
# THE EFFECTS OF SNAKE VENOMS AS CYTOTOXINS ON SELECTED PROSTATE CANCER CELL LINES

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**By**

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# Abstract

Cancer is expected to claim 9 million deaths world-wide by the year 2020. Although there has been an increase in the sophistication of current therapeutic strategies, 40% of patients are still likely to die from the disease. Novel anticancer compounds are needed.

Snake venoms may represent a relevant pool for selecting candidates that may have anticancer properties. Snake venoms are complex mixtures of unexplored sources of molecules with potential use in biomedical research and drug development. Some venom molecules are of low molecular weight and might trigger anticancer responses and could have potential therapeutic applications.

In this study, selected snake venoms obtained from *Naja pallida, Agkistrodon piscivorus conanti* and *Agkistrodon contortrix laticinctus* were evaluated for cytotoxicity and selectivity in vitro against a panel of prostate cancer cell lines (DU145, PC3, LNCaP) and a non-malignant prostate epithelial cell line (PNT2A). The cytotoxicity activity of each venom was initially assessed using the SYTOX Green assay. SYTOX Green is a fluorescent nucleic acid indicator dye that can be used as a marker for cell death.

The whole venoms, fractions and sub-fractions of *Naja pallida, Agkistrodon piscivorus conanti* and *Agkistrodon contortrix laticinctus* were tested and found to have activity ranging that from 0.1-20µg/ml against prostate cancer cell lines using an assay based on the release of lactate dehydrogenase (LDH).

Further characterization of cytotoxic activity, and chemical analysis, including High Performance Liquid Chromatography (HPLC) guided fractionation, gel filtration and ion exchange chromatography has been conducted. The investigation of the possible mode of activity of the most cytotoxic venoms, and prospects for future work and on-going isolation and identification of pure anticancer compounds have been detailed in this study.

The different fractions of low molecular weight (<15kDa) were further fractionated using a C18 reverse phase HPLC column and tested for activity against the prostate cell lines. The N-terminal amino acid sequences of the first residues of active pure fractions from *Naja pallida* venom were P4F3-2 LKXNQLIPPFWKTXP and P4F4-1 LKXNKLIPIA YKTXPEGKNLXYK. These N-terminal sequences show homology with the group of cobra cytotoxins/ cardiotoxins from the family Elapidae and are likely to be identical to two known cytotoxins, cardiotoxin γ from *N. pallida* and cardiotoxin 4 from *N. mossambica*. Another peptide had an N-terminal sequence similar to a phospholipase $A_2$.

These peptides were tested for specific cytotoxic activities against on the cell lines by the LDH assay. Fraction P4F4 at 5 and 10μg/ml had strong cytotoxic activity on DU145 and PC3 cells, but less activity on LNCaP cells and no activity on the non-cancer PNT2A cells. The sub-fractions P4F4-1 and P4F4-2 had marked effects on PC3 cells, lesser effect on LNCaP cells and no effect on DU145 and PNT2A cells.

Further characterisation is needed, but this may be the first report of cytotoxins with possibly selective anticancer cell activity isolated from the venom of red spitting cobra *Naja pallida*.

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## Contributions from this work

**Poster 1:** Basher M Abdalsaed, Alan L Harvey and Edward G Rowan (2011). Effect of snake venom *Naja pallida* on prostate cancer cell lines, Research day 2011 at Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS).

**Poster 2:** Basher M Abdalsaed, Alan L Harvey and Edward G Rowan (2012). Separation of proteins from three snake venoms by High performance liquid chromatography (HPLC) method and their effect on prostate cancer cell lines, Research day 2012 at Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS).

**Poster 3:** Basher M Abdalsaed, Alan L Harvey and Edward G Rowan. (2013) Toxicity and morphological of cell death induced by *Naja pallida* venom on selected types of human prostate cancer cell lines, Venoms 2013 Oxford Conference Venom 24-26$^{th}$ September 2013 St Hilda's College.

**Poster 4:** Basher M Abdalsaed, Alan L Harvey and Edward G Rowan (2014). Snake venom of *Naja pallida* as cytotoxins on selected prostate cancer cell lines, Higher Education Forum on 5 - 6$^{th}$ June 2014 at Hilton London Metropolis Hotel.

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## Author’s declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree. 

# Definitions

| | |
|---|---|
| ACE | Angiotensin-Converting Enzyme |
| AChR | Acetylcholine Receptor |
| AIDS | Acquired Immuno Deficiency Syndrome |
| AR | Androgen Receptor |
| BC | Before Christ |
| BLAST | Basic Local Alignment Search Tool |
| BPH | Benign Prostatic Hyperplasia |
| C | Degree Celsius |
| cm | Centimetre |
| CM-50 | Sephadex C-50 |
| CAM | Complementary and Alternative Medicine |
| CLP | C-type Lectin-like Protein |
| CNS | Central Nervous System |
| CRISP | Cysteine-Rich Secretory Protein |
| DHT | Dihydrotestosterone |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| $EC_{50}$ | Effective Concentration |
| ED | Erectile dysfunction |
| LDH | Lactate Dehydrogenase |
| FCS | Foetal Calf Serum |
| FIX/X | The binding site of FIX/FX-binding protein |
| G75 | Sephadex G75 |
| GI | Gastrointestinal |
| GP | Glycoproteins |
| GPIb/IX | Glycoproteins Ib and IIb/IIIa |
| HBSS | Hank's Balanced Salt Solution |
| HIV | Human Immunodeficiency Virus |
| HPLC | High Performance Liquid Chromatography |
| HRPC | Hormone Responsive Prostate Cancer |
| IEF | Isoelectric Focusing |
| KDa | Kilo Dalton |
| LCMS | Liquid Chromatography / Mass Spectrometry |
| LUTS | Lower Urinary Tract Symptoms |
| M | Molar concentration |
| mg | Milligram |
| µg | Microgram |
| µM | Micro molar |

| | |
|---|---|
| µl | Micro litre |
| Min | Minute |
| ml | Millilitre |
| MS | Mass spectrometry |
| MP | Mobile phase |
| L | Litre |
| LAAO | L-amino Acid Oxidase |
| NF- κB | Nuclear factor-κB |
| NIH | National Institutes of Health |
| Nm | Nanometre |
| PIN | Prostatic Intraepithelial Neoplastic |
| $PLA_2$ | Phospholipase A2 |
| PSA | Prostate Specific Antigen |
| PTM | Post-Translational Modification |
| RPMI 1640 | Roswell Park Memorial Institute medium |
| Rs | resolution |
| SD | Standard Deviation |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| TFA | Trifluoroacetic Acid |
| TM | Traditional Medicine |
| Triton X-100 | Polyethylene glycol p-(1,1,3,3-tetramethlybutyl)-phenyl ether |
| UGS | Urogenital Sinus |
| UV | Ultraviolet (spectroscopy) |

# CHAPTER ONE:

## GENERAL INTRODUCTION

# 1. CHAPTER ONE

## 1.1 Overview of prostate cancer

Scientists declared a war on cancer in 1971, but there still remain challenges to conquer the disease after more than four decades. The challenges in the study of cancer arise with the diversity in genetic composition, environmental factors and the ability of cancer cells to adapt and confront novel treatment methods. Cancer develops when cells fail to respond to conventional molecular cues for cell growth and development. This results in an abnormal accumulation of cells that can impair the function of the organ. Within diverse types of cancer, certain pathways and signalling molecules change function causing the transition to and the maintenance of neoplastic growth. The cancerous cell can be the result of deregulated pathways such as in DNA repair, cell cycle and apoptotic regulation, growth factors and their receptors (Hanahan and Weinberg 2000; Vogelstein and Kinzler 2004; Hanahan and Weinberg 2011).

Current records reveal a total of 220,800 new prostate cancer cases with 27,540 deaths in the USA, (Siegel, Miller et al. 2015) and there were 10,837 deaths due to prostate cancer and record a total of 41,736 new prostate cancer in the UK (Torre, Bray et al. 2015). Prostate cancer is the most frequently diagnosed cancer in men and the most common non-cutaneous malignant cancer in North America accounting for 27-29% of all new cases of cancer in males. It is also estimated to be the second (United States) or third (Canada) leading cause of cancer mortality. The Canadian Cancer Society estimates that one in eight men will develop prostate cancer in their lifetime. These figures support the fact that prostate cancer continues to create a significant public health concern which increases with the growth in the aging population (American Cancer Society).

Despite technological and scientific advancements, prostate cancer management remains a significant medical challenge. After the diagnosis of prostate cancer, the patient and physician have to make a complicated risk-benefit choice of intervention that ranges from conservative active surveillance to invasive surgical therapy (Klotz. 2006; Kantoff, Higano et al. 2010). It has been reported that interventions like radical prostatectomy or radiotherapy for a localized disease still

result in a 10 year recurrence of 80% (Pound, Partin et al. 1999; Han, Partin et al. 2001). The mortality rate goes up for patients who are diagnosed with advanced and metastatic stage of prostate cancer (Wirth, Hakenberg et al. 2007). Furthermore, the existing intervention procedures often have significant impact on human life. After radical prostatectomy, up to 70% of patients suffer from erectile dysfunction (ED) which is common even after less invasive therapies such as brachytherapy (Quek and Penson 2005).

There has been a little knowledge about the causes of prostate cancer. However, its increased prevalence with growing health concern has stimulated more research endeavour in discovering the biochemical and physiological functions associated with the disease. Although the pathophysiology is still not clearly elucidated, it is known to originate in the glandular tissue of the prostate. A population of precursor cells known as high-grade prostatic intraepithelial neoplasia (PIN) has been considered as the origin of prostate cancer. These precursor cells usually remain isolated within the prostate gland, but can eventually become malignant and form tumours (Bostwick 1995). It has been found that many genes or gene products have an association with the development and progression of prostate cancer. A mutation in oncogenes such as c-myc and ras yields abnormal cell division as a result of constitutive activation or gain-of-function (Jenkins, Qian et al. 1997; Nupponen, Kakkola et al. 1998; Shiraishi, Muneyuki et al. 1998; Carter, Epstein et al. 1990; Gumerlock, Poonamallee et al. 1991). Within a multistep prostrate carcinogenesis, a progressive accumulation of these genetic alterations facilitates the transformation from healthy prostate tissue to PIN which eventually leads to full blown prostate cancer.

Prostatectomy or radiation therapy can cure the cancer that manifests in an isolated mode within the capsule of the prostate gland. However, with the progression of the disease, it becomes metastatic where more surveillance and therapy are required for treatment. Metastatic prostate cancer is the heterogeneous composition of two types of malignant cells: androgen-dependent and androgen-independent (Gonzalgo and Isaacs 2003; Nelson, De Marzo et al. 2003). Androgens are steroid hormones that facilitate the development of male characteristics such as testes and spermatogenesis (Berg et al., 2002). In androgen-dependent cells, higher levels of androgens can repress the transcription of death-signalling genes necessary

for apoptosis (Denmeade, Lin et al. 1996). Androgen ablation therapy aims to reveal these death-signalling genes by suppressing the production of androgen (Kyprianou, English et al. 1990; Gao and Isaacs 1998). In contrast, androgen-independent cells are insensitive to any type of androgen ablation therapies as they show resistance to any levels of androgens (Feldman and Feldman 2001; Catz and Johnson 2003). These cells are considered as castrate-resistant which contribute to the lethal formation of the disease in advanced cancer prostate (Isaacs, 1999).

The above discussions show a significant progress in understanding the molecular biology of prostate cancer. However, treatment of the disease remains as a challenge and trade-off between risk and benefit as discussed before. Therefore, it remains as a major health concern for aging males which demands more research in medicine.

### *1.1.1 Description of the organ*

The male reproductive tract is developed from the urogenital sinus (UGS) in response to fetal androgens (Cunha, 1994). Testosterone hormone stimulates the development prostate epithelium from the UGS, which simultaneously expresses high levels of androgen receptor (AR) (Schulz, Burchardt et al. 2003). As the levels of testosterone rise by the $11^{th}$ week of development begins to differentiate the mesenchyme cells into the smooth muscle and fibroblasts that form the stroma (Wong, Wang et al. 2003;Orr, Riddick et al. 2012). The adult prostate maintains AR expression, and the signalling through the (AR) regulates new growth to replace the constant turnover of cells in the organ. In early adulthood, the testes are significant contributors of testosterone and subsequent AR activation in the prostate. In older men, the main source of AR ligand is the adrenal synthesis of dehydroepiandrosterone (DHEA) and its subsequent conversion to the potent androgen metabolite dihydrotestosterone (DHT) (Knudsen and Penning 2010).

A healthy adult prostate contains a glandular epithelial component and fibromuscular stroma. The epithelial cells in the glands express AR and are the source of prostate specific antigen (PSA), which is currently a biomarker for prostate health. These cells are arranged in a neatly delineated squamous basal layer and columnar secretory layer. The stromal region consists of fibroblasts as well as

predominant extent of smooth muscle cells organized as the orientation of fibres (Ayala, Tuxhorn et al. 2003).

In 1981, McNeal proposed the concept of a zone anatomy of the adult prostate based on locations and embryonic structure of origin. He partitioned adult prostate into 4 zones and defined them as the peripheral, central, transition, and periurethral zone (Mcneal 1981). The first 2 zones cover the glandular part of the prostate and account for ~95% of its total size. This zoning anatomy is currently still used to define and localize prostate cancers with the fact that the greatest percentage of tumours takes place in the large peripheral zone (Schulz, Burchardt et al. 2003).

The normal specimen demonstrates a clearly defined epithelial-lined gland surrounded by an organized fibro muscular stroma, whereas the prostate cancer (PC) specimen shows a loss of normal glandular architecture, a loss of structure in the basal and secretory epithelia, and infiltrating immune cells (stained red) (Figure 1-1).

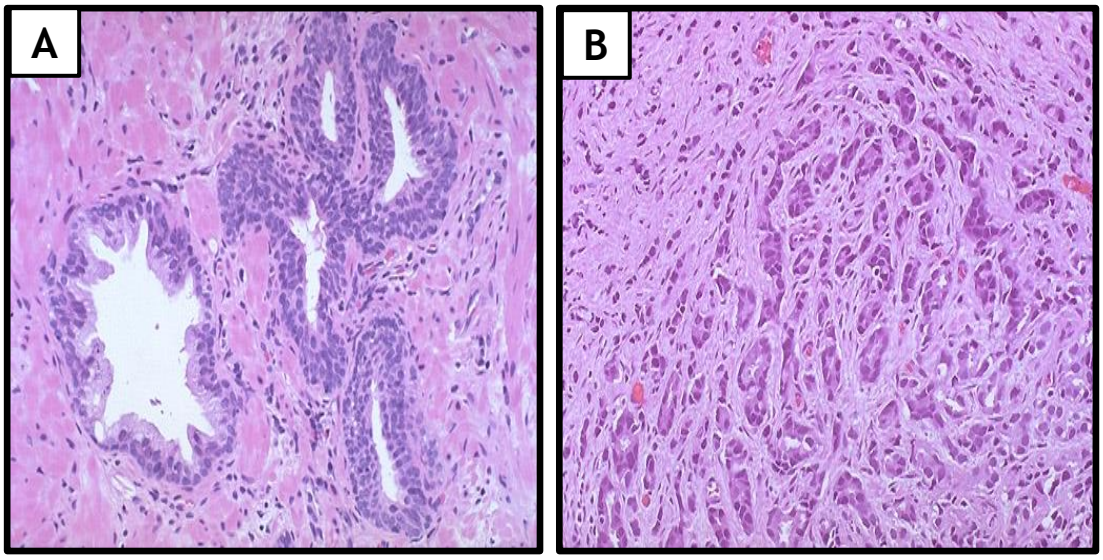



**Figure 1-1:** Tissue staining of normal and cancerous specimens from excised human prostate samples.

http://library.med.utah.edu/WebPath/TUTORIAL/PROSTATE/PROST020.html

**A.** Hematoxylin and eosin stain (H&E) stained microscopic slide of normal prostate gland demonstrating normal gland architecture in the left portion of the specimen. A squamous basal epithelial layer separates the secretory columnar epithelium from a ibromuscular stroma.

**B.** Hematoxylin and eosin stain (H&E) stained microscopic slide of a poorly differentiated prostate carcinoma. Note the loss of normal glands separated by areas of organized stroma. The glands appear smaller and have lost the basal epithelial layer. Red staining indicates infiltration by immune cells.

### *1.1.2 Prostate gland*

The prostate (Figure 1-2) is a walnut shaped and sized organ in men that is located at the base of the bladder through which the urethra passes. It is a tubuloalveolar gland composed of stroma and secretory acini, and produces fluids that contribute to semen (Marieb, Kollett et al. 2003). The development and functions of a healthy prostate are regulated by proper hormonal signalling (Marker, Donjacour et al. 2003).

