Anticancer effect of Moringa oleifera leaf extract on human breast cancer cell

A thesis submitted toward partial fulfilment of the requirements for the degree of **Master of Engineering in Biomedical Engineering**

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his **Master of Engineering in Biomedical Engineering** studies during academic session 2013-2014.

All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by this rules and conduct, I have fully cited and referred all material and results that are not original to this work.

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List of Figures

Fig 2.1: Leaves of *Moringa oleifera*
Fig 2.2: Many uses of *Moringa*
Fig 2.3: Selected phytochemicals from *Moringa* species
Fig 5.1: MTT assay on different soxlet fractions
Fig 5.2: MTT Assay on crude methanolic fraction for different doses
Fig 5.3: MTT Assay on MCF 7
Fig 5.4: MTT Assay on MDA MB 231
Fig 5.5: MTT Assay in the LD_{50} dose range of MLE on PBMC
Fig 5.6: Cell morphological changes on MCF 7
Fig 5.7: Cell morphological changes on MDA MB 231
Fig 5.8: Photomicrograh of scratch wound
Fig 5.9: Fluorescence image of adhered MDA MB 231 cells
Fig 5.10: Fluorescence image of adhered MCF 7 cells

List of Tables

Table 2.1 : Amino Acid Content of *Moringa* Leaves
Table 2.2 : Vitamin and Mineral Content of *Moringa* Leaves
Table 5.1 : Comparative effect of MLE on different cells

Contents

	Chapter Name	Page No
Chapter 1	Abstract	1
Chapter 2	Introduction and Literature review	3
Chapter 3	Objective and Proposed plan of work	22
Chapter 4	Materials and Methods	26
Chapter 5	Observations and Results	33
Chapter 6	Discussion and Future plan of work	46

Chapter 1

Abstract

Abstract:

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It has been reported that leaf extract of *Moringa oleifera* has anti-proliferative effect on the human cervical cancer cell line, KB cells and alveolar cancer cell line A549 cells. The object of the present work is to investigate the anti-cancer effect of *Moringa oleifera* leaf extract on breast cancer.

Soxlet fractions using different solvents and crude methanolic extract of *Moringa oleifera* leaf was prepared by standard extraction protocols. To check the anti-proliferative effect of the leaf extract, all the fractions were screened on the HeLa cell line. The extract chosen was tested for cell viability on the two breast cancer cells MCF 7 and MDA MB 231 in different concentrations. Cell viability was evaluated by MTT assay for 24 hour and 48 hours. The LD_{50} value was calculated and different *morphometric* assays were performed with the effective dose of the extract and compared with *cis*-Platinum. The effect of the extract on the normal cell was evaluated as well. Cell adhesion and scratch wound healing assays were performed.

The leaf extract showed a dose-dependent and time dependant inhibition on cell proliferation in the breast cancer cell lines. It showed low cytotoxicity in the normal cells and inhibited cellular adhesion and wound healing in treated cancer cells.

The present study suggests that the leaf extract from *M. oleifera* induces anti-cancer effect on the breast cancer cells. Further study might help to confirm it as an anti cancer drug.

Keywords: *Moringa oleifera*, anticancer drugs, breast cancer.

Chapter 2

Introduction and Literature Review

Background

Moringa oleifera is the most widely cultivated species of a monogeneric family, the Moringaceae that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. *Moringa oleifera* is the best known of the thirteen species of the genus Moringacae. This rapidly-growing tree (also known as the horseradish tree, drumstick tree, benzolive tree, moonga, mulangay, nébéday, saijhan, sajna tree), was utilized by the ancient Romans, Greeks and Egyptians; it is now widely cultivated and has become naturalized in many locations in the tropics. It is a perennial softwood tree with timber of low quality, but which for centuries has been advocated for traditional medicinal and industrial uses. It is already an important crop in India, Ethiopia, the Philippines and the Sudan, and is being grown in West, East and South Africa, tropical Asia, Latin America, the Caribbean, Florida and the Pacific Islands.

Fig 2.1: Leaves of *Moringa oleifera*

All parts of the *Moringa* tree are edible and have long been consumed by humans. According to Fuglie [1], the many uses for *Moringa* include: alley cropping (biomass production), animal forage (leaves and treated seed-cake), biogas (from leaves), domestic cleaning agent (crushed leaves), blue dye (wood), fencing (living trees), fertilizer (seed-cake), foliar nutrient (juice expressed from the leaves), green manure (from leaves), gum (from tree trunks), honey- and sugar cane juice-clarifier

(powdered seeds), honey (flower nectar), medicine (all plant parts), ornamental plantings, biopesticide (soil incorporation of leaves to prevent seedling damping off), pulp (wood), rope (bark), tannin for tanning hides (bark and gum), water purification (powdered seeds). *Moringa* seed oil (yield 30-40% by weight), also known as Ben oil, is a sweet non-sticking, non-drying oil that resists rancidity. It has been used in salads, for fine machine lubrication, and in the manufacture of perfume and hair care products. In the West, one of the best known uses for *Moringa* is the use of powdered seeds to flocculate contaminants and purify drinking water [2], but the seeds are also eaten green, roasted, powdered and steeped for tea or used in curries. This tree has in recent times been advocated as an outstanding indigenous source of highly digestible protein, Ca, Fe, vitamin C and carotenoids suitable for utilization in many of the so called "developing" regions of the world where under nourishment is a major concern.

Moringa trees have been used to combat malnutrition, especially among infants and nursing mothers. Three non-governmental organizations in particular—Trees for Life, Church World Service and Educational Concerns for Hunger Organization—have advocated *Moringa* as *"*natural nutrition for the tropics." Leaves can be eaten fresh, cooked, or stored as dried powder for many months without refrigeration, and reportedly without loss of nutritional value.

Moringa is especially promising as a food source in the tropics because the tree is in full leaf at the end of the dry season when other foods are typically scarce. A large number of reports on the nutritional qualities of *Moringa* now exist in both the scientific and the popular literature. *Moringa* leaves contain more Vitamin A than carrots, more calcium than milk, more iron than spinach, more Vitamin C than oranges, and more potassium than bananas, and that the protein quality of *Moringa* leaves rivals that of milk and eggs.

In fact, the nutritional properties of are now so well known that there seems to be little doubt of the substantial health benefit to be realized by consumption of *Moringa* leaf powder in situations where starvation is imminent. In many cultures throughout the tropics, differentiation between food and medicinal uses of plants (e.g. bark, fruit, leaves, nuts, seeds, tubers, roots, flowers), is very difficult since plant uses span both categories and this is deeply ingrained in the traditions and the

fabric of the community. The various nutritional values of fresh and dry leaves are charted below:

All values are per 100 grams of edible portion

	Fresh Leaves	Dried Leaves
Arginine	406.6 mg	1325 mg
Histidine	149.8 mg	613mg
Isoleucine	299.6 mg	825 mg
Leucine	492.2 mg	1950 mg
Lysine	342.4 mg	1325 mg
Methionine	117.7 mg	350 mg
Phenylalanine	310.3 mg	1388 mg
Threonine	117.7 mg	1188 mg
Tryptophan	107 mg	425 mg
Valine	374.5 mg	1063 mg

Table 2.1: *Amino Acid Content of Moringa Leaves [3]*

All values are per 100 grams of edible portion

	Fresh Leaves	Dried Leaves
Carotene (Vit. A)	6.78 mg	18.9 mg
Thiamin (B1)	0.06 mg	2.64 mg
Riboflavin (B2)	0.05 mg	20.5 mg
Niacin (B3)	0.8 mg	8.2 mg
Vitamin C	220 mg	17.3 mg
Calcium	440 mg	2003 mg
Calories	92 cal	205 cal
Carbohydrates	12.5 g	38.2 g
Copper	0.07 mg	0.57 mg
Fat	1.70 g	2.3 g
Fiber	0.90 g	19.2 g
Iron	0.85 mg	28.2 mg
Magnesium	42 mg	368 mg
Phosphorus	70 mg	204 mg
Potassium	259 mg	1324 mg
Protein	6.70 g	27.1g
Zinc	0.16 mg	3.29 mg

Table 2.2: *Vitamin and Mineral Content of Moringa Leaves [3]*

For centuries, people in many countries have used *Moringa* leaves as traditional medicine for common ailments. Clinical studies have begun to suggest that at least some of these claims are valid. With such great medicinal value being suggested by traditional medicine, further clinical testing is very much needed.

In India it is traditionally used for anemia, anxiety, asthma, blackheads, blood impurities, bronchitis, catarrh, chest congestion, cholera, conjunctivitis, cough, diarrhea, eye & ear infections, fever, glandular swelling, headaches, abnormal blood pressure, hysteria, pain in joints, pimples, psoriasis, respiratory disorders, scurvy, semen deficiency, sore throat, sprain, tuberculosis, skin infections and sores.

Over the past two decades, many reports have appeared in mainstream scientific journals describing its nutritional and medicinal properties. Its utility as a non-food product has also been extensively described. Every part of *Moringa* tree is said to have beneficial properties that can serve humanity. People in societies around the world have made use of these properties.

