In-vitro miRNA screening of breast cancer MDA MB 231 cell lines on treatment of lipids from medicinal plants

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Abstract:

MicroRNA – a key regulator of gene expression which are conserved across species. The regulation of miRNA biogenesis was influenced by precursors and regulated by their subsequent mature miRNA processing. These protein regulators are bound to the mature miRNA that directs degradation and preventing their expression. Most common regulatory protein is Lin28, which will bind let-7 miRNA and its target degradation. Cancer –a genetic disease, involves changes in structure and gene expression. The main mechanism of cancer cells is to attack and destroy normal cells due to body imbalance and can be treated by correcting the unevenness. A change in expression implicated miRNA down regulation in cancer cells and reintroducing miRNAs in animals have impaired cancer cell viability and cell death. Researchers in worldwide validated "miRNA replacement therapy" theory involves introduction of either miRNAs mimetic or synthetic miRNA in affected tissues, to restore the normal proliferation, cell cycle, apoptosis and cellular mechanism, which makes pharmaceutical industry to develop therapeutic and diagnostic targets. This study aimed to check miRNA stability and miRNA modulations on treatment with phyto-lipids isolated from medicinal plants. Calculated cell viability percentage showed increased cell stability in lipid treatment samples indicating the longevity property exhibited by the lipid extracts. In-vitro cytotoxicity assay on Breast cancer cell line MDA MB 231 treated with lipid extracts dissolved in DMSO, revealed the anti-cancerous property of *Alpinia galanga* exhibited at low IC50 Value of 774.3 µM/ ml.

Keywords: MicroRNA, Oncomir, Phyto- lipids, MDA MB 231, Breast cancer, *Alpinia galanga*

Introduction:

Micro RNA - small 19-23 nucleotide molecules acts as a transduction regulator of cellular, physiological processes and genetic networks, and. The coding sequences of miRNA were distributed widely throughout the genome located in untranslated regions (UTR) of protein coding

genes; non-coding regions and introns are regulated epigenetically by methylation (Lombardi et.al, 2016).

MicroRNA – a key regulator of gene expression, conserved across species of various cell types are active against larger proportion of transcriptome. In animals, multiple disease states abnormal miRNA has been identified that includes inflammatory disease, cardiovascular disease and malignancies. Multiple mRNA may be affected by miRNA and regulates its transcriptional activities (Chen NX et.al, 2013)

The miRNA biogenesis was regulated by precursors and subsequently processed to mature miRNA. These regulatory proteins bound to mature miRNA that directs degradation by preventing their expression. Most common regulatory Lin28 protein, binds with miRNA let-7 and target degradation (Leigh-Ann MacFarlane and Paul R. Murphyet et.al. 2010).

Micro RNA precursor's clusters were located in different regions of the genome within introns of protein coding genes and intergenic regions as "JUNK DNA" with unknown function. Discovery of miRNA genes of genome implies "JUNK DNA" is not useless. (Rodriguez et.al. 2004). In plants and Eukaryote animal branches, RNA polymerase II performs miRNA Transcription, whereas transcripts are capped and polyadenylated by array of encoded genes(Kim, 2005).

To prevent initiation and promotion of carcinogenesis, administration of naturally acquiring agents is being increasingly appreciated (Samarghandian et.al. 2014). 1, 8 – Cineole obtained from the ethanolic extract of *Alpinia galanga* have antibacterial activity against *Staphylococcus aureus* (Tachakittirungrod et.al, 2007). The essential oils extracts from rhizomes of greater galangal extensively proven to exhibit antitumor, antiulcer and anti-allergic activities (W.Y. Hsu et.al, 2010) (Mahae et.al , 2009). The information about anticancer activities of galangal is very limited. human T-cell leukemia JURKAT cells were exposed to various constituents like galanals A and B, initiated apoptosis through the mitochondrial pathway by enhancing proapoptotic protein Bax expression and down regulating antiapoptotic protein Bcl-2 (Ayman I. Elkady, 2012).

β- Element – a novel anticancer extract from *Zingiber officinale* induce activities caspase -3, -7 and -9, Bcl-2 decrease and increase of cleaved poly (ADP-ribose) polymerase and triggers apoptosis, associated with mitochondrial cytochrome. In human leukemia HL60, G1 arrest and apoptosis were induces by gingerdione by down-regulating G1- associated cyclin E, cdc 25A and up-regulateing p15, p27 (Ayman I. Elkady, 2012).It act as promising chemo preventive dietary agent inhibits

lipoxygenase, cyclooxygenase, (Huang et al., 1991) anti-tumorigenic and apoptosis induction (Lee et al., 1998; Chauhan, 2002).

Antitumor drugs developing from plant source have increased interest worldwide research due to high limitations and chemotherapeutic side effects is the second leading cause of cancer mortality. (Woo and Kin, 2011)Sphingolipids mediates signal transmission, cell growth, recognition, differentiation and cell death. Intermediates of sphingolipid metabolism, ceramides and sphingoid base, act as cell cycle mediated signaling molecules; apoptotic stress response (Petra sperling et.al, 2004)

In ceramide synthesis pathway, re-sensitizing resistant cancers up-regulate enzymes against drug-resistant colon cancer cells. CerS6 Over expression resistant cells, re-sensitized to increase C16-ceramide and apoptosis. CerS6 and C16-ceramide down-regulated to induce apoptosis and ER stress (Schiffmaan et.al, 2009).Apoptosis - cell suicide by controlled genes network, essential process development plays a crucial role in disease pathogenesis that include cancer (Ayman Al-Hendy, 2011).

Cancer - malignant disease, uncontrolled abnormal cell formation, cause death. (Porter, 2008). The main mechanism of cancer cells is to attack and destroy normal cells due to body imbalance and can be treated by correcting the unevenness. In women, increased rate of breast cancer exceeds when compared with other cancers globally, increased number of colorectal cancer and cervical cancer in developed countries and developing countries respectively (WHO, 2019). Treatment of cancer starts with surgery, chemotherapy and radiation (Lee et al., 2002). Conventional chemotherapeutic treatment cause serious side effects (Amir et al, 2009). The demand for utilization of alternative and specific drugs of natural origin is gaining importance (Shoeb, 2006).

