

**RECEPTOR-INTERACTING PROTEIN KINASE 3, A NECROPTOSIS PATHWAY  
GENE AS A CANDIDATE GENE TO TARGET BREAST CANCER**

**PROJECT WORK**

Submitted in partial fulfilment of the requirements for the award of  
Bachelor of Technology degree in Biotechnology

By

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**DEPARTMENT OF BIOTECHNOLOGY  
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

**SATHYABAMA**

**INSTITUTE OF SCIENCE AND TECHNOLOGY  
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

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**MAY - 2023**



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## DEPARTMENT OF BIOTECHNOLOGY

### BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **VISHWANATHAN J (39230058)** who carried out Project Phase-1 entitled "Receptor-interacting protein kinase 3, a necroptosis pathway gene as a candidate gene to target breast cancer" under my supervision from January 2023 to April 2023.

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## DECLARATION

I, Viswanathan. J (39230058), hereby declare that the Project Report entitled “**Receptor-interacting protein kinase 3, a necroptosis pathway gene as a candidate gene to target breast cancer**” done by me under the guidance of Dr. Usha Nandini and Dr. RP. Oviya at the Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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## TABLE OF CONTENTS

| S.NO      | CONTENTS                                                               | PAGE NO.  |
|-----------|------------------------------------------------------------------------|-----------|
|           | <b>ABSTRACT</b>                                                        |           |
|           | List of Abbreviations                                                  |           |
|           | List of Figures                                                        |           |
| <b>1.</b> | <b>INTRODUCTION</b>                                                    | <b>1</b>  |
| <b>2.</b> | <b>LITERATURE SURVEY</b>                                               | <b>6</b>  |
| 2.1       | EVADING APOPTOSIS                                                      | 6         |
| 2.2       | ROLE OF NECROPTOSIS IN CANCER                                          | 7         |
| 2.3       | APOPTOSIS AND NECROPTOSIS                                              | 8         |
| 2.4       | RIPK3 AND ITS ROLE                                                     | 9         |
| 2.5       | RIPK3 IN NECROPTOSIS                                                   | 10        |
| <b>3.</b> | <b>AIM AND SCOPE</b>                                                   | <b>5</b>  |
| <b>4.</b> | <b>MATERIALS AND METHODS</b>                                           | <b>13</b> |
| 4.1       | Insilco analysis of RIPK3 - Expression analysis of RIPK3 in pan-cancer | 13        |
| 4.2       | Selection of Natural Compounds                                         | 15        |
| 4.3       | Molecular Screening by Docking                                         | 28        |
| 4.4       | Mammalian cell culture                                                 | 31        |
| 4.5       | Preparation of Drug                                                    | 32        |

|                                                            |           |
|------------------------------------------------------------|-----------|
| 4.6 MTT ASSAY                                              | 33        |
| 4.7 Clonogenic Assay                                       | 34        |
| 4.8 Total RNA Isolation                                    | 36        |
| <b>5. RESULT AND DISCUSSION</b>                            | <b>42</b> |
| 5.1 Insilco analysis of RIPK3                              | 42        |
| 5.2 Selection of Natural compounds                         | 51        |
| 5.3 Preparation of RIPK3 3D structure for docking          | 51        |
| 5.4 Molecular docking of RIPK3 with the selected compounds | 52        |
| 5.5 <i>In-vitro</i> Cell Culture                           | 54        |
| 5.6 Cell Viability Assay                                   | 54        |
| 5.7 Clonogenicity observation                              | 56        |
| 5.8 Gene Expression on the Cancer                          | 57        |
| 5.9 Nanoparticle enhanced drug activity                    | 63        |
| <b>6. SUMMARY AND CONCLUSION</b>                           | <b>66</b> |
| <b>7. REFERENCES</b>                                       | <b>68</b> |
| <b>8. ANNEXURE</b>                                         | <b>72</b> |

## ABSTRACT

Cancer is a group of diseases that cause cells in the body to change and spread out of control. Most types of cancer cells eventually form a lump or mass called a tumor, and are named after the part of the body where the tumor originates. Breast cancer is a heterogeneous disease. It is the commonest malignancy among women globally. One in every four cancer patients among women had breast cancer in 2020 globally, as per the data from the International Agency for Research on Cancer. Most breast cancers begin either in the breast tissue made up of glands for milk production, called lobules, or in the ducts that connect the lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissues. The breast cancer is majorly treated by the chemotherapies. A major factor responsible for treatment failure in breast cancer is the development of resistance to commonly used chemotherapeutic drugs leading to disease relapse. Apoptosis resistance is to a large extent a major obstacle leading to chemotherapy failure during cancer treatment. Bypassing the apoptotic pathway to induce cancer cell death is considered to be a promising approach to overcoming this problem. Resistance to caspase-dependent apoptosis is often responsible for treatment failures in cancer. Finding novel therapeutic strategies that can activate alternative cell death programs appears to be appealing. A growing understanding of the molecular events triggering necroptosis helped to examine its implication in cancer development and to define new therapeutic strategies. Necroptosis is a programmed lytic cell death pathway, deregulation of which is linked to various inflammatory disorders. Escape from programmed cell death and inflammation play a significant role in cancer, and therefore, investigating the role of necroptosis in cancer has been of high interest. The early studies shown that Receptor-Interacting Protein 3 kinase (RIPK3) plays a major role in the necroptosis. This project aims to use the receptor-interacting protein kinase 3 to induce the necroptosis which targets the breast cancer.

**Keywords:** RIPK3, Apoptosis, Necroptosis, Breast cancer

## **List of Abbreviations**

Deoxyribonucleic acid (DNA)

The receptor interacting protein kinase 3 (RIPK3)

Receptor-interacting protein kinase 1 (RIPK1)

Mixed lineage kinase domain-like protein (MLKL)

Inhibitors of apoptosis proteins (IAPs)

Tumor necrosis factor (TNF)

B-Cell Leukemia/Lymphoma 2 (BCL2)

Bcl-2 Associated X protein (BAX)

Tumor protein p53 (p53)

Borane (BH<sub>3</sub>)

Deoxyadenosine monophosphate (DAMP)

RIP homotypic interaction motif (RHIMS)

False discovery rate (FDR)

The Cancer Genome Atlas Program (TCGA)

Clinical Proteomic Tumor Analysis Consortium (CPTAC)

Plating efficiency (PE)

Survival fraction (SF)

Doubling time (DT)

Gene Expression Profiling Interactive Analysis (GEPIA)

iGeneric Evolutionary Method Docking (iGEM)

Scanning Electron Microscopy (SEM)

Energy Dispersive X-Ray Analysis (EDX)

Polymerase Chain Reaction (PCR)

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Ultraviolet (UV)

Tris-borate-EDTA (TBE)

Tris-acetate-EDTA (TAE)

Carbon dioxide (CO<sub>2</sub>)

Eidetic acid and ethylenediaminetetraacetic acid (EDTA)

Dulbecco's Modified Eagle Medium (DMEM)

Dimethyl sulfoxide (DMSO)

Phosphate buffered saline (PBS)

## LIST OF FIGURES

| <b>FIGURE NO.</b> | <b>CONTENTS</b>                                                                                | <b>PAGE NO.</b> |
|-------------------|------------------------------------------------------------------------------------------------|-----------------|
| 1.1               | Stage of Tumor development                                                                     | 1               |
| 1.2               | A dividing breast cancer cell                                                                  | 2               |
| 5.1               | Fig: Expression of RIPK3 across TCGA cancers                                                   | 42              |
| 5.2               | Fig: Expression of RIPK3 across CPTAC cancer                                                   | 44              |
| 5.3               | RIPK3 expression validation by boxplot analysis                                                | 47              |
| 5.4               | Methylation status of RIPK3 in pan cancer                                                      | 48              |
| 5.5               | Survival analysis for Pan cancer                                                               | 50              |
| 5.6               | Ramachandran Plot of RIPK3                                                                     | 51              |
| 5.7               | Visualization of RIPK3 and Diosgenin                                                           | 52              |
| 5.8               | Effect of treatment on cytotoxicity                                                            | 55              |
| 5.9               | Growth Curve of cells                                                                          | 55              |
| 5.10              | Colonies formation of cells                                                                    | 56              |
| 5.11              | Effect of diosgenin on the cell lines                                                          | 57              |
| 5.12              | 1% Agarose Gel Electrophoresis of total RNA isolated from cells lines                          | 58              |
| 5.13              | 2% agarose gel electrophoresis showing PCR amplification of 6 genes run along with DNA ladder. | 58              |
| 5.14              | qRT-PCR based gene expression                                                                  | 60              |
| 5.15              | Morphology images of Ag and diosgenin by SEM analysis                                          | 63              |
| 5.16              | EDX pattern of Ag and diosgenin composite                                                      | 64              |
| 5.17              | Effect of Ag integrated diosgenin treated cell lines                                           | 65              |

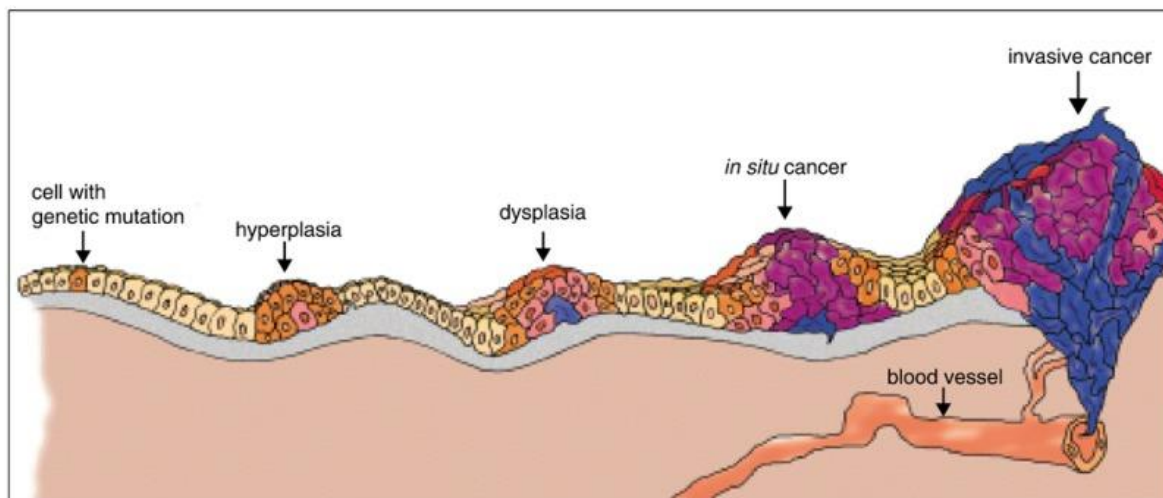
## LIST OF TABLES

| TABLE NO. | CONTENTS                                                             | PAGE NO. |
|-----------|----------------------------------------------------------------------|----------|
| 4.1       | List of natural compounds                                            | 16       |
| 4.2       | List of cell lines                                                   | 32       |
| 4.3       | Details of the primer used for PCR Amplification                     | 47       |
| 4.4       | Thermal Cycling parameters for samples                               | 48       |
| 4.5       | List of primers used for the q RT-PCR                                | 50       |
| 5.1       | Expression of RIPK3 across the TCGA cancers (Normal vs Tumor sample) | 53       |
| 5.2       | Expression of RIPK3 across CPTAG cancer samples (Normal vs tumor)    | 55       |
| 5.3       | Docking Validation of PyRx and iGem                                  | 63       |
| 5.4       | Gene expression analysis of qRT-PCR                                  | 71       |

## 1. INTRODUCTION

Cancer is a condition when a few of the body's cells grow out of control and spread to other bodily regions. In the millions of cells that make up the human body, cancer can develop practically anywhere. There are more than 100 different forms of cancer which are categorized based on the tissue or organ that is afflicted human body. Cancer, a multifactorial malady involves multifarious changes in the genome due to interactions with the individual's environment. When a cell rebels against the normal constraints on cell division and starts to proliferate according to its own plan, cancer develops.

The first, ancestral cell and all its progeny divide improperly, and all their cells exhibit this abnormal proliferation. Group or mass of cells formed because of these abnormal cells are called tumor which will remain within the originated tissue or it may invade into the nearby other organ tissue, the invasion is called malignant tumor which will results in the formation of new tumors by cells expelling on blood or lymph. The development of tumor undergoes different stages, commonly it undergoes four mutations but the mutation numbers will change accordingly to the cancer types.



**Fig 1.1: Stage of Tumor development**

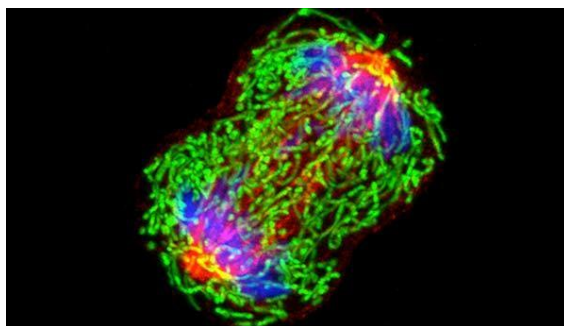
(Credits: <https://www.ncbi.nlm.nih.gov/books/NBK20362/>)

Approximately 12,00,000 new cancer cases are diagnosed in India every year. As per the latest National Cancer Registry data, one in eight men and one in nine women in India will develop some form of cancer. The incidence of cancer and cancer types are influenced by many factors such as age, sex, race, local environmental factors, diet, and genetics. In males, lung followed by oral cavity and throat cancers are the most common, while cervical and breast cancers are the commonest ones diagnosed in females in India.

### **1.1 BREAST CANCER**

Breast cancer is a malignant tumor that originates from cells in the breast. It is a heterogeneous disease. The commonest malignancy among women globally. One in every four cancer patients among women had breast cancer in 2020 globally, as per the data from the International Agency for Research on Cancer. In terms of mortality, 37.2 per cent of women died from this disease in India in 2020

Lobules (milk-producing glands), ducts and stroma make up the majority of the female breast (fatty tissue and connective tissue surrounding the ducts and lobules, as well as blood vessels and lymphatic vessels). The cells that line the ducts are where most breast tumors start (ductal cancers). Some (lobular cancers) start in the cells that line the lobules, while others start in other tissues. Breast cancer arises in the lining cells (epithelium) of the ducts (85%) or lobules (15%) in the glandular tissue of the breast. Genetic mutations, specifically BRCA 1 and 2, account for about 10% of breast cancers. A history of ductal carcinoma in situ, a high body mass index (BMI), a first child at a young age or nulliparity, early menarche (before age 13), a late menopause, and usage of postmenopausal hormone therapy are additional known risk factors.(Watkins, 2019). Breast cancer represents a set of highly heterogeneous diseases mainly classified on the basis of estrogen (ER) and progesterone receptor (PR) and HER2 status. Triple negative breast cancers (TNBCs), accounting for 15-20% of breast malignancies are phenotypically defined by the lack of expression of hormone receptors and HER2.



***Fig 1.2: A dividing breast cancer cell***

(Credit: National Cancer Institute / Univ. of Pittsburgh Cancer Institute)

Breast cancer is one of the major causes of cancer related deaths in women worldwide. A major factor responsible for treatment failure in breast cancer is the development of resistance to commonly used chemotherapeutic drugs leading to disease relapse. These therapies will activate the signaling pathway of which will trigger the apoptosis. Apoptosis is a cellular suicide program that organisms have evolved to eliminate unnecessary or unhealthy cells from the body in the course of development or following cellular stress. Chemotherapeutic agents are used primarily as adjuvant or neoadjuvant based approaches.

Several studies on breast cancer show that there is dysregulation of apoptotic program leading to escape from cell death. The evasion and resistance to apoptosis are considered to be an indisputable hallmark of cancer. Cancer cells experience ongoing stress, such as oncogenic stress, genomic instability, and cellular hypoxia, in contrast to normal cells. The intrinsic mechanism of apoptosis would ordinarily be initiated in response to such apoptotic stimuli. However, by blocking the apoptotic pathways, cancer cells can frequently avoid this biological reaction.

Apoptosis resistance mainly arises due to up regulation of anti-apoptotic factors like inhibitors of apoptosis proteins (IAPs), overexpression of Bcl2 and or downregulation of proapoptotic factors like FAS, FASL and caspase8 in cancer cells. Transcriptional, translational, and post-translational modifications of apoptotic pathways are possible in cancer cells. In some cases, the cancer cell can escape apoptosis by increasing or

decreasing the apoptotic genes and it can change the function of protein by post-transcriptional modifications such as phosphorylation.

So due to their ability to suppress the apoptosis process of programmed death of cells the therapies become effortless and it increases probability of recurrence of cancer on the same tissue or in the other parts of organ in the human body.

To combat the cancer cells which are showing resistant to the apoptosis process of programmed death cell, inducing necroptosis has been suggested as an alternative method of treatment. Necroptosis has been suggested to play a pivotal role in the regulation of cancer biology, including oncogenesis, cancer metastasis, cancer immunity, and cancer subtypes. Necroptosis is a programmed, caspase-independent cell death that is morphologically similar to necrosis, which can be executed by the receptor interacting protein kinase 3 (RIPK3) and with mixed lineage kinase domain-like protein (MLKL). Necroptosis was originally solely used to refer to non-apoptotic death receptor-initiated cell death; however, it has since been expanded to refer to any cell death process "that critically requires on MLKL and on the kinase activity of RIPK1 (in some situations) and RIPK3."

Necroptosis, as compared to apoptosis, causes inflammatory reactions by releasing molecular patterns linked to damage. The potential immunogenicity of necroptosis and its ability to kill cancer cells are two attractive characteristics for this type of cell death to be seen as a new therapeutic approach. The necroptotic pathway uses components that are different from apoptotic pathway, cancer cells that are resistant to apoptosis agents may be sensitive to necroptosis inducers. In other words, necroptosis can kill cancer cells when caspases are inhibited or damaged, whereas apoptosis requires caspase activation. According to recent research, tumors experience necroptosis in vivo, and this process may inhibit the growth and spread of cancer.

RIPK3 is an essential player in the necroptosis. RIPK3 is a serine/threonine-protein kinase, which stands for receptor-interacting protein kinase 3. The RIPK3 gene in humans can be found at chromosome 14q12. It spans roughly 40 kb of genomic DNA and has 10 exons. RIPK3 is a crucial mediator of infection-induced necroptosis, a type of programmed necrosis, and inflammatory cytokines (such TNF-tumor necrosis factor). The RIPK3

protein is characterized by an N-terminal kinase domain, with which it phosphorylates itself and other substrates, and a C-terminal domain that contains a receptor-interacting protein.

By interacting with and/or phosphorylating the essential regulators of the relevant signaling pathways, RIPK3 signaling is also involved in the control of apoptosis, cytokine/chemokine synthesis, mitochondrial metabolism, autophagy, and cell proliferation. It controls the signaling of TNF-tumor necrosis factor family. RIP3-dependent plasma membrane localization of MLKL is necessary for programmed necrotic cell death, and leads to plasma membrane disruption either indirectly or directly. By means of phosphorylating the proteins in the cell cycle the RIPK3 controls the cell cycle regulation. The majority of early investigations showed that RIPK3 signaling causes the generation of extrinsic cytokines and chemokines that are necroptosis-dependent.

## 2. LITERATURE SURVEY

### 2.1 EVADING APOPTOSIS

In order to maintain tissue homeostasis, apoptosis is a form of programmed cell death that eliminates undesired, unnecessary, and damaged cells(Kerr *et al.*, 1972). Deregulation of apoptosis is involved in the pathogenesis of several diseases such as cancer, which is characterized by accumulation of cells exhibiting insufficient engagement of the apoptotic machinery and evasion of apoptosis(Sharma *et al.*, 2019). One of the characteristics of cancer is the evasion of cell death(Hanahan and Weinberg, 2011). The intrinsic pathway of apoptosis and the extrinsic pathway of apoptosis are the two main processes that cause apoptosis. Both processes result in the activation of "caspases," or cysteine aspartyl-specific proteases, which are the apoptosis's final effectors and break a number of proteins to cause cell death(Riedl and Shi, 2004). The primary regulators of apoptosis are proteins belonging to a group known as the Bcl-2 family.

The BCL-2 family of proteins, which are broadly categorized as either anti-apoptotic or pro-apoptotic, are the main regulators of the intrinsic pathway of apoptosis. The release of pro-apoptotic BH3-only "activators" (BCL-2 interacting mediator of cell death (BIM), P53-upregulated modulator of apoptosis) triggers the intrinsic route of apoptosis. Antiapoptotic BCL-2 family protein overexpression is frequently linked to poor prognosis, recurrence, and resistance to cancer treatments (Emily *et al.*, 2001). The protein known as P53, sometimes known as "The Guardian of the Genome," is very significant. Its various tasks include detecting DNA damage, chromosome abnormalities, and stopping the cell cycle to start a repair process; if a repair cannot be completed, apoptosis is caused. By boosting the production of the pro-apoptotic Bcl-2 Associated X protein (Bax), P53 causes apoptosis. The most common method of cancer cells evading the apoptosis death is by loss of the apoptosis gatekeeper P53. Most of the human cancer cells will have the mutated or missing P53 protein which leads to the evasion of the cancer cells from the apoptosis(Enderling and Hahnfeldt, 2011).