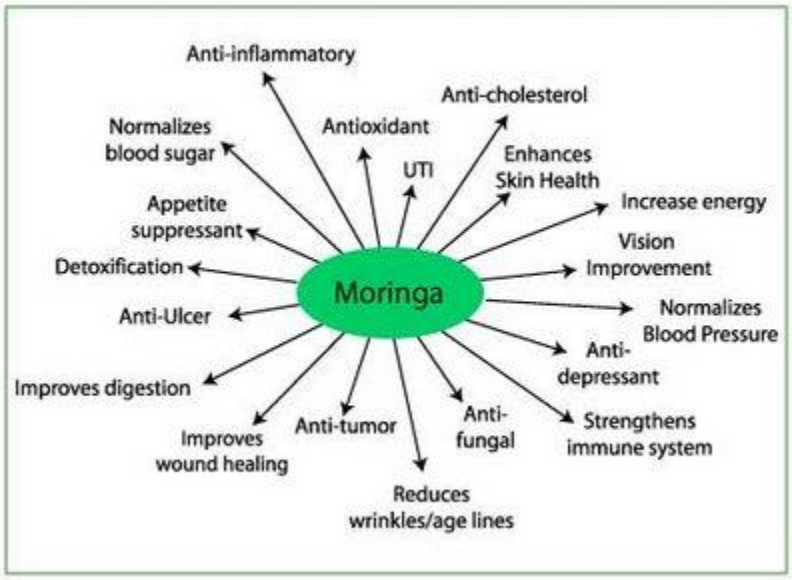

Fig 2.2: *Many uses of Moringa*

Nutritional analysis indicates that *Moringa* leaves contain a wealth of essential, disease preventing nutrients. They even contain all of the essential amino acids, which is unusual for a plant source. Since the dried leaves are concentrated, they contain higher amounts of many of these nutrients except Vitamin C.

Phytochemistry

Phytochemicals are, in the strictest sense of the word, chemicals produced by plants. Commonly, though, the word refers to only those chemicals which may have an impact on health, or on flavor, texture, smell, or color of the plants, but are not required by humans as essential nutrients.

An examination of the phytochemicals of *Moringa* species affords the opportunity to examine a range of fairly unique compounds. In particular, this plant family is rich in compounds containing the simple sugar, rhamnose, and it is rich in a fairly unique group of compounds called glucosinolates and isothiocyanates. For example, specific components of *Moringa* preparations that have been reported to have hypotensive, anticancer, and antibacterial activity include 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)benzylisothiocyanate,4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate , niazimicin , pterygospermin , benzyl isothiocyanate , and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate[4,5].

Along with these compounds the *Moringa* family, it is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as the carotenoids (including β-carotene or pro-vitamin A).

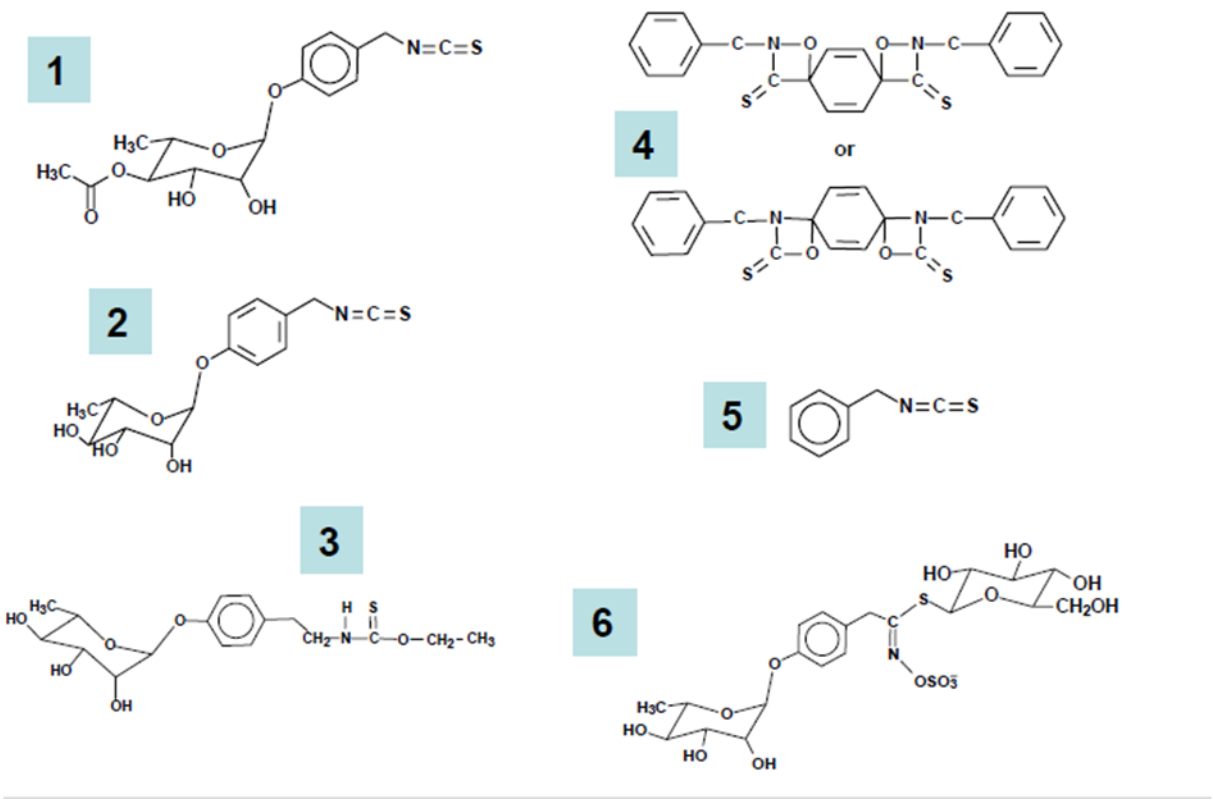

Fig 2.3: *Selected phytochemicals from Moringa species[4,5], 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)benzylisothiocyanate[1],4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate[2] , niazimicin[3] , pterygospermin[4] , benzyl isothiocyanate[5] , and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate[6]*

Literature Review

The benefits for the treatment or prevention of disease or infection that may accrue from either dietary or topical administration of *Moringa* preparations (e.g. extracts, decoctions, poultices, creams, oils, emollients, salves, powders, porridges) are not quite so well known. Although the oral history here is also voluminous, it has been subject to much less intense scientific scrutiny, and it is useful to review the claims that have been made and to assess the quality of evidence available for the more well-documented claims.

A plethora of traditional medicine references attest to its curative power, and scientific validation of these popular uses is developing to support at least some of the claims. *Moringa* preparations have been cited in the scientific literature as having antibiotic, antitrypanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypocholesterolemic, and hypoglycemic activities, as well as having considerable efficacy in water purification by flocculation, sedimentation, antibiosis and more. Unfortunately, many of these reports of efficacy in human beings are not supported by placebo controlled, randomized clinical trials, nor have they been published in high visibility journals. For example, on the surface a report published almost 30 years ago appears to establish *Moringa* as a powerful cure for urinary tract infection, but it provides the reader with no source of comparison (no control subjects).

The oldest scientific evidence of *Moringa* is that of its antibiotic property. In the late 1940's and early 1950's a team from the University of Bombay (BR Das), Travancore University (PA Kurup), and the Department of Biochemistry at the Indian Institute of Science in Bangalore (PLN Rao), identified a compound they called pterygospermin a compound which they reported readily dissociated into two molecules of benzyl isothiocyanate [4,56,7,8,].

Benzyl isothiocyanate was already understood at that time to have antimicrobial properties. This group not only identified pterygospermin, but performed extensive and elegant characterization of its mode of antimicrobial action in the mid 1950's. (They identified the tree from which they isolated this substance as "*Moringa pterygosperma*," now regarded as an archaic designation for "*M. oleifera*.") .

Although others were to show that pterygospermin and extracts of the *Moringa* plants from which it was isolated were antibacterial against a variety of microbes, the identity of pterygospermin has since been challenged [9] as an artifact of isolation or structural determination.

Subsequent elegant and very thorough work, identified a number of glyosylated derivatives of benzyl isothiocyanate (e.g. compounds containing the 6-carbon simple sugar, rhamnose). The identity of these compounds was not available in the refereed scientific literature until "re-discovered" 15 years later by Kjaer and co-workers [10]. Seminal reports on the antibiotic activity of the primary rhamnosylated compound then followed, from U Eilert and colleagues in Braunschweig, Germany [11]. They re-isolated and confirmed the identity of 4-(α-L-rhamnopyranosyloxy) benzylglucosinolate and its cognate isothiocyanate and verified the activity of the latter compound against a wide range of bacteria and fungi.

The antibacterial effect of aqueous and ethanolic *Moringa* leaf extracts (*Moringa oleifera*) on the growth of gram positive and negative bacteria was tested. All extracts were tested against *Escherichia coli, Staphylococcus aureus,Vibrio parahaemolyticus, Enterococcus faecalis,Pseudomonas aeruginosa, Salmonella enteritidis* and *Aeromonas caviae*. The strains *E. coli, P. aeruginosa* and *S. enteritidis* were resistant to all treatments. In general, the extract was efficient against *S. aureus, V. parahaemolyticus, E. faecalis* and *A. caviae*[12].

Extensive field reports and ecological studies forming part of a rich traditional medicine history, claim efficacy of leaf, seed, root, bark, and flowers against a variety of dermal and internal infections. Unfortunately, many of the reports of antibiotic efficacy in humans are not supported by placebo controlled, randomized clinical trials. Again, in keeping with Western medical prejudices, practitioners may not be expected to embrace *Moringa* for its antibiotic properties.

In many cases, published in-vitro (cultured cells) and in-vivo (animal) trials do provide a degree of mechanistic support for some of the claims that have sprung from the traditional medicine lore. For example, numerous studies now point to the elevation of a variety of detoxication and antioxidant enzymes and biomarkers as a

result of treatment with *Moringa* or with phytochemicals isolated from *Moringa* [13, 14, 15].

