64,1	0.8	
F13	-	-	-	0.61,1	-	

Table 4.5: The retention factor (Rf) for each of the thirteen fractions of *Cyprus rotundus* in different solvent system

Fraction No.	**RF Value (*Cyprus rotundus* fractions*)***					**Remarks**
	Solvent A	**Solvent B**	**Solvent C**	**Solvent D**	**Solvent E**	
F1	-	-	-	-	-	
F2	-	-	-	-	-	
F3	-	-	-	-	-	

F4	-	0.93	0.95	0.68,0.96	0.68	
F5	-	0.91,0.93	0.95	0.7,0.96	0.59,0.75	
F6	-	-	0.95	0.96	0.68	**No UV active compound was seen**
F7	-	-	0.63	0.96	0.68	
F8	-	-	-	0.94	0.91	
F9	-	-	0.22,0.71	0.9,0.98	0.8,0.95	
F10	-	-	0.64	0.7,0.92	0.66	
F11	-	-	1	0.94	0.66,0.93	
F12	-	-	-	0.94	-	
F13	-	-	-	0.96	-	

Table 4.6: The retention factor (Rf) for each of the thirteen fractions of *Carissa opaca* in different solvent system

Fraction No.	**RF Value (*Carissa opaca* fractions*)***					**Remarks**
	Solvent A	**Solvent B**	**Solvent C**	**Solvent D**	**Solvent E**	
F1	-	-	-	-	-	
F2	-	-	-	-	-	
F3	-	-	-	-	-	
F4	-	0.84,0.93	0.96	0.71,0.95	0.53,0.67	
F5	-	0.81,0.93	0.67,0.96	0.76,0.95	0.67,0.78	
F6	-	0.56,0.65, 0.72,0.81	0.93	0.95	0.78	**UV active**

F7	-	0.23,0.47	0.64,0.78, 0.93	0.98	0.84	**compound was seen in fractions 8,9 and 10**
F8	-	-	0.67	0.93	0.87	
F9	-	0.12,0.31, 0.81	0.17,0.33, 0.67,0.91	0.71,0.89, 0.98	0.7,0.87	
F10	-	0.1,0.31,0. 81	0.57,0.7,0. 91	0.22,0.67, 0.89,0.98	0.28,0.57, 0.83	
F11	-	0.31	-	0.69,0.96	0.67,0.8	
F12	-	0.31	-	0.73,0.96	0.8	
F13	-	0.31,0.83	-	0.96	0.78	

4.8 SCREENING PLANTS FRACTIONS FOR THEIR ANTICANCER ACTIVITY

Total 65 fractions were obtained from five plants and screen for their anticancer activity on HEP2 cancer cell line and their cytotoxic effect was checked by treating the fractions against HCEC cell line. IC_{50} values were calculated for each fraction of all plant extracts. The plant names were abbreviated as *F. cretica* (Fc), *A. mexicana* (Am), *C. intybus* (Ci), *C. rotundus* (Cr) *and C. opaca* (Co). The therapeutic level in the cell line is the range within which the fraction is expected to be effective and kill 50 percent of cells while toxic level is determined at which dose compound or fraction have a damaging effect to the organism so it should be significantly higher from therapeutic level.

F. cretica belongs to Zygophyllaceae family member, is found in tropical and arid regions of the world and is exploited for therapeutic purposes ranging from treating skin infections to curing fatal diseases (Qureshi et al., 2016). Among tested fractions of *F. cretica* it was observed that most of the fractions achieving toxic level first than therapeutic like FcF1, FcF2, FcF3, FcF4, FcF6, FcF7 and FcF10. These fractions have higher IC_{50} values for cancer cell line compared to normal HCEC cell

line. While FcF9 has no difference between both, it has IC_{50} 230 µg/ml for HEP2 and 232 µg/ml for HCEC cell line. Equally lethal to both cell lines. Rest of the fractions have difference less than 50 % between two values. For Example FcF5 have IC_{50} 155 µg/ml for HEP2 and 182 µg/ml for HCEC and FcF8, FcF11, FcF12 and FcF13 have 433,488,329,185 µg/ml vs 569, 562**,** 567 and 269 (µg/ml) in HEP2 Vs HCEC respectively.

So this plant excluded from further studies because of its cytotoxic nature to normal cells as shown in Figure 4.17. Literature studies showed that *F. cretica* is commonly used as a home remedy to treat liver and digestive issues, diarrhea, exhaustion, gastroenteritis, allergies, ulcers, and skin wounds (Hussain, Zia and Mirza, 2007).

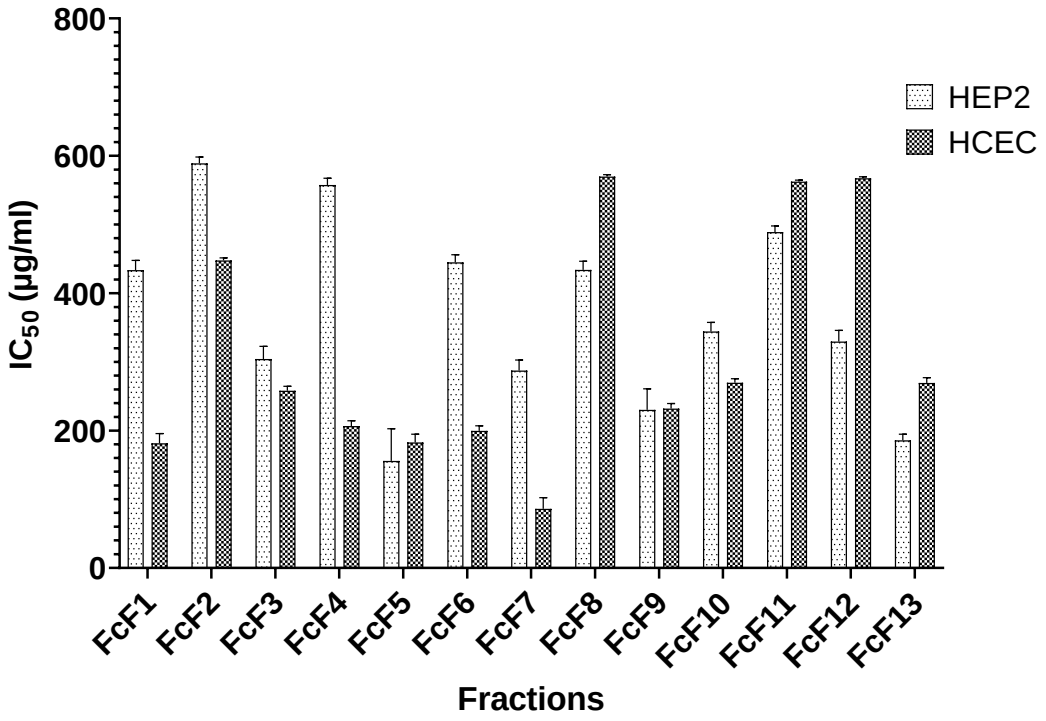

Figure 4.17: Cytotoxicity analysis of *F. cretica* (Fc) plant fractions on HEP2 and HCEC cell lines

C. rotundus also behaving like *F. cretica* plant fractions. Most of the fractions have higher IC_{50} in cancer cells compared to lower in HCEC cell line. For example CrF1 and CrF13 have IC_{50} in HCEC cell line 445 µg/ml and 895 µg/ml comparable to HEP2 IC_{50} which is 1343 µg/ml and 1952 µg/ml respectively. In a study conducted

by Mannarreddy et al., (2017) showed the noteworthy cytotoxic effect of *C. rotundus* rhizome extract against liver, breast, cervical and prostate cancer cell line elucidating the anticancer potential of this plant.

Two fractions which have highest anticancer activity and noticeable difference in both levels were selected for further studies. These are CrF8 and CrF10 having therapeutic concentration 22 µg/ml and 814 µg/ml respectively against HEP2 cancer cell line (Figure 4.18).

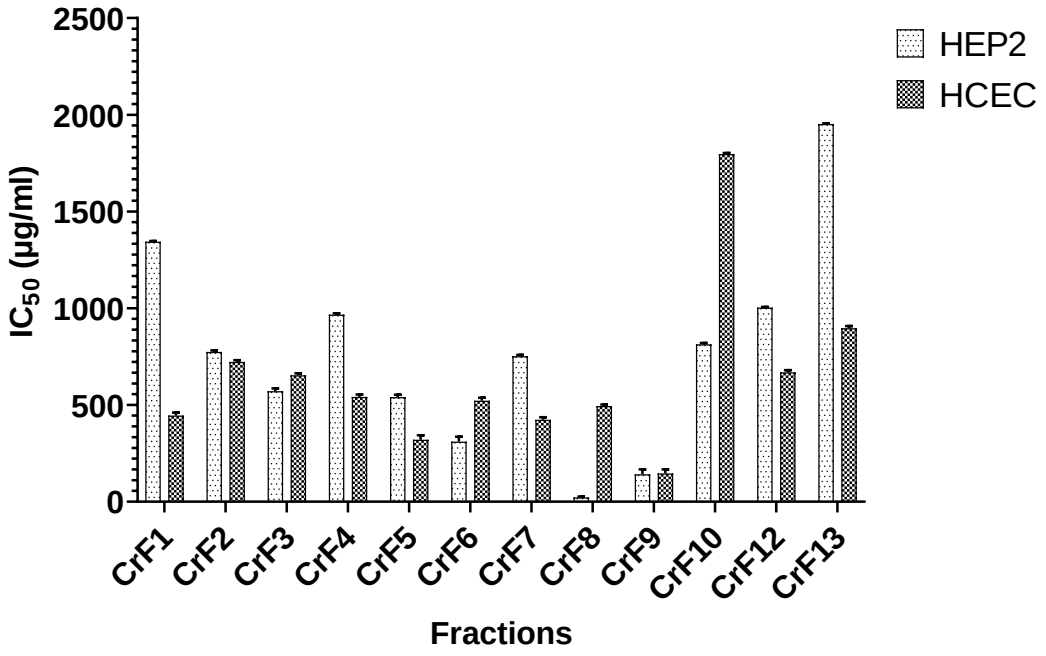

Figure 4.18: Cytotoxicity analysis of *C. rotundus* (Cr) plant fractions on HEP2 and HCEC cell lines

C. opaca fractions are showing more cytotoxic activity against normal cells. Fraction CoF9 of this plant was selected for further experiments. It has higher IC_{50} value in HEP2 cell line (1666 µg/ml) but relatively high difference is seen as compare to other fractions of this plant which is >30%. Crude methanolic, ethyl acetate and chloroform extract of this plant have significant high anti-proliferative activity against cancer cell lines as literature studies also showed the anticancer potential of this plant (Nisa et al., 2013). But tested CME fractions of this plant are also cytotoxic against normal cell line, so this plant was excluded from further studies (Figure 4.19).

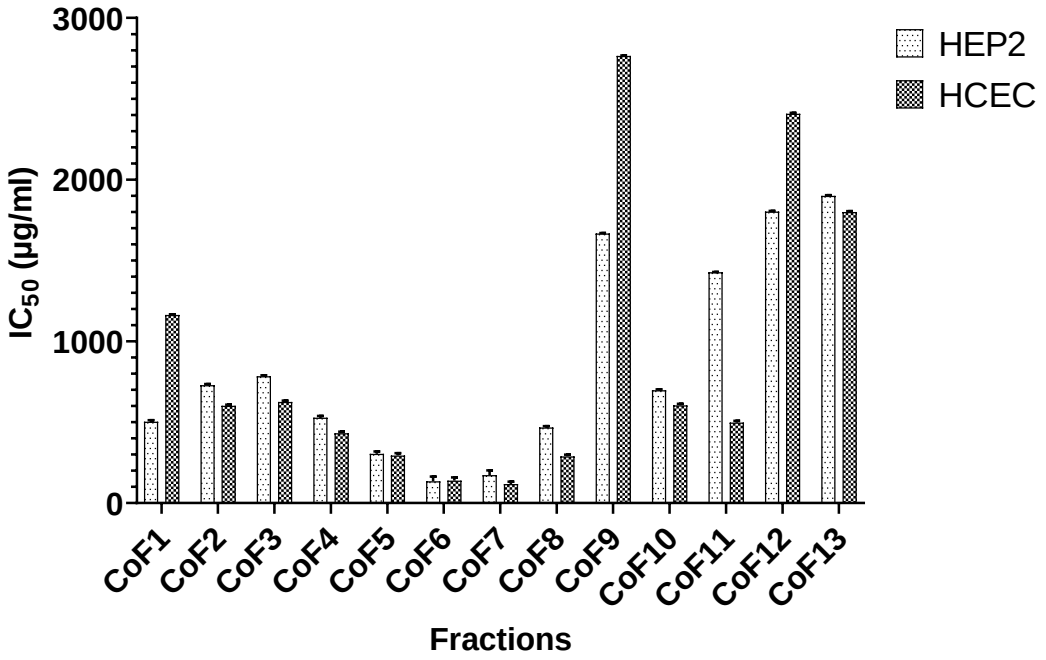

Figure 4.19: Cytotoxicity analysis *C. opaca* (Co) plant fractions on HEP2 and HCEC cell lines

A. mexicana, often known as the Mexican prickly poppy, is a stress-resistant plant in the Papaveraceae family that has been utilized for millennia in traditional medicine by indigenous populations in Mexico and the Western United States. With claimed antibacterial and antioxidant qualities, as well as cytotoxic effects against some human cancer cell lines, this plant has been used to treat a wide range of illnesses (Chang et al., 2003). *A. mexicana* has enormous potential as a drug discovery candidate because of its many medicinal applications and abundance of secondary metabolites.

A. mexicana has shown great effects on cancer cells and have significant differences in IC_{50} values of both cell lines except AmF7 has almost no difference in IC_{50} between HEP2 and HCEC which is 180 µg/ml and 177 µg/ml respectively while Mostly fractions achieving therapeutic level first as we can see in Figure 4.20. *A. mexicana* fractions showing the highest activity and significant difference between two doses are AmF4, AmF8 and AmF10. They have difference in activity in both cell lines more than 50 %. The IC_{50} values for HEP2 cell line of AmF4, AmF8 and AmF10 are 10 µg/ml, 31 µg/ml and 182 µg/ml respectively while for HCEC cell line their IC_{50} values are 47 µg/ml, 115 µg/ml and 355 µg/ml respectively. The fractions

having difference more than 20 % are selected for future experiments. Previous studies also showed the outer root methanol and seed hexane extracts of this plant also shown strong cytotoxic effect towards human colon carcinoma cells when tested using the MTT colorimetric technique for cytotoxicity analysis (Orozco-Nunnelly et al., 2021).