Another way of evading the process is by making transcriptional, translational, and post-translational modifications this involves gene deletion, DNA methylation-mediated gene silencing, gene copy number amplification, and the activation (or inactivation) of transcription factors that affect the expression of apoptotic regulators (Riedl and Shi, 2004). The chemotherapies are becoming less effective for treating the cancer cells because of their ability to evade the apoptosis process.

## **2.2 ROLE OF NECROPTOSIS IN CANCER**

Necroptosis is a controlled necrotic cell death mode that is caspase-independent mediated by Receptor-Interacting Protein 3 RIP3, and Mixed Lineage Kinase Domain-Like (MLKL). Necroptosis has been demonstrated to have significant roles in human disorders such cancer, infections, and reperfusion injury in recent years (Karlowitz and van Wijk, 2021). Necrosis is considered as an accidental death resulting from an overwhelming cytotoxic insult, and does not require specific molecular events in order for it to occur. In cancer treatment, necroptosis acts as a different method of overcoming apoptosis resistance and may activate and intensify antitumor immunity.

Due to the inactivation of apoptotic signaling pathways, certain cancer cells are resistant to these treatments. To combat cancer cells that are resistant to apoptosis, inducing necroptosis has been suggested as an alternative method of treatment (Gong, Fan, Luo, Yang, Huang, Fan, Cheng, Jin, Ni, Yu, *et al.*, 2019). The necroptotic cell death process is controlled by the post translational modifications and phosphorylation of the corresponding regulators in the signaling pathway which will results in the necroptosis. For example, in order to grow and invade the body, cancer cells subvert antitumoral immune responses. Induction of immunogenic cell death is an attractive approach to re-activate or enhance anti-tumor immune responses. Given that necroptosis induces the release of DAMPs, which initiate adaptive immunity, it became obvious to investigate its role in anticancer immune responses. A number of studies have shown that inhibition of caspases, in particular inhibition of caspase-8, switched cell death induced by some cytotoxic agents to necroptosis. For instance, Poly I:C and zVAD can induce necroptosis

in some cancer cells causing the release of DAMPs, which activate immune effectors to eliminate cancer (Takemura *et al.*, 2015).

Cancer cells that are resistant to apoptosis agents may be sensitive to necroptosis inducers because the necroptotic route utilizes components that are different from the apoptotic pathway. In other words, necroptosis can kill cancer cells when caspases are blocked or ineffective, whereas apoptosis requires caspase activation. Several research teams have verified this theory (Han *et al.*, 2007). The ultimate stage of a necroptotic process is the swelling and rupture of the cell membrane, releasing damage-associated molecular patterns (DAMPs), which can elicit immune responses (Kaczmarek *et al.*, 2013a).

### **2.3 APOPTOSIS AND NECROPTOSIS**

While necroptosis is differentiated by caspase independence, it resembles and shares a portion of the extrinsic apoptotic pathway and other molecular pathways that lead to apoptosis. However, necroptosis dramatically differs in the morphological and immunological effects. Similar to necrosis, necroptosis displays morphological characteristics like cellular membrane breakdown, gradually transparent cytoplasm, and enlargement of organelles (Rosenbaum *et al.*, 2010; Vandenabeele *et al.*, 2010).

Contrarily, membrane blebbing, cellular shrinkage, nucleus fragmentation, and chromatin condensation are representative of the morphological characteristics of apoptosis. Necroptotic cells are significantly more immunogenic than apoptotic cells because the rupture of the plasma membrane in necroptotic cells causes the discharge of cell contents, which can induce the exposure of damage-associated molecular patterns (DAMPs) and produce strong inflammatory reactions (Kaczmarek *et al.*, 2013b).

Which suggests that apoptosis typically does not induce an immune response as robust as that induced by necroptosis (Pasparakis and Vandenabeele, 2015) and that necroptotic cells are markedly more immunogenic than apoptotic cells

where latent DAMPs are confined to the relatively intact plasma membrane or are encased in apoptotic bodies, and their corpses are engulfed and dissolved by phagocytes. Because necroptosis has been shown to eliminate excessive and abnormal T cells in the absence of caspase-8 (Lenardo *et al.*, 1999), which is thought to aid apoptosis by functioning as the primary machinery countering abnormal lymphocyte proliferation, necroptosis was also discovered to be involved in the maintenance of T cell homeostasis (Ch'en *et al.*, 2011).

## **2.4 RIPK3 AND ITS ROLE**

RIPK3 is a serine/threonine-protein kinase, which stands for receptor-interacting protein kinase 3 for necroptosis which is a type of necrosis. The catalytic triad (Lys50, Glu60, and Asp160 in human RIPK3) and the DFG motif, which are necessary amino acids for normal protein kinases' enzymatic activity, are conserved in RIPK3 (Moriwaki and Chan, 2017).

RIPK3 performs a variety of non-canonical tasks, such as causing the inflammasome to activate and producing cytokines, boosting mitochondrial metabolism, and producing ROS (Shlomovitz *et al.*, 2017b). By means of phosphorylating the proteins in the cell cycle the RIPK3 controls the cell cycle regulation. The majority of early investigations showed that RIPK3 signaling causes the generation of extrinsic cytokines and chemokines that are necroptosis-dependent (Moriwaki and Chan, 2017). Numerous types of cancers have been linked to the pathogenesis of RIPK3 signaling (Najafov *et al.*, 2017).

Both caspase-8-mediated cleavage and ubiquitin-mediated degradation processes regulate the amount of RIPK3 protein in cells, by means of phosphorylating the proteins in the cell cycle the RIPK3 controls the cell cycle regulation (Feng *et al.*, 2007). Numerous cytokines and chemokines are produced under the control of RIPK3 signaling. The majority of early investigations showed that RIPK3 signaling causes the generation of extrinsic cytokines and chemokines that are necroptosis-dependent (Moriwaki *et al.*, 2017). Recent studies have also demonstrated that RIPK3

induces intrinsic inflammatory signaling, which plays a role in cytokine/chemokine synthesis independent of cell death (Najjar *et al.*, 2016).

RIPK3 expression in cancer cells by DNA methylation regulators may present a chance for new anti-cancer therapies that target the activation of necroptosis in cancer (Najafov *et al.*, 2017). RIPK3 includes several phosphorylation sites, only a few of them have known biological functions, given that caspase inhibition is necessary to avoid cell death when RIPK3 is overexpressed, it is reasonable to assume that overexpressing RIPK3 can also cause apoptosis (Newton *et al.*, 2014).

## **2.5 RIPK3 IN NECROPTOSIS**

Receptor-interacting protein 3 (RIP3) kinases plays an important role in the necroptosis, Necroptosis has been shown to be generally dependent on RIPK3, which is activated following phosphorylation by the serine/threonine kinase RIP1 (Cho *et al.*, 2009). RIP3 is thought to induce a switch in cell's metabolism, leading to the increase of mitochondrial ROS production that culminates in cell death (D.-W. Zhang *et al.*, 2009). The necroptotic cell death process is controlled by the post translational modifications and phosphorylation of the corresponding regulators in the signaling pathway which will results in the necroptosis. The receptor-interacting protein 3 (RIP3), a serine/threonine kinase, is necessary for tumor necrosis factor (TNF). The discovery of the molecular mechanism underlying programmed necrosis was initiated by -induced necrotic cell death (Holler *et al.*, 2000). Research has been done that as shown how the RIPK3 performs apoptosis and necroptosis process (Shlomovitz *et al.*, 2017a).

The majority of early investigations showed that RIPK3 signaling causes the generation of extrinsic cytokines and chemokines that are necroptosis-dependent. The balance of cytokines and chemokines produced determines whether RIPK3 signaling is pro- or anti-carcinogenic, respectively (Liu *et al.*, 2021). It controls the signaling of TNF-tumor necrosis factor family, RIPK3 phosphorylation is a key for the necroptosis and it phosphorylated at multiple sites upon necroptosis induction(He *et al.*, 2009). RIPK3 through their RIP homotypic interaction motif (RHIMS) leading to the formation necrosome complex - hetero dimeric amyloid structure, which is a key signaling

factor in the necroptosis (Li *et al.*, 2012).RIPK3 forms a positive feedback circuit by which the necroptosis is induced effectively (Y. Zhang *et al.*, 2017).

RIPK3 phosphorylates MLKL, a well-known functional substrate, in necrosomes. Following its oligomerization and translocation to the plasma membrane, MLKL triggers necroptosis (Cai *et al.*, 2014). Autophagy intracellular breakdown is a process of cytosolic components are delivered to the lysosome (Mizushima, 2007). Degradation of harmed organelles and outdated proteins, as well as the preservation of cellular homeostasis, are primary functions of autophagy (White, 2015). The autophagy protein ULK1, which controls both canonical and alternative autophagy, is one of RIPK3's most recent substrates (Torii *et al.*, 2020).

### **3.AIM AND OBJECTIVE**

#### **3.1 AIM**

The main objective of this study is to use the receptor-interacting protein kinase 3 (RIPK3), a necroptosis pathway gene as a candidate gene to target breast cancer.

#### **3.2 OBJECTIVE**

- To identify RIPK3 gene expression status and its methylation.
- To identify novel small molecule inhibitor targeting RIPK3.
- To validate the target small molecule inhibitor using invitro cell culture study.

## **4.MATERIAL AND METHODS**

### ***4.1 Insilco analysis of RIPK3 - Expression analysis of RIPK3 in pan-cancer***

#### ***4.1.1 Analysis of The Cancer Genome Atlas (TCGA)***

UACLAN <http://ualcan.path.uab.edu/> was used to analyses cancer data from The Cancer Genome Atlas (TCGA). The TCGA's gene expression and clinic-pathological data are included in the UALCAN open-access internet portal (*Chandrashekar et al., 2017a*) To make it easier to assess the gene expression patterns across the TCGA cancer data, we performed a pan-cancer study to determine the expression level of RIPK3 on clinical data from 24 different forms of cancer on the TCGA (*Chandrashekar et al., 2022*). The expression of RIPK3 is examined in clinical data from normal and cancer tissues. Gene expression levels may alter depending on the type of cancer.

#### ***Analysis of Clinical Proteomic Tumor Analysis Consortium (CPTAC)***

The UALCAN database was used to analyses RIPK3 protein expression in diverse cancer types (*Xu et al., 2021*). It analyses protein expression across different types of malignancies using data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) dataset (*Chen et al., 2019*). It is conceivable to connect proteomics to cancer genes and maybe phenotypes utilizing integrated analysis of cancer proteomics data using CPTAC results (*Wu et al., 2019*). I have supplied a Pan-cancer view to analyses the expression of RIPK3, where the values of the expression generally alter between cancer kinds.

#### ***4.1.2 Methylation status of RIPK3***

Using TCGA samples, the methylation status of RIPK3 was investigated in UACLAN. UALCAN is an interactive internet program that allows for in-depth analysis of TCGA gene expression data. It shows TCGA sample data from 11 different cancer types alongside relevant clinical data. The gateway allows researchers to compare the expression of one or more genes in normal samples with cancers of various stages, grades, and subtypes (*Sathyanarayanan et al., 2020*). We can then determine which genes are most up and

down regulated in which cancers. On the y-axis, beta values were utilized to show the degree of methylation and to compare normal (N=97) and primary tumor (N=793) samples. From 0 (unmethylated) to 1 (completely methylated), the Beta value represents the degree of DNA methylation. Several beta value cut-offs have been proposed to indicate either hypermethylation [Beta value: 0.7-0.5] or hypomethylation [Beta-value: 0.3-0.25]. (<http://ualcan.path.uab.edu/>)

### **4.1.3 GEPIA analysis of RIPK3**

#### **4.1.3.1 Boxplot analysis**

GEPIA (Gene Expression Profiling Interactive Analysis) is a web-based program that provides rapid and customizable capabilities. GEPIA can be found at <http://gepia.cancer-pku.cn/>. GEPIA dynamically plots the expression profiles of a specific gene using user-defined sample choices and methodologies (Song *et al.*, 2018). For the Boxplot study of RIPK3 on various malignancies, we used GEPIA. GEPIA illustrates gene expression by stage of the clinical condition based on the TCGA clinical annotation (Petryszak *et al.*, 2016). The GEPIA box depicts the critical gene expression level at which the gene is overexpressed in cancer samples versus normal samples.

#### **4.1.3.2 Survival Analysis**

The GEPIA datasets were used to examine the overall survival rate of RIPK3 in 20 distinct forms of cancer. GEPIA analyses overall survival (OS) or disease-free survival (DFS, also known as relapse-free survival and RFS) based on gene expression. We may collect survival information on different forms of cancer based on gene expression by using the survival plot on the GEPIA (Chandrashekar *et al.*, 2017b). Primary tumor samples were separated into two groups: "High expression group" and "Low expression group." The significance of survival analyses is established by taking the Log rank 'P' values into account. If the value of  $P > 0.05$ , it is considered significant; otherwise, it is considered non-significant.

#### ***4.1.4 Statistical significance***

For all analysis, p-values, and false discovery rate (FDR) <0.05 were considered statistically significant

#### ***4.2 Selection of Natural Compounds***

The list of natural compounds was taken from the knapsackfamily an open online website [http://kanaya.naist.jp/KNApSack\\_Family/](http://kanaya.naist.jp/KNApSack_Family/)

KNApSack is a database of natural chemicals and metabolites originating from plants, fungi, and bacteria. It has over 40,000 metabolites and natural chemicals, each with its own KNApSack ID.

The metabolites were searched by the natural plant species name, and their types.

**Table 4.1: List of natural compounds**

| <b>C_ID</b>                 | <b>CAS ID</b> | <b>METABOLITE</b>        | <b>MOLECULAR FORMULA</b>                                                      | <b>MOLECULAR WEIGHT</b> | <b>ORGANISM</b>         |
|-----------------------------|---------------|--------------------------|-------------------------------------------------------------------------------|-------------------------|-------------------------|
| <u>C0000367</u><br><u>4</u> | 83-48-7       | Stigmasterol             | C <sub>29</sub> H <sub>48</sub> O                                             | 412.3705162             | <i>Cyanara scolymus</i> |
| <u>C0000010</u><br><u>7</u> | 771-51-7      | Indole-3-acetonitrile    | C <sub>10</sub> H <sub>8</sub> N <sub>2</sub>                                 | 156.0687483             | <i>Brassicaceae</i>     |
| <u>C0000010</u><br><u>7</u> | 771-51-7      | Indole-3-acetonitrile    | C <sub>10</sub> H <sub>8</sub> N <sub>2</sub>                                 | 156.0687483             | <i>Brassicaceae</i>     |
| <u>C0000011</u><br><u>2</u> | 487-89-8      | Indole-3-carboxyaldehyde | C <sub>9</sub> H <sub>7</sub> NO                                              | 145.0527639             | <i>Brassicaceae</i>     |
| <u>C0000012</u><br><u>6</u> | 5187-84-8     | Neoglucobrassicin        | C <sub>17</sub> H <sub>22</sub> N <sub>2</sub> O <sub>10</sub> S <sub>2</sub> | 478.0715864             | <i>Resedaceae</i>       |
| <u>C0000012</u><br><u>6</u> | 5187-84-8     | Neoglucobrassicin        | C <sub>17</sub> H <sub>22</sub> N <sub>2</sub> O <sub>10</sub> S <sub>2</sub> | 478.0715864             | <i>Tovariaceae</i>      |
| <u>C0000012</u><br><u>6</u> | 5187-84-8     | Neoglucobrassicin        | C <sub>17</sub> H <sub>22</sub> N <sub>2</sub> O <sub>10</sub> S <sub>2</sub> | 478.0715864             | <i>Capparidaceae</i>    |
| <u>C0000012</u><br><u>8</u> | 83327-21-3    | 4-Methoxyglucobrassicin  | C <sub>17</sub> H <sub>22</sub> N <sub>2</sub> O <sub>10</sub> S <sub>2</sub> | 478.0715864             | <i>Brassicaceae</i>     |
| <u>C0000081</u><br><u>6</u> | 127-91-3      | beta-Pinene              | C <sub>10</sub> H <sub>16</sub>                                               | 136.1252005             | <i>Proteaceae</i>       |
| <u>C0000082</u><br><u>3</u> | 138-86-3      | Limonene                 | C <sub>10</sub> H <sub>16</sub>                                               | 136.1252005             | <i>Proteaceae</i>       |
| <u>C0000085</u><br><u>3</u> | 123-35-3      | beta-Myrcene             | C <sub>10</sub> H <sub>16</sub>                                               | 136.1252005             | <i>Proteaceae</i>       |
| <u>C0000097</u><br><u>0</u> | 520-26-3      | Hesperidin               | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub>                               | 610.1897704             | <i>Rutaceae</i>         |

|                             |            |                  |              |             |                                   |
|-----------------------------|------------|------------------|--------------|-------------|-----------------------------------|
| <u>C0000129</u><br><u>3</u> | 506-24-1   | Stearolic acid   | C18H32O2     | 280.2402303 | <i>Santalaceae</i>                |
| <u>C0000143</u><br><u>5</u> | 51-67-2    | Tyramine         | C8H11NO      | 137.084064  | <i>Apiaceae</i>                   |
| <u>C0000143</u><br><u>7</u> | 29883-15-6 | Amygdalin        | C20H27NO11   | 457.1584107 | <i>Rosaceae</i>                   |
| <u>C0000146</u><br><u>5</u> | 497-77-8   | Glucocapparin    | C8H15NO9S2   | 333.0188226 | <i>Capparidaceae</i>              |
| <u>C0000148</u><br><u>6</u> | 585-95-5   | Progoitrin       | C11H19NO10S2 | 389.0450373 | <i>Peltaria alliaceae</i>         |
| <u>C0000148</u><br><u>8</u> | 3952-98-5  | Sinigrin         | C10H17NO9S2  | 359.0344726 | <i>Brassica oleraceae</i>         |
| <u>C0000148</u><br><u>8</u> | 3952-98-5  | Sinigrin         | C10H17NO9S2  | 359.0344726 | <i>Peltaria alliaceae</i>         |
| <u>C0000177</u><br><u>2</u> | 83-94-3    | Tabernanthine    | C20H26N2O    | 310.2045135 | <i>family Apocinaceae spp.</i>    |
| <u>C0000178</u><br><u>5</u> | 3371-85-5  | Voacamine        | C43H52N4O5   | 704.3937708 | <i>family Apocynaceae spp.</i>    |
| <u>C0000192</u><br><u>5</u> | 27073-72-9 | Tiliacoline      | C36H36N2O5   | 576.2624223 | <i>family Menispermaceae spp.</i> |
| <u>C0000198</u><br><u>2</u> | 24282-31-3 | beta-Skytanthine | C11H21N      | 167.1673997 | <i>family Asteraceae spp.</i>     |
| <u>C0000203</u><br><u>4</u> | 454-14-8   | Cuscohygrine     | C13H24N2O    | 224.1888634 | <i>Cyphomandra betaceae</i>       |
| <u>C0000223</u><br><u>3</u> | 2122-29-4  | Retamine         | C15H26N2O    | 250.2045135 | <i>family Fabaceae spp.</i>       |
| <u>C0000229</u><br><u>3</u> | 101-31-5   | Hyoscyamine      | C17H23NO3    | 289.1677936 | <i>Cyphomandra betaceae</i>       |

|                             |             |                                  |           |             |                                    |
|-----------------------------|-------------|----------------------------------|-----------|-------------|------------------------------------|
| <u>C0000230</u><br><u>1</u> | 135-97-7    | Pseudotropine                    | C8H15NO   | 141.1153641 | <i>Cyphomandra betaceae</i>        |
| <u>C0000230</u><br><u>4</u> | 533-08-4    | Tigloidine                       | C13H21NO2 | 223.1572289 | <i>Cyphomandra betaceae</i>        |
| <u>C0000230</u><br><u>6</u> | 120-29-6    | Tropine                          | C8H15NO   | 141.1153641 | <i>Cyphomandra betaceae</i>        |
| <u>C0000241</u><br><u>0</u> | 26296-56-0  | Toxol                            | C13H14O3  | 218.0942943 | <i>family Asteraceae spp.</i>      |
| <u>C0000241</u><br><u>1</u> | 106928-36-3 | Toxyl angelate                   | C18H20O4  | 300.1361591 | <i>family Asteraceae spp.</i>      |
| <u>C0000244</u><br><u>6</u> | 34411-93-3  | Spathelia bischromene            | C20H20O4  | 324.1361591 | <i>family Rutaceae spp.</i>        |
| <u>C0000249</u><br><u>9</u> | 92-61-5     | Scopoletin                       | C10H8O4   | 192.0422587 | <i>Aceraceae nikoense</i>          |
| <u>C0000249</u><br><u>9</u> | 92-61-5     | Scopoletin                       | C10H8O4   | 192.0422587 | <i>Pterospermum lanceaefolium</i>  |
| <u>C0000268</u><br><u>0</u> | 101392-12-5 | Turricolol E                     | C21H30O3  | 330.2194948 | <i>family Hydrophyllaceae spp.</i> |
| <u>C0000272</u><br><u>4</u> | 327-97-9    | 3-O-Caffeoylquinic acid          | C16H18O9  | 354.0950822 | <i>Apiaceae</i>                    |
| <u>C0000278</u><br><u>2</u> | 158364-86-4 | 1,3,4,5-Tetracaffeoylquinic acid | C43H36O18 | 840.1901644 | <i>family Asteraceae spp.</i>      |
| <u>C0000292</u><br><u>6</u> | 60976-49-0  | Geraniin                         | C41H28O27 | 952.0817957 | <i>Aceraceae nikoense</i>          |
| <u>C0000336</u><br><u>2</u> | 26931-94-2  | Salonitenolide                   | C15H20O4  | 264.1361591 | <i>family Asteraceae spp.</i>      |
| <u>C0000337</u><br><u>3</u> | 22621-72-3  | Tetraneurin A                    | C17H22O6  | 322.1416384 | <i>family Asteraceae spp.</i>      |