Cancer –A genetic disease, involves changes in structure and gene expression. (Hadjzadeh et.al.2014). Oncomir - miRNA involved in cancer, miRNA deregulation plays primary role in regulation of miRNA biogenesis, methylation and transcriptional cleavage (Rupaimoole.et.al, 2016). miRNA act as a potential tumor suppressor and understanding mechanism of mRNA regulation in expression of various human pathological conditions, including cancer (Palmero et.al, 2011).

Breast cancer MDA-MB-231 cell lines widely used to study pathology, new therapies and molecularly hetero-generosity , Highly aggressive epithelial Breast cancer MDA-MB-231 cell lines,

poorly differentiated, invasive cell line lacks estrogen receptor, progesterone receptor expression, HER2 amplification established from pleural fluid effusion of 51 years old Caucasian female, extracellular matrix proteolytic degradation mediated metastatic mammary adenocarcinoma (ECACC catalog no. 92020424)

In recent years, miRNA emerged as a new regulatory component, complex mechanism of gene expression and powerful tools for studying therapeutic promises on clinical medicine to control cancer and more than half of miRNA genes were located in genomic regions are cancer associated (Palmero et.al. 2011). Studies also focused on saliva, plasma, serum, lymphocytes and other body fluids to determine, evaluate as prognostic, diagnostic and predictive cancer biomarker. The potential circulating miRNA as non-invasive diagnostic biomarkers has a greater attention (Charlootte et.al, 2016).

This study aimed to check miRNA stability and modulations on treatment with phyto lipids isolated from plants. It is based on the extraction of total phyto-lipid constituents from *Alpinia galangal (L.) Willd, Zingiber officinale Roscoe* and *Vigna radiata (L.) R. Wilczek* to check the effect of the extract on Normal Lymphocytes. Isolation of miRNA was also performed in the plant samples using the Lithium chloride method. The lipid extracts dissolved in DMSO were treated with Normal Lymphocytes at different concentrations and these lipids treated lymphocytes were subjected to miRNA isolation in order to check the stability of miRNA and Cytotoxicity assay.

Materials and Methods:

Sample collection, authentication and preparation:

Alpinia galangal and Zingiber officinale**:** The rhizomes of *Alpinia galangal (L.) Willd* (BSI/SRC/5/23/2019/Tech/3217) and *Zingiber officinale Roscoe* (BSI/SRC/5/23/2019/Tech/3218) were obtained from the cultivation farms in Sabarimala near Kerala, Tamil Nadu border, and authenticated from BSI – TNAU, Coimbatore. The collected rhizomes of *Alpinia galangal & Zingiber officinale* was washed with clean water, rinsed in sterile distilled water. The skin of rhizome was removed, sliced into pieces, shadow dried, powdered and stored at room temperature.

Vigna radiata**:** *Vigna radiata (L.) R. Wilczek* seeds (BSI/SRC/5/23/2019/Tech/3216) obtained from the department of millets, centre for plant breeding and genetics, Tamil Nadu agricultural University, Coimbatore, authenticated from BSI – TNAU, Coimbatore. The seeds of *Vigna radiata*

were washed with clean water, rinsed in sterile distilled water. The seeds were then shadow dried, powdered and stored in room temperature

Normal packed lymphocyte: The packed Lymphocyte was stored in deep freezer at - 20°C.

Extraction of total phyto-lipids

The total phyto-lipid content from the plant sample was extracted with reference to modified Bligh and dyer method (Aswini et.al. 2022). The extract lipids were evaporated using water bath upto 40-55°C to determine the dry weight residue by subtracting from the initial weight.

Extraction of small RNA from plant samples:

An amount of 0.1g freeze dried tissue in microcentrifuge tube and 200µl Licl extraction buffer, 500 µl phenols with PH 8.0 were added, mixed and place it on ice. Tubes were incubated at 60ºC and centrifuged for 10 min at 4 ºC. 600 µl Chloroform- isoamyl alcohol (24:1; v/v) were added to upper phase and centrifuged 10 min at 4ºC with, To the supernatant, add 50 µl 5M Sodium chloride, 63 µl 40% polyethylene glycol 8000 (w/v) mixes well and incubation on ice for 30 min, Transfer the supernatant to new tube, added 500 µl of phenol – chloroform – isoamyl alcohol (25:24:1; v/v/v) and centrifuge. Precipitate low molecular weight RNA by adding 50 µl of 3M sodium acetate pH 5.2 and 1.2 ml of absolute ethanol by incubating overnight at -20 ºC. Air dried the pellet and re-suspend pellets in Nuclease free water.

Extraction of small RNA from Normal blood lymphocyte using non-conventional method:

An equal volume of lysis buffer were added and mixed repeated inversions for 15-20 minutes. A volume of 7.2 µl of β – mercaptoethanol, one tenth volume of 2M Sodium acetate, equal volume of phenol and one fifth volume of Chloroform: Isoamyl alcohol (24:1) were added and centrifuged for 5 minutes at 13000 – 14000 rpm. Aqueous layer were transferred with the equal volume of ice-cold Isopropanol, Wash the pellet with 75% ethanol, air dried and re-suspended in TE buffer. By measuring their absorbance at 230,260 and 280 nm, RNA purity and concentration were determined and A_{260} / A_{280} and A_{260} / A_{230} ratio calculated. Normal lymphocytes were treated with extracted phyto lipids and plant miRNA in different concentrations of 10mg/ml, 30mg/ml, and 50mg/ml for different time intervals (5 minutes, 15minutes, 30 minutes). Extraction of small RNA was followed after incubation. The total small RNA extracted was quantified using Orcinol method and Denatured UREA PAGE followed by Silver staining procedure

Assessment of stability of viable human lymphocytes on treating with lipid extracts:

To calculate the viable cells present in a cell suspension - Trypan blue dye exclusion assay used, the principle behind is that live cells possess intact cell membranes which exclude dye, like trypan blue, Eosin or propidium, and not dead cells (NIH). A volume of 200 µl lymphocytes were added in each tube and lipid extract of plant sample were prepared at different concentrations and untreated lymphocyte sample as a control, were incubated at different time intervals. 20 µl trypan blue dye was added to each tube, counted using a hemocytometer and percentage viability was calculated. The differences in the cell stability according to the activity of lipid extracts at different concentrations were studied and a graph was plotted.