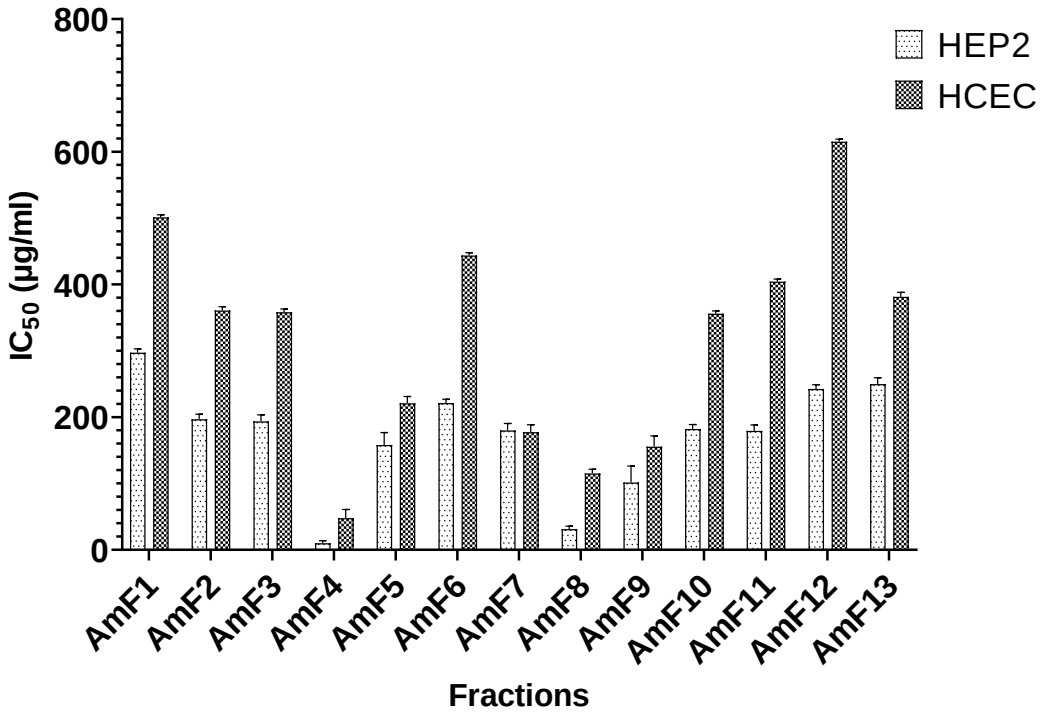

Figure 4.20: Cytotoxicity analysis of *A. mexicana* (Am) plant fractions on HEP2 and HCEC cell lines

There is a long history of using *Cichorium intybus* L., a member of the Cichorieae tribe of the Asteraceae family, as a food and medicine. This plant has attracted interest for its possible medicinal qualities due to its high concentration of important secondary metabolites such as phenols, flavonoids, and polyamines. C. intybus has shown remarkable anticancer efficacy against a variety of cancer cell lines, including Amelanoic melanoma C32, breast cancer MCF-7, renal adenocarcinoma, and prostate cancer (Bais and Ravishankar, 2001; Kandil, Abou-Elella and El Shemy, 2019).

The analysis of Figure 4.21 showed that *C. intybus* achieving therapeutic level dose for most of the fractions at higher IC_{50} concentration as compared to *A.*

mexicana fractions. But this plant extract fractions have also noteworthy difference between both cell lines. Similarly to *A. mexicana* plant fractions only two fractions CiF11 and CiF13 have low IC_{50} for HCEC i.e. 493 µg/ml and 664 µg/ml as compared to their HEP2 IC_{50} values of 224 µg/ml and 822 µg/ml respectively.

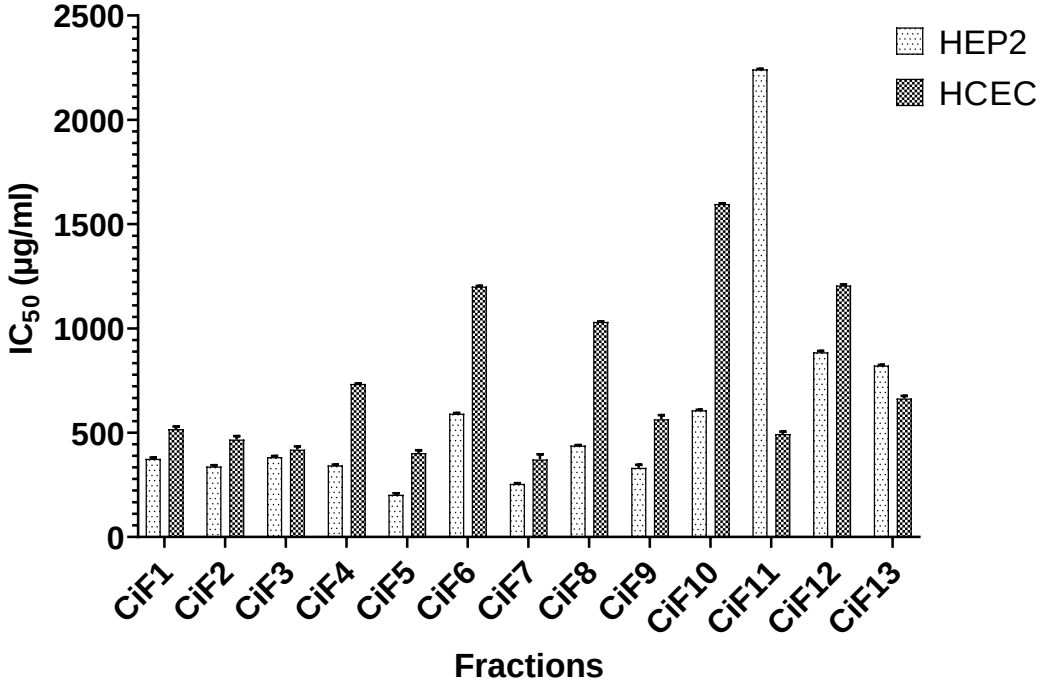

Figure 4.21: Cytotoxicity analysis of *C. intybus* (Ci) plant fractions on HEP2 and HCEC cell lines

If the IC_{50} is lower than it exhibit that the fraction is potent for anticancer activity. In case of *C. intybus* lowest IC_{50} for HEP2 cell line was observed by fraction CiF5 and CiF9 which is 202 µg/ml and 331 µg/ml respectively. All fractions which have >20% difference between both levels were subjected to relative expression studies of genes. The results are in accordance with previous cytotoxicity studies showing cytotoxic behavior of this plant primary constituent intybusins B and four other well-known chemicals clearly inhibited the growth of four different types of human cancer cells (Meng et al., 2022).

So those fractions which have noticeable difference between therapeutic and toxic level from all tested plant extracts were subjected to relative expression studies to check their effect on mRNA expression of genes included in TGFβ signaling

pathway. It is crucial to remember that natural (as compared to chemically synthesized) products, which include plant extracts, have been utilized in the treatment of cancer for a long time. In fact, the discovery of a number of the most potent cancer drugs in recent years has been attributed to the use of folk remedies. For instance, *Podophyllum peltatum* root extract, a substance rich in podophyllins, was traditionally used by American Indians to cure skin cancer and other ailments; this tradition served as inspiration for the quest for more advantageous and non-toxic derivatives of podophyllotoxin (Francesconi et al., 2010).

First, paclitaxel (Taxol) was made from *Taxus brevifolia* tree bark as part of a thorough screening strategy. Taxol underwent a protracted process from its development in 1967 to its first clinical application, but as a result of its effectiveness against breast, ovarian, and non-small cell lung cancers, it ended up going on to become the bestselling anticancer therapy in the early 2000s (Mann, 2002). The *Camptotheca acuminata* tree was the source of the pharmaceutical camptothecin, whose derivatives (irinotecan and tapotecan) are now often used to treat ovarian cancer, cervical cancer, small cell lung cancer, colon cancer, and other cancers (Oberlies and Kroll, 2004).

4.9 RELATIVE EXPRESSION STUDIES OF SELECTED FRACTIONS

TGFβ is a multifunctional cytokine that communicates with cells via intercellular SMAD transcription factors and cell membranes TGFβR1 and TGFβR2. The development of cancer can be aided by abnormal intracellular and intercellular TGFβ signaling. TGFβ may trigger an epithelial growth arrest and induce a tumor suppressor activity in healthy cells and in the early stages of cancer. TGFβ signaling, however, can operate as a tumor promoter in advanced cancer when the cytostatic effects of TGFβ in cancer cells are suppressed. This is because TGFβ signaling can boost the epithelial to mesenchymal transformation of cancer cells, drive revascularization, and support immune system escape (Huynh, Hipolito and Ten Dijke, 2019). So there is still need of drugs from natural resources targeting TGFβ pathway and having fewer side effects.

In current study we tested selected plant fractions having noticeable difference in therapeutic level and toxic level between cancer and normal cell line and then subjected to relative expression studies to test their role toward TGFβ signaling transduction. HEP2 cells treated with fractions for 24 hours and then mRNA extracted and relative expression studies were carried out using gene specific primer and results were presented in form of fold change with respect to control untreated (Figure 4.22 and 4.23).

Analysis of Figure 4.22 showed that CiF4, CiF8 and CaF9 have little or no effect on TGFβR1 and TGFβR2 mRNA level. A significant decrease in expression was seen by CiF6 and CiF9 (0.00058 $\pm$ 0.007 and 0.000148 $\pm$ 1.006 fold change respectively) in case of TGFβR1. An up regulation in mRNA level of TGFβR3 was observed by all fractions except CiF12 which is 0.5 $\pm$ 0.209 fold change.

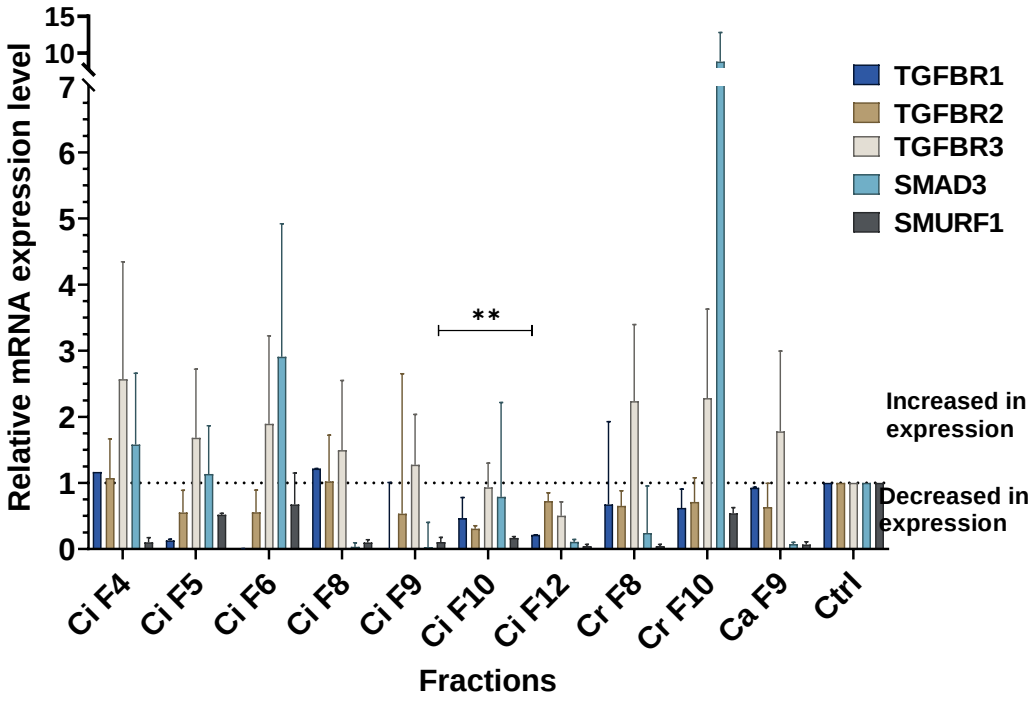

Figure 4.22: Relative expression studies of selected *C. intybus* (Ci), *C. rotundus* (Cr) and *C. opaca* (Ca) fractions. ** showing those fractions which successfully downregulated the expression of TGFBR (1, 2 &3) and SMAD 3 genes

CrF10 significantly increased the expression upto 8 $\pm$ 3.98 fold in case of SMAD3 gene while the lower expression level was observed in case of CiF9 which

is 0.025 ± 0.37. SMURF1 expression level of mRNA was decreased by all tested fractions <1 as compared to control. *C. intybus* fractions 10, 12 and *A. mexicana* 6, 8, 10 successfully inhibit the pathway by downregulating the gene expression fold change <1 of TGFβR 1, 2 & 3 and receptor associated complex protein SMAD3 as compared to control. *C. intybus* Fraction 9 also decreases the genes expression fold change of all genes except TGFβR3. CiF9, CiF10, AmF8 and AmF10 were selected for further studies (Figure 4.22). The Analysis of Figure 4.23 showed that AmF8 and AmF10 downregulated the expression of all three receptors TGFβR1, TGFβR2, TGFβR3 and downstream signaling protein SMAD3. The relative mRNA expression of all these genes was <1 as compared to control showing that their expression was decreased by *A. mexicana* plant fractions.