|                             |            |                        |           |             |                                   |
|-----------------------------|------------|------------------------|-----------|-------------|-----------------------------------|
| <u>C0000338</u><br><u>3</u> | 16983-23-6 | Vermeerin              | C15H20O4  | 264.1361591 | <i>family Asteraceae spp.</i>     |
| <u>C0000346</u><br><u>9</u> | 66107-37-7 | Pimelea factor P2      | C37H50O9  | 638.3454832 | <i>family Thymelaeaceae spp.</i>  |
| <u>C0000348</u><br><u>4</u> | 72963-56-5 | Spruceanol             | C20H28O2  | 300.2089301 | <i>family Euphorbiaceae spp.</i>  |
| <u>C0000348</u><br><u>7</u> | 66268-94-8 | Synaptolepis factor K1 | C36H54O8  | 614.3818687 | <i>family Thymelaeaceae spp.</i>  |
| <u>C0000367</u><br><u>1</u> | 521-03-9   | Schottenol             | C29H50O   | 414.3861662 | <i>Cactaceae spp.</i>             |
| <u>C0000367</u><br><u>2</u> | 83-46-5    | beta-Sitosterol        | C29H50O   | 414.3861662 | <i>Aceraceae nikoense</i>         |
| <u>C0000368</u><br><u>5</u> | 3877-86-9  | Cucurbitacin D         | C30H44O7  | 516.3087038 | <i>Cucurbitaceae</i>              |
| <u>C0000373</u><br><u>4</u> | 77182-69-5 | Trichilin A            | C35H46O13 | 674.2938416 | <i>family Meliaceae spp.</i>      |
| <u>C0000456</u><br><u>5</u> | 520-18-3   | Kaempferol             | C15H10O6  | 286.0477381 | <i>Pterospermum lanceaefolium</i> |
| <u>C0000456</u><br><u>5</u> | 520-18-3   | Kaempferol             | C15H10O6  | 286.0477381 | <i>Apocynaceae</i>                |
| <u>C0000456</u><br><u>5</u> | 520-18-3   | Kaempferol             | C15H10O6  | 286.0477381 | <i>Brassicaceae</i>               |
| <u>C0000456</u><br><u>5</u> | 520-18-3   | Kaempferol             | C15H10O6  | 286.0477381 | <i>Dilleniaceae</i>               |
| <u>C0000456</u><br><u>5</u> | 520-18-3   | Kaempferol             | C15H10O6  | 286.0477381 | <i>Ranunculaceae</i>              |
| <u>C0000463</u><br><u>1</u> | 117-39-5   | Quercetin              | C15H10O7  | 302.0426527 | <i>Aceraceae nikoense</i>         |
| <u>C0000463</u><br><u>1</u> | 117-39-5   | Quercetin              | C15H10O7  | 302.0426527 | <i>Solanaceae</i>                 |

|                             |            |                                   |              |             |                                    |
|-----------------------------|------------|-----------------------------------|--------------|-------------|------------------------------------|
| <u>C0000463</u><br><u>1</u> | 117-39-5   | Quercetin                         | C15H10O7     | 302.0426527 | <i>Rhamnaceae</i>                  |
| <u>C0000463</u><br><u>1</u> | 117-39-5   | Quercetin                         | C15H10O7     | 302.0426527 | <i>Passifloraceae</i>              |
| <u>C0000513</u><br><u>7</u> | 23627-87-4 | Trifolin                          | C21H20O11    | 448.1005615 | <i>Aceraceae negundo</i>           |
| <u>C0000537</u><br><u>2</u> | 482-36-0   | Quercetin-3-beta-galactoside      | C21H20O12    | 464.0954761 | <i>Aceraceae negundo</i>           |
| <u>C0000537</u><br><u>3</u> | 482-35-9   | Quercetin-3-O-glucoside           | C21H20O12    | 464.0954761 | <i>Aceraceae negundo</i>           |
| <u>C0000537</u><br><u>4</u> | 522-12-3   | Quercitrin                        | C21H20O11    | 448.1005615 | <i>Pterospermum lanceaefolium</i>  |
| <u>C0000537</u><br><u>4</u> | 522-12-3   | Quercitrin                        | C21H20O11    | 448.1005615 | <i>Aceraceae nikoense</i>          |
| <u>C0000552</u><br><u>4</u> | 6743-92-6  | Isorhamnetin 3-galactoside        | C22H22O12    | 478.1111262 | <i>Aceraceae negundo</i>           |
| <u>C0000620</u><br><u>6</u> | 35450-86-3 | Lutonarin                         | C27H30O16    | 610.1533849 | <i>Lemnaceae</i>                   |
| <u>C0000661</u><br><u>4</u> | 13306-05-3 | Cyanidin                          | C15H11O6+    | 287.0555631 | <i>Aceraceae macrophyllum</i>      |
| <u>C0000666</u><br><u>8</u> | 64963-54-8 | Cyanidin 3,3'-diglucoside         | C27H31O16    | 611.16121   | <i>Bromeliaceae</i>                |
| <u>C0000673</u><br><u>7</u> | 67286-40-2 | Malvidin 3-diglucoside            | C29H35O17    | 655.1874247 | <i>Cyphomandra betaceae</i> Sendt. |
| <u>C0000758</u><br><u>6</u> | 19041-09-9 | Gluconapin                        | C11H19NO9S2  | 373.0501227 | <i>Peltaria alliaceae</i>          |
| <u>C0000782</u><br><u>9</u> |            | 2-Methyl-2-propenyl glucosinolate | C11H19NO9S2  | 373.0501227 | <i>Peltaria alliaceae</i>          |
| <u>C0000786</u><br><u>4</u> | 19237-18-4 | Epiprogoitrin                     | C11H19NO10S2 | 389.0450373 | <i>Peltaria alliaceae</i>          |
| <u>C0001115</u><br><u>3</u> | 476-66-4   | Ellagic acid                      | C14H6O8      | 302.0062672 | <i>Aceraceae nikoense</i>          |

|                             |             |                               |           |             |                                                       |
|-----------------------------|-------------|-------------------------------|-----------|-------------|-------------------------------------------------------|
| <u>C0001930</u><br><u>8</u> | 474-58-8    | beta-Sitosterol glucoside     | C35H60O6  | 576.4389897 | <i>Aceraceae nikoense</i>                             |
| <u>C0002115</u><br><u>7</u> | 28587-45-3  | Tetraneurin B                 | C17H22O6  | 322.1416384 | <i>family Asteraceae spp.</i>                         |
| <u>C0002179</u><br><u>6</u> | 115873-01-3 | Ceratopicanol                 | C15H26O   | 222.1983655 | <i>Ceratocystis piceae</i>                            |
| <u>C0002197</u><br><u>0</u> | 62065-27-4  | Cerapicol                     | C15H26O   | 222.1983655 | <i>Ceratocystis piceae</i>                            |
| <u>C0002402</u><br><u>6</u> | 87686-76-8  | 6'-O-(p-Coumaroyl)-procumbide | C24H28O12 | 508.1580764 | <i>Harpagophytum procumbens</i><br>DC.(Pendialiaceae) |
| <u>C0002408</u><br><u>5</u> | 118075-30-2 | Celastranhydride              | C28H36O5  | 452.2562743 | <i>Kokoona zeylanica</i><br>THW.(Celastraceae)        |
| <u>C0002409</u><br><u>4</u> | 139954-00-0 | Palbinone                     | C22H30O4  | 358.2144095 | <i>Paeonia albiflora</i><br>PALLAS (paeoniaceae)      |
| <u>C0002930</u><br><u>6</u> | 59092-94-3  | (+)-Rhododendrol              | C10H14O2  | 166.0993797 | <i>Aceraceae nikoense</i>                             |
| <u>C0002945</u><br><u>1</u> | 4547-24-4   | 2alpha-Hydroxyursolic acid    | C30H48O4  | 472.35526   | <i>Rubus alceaefolius</i>                             |
| <u>C0002981</u><br><u>1</u> | 100-51-6    | Benzenemethanol               | C7H8O     | 108.0575149 | <i>Proteaceae</i>                                     |
| <u>C0003048</u><br><u>2</u> | 16930-96-4  | Hexyl tiglate                 | C11H20O2  | 184.1463299 | <i>Proteaceae</i>                                     |
| <u>C0003076</u><br><u>7</u> | 119-36-8    | Methyl salicylate             | C8H8O3    | 152.0473441 | <i>Proteaceae</i>                                     |
| <u>C0003130</u><br><u>6</u> | 78910-33-5  | Sarmentine                    | C14H23NO  | 221.1779644 | <i>Piperaceae</i>                                     |
| <u>C0003160</u><br><u>5</u> | 84575-10-0  | Aquillochin                   | C21H20O9  | 416.1107322 | <i>Aceraceae nikoense</i>                             |
| <u>C0003405</u><br><u>4</u> | 93-58-3     | Methyl benzoate               | C8H8O2    | 136.0524295 | <i>Proteaceae</i>                                     |

|                             |                 |                                       |             |             |                                       |
|-----------------------------|-----------------|---------------------------------------|-------------|-------------|---------------------------------------|
| <u>C0003445</u><br><u>2</u> | 100-52-<br>7    | Benzaldehyde                          | C7H6O       | 106.0418648 | <i>Proteaceae</i>                     |
| <u>C0003445</u><br><u>4</u> | 472-51-<br>5    | beta-Chaconine                        | C39H63NO10  | 705.4451972 | <i>Solanaceae</i>                     |
| <u>C0003796</u><br><u>0</u> | 532-24-<br>1    | Tropinone                             | C8H13NO     | 139.099714  | <i>Cyphomandra</i><br><i>betaceae</i> |
| <u>C0004801</u><br><u>9</u> | 871018-<br>31-4 | Niga-ichigoside F1                    | C36H58O11   | 666.3979127 | <i>Rubus alceaefolius</i>             |
| <u>C0004832</u><br><u>7</u> | 37526-<br>88-8  | Benzyl tiglate                        | C12H14O2    | 190.0993797 | <i>Proteaceae</i>                     |
| <u>C0005175</u><br><u>8</u> | 20312-<br>36-1  | Phenyllactic acid                     | C9H10O3     | 166.0629942 | <i>Cyphomandra</i><br><i>betaceae</i> |
| <u>C0005175</u><br><u>9</u> | 114-76-<br>1    | Phenylpyruvate                        | C9H8O3      | 164.0473441 | <i>Cyphomandra</i><br><i>betaceae</i> |
| <u>C0005742</u><br><u>6</u> | 534-69-<br>0    | 2-Propenyl<br>glucosinolate           | C10H17NO9S2 | 359.0344726 | <i>Brassica oleraceae</i>             |
| <u>C0005834</u><br><u>3</u> | 130233-<br>81-7 | Aceroside IX                          | C30H40O12   | 592.2519767 | <i>Aceraceae triflorum</i>            |
| <u>C0005834</u><br><u>4</u> | 130233-<br>82-8 | Aceroside X                           | C25H32O8    | 460.209718  | <i>Aceraceae triflorum</i>            |
| <u>C0005834</u><br><u>5</u> | 156312-<br>14-0 | Aceroside XII                         | C30H42O12   | 594.2676268 | <i>Aceraceae triflorum</i>            |
| <u>C0005834</u><br><u>6</u> | 156312-<br>15-1 | Aceroside XIII                        | C25H34O8    | 462.2253681 | <i>Aceraceae triflorum</i>            |
| <u>C0005846</u><br><u>3</u> | 102382-<br>31-0 | Elaeocarpusin                         | C47H34O32   | 1110.103319 | <i>Aceraceae nikoense</i>             |
| <u>C0000377</u><br><u>9</u> | 515-06-<br>0    | Mutatochrome                          | C40H56O     | 552.4331164 | <i>Cyanophyta sp.</i>                 |
| <u>C0000489</u><br><u>6</u> | 156298-<br>98-5 | 6-C-Methylquercetin<br>3-methyl ether | C17H14O7    | 330.0739528 | <i>Xerophyta retinervis</i>           |

|                             |            |                                        |           |             |                                           |
|-----------------------------|------------|----------------------------------------|-----------|-------------|-------------------------------------------|
| <u>C0000489</u><br><u>7</u> | 82318-51-2 | 8-C-Methylquercetin 3-methyl ether     | C17H14O7  | 330.0739528 | <i>Xerophyta retinervis</i>               |
| <u>C0001332</u><br><u>9</u> | 520-32-1   | Tricin                                 | C17H14O7  | 330.0739528 | <i>Xerophyta retinervis</i>               |
| <u>C0002323</u><br><u>1</u> | 472-62-8   | Isocryptoxanthin                       | C40H56O   | 552.4331164 | <i>Cyanophyta sp.</i>                     |
| <u>C0000067</u><br><u>4</u> | 491-70-3   | Luteolin                               | C15H10O6  | 286.0477381 | <i>Picris cyanocarpa</i>                  |
| <u>C0000107</u><br><u>1</u> | 529-44-2   | Myricetin                              | C15H10O8  | 318.0375673 | <i>Acacia cyanophylla</i>                 |
| <u>C0000217</u><br><u>7</u> | 484-08-2   | Kokusaginine                           | C14H13NO4 | 259.0844579 | <i>Glycosmis cyanocarpa</i>               |
| <u>C0000219</u><br><u>8</u> | 83-95-4    | Skimmianine                            | C14H13NO4 | 259.0844579 | <i>Glycosmis cyanocarpa</i>               |
| <u>C0000264</u><br><u>7</u> | 149-91-7   | Gallic acid                            | C7H6O5    | 170.0215233 | <i>Acacia cyanophylla</i>                 |
| <u>C0000324</u><br><u>7</u> | 65725-10-2 | 8-Deoxylactucin                        | C15H16O4  | 260.104859  | <i>Picris cyanocarpa</i><br><i>Boiss.</i> |
| <u>C0000377</u><br><u>9</u> | 515-06-0   | Mutatochrome                           | C40H56O   | 552.4331164 | <i>Cyanophyta sp.</i>                     |
| <u>C0000381</u><br><u>7</u> | 520-36-5   | Apigenin                               | C15H10O5  | 270.0528234 | <i>Picris cyanocarpa</i>                  |
| <u>C0000461</u><br><u>5</u> | 14965-09-4 | 5,7,4'-Trihydroxy-3,8-dimethoxyflavone | C17H14O7  | 330.0739528 | <i>Cyanostegia angustifolia</i>           |
| <u>C0000461</u><br><u>5</u> | 14965-09-4 | 5,7,4'-Trihydroxy-3,8-dimethoxyflavone | C17H14O7  | 330.0739528 | <i>Cyanostegia microphylla</i>            |
| <u>C0000461</u><br><u>9</u> | 6586-29-4  | Herbacetin 3,7,8-trimethyl ether       | C18H16O7  | 344.0896029 | <i>Cyanostegia angustifolia</i>           |

|                             |             |                                            |            |             |                                 |
|-----------------------------|-------------|--------------------------------------------|------------|-------------|---------------------------------|
| <u>C0000469</u><br><u>2</u> | 14965-20-9  | Chrysosplenol D                            | C18H16O8   | 360.0845175 | <i>Cyanostegia microphylla</i>  |
| <u>C0000473</u><br><u>3</u> | 4693-88-3   | Gossypetin 3,7,8-trimethyl ether           | C18H16O8   | 360.0845175 | <i>Cyanostegia angustifolia</i> |
| <u>C0000473</u><br><u>5</u> | 14965-08-3  | 5,7,4'-Trihydroxy-3,8,3'-trimethoxyflavone | C18H16O8   | 360.0845175 | <i>Cyanostegia angustifolia</i> |
| <u>C0000473</u><br><u>5</u> | 14965-08-3  | 5,7,4'-Trihydroxy-3,8,3'-trimethoxyflavone | C18H16O8   | 360.0845175 | <i>Cyanostegia microphylla</i>  |
| <u>C0000474</u><br><u>4</u> | 14965-12-9  | Gossypetin 3,7,8,3',4'-pentamethyl ether   | C20H20O8   | 388.1158176 | <i>Cyanostegia spp.</i>         |
| <u>C0000502</u><br><u>6</u> | 80370-37-2  | Rhynchospermin                             | C22H22O7   | 398.1365531 | <i>Rhynchosia cyanosperma</i>   |
| <u>C0000554</u><br><u>8</u> | 604-80-8    | Narcissoside                               | C28H32O16  | 624.169035  | <i>Acacia cyanophylla</i>       |
| <u>C0000864</u><br><u>2</u> | 119736-72-0 | Isotirumalin                               | C22H24O7   | 400.1522031 | <i>Rhynchosia cyanosperma</i>   |
| <u>C0000864</u><br><u>3</u> | 74514-46-8  | Tirumalin                                  | C22H24O7   | 400.1522031 | <i>Rhynchosia cyanosperma</i>   |
| <u>C0000881</u><br><u>7</u> | 970-73-0    | Gallocatechin                              | C15H14O7   | 306.0739528 | <i>Acacia cyanophylla</i>       |
| <u>C0001227</u><br><u>0</u> | 22419-78-9  | Jurineolide                                | C20H26O7   | 378.1678532 | <i>Jurinea cyanoides</i>        |
| <u>C0002080</u><br><u>7</u> | 7726-34-3   | Jacquinelin                                | C15H18O4   | 262.1205091 | <i>Picris cyanocarpa</i> Boiss. |
| <u>C0002323</u><br><u>1</u> | 472-62-8    | Isocryptoxanthin                           | C40H56O    | 552.4331164 | <i>Cyanophyta sp.</i>           |
| <u>C0002905</u><br><u>8</u> | 20824-29-7  | Strictosidine                              | C27H34N2O9 | 530.2264307 | <i>Palicourea cyanococca</i>    |

|                             |             |                           |             |             |                                                                            |
|-----------------------------|-------------|---------------------------|-------------|-------------|----------------------------------------------------------------------------|
| <u>C0003713</u><br><u>3</u> | 523-66-0    | Evolitrine                | C13H11NO3   | 229.0738932 | <i>Glycosmis cyanocarpa</i>                                                |
| <u>C0003821</u><br><u>3</u> | 102042-09-1 | 21-Hydroxyoligomycin A    | C45H74O12   | 806.5180278 | <i>Streptomyces cyaneogriseus</i><br><i>ssp.noncyanogenus</i><br>LL-F28249 |
| <u>C0004826</u><br><u>7</u> | 19974-12-0  | 22-Oxo-20-hydroxyecdysone | C27H42O7    | 478.2930537 | <i>Cyanotis arachnoidea</i>                                                |
| <u>C0005182</u><br><u>2</u> | 520-52-5    | Psilocybin                | C12H17N2O4P | 284.0925935 | <i>Conocybe cyanopus</i>                                                   |
| <u>C0005238</u><br><u>2</u> | 107622-37-7 | Phenaglydone              | C14H11NO    | 209.084064  | <i>Glycosmis cyanocarpa</i>                                                |
| <u>C0005572</u><br><u>7</u> | 21420-58-6  | Baeocystin                | C11H15N2O4P | 270.0769435 | <i>Conocybe cyanopus</i>                                                   |
| <u>C0000067</u><br><u>4</u> | 491-70-3    | Luteolin                  | C15H10O6    | 286.0477381 | <i>Picris cyanocarpa</i>                                                   |
| <u>C0000107</u><br><u>1</u> | 529-44-2    | Myricetin                 | C15H10O8    | 318.0375673 | <i>Acacia cyanophylla</i>                                                  |
| <u>C0000217</u><br><u>7</u> | 484-08-2    | Kokusaginine              | C14H13NO4   | 259.0844579 | <i>Glycosmis cyanocarpa</i>                                                |
| <u>C0000219</u><br><u>8</u> | 83-95-4     | Skimmianine               | C14H13NO4   | 259.0844579 | <i>Glycosmis cyanocarpa</i>                                                |
| <u>C0000264</u><br><u>7</u> | 149-91-7    | Gallic acid               | C7H6O5      | 170.0215233 | <i>Acacia cyanophylla</i>                                                  |
| <u>C0000324</u><br><u>7</u> | 65725-10-2  | 8-Deoxylactucin           | C15H16O4    | 260.104859  | <i>Picris cyanocarpa</i><br>Boiss.                                         |
| <u>C0000377</u><br><u>9</u> | 515-06-0    | Mutatochrome              | C40H56O     | 552.4331164 | <i>Cyanophyta sp.</i>                                                      |
| <u>C0000381</u><br><u>7</u> | 520-36-5    | Apigenin                  | C15H10O5    | 270.0528234 | <i>Picris cyanocarpa</i>                                                   |