The concentration effect and incubation time of cell viability was evaluated by two-way ANOVA with statistical software (GRAPHPAD PRISM 6). Tukey's HSD post hoc analysis was used to prove the statistical significant and differences among the groups. Statistical significance were set to $P< 0.05$.

Cytotoxicity assay (Igarashi and Miyazawa method, 2001):

The cytotoxicity effect of sample on cancer cells were determined by the 2-(4, 4-dimethyl-2-tetrazoyl)-2, 5-diphenyl-2, 4-tetrazolium salt dye reduction assay, live cells converted MTT into formazan derivative and amount of formazan formed were measured to know the number of viable cells. The formazan product formed solubilized with solvent to measure the cell viability. A volume of 100 µl treated cells were incubated with 50 µl MTT at 37° C. After incubation, 200µl PBS were added and aspirated to remove excess MTT. Incubate overnight in the dark by adding 200 µl acid-propanol for solubilization. Read the absorbance at 650nm using micro titer plate reader BIO RAD U.S.A. The optical density of control cells were fixed to 100% viable and the viability percentage of the treated cells were calculated using the formula.

Result and Discussion:

Extraction of total plant lipids – Bligh and dyer method (1959)

The plant phyto lipid wer extracted with reference to modified Bligh and dyer method (1959) indicates the amount of lipid constituents present in the plant samples. Arabidopsis lipids have most often extracted with Methanol:Chloroform (1:2) (Bligh et.al, 1959) (v/v), In phase separation, additional methanol will increase the water content that will leads to the concomitant loss of extraction that limits to 6.54 % .

Extraction of small RNA from plant samples and lymphocyte:

The small RNA extraction was performed in the plant samples *Alpinia galangal, Zingiber officinale, Vigna radiate* and normal blood lymphocyte. The isolated small RNA was dissolved in Nuclease free water, quantified using UV spec method and denaturing UREA PAGE. The RNA purity was determined by measuring the adsorption of UV light in UV spectrophotometer at wavelengths of 230, 260 and 280 nm, and reading 1.0 is equivalent to □ 40µg/ml single stranded RNA. The A_{260} / A_{280} Ratio assess RNA purity and 1.8 – 2.1 indicates highly purified RNA. Concentrated hydrochloric acid present in orcinol reagent dehydrates the ribose, pentose sugar, into furfural. Furfural then reacts with 3, 5 – dihydro toluene in the presence of ferric chloride to give a green colored complex and absorbance read at 665 nm. A graph plotted with standard concentration on X- axis, OD values in Y- axis and the concentration of unknown samples was calculated.

Cancer-associated micro RNA located in genomic regions plays an important role in pathogenesis of cancers. Third common cancer of blood, multiple myeloma of plasma cells, after lymphoma and leukemia has characteristic expression of miRNA in normal and MM patients. In which 57 % up regulation and 43% down regulation of miRNAs were identified. Recent studies implicate miRNAs as well known tumor suppressor or oncogenes, positive tumor suppressor effect or the negative oncogene effect depends upon the specific miRNA, whether it is up-regulated or down-regulated on specific cancer type. For example, miR-224 was up-regulated in thyroid tumors, renal cell carcinoma, colon cancers and prostate cancers, whereas down-regulated in ovarian, lung, breast cancer and oral carcinoma (KonstantiosMavridis et.al. 2013).

Assessment of stability of viable human lymphocytes on treating with lipid extracts

The lymphocytes when treated with the total lipids extracts and the plant miRNA, the miRNA content in the sample was increased when compared to the normal sample, by which variation has occurred. According to two-way ANOVA, a statistically significant ($P < 0.05$) difference was found between the groups when cell viability was considered. Further concentration evaluation and incubation time on the viability of the cell. The most significant effect on the cell viability was observed in *Alpinia galanga* when compared with *Zingiber officinale* and *Vigna radiata*

Cytotoxicity assay / MTT assay (Igarashi and Miyazawa method, 2001):

The antiproliferative assay of total phytolipids extracted from rhizomes of *Alpinia galanga*, *Zingiber officinale* and *Vigna radiata* was assessed with breast cancer MDA MB 231 cell line using

MTT assay for cell viability and cytotoxicity indices. Comparative results showed that control group of untreated cells with treated cells exhibited dose and time dependent decline in cell viability after 3 hours incubation.

Sphingolipids - a signaling paradigm receptor activated by agonists tumor necrosis factor-α and to elevate ceramide, platelet-derived growth factor induced by sphingomyelin, or downstream metabolites of sphingosine 1-phosphate or Sphingosine (Chia-Ling Chen et.al., 2011).

Treatment group	**Concentration (μg/ml)**	**Percentage viability**	**IC_{50} (μM)**
Control (Cells alone)	-	100%	-
Positive control (Etoposide drug)	100	23.36	606.27
Alpiniagalanga	100	67.71	774.3
	200	59.52	
	300	53.74	
	400	48.07	
	500	40.40	
Zingiberofficinale	100	64.97	1059
	200	62.13	
	300	59.23	
	400	56.37	
	500	50.49	
Vigna radiata	100	67.18	1010
	200	61.05	
	300	55.01	
	400	50.85	
	500	46.66	

The cell types and the dosages of stimulation depend on th cytopathic effects of ceramide, that are proapoptotic and necrotic-like, Thus, apoptotic signaling caused were diverged because of the involvement of intracellular organelles. A key strategy of tumorigenesis is to inhibit cell death by

interference on signaling pathway. Therefore, in the development of cancer therapy, metabolic pathways of ceramide as a candidate targets (Chiou-FengLin et.al, 2011).