It is seen that the aqueous extract of leaf (LE), fruit (FE) and seed (SE) of *Moringa oleifera* had the ability to inhibit the oxidative DNA damage, antioxidant and anti-quorum sensing (QS) potentials. It was found that these extracts could significantly inhibit the OH*- dependent damage of pUC18 plasmid DNA and also inhibit synergistically with trolox, with an activity sequence of LE > FE > SE. HPLC and MS/MS analysis was carried out, which showed the presence of gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin[16].

The antioxidant potency of different fractions of *Moringa oleifera* leaves has also been studied. On the basis of in vitro antioxidant properties, polyphenolic fraction of *M. oleifera* leaves (MOEF) was chosen as the potent fraction and used for the DNA nicking and in vivo antioxidant properties. MOEF shows concentration dependent protection of oxidative DNA damage induced by HO* and also found to inhibit the toxicity produced by CCl_4 administration as seen from the decreased lipid peroxides (LPO) and increased glutathione (GSH) levels. Among the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) levels were restored to almost normal levels compared to CCl_4 intoxicated rats [17].

Moringa species have long been recognized by folk medicine practitioners as having value in tumor therapy [18]. Recently the phytochemicals were shown to be potent inhibitors of phorbol ester (TPA)-induced Epstein-Barr virus early antigen activation in lymphoblastoid (Burkitt's lymphoma) cells [19, 20].

In one of these studies, it also inhibited tumor promotion in a mouse two-stage DMBA-TPA tumor model [20]. In an even more recent study, Bharali and colleagues have examined skin tumor prevention following ingestion of drumstick (*Moringa* seedpod) extracts [21]. In this mouse model, which included appropriate positive and negative controls, a dramatic reduction in skin papillomas was demonstrated.

Moringa oleifera contains a unique combination of isothiocyanate and glucosinolates. The effectiveness of the *Moringa* plant in treating ovarian cancer became evident after the publication of recent studies demonstrating that benzyl isothiocyanate (BITC) and phenethylisothiocyanate (PEITC) induce apoptosis in ovarian cancer cells in vitro[22,23].We knew that isothiocyanates have antitumor activity in cancers of the lung, breast, skin, esophagus, and pancreas, but we did not know that it can also induce apoptosis in ovarian cancer cell in vitro.

With regard to the use of the *Moringa* plant in cancer therapy, 4-(4'-O-acetyl-alpha-Lrhamnopyranosyloxy) benzyl isothiocyanate and the related compound niazimicin have been shown to be potent inhibitors of phorbol ester in lymphoblastoid cells. In a mouse study, niazimicin inhibited tumor production. Niaziminin and beta-sitosterol-3-O-beta-D-glucopyranoside have also been shown to be associated with antitumor activity [19]. Bharali and colleagues[21] even showed chemopreventive potential of *Moringa oleifera* drumstick extract against chemical carcinogens via the hepatic pathway.

With regard to the female reproductive system, *Moringa oleifera* root is shown to have unique estrogenic, antiestrogenic, progestational, and antiprogestational activities[24].Root-bark yields alkaloids: moringine and moringinine. Moringinine acts as a cardiac stimulant, produces a rise in blood pressure, acts on sympathetic nerve endings as well as smooth muscles throughout the body, and depresses the sympathetic motor fibers of vessels in large doses only. *M oleifera* confers significant radiation protection as well. It appears to be acting on some receptor, most likely the follicle-stimulating hormone receptor (FSHR), because it initially increases estrogenic action when the uterus is enlarged. Then, it may inactivate thisreceptor locally or with the help of a central mechanism through nerve growth factor (NGF)-mediated pathways[25].

The central inhibitory effect of *Moringa oleifera* root extract and a possible role of neurotransmitters have also been proposed. Dopamine and norepinephrine levels were studied in Holtzman strain adult albino rats. The results revealed that pretreatment with *Moringa oleifera* inhibited penicillin-induced seizure and

markedly reduced locomotor activity. Chronic treatment with *Moringa oleifera* significantly increased the 5-HT and decreased the dopamine level in the cerebral cortex, midbrain, caudate nucleus, and cerebellum. The norepinephrine level was significantly decreased in the cerebral cortex[26,27]. As dopamine and norepinephrine influence NGF and FSHR through central mechanisms[25], this result may support and corroborate their possible role in epithelial ovarian Cancer.

A hormonal etiology of epithelial ovarian cancer has long been suspected, and now the role of FSHR has also been elucidated[28]. *Moringa oleifera* can interfere with hormone receptor-related and neoplastic growth-related cytokine pathways via centrally acting mechanisms. It appears to have a tremendous effect on G protein-linked signal transduction system as well. Thus, the effects of *Moringa oleifera* Lam. in the treatment of epithelial ovarian cancer are worth investigating.

Moringa oleifera inhibits maintenance and growth of reproductive organs. In fact, in rural and tribal areas of West Bengal province in India, the root of this plant is taken by women, especially prostitutes, as permanent contraception, and it has been shown to totally inactivate or suppress the reproductive system.

Different parts of the plant have different pharmacological actions and toxicity profiles, which have not yet been completely defined. However, several toxicities have been described and are worth mentioning. The root bark contains 2 alkaloids as well as the toxic hypotensive moringinine. At lower concentrations, it produces a dose-dependent positive inotropic effect, and at higher concentrations, a dose-dependent negative inotropic effect, as was demonstrated in a study using an isolated frog heart. Niazinin A, niazimicin, and niaziminin A+B resulted from bioassay-directed fractionation of the ethanolic extract of *Moringa oleifera* leaves[13].

Intravenous administration (1-10 mg/kg) produced hypotensive and bradycardiac effects in anesthetized rats and negative inotropic and chronotropic effects in isolated guinea pig atria. The direct depressant action of these compounds exhibited on all of the isolated preparations tested is thought to be responsible for its hypotensive and bradycardiac effects observed *in vivo*.

Methanolic extract of *Moringa oleifera* root was found to contain 0.2% alkaloids. Effects of multiple weekly doses (35, 46, 70mg/kg) and daily therapeutic (3.5, 4.6, and 7.0 mg/kg) intraperitoneal doses of the crude extract on liver and kidney function and hematologic parameters in mice have been studied. The results indicate that weekly moderate and high doses (> 46 mg/kg body weight) and daily/therapeutic high doses (7mg/kg) of crude extract affect liver and kidney function and hematologic parameters, whereas a weekly dose (3.5 mg/kg) and low and moderate daily/therapeutic doses (3.5 and 4.6 mg/kg) did not produce adverse effects on liver and kidney function[29].LD_{50} and lowest published toxic dose of root bark extract *Moringa oleifera* Lam. are 500 mg/kg and 184 mg/kg, respectively, when used intraperitoneally in rodents (mice). Changes in clotting factor, changes in serum composition (eg, total protein, bilirubin, cholesterol), along with enzyme inhibition, induction, or change in blood or tissue levels of other transferases have also been noted[30].

However, the interior flesh of the plant can also be dangerous if consumed too frequently or in large amounts. Even though the toxic root bark is removed, the flesh has been found to contain the alkaloid spirochin, which can cause nerve paralysis[31].

S. Sreelatha and colleagues [32] in their study indicated induction of apoptosis as the key success of plant products as anticancer agents. Their study was designed to determine the antiproliferative and apoptotic events of *Moringa oleifera* leaf extract using human tumor (KB) cell line as a model system. It showed a dose-dependent inhibition of cell proliferation of KB cells. They also studied that the antiproliferative effect was associated with induction of apoptosis through morphological changes and DNA fragmentation. The morphology of apoptotic nuclei was quantified using DAPI and propidium iodide staining. The degree of DNA fragmentation was analyzed using agarose gel electrophoresis. In addition, they found that MLE at various concentrations was found to induce ROS production suggesting modulation of redox sensitive mechanism. Eventually, HPTLC analysis indicated the presence of phenolics such as quercetin and kaempferol. Thus, their findings suggest that the leaf extracts from *M. oleifera* had strong antiproliferation and potent induction of apoptosis. Thus, their work

indicates that *M. oleifera* leaf extracts has potential for cancer chemoprevention and can be claimed as a therapeutic target for cancer.

Also, Charlette Tiloke[33] and colleagues prepared a crude aqueous leaf extract of *Moringa oleifera(MOE)* and the A549 cells were treated with 166.7 µg/ml MOE (IC_{50}) for 24 h and assayed for oxidative stress (TBARS and Glutathione assays), DNA fragmentation (comet assay) and caspase (3/7 and 9) activity. In addition, they studied the expression of Nrf2, p53, Smac/DIABLO and PARP-1 .The mRNA expression of Nrf2 and p53 was also assessed. A significant increase in reactive oxygen species with a concomitant decrease in intracellular glutathione levels in MOE treated A549 cells was observed. MOE showed a significant reduction in Nrf2 protein expression and mRNA expression. A higher level of DNA fragmentation was seen in the MOE treated cells. MOE's pro-apoptotic action was confirmed by the significant increase in p53protein expression, p53 mRNA expression, caspase-9, caspase-3/7 activities and an enhanced expression of Smac/DIABLO. MOE also caused the cleavage and activation of PARP-1 into 89 KDa and 24 KDa. Hence, MOE exerts antiproliferative effects in A549 lung cells by increasing oxidative stress, DNA fragmentation and inducing apoptosis.