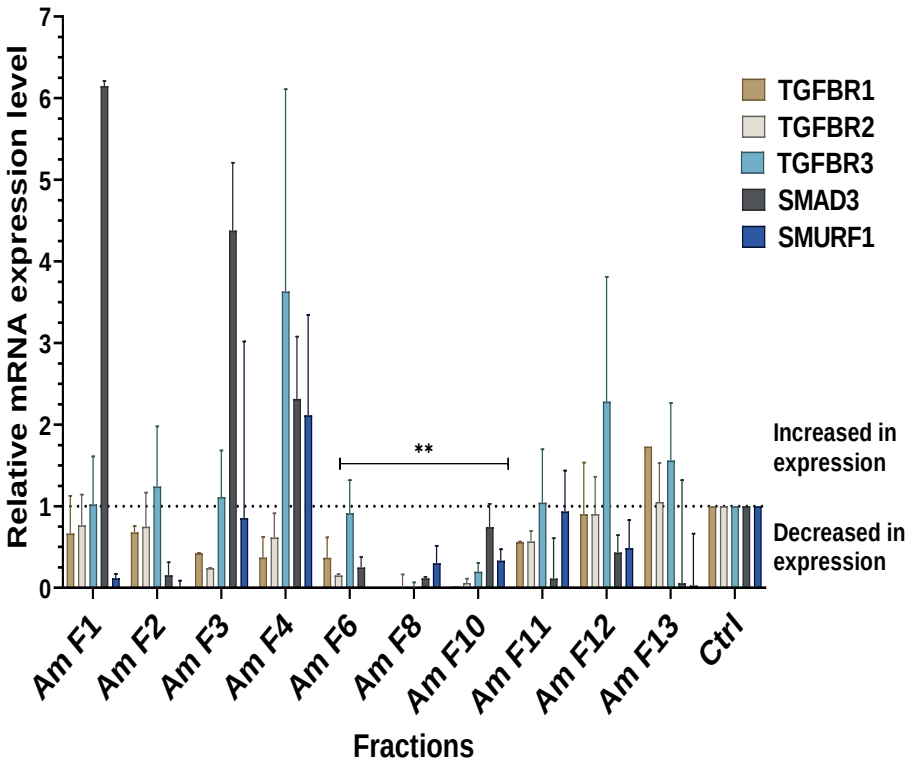

Figure 4.23: Relative expression studies of selected *A. mexicana* (Am) fractions. ** showing those fractions which successfully downregulated the expression of TGFBR (1,2 &3) and SMAD 3 genes

TGFβR1 mRNA level was decreased by all tested fractions of *A. mexicana* except AmF13 and AmF12 exhibiting that these fractions have no effect on this gene

as compared to control. Same pattern was seen in case of TGFβR2. TGFβR3 mRNA level was decreased only by two fractions of *A. mexicana* (AmF8 and AmF10) which is 0.0001 ± 0.067 and 0.1948 ± 0.112 fold change respectively. *A. mexicana* fractions AmF1, AmF3 and AmF4 have no effect on SMAD3 gene expression. An increase in expression was observed by fraction AmF4 in case of ubiquitous protein SMURF1. Rest of the fractions of this plant decreased mRNA level of targeted genes as presented in Figure 4.23.

Our findings are supporting the idea of inhibition of tumor growth as studied by Qin et al., (2017) when a new TGFβ receptor trap with the TGFβRII and TGFβRIII domains was created. The receptor trap totally prevented TGFβ to TGFβRII binding and, as a result, TGFβ1 and TGFβ3 activation in cultivated epithelial cells in this study. However, in a prostate-specific knockout mouse model, one of the most frequent oncogene altered in prostate cancer, systemic administration of this TGFβ receptor trap inhibited tumor cell proliferation and the invasion potential of tumor cells in high grade prostatic intraepithelial neoplasia lesions.

Other studies also showed the reduction in tumor growth when Receptors action was blocked. Among them Small molecule inhibitors are a broad and varied class of TGFβ inhibitors that aim to obstruct TGFβ receptor kinase activity and subsequently obstruct signal transmission via canonical or non-canonical pathways. This hypothesis has received support from a number of preclinical experiments utilizing these small inhibitors. When scientist created the TGFβRI kinase Inhibitor SB-431542, which has been extensively employed in basic research. It prevents renal epithelial carcinoma cells from expressing ECM (fibronectin and collagen) when TGFβ is present as a result decreases human malignant tumor cell motility, angiogenesis, and proliferation (Hjelmeland et al., 2004; Colak and Ten Dijke, 2017).

4.10 ANTICANCER ACTIVITY OF PLANT EXTRACTS AND SELECTED FRACTIONS ON SB28 CELL LINE

Cancer therapy is still hampered by drug resistance, which results in the ineffective treatment of tumors. Oncologists' top priority has always been the

development of tumor treatment resistance. As a result, the field of tumor research is heavily focused on overcoming tumor therapeutic resistance and seeking out novel methods for tumor treatment. Natural products' varied chemical compositions and pharmacological properties make them potent weapons against drug resistance and cancer treatment (Yang et al., 2022).

Two plant extracts i.e. *A. mexicana* and *C. intybus* and their selected fractions were administered at different concentration to SB28 cell line (Figure 4.24) to check anticancer activity against brain cancer cell line. The 10th leading cause of mortality for cancer patients globally is brain cancer. Despite the enormous effort put out to extract, design, and produce novel chemotherapeutic medications, scientist still encounter substantial challenges as a result of significant side effects, rising tumor resistance, and poor selectivity. Much focus has been placed on the anti-cancer potential of many natural substances in recent years (Mustafa and Abdulaziz, 2021). Etoposide, a drug currently used to treat GBM and several other malignancies, was created as a result of the research (Hande, 1998).

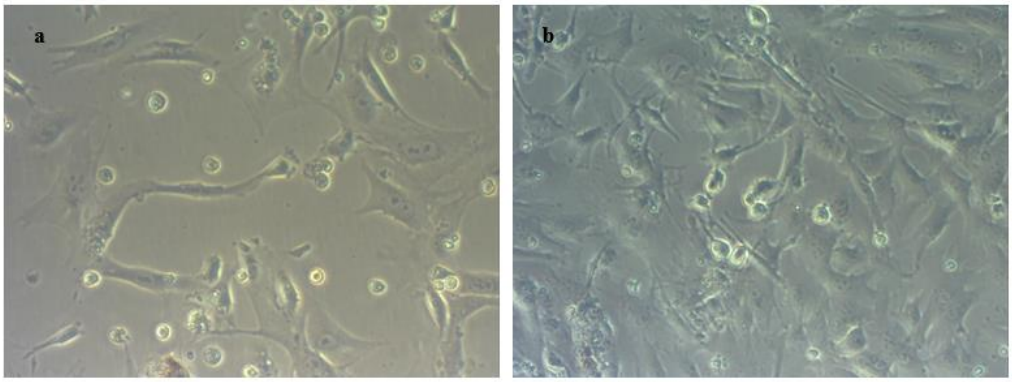

Figure 4.24: SB28 cells at 10X, a) is showing day 1 after culturing while b) is showing day 2 after culturing

In present study SB28 cells were treated with selected fractions at different concentration to study dose dependent cell morphological changes and to calculate IC_{50} values. Cells were treated at 60, 120, 250 and 500 (µg/ml) concentration and it was observed that cell number was decreased while increasing concentration of AmF8 of *A. mexicana* and CiF9 of *C. intybus*. Dose dependent cell morphological

changes were also observed at both above mentioned fractions (Figure 4.25 and 4.26). So these fractions were selected for further studies.

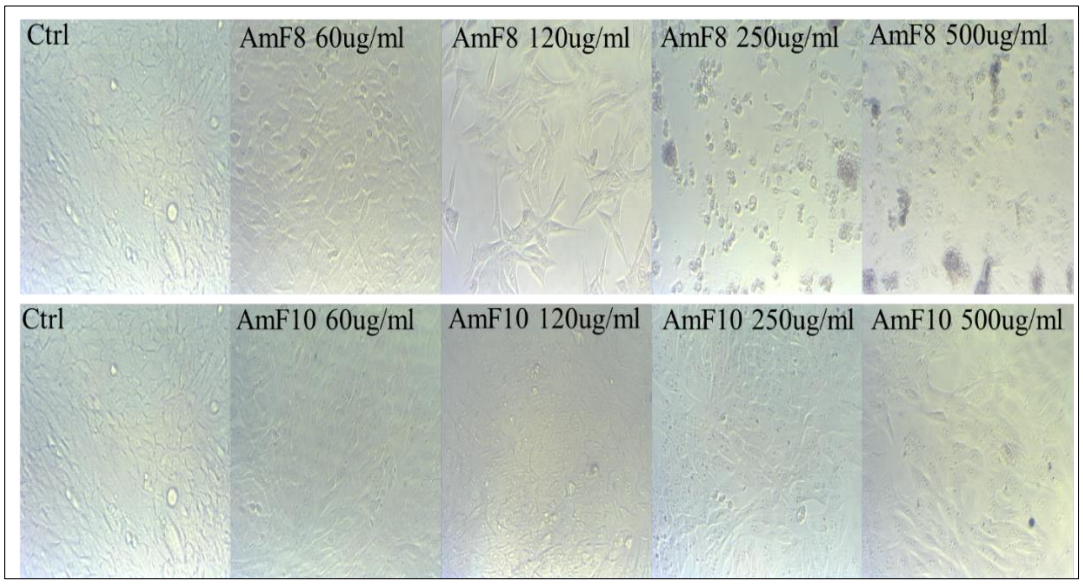

Figure 4.25: Dose dependent cell morphological changes of *A. mexicana* fractions on SB28 cell line at different concentrations

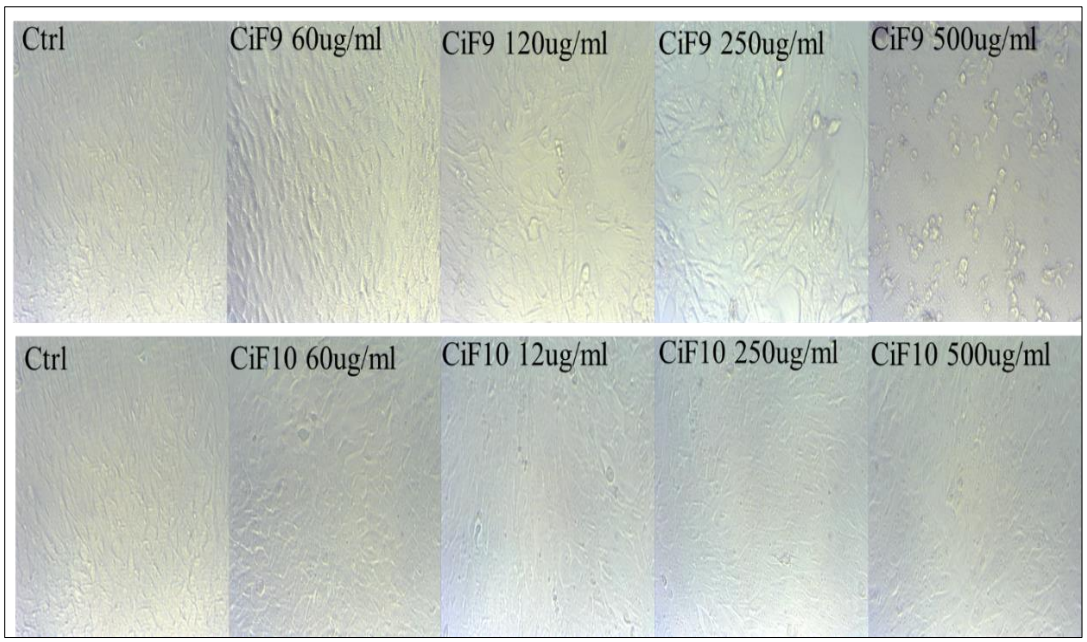

Figure 4.26: Dose dependent cell morphological changes of *C. intybus* fractions on SB28 cell line at different concentrations

Cells were also treated with CMEs of both plants at 15, 30, 60 and 120 (µg/ml) conc. and it was observed that inhibition percent was increased while increasing concentration as shown in Figure 4.27a. Among tested plant extracts *A.*

mexicana CME was more cytotoxic against SB28 cancer line having IC_{50} value of 30 µg/ml as compared to *C. intybus* CME which has IC_{50} value of 92 µg/ml significantly high as compared to *A. mexicana* plant extract (Figure 4.27b).

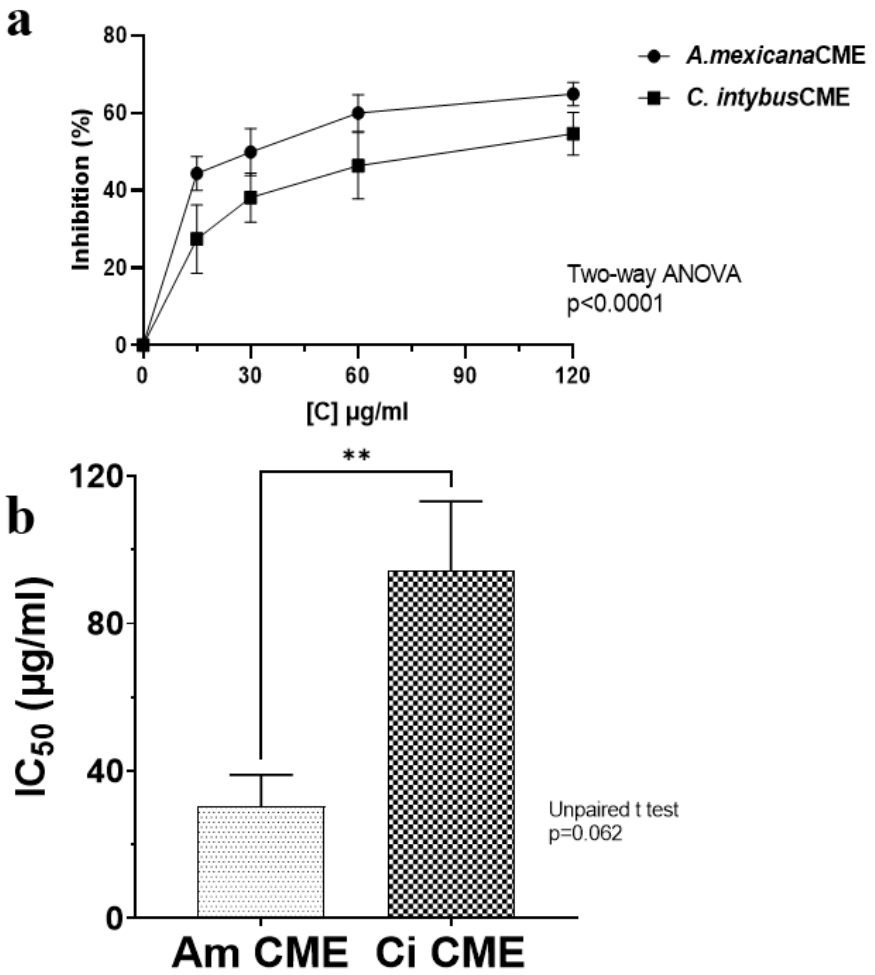

Figure 4.27: Anticancer activity of crude methanolic extracts on SB28 cell line. Part a) is showing that cell death increased while increasing the concentration of plant extracts while b) is showing IC_{50} concentration of CMEs of *A. mexicana* and *C. intybus* on SB28 cell line

Same was observed in case of fractions that cell death was increased while increasing concentration. Among tested fraction AmF8 and CiF9 have higher cytotoxicity against SB28 cell line as shown in Figure 4.28a. The analysis of figure 4.28b showed that AmF8 has lower IC_{50} value (62 µg/ml) as compared to other

fractions having IC_{50} value of 174 µg/ml (AmF8), 185 µg/ml (CiF9) and 298 µg/ml (CiF10).