The major constituents of *Catharanthus roseus was the* forerunner of anticancer agents Vincristine, Vinca alkaloids and Vinblastine were introduced to have long term remissions and cures for testicular teratoma, malignant lymphoma, lymphoblastic leukemia and other cancers. Natural products and its derivatives comprise more than 50% of drugs that are used in the cancer treatment and chemotherapy which leads to the discovery of novel anticancer drugs from natural products (Bardwaj .R.G et al., 2017)

Summary and Conclusion

The miRNAs molecular biology, how it acts in organisms were still in beginning to understood, increasing number of studies revealed small RNAs with diverse biological processes. The overall cellular gene expression and regulation associations with mechanistic pathway indicate miRNAs involved in various human diseases (Palmero et.al, 2011). This shows significant scientific research findings indicating miRNA a cancer biomarkers for diagnosis, prediction and prognosis, suggests substitution of tumor suppressive miRNAs or inhibition of oncogenic miRNA to develop novel treatment strategies (kaladar et.al, 2015).

A change in expression of single miRNA has implicated in cancer, down regulation of microRNA might be critical for maintaining the cells, in animals reintroducing miRNAs shows impairments in the cancer cells viability that results in cell death. Misregulation of several miRNAs may also have chance to develop diseases in human; reverting back mis-regulated miRNAs to its normal levels may reduce or eliminate disease have advantages in application as therapeutic agents (Christopher et.al, 2010).

In this present study, extraction of total phytolipids was performed in the plant samples using methanol: Chloroform Bligh and Dyer method were quantified. Trypan blue dye exclusion assay was performed with normal lymphocytes treated with the lipid extracts and miRNA from *Alpinia galanga, Zingiber offcicinale* and *Vigna radiata* dissolved in DMSO, percentage cell viability were calculated showed increased cell stability indicating the longevity property exhibited by lipid extracts. The conclusion draw from these findings showed significant miRNA modulation and mechanism regulate gene expression varies in response to both phyto lipids and miRNA from plants (George et al., 2007).

In the present study, the extraction of lipids was performed in the medicinal plants *Alpinia galangal (L.) Will*, *Zingiber officinale Roscoe* and *Vigna radiata (L.) R. Wilczek* using Bligh and Dyer method and quantified. In vitro cytotoxicity assay were performed on breast cancer MDA MB 231 cell line treated with lipid extracts dissolved in DMSO, which revealed the anti-cancerous property of *Alpinia galanga* exhibited at low IC_{50} Value of 774.3 µM/ml

The relationship between miRNA expression factor and onset of cancer make them a primary research focus with promising future in medicine associated with its detection. Northern blotting procedure, PCR and microarray make them suitable for detection. miRNA based technology are reliable and cost-effective method to developed early diagnosis and therapeutic assessment of cancer. Lymphocyte miRNA are stable and consistent among different blood samples. Their expression profiling could be used as a novel lymphocyte-based potential biomarker offering sensitive and specific tools for early diagnosis and monitoring of cancer.

Conflict of interest

The research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

Grand information

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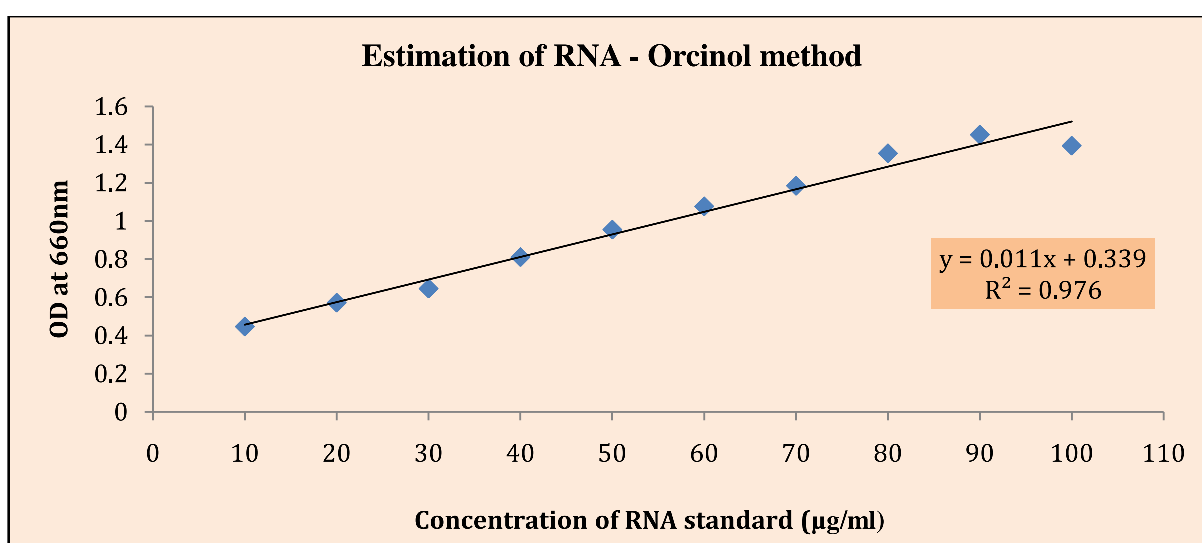
Tables and graphs:



Graph 1: Estimation of RNA – Orcinol method: The linear regression plotted with concentration of RNA standard and Optical density at 660nm. The R^2 value 0.976 were calculated.