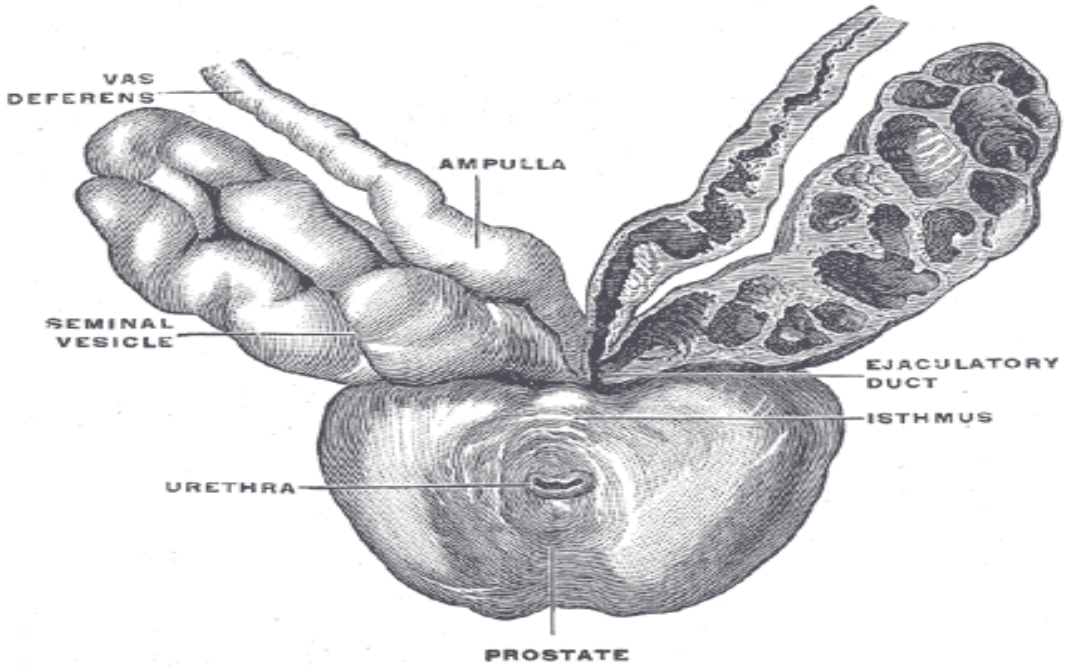



**Figure 1-2:** Prostate with seminal vesicles and seminal ducts, viewed from in ront and above (Gray's Anatomy, Book, 1918).
http://www.enlargedprostateremedy.com

The prostate is comprised of five zones (Marieb, Kollett et al. 2003). The anterior zone predominantly consists of smooth muscle. The pre-prostatic zone plays a role in ejaculation, preventing the flow of semen back up the urethra. The central zone, adjacent to the seminal vesicle, is the least likely to develop prostate cancer. The area surrounding the proximal urethra makes the transitional zone which accounts for BPH and about one quarter of prostate cancer (Walsh and Worthington 2007). With the highest concentration of prostate glands, the peripheral zone lies posterior to the urethra and is known for being the most common zone for prostate cancer development (Walsh and Worthington 2007).

### *1.1.3 Current treatment approaches for prostate cancer*

There are multiple options to treat cancer prostate (Yoo et al. 2016). Physicians most often adopt a strategy for treatment based on age and overall health status of the patient. A notable consideration in treatment procedure is made based on the stage and grade of the disease. Treatment approach for the case with localized cancer prostate is different than the case with a more advanced progression of the disease (Bracarda, de Cobelli et al. 2005).

Treatment option for the case with localized cancer prostate include: an active surveillance approach, radical prostatectomy, and radioactive therapy. The active surveillance approach is adopted for patients exhibiting a low risk of progression or for patients with a high risk of side effects from certain treatments (Klotz 2007). This approach postpones an immediate therapy until the patient is at increased risk symptoms of disease progression (Thompson, Thrasher et al. 2007). This surveillance method can drastically reduce the detriments of over-treatment or unnecessary risk of harm to the patient. With the progression to a more advanced stage, cancer is more likely to become metastatic affecting other tissues or organs. The bone is one of the common parts that get involved in the metastatic form of prostate cancer (Roodman 2001).

#### 1.1.3.1 Radiation and hormone therapy

An aggressive form of therapy needs to be administered for men with an advanced or recurring form of cancer prostate (Kapoor et al. 2016). Apart from a prostatectomy and localized radiation therapy, systemic (whole body) therapy would be an option to follow up. Since androgens stimulate the growth of normal and cancerous prostate cells, a systemic therapy approach to metastatic cancer prostate would be androgen deprivation therapy (ADT). ADT can be achieved through surgical castration, chemical castration or a combination of both. Unfortunately, there is a form of cancer prostate that is androgen-independent which would make ADT useless. This castrate-resistant prostate cancer (CRPC) is most often treated with chemotherapy (Yagoda and Petrylak 1993). However, the use of immunotherapy has been found effective in a new treatment option for CRPC (Vishnu and Tan 2010). Provenge (Sipuleucel-T) is a form of therapeutic cancer vaccine that utilizes a subset of a patient’s leukocytes to express an immune-

activating antigen. The immune response activates T-cells and is clinically shown to reduce the risk of death by 22% compared to placebo (Kantoff, Higano et al. 2010). Other androgen receptor-directed therapies also show promise (Yi et al. 2016).

#### 1.1.3.2 Chemotherapy

The era of cancer chemotherapy began with the use of nitrogen mustards and folic acid antagonist drugs in the 1940s (Goodman, Wintrobe et al. 1946; Farber, Diamond et al. 1948). Nitrogen mustard was discovered as a chemical warfare agent during World War I and was later found to be effective in treating cancer. Subsequent investigations on mustard gas revealed its significant therapeutic actions against lymphoma, a cancer of the lymph nodes. This discovery allowed the first documented treatment of a lymphoma patient in 1943 (Gilman 1963).

Chemotherapy with nitrogen mustard showed dramatic reduction in the patient's cancer masses. It was the first step to realize that cancer can be treated by pharmacological agents (Goodman, Wintrobe et al. 1946) although this effect lasted only a few weeks. Further studies on the toxic effects of mustard gas lead to more effective agents called (alkylating) agents that destroyed rapidly proliferating cancer cells by damaging their DNA.

Not long after the discovery of nitrogen mustard, aminopterin, an antagonist to the vitamin, folic acid, was tested as a drug for cancer chemotherapy. It demonstrated remission in acute leukaemia in children (Farber, Diamond et al. 1948) . The characteristic that makes aminopterin (Figure 1-5) useful for cancer chemotherapy is its ability to block a critical chemical reaction needed for DNA replication (Nichol and Welch 1950). Although the remission in cancer was brief, it was clearly evident that anti-folates could suppress proliferation of malignant cells which in turn could restore normal bone marrow function (Goldin, Venditti et al. 1955; Cole, Drachtman et al. 2005). Aminopterin was the predecessor of methotrexate (Warkany 1978), which is most commonly used for cancer chemotherapy today. As of now, several drugs are available for cancer chemotherapy that can block different cellular functions involved in cell growth and replication.

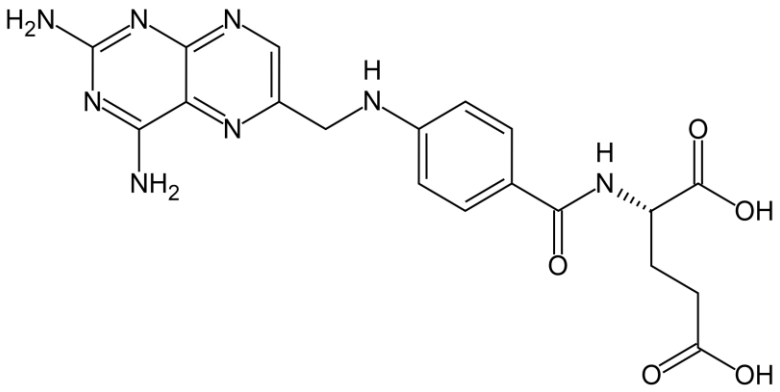


**Figure 1-3:** Structure of Aminopterin

Combination therapy was a major break-through in cancer therapy. Since different chemotherapy drugs possess different mechanisms of action, an optimal efficacy can be achieved when they are administered in combination than individually. Cancer cells may easily grow resistance to a single agent, but supposedly it would be more difficult to develop such resistance to a combination of agents. A number of regimens became widely used for their considerable effects in treating lymphomas. This approach has been extended to a number of different cancers with success which made combination therapy a common approach in cancer chemotherapy today (Bonadonna, Brusamolino et al. 1976).

Chemotherapy was not effective on all cancers; however, if the cancer burden could be reduced first by surgery, chemotherapy could be more effective in eradicating the remaining malignant cells. This approach introduced the strategy known as adjuvant therapy which is given in addition to primary treatment (Jaffe, Link et al. 1981).

Adjuvant therapy was tested first in breast cancer and found to be effective. In adjuvant therapy, it was found that the recurrence of osteosarcoma was prevented by administering high doses of methotrexate after surgical removal of the primary cancer. Following a surgery in treating patients with colon cancer, the application of adjuvant 5-fluorouracil, an inhibitor of DNA synthesis showed improvement in survival rates. There are a number of other cases where adjuvant drugs demonstrated effectiveness in treatment of cancer (Bellamy, Dalton et al. 1991).

Other treatment options such radiation therapy, hormone therapy, and chemotherapy are common in cases where the cancer is still confined within the prostate or if there is an evidence that cancer has relapsed with rising PSA following surgery (Walsh and Worthington 2007). Since radiation therapy targets a particular area, it is most useful when metastases are local to the seminal vesicles or pelvic lymph nodes. Hormone therapy, also known as androgen-deprivation therapy (ADT), has been found to make substantial reduction in prostate size and tumour burden for most patients as a result of significant androgen-dependent apoptosis (Litvinov, De Marzo et al. 2003). ADT targets three main points in the signalling cascade leading to androgen action in the prostate: by disrupting the pulsatile manner of the hypothalamic-pituitary connection (LHRH agonists), by inhibiting testosterone synthesis (5α reductase inhibitors like finasteride), or by blocking androgen AR signaling using AR antagonists (Figure 1-6) (Hellerstedt and Pienta 2002). However, progression to castrate-resistant disease generally occurs within 12-33 months (Hellerstedt and Pienta 2002).

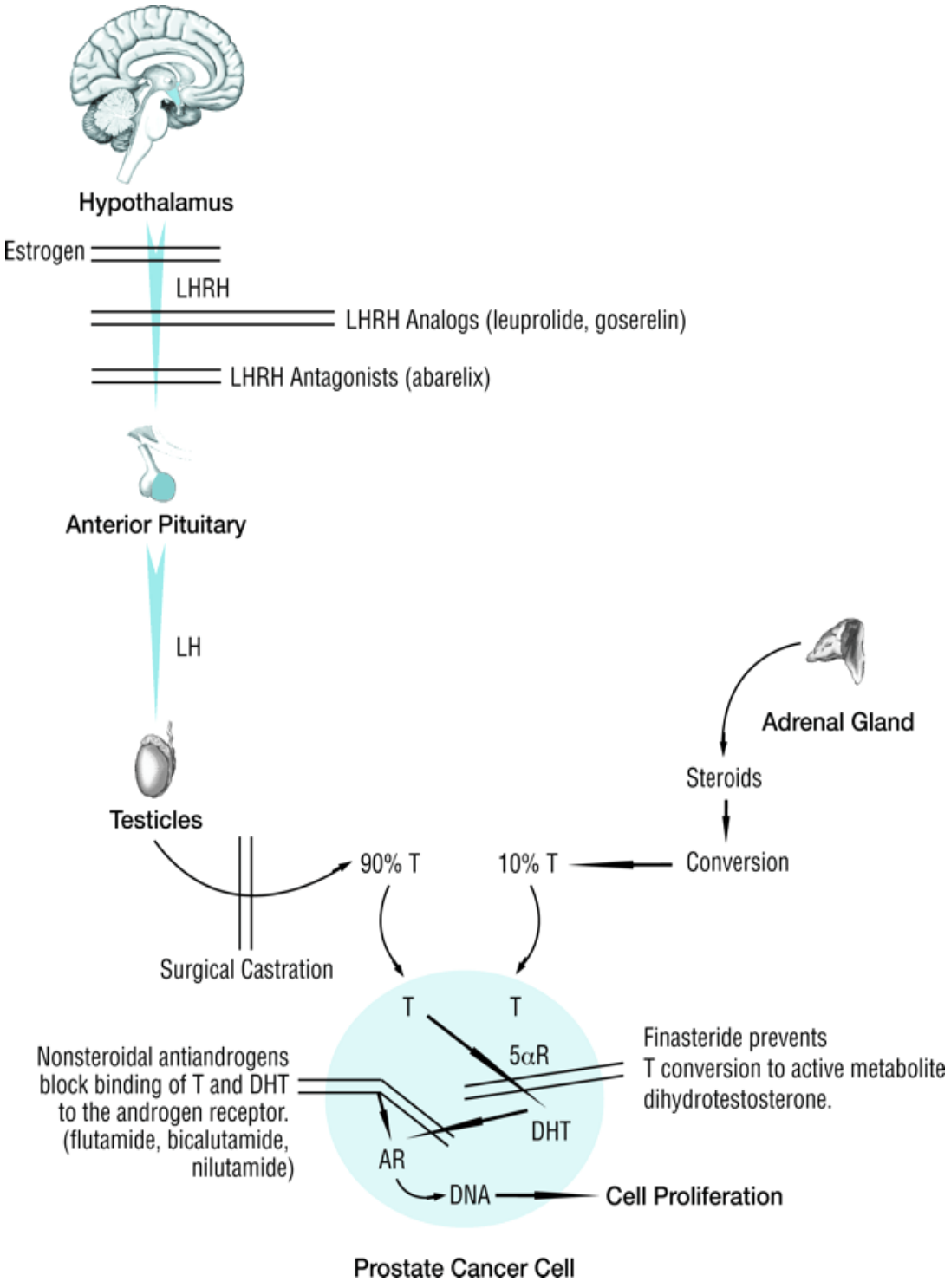



**Figure 1-4:** Treatment strategies for androgen deprivation (T=Testosterone) (Hellerstedt and Pienta 2002)

#### 1.1.3.3 Current status of cancer therapy

In past, there was a common notion that cancer is not curable once it had spread out and any intervention might be more harmful than no treatment at all. However, despite decades of research, development and discovery of sophisticated new drugs at the expense of billion dollars, there has not been much satisfactory progress towards a cure for cancer, but the development of advance tools and techniques help for early detection of cancer which helps to minimise the incidence and death rates in the community. The conventional therapies basically based on one or more modalities such as surgery, radiotherapy, and chemotherapy which are treating approximately half of the cancer patients, while the other half of the affected individuals may have only prolonged survival or even no benefit at all. But, there is need to develop more advanced techniques, methods and targeted chemotherapy including Molecular Medicine, Nanomedicine, and non-invasive therapeutic (Kapoor et al. 2016; Yi et al. 2016; Yoo et al. 2016; Taghipour, 2016).

Although more than 50 of all cancer patients receive chemotherapy, such as toxic drugs which help only 5% of these cases to recover (Ramirez, Craig et al. 1989; Morgan, Ward et al. 2004). Overall chemotherapy helps in reducing the mortality in childhood leukaemia, Hodgkin's disease and other rare cancers, it has limited success in prolonging survival from large deadly cancers of the lung, breast, colon, rectum and prostate (Ramirez, Craig et al. 1989).

It is well understood that many chemotherapy drugs work by killing cancer cells which also kill healthy cells within the process and depress the immune system-making the recipient more susceptible to various infectious diseases. This harmful effect of chemotherapy on the immune system can lead to a secondary cancer 25 times more often than the expected rate. Another major burgeoning challenge encountered in chemotherapy treatment of cancer is multi-drug resistant (MDR) cancer cells. This occurs when cancer cells develop cellular resistance mechanisms to chemotherapy and drug targets due to changes in cellular metabolic processes, genetic or stimulated protein expression and alterations in DNA repair mechanisms. These changes affect localized drug concentration and subsequently, such cancers will continue to progress despite treatment (Bellamy, Dalton et al. 1991). New therapeutic agents that do not target rapidly proliferating cells need special

consideration in the treatment of prostate cancer, because the proliferative fraction of prostate cancer cells is usually less than 10% (Berges, Vukanovic et al. 1995).