|                             |             |                                            |           |             |                                 |
|-----------------------------|-------------|--------------------------------------------|-----------|-------------|---------------------------------|
| <u>C0000461</u><br><u>5</u> | 14965-09-4  | 5,7,4'-Trihydroxy-3,8-dimethoxyflavone     | C17H14O7  | 330.0739528 | <i>Cyanostegia angustifolia</i> |
| <u>C0000461</u><br><u>5</u> | 14965-09-4  | 5,7,4'-Trihydroxy-3,8-dimethoxyflavone     | C17H14O7  | 330.0739528 | <i>Cyanostegia microphylla</i>  |
| <u>C0000461</u><br><u>9</u> | 6586-29-4   | Herbacetin 3,7,8-trimethyl ether           | C18H16O7  | 344.0896029 | <i>Cyanostegia angustifolia</i> |
| <u>C0000469</u><br><u>2</u> | 14965-20-9  | Chrysosplenol D                            | C18H16O8  | 360.0845175 | <i>Cyanostegia microphylla</i>  |
| <u>C0000473</u><br><u>3</u> | 4693-88-3   | Gossypetin 3,7,8-trimethyl ether           | C18H16O8  | 360.0845175 | <i>Cyanostegia angustifolia</i> |
| <u>C0000473</u><br><u>5</u> | 14965-08-3  | 5,7,4'-Trihydroxy-3,8,3'-trimethoxyflavone | C18H16O8  | 360.0845175 | <i>Cyanostegia angustifolia</i> |
| <u>C0000473</u><br><u>5</u> | 14965-08-3  | 5,7,4'-Trihydroxy-3,8,3'-trimethoxyflavone | C18H16O8  | 360.0845175 | <i>Cyanostegia microphylla</i>  |
| <u>C0000474</u><br><u>4</u> | 14965-12-9  | Gossypetin 3,7,8,3',4'-pentamethyl ether   | C20H20O8  | 388.1158176 | <i>Cyanostegia spp.</i>         |
| <u>C0000502</u><br><u>6</u> | 80370-37-2  | Rhynchospermin                             | C22H22O7  | 398.1365531 | <i>Rhynchosia cyanosperma</i>   |
| <u>C0000554</u><br><u>8</u> | 604-80-8    | Narcissoside                               | C28H32O16 | 624.169035  | <i>Acacia cyanophylla</i>       |
| <u>C0000864</u><br><u>2</u> | 119736-72-0 | Isotirumalin                               | C22H24O7  | 400.1522031 | <i>Rhynchosia cyanosperma</i>   |
| <u>C0000864</u><br><u>3</u> | 74514-46-8  | Tirumalin                                  | C22H24O7  | 400.1522031 | <i>Rhynchosia cyanosperma</i>   |
| <u>C0000881</u><br><u>7</u> | 970-73-0    | Gallocatechin                              | C15H14O7  | 306.0739528 | <i>Acacia cyanophylla</i>       |

|                             |             |                           |             |             |                                                                     |
|-----------------------------|-------------|---------------------------|-------------|-------------|---------------------------------------------------------------------|
| <u>C0001227</u><br><u>0</u> | 22419-78-9  | Jurineolide               | C20H26O7    | 378.1678532 | <i>Jurinea cyanoides</i>                                            |
| <u>C0002080</u><br><u>7</u> | 7726-34-3   | Jacquinelin               | C15H18O4    | 262.1205091 | <i>Picris cyanocarpa</i><br>Boiss.                                  |
| <u>C0002323</u><br><u>1</u> | 472-62-8    | Isocryptoxanthin          | C40H56O     | 552.4331164 | <i>Cyanophyta</i> sp.                                               |
| <u>C0002905</u><br><u>8</u> | 20824-29-7  | Strictosidine             | C27H34N2O9  | 530.2264307 | <i>Palicourea cyanococca</i>                                        |
| <u>C0003713</u><br><u>3</u> | 523-66-0    | Evolitrine                | C13H11NO3   | 229.0738932 | <i>Glycosmis cyanocarpa</i>                                         |
| <u>C0003821</u><br><u>3</u> | 102042-09-1 | 21-Hydroxyoligomycin A    | C45H74O12   | 806.5180278 | <i>Streptomyces cyaneogriseus</i><br>ssp.noncyanogenus<br>LL-F28249 |
| <u>C0004826</u><br><u>7</u> | 19974-12-0  | 22-Oxo-20-hydroxyecdysone | C27H42O7    | 478.2930537 | <i>Cyanotis arachnoidea</i>                                         |
| <u>C0005182</u><br><u>2</u> | 520-52-5    | Psilocybin                | C12H17N2O4P | 284.0925935 | <i>Conocybe cyanopus</i>                                            |
| <u>C0005238</u><br><u>2</u> | 107622-37-7 | Phenaglydone              | C14H11NO    | 209.084064  | <i>Glycosmis cyanocarpa</i>                                         |
| <u>C0005572</u><br><u>7</u> | 21420-58-6  | Baeocystin                | C11H15N2O4P | 270.0769435 | <i>Conocybe cyanopus</i>                                            |
| <u>C0000118</u><br><u>3</u> | 110-17-8    | Fumaric acid              | C4H4O4      | 116.0109586 | <i>Cladonia rangiferina</i>                                         |
| <u>C0000118</u><br><u>3</u> | 110-17-8    | Fumaric acid              | C4H4O4      | 116.0109586 | <i>Cladonia fallax</i>                                              |
| <u>C0000241</u><br><u>3</u> | 125-46-2    | Usnic acid                | C18H16O7    | 344.0896029 | <i>Cladonia stellaris</i>                                           |
| <u>C0000241</u><br><u>3</u> | 125-46-2    | Usnic acid                | C18H16O7    | 344.0896029 | <i>Cladonia</i> spp.                                                |

|                             |                  |                                             |          |             |                             |
|-----------------------------|------------------|---------------------------------------------|----------|-------------|-----------------------------|
| <u>C0000241</u><br><u>3</u> | 125-46-<br>2     | Usnic acid                                  | C18H16O7 | 344.0896029 | <i>Cladonia fallax</i>      |
| <u>C0000364</u><br><u>6</u> | 474-67-<br>9     | Brassicasterol                              | C28H46O  | 398.3548661 | <i>Cladonia gonecha</i>     |
| <u>C0002330</u><br><u>0</u> | 6832-<br>60-6    | Imbricatolic acid                           | C20H34O3 | 322.250795  | <i>Cladonia rangiferina</i> |
| <u>C0002330</u><br><u>3</u> | 41787-<br>69-3   | Junicedric acid                             | C20H32O4 | 336.2300595 | <i>Cladonia rangiferina</i> |
| <u>C0002378</u><br><u>7</u> | 22570-<br>53-2   | Zeorin                                      | C30H52O2 | 444.3967309 | <i>Cladonia deformis</i>    |
| <u>C0002976</u><br><u>0</u> | 526-37-<br>4     | 4-Methyl-2,6-<br>dihydroxy-<br>benzaldehyde | C8H8O3   | 152.0473441 | <i>Cladonia rangiferina</i> |
| <u>C0002978</u><br><u>8</u> | 17636-<br>16-7   | Barbatic acid                               | C19H20O7 | 360.120903  | <i>Cladonia rangiferina</i> |
| <u>C0003001</u><br><u>0</u> | 79994-<br>21-1   | Condidymic acid                             | C24H30O5 | 398.2093241 | <i>Cladonia rangiferina</i> |
| <u>C0003012</u><br><u>7</u> | 436-74-<br>8     | Didymic acid                                | C22H26O5 | 370.1780239 | <i>Cladonia rangiferina</i> |
| <u>C0003045</u><br><u>3</u> | 103881<br>3-57-8 | Hanagokenol A                               | C20H26O3 | 314.1881947 | <i>Cladonia rangiferina</i> |
| <u>C0003045</u><br><u>4</u> | 103881<br>3-60-3 | Hanagokenol B                               | C20H26O5 | 346.1780239 | <i>Cladonia rangiferina</i> |
| <u>C0003048</u><br><u>9</u> | 486-36-<br>2     | Homosekikaic acid                           | C24H30O8 | 446.1940679 | <i>Cladonia rangiferina</i> |
| <u>C0003079</u><br><u>2</u> | 140923-<br>35-9  | Montbretol                                  | C20H26O3 | 314.1881947 | <i>Cladonia rangiferina</i> |
| <u>C0003087</u><br><u>5</u> | 209863-<br>21-8  | Obtuanhydride                               | C20H26O4 | 330.1831093 | <i>Cladonia rangiferina</i> |
| <u>C0003678</u><br><u>2</u> | 479-20-<br>9     | Atranorin                                   | C19H18O8 | 374.1001676 | <i>Cladonia rangiferina</i> |
| <u>C0003769</u><br><u>1</u> | 73036-<br>29-0   | Protoconstipatic acid                       | C21H36O5 | 368.2562743 | <i>Cladonia furcata</i>     |

|                  |             |                                        |              |             |                                         |
|------------------|-------------|----------------------------------------|--------------|-------------|-----------------------------------------|
| <u>C00039870</u> | 571-67-5    | Norstictic acid                        | C18H12O9     | 372.048132  | <i>Cladonia verticillata</i>            |
| <u>C00040391</u> | 549-06-4    | Stictic acid                           | C19H14O9     | 386.0637821 | <i>Cladonia verticillata</i>            |
| <u>C00000911</u> | 502-65-8    | Lycopene                               | C40H56       | 536.4382018 | <i>Rosa canina</i>                      |
| <u>C00002668</u> | 99-50-3     | Protocatechuic acid                    | C7H6O4       | 154.0266087 | <i>Rosa canina</i>                      |
| <u>C00002682</u> | 121-34-6    | Vanillic acid                          | C8H8O4       | 168.0422587 | <i>Rosa canina</i>                      |
| <u>C00002783</u> | 61276-17-3  | Acteoside                              | C29H36O15    | 624.2054205 | <i>Scrophularia canina</i>              |
| <u>C00003467</u> | 150-86-7    | Phytol                                 | C20H40O      | 296.3079159 | <i>Scrophularia canina</i>              |
| <u>C00003785</u> | 3763-55-1   | Rubixanthin                            | C40H56O      | 552.4331164 | <i>Rosa canina</i>                      |
| <u>C00029601</u> | 82509-41-9  | 8-Epiloganic acid                      | C16H24O10    | 376.136947  | <i>Scrophularia canina</i>              |
| <u>C00000138</u> | 31456-68-5  | (R)8Methylsulfinyloctyl isothiocyanate | C10H19NOS2   | 233.0908057 | <i>Rorippa islandica</i> (Oeder) Borbas |
| <u>C00007352</u> | 112572-51-7 | 7(Methylsulfinyl)heptyl glucosinolate  | C15H29NO10S3 | 479.0953584 | <i>Rorippa islandica</i>                |
| <u>C00007355</u> | 21973-60-4  | 8-Methylsulfinyloctyl glucosinolate    | C16H31NO10S3 | 493.1110084 | <i>Rorippa islandica</i>                |
| <u>C00007586</u> | 19041-09-9  | Gluconapin                             | C11H19NO9S2  | 373.0501227 | <i>Rorippa islandica</i>                |
| <u>C00007805</u> | 19253-84-0  | 4-Hydroxybenzyl glucosinolate          | C14H19NO10S2 | 425.0450373 | <i>Rorippa islandica</i>                |

### **4.3 Molecular Screening by Docking**

Finding the right target group is crucial for any virtual screening attempt to be successful. We use docking algorithms to look for the optimal binding mode when the 3D (three-dimensional) structure of a target is known by X-ray crystallography, NMR spectroscopy, or any other method between the ligand and the target macromolecule

The RIPK3 structure was generated from the RCSB Protein Data Bank ([rcsb.org](http://rcsb.org)). The generated RIPK3 bound ligands and the meta ions are removed and it was energy minimized using Pyrx Software (Dallakyan and Olson, 2015). The energy minimized model was used for the docking studies. The compounds which are previously known to have the tendency to modify the other proteins of the RIPK3 family are shortlisted and docked to study its binding affinity efficiency with the RIPK3

The input files were taken from publicly available websites where user can download files, those websites are PubChem and Chempider

PubChem - <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=5988>

Chempider- <http://www.chemspider.com/Chemical-Structure.1906.html>.

The Mol type and SDF types were converted into PDB file type by using the open source called Open Babel <http://openbabel.org>.

#### **4.3.1 Hardware and Software**

Dell Inspiron 3505 with AMD Ryzen 3 3250U with Radeon Graphics 2.60 GHz, 8GB (RAM) and 64-bit operating system, x64-based processor.

PyRx version 0.8 for Windows is available as a free binary download at <http://pyrx.sourceforge.net>.

### **4.3.2 Virtual screening of molecule by PyRx docking**

Python is a programming language that may be used to create PyRx, which is compatible with almost all current computers, including PCs. The Windows PC used in the Methods section is described in full below, while same techniques also apply to Linux and Mac OS.

#### **4.3.2.1 Preparing Input files for PyRx docking**

We must convert input files to a PDBQT file format compatible with docking using AutoDock Vina before using them for virtual screening (Trott and Olson, 2010).

1. Double-click the PyRx icon on the desktop to get started.
2. Click the first icon with a plus (+) symbol on the toolbar of the Controls panel's Open Babel tab. Go to the Downloads folder and choose RIPK3 PDB file.
3. Now select the corresponding RIPK3 file and click right select the minimization option. Click ok and wait for few minutes to complete the minimization. After the minimization the name would have changed to RIPK3\_uff\_E=197.68.
4. Convert it into the autodock and select it as macromolecule
5. Similarly for ligand, once again click the first icon on the Open Babel toolbar locate, and open the ligand files and add them to the open babel.
6. Selected all the ligand click right and select the energy minimization option and wait until all the ligand gets minimized.
7. After the energy minimization, click any row in the Open Babel table with the right mouse button, then select Convert All to AutoDock Ligand (pdbqt). Under the Ligands folder, pdbqt files corresponding to the molecules will be generated.

#### **4.3.2.2 Virtual Screening Using Vina Wizard**

1. Selected the Vina Wizard tab from the Controls panel and pressed Start button.
2. Selected the multiple ligand files under the ligand folder
3. RIPK3 energy minimized file under macromolecule was selected and forward option was selected on Vina Wizard.
4. Maximized the size of the bar on the 3D structure on the Vina search space and forwarded.

5. This starts the autodock vina and docks each ligand one by one to the RIPK3.
6. Repeatedly the process was continued and cycled until thousand natural compounds were docked.
7. When virtual screening is finished, PyRx advances to the Analyze Results page, where you can view the results of the virtual screening computation. By default, AutoDock Vina generates top optimum binding modes for each docking run. To arrange this table by predicted binding affinity (kcal/mol), left-click on the Binding Affinity (kcal/mol) table header cell under the Analyze Results tab.
8. The results were stored in .xls file type for future analyses.
9. With the binding affinity values analyses were made and the compounds was shortlisted.

Furthermore, shortlisting of the compound was carried out with the iGEM docking software tool.

#### ***4.3.2.1 Virtual screening by iGEM Docking***

iGEM (iGeneric Evolutionary Method Docking) software was used to shortlist the compounds which are filtered by PyRx.

iGemdock is a virtual screening (VS) environment that includes preparations, post-screening analyses, and pharmaceutical interactions. iGemdock provides interactive interfaces for preparing both the target binding site and the screening chemical library. The in-house docking tool iGemdock is then used to dock each compound in the library into the binding site.

Following that, iGemdock generates electrostatic (E), hydrogen-bonding (H), and Van der Waal's (V) protein-compound interaction profiles. iGemdock infers pharmacological interactions and clusters the screening compounds for post-screening analysis based on these profiles and compound structures. Finally, by integrating the pharmacological interactions and the energy-based scoring mechanism of iGemdock, iGemdock ranks and visualizes the screening compounds.

iGEM dock was carried out by preparing binding site of macromolecule which RIPK3 structure in a form of PDB file format and the ligand compounds were prepared as in a form of previously mentioned manner. Both were docked and the post docking analysis were made and shortlist of the compound were made by the values.

#### **4.4 Mammalian cell culture**

Human derived cell line (Table 4.1) was grown in the T- 25cm<sup>2</sup> (Hi Media, India) in Dulbecco's Modified Eagle Medium (DMEM) (Hi Media, India) were supplemented with 10% Fetal bovine serum (FBS) (Hi Media, India) and maintained at 37°C and 5% of CO<sub>2</sub> in a humidified incubator. Sub-culturing was done with the 80-90% confluent cells, they were washed in sterile phosphate buffered saline (PBS) which was prepared by adding 8g of sodium chloride (NaCl), 0.2g of Potassium Phosphate (KH<sub>2</sub>PO<sub>4</sub>), and 1.44g of Disodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>) to 1liter graduated cylinder and add distilled water to the cylinder until the volume reaches 1L. Place the cylinder in the magnetic stirrer until all the salts dissolve. Then using the pH meter (susima pH meter) adjust the pH to 7.4 by adding small amount of HCl or NaOH according to the need. Then autoclave the PBS for 20mins to ensure it was sterile. Then 0.5 to 2mL of trypsin-EDTA solution was added [ 0.25% Trypsin and 0. 02% EDTA]. After for the cell counting the cells were centrifuged at 300 to 500 rpm for 2 min and the pellet was suspended in a growth media containing 10% FBS to inactivate the trypsin. The cell suspension was stained with 10 µL of 0.4% trypan blue solution, and the cell counting were done by using traditional hemocytometers cell counting method. The cell line supplies were frozen in liquid nitrogen using a 70% FBS, 20% DMEM freezing mixture. The contamination analyses were frequently analyzed using Gram Stain and PCR.

**Table 4.2 List of cell lines**

| Type of Cell line    | Cell line | Medium used       | Source of cell line                    |
|----------------------|-----------|-------------------|----------------------------------------|
| Control cells        | HEK293    | DMEM with 10% FBS | Cancer Institute (WIA), Adyar, Chennai |
| Control breast cells | MCF7      | DMEM with 10% FBS | Cancer Institute (WIA), Adyar, Chennai |
| Breast cancer cells  | SKBR3     | DMEM with 10% FBS | Cancer Institute (WIA), Adyar, Chennai |

#### **4.5 Preparation of Drug**

The Diosgenin (Cell-Chem, Canada) compound was commercially brought in the powdered form, the molecular weight of diosgenin 414.62 g/mol. Then the 100mM stock was prepared by dissolving the 41mg of diosgenin in the 1mL of 99.9% of ethanol, then vortex mixer was used for 20-30 minutes to dissolve the particle in the solute. After the dilution the stock solution of 100mM was serial diluted to prepare the working standard in the concentration of 1:1000(mM), by adding 10  $\mu$ L of stock diosgenin solution in the 990  $\mu$ L of 70% ethanol which is then vortexed for 20 minutes to mix the diluted solution, then it was stored in the -20°C freezer for further usage.

##### **4.5.1 Preparation of nanoparticle**

The silver nanoparticle of size 30nm was brought commercially. The silver nanoparticle was agitated and solution was prepared, this process was carried out by adding the nanoparticle of 50mg in the 5mL of deionized water which is then sonicated (Rivotek, India) for 1 hour to agitate the nanoparticle in the solution.

#### **4.5.2 Conjugation of Nanoparticle with Drug**

The conjugation of silver nanoparticle with the diosgenin drug formation was formed by adding 300  $\mu$ L of nanoparticle solution to the 700  $\mu$ L of 1:1000(mM) diluted diosgenin which was then vortexed for 5-6 hours to make them mix well. Then it was stored in the freezer (-20°C) for the future usage.

#### **4.5.3 Characterization of Drug**

The chemical composition of the drug was analyzed by using the scanning electron microscope (SEM) integrated with windowless Energy dispersive x-ray spectroscopy (EDX) (Oxford instruments, Germany).