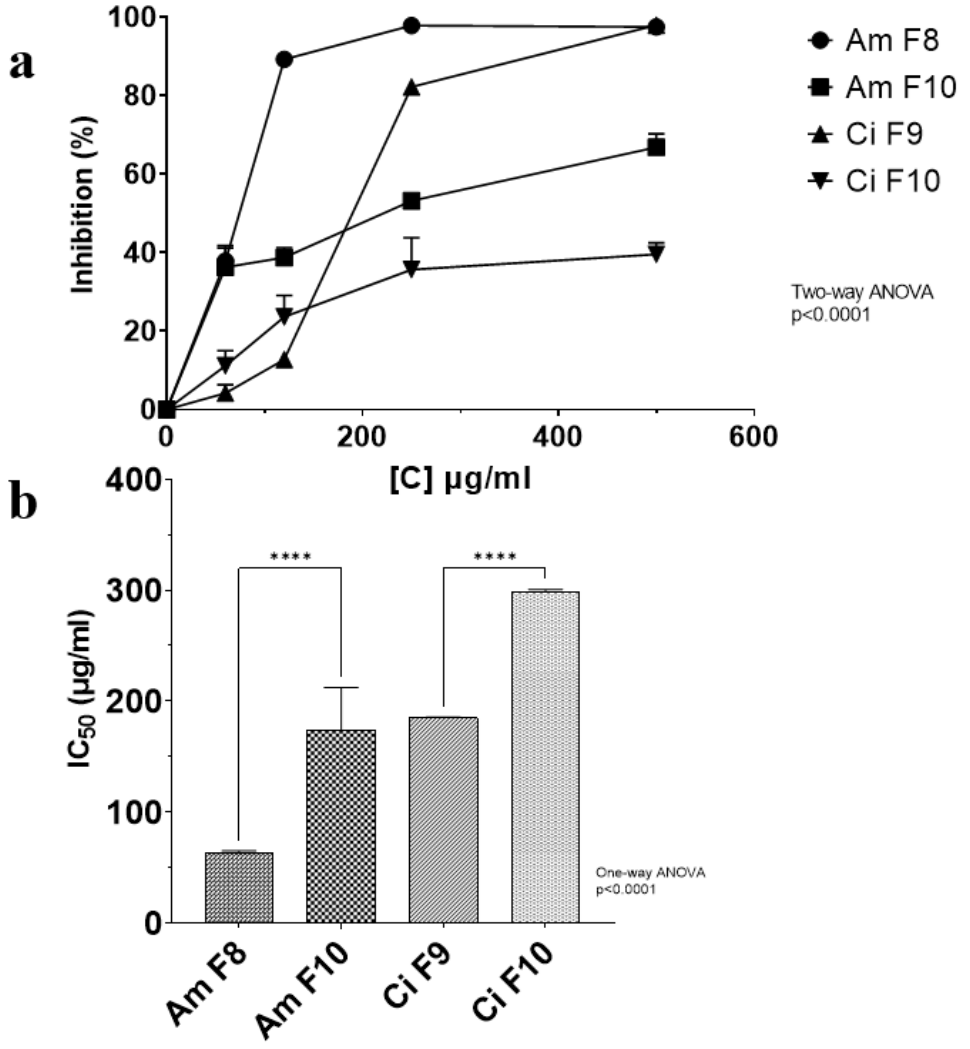

Figure 4.28: Anticancer activity of *A. mexicana* fractions (AmF8, AmF10) and *C. intybus* fractions (CiF9, CiF10) on SB28 cell line. Part a) is showing percent inhibition increased while increasing the concentration of plant extracts while b) is showing IC_{50} concentration of *A. mexicana* and *C. intybus* fractions on SB28 cell line

So one fraction from each plant extract was selected according to dose dependent studies and having lower IC_{50} value includes AmF8 and CiF9 were subjected to flow cytometry and protein expression studies.

Literature studies revealed that coumarin-based natural compounds stand out for their vast range of pharmacological properties and structural variation. Hymecromone, also known as 7-hydroxy-4-methylcoumarin, and its derivatives have among them demonstrated promise in the treatment of multi-drug resistance in cancer, the reduction of chemotherapeutic drug side effects, and the development of photo-directed cancer therapy. In addition, a range of artificial hymecromone derived agents have been shown to possess potent anticancer properties, making them useful against leukemia, breast cancer, lung cancer, prostate cancer, and brain cancer (Mustafa and Abdulaziz, 2021).

4.11 WESTERN BLOTTING

Western blot analyses were carried out to investigate the *A. mexicana* and *C. intubus* CMEs and their selected fractions to elucidate their modes of action. SB28 cells were treated with samples according to IC_{50} values as determined from MTT assay while control was treated with DMSO having same concentration as treated sample. The analysis of Figure 4.22 showed that cells treated with Am CME and its fraction 8 lowered the expression of TGFBR2 as compared to control and other treated samples while successful decreased in protein expression of TGFBR1 was seen in all treated samples as compared to control.

Literature studies showed significant increase of TGFβ pathway in cancer and its key regulated mechanism involve When TGFβRII and TGFβRI link up to generate an activated receptor complex for the Smad-dependent pathway, Smad2/3-dependent transmission is induced. TGFβRI kinase then phosphorylates Smad2 and Smad3, binds to Smad4, and forms complexes that go to the nucleus to control transcription of the target genes (Kittiwattanokhun et al., 2022).

It was revealed that Rhoifolin dramatically reduced TGFβ2 protein expression in pancreatic cells, which explain why it has an inhibitory role on the migration and invasion of cancer cells (Zheng et al., 2022). So decrease in protein expression of receptors play a significant role for cancer treatment.

Beta actin was used as control to validate our process of blotting showing successful transfer and extraction of proteins from each treated sample and control. No bands were seen in case of pSMAD2, so further experiment was carried out to elucidate the expression of pSMAD2 i.e. Flow cytometry. Among tested samples Am CME is most potent plant extract as shown in Figure 4.29.

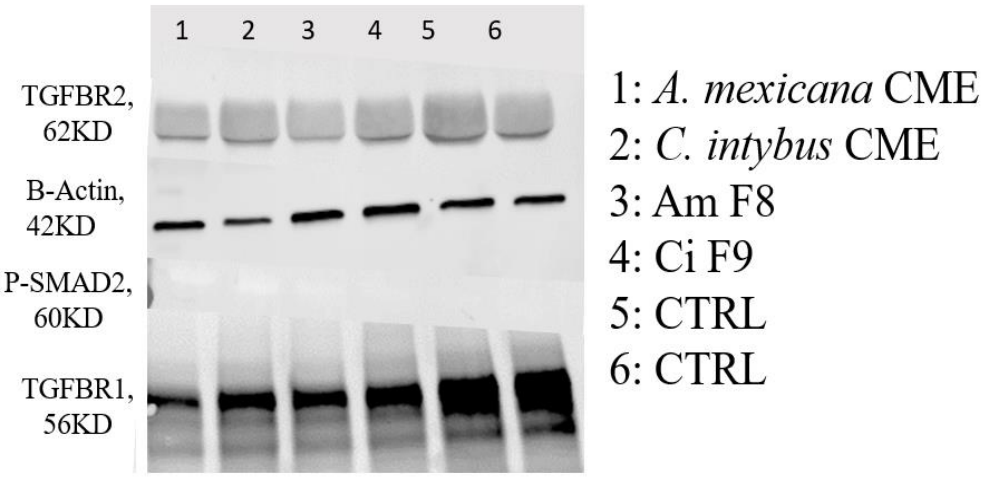

Figure 4.29: Protein expression studies of selected CMEs and fractions against selected protein of interest.

4.12 FLOW CYTOMETRY

When SB28 cells were treated with CMEs of *A. mexicana* and *C. intybus* and their fraction respectively to elucidate the mode of action towards cancer cells and the frequency of cells in each phase of cell cycle was measured. It was observed that there is no significant difference was seen in treated and untreated cells. That's confirm the cytotoxic behavior of sample exhibiting that they don't possess cytostatic properties Figure 4.30 and 4.31.

Our findings are in accordance with previous work carried out by Barhoi et al., 2021 also showed that no cell cycle arrest were seen in cancer cells treated with aqueous extract of *Moringa oleifera.* This implies that the treatment kills the cells without stopping the cell cycle.

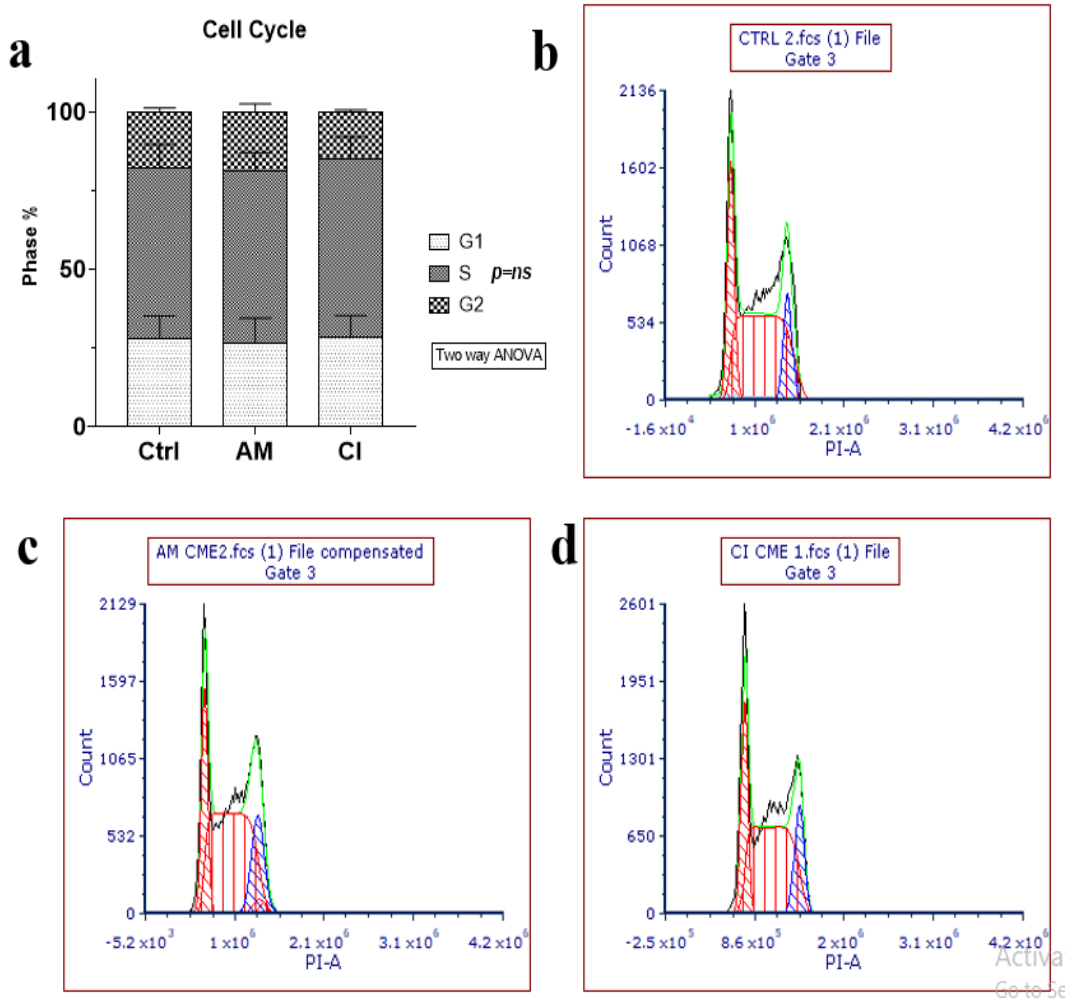

Figure 4.30: Cell cycle analysis of crude methanolic extract. Part a) is showing percent of cells present in each phase. b), c) and d) are showing multi-cycle DNA graph of control, Am CME and Ci CME respectively.

Cell cycle analysis was done using FCS Express 7 software and percent of cells in each cell cycle were calculated but no significant difference were found as shown in Figure 4.30 and 4.31.

The pSMAD2 is key regulator of downstream signaling transduction in TGFβ signaling pathway as mentioned in earlier studies (Massague, 2008). So if number of pSMAD2 positive cells reduced than it can help to inhibition of pathway and ultimately suppress the cancer cell growth. Large nuclear protein Ki67 is selectively expressed during all phases of the cell cycle that are active (G1, S, and G2/M), but it is absent from cells that are at rest (G0).