### *1.1.4 Rationale or failures in treatment leading to need for new development of treatment prostate cancer*

As mentioned before, prostate cancer is the most common form of malignancy and the second leading cause of cancer mortality in men of the western world (Jemal, Siegel et al. 2008). The majority of prostate cancer patients are aged and diagnosed with clinically localized, low-risk prostate cancer that can be effectively treated with surgery and radiation.

Today, natural products directly or indirectly contribute about 50% of the most prescribed medications in the United States (Bernstein and Ludwig 2008). Equally important, these natural compounds have high affinities for molecular targets which facilitate the explanation of various physiological processes in cell systems (Bernstein and Ludwig 2008).

Over the last twenty years, combinatorial chemistries have been used to make rapid development of synthetic agents that are more powerful and effective analogs of the existing agents (Lewis and Garcia 2003; Molinski, Dalisay et al. 2009). The advancement in computer based modelling and structure determination has created an enormous potential for the design and development of smart drugs. However, this reductionist approach only provides partial models of the molecular interaction with an isolated protein molecule or target (Molinski, Dalisay et al. 2009).

The lack of knowledge about the molecular interaction with other proteinaceous such as bradykinin potentiating peptide (BPP), natriuretic peptide (NP), etc. or nonproteinaceous components in a complex cellular environment may lead to poor efficacy with undesirable effects. This highlights a major need for further examination of the structure of the cellular targets. The intense review on the structural diversity of natural compounds may be valuable source against new or existing therapeutic targets.

A compelling reason for this re-examination is to take benefit of the tremendous advances in analytical techniques, which make this study of natural products much sophisticated. These new methods facilitate the analysis of structural diversity and offer immense opportunities for finding novel molecular interactions that are active against a wide range of targets. Since only a small fraction of the biologically diverse and active compounds are known, a myriad of valuable natural compounds are awaiting discovery. Prior research indicates that compounds in some snake venoms may prevent the growth of cancerous cells. One of the most common reactions to snake bite is the immediate fear of envenomation and death. It is interesting to note that some snake venom has specific proteins that are responsible to lowering the blood pressure of their victim. These proteins include the bradykinin potentiating peptide (BPP), natriuretic peptide (NP), blocker of L type (CaV1) voltage gated calcium channel etc.

Bradykinin is a proteolytic product of plasma kininogen with potent vasodilator effects. This molecule was identified through the study of envenomation by the South American pit viper *Bothrops jararca*. It was shown that bradykinin having strong hypotensive effects. This initial discovery lead to development of Captopril, one of the most successful examples of venom derived therapeutics. (Cushman, et al.1987 Cardiovascular pharmacology S17-S30).

Until now, about a dozen diagnostic tests and drugs are derived from snake venom. Drugs used to treat high blood pressure and other cardiovascular disorders, were developed from the venom of *Agkistrodon contortrix contortrix* (Southern Copperhead) (Minea, Swenson et al. 2005). Researchers have obtained the venom from the snake to cut off the blood supply to the cancers to kill the cancerous cells. This is because the venom disrupts endothelial cells which line the inner surface of the blood cells (Schmitmeier, Markland et al. 2003). This separates the cell from one another to interfere with the blood supply of the cancer. Venoms have also been tested in the treatment of neural, blood, muscle, heart and psychological disorders including multiple sclerosis, epilepsy, Alzheimer's and, more recently, in AIDS research (Hile 1986; Opie and Kowolik 1995; Camargo, Ianzer et al. 2012; Harvey 2014).

Bleak statistics and growing projections of worldwide mortality at an alarming rate indicate a lapse in current strategies. In spite of the availability of large number of anticancer drugs and various therapeutic options, there remains a challenge to develop less toxic and more potent cancer therapy. Novel molecules that are structurally unique and potent can play an important role in the development of cancer drug. Studying toxin composition of venoms can help generate toxin-specific anticancer with greater specificity and effectiveness. In addition, a number of protein components of snake venom have been shown to have therapeutic value. While a large number of venom proteins have been identified, sensitive mass spectrometry-based proteomic technologies may still allow identification of yet unknown venom proteins.

### *1.1.5 Model of prostate cancer cell lines*

Cancer is a complex disease that is ascribed to several causes related to cellular progression such as proliferation, cell transformation, and escape of apoptosis, invasion, angiogenesis, and metastasis. Prostate cancer can be studied in a limited manner using cells or tissue cultures. However, in vitro models are needed to investigate cells related to metastasis.

An appropriate cell model mimics the key features of human prostate adenocarcinoma, such as response to androgens, relatively slow growth and metastases. The optimal model should also include similar embryological origin, biological behaviour, histology, biochemical properties and molecular and genetic characteristics for the disease. In addition, these key features in the cell model description should be stable and easy to maintain even in large numbers. Tumours should be easily and successfully implanted or induced.

The widely used cell models of prostate cancer are androgen-independent PC3 and DU145 cell lines and androgen-dependent LNCaP cells (van Bokhoven, Varella-Garcia et al. 2003; van Weerden, Bangma et al. 2009). PC3 and DU145 lack the expression of AR as well as secretion of PSA, which both are characteristic of hormone-responsive prostate cancer (HRPC). LNCaP cell is more close to HRPC expressing AR and PSA but have limited tumorigenicity. LNCaP response to anti androgens is aberrant due to a point mutation in a ligand-binding domain of the AR,

whereas they are also sensitive to other hormones such as progesterone and estragon (Veldscholte, Risstalpers et al. 1990; Veldscholte, Berrevoets et al. 1992).

In this study, four cell lines DU145, PC3, LNCaP and PNT2A were used. Cells were chosen because they represented different kinds of prostate cancer as well as the normal prostate cell line (PNT2A) that served as a control.

## 1.2 Classification of snake venom

Snakes are cold blooded vertebrate and they are classified in Phylum-chordate, subphylum- vertebratea, class Reptilia, sub class Synaptosuria, order Squamata, suborder Serpentes. About 3,300 species are recognized and classified in 11 snake families. Venomous snakes are identified in only five families: Elapidae, Hydrophiidae, Viperidae, Crotalidae, and Colubridae.

Much of the snake venom taxonomy to this date is based upon dentition and external features. The classification and evolution of higher snakes has been in terms of progression from harmless (non-poisonous) forms with simple teeth and no venom glands, through various intermediate stages, to the vipers with their elaborate venom injection apparatus.

In snakes, venom is produced by specialized glands which are evolutionarily related to salivary glands (Kochva 1987). Over 100 million years ago, snakes evolved from lizards and ever since they have become separate reptiles with their venom apparatus in ophidian evolution at the base of the Colubridae radiation (Kochva 1987; Heise, Maxson et al. 1995; Fry and Wuster 2004). All known advanced snake species are venomous and belong to the super family Colubridae which include the families Elapidae such as cobras, kraits, coral snakes, sea snakes and Viperidae such as vipers and pit vipers (Fry and Wuster 2004).

Venoms from the families Elapidae and Viperidae contain a wide mixture of biologically and pharmacologically active proteins and polypeptides which constitute 90-95% of their venom. About 5% of the venom constitution includes amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (Heise, Maxson et al. 1995; Fry and Wuster 2004). About one-fifth of the existing

species of snakes are venomous (Mebs 1985). Each snake family may have similar venom proteins. Nevertheless, amino acid sequences, protein abundances, protein characteristics, biological, pharmacological and pathological activities of shared venom proteins in each individual family are different.

Snake venoms can also be broadly classified as haemorrhagic or neurotoxic based on the toxic effect of venom in animals (Juarez, Sanz et al. 2004). Most Viperidae (vipers, pit vipers, and rattlesnakes) venoms have little neurotoxic activity, but have an abundance of serine proteinases, metalloproteinase and C-type lectins activities that hinder the coagulation cascade, the normal haemostatic system and tissue repair. Therefore, envenomation by these snakes generally results in excessive bleeding, lack of clotting, hypofibrinogenemia, hypotensive, local tissue necrosis, and inflammatory effects (Meier and Stocker 1991; Markland 1997; Morita 2004).

In contrast, the snakes of Elapidae family (mambas, cobras, coral snakes, and especially Australian snakes) contain neurotoxins in their venoms that disable muscle contraction and cause paralysis. Venoms of the Colubridae snakes share some activities common with both the Viperidae and Elapidae snakes. On the other hand, snake venoms of the Atractaspididae family have a variety of peptide toxins that affect the cardiovascular system. Venom toxins, secreted by a pair of specialized glands connected to the fangs by ducts, are likely to be evolved from proteins with normal physiological functions. These toxins are recruited into the venom proteome before the diversification of the advanced snakes (Vidal 2002; Fry and Wuster 2004).

Venom represents a key adaptation in ophidian evolution that made the transition from a mechanical (constriction) to a chemical (venom) means of subduing and digesting large prey. In spite of the fact that snake venoms contain many distinctive proteins, they belong to only a few families such as enzymes (serine proteinases, $Zn^{2+}$ metalloproteinases, phospholipase $A_2$, L-amino acid oxidase) and proteins without enzymatic activity (C-type lectins, disintegrins, natriuretic peptides, myotoxins, cysteine-rich secretory protein toxins (CRISP), nerve and vascular endothelium growth factors, cystatin, and Kunitz-type proteinase inhibitors). Snake venom serine proteinases can cause platelet aggregation, coagulation, or fibrinolysis either by triggering or inhibiting the specific blood factors (Kini and Evans 1992).

$Zn^{2+}$ metalloproteinases induce local haemorrhage by degrading extracellular matrix proteins, while $PLA_2$ causes severe local swelling followed by necrosis. Snake venom $PLA_2$ isozymes are multi-functional enzymes that have many diverse activities such as presynaptic and postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant effects, platelet aggregation (inhibition or initiation), antihaemorrhagic activities, convulsant activities, hypotensive activities, edema-inducing activities, and organ or tissue damage activities (Fry 1999). C-type lectin-like proteins (CLP) are multimeric molecules (Mizuno, Kimura et al. 1997) which include inhibitors and activators of coagulation factors V (AaACP), IX, and X (botrocetin, fIX/X-binding protein). CLP can also compete with von Willebrand factor VWF binding to the platelet membrane GPIb/IX complex and either block (echicetin, agkicetin, flavocetin, and tokarecetin) or promote (alboaggregins A and B) platelet aggregation. Disintegrins are released from venoms by proteolytic processing of PII $Zn^{2+}$ metalloproteinases and inhibit integrin-ligand interactions (McLane, Marcinkiewicz et al. 1998).

Each protein family differs from another in protein quantities, sequences, and biological activities (Collado, Gil et al. 2005). The ophidian evolutionary history retained in the venom composition provides insight into potential taxonomical value. Research endeavours in the characterization of proteins and peptides of snake venoms can open the pathways for a numerous potential areas from basic research, clinical diagnosis to the development of novel clinical tools, drugs as well as anti-venom production strategies (Menez, Stocklin et al. 2006). For instance, snake venom components affecting thrombosis and haemostasis have been widely used for developing diagnostic tests because of their usefulness in studying blood coagulation mechanisms (Juarez, Sanz et al. 2004).

Disintegrins are valuable tools for identifying integrin-binding sequence motifs that require selective integrin inhibition. Also, disintegrins have numerous applications in platelet thrombosis, angiogenesis, cancer, bone destruction, and inflammation. Integrilin, a synthetic derivative of the disintegrin barbourin, has been developed as a drug that prevents arterial thrombosis after angioplasty (McLane, Marcinkiewicz et al. 1998). Furthermore, some proteins in animal sera naturally resist envenomation and can be grouped as inhibitors of either $PLA_2$ (antimyotoxic and antineurotoxic factors) or metalloproteinases of the reprolysin family

(antihemorrhagic factors) (Dunn and Broady 2001; Neves-Ferreira, Perales et al. 2002). Therefore, establishing structure-function relationships of isolated venom toxins may lead to the design of novel nontoxic drugs for future clinical use.

### *1.2.1 Selection and collection of snake venoms*

In this study, venoms of three snakes *Naja pallida*, *Agkistrodon piscivorus conanti*, and *Agkistrodon contortrix laticinctus* were selected based on high toxicity and ready availability. The details of all three snakes' venom *Naja pallida* (Red spitting cobra), *Agkistrodon piscivorus conanti* (Florida cottonmouth), and *Agkistrodon contortrix laticinctus* (Northern Copperhead) are given below.

#### 1.2.1.1 Florida Cottonmouth (*Agkistrodon piscivorus conanti*)

Figure (1-5) the Florida cottonmouth, scientifically known as *Agkistrodon piscivorus conanti*, is a poisonous snake abundant throughout much of Florida as well as in some of the offshore islands.

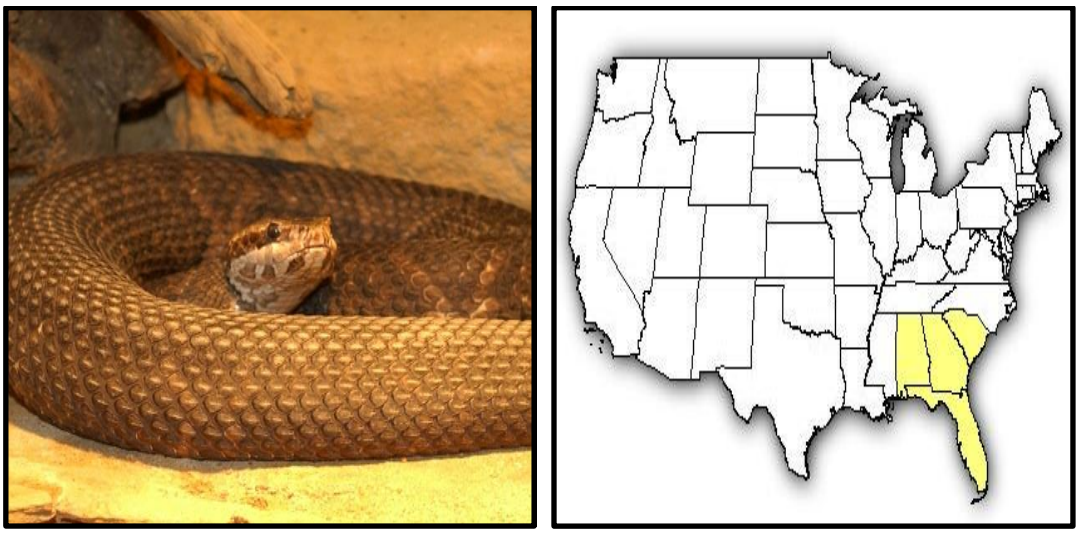


**Figure 1-5:** *Agkistrodon piscivorus conanti*
http://www.venombyte.com/venom/snakes/florida_cottonmouth.asp

The success of this species in varied habitats is related, in part, to the wide range of prey items that provide a potential energy base for populations (Fritts 1988), that is a pit viper without rattles. It can grow more than 5 feet in length with an average about 3 feet. Cottonmouth varies from olive to brown to black in colour with dark crossbands around the body and is classified as follows:

Kingdom: Animalia, Phylum: Chordata, Class: Reptilia, Order: Squamata, Suborder: Serpentes, Family: Viperidae, Subfamily: Crotalinae, Genus: Agkistrodon, Species: Agkistrodon piscivorus, Subspecies: Agkistrodon piscivorus conanti, *Agkistrodon piscivorus conanti*

#### 1.2.1.2 Red spitting cobra (*Naja pallida*)

Cobra is a common snake throughout the Africa and Asia. Each species is fairly distinct and there is a little difference in their classification. The cobras of Southeast Asia are generally smaller than the African counterparts and can spread their hoods to greater width. Southeast Asian cobras, however, have few distinct external characteristics. There is considerable variation in both colour and pattern, even among individual specimens within the same geographic area.