#### **4.6 MTT ASSAY**

The confluent cells were taken out of media and washed with the in sterile phosphate buffered saline (PBS) after that 2mL of trypsin-EDTA solution was added [0.25% Trypsin and 0.02% EDTA]. After that the cells were centrifuged at 200 - 300 rpm for 2 mins and then the pellet was suspended in the growth media Dulbecco's Modified Eagle Medium (DMEM) with 10% of FBS. Then the cell line with the media is seeded in the 96 well plate at the volume of 100  $\mu$ L with the density of 2,000 to 5,000 cells per well and incubate overnight at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. For analyzing the cytotoxicity and cell viability the HEK293 is used as a control cell and the SKBR3 is used as a cancer cell. After the incubation the 1:1000 diluted drug of 100  $\mu$ L was added to the cells in the each well and incubated for 24 hours, after the incubation 10  $\mu$ L of MTT reagent which was prepared by dissolving the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Hi Media, India) in PBS on the concentration of 1:1000 dilution then it was added and incubate the cells for 4 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. After the incubation add 100  $\mu$ L of DMSO (Dimethyl Sulfoxide) to each well. Incubate the plates for 15 minutes at room temperature on a shaker to dissolve the formazan crystals. Then measure the absorbance of the each well at 570 nm using the microplate reader (Bioteck, Germany). With the varying concentration of drug and different time of incubation the readings were observed.

Likewise, the nanoparticle conjugated diluted drug was also added separately to 96 well plate seeded with the cells and observed at the same time period interval measured at the 570nm. Calculate the percentage of viable cells and cytotoxicity by comparing the absorbance of the experimental groups with the control group.

#### **4.7 Clonogenic Assay**

##### **4.7.1 Cell lines**

The cell line clonogenic experiment was based on techniques previously described (Franken *et al.*, 2006). At about 70% confluence, growth media was evacuated from a cell monolayer, and the cells were washed with PBS. 1 ml of 0.25% trypsin was added to the flask and incubated for 5 minutes at 37°C until the cells rounded and detached from the culture flask. To stop the trypsinization process, 9 ml of growth media was introduced. To break up clumps and form a single cell suspension, the cell suspension was pipetted up and down many times. 50µl of cell suspension was diluted with trypan blue at a ratio of 1:1 (v/v) and 10µl was added to the hemocytometers.

The number of viable cells was counted in each of the hemocytometers' eight squares. Viable cells are phase bright and dark cells that took up trypan blue as a result of a permeable cell membrane. Non-viable cells are phase bright and dark cells that took up trypan blue as a result of a permeable cell membrane. Based on the assumption that each big square is comparable to 0.1µl, the number of cells per ml may be computed as the number of cells per grid  $\times 10^4$  ml. The viable cell count per ml was calculated by multiplying the average number of live cells per grid by the dilution factor by  $10^4$ . Cell suspensions with less than 95% single, live cells were excluded.

##### **4.7.2 Colony formation**

As previously described, single cell suspensions of HEK293 and SKBR3 cells were produced and quantified. 250 cells were aliquoted into each well of the 6 well plates in a volume of 2mls of medium and incubated overnight. After the incubation, once the cell adhered and attached to the well plates it was treated with the diosgenin drug, and incubated for further 7-15 days. The control plates were also subjected to the same number and timing of medium changes throughout the experiment. After the incubation

were fixed and stained. Medium was aspirated and cells were fixed in 1ml of 70% of methanol – PBS aliquot into each well for 15 minutes. Then the aliquot was replaced by 1ml of 70% of methanol and the plates were left out for further 10 minutes. Following methanol fixation, colonies were stained for 3 hours with a 1:25 solution of crystal violet after that stain was removed and the plates were dried overnight. Colonies of >50 were counted under the Gel Dock (Bio-Rad, India) with Epic white.

#### **4.7.3 Analysis of assay**

For each condition, colony counts were collected from triplicate wells, and each independent experiment was performed at least three times. Only colonies with more than 50 cells were counted. From these data, a mean colony number for each experimental condition was computed, and plating efficiency (PE) was derived by dividing the mean number of colonies by the number of cells plated (250 cells per well). The SF were then computed by dividing the PE of the experimental condition by the PE of the control cells and diosgenin drug treated cells.

**Plating efficiency (PE) = (number of colonies formed / number of cells plated) x 100%**

This formula calculates the percentage of cells that were plated that went on to form colonies.

**Survival fraction (SF) = (number of colonies formed after treatment / number of cells plated x PE of control) x 100%**

This formula calculates the percentage of cells that survived the treatment, considering the plating efficiency of the control sample.

**Doubling time (DT) = (log2 x culture time) / (log final cell count - log initial cell count)**

This formula calculates the time it takes for a population of cells to double in number.

**Mean colony size = (sum of the areas of all colonies / number of colonies counted)**

This formula calculates the average size of the colonies that formed.

#### **4.8 Total RNA Isolation**

At 80% confluent the growth media was removed from the cell's monolayers and 1ml of trizol reagent added to the cells. The trizol reagent TRIzol™ Reagent (Ambion life technologies, India) was used to isolate total RNA. Cells suspended in trizol were mixed gently with an equivalent amount of chloroform before being centrifuged at 12,000 rpm for 10 minutes at 4°C. The aqueous phase was pipetted out without disturbing the contents and put on ice in a new 1.5 mL tube. The aqueous phase was then supplemented with isopropanol and centrifuged at 12,000 rpm for 10 minutes at 4°C. The resulting white pellet was cleaned twice with 70% ethanol. The pellet was then air-dried before being dissolved in 20 µL of sterile nuclease-free water. The RNA concentration was determined using a Thermo Scientific Nanodrop ND 2000 spectrophotometer. As a blank, sterile nuclease-free water was utilized, and 1 µL of the sample was used to estimate the concentration of RNA in ng/µL.

##### **4.8.1 Agarose Gel Electrophoresis**

Agarose gel electrophoresis was used to test the integrity of the RNA. 1% agarose ( ) was dissolved in 100 mL of 1x Tris/Borate/EDTA (TBE) buffer (0.13 M Tris, 5 mM boric acid, and 2.5 mM EDTA, pH 7.6). The agarose was heated up until completely dissolved, then 3 µl of ethidium bromide (chelating agent) was added with caution as it is a carcinogenic agent to the cooled agarose solution, after that the gel was poured into the gel tray and the comb was inserted. After solidifying the agarose for 30 minutes, the gel tray was submerged in a gel tank containing 1x TBE buffer. 1 g total RNA was combined with 6x clear safe dye (Qiagen, India) and put onto an agarose gel for 30 minutes at 100 V. The ChemiDoc™ (BioRad,US) MP imaging equipment was used to see and document the gel.

##### **4.8.2 PCR Amplification**

All PCR amplification reactions were carried out in a final volume of 30 µl, which included 1 µl of each primer (Forward and Reverse), DNA of the specimen of 4 µl, 10.5 µl nuclease-free water, and 13.5 µl Taq DNA polymerase. Amplifications were performed with the following cycling conditions: The first round of amplification included initial denaturation

followed by 35 cycles of Denaturation, Annealing, and Elongation. Then at last final elongation followed up with an infinite hold.

**Table 4.3: Details of the primer used for PCR Amplification**

| <b>Gene target</b> | <b>Primer Name</b> | <b>5' Sequence 3'</b>               | <b>Length (bp)</b> |
|--------------------|--------------------|-------------------------------------|--------------------|
| RIPK3              | RIPK3 (Forward)    | <b>5'-ACATTGCGGCCTTGGGTCTT-3'</b>   | 100 bp             |
|                    | RIPK3 (Reverse)    | <b>5'-TCGCCTGATCTGAGGTCGAC - 3'</b> | 100 bp             |
| CIAP1              | CIAP1(Forward)     | <b>5'-TCAGATTGGACAAACAGCAAC-3'</b>  | 100 bp             |
|                    | CIAP1(Reverse)     | <b>5'-AAAACCAGCACGAGCAAG-3'</b>     | 100 bp             |
| E-CADHERIN         | E-CAD (Forward)    | <b>5'-CCAGAATAAAGACCAAGTGACC-3'</b> | 100 bp             |
|                    | E-CAD (Reverse)    | <b>5'-CGAAGAAACAGCAAGAGCAG-3'</b>   | 100 bp             |
| VIMENTIN           | VIMENTIN (Forward) | <b>5'-CTAACCAACGACAAAGCCC-3'</b>    | 100 bp             |
|                    | VIMENTIN (Reverse) | <b>5'-CCTCTCTCTGAAGCATCTCC-3'</b>   | 100 bp             |
| TP53               | TP53(Forward)      | <b>5'-ACCACCATCCACTACAACCTAC-3'</b> | 100 bp             |
|                    | TP53(Reverse)      | <b>5'-AAACACGCACCTCAAAGC-3'</b>     | 100 bp             |
| TIMP3              | TIMP3(Forward)     | <b>5'-TGGAGGTCAACAAGTACCAG-3'</b>   | 100 bp             |
|                    | TIMP3(Reverse)     | <b>5'-AAGTCACAAAGCAAGGCAG-3'</b>    | 100 bp             |

**Table 4.4: Thermal Cycling parameters for samples**

| <b>SAMPLE ID</b> | <b>Initial Denaturation (Temp./Time)</b> | <b>Denaturation (Temp./Time)</b> | <b>Annealing (Temp./Time)</b> | <b>Extension (Temp./Time)</b> | <b>Number of cycles</b> | <b>Final Extension (Temp./Time)</b> |
|------------------|------------------------------------------|----------------------------------|-------------------------------|-------------------------------|-------------------------|-------------------------------------|
| RIPK3            | 72/00:30                                 | 94/00:30                         | 59/00:30                      | 72/00:30                      | 30X                     | 72/2:00                             |
| CIAP1            | 72/00:30                                 | 94/00:30                         | 56/00:30                      | 72/00:30                      | 30X                     | 72/2:00                             |
| E-CADHERIN       | 72/00:30                                 | 94/00:30                         | 59/00:30                      | 72/00:30                      | 30X                     | 72/2:00                             |
| VIMENTIN         | 72/00:30                                 | 94/00:30                         | 58/00:30                      | 72/00:30                      | 30X                     | 72/2:00                             |
| TP53             | 72/00:30                                 | 94/00:30                         | 56/00:30                      | 72/00:30                      | 30X                     | 72/2:00                             |
| TIMP3            | 72/00:30                                 | 94/00:30                         | 57/00:30                      | 72/00:30                      | 30X                     | 72/2:00                             |

## **4.8.2 Agarose Gel Electrophoresis**

### **4.8.2.1 Casting an Agarose gel**

Depending upon the number of PCR products, the number of wells in the agarose gel is decided. For 6 wells 1% gel is prepared by carefully weighing and transferring 1 gm of agarose to 50 ml of 1X TBE buffer (pH-8) and for 15 to 20 wells 2% of agarose gel was prepared by weighing 0.8gm of agarose and adding it to the flask containing 80 ml of 1X TBE buffer and the mixture was microwaved for 1 minute 50 seconds, until agarose was completely dissolved. Then 3 µl of ethidium bromide (chelating agent) was added with caution as it is a carcinogenic agent to the cooled agarose solution. Ethidium bromide is

added to the agarose solution to view the DNA bands under the UV light source. The gel casting tray was assembled and the gel casting apparatus was wiped with 100% ethanol and the comb was inserted. The gel solution was poured into the gel boat and left for 45 minutes for the solidification of the gel (Figure 25).

#### **4.8.2.2 Gel Electrophoresis run**

The Gel boat was fixed inside the Gel tank containing 1X TBE buffer and the comb was removed. 5 µl of samples were loaded into the consecutive wells and finally, 2 µl of concentrated 100 bp Ladder was added to compare the base pair length of samples. The leads were connected and the gel was run at a constant voltage, 70 Volts until the colored dye reached the end of the gel.

#### **4.8.2.3 Gel Documentation**

The gel was visualized and documented using the ChemiDoc™ MP imaging system (Biorad, USA). under UV absorbance.

#### **4.8.3 cDNA Conversion**

In a two-step technique, 1 g of total RNA was transcribed to cDNA using the Two step RT-PCR Kit (Qaigen, Germany) according to the manufacturer's instructions. 1 g total RNA, 1 L of primer (2.5 M), and 1 L dNTP mixture (10 mM) were added, and the volume was adjusted to 10 µL using sterile nuclease-free water. The tube was placed in a thermal cycler for denaturation and annealing at 65°C for 5 minutes, followed by 10 minutes at 4°C. The aforementioned reaction mixture was then mixed with 4 µL 5X buffer, 0.5 µL RNase inhibitor (40 U/µL), 0.5 µL Reverse Transcriptase, and 20 µL sterile nuclease-free water. The tube was placed on a thermal cycler, and the cycling temperature is 30°C at 5 min, 42°C for 30 min, 95°C for 5 min, and 4°C.

**Table 4.5: List of primers used for the q RT-PCR**

| <b>Gene target</b> | <b>Primer Name</b>    | <b>5' Sequence 3'</b>                |
|--------------------|-----------------------|--------------------------------------|
| RIPK3              | RIPK3 (Forward)       | <b>5'-ACATTGCGGCCTTGGGTCTT-3'</b>    |
|                    | RIPK3 (Reverse)       | <b>5'-TCGCCTGATCTGAGGTCGAC - 3'</b>  |
| CIAP1              | CIAP1(Forward)        | <b>5'-TCAGATTGGACAAACAGCAAC-3'</b>   |
|                    | CIAP1(Reverse)        | <b>5'-AAAACCAGCACGAGCAAG-3'</b>      |
| E- CADHERIN        | E-CAD (Forward)       | <b>5'-CCAGAATAAAGACCAAGTGACC-3'</b>  |
|                    | E-CAD (Reverse)       | <b>5'-CGAAGAAACAGCAAGAGCAG-3'</b>    |
| VIMENTIN           | VIMENTIN<br>(Forward) | <b>5'-CTAACCAACGACAAAGCCC-3'</b>     |
|                    | VIMENTIN<br>(Reverse) | <b>5'-CCTCTCTCTGAAGCATCTCC-3'</b>    |
| TP53               | TP53(Forward)         | <b>5'-ACCACCATCCACTACAACACTAC-3'</b> |
|                    | TP53(Reverse)         | <b>5'-AAACACGCACCTCAAAGC-3'</b>      |
| TIMP3              | TIMP3(Forward)        | <b>5'-TGGAGGTCAACAAGTACCAG-3'</b>    |
|                    | TIMP3(Reverse)        | <b>5'-AAGTCACAAAGCAAGGCAG-3'</b>     |

#### **4.8.4 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)**

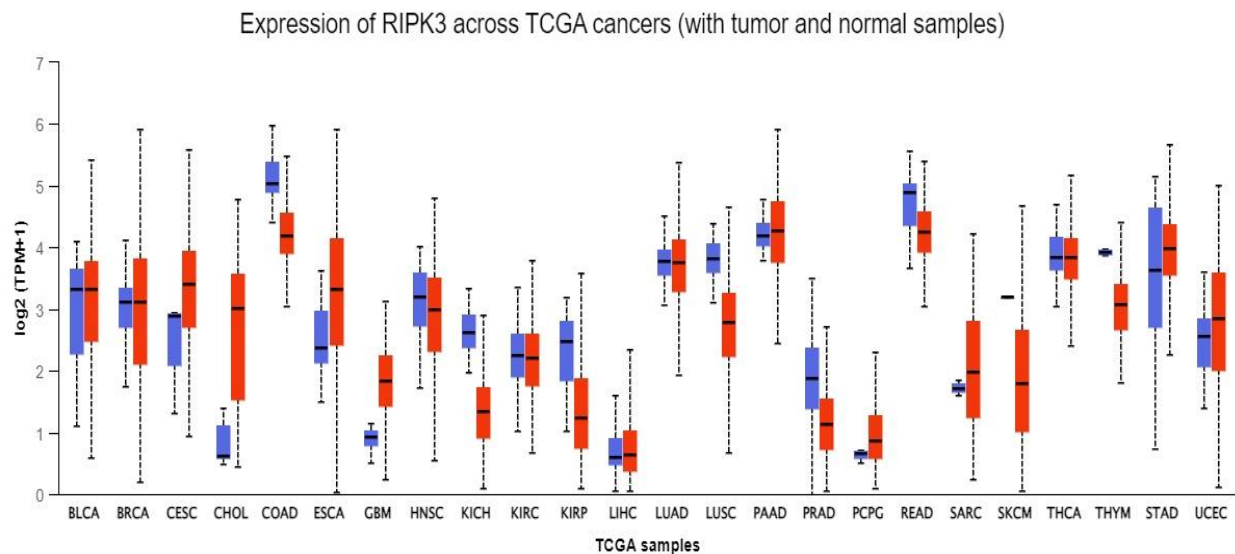
For 7 Gene, qRT-PCR was done using gene-specific primers (RIPK3, CIAP1, E-CADHERIN, VIMENTIN, TP53, TIMP3) and  $\beta$ -actin as the control gene, using the two step RT-PCR kit (Qiagen, Germany) according to the manufacturer's protocol. 10  $\mu$ L of master mix, 1  $\mu$ L of forward primer (10  $\mu$ M), 1  $\mu$ L of reverse primer (10  $\mu$ M), 1  $\mu$ L of cDNA template (50 ng) was added and the volume was made to 20  $\mu$ L with nuclease-free water. PCR amplification was performed. In the Eppendorf tubes 10  $\mu$ L of master mix (HI- SYBR RT PCR kit, Qiagen, Germany) 1  $\mu$ L of template, 1.5  $\mu$ L of forward primer, 1.5  $\mu$ L of reverse primer and by using sterile nuclease free water it was made up to 20  $\mu$ L and the beta actin primers were added according. The tubes were centrifuged at 100rpm to ensure that contents are present at the bottom. Then the reaction mixer was transferred into the RT PCR tubes and analyzed using CFX Real-Time PCR (Bio-Rad, USA). The program was set up to calculate the cycle threshold value (CT-value) and 40 cycles. The relative expression levels of the target and control were determined using the  $2^{-\Delta\Delta CT}$  technique. In this approach, the reference sample's relative gene expression is considered to be one, hence a value of  $2^0$  yields a value of one. It is based on the assumption of 100% efficiency and uniform PCR amplification across all samples. The results were reported as a fold change in expression when compared to the control. Gene expression was termed upregulated if the fold change was greater than 1.5, and downregulated if the fold change was less than 0.5.

## 5. RESULT AND DISCUSSION

### 5.1 Insilco analysis of RIPK3

#### 5.1.1 RIPK3 is differentially gene expressed in cancer:

The RIPK3 gene expression was analyzed using UALCAN TCGA datasets. It showed that in Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Uterine Corpus Endometrial Carcinoma (UCEC), Cholangio carcinoma (CHOL), in Glioblastoma multiforme (GBM), Pheochromocytoma and Paraganglioma (PCPG), RIPK3 was upregulated significantly. Moreover, in Colon adenocarcinoma (COAD), Kidney Chromophobe (KICH), Kidney renal papillary cell carcinoma (KIRP), Lung squamous cell carcinoma (LUSC), Breast invasive carcinoma (BRCA), Prostate adenocarcinoma (PRAD), RIPK3 was downregulated significantly [Table 5.1] [Fig 5.1]. The analysis TCGA datasets have revealed that the RIPK3 to be differentially expressed, and majority of cancers showing upregulation, indicating its involvement in the tumor progression.