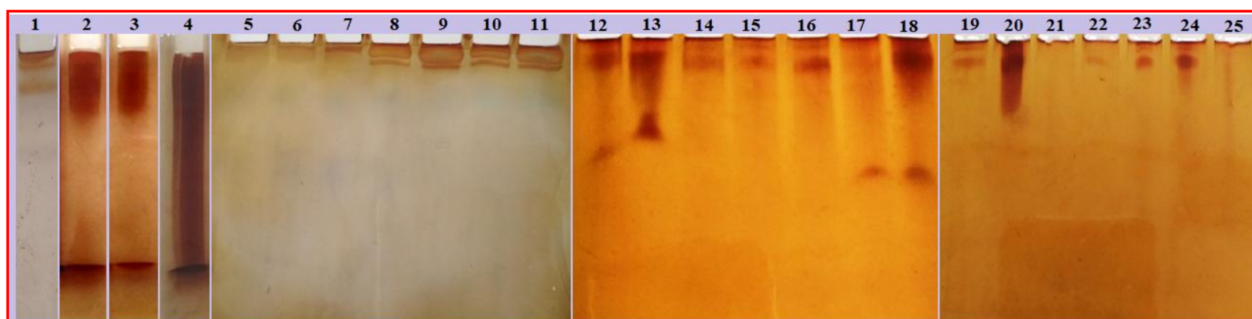
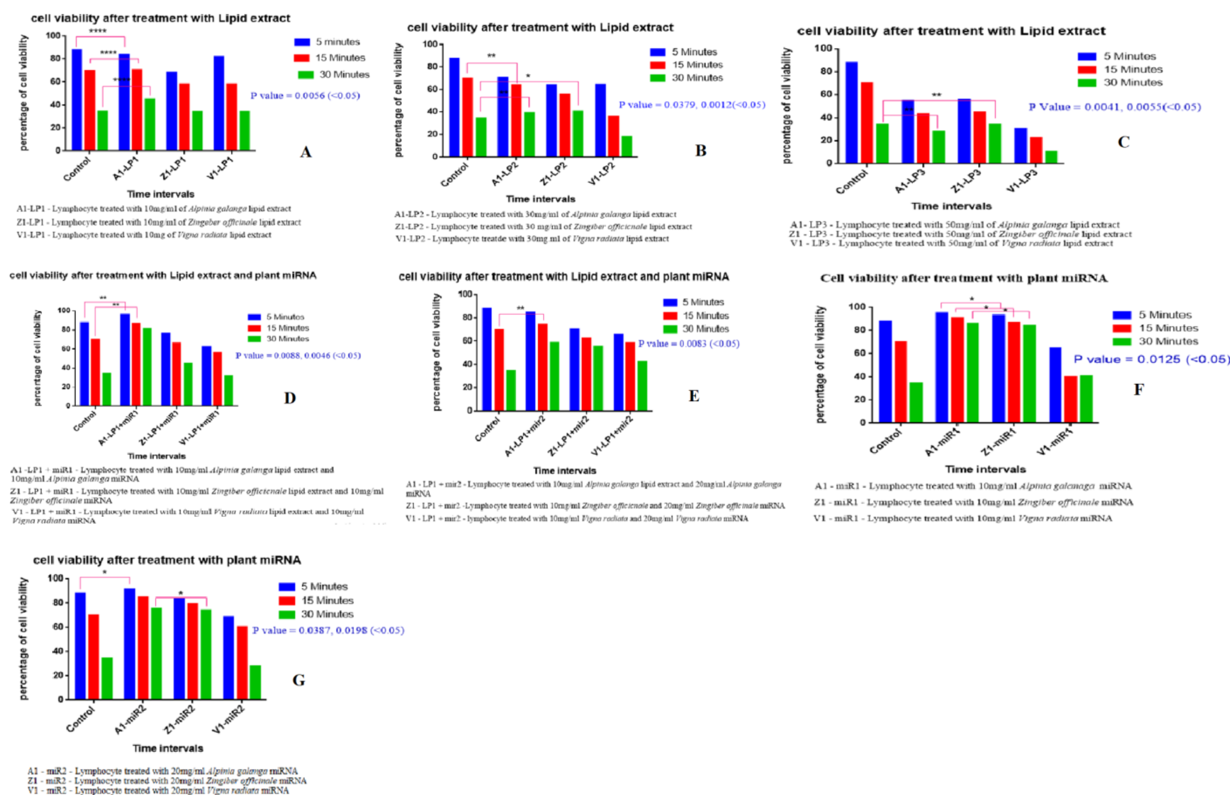
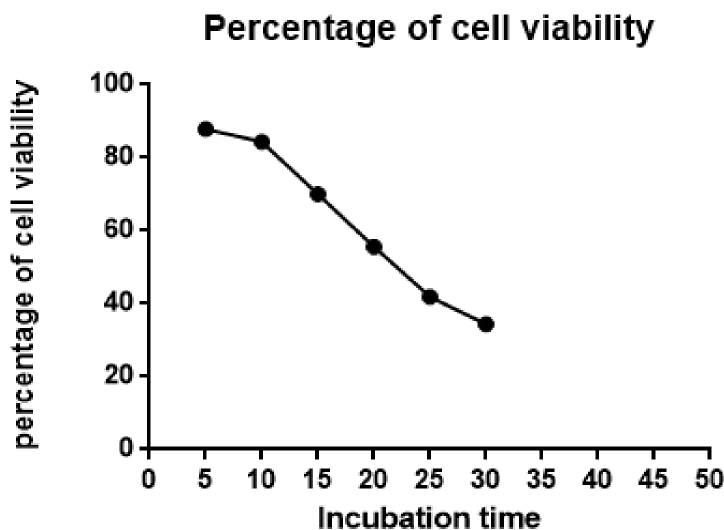