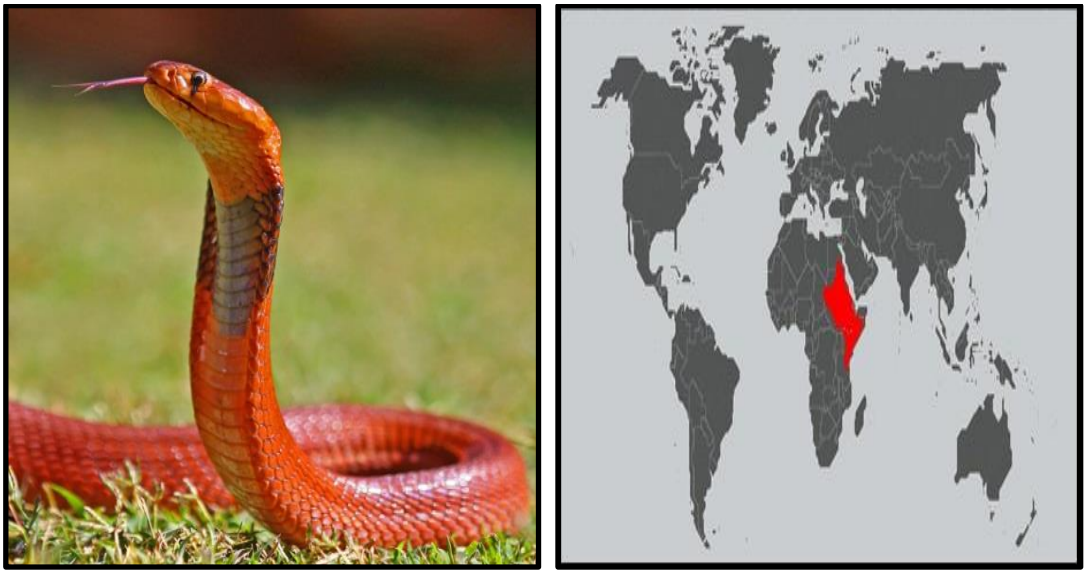


**Figure 1-6:** *Naja pallida*
http://animalesalbatice.ro/serpi-veninosi-cobra-rosie-scuipatoare-naja-pallida

Most adult red spitting cobras have length within the range between 70-120 cm and a maximum of 150 cm. This cobra is relatively small and quite slender in body proportions. They usually have a small head, but some specimens can have big heads with huge swollen venom glands clearly seen at the side of the head with large eyes. The one with a long tail has a body in cylindrical shape. The colour of this cobra can vary depending on the origin of the snake.

The specimens from southern Kenya and northern Tanzania have an orange red colour, with a broad steel blue or black throat band. Some specimens show 2 or 3 throat bands which are not common for specimens from East Africa. The true red specimens will turn red-brown with the increase of their size. Finally, that is classified as follows:

Kingdom: Animalia, Phylum: Chordata, Class: Reptilia, Order: Squamata, Suborder: Serpentes, Family: Elapidae, Genus: Naja, Species: pallida *Naja pallida*

#### 1.2.1.3 Northern copperhead (*Agkistrodon contortrix laticinctus*)

The broad-banded copperhead is found within the region from central Texas north to the southern border of Kansas and Oklahoma (Wright and Wright 1957), The most of adult have length within the range between 60-90 cm and a maximum of 120 cm. Its head is reddish-brown in colour and its body is brown, which is classified as follows:

Kingdom: Animalia, Phylum: Chordata, Class: Reptilia, Order: Squamata, Suborder: Serpentes, Family: Viperidae, Subfamily: Crotalinae, Genus: Agkistrodon, Species: A. contortrix, Subspecies: Agkistrodon contortrix laticinctus *Agkistrodon contortrix laticinctus*

**Figure 1-7:** *Agkistrodon contortrix laticinctus*
https://www.google.co.uk/search?q=Agkistrodon+contortrix+laticinctus&source

## 1.3 Proteins and polypeptides in snake venoms

### *1.3.1 Enzymes in snake venoms*

Venoms contain many different compounds, mostly protein and polypeptide. The venom composition may vary depending on age, (Meier 1986) geographical origin, (Meier, Stocker et al. 1985; Stocker 1990) and individual snake (Taborska and Kornalik 1985). Snake venoms are complex mixtures composed chiefly of varied enzymatic and non-enzymatic toxins. Venom enzymes play an important role in immobilisation the prey, digestion of prey and defence mechanism against the pray.

Many of the proteins in venom are water-soluble enzymes. Many of these enzymes are hydrolases and possess a digestive role such as proteinase, exo and endopeptidases and phosphodiesterase. Out of 26 detected enzymes in snake venom, 12 are commonly found in all venoms, although venom contents vary significantly with snake to snake species. Such variation in enzymes may depend on different factors such as age, nutrition, sex, living space or circumstances of the snake. Enzyme levels of Viperidae and Crotalidae venoms are in the range of 80-90% of total dry matter, whereas the corresponding range for elapid venom is 25-70% (Barrett, Rawlings et al. 2004; Mackessy 2010; Calvete 2014).

The structure, immunological properties, and biological characteristics of snake venom enzymes are specific to most of the animal protein. Accordingly, the interaction of venom protein and their targets in cells depends on specific features of both venom and cells (Table 1-1).

**Table 1-1:** Application of snake venom enzymes (Vagish Kumar Laxman Shanbhag 2015)

| Application | Snake venom enzymes |
|---|---|
| **Haemostatic** | Activators of factor II, X, thrombin-like enzymes |
| **Antithrombotic** | Thrombin-like enzymes, fibrinogenase |
| **Tissue adhesives** | Thrombin-like enzymes |
| **Plasma processing** | Thrombin-like enzymes |
| **Haemostasis testing** | Activators of factor II, V, X, thrombin-like proteinases, fibrinogenase, serpinase, phospholipases, Heparinase |
| **Neurobiology** | Phospholipases $A_2$ |
| **Complement system research** | Proteinases |
| **DNA/ RNA sequencing** | Phosphodiesterase |
| **Phospholipid analysis** | Phospholipases $A_2$ |

### *1.3.2 Non Enzymatic snake venoms proteins*

#### 1.3.2.1 Neurotoxins

The venom of many snake species contains agents that affect nervous system of the prey animal, causing cramps, convulsions, or muscle paralysis and subsequent death.

##### 1.3.2.1.1 *Postsynaptic neurotoxins*

These neurotoxins are low-molecular weight basic proteins that are found predominantly in elapid venoms. They were divided into two types on the basis of their size and disulphide linkages. Short neurotoxins contain 60-62 amino acid residues and 4disulphide cross linked bridges. Most of the neurotoxins consist of long 70-74 amino acid residues and 5 disulphides bridges or sometime 4 disulphide bridges. Both of the types have a common mode of action. They block neuromuscular transmission by binding specifically with high affinity to the nicotinic acetylcholine receptor in the postsynaptic membranes of skeletal muscles prevent the binding of chemical neurotransmitter acetylcholine and thereby blocking the excitation of muscle. This block at the neuromuscular junction leads to flaccid paralysis which is similar to the action of d-tubocurarine, so called curare mimetic or curariform toxins or α-neurotoxin. Whereas the action of blocking due to d-tubocurarine is easily reversible by physostigmine, the effect of most α-neurotoxin is generally irreversible or very slowly reversible.

##### 1.3.2.1.2 *Presynaptic neurotoxins*

The presynaptic neurotoxins are mostly toxic phospholipase $A_2$ and they exert the catalytic function of this type of enzyme. The significance of phospholipid cleavage for the neurotoxic effect is not yet fully understood. All of these neurotoxins found in Elapidae and some Viperidae snake venoms which have a basic phospholipase $A_2$ in common that may be composed of acidic, basic or neutral protein units.

#### 1.3.2.2 Cytotoxins

Cytotoxins are toxic polypeptides consisting of 60-62 amino acid residues with 4 intermolecular disulphide bonds. The pharmacological actions of cytotoxins include haemolysis, cytolysis, depolarization of muscle membrane, and specific cardiotoxicity.

#### 1.3.2.3 Myotoxins

Myotoxins are snake venom polypeptides that induce skeletal muscle contracture or produce local myonecrosis or myoglobinuria. Myonecrosis, although is common in most cases of snake envenomation, is most pronounced with Crotalidae and Viperidae venoms and can also be observed with Hydrophiidae and Elapidae envenomation (Bober, Glenn et al. 1988; Ownby 1998).

#### 1.3.2.4 Cardiotoxins

Cardiotoxins are single-chain polypeptides which are chemically and structurally related to the neurotoxins. All of these toxins are highly basic polypeptides consisting of about 60-62 amino acid residues with 4 disulphide linkages in the molecules (Ownby, Fletcher et al. 1993). Cardiotoxins have lytic effects on a wide range of cells, so they have other names as a direct lytic factor, cytotoxin, membrane-disruptive polypeptide or membrane toxin. These have different other effects including systolic contractions, haemolysis, cytolysis, and muscle depolarization (Potts, Nussbaum et al. 1989; Menn, Kelly et al. 1991; Jones, Ndhlovu et al. 2008).

Other nonenzymic snake venom proteins act as proteinase inhibitors or represent structure analogues of proteinase inhibitors, bradykinin-potentiating peptides, choline esterase inhibitor, phospholipase inhibitors, nerve growth factors, lectins, proteins affecting platelet functions and proteins acting on the complement system.

## 1.4 Nonproteinous snake venom components

The nonprotein portion of the venom is biologically less active and much smaller than the proteins. The nonprotein fraction includes metal ions, inorganic anions, and some small organic molecules such as peptides, lipids, nucleosides, carbohydrate and amines (Stocker 1990).

Since all snake venoms contain multiple components with different mechanisms of action, the pathogenesis developing after a bite is very complex in nature. It is not only dependent on the qualitative composition, but also on their quantitative distribution of different components in particular venom.

## 1.5 Summary of enzymes found in snake venomous

The enzymes present in snake venoms act in the following ways:

**(A)** Effect as local capillary damage and tissue necrosis by protease, phospholipase, arginine ester hydrolases, and hyaluronidase.

**(B)** Cause diverse coagulant and anticoagulant actions by various proteases and phospholipase.

**(C)** Acute hypotension and pain are induced due to a release due to release of vasoactive peptides by kinin-releasing enzyme, kininogenase (Barrett, Rawlings et al. 2004; Mackessy 2010).

The other enzymes proposed as toxic elements in snake venoms, are 5-nucleotidase, phosphodiesterase, cholinesterase and L-amino acid oxidase. However, numerous studies have shown that these enzymes are not responsible for the acute toxicity of snake venoms. At least 26 enzymes, most of them hydrolases, have been detected in snake venoms (Table 1-2). Of these enzymes 12 are found in all snake venom with significantly variable contents and quantities. Unlike Elapid venoms, Crotalidae and Viperidae venoms possess different types of proteases which render coagulant activity (Barrett, Rawlings et al. 2004; Mackessy 2010).

**Table 1-2:** Enzymes found in snake venoms (Vagish Kumar Laxman Shanbhag 2015)

| Group | Enzymes |
|---|---|
| **Group 1**<br>**Enzymes found in all snake venoms** | *Phospholipase $A_2$, L-amino acid oxidase, Phosphodiesterase, 5-Nucleotidase, Phosphomonoesterase, Deoxyribonuclease, Ribonuclease, Adenosine triphosphatase, Hyaluronidase, DNA-nucleosidase, Arylamidase, Peptidase.* |
| **Group 2**<br>**Enzymes found in crotalid and viperid venoms** | *Endopeptidases, Arginine ester hydrolase, Kininogenase, Thrombin-like enzyme, Factor X activator, Prothrombin activator.* |
| **Group 3**<br>**Enzymes found mainly in elapid venoms** | *Acetylcholinesterase, Phospholipase B, Glycerophosphate.* |
| **Group 4**<br>**Enzymes found in some venoms** | *Glutamic-pyruvic transaminase, Catalase, Amylase, β-Glucosaminidase, Lactate dehydrogenase, Heparinase-like enzyme* |

## 1.6 Pharmacology

A cytotoxic compound is toxic to cells which can potentially be of therapeutic interest. However, as explained by Paracelsus logic, 'the dose makes poison' and this is the premise of our characterization of what may be an effective anticancer agent. In other words, although cytotoxicity can be related to anticancer activity, the dosage and concentration are key criteria in determining the potential of a compound a drug candidate. While the primary objective is to use the cytotoxicity of the compound to cancer cells, ideally the cytotoxic effects should be within a tolerable dosage in such a mechanism that selectively targets cancer cells while permitting the survival of normal cells (Fox, Curt et al. 2002).

Most chemotherapeutic agents in use are cytotoxic to all cells causing a number of side effects. The half maximal inhibitory concentration ($IC_{50}$) quantitative measure indicates how much of a particular drug or other substance is needed to inhibit a given biological process or system by 50%. The $IC_{50}$ values can, therefore, be translated into a substance's effectiveness and relative toxicity. Cytotoxic response of potentially useful agents in plant or animal extract can be detected in an in vitro cell culture system (Ghose and Blair 1978; Fox, Curt et al. 2002).

In addition, cytotoxins from snake venom inhibit platelet aggregation and cause extensive haemolysis (Shier and Mebs 1990). Similarly, cardiotoxins isolated from *Naja naja atra* venom inhibit the growth of cells and induce apoptosis in cancer cells while drC-T1 isolated from Indian Russell's viper *Daboia russelli russelli* venom shows anti-proliferative, cytotoxic and apoptotic activity to cancer cell lines (Gomes, Choudhury et al. 2007).

In contrast to this, $PLA_2$ isolated from *Bothrops neuweildii* venom shows cytotoxic activity in melanoma cells while same enzyme from *Naja naja* venom shows cytotoxic effects in Ehrlichs ascites cancer cells (Basavarajappa and Gowda 1992). More specifically, L-amino oxidase occurs in snake venom inhibit platelet aggregation and induce anti-coagulant and antimicrobial activity while disintegrins isolated from snake venom obstruct cell-cell interactions and signal transduction (Li, Yu et al. 1994).

Similarly, a disintegrin, saxatilin isolated from Korean snake venom *Gloydius saxatilis* which inhibit the proliferation of ovarian cancer cells (Kim, Jang et al. 2007) while colombistatin isolated from *Bothrops colombiensis* inhibit ADP induced platelet aggregation and obstruct the growth of human skin melanoma cells (Sanchez, Rodriguez-Acosta et al. 2009).

### *1.6.1 Medical use of snake venom enzymes*

Snake venoms have been used in occult and public medicine in different places based on the regional venomous snakes. Snake venoms have been used to treat pathological conditions such as haemophilia, hypertension, rheumatic diseases, neuralgia, hay fever, cancer, yellow fever, epilepsy and rabies. Long before understanding the biochemistry of snake venoms and the haemostatic system was understood, unfractionated venoms of various snake species, including *Agkistrodon piscivorus, Notechis scutatus, Cerastes vipera, Naja naja* and *Bothrops atrox*, were empirically tried as local and systemic haemostatics in humans (Stocker 1990).

Some of these observations made in the course of such therapeutic trials intrigued systematic research. One of the fascinating observations came out of these trials is the blood clotting in vitro upon the addition of snake venom, while patients bitten by the same snake species has unclotted blood. This paradoxical phenomenon intrigued scientists to elucidate the composition, properties and actions of snake venoms and to try venoms or venom fractions for the therapy of haemorrhagic and thromboembolic diseases. Purified snake venom enzymes with a known mode of action and a narrow substrate specificity are resistant to the inhibitor systems in blood and tissues. These have enabled multiple applications in therapeutic, diagnostic or preparative procedures in hemostaseology (Table 1-1), whereas similar practical applications of snake venom enzymes (Elliott and Christensen 1978; Stocker 1990).

### *1.6.2 Anticancer pharmacology of snake venom*

Claude Bernard was the first one to realize the involvement of some components of snake venom in different therapeutic potential. The use of venom for the treatment of cancer in laboratory animal was first reported by Calmette, 1933 and later on (Hawgood 1999). Recently, Caldron et al. 2014 published a review article entitled "Antitumoral activity of snake venom proteins: New trend in cancer therapy". The authors focused on the new paradigms of both cytotoxic and pharmacological actions of snake venom toxins.

Elapid, Crotalid and Viperidae venoms were subjected to a comparative screening for in vivo and in vitro cytotoxicity towards B16F10 melanoma and chondrosarcoma cell lines. It was found that elapid venom possess considerably higher cytotoxic activity than that of Viperid and Crotalid venoms (Chaimmatyas and Ovadia 1987). Except Hydrophiidae snake venoms, venoms from the snake family Elapidae, Crotalidae and Viperidae cause lysis of Yoshida sarcoma cells (Braganca and Patel 1972). The venoms from two Viperidae species such as *Bothrops jararaca* and *Crotalus durissus terrificus* act directly on cancer cell. Their anticancer activity may be due to the snake venoms mainly from members of family Elapidae, Crotalidae and Viperidae showed cytotoxicity to kill sarcoma and melanoma cells as well as growth inhibitory activity in chondrosarcoma cell lines mediated for example, II2, IL8 and TNF-α (da Silva, da Silva et al. 2002; Gomes, Bhattacharjee et al. 2010).

The Southern Copperhead *Agkistrodon contortrix contortrix* is a pale brown snake found in the eastern and southern portions of the United States. The bite from a Southern Copperhead is seldom fatal due to its short fangs. The Southern Copperhead often produces a warning bite with little to no venom being released. However, Southern Copperhead possesses cytotoxic and neurotoxic venoms that can cause bone and tissue damage in human beings (Brownlee 2001). Researchers at the University of Southern California Keck School of Medicine have been searching for compounds that might stop the spread of breast cancer. They focused on contortrostatin, a protein derived from Southern Copperhead snake venom, because of its potent anticancer activity (Swenson, Costa et al. 2005). While studying the effect of this protein in mice, the researchers found that breast cancer spread was

60% to 70% smaller in contortrostatin-treated mice than in untreated mice. Markland and Zhou, the lead researchers, believe that the compound works by preventing cancer cells from connecting to healthy cells. In other words, this prevents the cancer cell from invading the healthy tissue as well as preventing the growth of new blood vessels that cancers need to survive. They believe that the use of the compound could also benefit cancer patients with other cancers including prostate, bladder, melanoma, brain, and Kaposi's sarcoma (Koh, Armugam et al. 2006).