**Fig 5.1: Expression of RIPK3 across TCGA cancers**

(Credits: <http://ualcan.path.uab.edu/> )

**Table 5.1: Expression of RIPK3 across the TCGA cancers (Normal vs Tumor sample)**

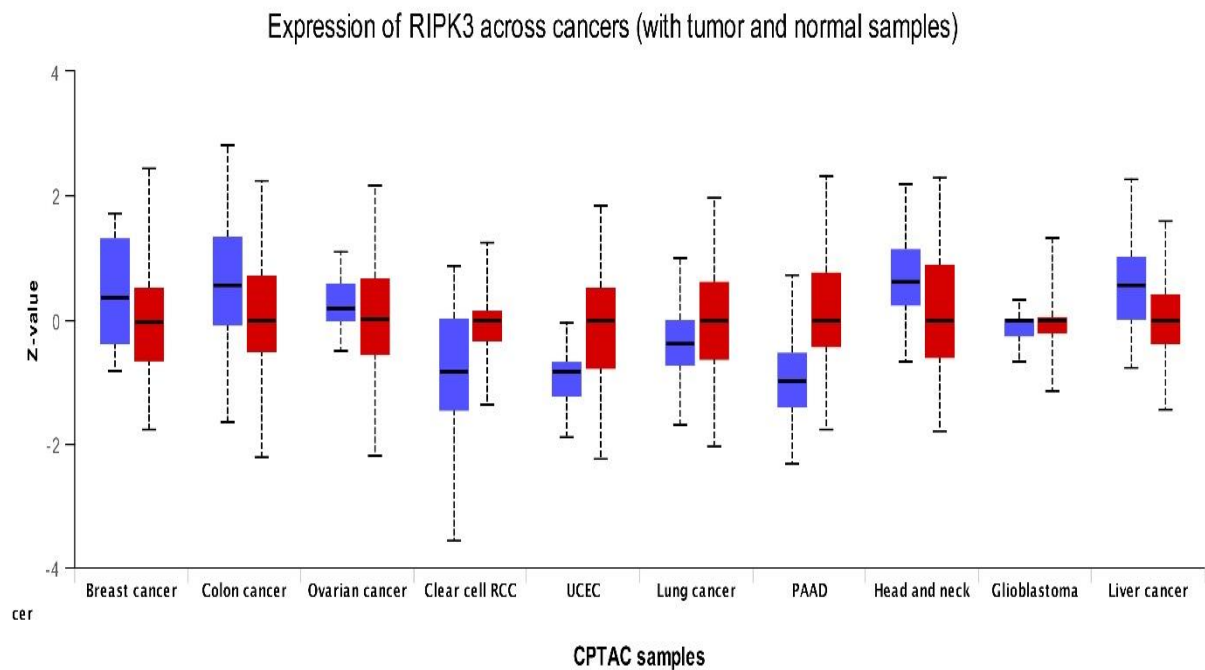
| Cancer Name | Normal Samples<br>Median Value | Tumor Cancer<br>Median Value | Statistical<br>significance<br>(P value) |
|-------------|--------------------------------|------------------------------|------------------------------------------|
| BLCA        | 3.327                          | 3.328                        | 1.55                                     |
| BRCA        | 3.11                           | 3.127                        | 7.8E <sup>-08</sup>                      |
| CESC        | 2.899                          | 3.41                         | 0.2034800                                |
| CHOL        | 0.618                          | 3.01                         | 1.12E <sup>-06</sup>                     |
| COAD        | 5.034                          | 4.187                        | 2.48E <sup>-10</sup>                     |
| ESCA        | 2.371                          | 3.323                        | 0.3894000                                |
| GBM         | 0.921                          | 1.841                        | 3.07E <sup>-06</sup>                     |
| HNSC        | 3.206                          | 2.991                        | 0.07110400                               |
| KICH        | 2.624                          | 1.332                        | 5.44E <sup>-08</sup>                     |
| KIRC        | 2.248                          | 2.213                        | 0.9960600                                |
| KIRP        | 2.47                           | 1.243                        | 6.06E <sup>-05</sup>                     |
| LIHC        | 0.595                          | 0.641                        | 0.09967600                               |
| LUAD        | 3.774                          | 3.75                         | 0.6356000                                |
| LUSC        | 3.822                          | 2.794                        | <1E <sup>-12</sup>                       |
| PAAD        | 4.186                          | 4.282                        | 0.9306200                                |
| PRAD        | 1.886                          | 1.144                        | 2.22E <sup>-06</sup>                     |
| PCPG        | 0.658                          | 0.872                        | 0.4982800                                |
| READ        | 4.89                           | 4.254                        | 0.002681900                              |
| SARC        | 1.723                          | 1.982                        | 0.5175600                                |
| SKCM        | 3.198                          | 1.805                        | 3.1008E <sup>-01</sup>                   |
| THCA        | 3.843                          | 3.835                        | 0.2698200                                |
| THYM        | 3.922                          | 3.087                        | 0.005943700                              |
| STAD        | 3.636                          | 3.98                         | 0.5182600                                |
| UCEC        | 2.565                          | 2.854                        | 1.18E <sup>-06</sup>                     |

### **5.1.2 RIPK3 Proteomic is differentially Protein expressed in cancer**

**Table 5.2: Expression of RIPK3 across CPTAG cancer samples (Normal vs tumor)**

The RIPK3 proteomic expression was analyzed using the UALCAN CPTAC datasets. It has showed that the Upregulation of RIPK3 in Clear cell RCC, Uterine Corpus Endometrial Carcinoma (UCEC), Pancreatic adenocarcinoma (PAAD), Lung cancer and downregulation in the Breast cancer, colon cancer, ovarian cancer and in liver cancer. The differential expression of RIPK3 indicates its involvement in the tumor progression and can be potential as a target for therapeutic intervention.

The analysis approach is to compare the expression levels of RIPK3 between tumor and normal tissues within each cancer type. Here I have used analyzed the Box whisker plot to show the expression level and significance of difference was estimated by the t-test.

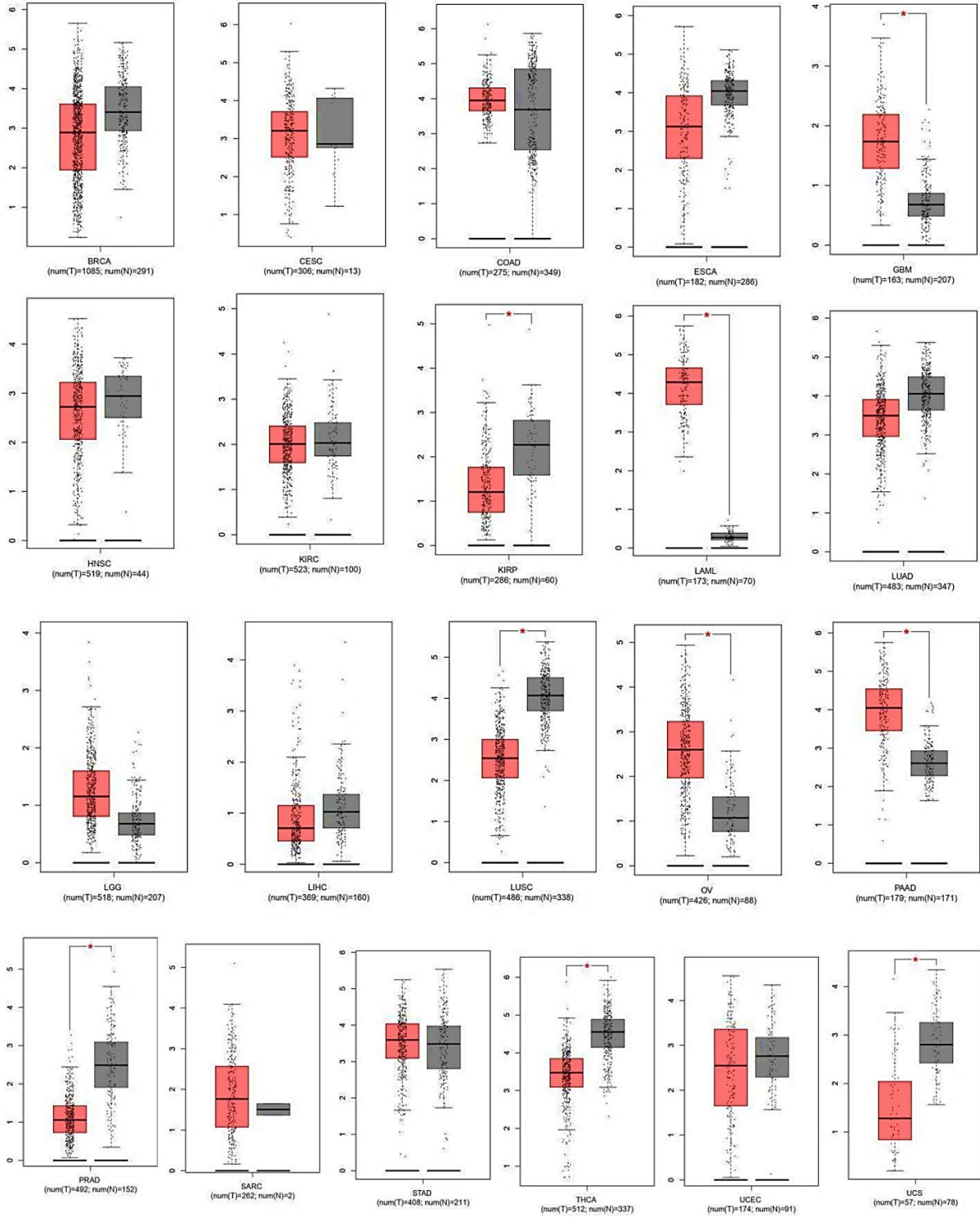


**Fig 5.2: Expression of RIPK3 across CPTAC cancer**

| <b>CANCER NAME</b>                   | <b>Normal Sample Media Value</b> | <b>Tumor Sample median vale</b> | <b>Statistical significance (P value)</b> |
|--------------------------------------|----------------------------------|---------------------------------|-------------------------------------------|
| Breast Cancer                        | 0.352                            | -0.045                          | 0.09749621                                |
| Colon Cancer                         | 0.556                            | 0                               | 0.002918914                               |
| Ovarian Cancer                       | 0.193                            | 0.004                           | 0.07331065                                |
| Clear cell RCC                       | -0.838                           | 0                               | 3.58E <sup>-11</sup>                      |
| Uterine corpus endometrial carcinoma | -0.833                           | -0.01                           | 1.019E <sup>-09</sup>                     |
| Lung adenocarcinoma                  | -0.38                            | -0.01                           | 0.006257583                               |
| Pancreatic adenocarcinoma            | -0.982                           | -0.006                          | 1.094E <sup>-19</sup>                     |
| Head and neck squamous carcinoma     | 0.609                            | 0                               | 1.94E <sup>-05</sup>                      |
| Glioblastoma Multiforme              | 0                                | 0                               | 0.03426241                                |
| Hepatocellular carcinoma             | 0.565                            | 0                               | 1.403E <sup>-12</sup>                     |

### ***5.1.3 Validation of RIPK3 gene expression in cancer by Boxplot analysis of GEPIA***

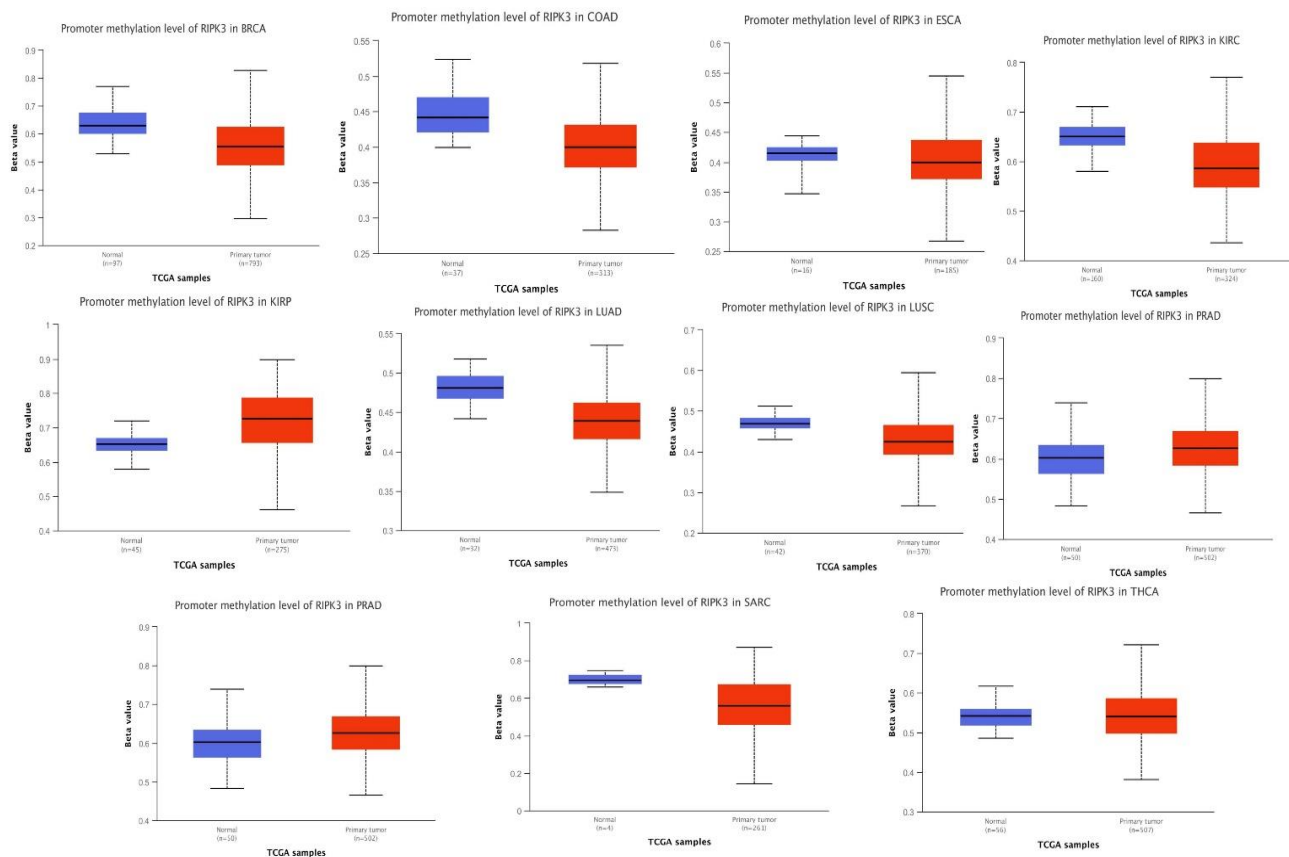
The gene expression of the RIPK3 was also analyzed by Boxplot analysis of TCGA datasets in GEPIA. It showed that the RIPK3 upregulation significance of the Glioblastoma multiforme (GBM) and the Kidney renal papillary cell carcinoma (KIRP), Lung squamous cell carcinoma (LUSC), Prostate adenocarcinoma (PRAD) were downregulated significantly [Fig 2]. The results of the gene expression analysis of RIPK3 by using the TCGA dataset has shown the similar regulations. Since the gene expression showed the upregulation, we investigated its protein expression.



**Fig 5.3: RIPK3 expression validation by boxplot analysis**

### 5.1.4 Methylation of RIPK3 among cancers

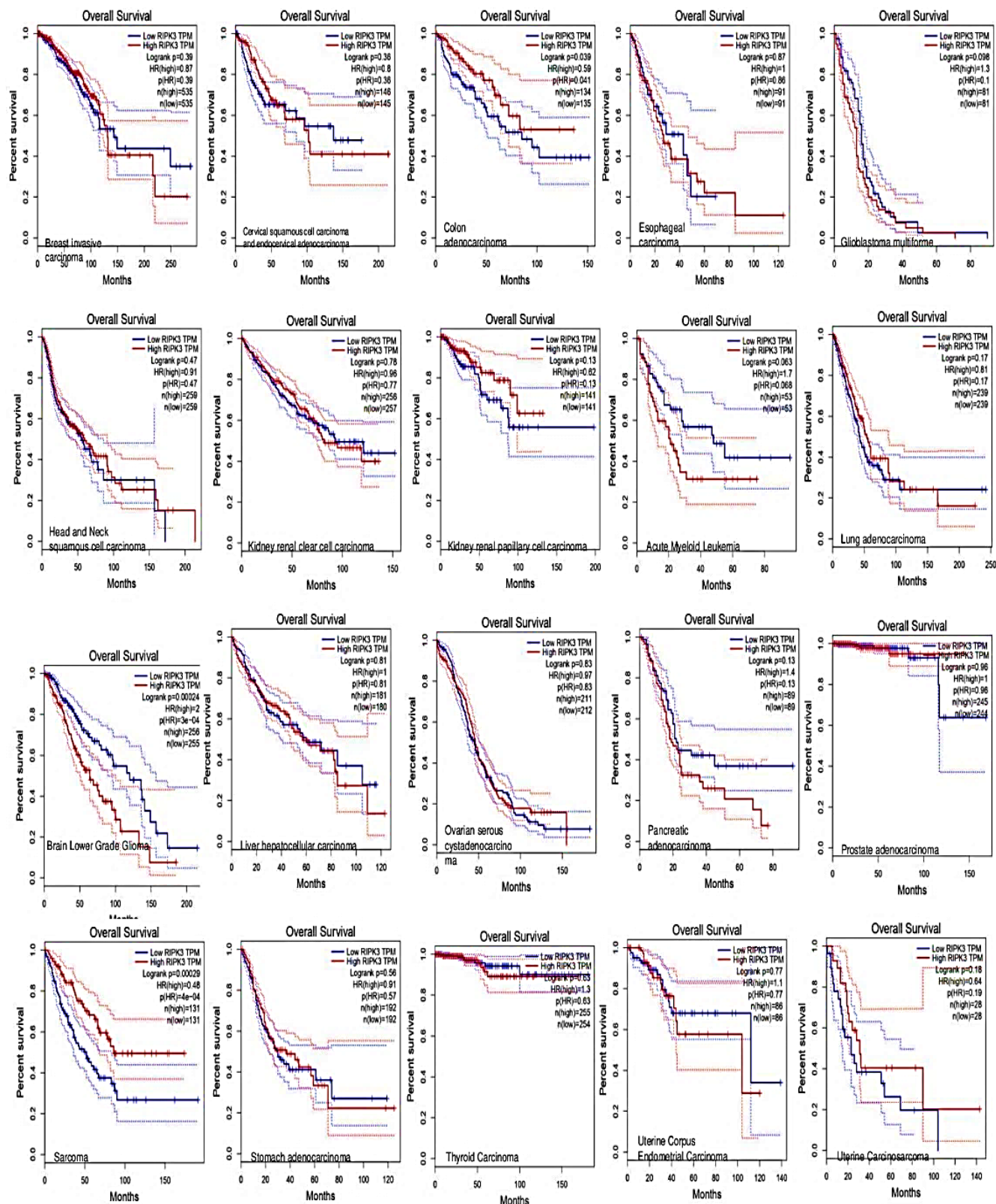
To check if the methylation plays important role in RIPK3 differential expression among cancer. The methylation status analysis is done by UALCAN with the TCGA datasets which revealed RIPK3 is hypomethylated in Breast invasive carcinoma (BRCA), Colon adenocarcinoma (COAD), Kidney renal clear cell carcinoma (KIRC), Lung adenocarcinoma (LUAD), Sarcoma (SARC) and it is significant which indicates increased expression in cancer. Hypomethylation status in cancer is the indication of RIPK3 is involved in cancer development unlike its role as important part of necroptotic pathway. As RIPK3 showed differential expression of hypomethylated status, we further analyze to investigate the survival status based on expression of RIPK3.



**Fig 5.4: Methylation status of RIPK3 in pan cancer**

#### ***5.1.5 RIPK3 survival analysis in differential cancer***

Survival analysis using GEPIA datasets showed that the majority of the cancers did not show a significant differentiation based on the RIPK3 expression. However, Glioblastoma multiforme ( $p = 0.0002$ ) and Sarcoma ( $p = 0.0002$ ) have showed significant survival status. On conclusion, the higher the expression of RIPK3 will results lower the survival rate.



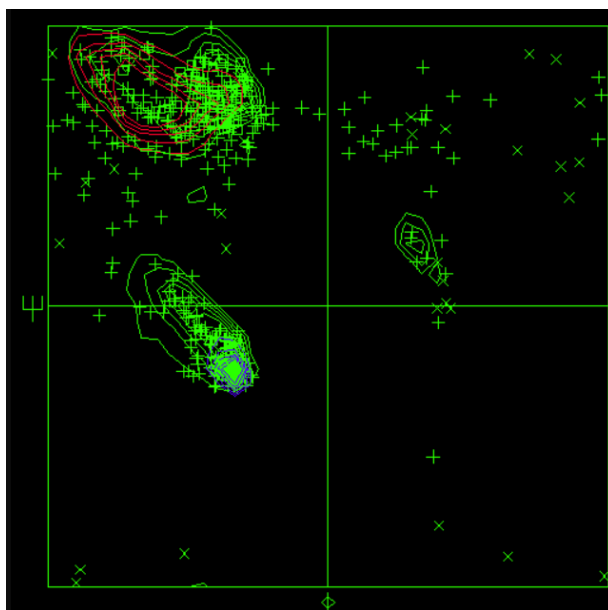
**Fig 5.5: Survival analysis for Pan cancer**

## 5.2 Selection of Natural compounds

Natural compounds were selected from the various kind of plant species and listed up to thousand metabolites with their molecular weight.