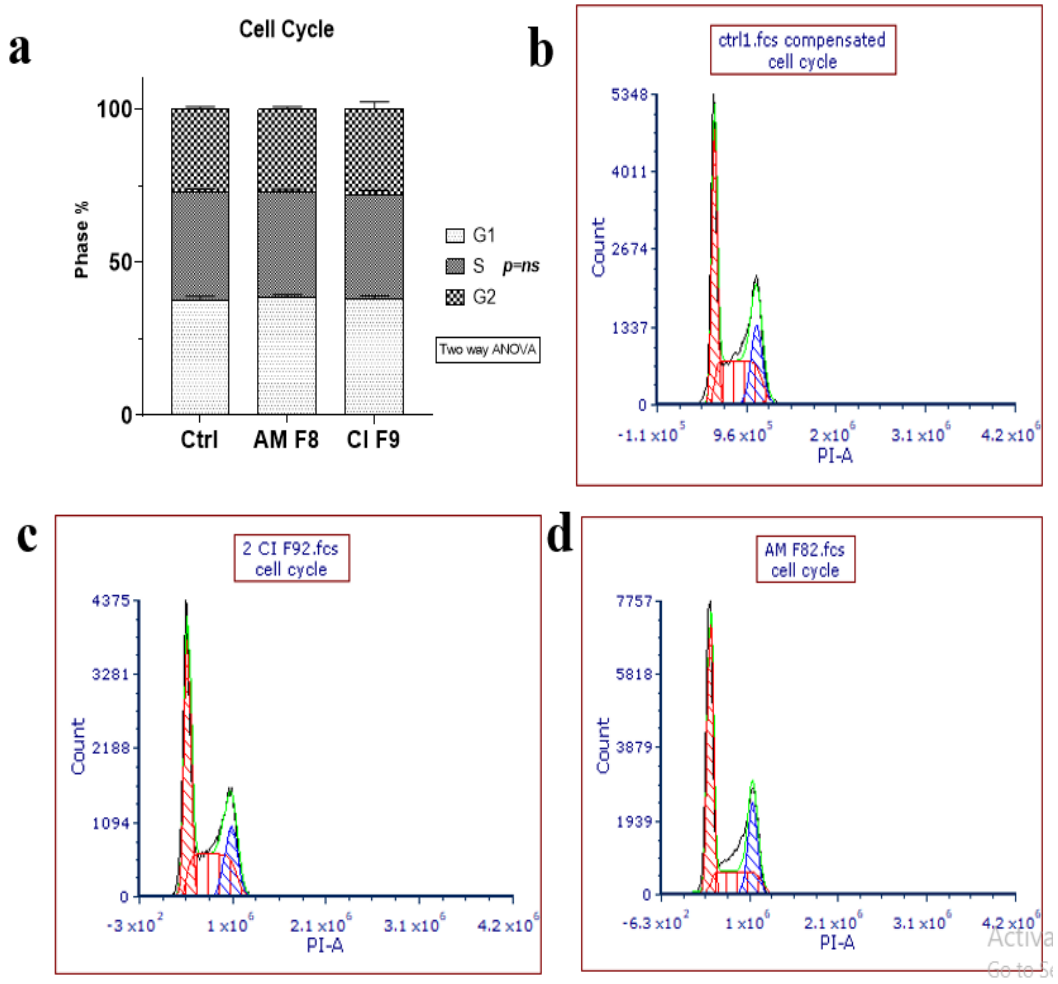

Figure 4.31: Cell cycle analysis of fractions. Part a) is showing percent of cells present in each phase. b), c) and d) are showing multi-cycle DNA graph of control, Ci F9 and Am F8 respectively.

SB28 cells treated with CMEs of both plant for 24 hours and then stained for pSMAD2 positive cells, live dead and cell proliferation. After this run on machine and analyzed by using FCS Express-7 software. Following gating strategy was used for analysis (Figure 4.32). First single cells were separated and then live dead cells were taken from singlets and from lie cells pSMAD2 positive cells and proliferating cells expressing ki67 were measured and in pSMAD2 positive cells proliferation was checked. The analysis of figure 4.33 showed that among tested CMEs of both plants the CME of *C. intybus* significantly lowered the number of pSMAD2 positive cells as compared to control untreated and cells treated with positive control TGFβ while cell proliferation was increased. It was observed that no significant effect was seen

in case of *A. mexicana* plant extract as showed in figure 4.26. It is indicated that this plant is killing cells by some other mean and effecting the expression of other genes (Figure 4.33).

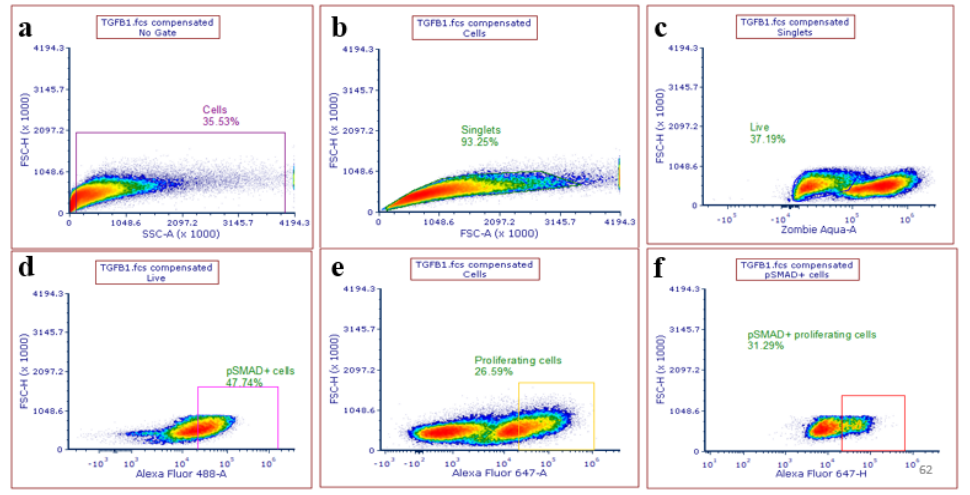

Figure 4.32: part a-f) are showing gating strategy used for analysis of psmad2 positive cells and percent of proliferating cells after treatment.

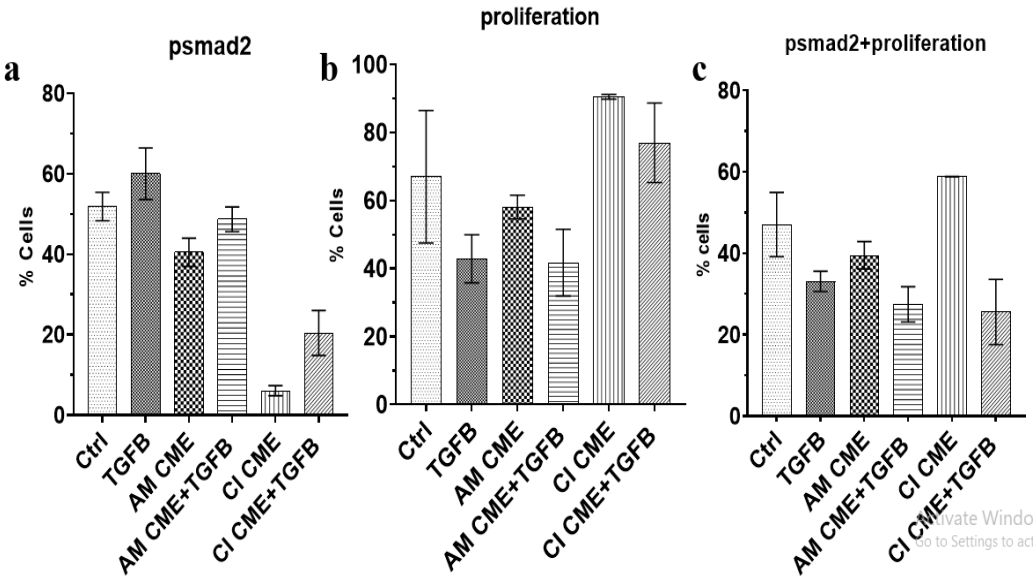

Figure 4.33: Part a) is showing percent of pSMAD2 positive cells in treated sample with extract and control while b) is showing percent of proliferating cells in each sample. C is showing percent of proliferation in pSMAD2 positive cells.

4.13 *IN VIVO* MICE STUDIES

The *A. mexicana* CME was injected intra-peritoneally into mice that had V291LK xenografts and mice without tumor to see if it had the same impact *in vivo*. When compared to the control group, mouse weight measurements did not differ significantly between the Am CME treated groups and the control group (Figure 4.34), but the size of the xenograft tumor (Figure 4.35) was significantly ($P < 0.05$) smaller in the Am CME treated groups, particularly in the 15, 120 and 240 mg/kg group (Figure 4.36).

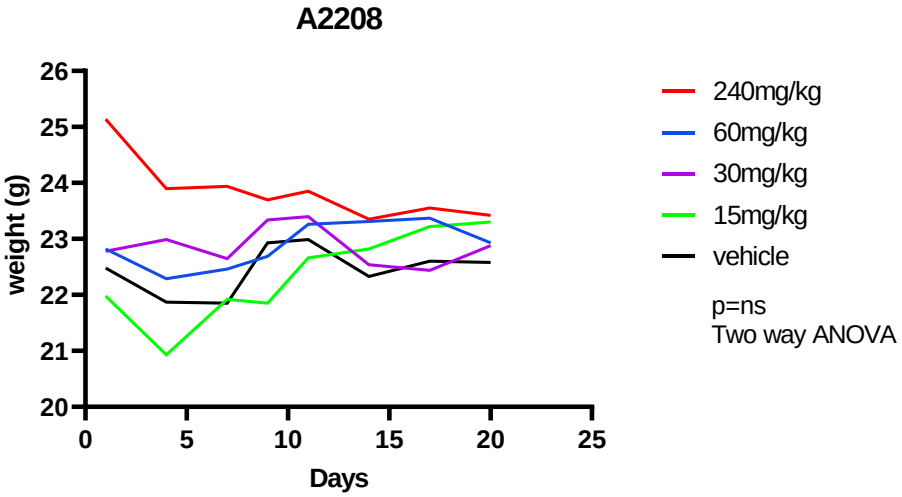

Figure 4.34: Am CME have no significant effect on mice (without tumor) weight.

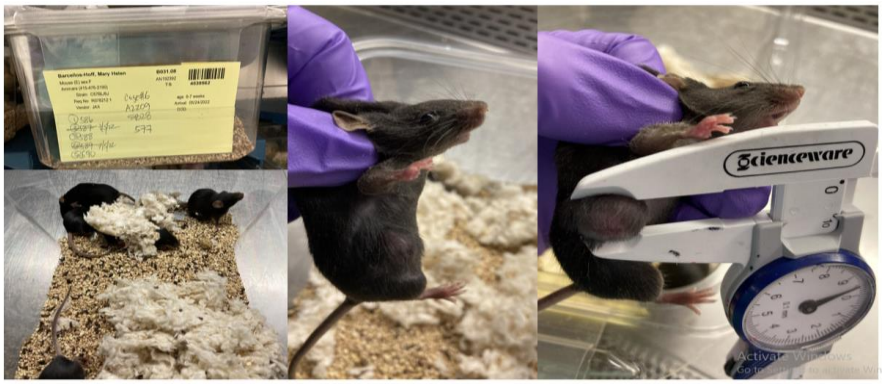

Figure 4.35: Measuring mice tumor size

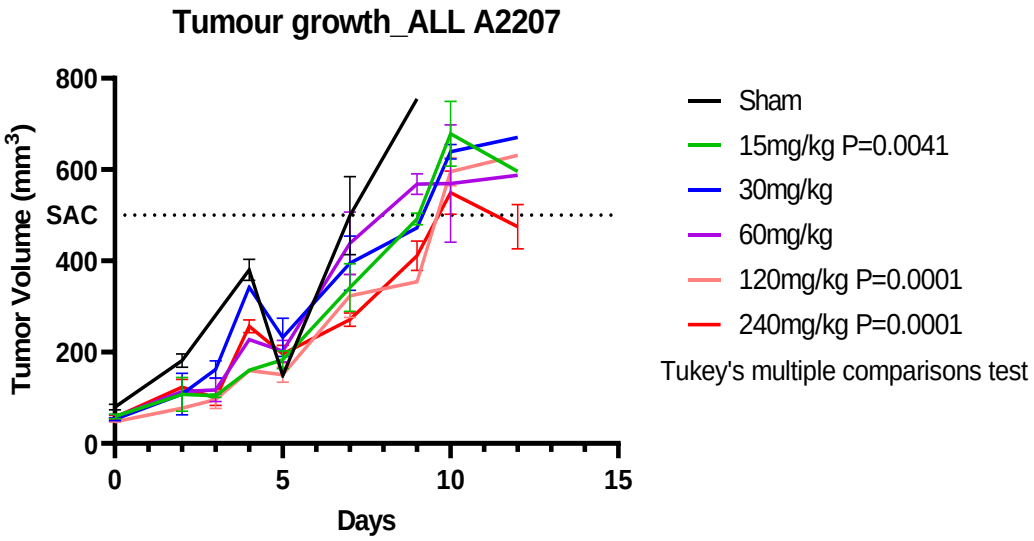

Figure 4.36: Am CME inhibits the growth of V291LK xenograft mice model for breast cancer

No previous studies were done in this regard. It's interesting to note that none of the negative effects, including weight loss, eye color and behavioral changes were seen in the current trial. It is important to note that chemotherapy can cause both acute and chronic organ toxicity, including damage to the liver and kidneys (Ramadori and Cameron, 2010).

Survival time was also significantly ($P < 0.05$) increased in tumor bearing mice treated with different concentration of Am CME, particularly in the 15, 120 and 240 mg/kg groups as presented in figure 4.37. The study was reported first time. To the best of our knowledge, no research have been published that investigate the *in vivo* anticancer effects of any extract from the *A. mexicana* species. However, a significant number of studies has addressed *in vivo* potentially anticancer effects of plant extracts. According study conducted by Bar-Shalom et al., (2019) to *in vivo* research, giving mice with MC38 cell transplants 150 or 300 mg/kg *Inula viscosa* extract prevented tumor growth. When compared to the untreated control group, the weight and volume of the tumors were dramatically ($P < 0.001$) decreased.