However, contortrostatin was primarily isolated from venom because of its clot busting properties. Markland and his colleagues believe that this protein disrupts integrins. Integrins are transmembrane receptor proteins that bind to components of the extracellular matrix. They provide traction and allow cells to migrate from one place to another. Contotrostatin is believed to disrupt this by binding to cell-surface proteins in the integrin family which prevents it from gripping and scrambling its signals to the cytoskeleton. In order to test the effectiveness of contortrostatin as a treatment, researchers used human breast cancer and ovarian cancer cells in immunodeficient mice. They were given daily injections of the protein. With a few side effects, it showed that the protein prevented the movement of the cancers. It was found that contortrostatin causes the intracellular signs to be inappropriate and thus disrupt adhesions and the cytoskeleton. This indicates that not only does the protein keep things from binding; it also sends faulty signals (Finn, Weil et al. 2000). In general, contortrostatin has been proven useful in cancer treatment as it prevents metastasis by terminating blood supply to the cancer cells causing them to slowly die. The emphasis of research undertaken during past four decades has been on isolation and characterization of snake venom cytotoxins (Hayashi, Sasaki et al. 2008; Takechi, Hayashi et al. 1972; Zhong, Liu et al. 1993).

Cytotoxins isolated from different venom sources exhibited various physiological effects such as modulation of the activity of membrane enzymes, depolarization of excitable membrane inhibition, and cytotoxicity (Shier and Mebs 1990). These natural substances can be used as important pharmacological tools, probes, diagnostic techniques for the elucidation of complex biological processes.

For this thesis, the peptide components of venoms from three snakes *Naja pallida* (Red spitting cobra), *Agkistrodon piscivorus conanti* (Florida cottonmouth)

and *Agkistrodon contortrix laticinctus* (Northern Copperhead) were examined for cytotoxic effects on prostate cancer cell lines.

## 1.7 Purpose of the study

### *1.7.1 General objectives*

A. To test *N. pallida, A. contortrix laticinctus and A. piscivorus conanti* snake venoms for selective cytotoxic effects on DU145, PC3, LNCaP and PNT2A cell lines in vitro.

B. To explore the possible mechanisms of the venoms' cytotoxic actions.

C. To identify the components responsible for the cytotoxic actions of the venoms.

### *1.7.2 Specific objectives*

A. Separation and purification crude venom of *Naja pallida, Agkistrodon contortrix laticinctus* and *Agkistrodon piscivorus conanti* were carried out by using High Performance Liquid Chromatography (HPLC), Ion Exchange chromatography and reverse phase column C18 chromatography.

B. All crude venom and isolated fractions were tested for anticancer activity, phospholipase ($PLA_2$) activity in vitro prostate cell lines.

C. To determine the amino-acid sequence of the isolated peptide by N-terminal analysis followed by BLAST analysis.

The information gained could identify venom components worthy of further investigation as possible leads for anticancer therapeutics.

# CHAPTER TWO:

## TOXICITY AND MORPHOLOGICAL CHANGES INDUCED BY THREE SNAKE VENOMS ON PROSTATE CANCER CELL LINES AFTER 24 HOURS EXPOSURE

# 2. CHAPTER TWO

## 2.1 Introduction

An adult human body replaces approximately one per million of the 50-70 trillion cells on a daily basis to facilitate the biological processes for survival and reproduction. The balance between cell division and cell death maintains a relatively constant body size where each cell obediently serves the benefit of the organism, even at the sacrifice the life of the cell. A process called apoptosis is a mode of programmed cell death that enables cells to die in a well-coordinated manner and the remains to be cleared away by the neighbouring cells. The signals that trigger this cellular suicide program can originate from within the cell in response to cellular injuries, such as accumulating DNA damage, or from the exterior of the cell in the form of death ligands. In an adult, the cells of the immune system primarily use death ligands to activate apoptosis in cells that the body no longer needs, or in cells that are considered detrimental to the organism. The activation proceeds by binding of the death ligands to cell surface proteins, termed as death receptors which are capable of transmitting the signal to the interior of the cell (Hengartner 2000; Golstein and Kroemer 2007).

In cancer, individual cells continue to grow and divide disregarding the internal and external signals that demand the cell to commit suicide. This behaviour stems from critical mutations in the cancer cell genome that disrupt the mechanisms of normal cell division or apoptotic cell death. This turns the survival and growth of the cancer cell independent of external signalling. The internal differences in cell signalling can also be manifested at the cancer cell surface as altered expression of molecules, like receptors. Such manifestation in cell surface composition can be identified and used for distinction of cancer cells from healthy cells. However, current cancer therapies rely heavily on the induction of apoptosis in dividing cells which consequently damage healthy cells in tissues that are being rapidly replaced. The second major challenge in treating cancer is that the cancer cells adapt to new circumstances and, therefore, evade applied treatments by becoming resistant. These critical issues are posing major challenges to the ageing populations and in discovering novel, more powerful and cost-efficient treatments as well as improved

modes of cancer drug administration (Majno and Joris 1995; Kroemer and Jaattela 2005; Galluzzi, Maiuri et al. 2007).

Mammalian cell death is widely classified into two types: apoptosis and necrosis (Majno and Joris 1995; Galluzzi, Maiuri et al. 2007; Kroemer, Galluzzi et al. 2009). Autophagy, a third mode of cell death, is a process in which cells generate energy and metabolites by digesting their own organelles and macromolecules (Kroemer and Jaattela 2005; Klionsky 2007; Levine and Deretic 2007; Levine and Abrams 2008). Autophagy allows to growth limited cells (Kroemer and Jaattela 2005; Klionsky 2007; Levine and Deretic 2007; Levine and Abrams 2008). However, cells that do not receive nutrients for extended periods ultimately digest all available substrates and die (autophagy-associated cell death). The types of cell death such as apoptosis, necrosis, and autophagy are different in the mode of death and morphological, biochemical, and molecular attributes (Majno and Joris 1995; Galluzzi, Maiuri et al. 2007; Kroemer, Galluzzi et al. 2009).

Programmed cell death is an important concept. A programmed cell death can happen in two fundamental mechanisms such as genetically controlled apoptosis and autophagy-associated cell death (Klionsky 2007; Kroemer, Galluzzi et al. 2009). The discovery that genetically controlled processes can cause cell death has enabled advances in unravelling the mechanisms of many diseases. This novel knowledge has facilitated the development of pharmacological agents that initiate or inhibit programmed cell death (Thompson 1995; Green and Kroemer 2005; Reed 2006). Moreover, it is evident that necrosis, traditionally considered an accidental form of cell death, can be initiated or modulated by programmed control mechanisms in certain instances (Zong and Thompson 2006; Golstein and Kroemer 2007). The intracellular signalling amplifies the received message and ultimately leads to enzymatic decomposition of the cellular structures (Cooper, Sykes et al. 2007).

### *2.1.1 Apoptosis induced cell death*

Programmed cell death (PCD) was first described by Kerr, Wyllie and Currie in 1972. They named the natural ordered elimination of cells from a tissue. Apoptosis is derived from an ancient Greek word that suggests leaves falling from a tree (Kerr, Wyllie et al. 1972; Strasser 2005).

Apoptosis is a genetically mediated form of cell death and plays an integral role in normal tissue development. It is involved in organogenesis, tissue homeostasis and remodelling, and the removal of auto-reactive clones by modifying the immune system. In contrast to necrosis, which affects a large number of cells in one area, apoptosis affects scattered individual cells and usually does not cause an inflammatory response (D'Amico and McKenna 1994). The process of apoptosis has been described as an active bio-energy saving cell-elimination mechanism, which removes aged, unwanted or damaged cells. The cellular contents with precious caloric value are phagocytosed by adjacent cells or macrophages and recycled (Allen, Hunter et al. 1997).

Apoptosis is a special kind of cellular suicide mediated by various intracellular molecules. The susceptibility of any cell to death signal is determined by ratio of proapoptotic protein Bax/anti apoptotic protein Bcl2 (Das Gupta, Gomes et al. 2010). Although, apoptosis, autophagy, and necrosis are considered the main types of cell death, several other subtypes can be distinguished based mostly on biochemical and functional criteria (Galluzzi, Vitale et al. 2012).

In contrast to the swelling of the cell and its organelles that defines necrosis, the principal morphological feature of apoptosis is shrinkage of the cell and its nucleus. The distinction between necrosis and apoptosis can be partially attributed to the differences in how the plasma membrane participates in these processes. In necrosis, early loss of integrity of the plasma membrane allows an influx of extracellular ions and fluid which results in swelling of the cell and its organelles (Nishimura and Lemasters 2001; Zong and Thompson 2006; Malhi, Gores et al. 2006; Golstein and Kroemer 2007).

In apoptosis, plasma-membrane integrity persists until late in the process. A key feature of apoptosis is the cleavage of cytoskeletal proteins by aspartate-specific proteases, which thereby collapses subcellular components (Marsden and Strasser 2003; Green and Kroemer 2005; Strasser 2005; Adams and Cory 2007). Other characteristic features are chromatin condensation, nuclear fragmentation, and the formation of plasma-membrane blebs. Finally, apoptosis is programmed cell death that occurs regularly in old or unhealthy cells, but cancer cells are able to escape this programmed cell death in order to live and divide indefinitely (Reddy, Shapiro et al. 2003).

### *2.1.2 Necrosis induced cell death*

Necrosis (from the Greek nekros, for corpse) is best defined by light or electron microscopic detection of cell and organelle swelling or rupture of surface membranes with spillage of intracellular contents (Majno and Joris 1995; Lemasters 2005). The term oncosis (Greek for swelling) is preferred by some investigators, and "oncotic necrosis" has also been used (Majno and Joris 1995). The compromise of organelle membranes allows proteolytic enzymes to escape from lysosomes, enter the cytosol, and cause cell destruction (Zong and Thompson 2006; Luke, Pak et al. 2007; Leist, Single et al. 1997; Liu, Van Vleet et al. 2004; Turk and Stoka 2007; Conus, Perozzo et al. 2008; Conus and Simon 2008).

Necrosis usually results from metabolic failure that coincides with rapid depletion of ATP; it classically occurs in ischemia (Leist, Single et al. 1997; Malhi, Gores et al. 2006). Necrosis is usually considered as an accidental form of cell death that occurs in response to acute hypoxic or ischemic injury, such as myocardial infarction and stroke. It occurs spontaneously in neoplasms when cell proliferation outpaces angiogenesis. The exposure of cells to supraphysiologic conditions for example, mechanical force, heat, cold, and membrane permeabilizing and toxins also initiates necrosis.

Random DNA degradation and further membrane disruption occurs with the activation of $Ca^{2+}$-dependent degradative enzymes, such as phospholipases, hydrolases, proteases and endonucleases. The cell eventually bursts and releases lysosome and degradative enzymes due to the osmotic imbalance. The cell

eventually bursts the lysosome due to the osmotic imbalance and releases degradative enzymes. These elicit an inflammatory response in the adjacent viable cells and can result in a zone of necrotic cells radiating from a necrotic centre in a tissue (Gilloteaux, Jamison et al. 1998; Walsh, Dewson et al. 1998).

### *2.1.3 Differentiation between apoptosis and necrosis*

**A.** Apoptosis, also called programmed cell death, is a physiological process that occurs under normal conditions by which unwanted or useless cells are eliminated during development or other biological process. Apoptosis is an energy requiring process.

**B.** Necrosis also known as accidental cell death is a passive, catabolic pathological process that occurs when cells are exposed to serious physical or chemical insult like heat stress or toxic agents.

**C.** As apoptosis marks the onset of cell death, it is important to accurately distinguish between the apoptotic and necrotic states from the stand-point of the current work. Table 2-1 and Figure 2-1 list the differences between the two modes of cell death based on morphology (Ameisen, Idziorek et al. 1995; Budd 2002).

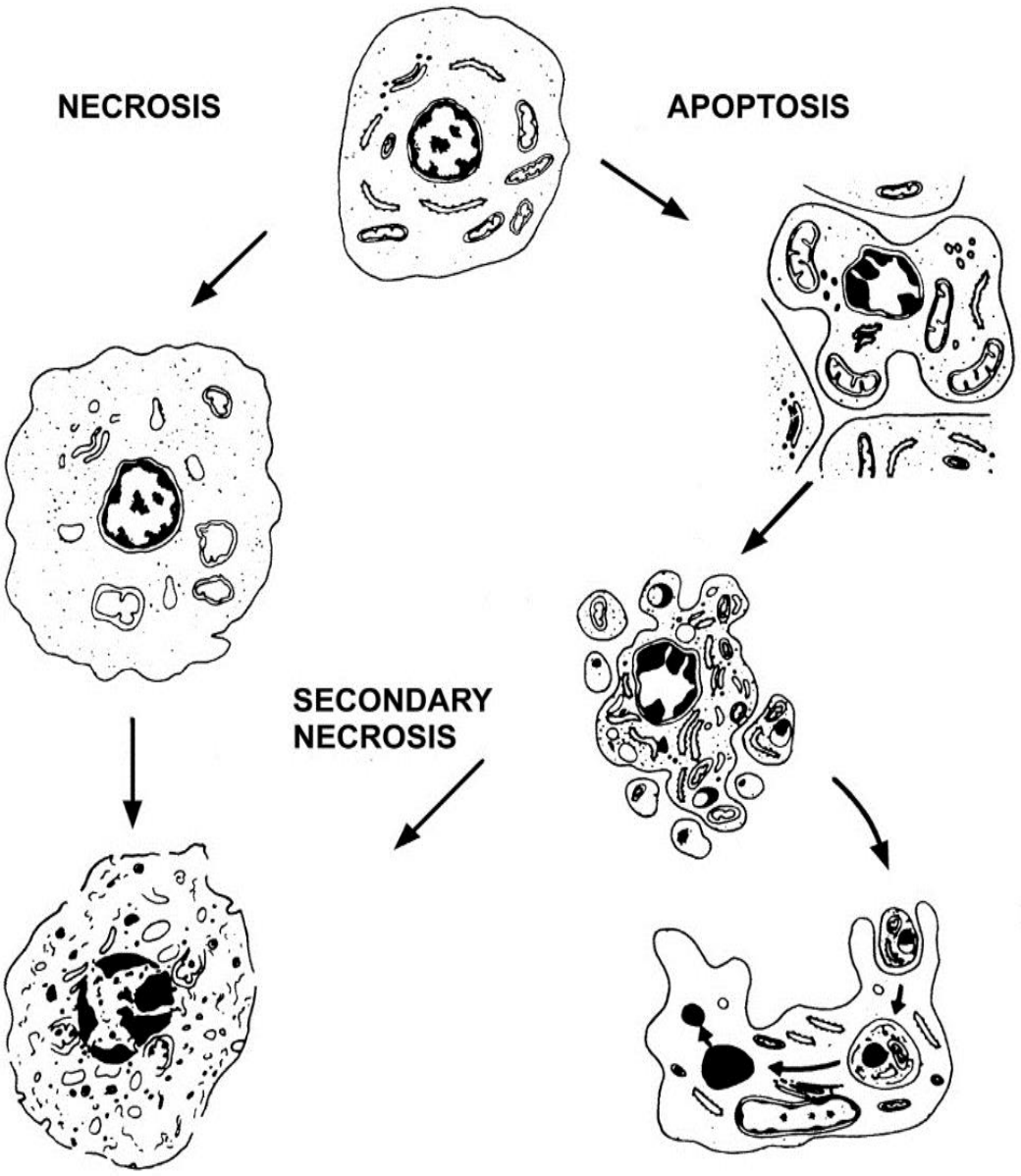



**Figure 2-1:** Different morphological between necrosis and apoptosis (Gold, Schmied et al. 1994).

**Table 2-1:** Differential Morphological Feature and Significance of Apoptosis and Necrosis adapted from (Kerr, Wyllie et al. 1972; Wyllie 1997)

| Morphological Features | |
|---|---|
| **Apoptosis** | **Necrosis** |
| • Protrusions on the cell membrane, but no loss of membrane integrity | • Loss of membrane integrity |
| • Aggregation of chromatin in the nuclear membrane | |
| • Begins with shrinking of cytoplasm and condensation of nucleus | |
| • Ends with fragmentation of cell into smaller bodies | • Begins with swelling of cytoplasm and mitochondria |
| • Formation of membrane bound vesicles (apoptotic bodies) | • Ends with total cell lysis |
| • Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family | • No vesicle formation, complete lysis |
| • Rounding-up of the cell and Plasma membrane blebbing | • Swelling of cytoplasmic organelles |
| • Reduction of cellular and nuclear volume (pyknosis) | • Cytoplasmic swelling (oncosis) |
| • Retraction of pseudopods | • Rupture of plasma membrane |
| • Nuclear fragmentation (karyorrhexis) | • Moderate chromatin condensation |
| • Minor modification of cytoplasmic organelles | |
| • Engulfment by resident phagocytes, in vivo | |

### 2.1.4 Causes of apoptosis

The following factors have been found responsible for the incidence of apoptosis (Mercille and Massie 1994; Singh, Alrubeai et al. 1994) supporting the incidence of apoptosis due to the following factors:

- Nutrient limitation (namely, glucose and glutamine)
- Oxygen deprivation
- Cysteine deprivation
- Serum limitation
- Hypoxia

### 2.1.5 Causes of necrosis

- Ammonia accumulation (lowest concentration reported = 2mM)
- Lactate accumulation (lowest concentration reported = 20mM)
- Osmotic pressure
- pH

## 2.2 Materials and Methods

### *2.2.1 Chemicals, reagents and venoms*

Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640, foetal calf serum (FCS), TrypLE Express, L-glutamine, Sodium pyruvate, Kanamycin, Gentamicin, Non-essential amino acids, $Mg^{2+}$-$Ca^{2+}$ -free Hank's balanced salt solution (HBSS), and SYTOX Green were purchased from Invitrogen™ Thermo Fisher Scientific, 3 Fountain Drive, Inchinnan Business Park, Inchinnan, Paisley, UK. Snake venoms: *Naja pallida* (Red spitting cobra), *Agkistrodon contortrix laticinctus* (Northern Copperhead) and *Agkistrodon piscivorus conanti* (Florida cottonmouth) were supplied by National Natural Toxins Research Centre (Texas A&M University-Kingsville, USA).