## 5.3 Preparation of RIPK3 3D structure for docking

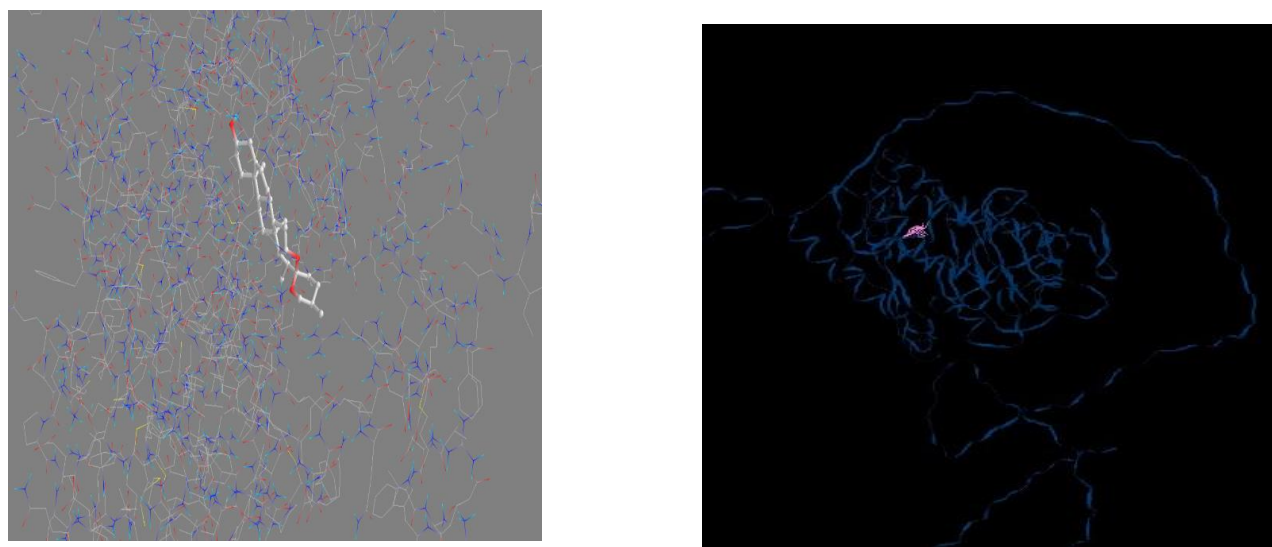
Human RIPK3 structure was downloaded from the Uniprot (<https://www.uniprot.org/>) which is comprised of characteristic structure and catalytic activity domains with N-terminal kinase domain C-terminal homotypic interaction. The Ramachandran Plot indicates the predicted 3D structure of protein contains phi and psi angles that are feasible and it showed 85% of favored configuration. The RIPK3 3D structure was saved as PDB file. The selected natural compounds were docked with the RIPK3 using the PyRx and iGem dock.



**Fig 5.6: Ramachandran Plot of RIPK3**

#### **5.4 Molecular docking of RIPK3 with the selected compounds**

The selected compounds were docked with RIPK3 by using the PyRx docking software. The docked results were analyzed using the Root Mean Square Deviation and the binding affinity. The lower the RMSD and higher the negative value of binding affinity which indicates the better agreement between the docked pose and the crystal structure. From the thousand compounds with the above-mentioned criteria, it was shortlisted as top fifty compounds. The validation of the top 50 compounds was carried out by iGem dock. The RIPK3 was docked with the shortlisted 50 compounds and the results output was delivered by the means of Hydrogen bonding and the Vander Waal's cut off and the interacting amino acids. Natural metabolites such as (-)-Carene (-)-3-Carene, (-)-Roemerin, (+)-Coclaurin, (+)-Homoaromoline, (+)-Taxusin, (+)-Veraguensin, (Z)-Jasmone, 11-Octadecenoic acid, 17 beta-hydroxyazadiradione were identified. In addition, with repositioned drug category manner the diosgenin was found to have binding affinity and energy of -10.2 and -84.61 Kcal/mol respectively. Although, Diosgenin had moderate binding energy toward RIPK3 compared to other metabolites, it was included due to its promising anti-cancer activity and anti-inflammatory properties against the breast cancer in previous reports and studies. It also showed the highest affinity among the other re-pursuable drug candidates included in the studies.



**Fig 5.7: Visualization of RIPK3 and Diosgenin**

**Table 5.3: Docking Validation of PyRx and iGem**

| Ligand                                         | Docking score using PyRX | Docking score using iGEM |
|------------------------------------------------|--------------------------|--------------------------|
| (-)-Carene(-)-3-Carene                         | -10.5                    | -6.48222                 |
| (-)-Roemerin                                   | -9.8                     | -18.0266                 |
| (+)-Coclaurin                                  | -10.7                    | -13.1668                 |
| (+)-Homoaromoline                              | -12.5                    | -9.53713                 |
| (+)-Taxusin                                    | -9.9                     | 0                        |
| (+)-Veraguensin                                | -10                      | -18.1153                 |
| (Z)-Jasmone                                    | -10.2                    | -21.9582                 |
| 11-Octadecenoic acid                           | -12.3                    | 0                        |
| 17-Acetoxylophotoxin                           | -10.3                    | -10.0307                 |
| 21-Hydroxyoligomycin_A                         | -10.2                    | 0                        |
| 22,23-Dihydronimocinol                         | -10.4                    | 0                        |
| 2alpha- 3beta-Dihydroxyolean-12-en-28-oic_acid | -11                      | -10.7587                 |
| 3,7-Dimethoxyflavone                           | -10.4                    | 0                        |
| 3'-N-Debenzoyltaxol                            | -11.9                    | -14.0157                 |
| 5 7-Dihydroxy-8 2'-dimethoxyflavone            | -10.8                    | -11.2037                 |
| 8-Methylsulfinyloctyl glucosinolate            | -9.7                     | -18.7479                 |
| 9 15-Octadecadienoic_acid                      | -10.7                    | 0                        |
| 9-Ribosyl-trans-zeatin                         | -10.8                    | -14.5976                 |
| Ambonic_acid                                   | -11.1                    | -2.5                     |
| Anhydrocinanzeylanine                          | -11.9                    | -7.61623                 |
| aristolochic_acid                              | -10                      | -23.437                  |
| Aureusidin                                     | -10                      | -14.7752                 |
| Azadirachtin A1                                | -11.6                    | -14.6446                 |
| Azadirachtin B2                                | -12                      | -10.0174                 |
| Azadirachtol                                   | -11.4                    | -3.5                     |
| Azadiradione                                   | -11.2                    | -11.1238                 |
| Azadiradionolide 1                             | -11.7                    | -14.6647                 |
| Azadiradionolide 2                             | -10.8                    | -11.7134                 |
| Azadirone                                      | -11.7                    | -16.8196                 |
| Azan                                           | -10.7                    | -2.79676                 |
| Cannabidiolic_acid 2                           | -10                      | 0                        |
| Cannabigerolic_acid                            | -10.4                    | -3.5                     |
| Cannflavin_A                                   | -10.1                    | -15.658                  |
| Cyclo-DOPA                                     | -9.9                     | -18.6762                 |
| Diosgenin                                      | -10.2                    | -4.88252                 |
| Hypericin                                      | -11.2                    | -26.1173                 |
| Lactucain A                                    | -11.4                    | -20.3418                 |
| Lactucain_B                                    | -9.9                     | -11.0393                 |
| Limonin                                        | -10.1                    | -5.92995                 |
| Lupeol_acetate                                 | -9.9                     | 0                        |

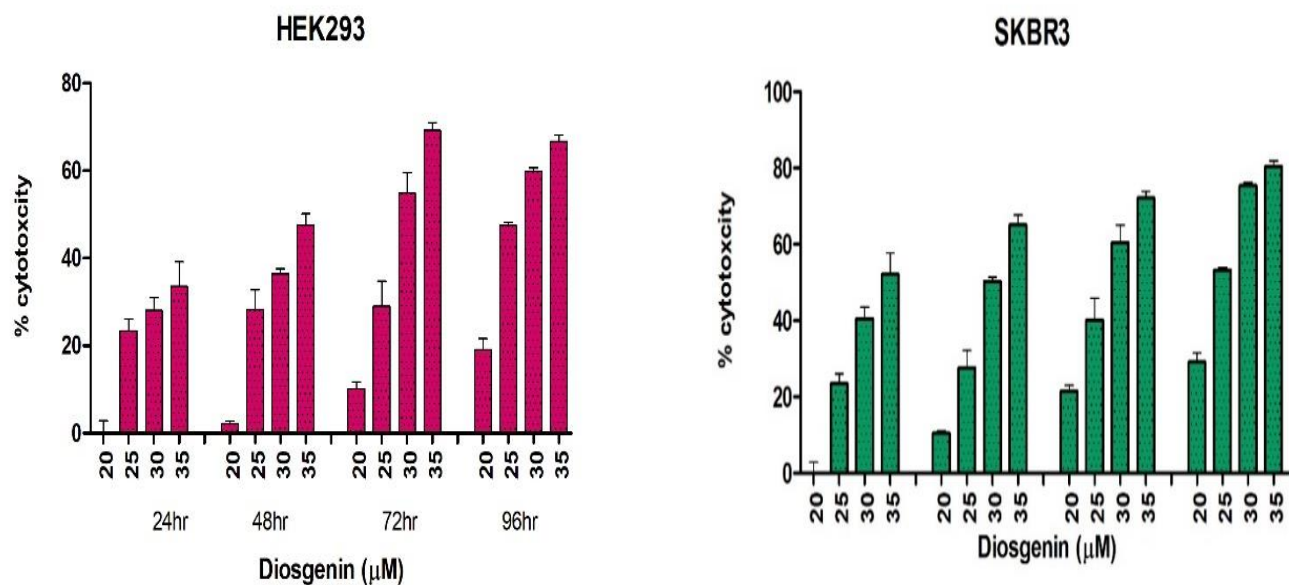
|                               |       |          |
|-------------------------------|-------|----------|
| Oleanolic acid                | -10   | -5.21879 |
| Prunin 6"-p-coumarate         | -10   | -14.8518 |
| Pseudohypericin               | -10.8 | -29.5851 |
| psi-Taraxastanonol- 3,20-diol | -10.4 | -8.72841 |

### **5.5 Invitro Cell Culture**

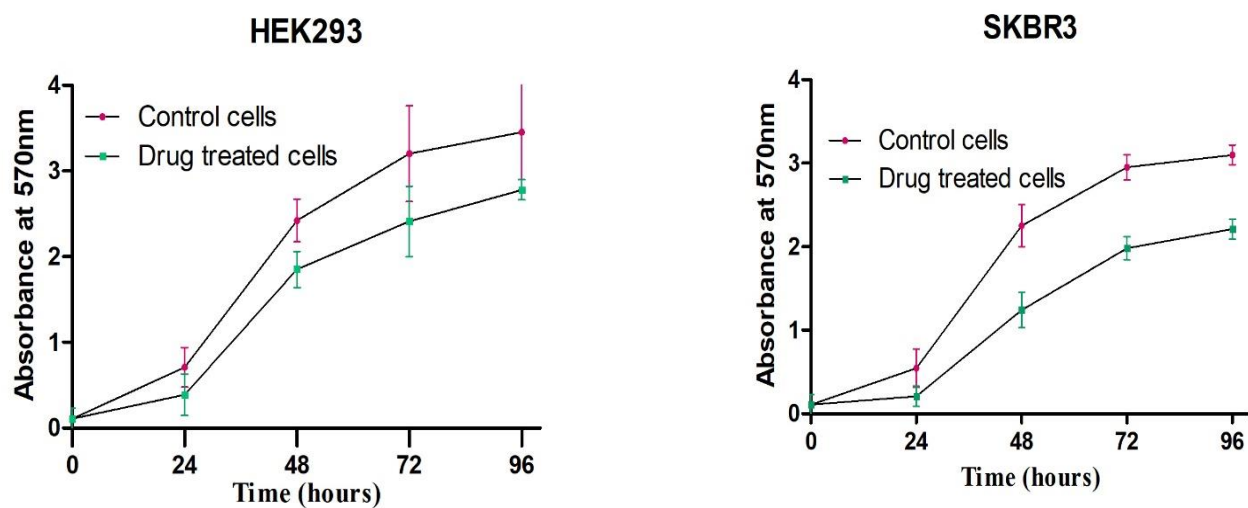
Human breast cancer cells (SKBR3) and human embryonic kidney 293 (HEK293) cells were obtained from the Cancer Institute (WIA), Adyar, Chennai. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) FBS, Cells were kept at 37°C in a humid atmosphere in a 5% CO<sub>2</sub> incubator. The cell line supplies were frozen in liquid nitrogen using a 70% FBS, 20% DMEM freezing mixture. The contamination analyses were frequently analyzed using Gram Stain and PCR.

### **5.6 Cell Viability Assay**

The effect of lead compounds on cell viability was measured by MTT assay following the method by (Bahuguna *et al.*, 2017). Briefly, the cells (2000 cells/well) were seeded in a 96 well microtiter plate (100 µl/well). Treatments were done for different time intervals at different concentrations. After incubation, 10 µl of MTT solution (5mg/ml) was added to each well and incubated for 4h at 37°C. Formazan crystals were solubilized with 100 µl dimethyl sulfoxide (DMSO) and the absorbance was measured at 590 nm using a microplate reader (BioTek Instruments, Inc. USA). The results were shown that the drug treatment have significantly reduced the cell viability of the SKBR 3 cancer cell line and has less effect on the normal control cell line (HEK293). The drug treated cells have shown significant cytotoxicity when compared to the normal control cell lines. The IC<sub>50</sub> value was 30mM and it was analyse using the GRAPHPAD prism software.



**Fig 5.8: Effect of treatment on cytotoxicity**

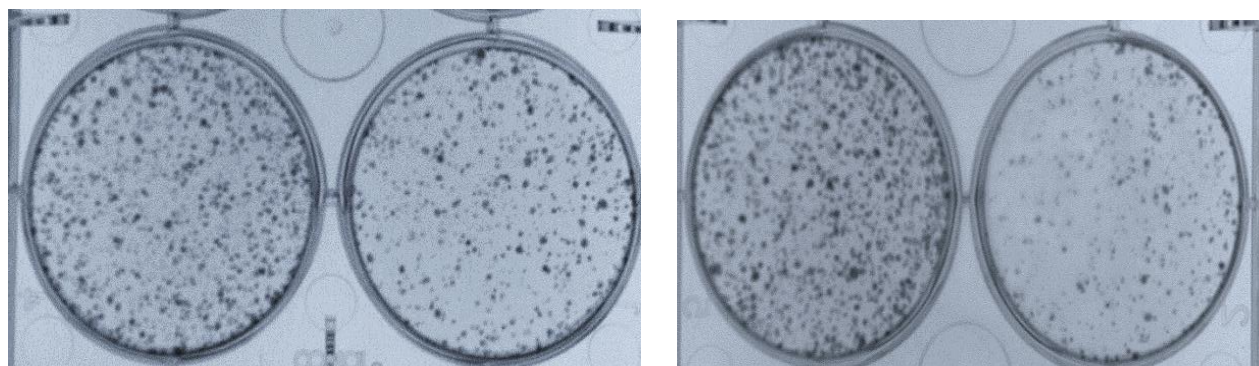


**Fig 5.9: Growth Curve of cells**

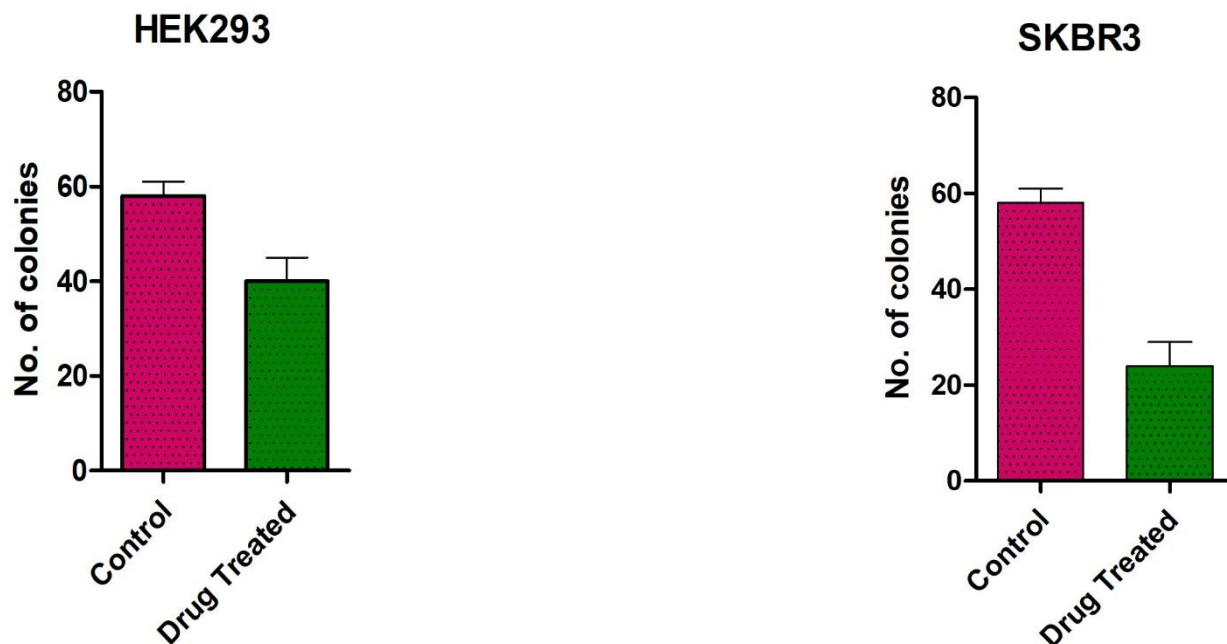
### **5.7 Clonogenicity observation**

A clonogenic assay was used to determine and test the proportion of HRK293 and SKBR3 cells that are capable of producing colonies of more than 32 cells when cultivated in drug treated media. were the media evaluated. Before measuring CFE, the cells were exposed to various conditions and some dramatic impacts on CFE were observed. The cells were seeded and grown in the Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) FBS which is treated with 10 $\mu$ l of drug.

After the incubation, the colonies were treated with methanol and crystal violet observed under Epic white by Gel dock. Diosgenin treated wells have shown less growth significance when compared to the untreated cells. The drug treated cell line have shown major difference when compared to the control cells. The drug had major effect on the SKBR3 cell where it approximately formed only 20 colonies whereas untreated cells have formed 60 colonies and the drug has less effect on the HEK293.



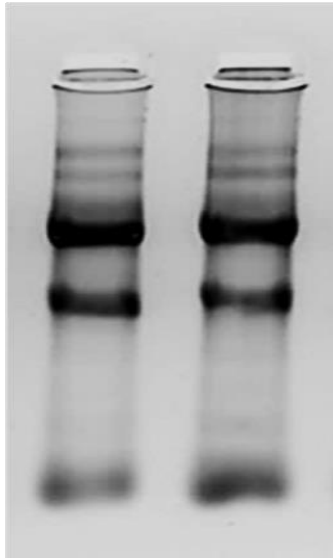
***Fig 5.10: Colonies formation of cells***



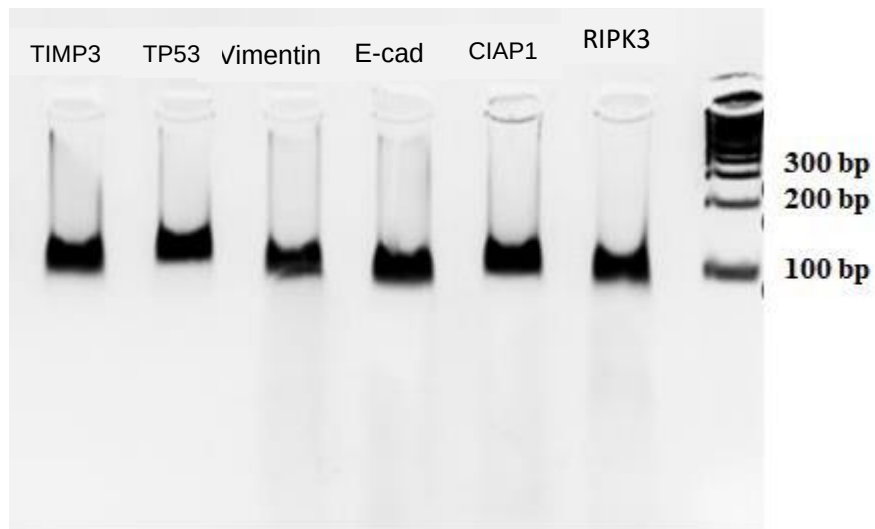
**Fig 5.11: Effect of diosgenin on the cell lines**

### **5.8 Gene Expression on the Cancer**

Evidence from the literature suggested that RIPK3 genes could play a potential role against the breast cancer. Hence, RIPK3 and other important protein markers like CIAP1, E-cadherin, Vimentin, TP53 and TIMP3 were also analyzed to identify the effect of the diosgenin. qRT-PCR study of genes was carried on the HER+ breast cancer cell. The study on the HER+ breast cancer cells revealed significant changes ( $\geq$  fold change) in the gene expression. Agarose gel electrophoresis was used to confirm the stability and integrity of total RNA from each cell line. cDNA conversion was carried out for the cell line, and the primer annealing temperature was standardized. The annealing temperature for the genes was found to be RIPK3 (58°C), CIAP1 (56°C), E-cadherin (60°C), Vimentin (59°C), TP53 (56°C), TIMP3 (57°C). A prominent band in agarose gel electrophoresis indicated the ensuing single amplicon.