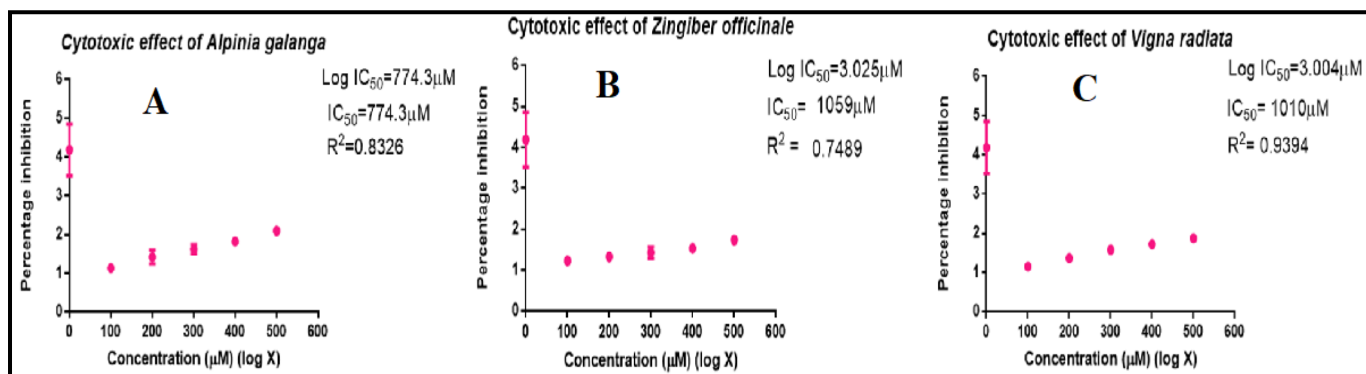
Figure 1: Total plant treatment with the Normal lymphocyte: 1. miRNA isolated from normal packed Lymphocyte*,* **2.** miRNA isolated from *Alpinia galanga,* **3.** miRNA isolated from *Zingiber officinale,* **4.** miRNA isolated from *Vigna radiata,* **5.** miRNA isolated from the normal lymphocyte after treatment with lipid (10mg/ml), **6.** miRNA isolated from the normal lymphocyte after treatment with lipid (30mg/ml), **7.** miRNA isolated from the normal lymphocyte after treatment with lipid (50mg/ml), **8.** miRNA isolated from the normal lymphocyte after treatment with lipid and plant miRNA (10µg of plant miRNA and 10mg/ml of lipid), **9.** miRNA isolated from the normal lymphocyte after treatment with lipid and plant miRNA (20µg of plant miRNA and 10mg/ml of lipid), **10.** miRNA isolated from the normal lymphocyte after treatment with plant miRNA (10µg of plant miRNA), **11.** miRNA isolated from the normal lymphocyte after treatment with plant miRNA (20µg of plant miRNA), **12.** miRNA isolated from the normal lymphocyte after treatment with lipid (10mg/ml), **13.** miRNA isolated from the normal lymphocyte after treatment with lipid (30mg/ml), **14.** miRNA isolated from the normal lymphocyte after treatment with lipid (50mg/ml), **15.** miRNA isolated from the normal lymphocyte after treatment with lipid and plant miRNA (10µg of plant miRNA and 10mg/ml of lipid), **16.** miRNA isolated from the normal lymphocyte after treatment with lipid and plant miRNA (20µg of plant miRNA and 10mg/ml of lipid), **17.** miRNA isolated from the normal lymphocyte after treatment with plant miRNA (10µg of plant miRNA), **18.** miRNA isolated from the normal lymphocyte after treatment with plant miRNA (20µg of plant miRNA), **19.** miRNA isolated from the normal lymphocyte after treatment with lipid (10mg/ml), **20.** miRNA isolated from the normal lymphocyte after treatment with lipid (30mg/ml), **21.** miRNA isolated from the normal lymphocyte after treatment with lipid (50mg/ml), **22.** miRNA isolated from the normal lymphocyte after treatment with lipid and plant miRNA (10µg of plant miRNA and 10mg/ml of lipid), **23.** miRNA isolated from the normal lymphocyte after treatment with lipid and plant miRNA (20µg of plant miRNA and 10mg/ml of lipid), **24.** miRNA isolated from the normal lymphocyte after treatment with plant miRNA (10µg of plant miRNA), **25.** miRNA isolated from the normal lymphocyte after treatment with plant miRNA (20µg of plant miRNA).



Graph 2: A. Cell viability percentage on treatment with Lipid extract - 10mg/m, **B.** Cell viability percentage on treatment with 30 mg/ml lipid extract, **C.** Cell viability percentage on treatment with 50 mg/ml lipid extract, **D.** Cell viability percentage on treatment with 10mg/ml lipid extract and 10mg/ml plant miRNA, **E.** Cell viability percentage on treatment with 10mg/ml lipid extract and 20mg/ml plant miRNA, **F.** Cell viability percentage on treatment with 10mg/ml plant miRNA, **G.** Cell viability percentage on treatment with 20mg/ml plant miRNA



Graph 3: Percentage of normal lymphocytes cell viability



Graph 4: Percentage of cell inhibition of total lipids on Breast cancer cell line MDA MB 231:

A- The cytotoxic assay of lipids extracts of *Alpinia galanga* on Breast cancer MDA MB 231 cell line was expressed as percentage viability of cell. Breast cancer MDA MB 231 cell line morphology changes showed progressively from 100µg/ml – 500µg/ml concentration of the extract and were compared with control. The IC_{50} for *Alpinia galanga* lipid extract was found to be 774.3µM. The lipid extract was found to have cytotoxic effect against Breast cancer MDA MB 231 cell line, shows concentration - dependent cell proliferation inhibition, **B-** The cytotoxic assay of lipid extracts from *Zingiber officinale* on Breast cancer MDA MB 231 cell line was expressed as percentage cell viability. Breast cancer MDA MB 231 cell line morphology changes showed progressively from 100µg/ml – 500µg/ml concentration of the extract and were compared with the control. The IC_{50} for *Zingiber officinale* lipid extract was found to be 1059 µM. The lipid extract was found to have cytotoxic effect against Breast cancer MDA MB 231 cell line, showing cell proliferation inhibition in a concentration - dependent manner, **C-**The cytotoxic assay of lipid extracts of *Vigna radiata* on Breast cancer MDA MB 231 cell line was expressed as percentage cell viability. Breast cancer MDA MB 231 cell line morphology changes showed progressively from 100µg/ml – 500µg/ml concentration of the extract and were compared with the control. The IC_{50} for *Vigna radiata* lipid extract was found to be 1010 µM. The lipid extract was found to have cytotoxic effect against Breast cancer MDA MB 231 cell line, showing cell proliferation inhibition in concentration - dependent manner.