### *2.2.2 Cell lines and cell culture*

Assays were carried out using prostate cancer cell lines, particularly DU145, PC3, and LNCaP and a normal prostate epithelial line PNT2A to look for selectivity against cancer cells. The DU145 and PC3 human prostate cancer cell lines are the classical cell lines of prostate cancer. DU145 cells have moderate metastatic potential compared to PC3 cells which have high metastatic potential. DU145 and PC3 cell lines were maintained in DMEM supplemented with 10% foetal calf serum (FCS), 1% sodium pyruvate, 1% L-glutamine, 1% kanamycin, 1% gentamicin, 1% non-essential amino acids, in tissue culture incubator (37°C and 5% $CO_2$). Cells were reseeded on to new (T75) flasks with fresh medium before becoming confluent (80-90%). To reseed, the cells were washed with HBSS, before being exposed to TrypL E Express for 2 minutes at 37$^{o}$C, after which the complete medium containing 10% FCS was added to the cells suspension. The media containing the cell suspension was transferred to 15 ml centrifuge tubes and spun at 1000 rpm for 1 min. The resulting pellet was resuspended in 1ml of media and cells were counted using an improved Neubauer haemocytometer and reseeded at appropriate concentration (number of cells/cm$^2$). LNCaP cell lines are androgen sensitive human prostate adenocarcinoma cells derived from the lymph node. PNT2A is a human normal prostatic cell line. The LNCaP and PNT2A cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 1% sodium pyruvate, 1% L-glutamine, 1% kanamycin, 1% gentamicin 1% non-essential amino acids. The growth conditions and the

reseeding protocol was the same as was used for the DU145 and PC3 cell lines. For all cells, the media was refreshed every three days and cells passaged when 80-90% confluence was achieved.

### *2.2.3 Cisplatin*

Cisplatin is an anticancer chemotherapy drug which is classified as an "alkylating agent". DU145, PC3, LNCaP, and PNT2A cells were seeded onto 96-well plates at around 10,000 - 40,000 cells per well and sub confluent cells were subsequently treated with various concentration of Cisplatin (3, 10, 30, 100 and 300µM) for 24 hours. After the incubation period treatment, SYTOX Green (0.5µM) was added to each well and incubated for 15 mins before being read on a Wallac2 Victor2 multilabel plate reader (PerkinElmer, Cambridge, UK) (excitation 455nm, emission 535 nm) for 0.1s per well.

### *2.2.4 Preparation of frozen stock of cells*

Sub confluent cells were harvested from 75 $cm^2$ flasks as above, suspended in culture media containing 10% DMSO, 90% culture medium and aliquoted into 1 ml cryovials. The vial was wrapped in polystyrene (to ensure a rate of cooling equivalent to $1^oC$ per min) and vials were placed in a $-80^oC$ deep freezer overnight before being transferred into a liquid nitrogen storage container for long term storage.

### *2.2.5 Growth characteristics of the DU145, PC3, LNCaP, and PNT2A cell lines*

Growth curves of cells were generated by seeding 6 flasks (T25) at the same density ($0.5x10^4$cells/$cm^2$). Flasks were cultured in tissue culture incubator and duplicate flasks were counted every 24 hours using a haemocytometer.

### *2.2.6 Determination of cytotoxicity*

#### 2.2.6.1 SYTOX Green assay

Prostate cancer cells were seeded onto 96-well plates at around 10,000-40,000 cells per well and sub confluent cultures were subsequently treated with

various snake venoms at concentration of 3, 10, 30, 100 and 300µg/ml for 24 hours. After the incubation period treatment, SYTOX Green (0.5µM) was added to each well and incubated for 15 mins before being read on a Wallac2 Victor2 multilabel plate reader (Perkin Elmer, Cambridge, UK) with excitation 455nm, emission 535 nm for 0.1s per well.

#### 2.2.6.2 SYTOX Green activity measurement

The fluorescent nucleic acid dye SYTOX Green, an indicator of cell death, was employed to determine the cytotoxicity of compound. The dye is excluded from live cells but penetrates cells with compromised plasma membranes (dying cells). The dye binds to double stranded DNA resulting in a large increase in relative fluorescence which can be detected by a spectrophotometer, allowing for the rapid quantification of cell death (Jones and Singer 2001).

### *2.2.7 Microscopy*

Cell were visualised routinely using a Nikon TMS-F inverted Phase Contrast Microscope. The Nikon TMS-F is a widely used inverted microscope in cell culture laboratories. Digital images of cells were acquired by a microscope-mounted digital camera (Moticam 1000; Motic Germany), visualised using Motic Images Plus, 2.0 and images stored on a desktop computer running Microsoft Windows XP. All cell cultures were directly used for visualisation at 400X magnification without any use of staining.

### *2.2.8 Statistical analysis*

The data was plotted and statistical analysis carried out using GraphPad Prism version 4.00 for Windows, (GraphPad Software, San Diego, California, USA). Data was plotted as mean ± standard error of the mean with N representing the number of independent experiments. Unless stated in the text individual experiments were carried out in triplicate. Means were compared by one-way analysis of variance (ANOVA) and $P<0.05$ was considered to be statistically significant.

## 2.3 Results

### *2.3.1* Effect of *Naja pallida* venom on DU145, PC3, LNCaP, and PNT2A cell lines for 24 hours

The cytotoxic effect of *N. pallida* venom on DU145, PC3, LNCaP and PNT2A cells treated with various concentrations (3, 10, 30, 100 and 300µg/ml) over a period of 24 hours is shown in Figure 2-2.

In DU145 cells SYTOX Green fluorescence reduced as the venom concentration increased. In the positive controls (Triton-X 100, 0.1%), highest fluorescence (AU) was found, and in the negative controls (without venom) it was lowest.

There were no major changes observed in fluorescence in PC3 cells after exposure to the various concentration of *N. pallida* venom with SYTOX Green. The SYTOX Green fluorescence was almost similar to negative control (without venom) in PC3 cell line.

The cell line LNCaP showed an increase in fluorescence with increasing concentration of venom from 10 to 100µg/ml, but at the highest concentration (300µg/ml) the fluorescence decreased.

With PNT2A cells, the effect of *N. pallida* venom was a triphasic change in fluorescence associated: there was an initial high increase in fluorescence with the lowest concentration of venom tested followed by a marked reduction in fluorescence which in subsequently increased with higher concentrations of venom and it was a statistically significant difference.

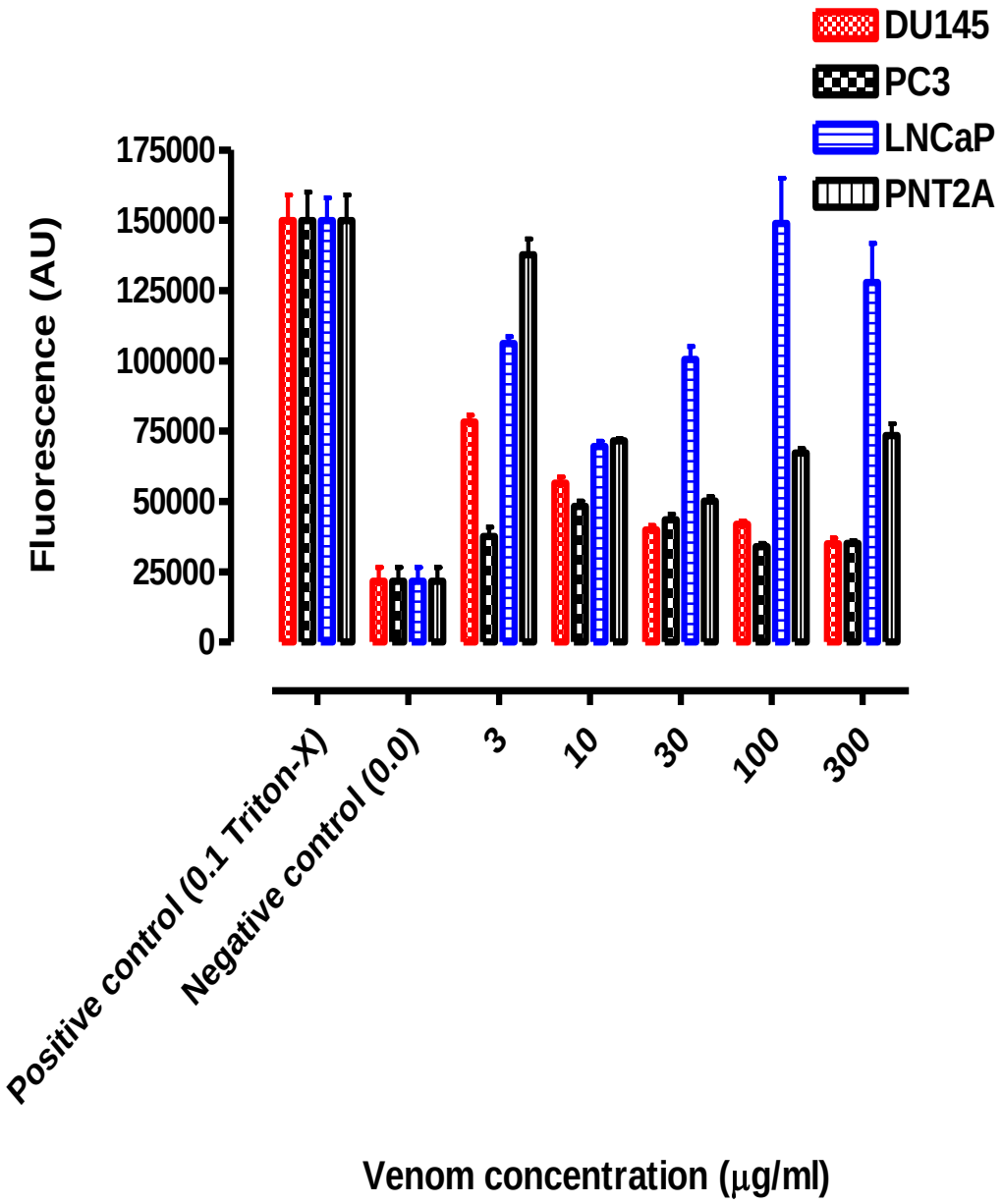



**Figure 2-2:** Changes in SYTOX green (0.5µM) intensity in cultured cell lines DU145, PC3, LNCaP, and PNT2A following 24 hour exposure to 3, 10, 30, 100 and 300µg/ml of *Naja pallida* snake venom in complete media at 37$^{o}$C. The data is represented as means ± S.E.M of 3 independent experiments and in most cases the S.E.M are smaller than the symbols. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 2.3.1.1 Morphological analysis

Figure 2-3; 2-4; 2-5 and 2-6 illustrate the morphological changes observed in DU145, PC3, LNCaP and PNT2A cells after 24 hours of incubation with venom. The untreated control cells were homogeneously distributed in the culture field and were polygonal in shape with distinct boundaries and homogeneous or slightly granulated intracellular contents.

After incubating the cell lines with *N. pallida* venom for 24 hours, various morphological abnormalities were observed. A dead cell morphology (swelling and apoptotic shrinkage) was observed in *N. pallida* treated DU145, PC3, LNCaP and PNT2A cells lines as compared with untreated control as shown in Figures 2-3; 2-4; 2-5 and 2-6. For example, at the lowest concentration (3µg/ml) of *N. pallida* venom, the cells have lost some of their typical morphology and were more rounded in appearance. With higher concentrations of *N. pallida* venom, cell morphology is further deranged and the cells have an increasingly granular appearance.

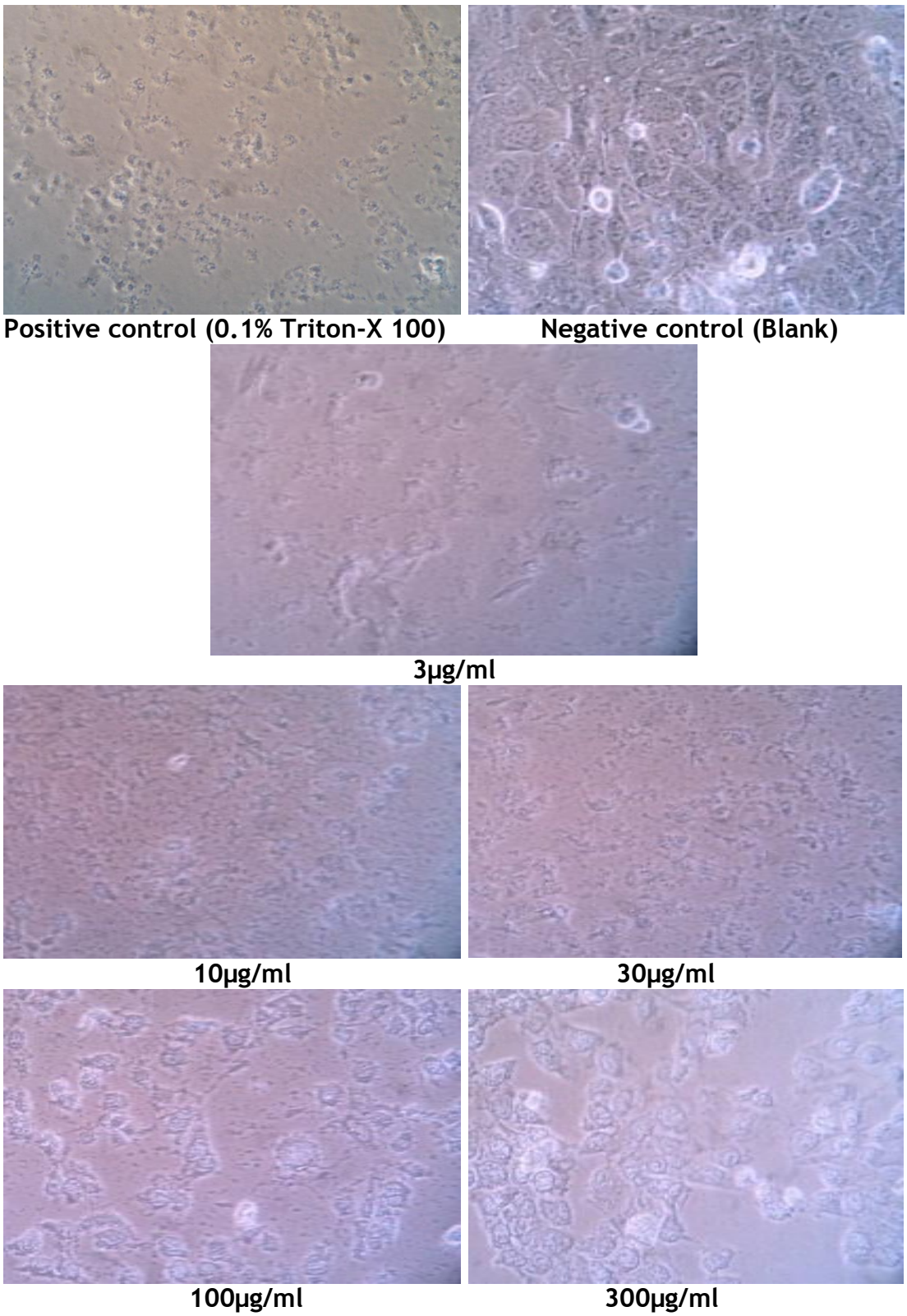



**Figure 2-3:** Light micrographs of DU145 cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *Naja pallida* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