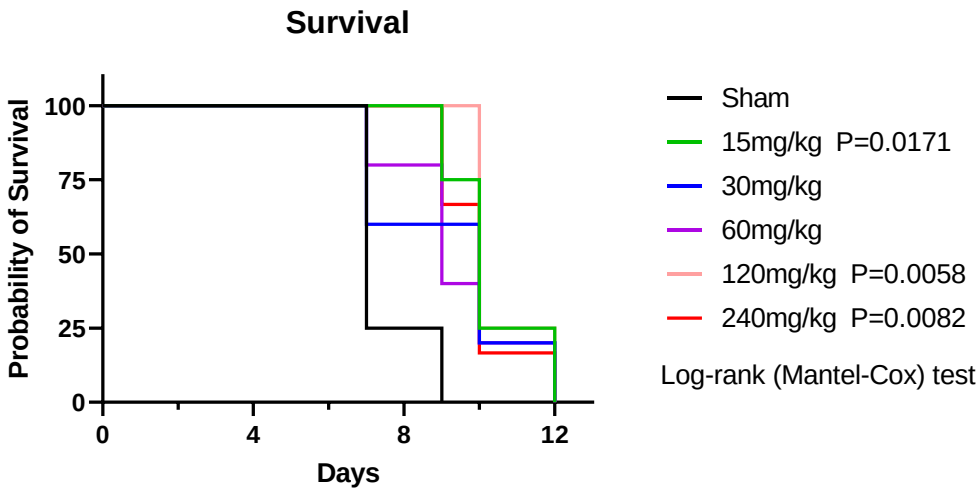

Figure 4.37: Am CME increased survival rate in V291LK xenograft mice model for breast cancer

Other researchers conducted comparable findings using a mouse model. In a study conducted by Barhoi et al., (2021) demonstrated the ability of aqueous extract of *Moringa oleifera* to treat solid tumors in mice. Our research showed that aqueous extract of this plant administration increased the survival period of tumor-bearing mice while attenuating Tumor volume in a dose and time dependent manner. Other studies revealed quercetin-mediated decrease of tumor volume in mice with solid tumors (Srivastava et al., 2016). Thomas et al. (2016) demonstrated that *Vernonia condensata* extracts inhibited tumor growth in the mice test system. Our findings, which showed a reduction of tumor volume in mice with solid tumors, are consistent with Noaman et al., 2008 observation that tumor weight was significantly and progressively reduced in arabinoxylan rice bran, MGN-3, and biobran-treated tumor cell mice.

The use of natural substances and their derivatives in the treatment of cancer has a long past and a wide present; some of the most well-known and efficient cancer medications on the market today have been derived from natural sources, such as plants. Therefore, it is understandable that employing alternative and complementary treatments many of which are plant-derived is considered with some trepidation or even mistrust, especially when it comes to the treatment of cancer. Clinicians should be aware that certain plant-based chemotherapy regimens are completely mainstream

and backed by significant clinical research (Tewari, Priya, Bishayee and Bishayee, 2022). Relatedly, one goal of this research is to pinpoint the plants and plant extracts that have real potential for treating cancer. We do this in the hopes that researchers will be better able to distinguish these more promising agents from other natural products whose use may not be well-supported by science, if at all.

SUMMARY

Cancer is still leading cause of mortality and major hurdle of increased life expectancy worldwide with increasing rate day by day. Cancer is worst diseases in existence, whose incidence is unpredictably rising as a result of rapid urbanization, environmental problems, and significant changes in lifestyle. Despite multiple important medical and technological advancements in the fight against this illness, many traditional cancer-targeted treatments have serious drawbacks and consequences, such as the risk of being exposed to toxicities and the emergence of drug resistance.

New powerful medications are required for the treatment of cancer in order to address the issues of side effects, selectivity, and drug resistance. Anticancer pharmaceuticals compounds can be found in natural compounds, which frequently appear to be more efficient and/or less hazardous. About 60% of the drugs used to treat cancer today have been extracted from natural sources, many of which are taken from plants. Medicinal plant extracts are a popular, non-toxic alternative to chemotherapy in many nations across the world.

We have focused our research on the TGFβ signaling system, even though cancer medications can cure different people via a variety of strategies involving numerous signaling pathways. When it comes to cell growth, apoptosis, motility, invasion, angiogenesis, and differentiation, the TGFβ signaling pathway is essential. Although TGFβ is mostly investigated for its ability to prevent tumor growth in the early stages of cancer, it is also known to play a role in the disease's progression, particularly in its later stages. Downregulation of TGFβR signaling pathway can provides better therapeutic strategy to control oncogenesis.

A vast variety of natural compounds has been reported for antitumor activities making them a potential candidates for inhibition of TGFβR signaling pathway. The present study is designed to investigate the inhibitory effects of extracts and natural compounds from reported Pakistani medicinal plant species against TGFβR signaling pathway. Maceration was used to create CMEs of sixteen different plant species. These CMEs were then subjected to phytochemical analyses in order to

identify the main phytometabolites, notably the components that are cancer chemopreventive. *Cichorium intybus* has the highest total flavonoid concentration (0.53 mg/gQE), compared to all other plants extracts. All plant extracts were tested for their ability to scavenge free radicals, and their IC_{50} values ranged from 2.03 to 88 µg/ml. Using the MTT colorimetric test, the comparative cytotoxicity of plant extracts was assessed in the HUH and MCF-7 cell lines. The nine active plant extracts were identified based on their high antiproliferative activity > 50% inhibition against cancer cell lines and were further subjected to relative expression studies.

Modulation of TGFβ signaling molecules (i.e. TGFβR1, 2&3, SMAD3, SMAD5) and ubiquitous proteins (SMURF1 and SMURF2) genetic expression by selected nine active extracts was determined by RT-PCR using GAPDH as gene of reference. Expression studies revealed that CMEs of five plant extracts significantly inhibit TGFβ mediated signaling cascade by downregulating the gene expression fold change <1.0. These plant extracts were further subjected to column chromatography and column fractions were obtained using various solvent combinations. Thin layer chromatography showed the presence of UV active compounds in *A. mexicana* and *C. opaca* sample and RF values were calculated and cytotoxic and relative expression studies were performed again on active fractions.

Two plants extracts i.e. *C. intybus* and *A. mexicana* and their most potent column fractions were selected for further *in vitro* anticancer activities using SB28 cell line which showed significant anticancer activity in Am CME having IC_{50} of 30 µg/ml. A significant decreased in expression was observed by all tested sample against TGFβR1 in western blotting studies while flow cytometry studies showed no significant difference in each phase of cell cycle as compared to control. The most potent plant extract (*Argemone mexicana* CME) was subjected to *in vivo* studies using mice model for breast cancer (MTDT) and mice with no tumor.

Dose finding studies in MTDT mice model showed significance delay in tumor growth at 250 mg/kg with respect to control SHAM group. Survival time increased in mice treated with *A. mexicana* crude extract $P < 0.05$. While interestingly no side effects were seen in mice treated with plant extract from low to high concentration.

Among tested plant extracts, the crude methanolic extract of *A. mexicana* significantly inhibited TGFβ mediated signaling cascade and increased life expectancy in MTDT mice. The active constituents of *A. mexicana* CME can be used for further studies as potent cancer therapeutic agents paving a new way to treat cancer.

Additional clinical investigations are urgently required because there have been so few studies conducted on the effects of different natural products on TGFβ signaling. Future research using cutting-edge pharmacological techniques, such as multi-omic studies, is also encouraged. The understanding of TGFβ participation at genomic levels likewise has a huge range. Additionally, methods like metabolomics can be used to comprehensively explore the effects of different plant secondary metabolites. TGFβ targeting bioactive substances from natural sources have the potential to be used as chemotherapeutic drugs for the treatment and prevention of various human cancers, according to newly available evidence that is provided in this thesis.

LITERATURE CITED

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., ... & Piccolo, S. (2009). A mutant-p53/Smad complex opposes p63 to empower TGFβ-induced metastasis. *Cell*, *137*(1), 87-98.

Agbarya, A., Ruimi, N., Epelbaum, R., Ben-Arye, E., & Mahajna, J. (2014). Natural products as potential cancer therapy enhancers: A preclinical update. *SAGE Open Medicine*, *2*, 2050312114546924.

Akhtar, M. F., Saleem, A., & Saleem, M. (2019). A comprehensive review on ethnomedicinal, pharmacological and phytochemical basis of anticancer medicinal plants of Pakistan. *Current Cancer Drug Targets*, *19*(2), 120-151.

Amber, A., Bokhari & Syed, V. (2016). Inhibition of Transforming Growth Factor-β (TGF-β) Signaling by *Scutellaria baicalensis* and *Fritillaria cirrhosa* Extracts in Endometrial Cancer. *Journal of Cellular Biochemitry*, 116: 1797-1805.

Apostolou, A., Stagos, D., Galitsiou, E., Spyrou, A., Haroutounian, S., Portesis, N., ... & Kouretas, D. (2013). Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of Vitis vinifera stem extracts. *Food and Chemical Toxicology*, *61*, 60-68.

Araki, S., Eitel, J. A., Batuello, C. N., Bijangi-Vishehsaraei, K., Xie, X. J., Danielpour, D., ... & Mayo, L. D. (2010). TGF-β1–induced expression of human Mdm2 correlates with late-stage metastatic breast cancer. *The Journal of Clinical Investigation*, *120*(1), 290-302.

Aromokeye, R., & Si, H. (2022). Correction: Aromokeye, R.; Si, H. Combined Curcumin and Luteolin Synergistically Inhibit Colon Cancer Associated with Notch1 and TGF-β Signaling Pathways in Cultured Cells and Xenograft Mice. Cancers 2022, 14, 3001. *Cancers*, *14*(17), 4238.

Arun, N., & Singh, D. P. (2012). Punica granatum: a review on pharmacological and therapeutic properties. *International Journal of Pharmaceutical Sciences and Research*, *3*(5), 1240.

Bais, H. P., & Ravishankar, G. A. (2001). Cichorium intybus L–cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. *Journal of the Science of Food and Agriculture*, *81*(5), 467-484.

Barhoi, D., Upadhaya, P., Barbhuiya, S. N., Giri, A., & Giri, S. (2021). Aqueous extract of Moringa oleifera exhibit potential anticancer activity and can be used as a possible cancer therapeutic agent: a study involving in vitro and in vivo approach. *Journal of the American College of Nutrition*, *40*(1), 70-85.

Bar-Shalom, R., Bergman, M., Grossman, S., Azzam, N., Sharvit, L., & Fares, F. (2019). Inula viscosa extract inhibits growth of colorectal cancer cells in vitro and in vivo through induction of apoptosis. *Frontiers in Oncology*, *9*, 227.

Batool, S., Ullah, S., Tabassum, S., Bilal, A., Faisal, A., Saleem, R. S. Z., & Ahmad, M. S. (2019). Effect of extraction methodologies on antioxidant potential and synergistic anticancer behavior of leaf extract of Polygonum amplexicaule against HCT-116 cells. *Iranian Journal of Science and Technology, Transactions A: Science*, *43*(3), 709-714.

Batra, P., & Sharma, A. K. (2013). Anti-cancer potential of flavonoids: recent trends and future perspectives. *3 Biotech*, *3*(6), 439-459.

Biswas, S., Guix, M., Rinehart, C., Dugger, T. C., Chytil, A., Moses, H. L., ... & Arteaga, C. L. (2007). Inhibition of TGF-β with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. *The Journal of Clinical Investigation*, *117*(5), 1305-1313.

Bogdahn, U., Hau, P., Stockhammer, G., Venkataramana, N. K., Mahapatra, A. K., Suri, A. A., ... & Trabedersen Glioma Study Group. (2010). Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: results of a randomized and controlled phase IIb study. *Neuro-oncology*, *13*(1), 132-142.

Budi, E. H., Mamai, O., Hoffman, S., Akhurst, R. J., & Derynck, R. (2019). Enhanced TGF-β signaling contributes to the insulin-induced angiogenic responses of endothelial cells. *Iscience*, *11*, 474-491.

Capper, D., Von Deimling, A., Brandes, A. A., Carpentier, A. F., Kesari, S., Sepulveda-Sanchez, J. M., ... & Wick, W. (2017). Biomarker and histopathology evaluation of patients with recurrent glioblastoma treated with galunisertib, lomustine, or the combination of galunisertib and lomustine. *International Journal of Molecular Sciences*, *18*(5), 995.

Cedar, H., & Bergman, Y. (2012). Programming of DNA methylation patterns. *Annual Review of Biochemistry*, *81*, 97-117.

Chang, Y. C., Chang, F. R., Khalil, A. T., Hsieh, P. W., & Wu, Y. C. (2003). Cytotoxic benzophenanthridine and benzylisoquinoline alkaloids from Argemone mexicana. *Zeitschrift für Naturforschung C*, *58*(7-8), 521-526.

Chart, R. S., Price, D. T., Sue, S. R., Meyers, W. C., & Jirtle, R. L. (1995). Down-regulation of transforming growth factor beta receptor type I, II, and III during liver regeneration. *The American Journal of Surgery*, *169*(1), 126-132.

Cheifetz, S., Hernandez, H., Laiho, M., Ten Dijke, P., Iwata, K. K., & Massagué, J. (1990). Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *Journal of Biological Chemistry*, *265*(33), 20533-20538.

Chen, L., & Flies, D. B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature Reviews Immunology*, *13*(4), 227-242.

Chen, X. H., Liu, Z. C., Zhang, G., Wei, W., Wang, X. X., Wang, H., ... & Du, J. (2015). TGF-β and EGF induced HLA-I downregulation is associated with epithelial-mesenchymal transition (EMT) through upregulation of snail in prostate cancer cells. *Molecular Immunology*, *65*(1), 34-42.

Chen, Y., Di, C., Zhang, X., Wang, J., Wang, F., Yan, J. F. & Zhang, H. (2020). Transforming growth factor β signaling pathway: a promising therapeutic target for cancer. *Journal of Cellular Physiology*, *235*(3), 1903-1914.

Colak, S., & Ten Dijke, P. (2017). Targeting TGF-β signaling in cancer. *Trends in cancer*, *3*(1), 56-71.

Cui, W., Fowlis, D. J., Bryson, S., Duffie, E., Ireland, H., Balmain, A. & Akhurst, R. J. (1996). TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell,* 86: 531-542.

Darland, D. C., & D'amore, P. A. (2001). TGFβ is required for the formation of capillary-like structures in three-dimensional cocultures of 10T1/2 and endothelial cells. *Angiogenesis*, *4*(1), 11-20.

Deshpande, S. S. (2002a). Food Additives. *In Handbook of Food Toxicology*, 219-284.