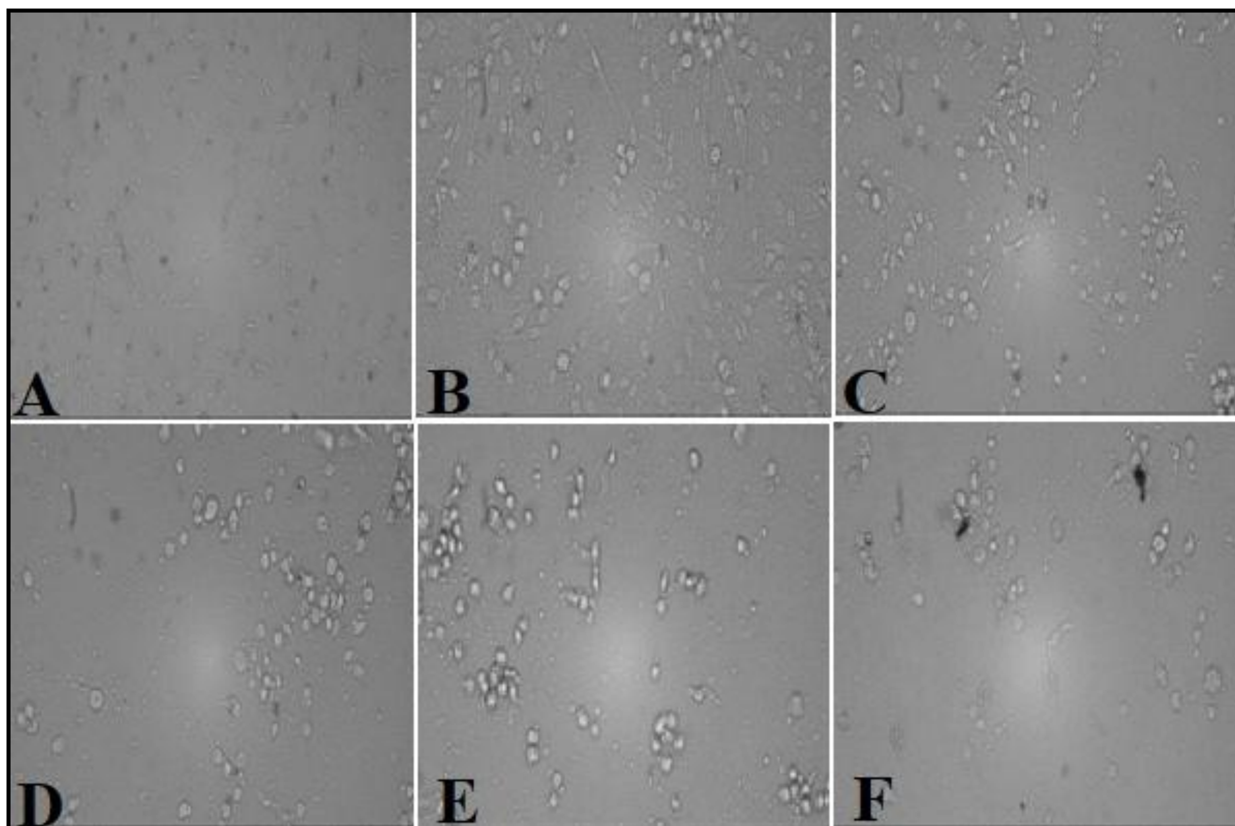


Figure 2: Cytotoxic assay - *Alpinia galanga* lipid extract on Breast cancer MDA MB 231 cell line: A. Control (Breast cancer MDA MB 231 cell line), B. 100µg *Alpinia galanga* lipid treated to Breast cancer MDA MB 231 cell line, C. 200µg *Alpinia galanga* lipid treated to Breast cancer MDA MB 231 cell line, D. 300µg *Alpinia galanga* lipid treated to Breast cancer MDA MB 231 cell line, E. 400µg *Alpinia galanga* lipid treated to Breast cancer MDA MB 231 cell line, F. 500µg *Alpinia galanga* lipid treated to Breast cancer MDA MB 231 cell line.