Thus, traditional practice has long suggested that cancer prevention and therapy may be achievable with native plants. Modern practitioners have used crude extracts and isolated bioactive compounds. The proof required by modern medicine has not been realized because neither the prevention of cancer nor the modification of relevant biomarkers of the protected state has been adequately demonstrated in human subjects. More rigorous study is required in order to achieve a level of proof required for full biomedical endorsement of *Moringa* as, in this case, a cancer preventative plant.

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Chapter 3

Objective and Proposed plan of work

Objective

Moringa oleifera (MO) was chosen as the plant to be studied further as it is an indigenous tree to India, belonging to the family Moringaceae and is widely cultivated for medicinal and industrial purposes. It is commonly referred to as the 'tree of life' or Drumstick tree. All parts of the MO plant possess medicinal properties, but the leaves have high nutritional value (high levels of vitamins C and A, potassium, proteins, calcium and iron), phytochemicals like carotenoids, alkaloids and flavonoids and is rich in amino acids such as cystine, lysine, methionine and tryptophan. MO is used in traditional treatment of diabetes mellitus, cardiovascular and liver disease.

Breast cancer is the second leading cause of cancer death in women, exceeded only by cervical cancer in India. Breast cancer though has better medications available than other cancers in recent times, but current drug therapies have many side-effects and alternate therapy is actively being sought.

If traditional medicine can provide an alternate source for treatment or control, the number of breast cancer incidences can be reduced as it is a much more cost effective treatment.

The antiproliferative effect of aqueous *Moringa oleifera* leaf extract is already demonstrated on the KB cell line, A549 and HeLa cell line[1,2,3].

The major objective of the present work was to evaluate the anticancer potential of *Moringa oleifera* Leaf extract on the human breast cancer cell as it has not yet been demonstrated.

Proposed Plan of work

- The initial work was to prepare the various fractions of the *Moringa oleifera* leaf extract by two methods :

    (i)Successive extraction procedure using soxlet apparatus

    (ii)Crude methanolic extract prepararion by maceration

- Standardization of the extract which has the maximum cytotoxity in the lowest dose using the HeLa cell line as the model.
- Dose dependant and time dependant cytotoxic effect of the extract on the two Breast cancer cell lines MCF 7 and MDA MB 231.

- Determination of LD_{50} value of extract for both the cell lines.

- Effect of extract on the human peripheral mononuclear lymphocytes.

- Effect of extract on cell morphology as viewed under Phase Contrast Microscope.

- Effect of extract on Scratch Wound Healing.

- Effect of extract on Cellular adhesion.

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Chapter 4

Materials and Methods

Sample collection and Preparation:

Fresh leaves of *Moringa oleifera* was collected, washed twice in distilled water and shade dried for 72 hours. The dried leaves were powdered using a domestic mixer grinder into a coarse powder and its raw weight was taken.

Extraction Protocol:

Two methods of extraction were followed:

1. Extract preparartion using soxlet apparatus: 75g of the dried leaf powder was re-extracted using petroleum ether, chloroform, ethyl acetate, methanol, and water in succession using soxhlet apparatus. Each extract obtained following successive extraction was filtered using Whatman No 1 filter paper, dried to a semisolid mass using water bath and the yield of each extract thus obtained was recorded and stored in a refrigerator at -20°C till further use.

2. Crude Methanolic Extract (MLE): 100 gms of air dried *Moringa oleifera* leaf powder was taken in 1000ml pure methanol in a round bottomed flask. The solution was masticated with occasional stirring and kept in room temperature for 48 hours. The supernatant was recovered after filtration through folded paper using suction apparatus. The respective solvent was completely removed by rotary vacuum evaporator and the extract was freeze dried and stored in a -20°C freezer for further use.

Sample Preparation:

The stock solution of each extract was prepared by taking 10mg of extract in 500µl of filter sterilized (0.22µm) DMSO and stored at 4°C. The other working concentrations are prepared using serial dilutions of the stock solution.

Culture of cells:

HeLa culture: Cervical cancer cell line HeLa was cultured in complete DMEM(Dulbecco's modified eagle medium) with 10% Fetal bovine serum and 2% Penicillin Streptomycin and maintained at 37^0C in a humidified incubator with 5% CO_2. The cells were grown in T-25 flasks till about 80% confluence and split to 1:3 ratio in 60 mm culture dishes before any assay was performed.

MCF 7 culture: Epithelial breast cancer cell line MCF 7 was cultured in complete DMEM(Dulbecco's modified eagle medium) with 10% Fetal Bovine Serum and 2% Penicillin Streptomycin and maintained at 37^0C in a humidified incubator with 5% CO_2. The cells were grown in T-25 flasks till about 80% confluence and split to 1:3 ratio in 60 mm culture dishes before any assay was performed.

MDA MB 231 culture: Mesenchymal breast cancer cell line MDA MB 231 was cultured in L-15(Leibovitz-15) medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin and maintained at 37^0C in a humidified incubator without CO_2. The cells were grown in T-25 flasks till about 80% confluence and split to 1:3 ratio in 60 mm culture dishes before any assay was performed.

Isolation of Peripheral Blood Mononuclear Lymphocyte cell:

1. Fresh blood (3ml) was collected in EDTA coated vials and stored in room temperature and processed within a maximum of 2 hours.
2. 0.9% saline (0.9mg of NaCl in 100ml Millipore water) was prepared, autoclaved and cooled.
3. A 1:3 dilution of the whole blood was made with saline and care was taken so as to prevent the formation of any blood clots.
4. 2.5ml of HiSep LSM 1077(Himedia) was taken in a 15ml centrifuge tube and 7.5ml of diluted blood was layered over it. Care was taken to ensure there is no mixing of the two layers and there is a sharp interphase between them.
5. Centrifugation was done at room temperature for 30 mins.
6. The blood gets separated into 4 layers post centrifugation. The topmost lightish yellow layer of plasma thrombocyte layer was aspirated using a Pasteur pipette

and discarded. The following whitish ring like layer is that of the lymphocytes. This white ring layer together with half of the HiSep layer was taken in another 15ml tube. The lowermost red pellet of RBC's was discarded.

7. The lymphocytes and HiSep mixture was added to an equal volume of serum free RPMI 1640 medium, mixed well and centrifuged at 800 rpm for 10 minutes.
8. The same process of washing was repeated to remove excess platelets, HiSep and plasma.
9. The cell pellet obtained was suspended in complete RPMI 1640 medium with 10% Fetal Bovine Serum and 2% Penicillin Streptomycin and maintained at 37^0C in a humidified incubator with 5% CO_2.

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) Assay:

- Cells were split from T 25 flasks to 60 mm dishes.
- When the cells have reached around 80% confluence, cells were trypsinized and seeded in 24 well culture dishes in a seeding density of $10x10^4$cells per well in 500µl of complete DMEM per well; complete media containing 10% FBS and 2% Penicillin Streptomycin and incubated overnight.
- After the cells have reached 60% confluence, complete media was removed and replaced with serum free media with 1% ITS.
- The cells were treated with 10 µl of the different concentrations of the drug for the incubation time at 37^0C in a humidified incubator with 5% CO_2.
- Cells were kept as control for normal (without treatment) and vehicle (DMSO).
- After the incubation period with the drug MTT solution was added. (conc of MTT used: 5mg/ml in PBS)
- Incubated for 3 hours.
- Media removed after incubation of 3 hours with MTT.
- 500µl of DMSO was added to each of the wells, plate covered and agitated on gel rocker for 15 minutes.
- Volume made upto1.5ml with DMSO.
- Read spectrophotometrically at 590 nm.

- The % of cell viability = $\frac{\text{Absorbance of treated cells} \times 100}{\text{Absorbance of control cells}}$

Screening of Different extract fractions:

To determine which extract has the maximum amount of cytotoxicity in the lowest dose MTT assay was performed for all the extract fractions using the HeLa cell line.

Dose finding:

After the standardization and selection of the extract to be used for further studies the MTT assay was repeated on the breast cancer cell lines MCF 7 and MDA MB 231 to find the LC_{50} value of the drug on these two cell lines for both the time durations of 24 and 48 hours keeping cis Platinum as a positive control for the drug used.

Effect of extract on normal cell (PBMC):

The effect of LC_{50} dose of extract on the normal cell (PBMCs) is evaluated using the MTT assay and the percentage of cell viability is calculated by the procedure discussed above. Before proceeding with the MTT assay the following steps are performed for PBMC.

1. The cell viability of the isolated lymphocytes was tested using trypan blue cell staining test and counted under phase contrast microscope.
2. The stain was prepared using 40mg trypan in 10 ml PBS and warmed a little.
3. % dead cells calculated = $\frac{\text{Dead cells} * 100}{\text{Total cells}}$

The cell viability of the PBMC should be atleast 95% before we begin with the MTT assay protocol.

Effect of extract on cell morphology as viewed under Phase Contrast Microscope:

Cells were incubated for 24 and 48 h in the absence or presence of extract in 24-well plates. After incubation, the medium was removed and cells in wells were washed once .They was then observed by phase contrast inverted microscope at 20X magnification.

Scratch Wound Healing Assay:

- Cells were seeded in 24 well plates with seeding density of $25x10^4$cells/well and incubated overnight in 500µl of complete media.
- When the cells have reached nearly 60% confluence , complete media was replaced with serum free media containing 1% ITS and incubated in this serum starved condition overnight.
- When the cells have reached 80-90 % confluence a scratch was made using a sterile pipette.
- The media was replaced with fresh serum free media to remove the unseeded cells.
- Different concentrations of the drug was added and incubated for 24 and 48 hours. Groups are kept for control and vehicle.
- Photo micrographs of the scratch are taken using phase contrast microscope at 0, 24 and 48 hours.