Dodagatta-Marri, E., Meyer, D. S., Reeves, M. Q., Paniagua, R., To, M. D., Binnewies, M., ... & Akhurst, R. J. (2019). α-PD-1 therapy elevates Treg/Th balance and increases tumor cell pSmad3 that are both targeted by α-TGFβ antibody to promote durable rejection and immunity in squamous cell carcinomas. *Journal for Immunotherapy of Cancer*, *7*(1), 1-15.

Dolan, L. C., Matulka, R. A., & Burdock, G. A. (2010). Naturally occurring food toxins. *Toxins*, *2*(9), 2289-2332.

Dvorchik, B. H. (2000). The disposition (ADME) of antisense oligonucleotides. *Current Opinion in Molecular Therapeutics*, 2: 253-257.

Edlund, S., Landstrom, M., Heldin, C. H., & Aspenstrom, P. (2002). Transforming growth factor-β–induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. *Molecular Biology of the Cell*, *13*(3), 902-914.

Farooq, F., Rai, M., Tiwari, A., Khan, A. A., & Farooq, S. (2012). Medicinal properties of Moringa oleifera: An overview of promising healer. *Journal of Medicinal Plants Research*, *6*(27), 4368-4374.

Fessler, E., Drost, J., van Hooff, S. R., Linnekamp, J. F., Wang, X., Jansen, M., ... & Medema, J. P. (2016). TGFβ signaling directs serrated adenomas to the mesenchymal colorectal cancer subtype. *EMBO Molecular Medicine*, *8*(7), 745-760.

Formenti, S. C., Hawtin, R. E., Dixit, N., Evensen, E., Lee, P., Goldberg, J. D., ... & Demaria, S. (2019). Baseline T cell dysfunction by single cell network profiling in metastatic breast cancer patients. *Journal for Immunotherapy of Cancer*, *7*(1), 1-8.

Francesconi, A. B., Dupre, S., Matos, M., Martin, D., Hughes, B. G., Wyld, D. K., & Lickliter, J. D. (2010). Carboplatin and etoposide combined with bevacizumab for the treatment of recurrent glioblastoma multiforme. *Journal of Clinical Neuroscience*, *17*(8), 970-974.

Gatto, B. (2004). Monoclonal antibodies in cancer therapy. *Current Medicinal Chemistry-Anti-Cancer Agents*, *4*(5), 411-414.

Giaccone, G., Bazhenova, L. A., Nemunaitis, J., Tan, M., Juhász, E., Ramlau, R., & Fakhrai, H. (2015). A phase III study of belagenpumatucel-L, an allogeneic tumour cell vaccine, as maintenance therapy for non-small cell lung cancer. *European journal of cancer*, *51*(16), 2321-2329.

Giffin, R., Pool, R., & Robinson, S. (2008). *Emerging safety science: workshop summary*. National Academies Press.

Gobbi, H., Arteaga, C. L., Jensen, R. A., Simpson, J. F., Dupont, W. D., Olson, S. J., ... & Page, D. L. (2000). Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. *Histopathology*, *36*(2), 168-177.

Golden, E. B., Frances, D., Pellicciotta, I., Demaria, S., Helen Barcellos-Hoff, M., & Formenti, S. C. (2014). Radiation fosters dose-dependent and

chemotherapy-induced immunogenic cell death. *Oncoimmunology*, *3*(4), e28518.

Gómez-Gil, V. (2021). Therapeutic implications of TGFβ in cancer treatment: a systematic review. *Cancers*, *13*(3), 379.

Gunasekera, R. S., Sewgobind, K., Desai, S., Dunn, L., Black, H. S., McKeehan, W. L., & Patil, B. (2007). Lycopene and lutein inhibit proliferation in rat prostate carcinoma cells. *HNUC*, *58*(2), 171-177.

Gutiérrez, R. M. P., Mitchell, S., & Solis, R. V. (2008). Psidium guajava: a review of its traditional uses, phytochemistry and pharmacology. *Journal of Ethnopharmacology*, *117*(1), 1-27.

Hahn, S. A., Schutte, M., Shamsul Hoque, A. T. M., Moskaluk, C. A., Da Costa, L. T., Rozenblum, E., ... & Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21. 1. *Science*, *271*(5247), 350-353.

Hanafy, N. A. N., Quarta, A., Ferraro, M. M., Dini, L., Nobile, C., De Giorgi, M. L., ... & Leporatti, S. (2018). Polymeric nano-micelles as novel cargo-carriers for LY2157299 liver cancer cells delivery. *International Journal of Molecular Sciences*, *19*(3), 748.

Hande, K. R. (1998). Etoposide: four decades of development of a topoisomerase II inhibitor. *European Journal of Cancer*, *34*(10), 1514-1521.

Hata, A., & Chen, Y. G. (2016). TGF-β signaling from receptors to Smads. *Cold Spring Harbor Perspectives in Biology*, *8*(9), a022061.

Hau, P., Bogdahn, U., Stauder, G., Kunst, M. & Schlingensiepen, K. H. (2003). TGF-β2 specific antisense oligonucleotide (AP-12009) as continuous intratumoural treatment of recurrent high-grade glioma patients. *Arzneimittel Forschung_Drug Research*, *53*, 464.

Hjelmeland, M. D., Hjelmeland, A. B., Sathornsumetee, S., Reese, E. D., Herbstreith, M. H., Laping, N. J., ... & Rich, J. N. (2004). SB-431542, a small molecule transforming growth factor-β-receptor antagonist, inhibits human

glioma cell line proliferation and motility. *Molecular cancer therapeutics*, *3*(6), 737-745.

Huang, C. Y., Chung, C. L., Hu, T. H., Chen, J. J., Liu, P. F., & Chen, C. L. (2021). Recent progress in TGF-β inhibitors for cancer therapy. *Biomedicine & Pharmacotherapy*, *134*, 111046.

Huang, S., Hölzel, M., Knijnenburg, T., Schlicker, A., Roepman, P., McDermott, U., ... & Bernards, R. (2012). MED12 controls the response to multiple cancer drugs through regulation of TGF-β receptor signaling. *Cell*, *151*(5), 937-950.

Hussain, A., Zia, M., & Mirza, B. (2007). Cytotoxic and antitumor potential of Fagonia cretica L. *Turkish Journal of Biology*, *31*(1), 19-24.

Huynh, L. K., Hipolito, C. J., & Ten Dijke, P. (2019). A perspective on the development of TGF-β inhibitors for cancer treatment. *Biomolecules*, *9*(11), 743.

Imamura, T., Hikita, A., & Inoue, Y. (2012). The roles of TGF-β signaling in carcinogenesis and breast cancer metastasis. *Breast Cancer*, *19*(2), 118-124.

Izzi, L., & Attisano, L. (2004). Regulation of the TGFβ signalling pathway by ubiquitin-mediated degradation. *Oncogene*, *23*(11), 2071-2078.

Jia, L., Jin, H., Zhou, J., Chen, L., Lu, Y., Ming, Y., & Yu, Y. (2013). A potential anti-tumor herbal medicine, Corilagin, inhibits ovarian cancer cell growth through blocking the TGF-β signaling pathways. *BMC Complementary and Alternative Medicine*, *13*(1), 1-11.

Kandil, A. S., Abou-Elella, F., & El Shemy, H. A. (2019). Cytotoxic profile activities of ethanolic and methanolic extracts of chicory plant (Cichorium intybus L.). *Journal of Radiation Research and Applied Sciences*, *12*(1), 106-111.

Kim, B. G., Malek, E., Choi, S. H., Ignatz-Hoover, J. J., & Driscoll, J. J. (2021). Novel therapies emerging in oncology to target the TGF-β pathway. *Journal of Hematology & Oncology*, *14*(1), 1-20.

Kittiwattanokhun, A., Innajak, S., Tashiro, E., Imoto, M., & Watanapokasin, R. (2022). Cinnamomum bejolghota Extract Inhibits Colorectal Cancer Cell Metastasis and TGF-β1-Induced Epithelial-Mesenchymal Transition via Smad and Non-Smad Signaling Pathway. *Scientia Pharmaceutica*, *90*(2), 30.

Koleva, I. I., Van Beek, T. A., Linssen, J. P., Groot, A. D., & Evstatieva, L. N. (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, *13*(1), 8-17.

Kumar, V. S., & Navaratnam, V. (2013). Neem (Azadirachta indica): prehistory to contemporary medicinal uses to humankind. *Asian Pacific Journal of Tropical Biomedicine*, *3*(7), 505-514.

Lamora, A., Talbot, J., Bougras, G., Amiaud, J., Leduc, M., Chesneau, J. & Verrecchia, F. (2014). Overexpression of smad7 blocks primary tumor growth and lung metastasis development in osteosarcoma. *Clinical Cancer Research*, *20*(19), 5097-5112.

Larsson, C., Cordeddu, L., Siggens, L., Pandzic, T., Kundu, S., He, L., ... & Sjöblom, T. (2020). Restoration of KMT2C/MLL3 in human colorectal cancer cells reinforces genome-wide H3K4me1 profiles and influences cell growth and gene expression. *Clinical Epigenetics*, *12*(1), 1-12.

Lee, C. Y., Hsu, Y. C., Wang, J. Y., Chen, C. C., & Chiu, J. H. (2008). Chemopreventive effect of selenium and Chinese medicinal herbs on N-nitrosobis (2-oxopropyl) amine-induced hepatocellular carcinoma in Syrian hamsters. *Liver International*, *28*(6), 841-855.

Lee, C., Zhang, Q., Kozlowski, J., Brendler, C., Soares, M. B., Dash, A., ... & Mercola, D. (2013). Natural products and transforming growth factor-beta (TGF-β) signaling in cancer development and progression. *Current Cancer Drug Targets*, *13*(5), 500-505.

Lee, J. D., Hempel, N., Lee, N. Y., & Blobe, G. C. (2010). The type III TGF-β receptor suppresses breast cancer progression through GIPC-mediated inhibition of TGF-β signaling. *Carcinogenesis*, *31*(2), 175-183.

Lee, S., Ra, J., Song, J. Y., Gwak, C., Kwon, H. J., Yim, S. V., ... & Ahn, H. J. (2011). Extracts from Citrus unshiu promote immune-mediated inhibition of tumor growth in a murine renal cell carcinoma model. *Journal of Ethnopharmacology*, *133*(3), 973-979.

Lee, T. K., Lee, J. Y., Kim, D. I., Lee, Y. C., & Kim, C. H. (2005). Differential regulation of protein kinase C activity by modulating factors and Euonymus alatus (Thunb.) Sieb in human myometrial and uterine leiomyomal smooth muscle cells. *International Journal of Gynecologic Cancer*, *15*(2).

Levy, L., & Hill, C. S. (2006). Alterations in components of the TGF-β superfamily signaling pathways in human cancer. *Cytokine & Growth Factor Reviews*, *17*(1-2), 41-58.

Lin, H. H., Tsai, C. W., Chou, F. P., Wang, C. J., Hsuan, S. W., Wang, C. K., & Chen, J. H. (2011). Andrographolide down-regulates hypoxia-inducible factor-1α in human non-small cell lung cancer A549 cells. *Toxicology and Applied Pharmacology*, *250*(3), 336-345.

Liu, S., Ren, J., & Ten Dijke, P. (2021). Targeting TGFβ signal transduction for cancer therapy. *Signal Transduction and Targeted Therapy*, *6*(1), 1-20.

Lonning, S., Mannick, J., & M McPherson, J. (2011). Antibody targeting of TGF-β in cancer patients. *Current Pharmaceutical Biotechnology*, *12*(12), 2176-2189.

Mann, J. (2002). Natural products in cancer chemotherapy: past, present and future. *Nature Reviews Cancer*, *2*(2), 143-148.

Mannarreddy, P., Denis, M., Munireddy, D., Pandurangan, R., Thangavelu, K. P., & Venkatesan, K. (2017). Cytotoxic effect of Cyperus rotundus rhizome extract on human cancer cell lines. *Biomedicine & Pharmacotherapy*, *95*, 1375-1387.

Markowitz, S. D., & Roberts, A. B. (1996). Tumor suppressor activity of the TGF-β pathway in human cancers. *Cytokine & Growth Factor Reviews*, *7*(1), 93-102.

Massagué, J. (2008). TGFβ in cancer. *Cell*, *134*(2), 215-230.

Massagué, J. (2012). TGFβ signalling in context. *Nature Reviews Molecular Cell Biology*, *13*(10), 616-630.

Matsumura, N., Huang, Z., Mori, S., Baba, T., Fujii, S., Konishi, I., ... & Murphy, S. K. (2011). Epigenetic suppression of the TGF-beta pathway revealed by transcriptome profiling in ovarian cancer. *Genome Research*, *21*(1), 74-82.

Mawa, S., Husain, K., & Jantan, I. (2013). Ficus carica L.(Moraceae): phytochemistry, traditional uses and biological activities. *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Meghwal, M., & Goswami, T. K. (2013). Piper nigrum and piperine: an update. *Phytotherapy Research*, *27*(8), 1121-1130.

Meng, X. H., Lv, H., Ding, X. Q., Jian, T. Y., Feng, X. J., Ren, B. R., & Chen, J. (2022). Sesquiterpene lactones with anti-inflammatory and cytotoxic activities from the roots of Cichorium intybus. *Phytochemistry*, *203*, 113377.

Miyazono, K., Maeda, S., & Imamura, T. (2005). BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine & Growth Factor Reviews*, *16*(3), 251-263.

Mo, N., Li, Z. Q., Li, J., & Cao, Y. D. (2012). Curcumin inhibits TGF-β1-induced MMP-9 and invasion through ERK and Smad signaling in breast cancer MDA-MB-231 cells. *Asian Pacific Journal of Cancer Prevention*, *13*(11), 5709-5714.

Morris, J. C., Tan, A. R., Olencki, T. E., Shapiro, G. I., Dezube, B. J., Reiss, M., ... & Lawrence, D. P. (2014). Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGFβ) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. *PloS One*, *9*(3), e90353.

Moustakas, A., & Heldin, C. H. (2016). Mechanisms of TGFβ-induced epithelial–mesenchymal transition. *Journal of Clinical Medicine*, *5*(7), 63.

Mustafa, Y. F., & Abdulaziz, N. T. (2021). Hymecromone and its products as cytotoxic candidates for brain cancer: A brief review. *NeuroQuantology*, *19*(7), 175-186.