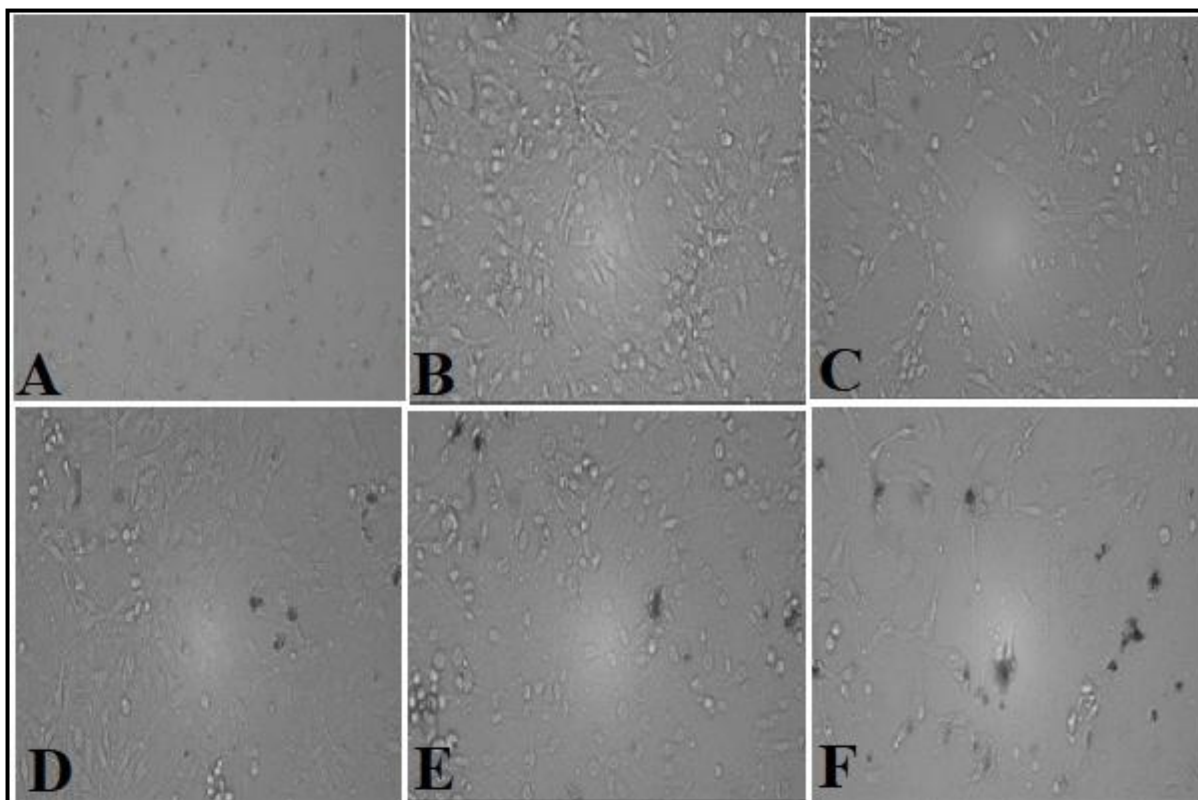


Figure 3: Cytotoxic assay - *Zingiber officinale* lipid extract on Breast cancer MDA MB 231 cell line A. Control (Breast cancer MDA MB 231 cell line), B. 100µg *Zingiber officinale* lipid treated to Breast cancer MDA MB 231 cell line, C. 200µg *Zingiber officinale* lipid treated to Breast cancer MDA MB 231 cell line, D. 300µg *Zingiber officinale* lipid treated to Breast cancer MDA MB 231 cell line, E. 400µg *Zingiber officinale* lipid treated to Breast cancer MDA MB 231 cell line, F. 500µg *Zingiber officinale* lipid treated to Breast cancer MDA MB 231 cell line**.**

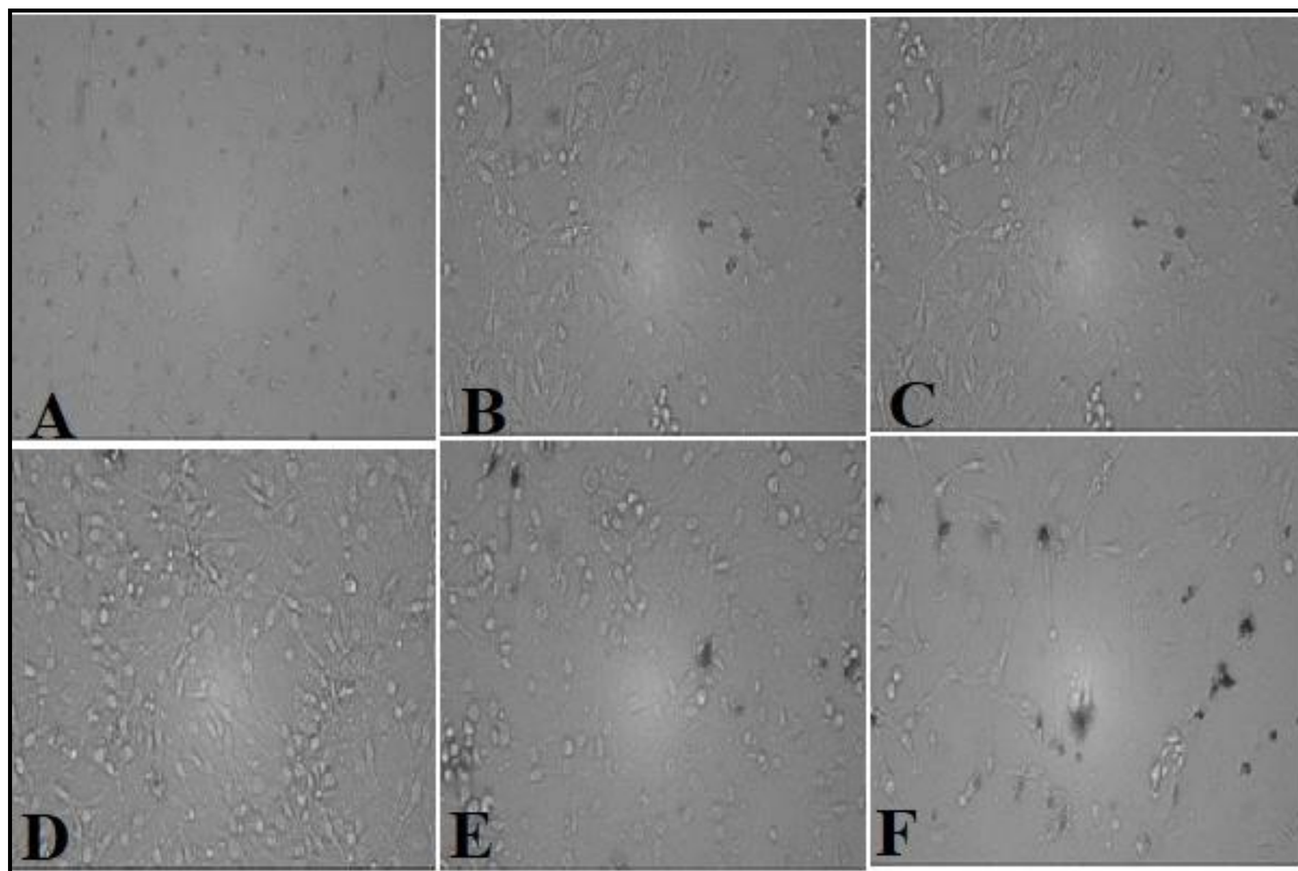


Figure 4: Cytotoxic assay - *Vigna radiata* lipid extract on Breast cancer MDA MB 231 cell line: A. Control (Breast cancer MDA MB 231 cell line), B. 100µg *Vigna radiata* lipid treated to Breast cancer MDA MB 231 cell line, C. 200µg *Vigna radiata* lipid treated to Breast cancer MDA MB 231 cell line, D. 300µg *Vigna radiata* lipid treated to Breast cancer MDA MB 231 cell line, E. 400µg *Vigna radiata* lipid treated to Breast cancer MDA MB 231 cell line, F. 500µg of *Vigna radiata* lipid treated to Breast cancer MDA MB 231 cell line**.**