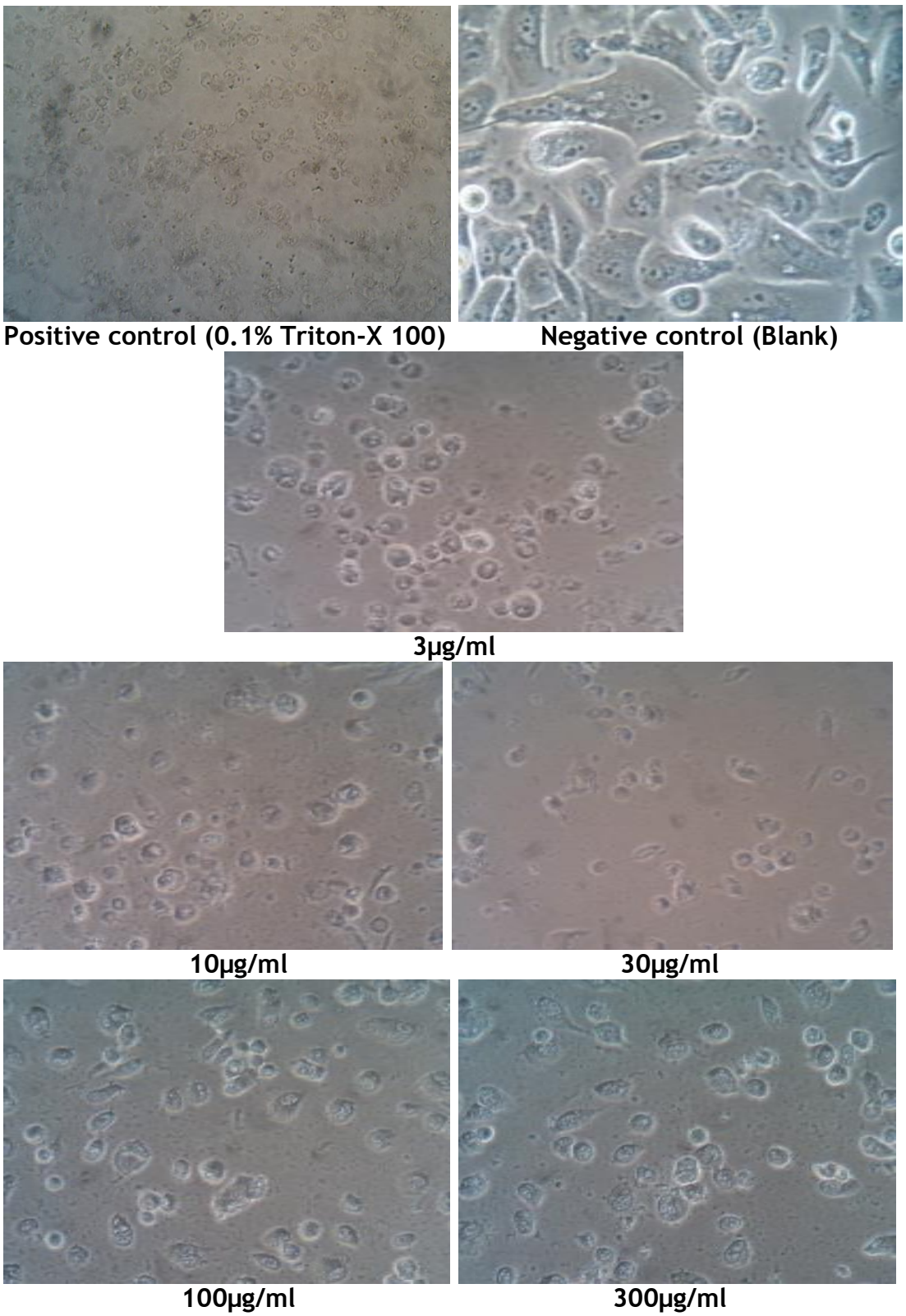



**Figure 2-4:** Light micrographs of PC3 cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *Naja pallida* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using aphase-contrast microscope.

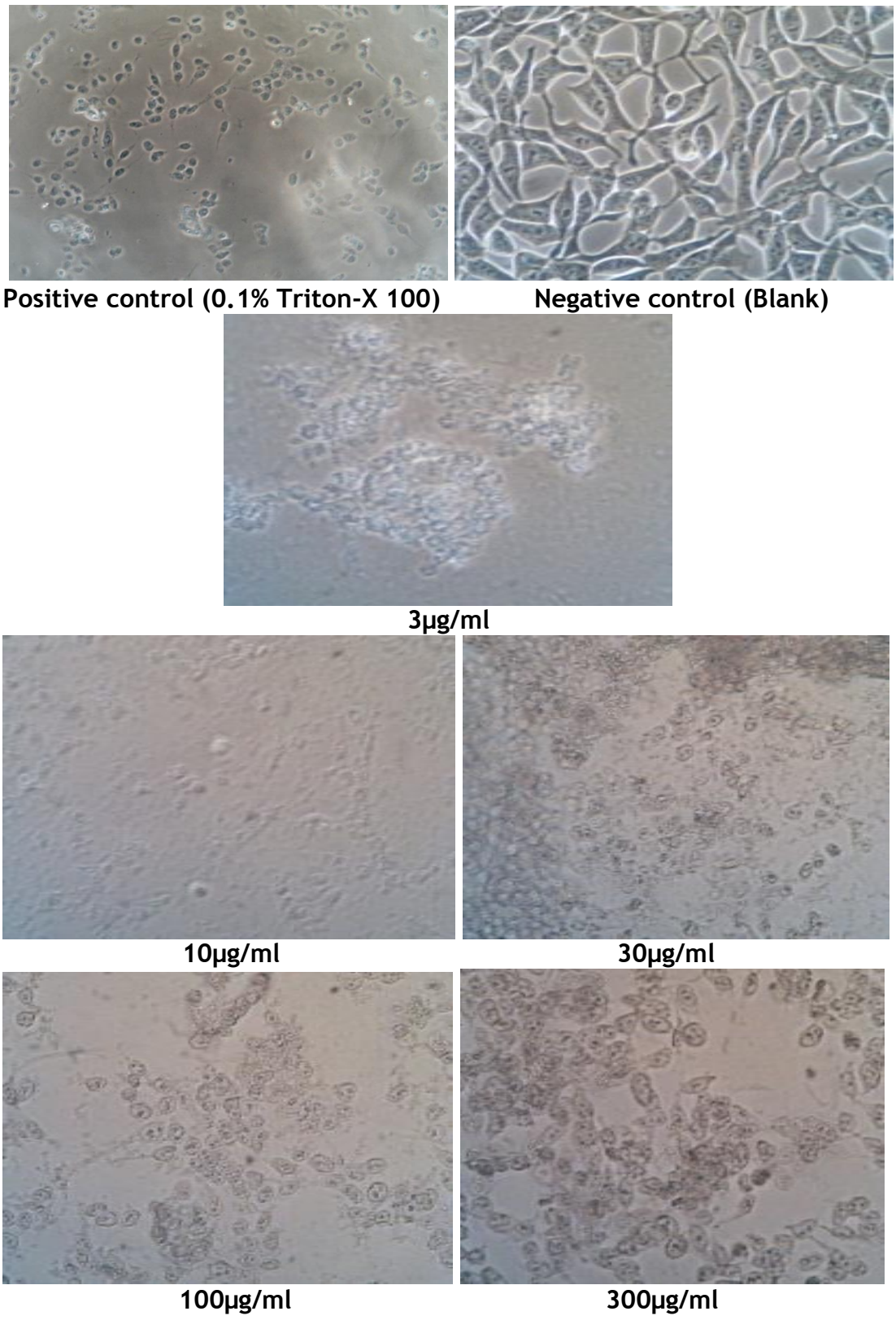



**Figure 2-5:** Light micrographs of LNCaP cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *Naja pallida* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using aphase-contrast microscope.

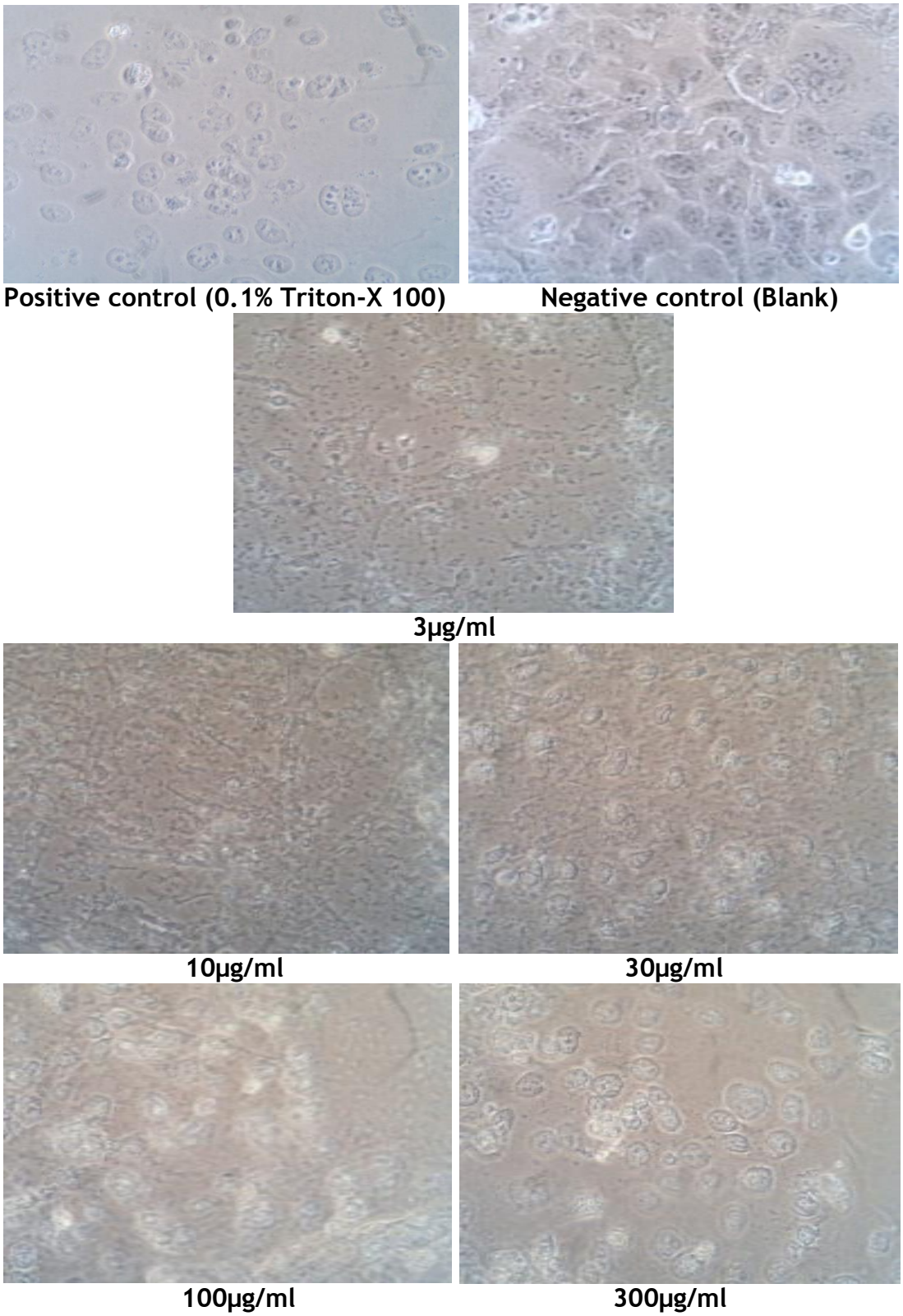



**Figure 2-6:** Light micrographs of PNT2A cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *Naja pallida* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### *2.3.2 Effect of Agkistrodon contortrix laticinctus venom on DU145, PC3, LNCaP, and PNT2A cell lines for 24 hours*

The cytotoxic effects of *A. contortrix laticinctus* venom (3, 10, 30, 100 and 300µg/ml) on cell lines DU145, PC3, LNCaP, and PNT2A were determined using SYTOX Green as an indicator of cellular death after 24 hours exposure to venom. As shown in Figure 2-7, there is a clear and concentration dependent slight increase in SYTOX Green fluorescence associated with DU145, and PNT2A cells lines as compared to positive control fluorescence, and in negative control experiments, fluorescence was very much less. In LNCaP cell line there was a large concentration dependent increase in SYTOX Green fluorescence and decreases with high concentration 100 to 300µg/ml. There were no significant changes in observed PC3 cell line fluorescence.

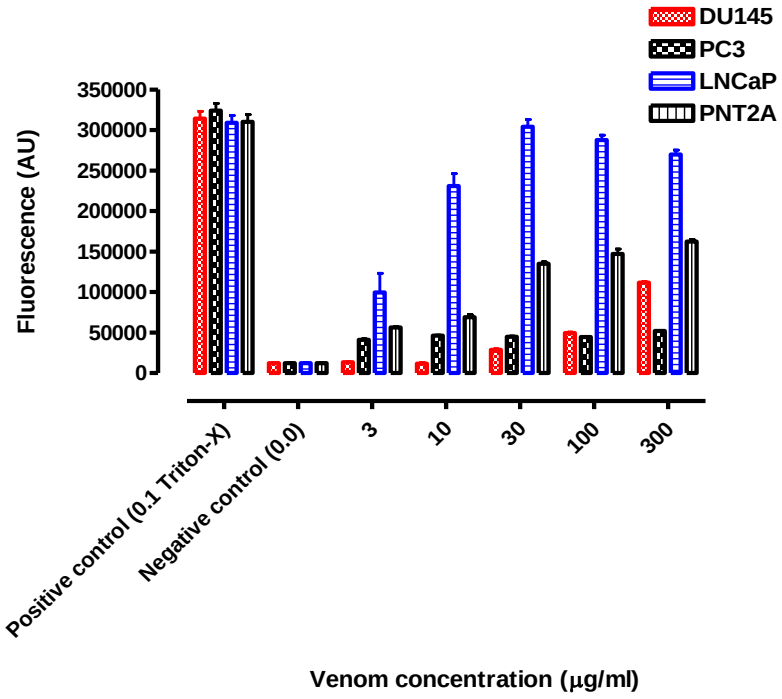



**Figure 2-7:** Changes in SYTOX green (0.5µM) intensity in cultured cell lines DU145, PC3, LNCaP and PNT2A following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. contortrix laticinctus* snake venom in complete media at 37$^{o}$C. The data is represented as means ± SEM of 3 independent experiments. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 2.3.2.1 Morphological analysis

The morphological changes were observed in DU145, PC3, LNCaP and PNT2A cell line after 24 hours exposure to *A. contortrix laticinctus* venom with 3-300μg/ml as shown in Figures 2-8; 2-9; 2-10 and 2-11. In control, the untreated cells were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents.

However, after incubating the cell lines with *A. contortrix laticinctus* venom for 24 hours, various morphological abnormalities were observed. There is clear evidence of cell rounding, clumping and loss of adhesion across all cell lines and at all concentrations. However, DU145 cells seem to be less affected in comparison with control and the other cell lines. In LNCaP and PNT2A cells, the higher concentrations of venom resulted in complete loss cellular contents and the field of view was stained with cellular debris.