Nagata, H., Hatano, E., Tada, M., Murata, M., Kitamura, K., Asechi, H., ... & Uemoto, S. (2009). Inhibition of c-Jun NH2-terminal kinase switches Smad3 signaling from oncogenesis to tumor-suppression in rat hepatocellular carcinoma. *Hepatology*, *49*(6), 1944-1953.

Nemunaitis, J., Dillman, R. O., Schwarzenberger, P. O., Senzer, N., Cunningham, C., Cutler, J., ... & Fakhrai, H. (2006). Phase II study of belagenpumatucel-L, a transforming growth factor beta-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer. *Journal of Clinical Oncology*, *24*(29), 4721-4730.

Neu, J. L., Prather, M. J., & Penner, J. E. (2007). Global atmospheric chemistry: Integrating over fractional cloud cover. *Journal of Geophysical Research: Atmospheres*, *112*(D11).

Nisa, S., Bibi, Y., Zia, M., Waheed, A., & Chaudhary, M. F. (2013). Anticancer investigations on Carissa opaca and Toona ciliata extracts against human breast carcinoma cell line. *Pakistan Journal of Pharmaceutical Sciences*, *26*(5), 1009-12.

Nishida, N., Yano, H., Nishida, T., Kamura, T., & Kojiro, M. (2006). Angiogenesis in cancer. *Vascular Health and Risk Management*, *2*(3), 213.

Noaman, E., El-Din, N. K. B., Bibars, M. A., Abou Mossallam, A. A., & Ghoneum, M. (2008). Antioxidant potential by arabinoxylan rice bran, MGN-3/biobran, represents a mechanism for its oncostatic effect against murine solid Ehrlich carcinoma. *Cancer Letters*, *268*(2), 348-359.

Oberlies, N. H., & Kroll, D. J. (2004). Camptothecin and taxol: historic achievements in natural products research. *Journal of Natural Products*, *67*(2), 129-135.

Orozco-Nunnelly, D. A., Pruet, J., Rios-Ibarra, C. P., Bocangel Gamarra, E. L., Lefeber, T., & Najdeska, T. (2021). Characterizing the cytotoxic effects and

several antimicrobial phytocompounds of Argemone mexicana. *Plos One*, *16*(4), e0249704.

Pastushenko, I., & Blanpain, C. (2019). EMT transition states during tumor progression and metastasis. *Trends in Cell Biology*, *29*(3), 212-226.

Patel Rajesh, M., & Patel Natvar, J. (2011). In vitro antioxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging methods. *Journal of Advanced Pharmacy Education & Research*, *1*, 52-68.

Pottier, C., Fresnais, M., Gilon, M., Jérusalem, G., Longuespée, R., & Sounni, N. E. (2020). Tyrosine kinase inhibitors in cancer: breakthrough and challenges of targeted therapy. *Cancers*, *12*(3), 731.

Pourmorad, F., Hosseinimehr, S. J., & Shahabimajd, N. (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African Journal of Biotechnology*, *5*(11).

Qin, T., Barron, L., Xia, L., Huang, H., Villarreal, M. M., Zwaagstra, J., ... & Sun, L. Z. (2017). Correction: A novel highly potent trivalent TGF-β receptor trap inhibits early-stage tumorigenesis and tumor cell invasion in murine Pten-deficient prostate glands. *Oncotarget*, *8*(34), 57905.

Qureshi, H., Asif, S., Ahmed, H., Al-Kahtani, H. A., & Hayat, K. (2016). Chemical composition and medicinal significance of Fagonia cretica: a review. *Natural Product Research*, *30*(6), 625-639.

Ramadori, G., & Cameron, S. (2010). Effects of systemic chemotherapy on the liver. *Annals of Hepatology*, *9*(2), 133-143.

Ramirez-Erosa, I., Huang, Y., Hickie, R. A., Sutherland, R. G., & Barl, B. (2007). Xanthatin and xanthinosin from the burs of Xanthium strumarium L. as potential anticancer agents. *Canadian journal of Physiology and Pharmacology*, *85*(11), 1160-1172.

Sadeghi Najafabadi, S. A., Bolhassani, A., & Aghasadeghi, M. R. (2022). Tumor cell-based vaccine: an effective strategy for eradication of cancer cells. *Immunotherapy*, *14*(8), 639-654.

Sahreen, S., Khan, M. R., Khan, R. A., & Shah, N. A. (2013). Estimation of flavoniods, antimicrobial, antitumor and anticancer activity of Carissa opaca fruits. *BMC Complementary and Alternative Medicine*, *13*(1), 1-7.

Sakanaka, S., Tachibana, Y., & Okada, Y. (2005). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). *Food Chemistry*, *89*(4), 569-575.

Sandoval, J., & Esteller, M. (2012). Cancer epigenomics: beyond genomics. *Current Opinion in Genetics & Development*, *22*(1), 50-55.

Satpute, R., Bhattacharya, R., Kashyap, R. S., Purohit, H. J., Deopujari, J. Y., Taori, G. M., & Daginawala, H. F. (2012). Antioxidant potential of Fagonia arabica against the chemical ischemia-induced in PC12 cells. *Iranian Journal of Pharmaceutical Research: IJPR*, *11*(1), 303.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*(6), 1101-1108.

Sengupta, S., Jana, S., & Bhattacharyya, A. (2014). TGF-β-Smad2 dependent activation of CDC 25A plays an important role in cell proliferation through NFAT activation in metastatic breast cancer cells. *Cellular Signalling*, *26*(2), 240-252.

Sharma, N., & Vijayvergia, R. (2013). A Review on Digera muricata (L.) Mart-a great versatile medicinal plant. *International Journal of Pharmaceutical Sciences Review and Research, 20*(1), 114-119.

Shinto, O., Yashiro, M., Kawajiri, H., Shimizu, K., Shimizu, T., Miwa, A., & Hirakawa, K. (2010). Inhibitory effect of a TGFβ receptor type-I inhibitor, Ki26894, on invasiveness of scirrhous gastric cancer cells. *British Journal of Cancer*, *102*(5), 844-851.

Singh, A., Jain, D., Upadhyay, M. K., Khandelwal, N., & Verma, H. N. (2010). Green synthesis of silver nanoparticles using Argemone mexicana leaf extract and evaluation of their antimicrobial activities. *Digest Journal of Nanomaterials and Biostructures, 5*(2), 483-489.

Singh, P., Singh, R. S., Rani, A., & Bast, F. (2016). Homology modeling of chemokine CCR7, molecular docking, and in vitro studies evidenced plausible immunotherapeutic anticancer natural compounds. *Medicinal Chemistry Research*, *25*(10), 2410-2424.

Srivastava, S., Somasagara, R. R., Hegde, M., Nishana, M., Tadi, S. K., Srivastava, M., ... & Raghavan, S. C. (2016). Quercetin, a natural flavonoid interacts with DNA, arrests cell cycle and causes tumor regression by activating mitochondrial pathway of apoptosis. *Scientific Reports*, *6*(1), 1-13.

Stevenson, J. P., Kindler, H. L., Papasavvas, E., Sun, J., Jacobs-Small, M., Hull, J. & Albelda, S. M. (2013). Immunological effects of the TGFβ-blocking antibody GC1008 in malignant pleural mesothelioma patients. *Oncoimmunology*, *2*(8), e26218.

Street, R. A., Sidana, J., & Prinsloo, G. (2013). Cichorium intybus: Traditional uses, phytochemistry, pharmacology, and toxicology. *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209-249.

Tabassam, S., Anwar, M. A., Gulfraz, M., Arshad, M., Sabitaliyevich, U. Y., Nurmurzayevich, S. B., & Ahmad, M. S. (2019). Bioactivity evaluation and HPLC UV-VIS based quantification of antioxidant secondary metabolites from extract and fractions of Bistorta amplexicaulis rhizome. *Cellular and Molecular Biology*, *65*(1), 19-26.

Talukdar, A. D., Choudhury, M. D., Chakraborty, M., & Dutta, B. K. (2010). Phytochemical screening and TLC profiling of plant extracts of Cyathea gigantea (Wall. Ex. Hook.) Haltt. and Cyathea brunoniana. Wall. ex. Hook (Cl. & Bak.). *Assam University Journal of Science and Technology*, *5*(1), 70-74.

Tan, A. R., Alexe, G., & Reiss, M. (2009). Transforming growth factor-β signaling: emerging stem cell target in metastatic breast cancer?. *Breast Cancer Research and Treatment*, *115*(3), 453-495.

Tang, D. S., Zhang, L., Chen, H. L., Liang, Y. R., Lu, J. L., Liang, H. L., & Zheng, X. Q. (2007). Extraction and purification of solanesol from tobacco:(I). Extraction and silica gel column chromatography separation of solanesol. *Separation and Purification Technology*, *56*(3), 291-295.

Tauriello, D. V., Palomo-Ponce, S., Stork, D., Berenguer-Llergo, A., Badia-Ramentol, J., Iglesias, M., ... & Batlle, E. (2018). TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature*, *554*(7693), 538-543.

Tauriello, D. V., Sancho, E., & Batlle, E. (2022). Overcoming TGFβ-mediated immune evasion in cancer. *Nature Reviews Cancer*, *22*(1), 25-44.

Tewari, D., Priya, A., Bishayee, A., & Bishayee, A. (2022). Targeting transforming growth factor-β signalling for cancer prevention and intervention: Recent advances in developing small molecules of natural origin. *Clinical and Translational Medicine*, *12*(4), e795.

Thomas, E., Gopalakrishnan, V., Somasagara, R. R., Choudhary, B., & Raghavan, S. C. (2016). Extract of vernonia condensata, inhibits tumor progression and improves survival of tumor-allograft bearing mouse. *Scientific Reports*, *6*(1), 1-12.

Vaishnav, K., George, L. B., & Highland, H. N. (2015). Antitumour activity of Xanthium strumarium L. on human cervical cancer HeLa cells. *Journal of Cancer and Tumor International*, *2*, 1-13.

Vander Ark, A., Cao, J., & Li, X. (2018). TGF-β receptors: In and beyond TGF-β signaling. *Cellular Signalling*, *52*, 112-120.

Wang, H., Zhang, H., Tang, L., Chen, H., Wu, C., Zhao, M., ... & Liu, G. (2013). Resveratrol inhibits TGF-β1-induced epithelial-to-mesenchymal transition

and suppresses lung cancer invasion and metastasis. *Toxicology*, *303*, 139-146.

Wang, S. D., Chen, B. C., Kao, S. T., Liu, C. J., & Yeh, C. C. (2014). Genistein inhibits tumor invasion by suppressing multiple signal transduction pathways in human hepatocellular carcinoma cells. *BMC Complementary and Alternative Medicine*, *14*(1), 1-12.

Xu, J., Acharya, S., Sahin, O., Zhang, Q., Saito, Y., Yao, J., ... & Yu, D. (2015). 14-3-3ζ turns TGF-β's function from tumor suppressor to metastasis promoter in breast cancer by contextual changes of Smad partners from p53 to Gli2. *Cancer Cell*, *27*(2), 177-192.

Xu, X., Chen, R., Li, Z., Huang, N., Wu, X., Li, S. & Wu, S. (2015). MicroRNA-490-3p inhibits colorectal cancer metastasis by targeting TGFβR1. *BMC Cancer*, *15*(1), 1-10.

Yang, C., Mai, Z., Liu, C., Yin, S., Cai, Y., & Xia, C. (2022). Natural Products in Preventing Tumor Drug Resistance and Related Signaling Pathways. *Molecules*, *27*(11), 3513.

Yingling, J. M., Blanchard, K. L. & Sawyer, J. S. (2004). Development of TGF-beta signaling inhibitors for cancer therapy. *Nature Reviews Drug Discovery*, 3: 1011-1022.

Yu, J. R., Tai, Y., Jin, Y., Hammell, M. C., Wilkinson, J. E., Roe, J. S., ... & Van Aelst, L. (2015). TGF-β/Smad signaling through DOCK4 facilitates lung adenocarcinoma metastasis. *Genes & Development*, *29*(3), 250-261.

Zhang, J., Li, C., Zheng, Y., Lin, Z., Zhang, Y., & Zhang, Z. (2017). Inhibition of angiogenesis by arsenic trioxide via TSP-1–TGF-β1-CTGF–VEGF functional module in rheumatoid arthritis. *Oncotarget*, *8*(43), 73529.

Zhang, Q., Chen, L., Helfand, B. T., Zhu, L. J., Kozlowski, J., Minn, A., ... & Lee, C. (2011). Transforming Growth Factor-β-induced DNA methyltransferase contributes to aggressive prostate cancer phenotypes and predicts biochemical recurrence after radical prostatectomy. *PLoS One*, *6*, e25168.

Zhang, Q., Hou, X., Evans, B. J., VanBlaricom, J. L., Weroha, S. J., & Cliby, W. A. (2018). LY2157299 monohydrate, a TGF-βR1 inhibitor, suppresses tumor growth and ascites development in ovarian cancer. *Cancers*, *10*(8), 260.

Zhang, Y. E. (2009). Non-Smad pathways in TGF-β signaling. *Cell Research*, *19*(1), 128-139.

Zhao, G., Yin, Z., & Dong, J. (2009). Antiviral efficacy against hepatitis B virus replication of oleuropein isolated from Jasminum officinale L. var. grandiflorum. *Journal of Ethnopharmacology*, *125*(2), 265-268.

Zhao, X., Wang, K., Hu, F., Qian, C., Guan, H., Feng, K., ... & Chen, Z. (2015). MicroRNA-101 protects cardiac fibroblasts from hypoxia-induced apoptosis via inhibition of the TGF-β signaling pathway. *The International Journal of Biochemistry & Cell biology*, *65*, 155-164.

Zheng, B., Zheng, Y., Zhang, N., Zhang, Y., & Zheng, B. (2022). Rhoifolin from Plumula Nelumbinis exhibits anti-cancer effects in pancreatic cancer via AKT/JNK signaling pathways. *Scientific Reports*, *12*(1), 1-14.

Zia-Ul-Haq, M., Riaz, M., De Feo, V., Jaafar, H. Z., & Moga, M. (2014). Rubus fruticosus L.: constituents, biological activities and health related uses. *Molecules*, *19*(8), 10998-11029.