Adhesion Assay:

- 24 well plates were coated with 200 µl of diluted Gelatin (250µg/ml) and incubated at 37^0C for 1 hour.
- The gelatin was aspirated out and incubated again at 37^0C for half an hour to let the gelatin polymerize.
- After polymerization treated cells were seeded in gelatin coated wells with a seeding density of $5x10^4$ cells in 500 µl of serum free media.
- Cells were allowed to adhere on the gelatin for 2hrs.
- After the incubation time, the media was removed and the non adherent cells were washed off with 1X PBS twice.
- Adherent cells were fixed with 500µl of 100% methanol for 5 minutes and washed with 1X PBS again.
- 300µl of 1X PBS is added to the wells
- 10µl of 4', 6-diamidino-2-phenylindole (DAPI) stain in working concentration of 25µg/ml in PBS was added.
- The well plate was covered using aluminium foil as DAPI is light sensitive.
- Observed under the fluorescence microscope using UV filter.
- The number of adhered cells was observed under fluorescence microscope and counted.

Chapter 5

Observations and Results

I. Screening of soxlet extract fractions in different concentrations using MTT Assay on the HeLa cell line

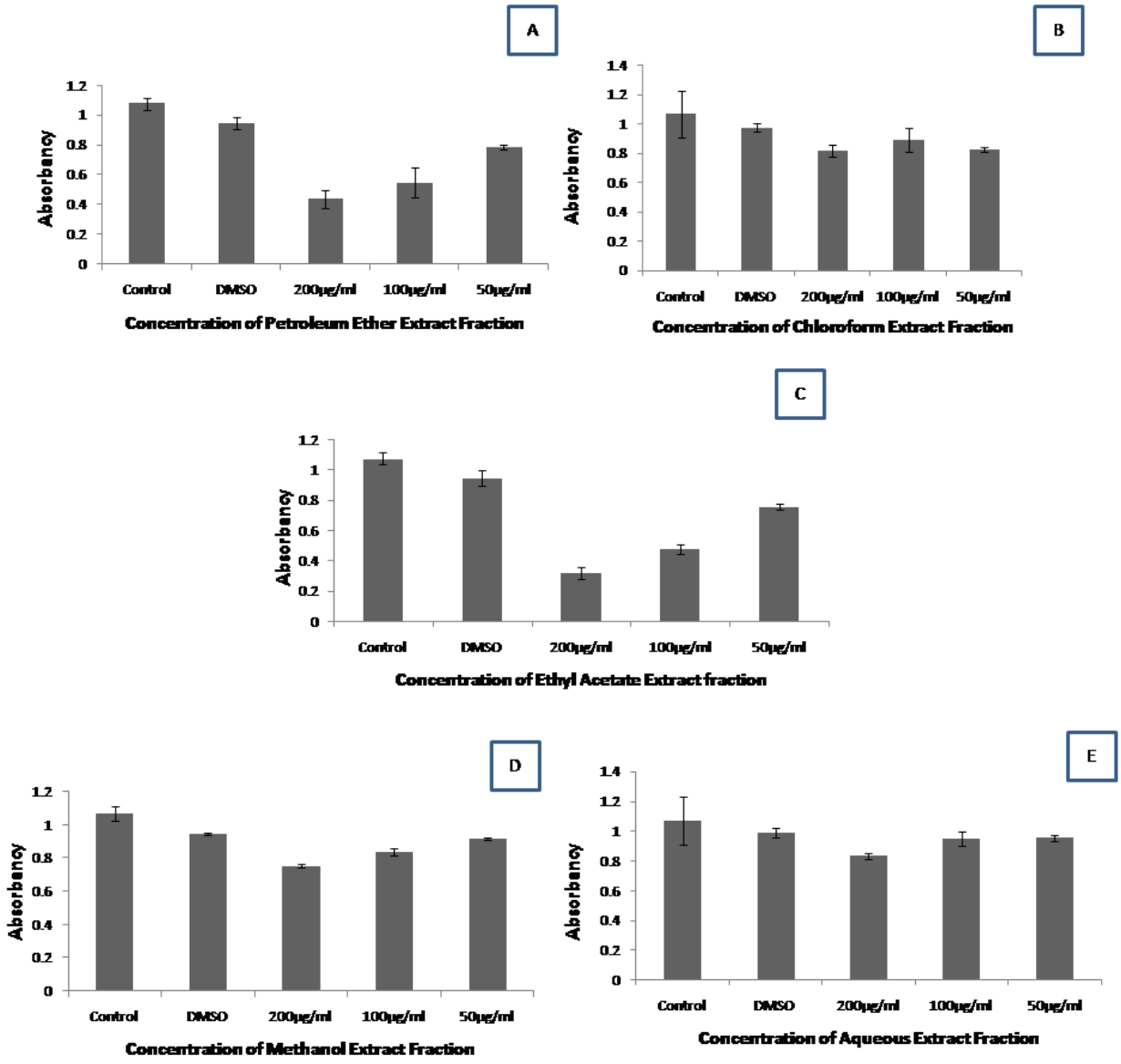


Fig 5.1: *MTT assay on different soxlet fractions A: Petroleum ether, B: Chloroform , C: Ethyl acetate, D: Methanol , E : Aqueous.*

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The MTT assay measures cell viability based on the generation of reducing equivalents in metabolic active cells. The higher the absorbance measured the higher is the cell viability. The percentage cell viability is calculated.

It was seen that the Ethyl acetate fraction has the higest cytotoxicity in the lowest dose as compared to the other fractions in the MTT assay.

Petroleum ether fraction has the next highest toxicity. Choloroform, Methanol and Aqueous fractions have nearly comparable cytotoxicities.

II.Screening of Crude Methanolic fraction in different concentrations using MTT Assay on the HeLa cell line

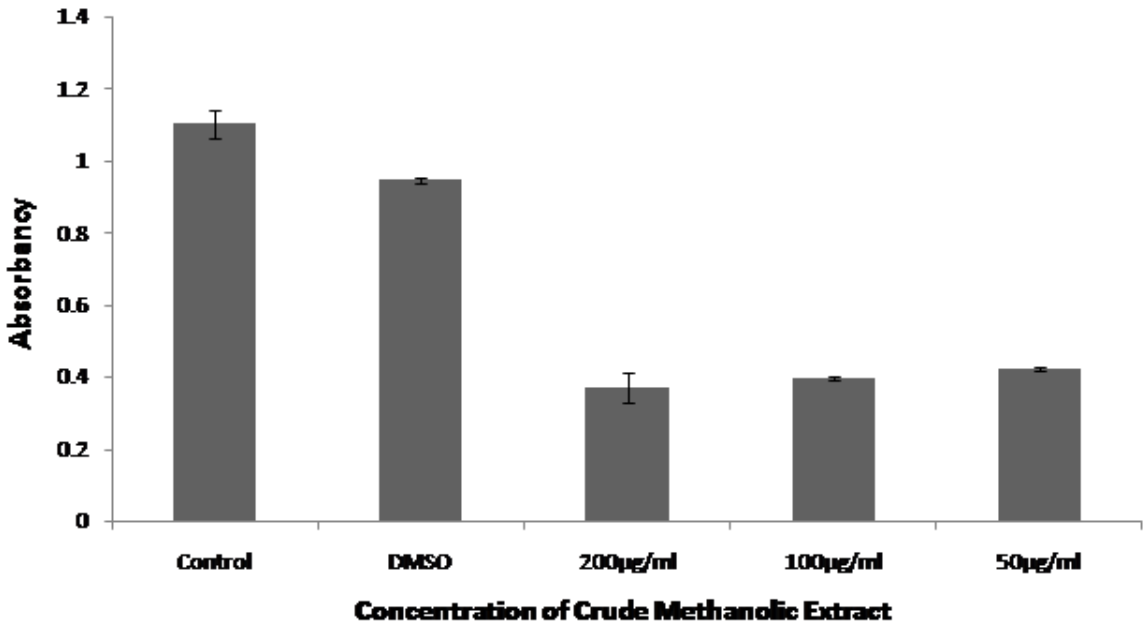


Fig 5.2: *MTT Assay on crude methanolic fraction for different doses*

The MTT assay measures cell viability based on the generation of reducing equivalents in metabolic active cells. The higher the absorbance measured the higher is the cell viability.

It is seen that the crude methanolic fraction has a dose dependant cytototoxic effect on the HeLa cells, as with increasing dose the absorbance of the cells decrease proportionally and the percentage of cell viability was calculated and compared to the control.

III.Selection of Extract for Further Studies on the Breast Cancer Cell Line

The crude methanolic extract gave the maximum cytotoxicity at the lowest dose as compared to the Ethyl acetate fraction(MTT assay results) and hence all further testing was carried out by using this crude methanolic extract as the drug.

IV. MTT assay of Crude Methanolic *Moringa oleifera* leaf extract (MLE) on the Breast cancer cell lines

The cell lines which are being used for measuring the anti –proliferative capacity of MLE are :

(i) MCF 7 : Epithelial breast cancer cell line.
(ii) MDA MB 231: Mesenchymal breast cancer cell line.

The dose dependant as well as time dependant effect of MLE is checked on both the above cell lines. Groups were kept for both Control and Vehicle (DMSO). Cis Platinum is kept as a positive control for both the cell lines.