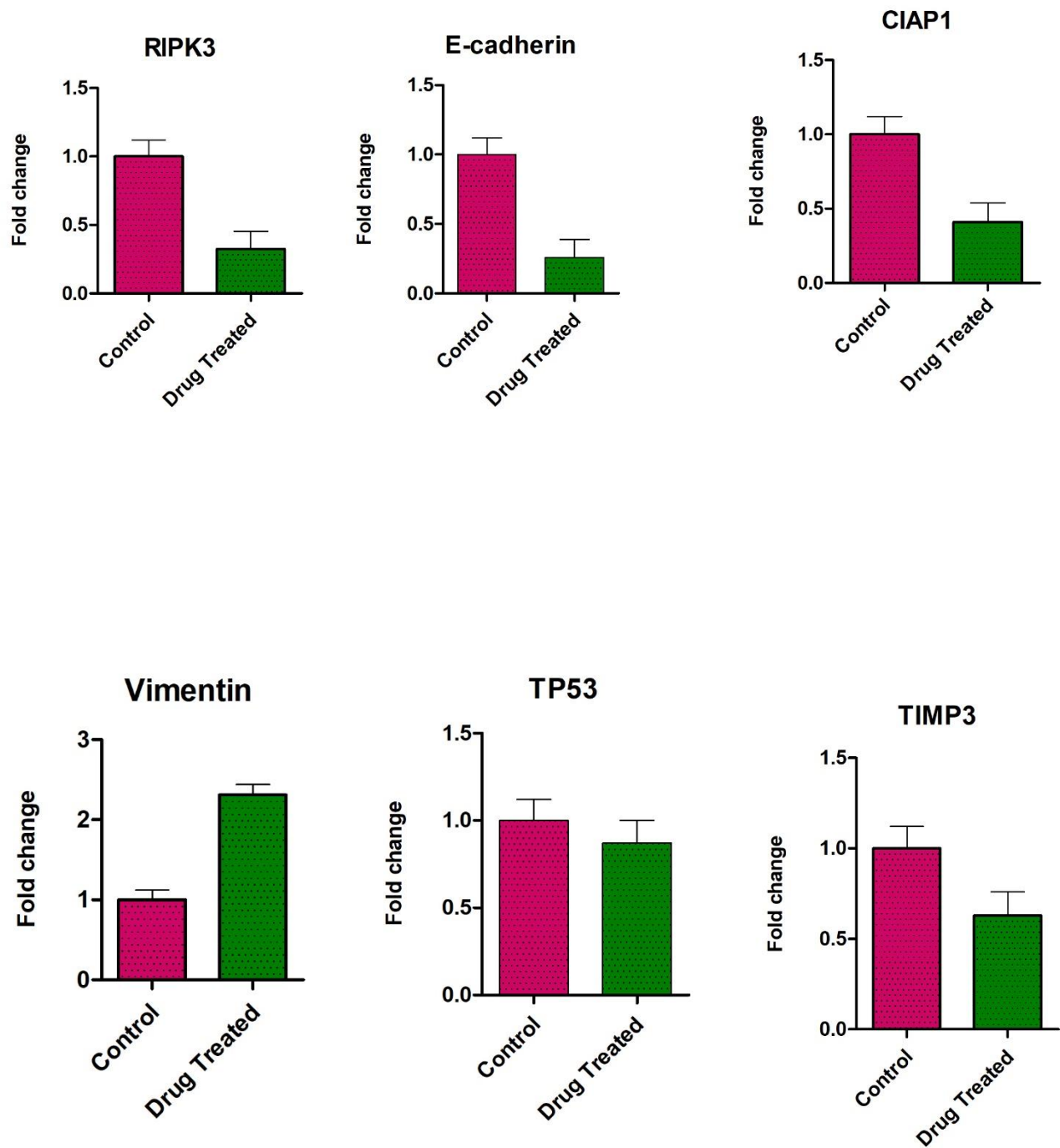


***Fig 5.12: 1% Agarose Gel Electrophoresis of total RNA isolated from cells lines***



***Fig 5.13: 2% agarose gel electrophoresis showing PCR amplification of 6 genes run along with DNA ladder.***

All the Diosgenin drug treated genes tested showed differential expression in comparison to control cells in the cell lines. 5/6 genes were found to be upregulated, whereas 1/6 were found to be downregulated. The following genes treated with the diosgenin RIPK3, CIAP1, E-cadherin, TP53 and TIMP3 have been significantly downregulating the breast cancer cells when compared to the control cells in this study and the Vimentin was upregulating the cancer cells when compared to the control cells. Gene expression studies show a broad change in the expression levels of genes in cancer cell lines. All the Diosgenin drug treated genes tested showed differential expression in comparison to control cells in the cell lines. 5/6 genes were found to be downregulated, whereas 1/6 were found to be upregulated. The following genes, RIPK3, CIAP1, E-cadherin, TP53 and TIMP3 showed significant Upregulated in breast cancer cells in diosgenin treated cells when compared to control. Vimentin showed downregulated in the cancer cells. Gene expression studies shown a broad change in the expression levels of cancer associated genes in diosgenin treated cancer cell lines.



**Fig 5.14: qRT-PCR based gene expression.** HER+ cell line of breast cancer was analyzed with RIPK3 and other important genes. The fold change was based on the control cell line HEK293. Beta-actin was used as the house keeping gene.

**TABLE 5.4: Gene expression analysis of qRT-PCR**

| Control Cell line |        |        |        | Beta-actin | Beta-actin | Beta-actin | del ct | del ct | del ct |
|-------------------|--------|--------|--------|------------|------------|------------|--------|--------|--------|
| RIPK3             | 22.498 | 25.625 | 23.529 | 21.119     | 21.409     | 21.177     | 1.379  | 4.216  | 2.352  |
| E-cadherin        | 22.748 | 23.881 | 22.861 | 21.119     | 21.409     | 21.177     | 1.629  | 2.472  | 1.684  |
| CIAP 1            | 23.178 | 22.266 | 23.169 | 21.119     | 21.409     | 21.177     | 2.059  | 0.857  | 1.992  |
| Vimentin          | 22.156 | 23.294 | 22.387 | 21.119     | 21.409     | 21.177     | 1.037  | 1.885  | 1.210  |
| TP53              | 22.225 | 24.135 | 26.996 | 21.119     | 21.409     | 21.177     | 1.106  | 2.726  | 5.819  |
| foxc2             | 23.056 | 31.121 | 25.888 | 21.119     | 21.409     | 21.177     | 23.056 | 9.712  | 4.711  |

| Cancer cell line |        |          |        | Beta-actin | Beta-actin | Beta-actin | $\Delta$ CT | $\Delta$ CT | $\Delta$ CT | $\Delta\Delta$ CT | $\Delta\Delta$ CT | $\Delta\Delta$ CT |
|------------------|--------|----------|--------|------------|------------|------------|-------------|-------------|-------------|-------------------|-------------------|-------------------|
| RIPK3            | 34.23  | 33.814   | 35.23  | 21.258     | 21.628     | 21.062     | 12.972      | 12.186      | 14.168      | 2.035             | 2.473999          | 2.456999          |
| E-cadherin       | 28.369 | 30.144   | 32.404 | 21.258     | 21.258     | 21.062     | 7.111       | 8.885999    | 11.342      | -1.995            | 0.159999          | 2.522999          |
| CIAP1            | 24.161 | 24.889   | 24.469 | 21.258     | 21.628     | 21.062     | 2.903       | 3.261       | 3.407       | -0.156            | 0.403999          | 0.414999          |
| TIMP3            | 19.154 | 19.436   | 20.264 | 21.258     | 21.628     | 21.062     | -2.104      | -2.192      | -0.798      | -0.483            | -0.408            | 0.85              |
| TP53             | 28.342 | 29.145   | 28.334 | 21.258     | 21.628     | 21.062     | 7.084001    | 7.517       | 7.271999    | -0.545            | 0.045             | -0.412            |
| Vimentin         | 24.826 | 24.94065 | 24.826 | 21.258     | 21.628     | 21.258     | 3.568001    | 3.312654    | 3.568       | -1.469            | -1.57235          | -1.642            |

## Calculation of fold change

The fold change of the gene expression is calculated by:

$$\text{Fold change} = 2^{(-\Delta\Delta CT)}$$

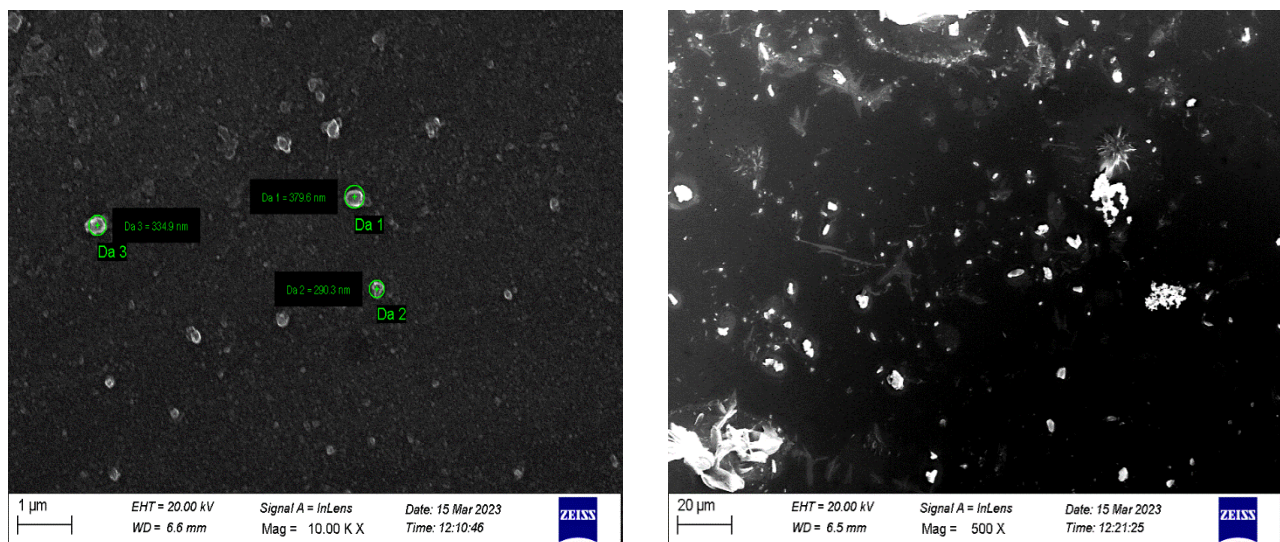
For example, the fold change value of the RIPK3 is calculated from del del ct value is 0.244007966

$$\text{Fold change} = 2^{-(2.035)} = 0.244$$

Then the standard deviation of all the fold change was calculated, to calculate the gene expression values of <0.5.

## 5.9 Nanoparticle enhanced drug activity

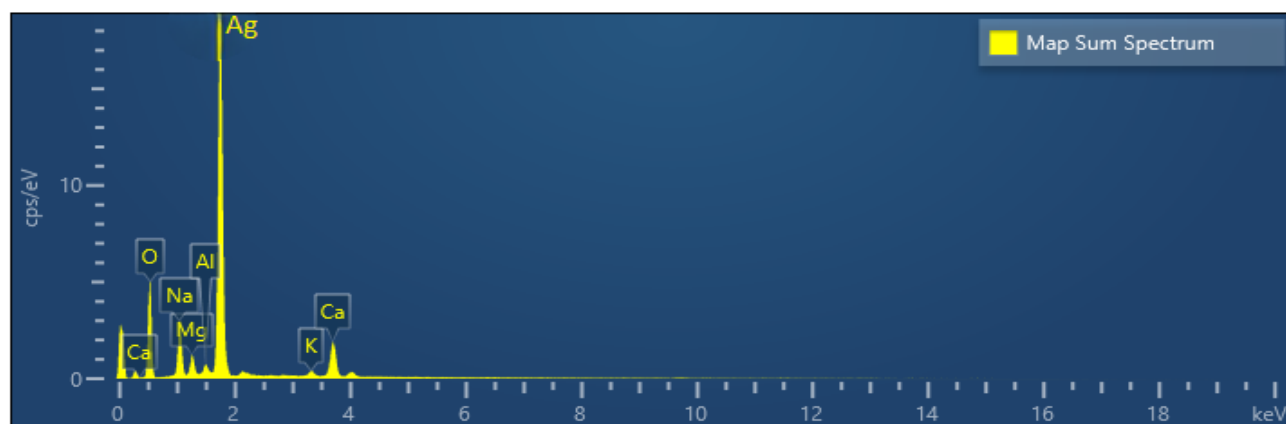
The activity of the diosgenin was confirmed by the above carried out experiment results, with that evidence the Silver (Ag) nanoparticle was added with the diosgenin. SEM were used to analyze the morphology of silver conjugated diosgenin. The figure shows the morphology of the Ag with the diosgenin. The Ag particles were integrated with diosgenin surface.



**Fig 5.15: Morphology images of Ag and diosgenin by SEM analysis**

### 5.12.1 EDX analysis

In order to confirm the formation of Ag nanoparticle with the diosgenin complex EDX analysis was performed. During the EDX measurements different areas were focused and corresponding peaks are shown in the figure. Details of the EDX spectra values measured in atomic and weight % are listed in the table

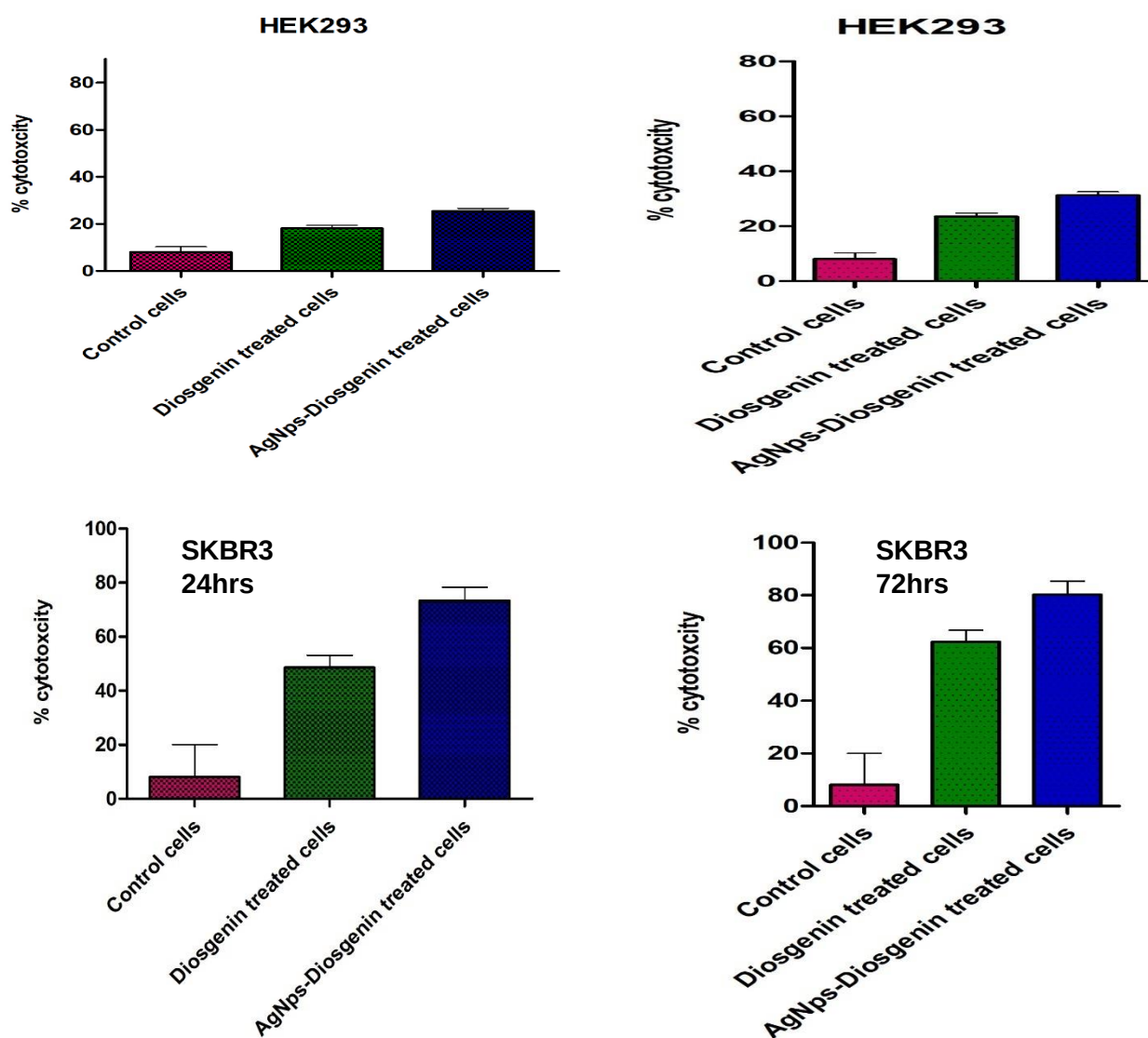


| Element | Line Type | Weight % | Weight % Sigma | Atomic % |
|---------|-----------|----------|----------------|----------|
| O       | K series  | 42.08    | 0.29           | 56.00    |
| Na      | K series  | 8.75     | 0.13           | 8.10     |
| Mg      | K series  | 2.57     | 0.08           | 2.25     |
| Al      | K series  | 0.99     | 0.06           | 0.78     |
| Ag      | K series  | 38.00    | 0.23           | 28.81    |
| K       | K series  | 0.86     | 0.06           | 0.47     |
| Ca      | K series  | 6.75     | 0.10           | 3.58     |
| Total   |           | 100.00   |                | 100.00   |

**Fig 5.16: EDX pattern of Ag and diosgenin composite**

### 5.12.2 Cell viability assay

The silver nanoparticle conjugated diosgenin was used to treat the cells in the cell viability assay by previously discussed way. Treatments were done for different time intervals at different concentrations. The results have shown significantly increased effect on the cells by improved reduction of cell viability and proliferation when compared to the pure diosgenin treatment. From the result I conclude that the silver (Ag) particles conjugated with the drug can improve and enhance the activity of the drug against the cancer cells which needs a further more study.



**Fig 5.17: Effect of Ag integrated diosgenin treated cell lines**

## 6.SUMMARY AND CONCLUSION

The effect of the diosgenin on RIPK3 and its associated pathways was studied using HER2+ Breast cancer cells. When the diosgenin treated cells compared with the control cells, we could see significance downregulation of RIPK3 indicating that diosgenin can directly affect the RIPK3 associated inflammatory pathways. Some of the important protein markers were also analyzed to identify the effect of diosgenin treatment on HER2+ breast cancer

The inhibitor of RIPK3 associated with pathway CIAP1 also shown downregulation in drug treated cells indicating significant targeting of necroptotic pathway.

To analyze the effect of Epithelial-to-Mesenchymal Transition (EMT) in the cancer the E-Cadherin and Vimentin was also analyzed. The diosgenin treated cells showed downregulation of the E- cadherin when compared to the control cells. In the case of vimentin, it has shown upregulation when compared to the control. So, the results indicate direct effect of the diosgenin on modulation of gene expression which affecting the EMT transmission.

To analyze the effect of apoptotic signaling pathway we chose TP53 as a protein marker to check whether diosgenin induce TP53 release from the mitochondria and induce apoptosis and its mRNA expression was quantified. Diosgenin treated cells did not shown significance when compared to the control, further experiment needed to analyze the TP53 dependent and independent pathway.

TIPM3 was chosen to check whether the diosgenin can alter mitochondria and affects its energy production in cancer. TIMP3 gene expression was analyzed in the drug treated cells. TIMP3 was shown moderate downregulation in the diosgenin treated cells when compared to the control cells which indicates that the diosgenin can modulate the mitochondrial metabolism. However, further analysis is required to check the immediate effect on the mitochondrial membrane protein n and its energy production.

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## ANNEXURE

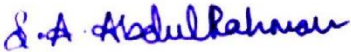
### Number of Patent applied

### Patent 1: Method to prepare natural sprinkling powder to treat open wounds

TITLE : METHOD TO PREPARE NATURAL SPRINKLING POWDER TO TREAT OPEN WOUNDS

ALLOTTED REF.NO – B1

WE NEED THE FOLLOWING DETAILS (MUST) FOR EVERY SINGLE APPLICATION

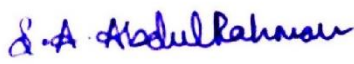
| S.NO | INVENTOR NAME                                   | AFFILIATION WITH FULL ADDRESS                           | SIGNATURE SOFTCOPY<br>(USE BLUE PEN WITH WHITE BACKGROUND)                            |
|------|-------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------------------------------------|
| 1    | Sathyabama Institute of Science and Technology, |                                                         |                                                                                       |
| 2    | Syed Ali Abdul Rahman                           | Sathyabama Institute of Science and Technology, Chennai |   |
| 3    | VISHWANATHAN. J                                 | Sathyabama Institute of Science and Technology, Chennai |   |
| 4    | Dr.R.P.Oviya                                    | Sathyabama Institute of Science and Technology, Chennai |  |

## Patent 2: Method for preparation of lip balm using natural extracts

**TITLE :** Method for Preparation of Lip balm using Natural Extracts

**ALLOTTED REF.NO – A10**

**WE NEED THE FOLLOWING DETAILS (MUST) FOR EVERY SINGLE APPLICATION**

| S.NO | INVENTOR NAME                                            | AFFILIATION WITH FULL ADDRESS                                    | SIGNATURE SOFTCOPY<br>(USE BLUE PEN WITH WHITE BACKGROUND)                           |
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Paper Manuscript was submitted to the journal