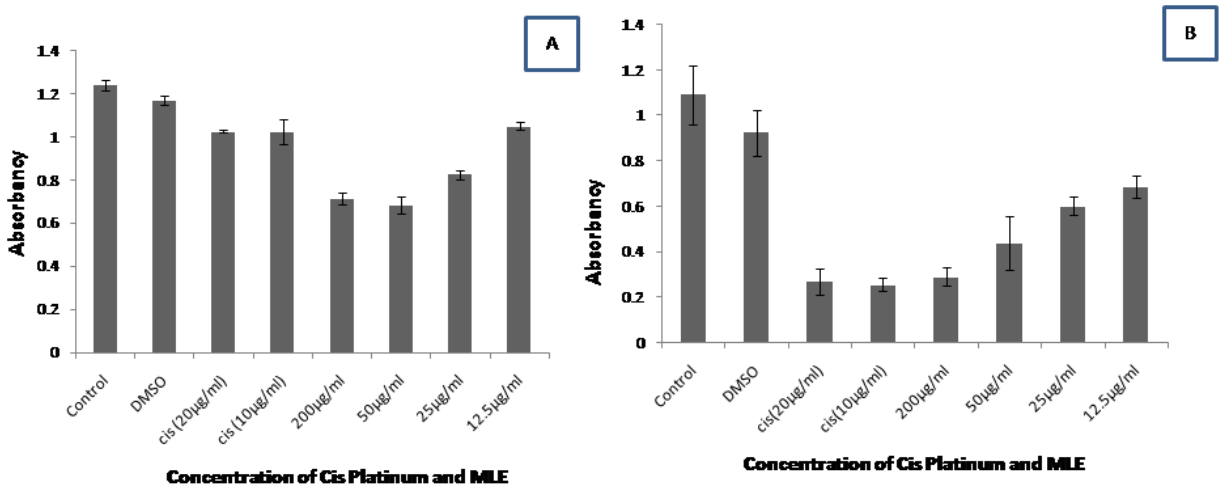


Fig 5.3 :*MTT Assay on MCF 7, A: 24 hours B: 48 hours*

MTT Assay is performed on the MCF 7 cell line for both 24 hours and 48 hours for different doses. It is seen that the cytotoxic effect of MLE is less pronounced at 24 hours than it is at 48 hours. It is seen that even for cis Platinum which is used as the standard positive control for the experiment, the effect is seen mainly after 48 hours.

Hence all assays for MCF 7 henceforth were conducted for 48 hours.

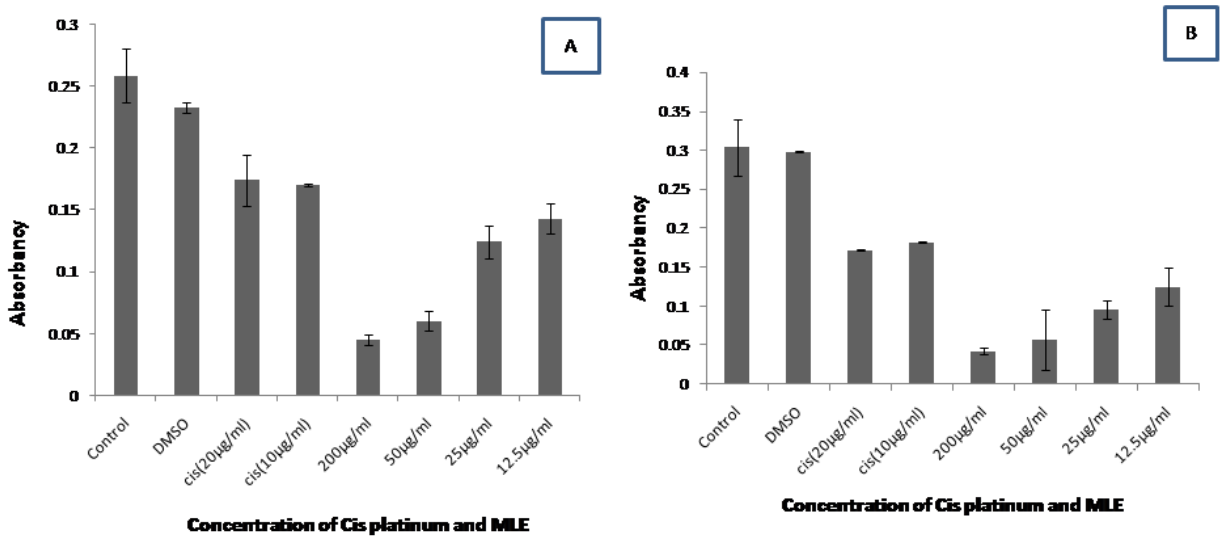


Fig 5.4: *MTT Assay on MDA MB 231, A: 24 hours B: 48 hours*

MTT Assay is performed on the MDA MB 231 cell line for both 24 hours and 48 hours for different doses. It is seen that the cytotoxic effect of MLE is only slightly higher at 48 hours than it is at 24 hours.

It is also seen, that for cis Platinum which is used as the standard positive control for the experiment, the effect is not much pronounced at both 24 and 48 hours.

Hence all assays for MDA MB 231henceforth were conducted for 48 hours.

V. Calculation of LD_{50} value for both the cell lines

Acute toxicity of a drug can be determined by the calculation of LD_{50}value, i.e., the dose that will kill 50% of cells of a particular cell line. The LD_{50} value of MLE was determined for both the cell lines and sub lethal dose for assigned for all further assays.

MCF 7: The sub lethal dose was determined using the absorbancy of DMSO treated cells as the standard control(100% cell viability) for the calculation. It was found out to be 25µg/ml for MCF 7 cell line.

MDA MB 231: The sub lethal dose was determined using the absorbancy of DMSO treated cells as the standard control(100% cell viability) for the calculation. It was found out to be 12.5µg/ml for MDA MB 231 cell line.

VI. Effect of MLE on normal cell (PBMC)

The effect of MLE was checked on peripheral blood mononuclear lymphocyte cells to evaluate its potential as a prospective anti cancer agent.

The MTT Assay was performed on PBMC after 24 hours of treatment with the MLE. Groups were kept for Control and Vehicle (DMSO). The doses were chosen in the range of the LD_{50} values used.

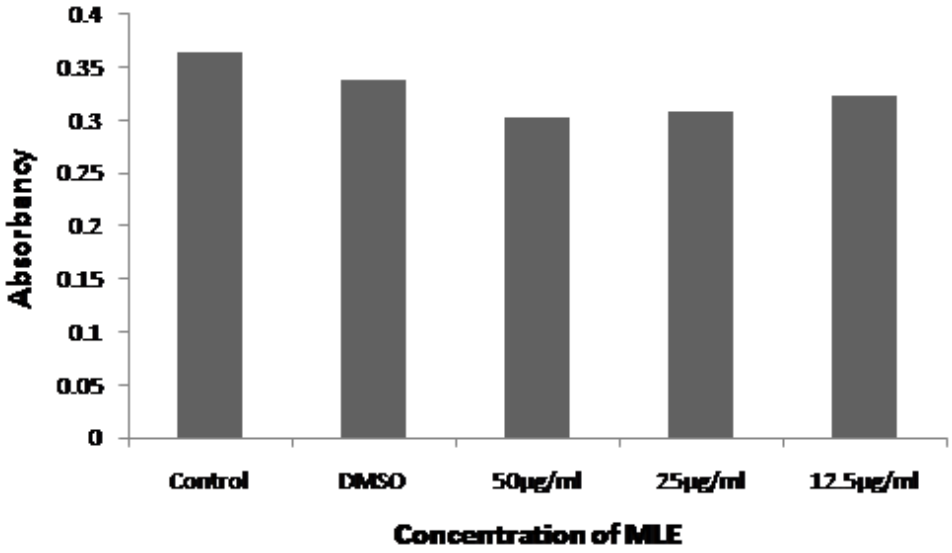


Fig 5.5: *MTT Assay in the LD_{50} dose range of MLE on PBMC*

It is seen that the cell viability is around 70-90% for PBMC's after MLE treatment.

Comparative effect of MLE on Normal and the Cancer cells:

Cell lines	Dose(µg/ml)	%Live cell	%Dead cell
MCF 7	50	47	53
	25	74	26
MDA MB 231	50	28	72
	25	48	52
PBMC	50	77	23
	25	88	12

Table 5.1:*Comparative effect of MLE on different cells*

So here we can see that MLE induces very low cytotoxic effect on PBMC's in comparison to the Breast cancer cell line in the above determined doses.

Hence MLE has the potential to be evaluated as a potent anticancer drug since it inhibits only the cancer cells and does not greatly inhibit the proliferation of normal cells.

VII.Effect of MLE on Cell Morphology

Untreated control cells as well treated cells for the dose determined were viewed under phase contrast microscope to note down the morphological changes.

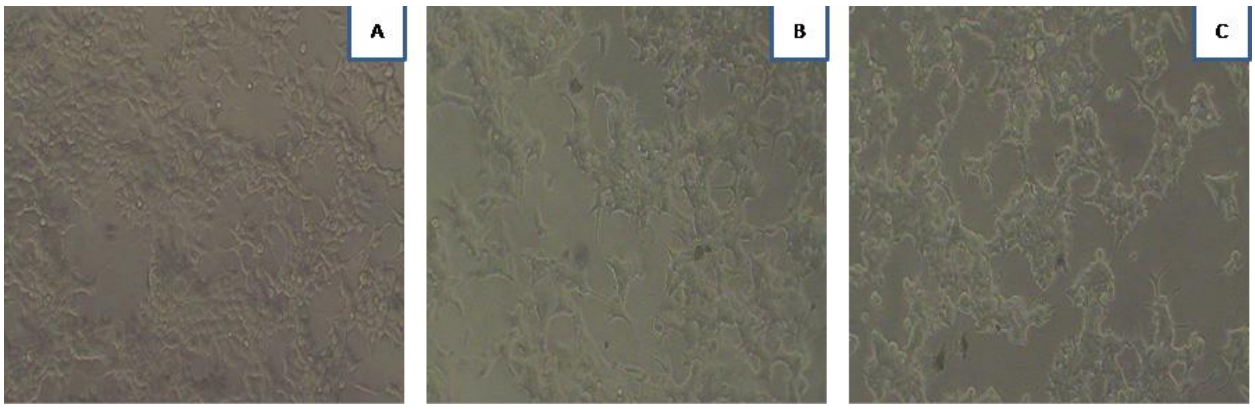


Fig 5.6: *Cell morphological changes A: Untreated control MCF 7 cells, B: Treated cells at 24 hours, C: Treated cells at 48 hours.*

It is seen that the control cells are healthy and growing well with sharp morphology both at 24 and 48 hours. At 24 hours post treatment nearly 10-20% of cells are rounded up and floating. Cells which are seeded, cell morphology is sharp.

At 48 hours around 20-30% of cells are rounded up and deposited on cells which are seeded. Few cells are dead and floating. The seeded cells have sharp morphology.

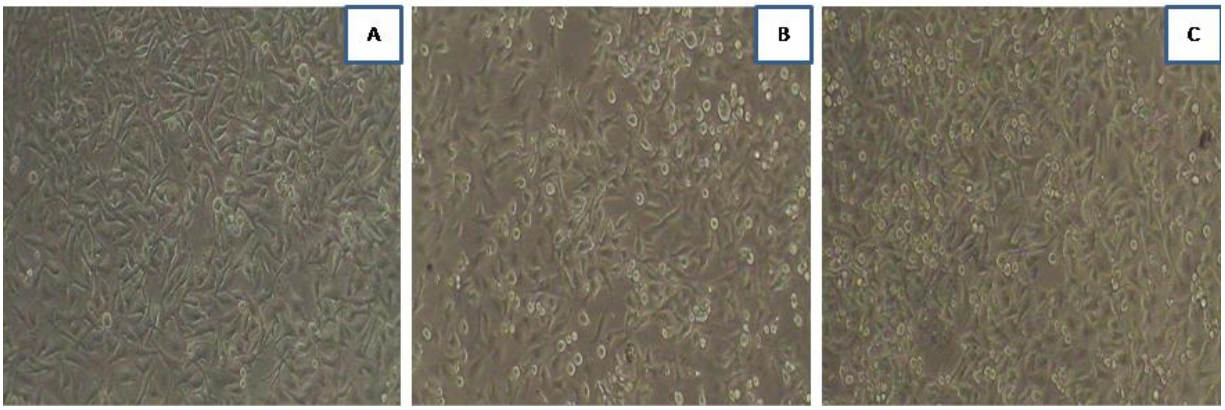


Fig 5.7: *Cell morphological changes A: Untreated control MDA MB 231 cells, B:Treated cells at 24 hours, C: Treated cells at 48 hours.*

It is seen that the untreated control MDA MB 231 cells are healthy and growing well both at 24 and 48 hours with sharp cell morphology.

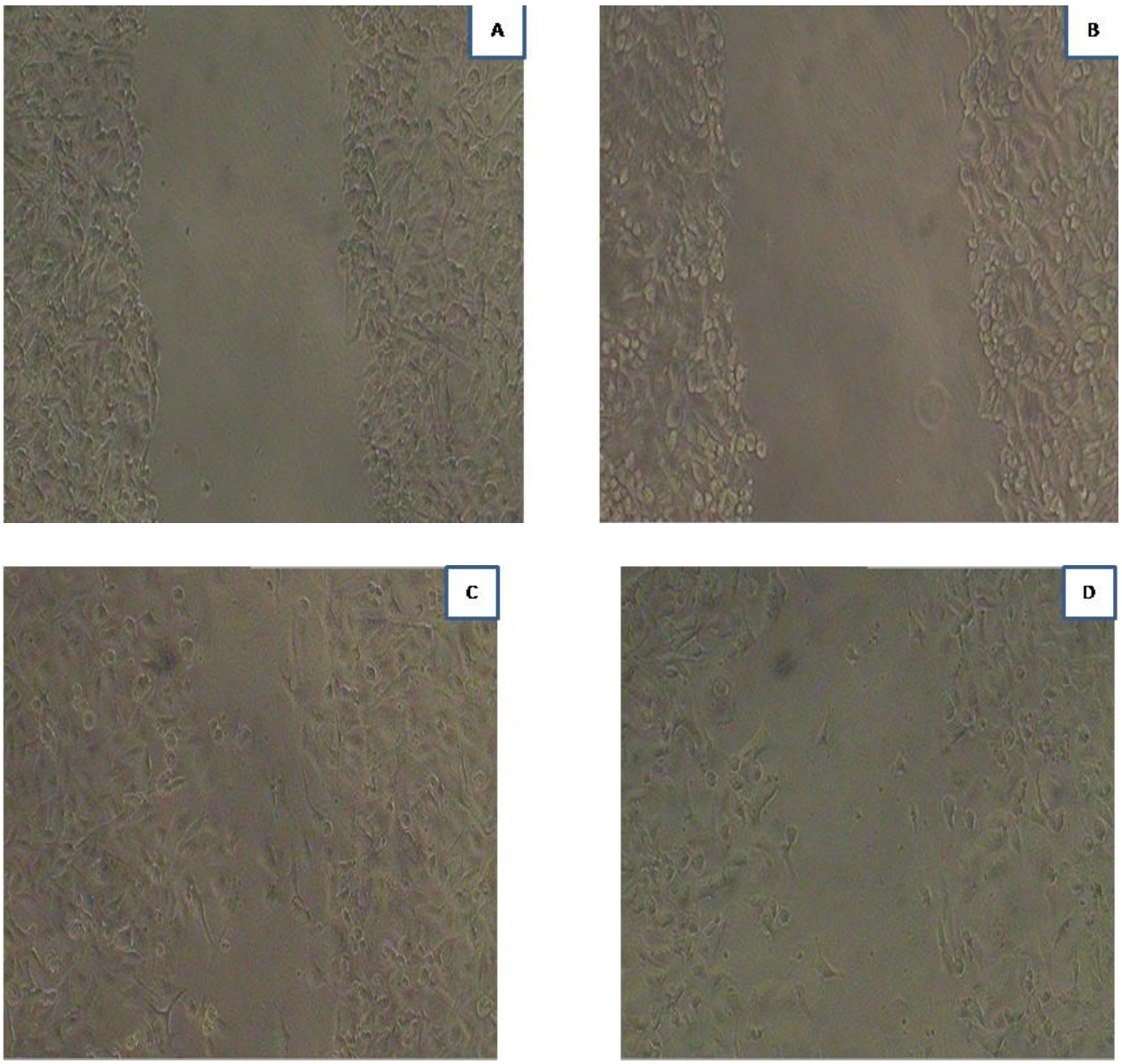
For the treated cells 30-40% of the cells rounded up and floating at 24 hours. Few cells are shrinked. Cells which are seeded have sharp morphology.

At 48 hours 50-60% of the treated cells are rounded up and floating. Few cells are shrinked. Cells which are seeded have sharp morphology.

Hence, from the morphological analysis of the cells under the phase contrast microscope we can predict that maybe MLE either arrests growth or induces apoptosis in both the cell lines.

VIII. Effect of MLE on Scratch Wound

Effect on the wound healing capacity of the cells after MLE treatment for 48 hours: Scratch wound healing assay is performed to see whether cells proliferate and migrate post the formation of the wound.



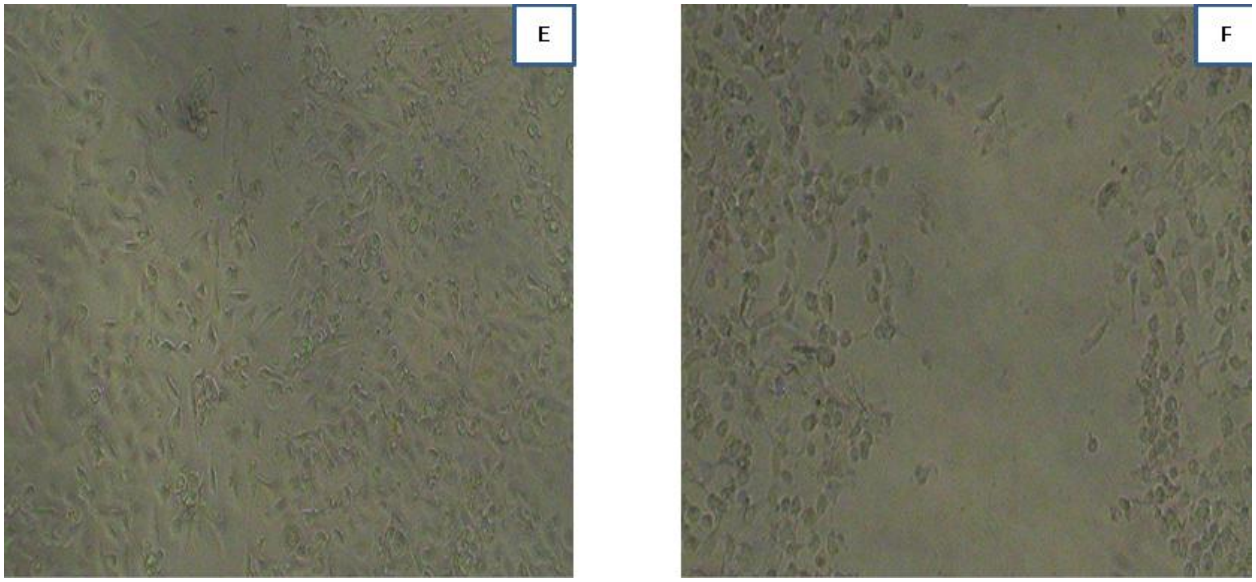


Fig 5.8: *<u>Photomicrograh of scratch at 0(A,B),24(C,D) and 48(E,F) hour</u> : A,B: Scratch wound on MDAMB 231 cells at 0 hour C: The wounded area in the control cells(untreated) started to heal up after 24hrs of scratching. E : At 48hrs the scratch almost healed up in the control cells. D, F : The wound in the treated cells(LD_{50} dose) heal up at a very slow rate with almost negligible decrease in the area of the wound, the wound in the treated cells remained with few cells filling up the denuded area at 24hrs and also at 48 hours.*

It is seen that MLE prevents wound healing in MDA MB 231 cells. This indicates that MLE inhibits cellular migration and proliferation in cancer cells.

Maybe, MLE has an anti metastatic effect on MDA MB 231 cells.

IX.Effect of MLE on Cellular Adhesion

Effect on the adhesive capacity of the cells after MLE treatment on both the cell lines:

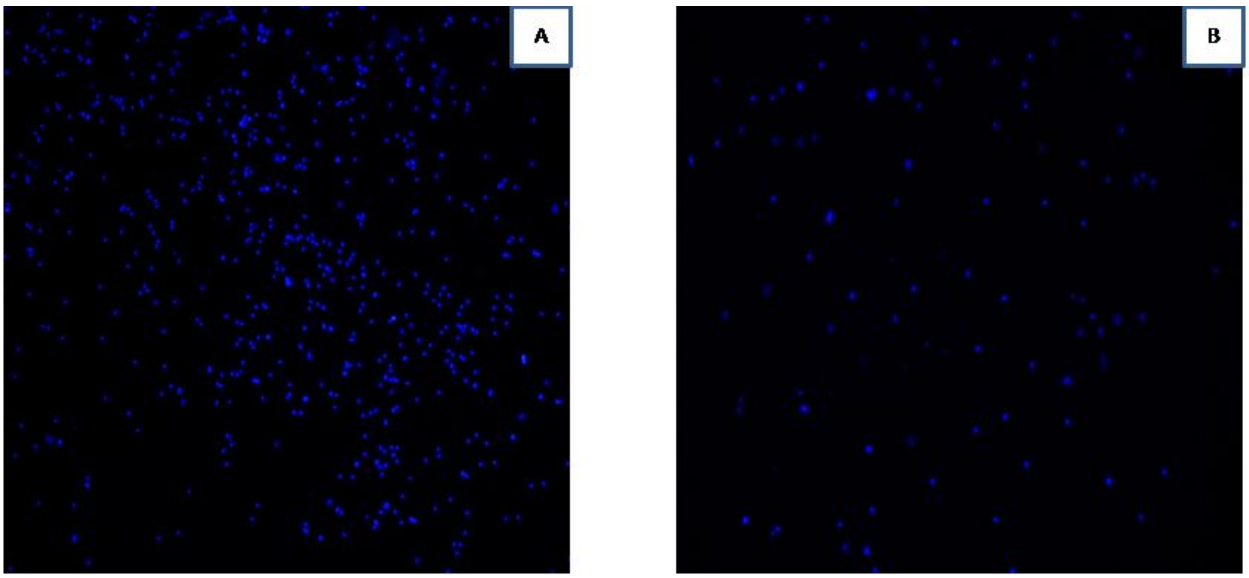


Fig 5.9: A: *Fluorescence image of adhered MDA MB 231 cells without any treatment, B: after MLE treatment.*

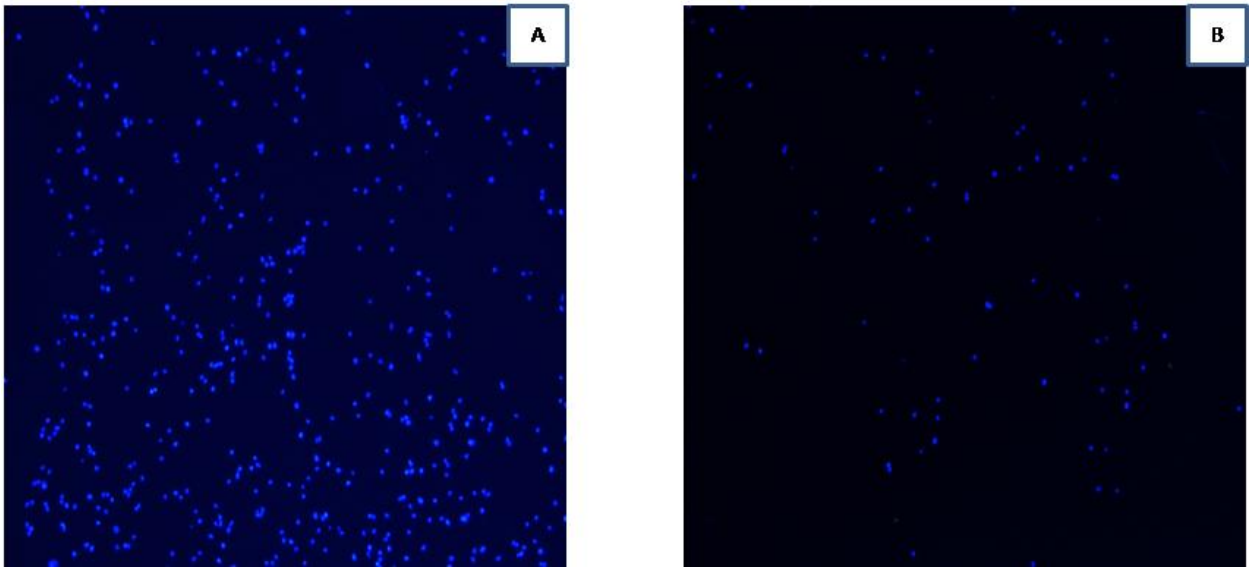


Fig 5.10: A :*Fluorescence image of adhered MCF 7 cells without any treatment, B: after MLE treatment*.

There was decrease of nearly 40% of the adhered cells of MDA MB 231and nearly 50% decrease of MCF 7 cells in comparison to the untreated control cells.

The adhesive capacity of MDA MB 231 and MCF 7 cells was decreased after MLE treatment.

These results indicate that MLE might have an anti metastatic effect on these breast cancer cells.

Chapter 6

Discussion and Future plan of work

Discussion:

After evaluating the various extracts using different exacting solvents in increasing polarities, it was seen that the crude extract of *Moringa oleifera* has the maximum cytotoxicity in the lowest dose.

The ethyl acetate fraction also has high cytotoxicity and hence it maybe possible that this fraction contains the principal component(lead compound) responsible for the cytotoxic activity of *Moringa*.

It was for the first time that the anti proliferative effect of *Moringa* was studied on two human breast cancer cell lines MDA MB 231 and MCF 7 and it showed both a dose and time dependant effect. It was also compared to a standard drug, cis Platinum and comparable LD_{50} values were obtained.

The effect of *Moringa* on the normal cells were also studied for the first time in our experiment. *Moringa* induces very low cytotoxicity in PBMC as compared to cancer cells. This is an encouraging result as most cancer drugs affect both normal and cancer cells considerably. Hence, MLE can be further evaluated as a potential anticancer agent which kills cancer cells without affecting normal healthy body cells.

When viewed under the phase contrast microscope treated cells show cell morphological changes which suggest that maybe MLE arrests growth or induces apoptosis.

MLE inhibits wound healing and cellular adhesive properties. Thus MLE might have anti metastatic effect on breast cancer cells.

Hence, the anti proliferative effect of MLE has been demonstrated on the breast cancer cell line and its low cytotoxic effect on PBMC has also been evaluated in this study. Further study is required to confirm whether the cells follow an apoptotic or anti metastatic pathway to inhibit the cancer cells.

Future Plan of Work:

- Effect on Cell cycle and apoptosis.
- Effect on cellular invasion and migration.
- Effect on different molecular parameters like various apoptotic and metastatic markers using real time PCR and western blot analysis.
- In vivo experiment with nude mice model.
- Identification, isolation and quantification of Lead compound in crude extract using HPLC and HPTLC.
- Formulation of nano form of the lead compound in the extract.
- Characterization of the nano form using UV VIS spectroscopy, TEM and AFM.
- Comparison of the nano form to the macro form of the drug using the various cell morphometric assays.

List of Publications:

1. Nanoparticles of *Moringa oleifera*- Characterization and its Anti Microbial Efficacy ,International Conference on Functional Materials- Materials Science Centre, at IIT Kharagpur, 2014.

2. Anti-Proliferative effect of the *Moringa oleifera* Leaf extract on Human Cancer Cell ,International conference on "Molecular Biology and its applications" at Department of Life Science and Biotechnology, Jadavpur University, Kolkata , 2014.

3. Anti-Proliferative effect of the *Moringa oleifera* Leaf extract onHuman Breast Cancer Cell in 21st West Bengal State Science &Technology Congress, 2014.

Journal Publications:

Silver nanoparticles of *Moringa oleifera* and its antimicrobial efficacy. *To be communicated.*