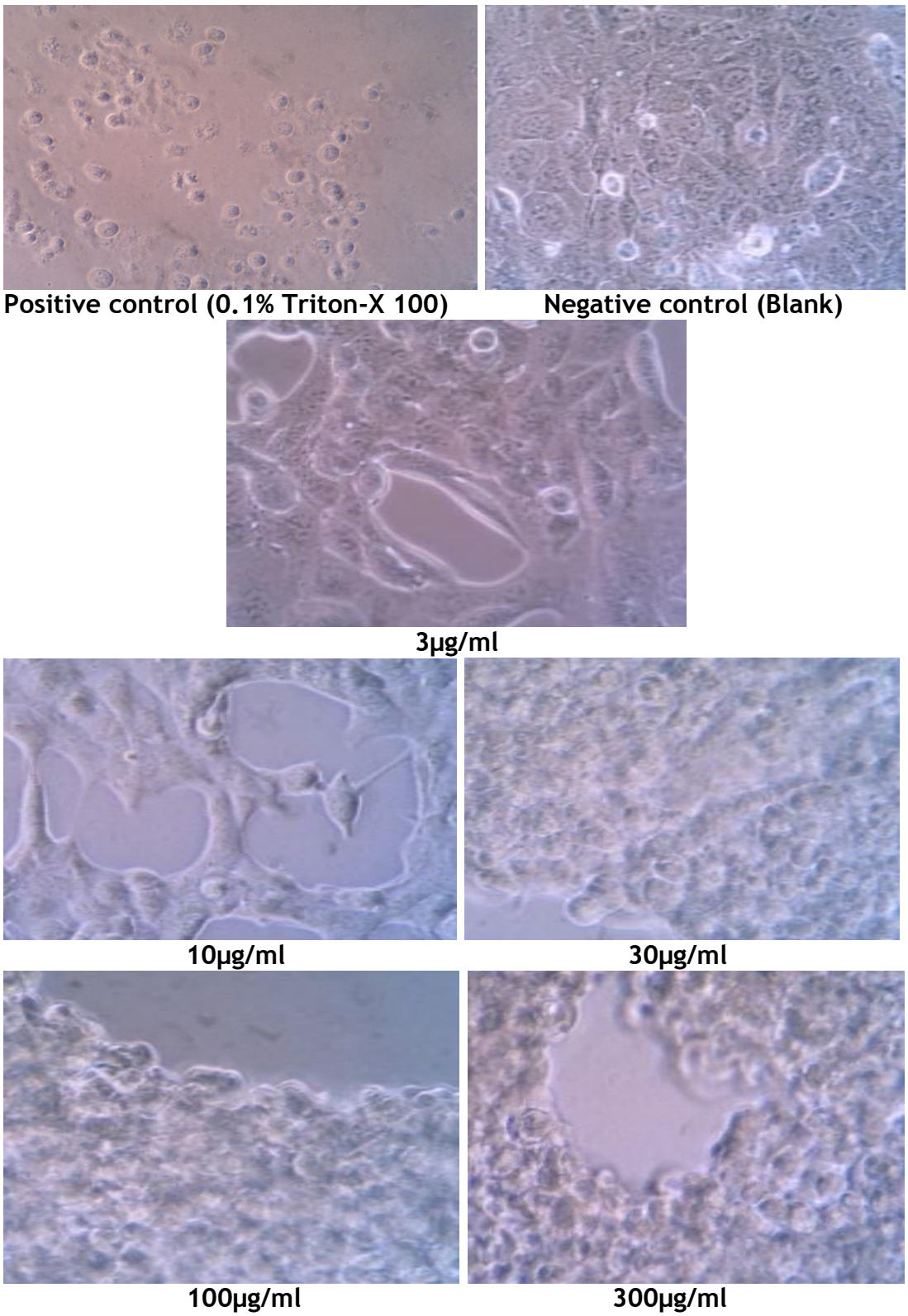



**Figure 2-8:** Light micrographs of DU145 cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. contortrix laticinctus* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using aphase-contrast microscope.

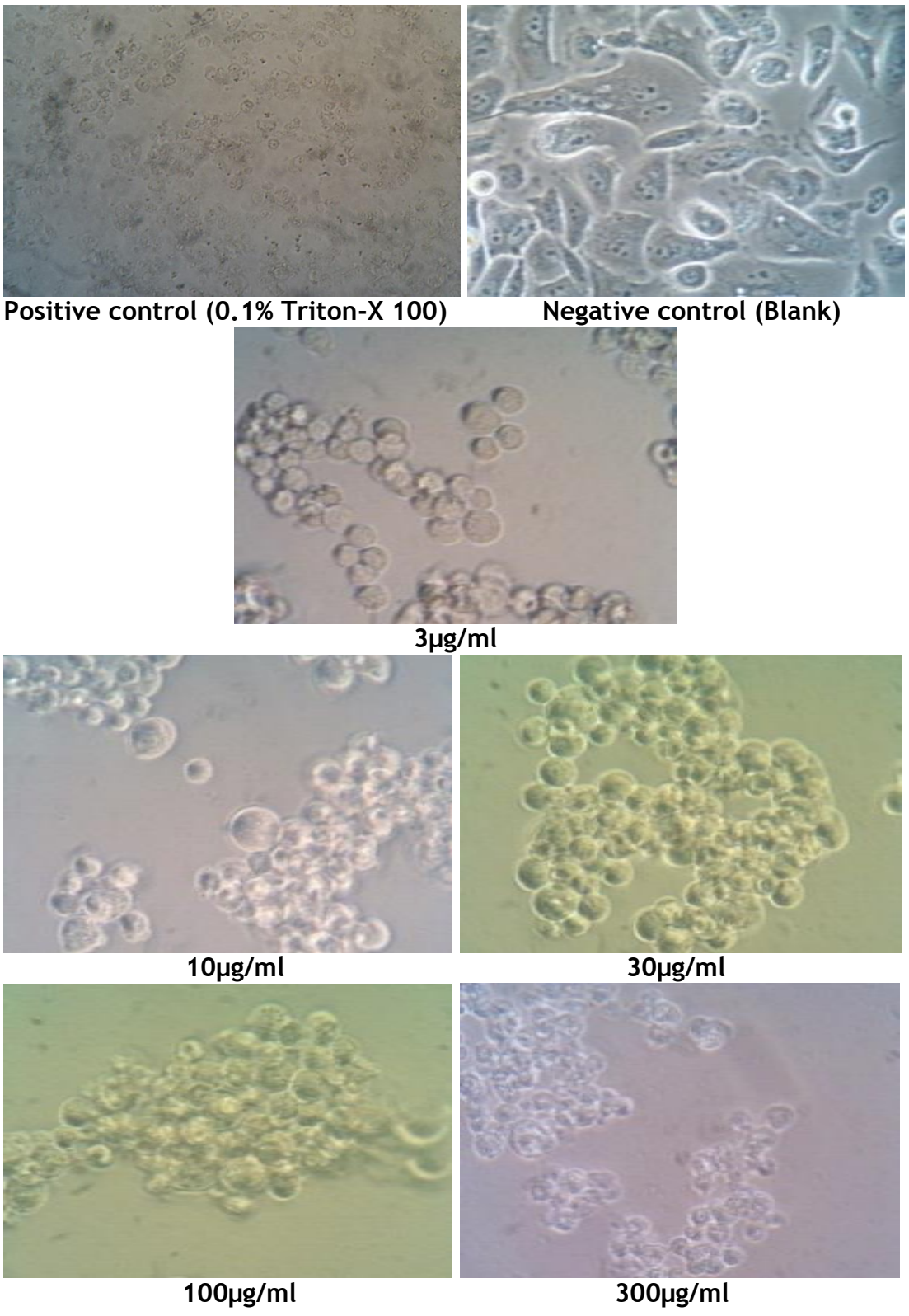



**Figure 2-9:** Light micrographs of PC3 cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. contortrix laticinctus* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using aphase-contrast microscope.

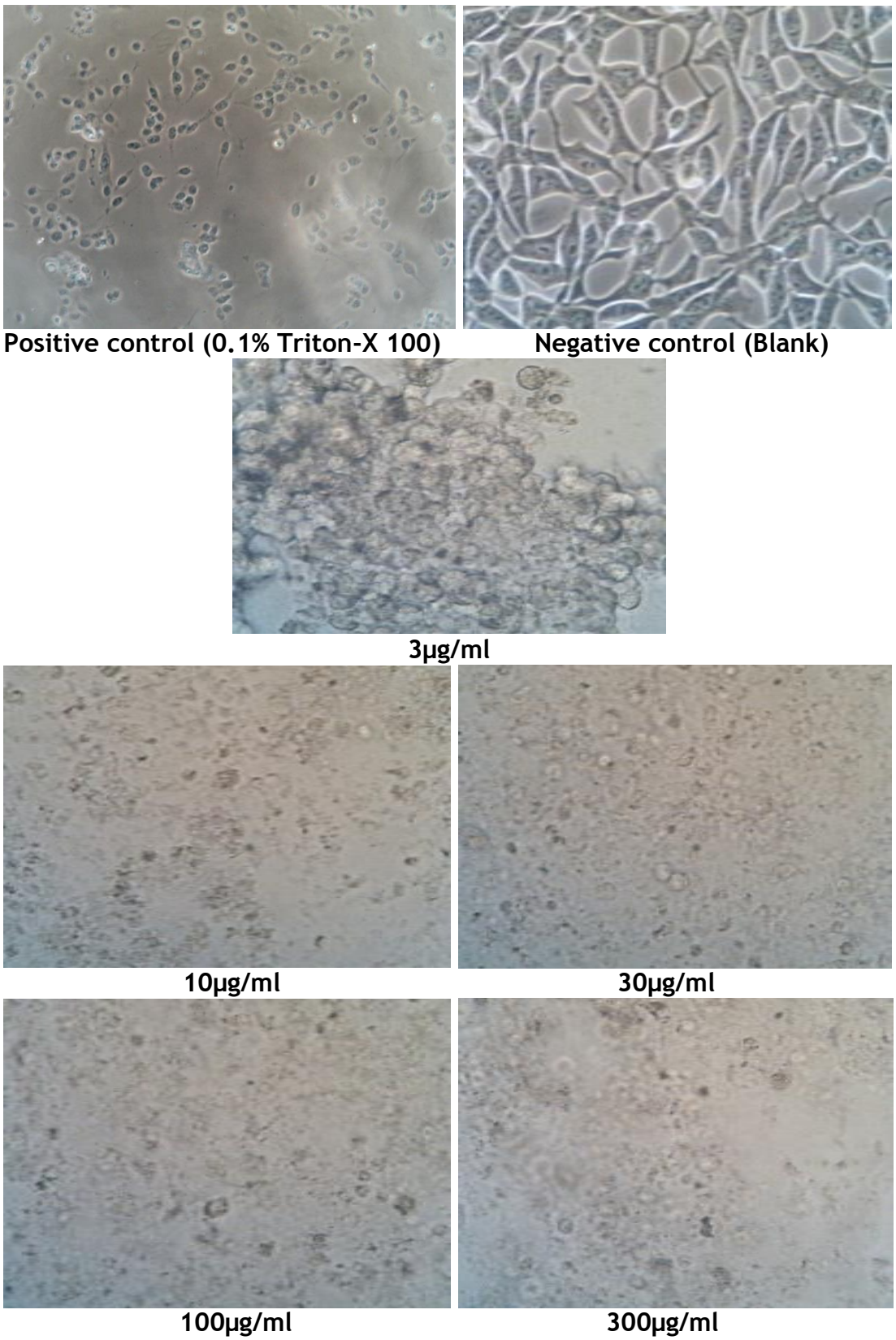
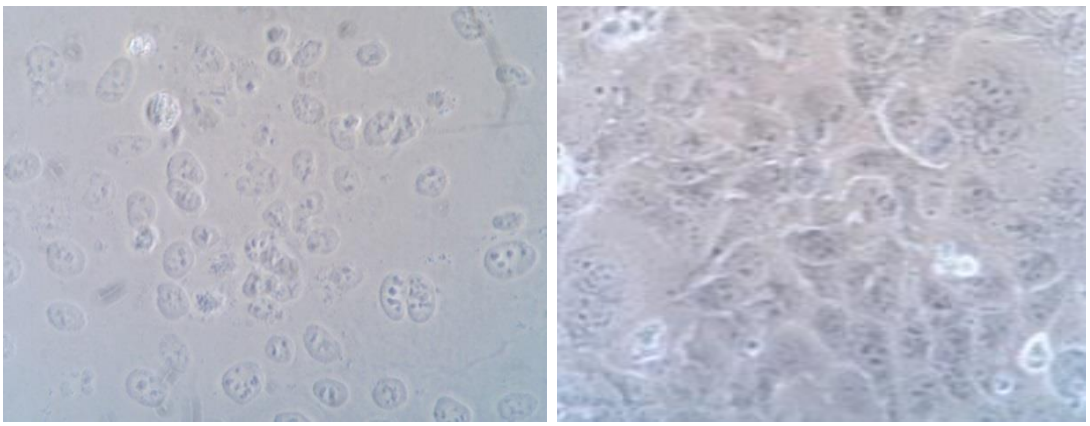
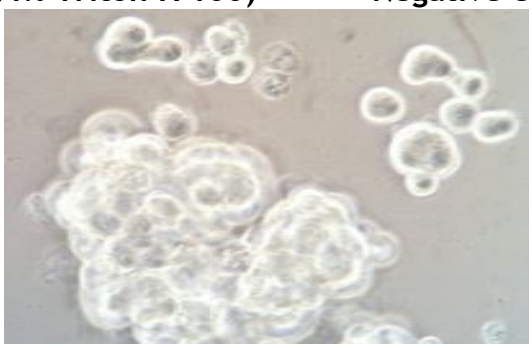


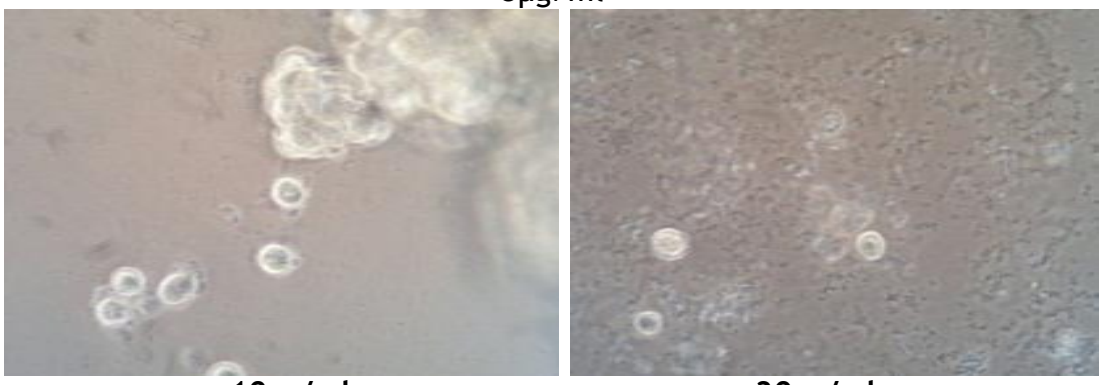

**Figure 2-10:** Light micrographs of LNCaP cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. contortrix laticinctus* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using aphase-contrast microscope.



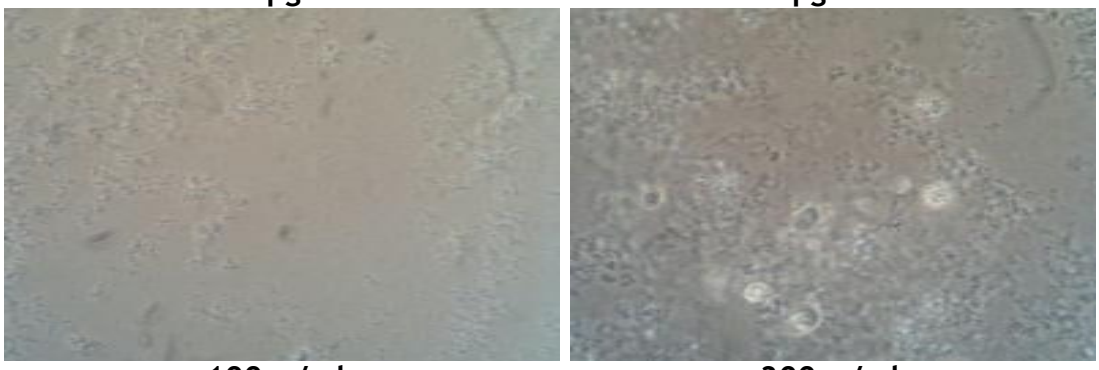
Positive control (0.1% Triton-X 100) Negative control (Blank)



3µg/ml



10µg/ml 30µg/ml



100µg/ml 300µg/ml

**Figure 2-11:** Light micrographs of PNT2A cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. contortrix laticinctus* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using aphase-contrast microscope.

### *2.3.3* Effect of *Agkistrodon piscivorus conanti* venom on DU145, PC3, LNCaP and PNT2A cell lines for 24 hours

SYTOX Green assay was performed to investigate cytotoxic effects on DU145, PC3, LNCaP and PNT2A cells treated with various concentrations (3, 10, 30, 100 and 300 µg/ml) of *A. piscivorus conanti* venom for 24 hours. As shown in Figure 2-12, there was a clear concentration dependent increase in SYTOX Green fluorescence in the PC3 cell line but LNCaP and PNT2A showed decreases in fluorescence as the concentrations were increased. There were no obvious changes found in fluorescence on the DU145 cell line. Positive control (Triton-X 100) showed the maximum fluorescence while the negative control (complete medium only) showed lowest fluorescence.

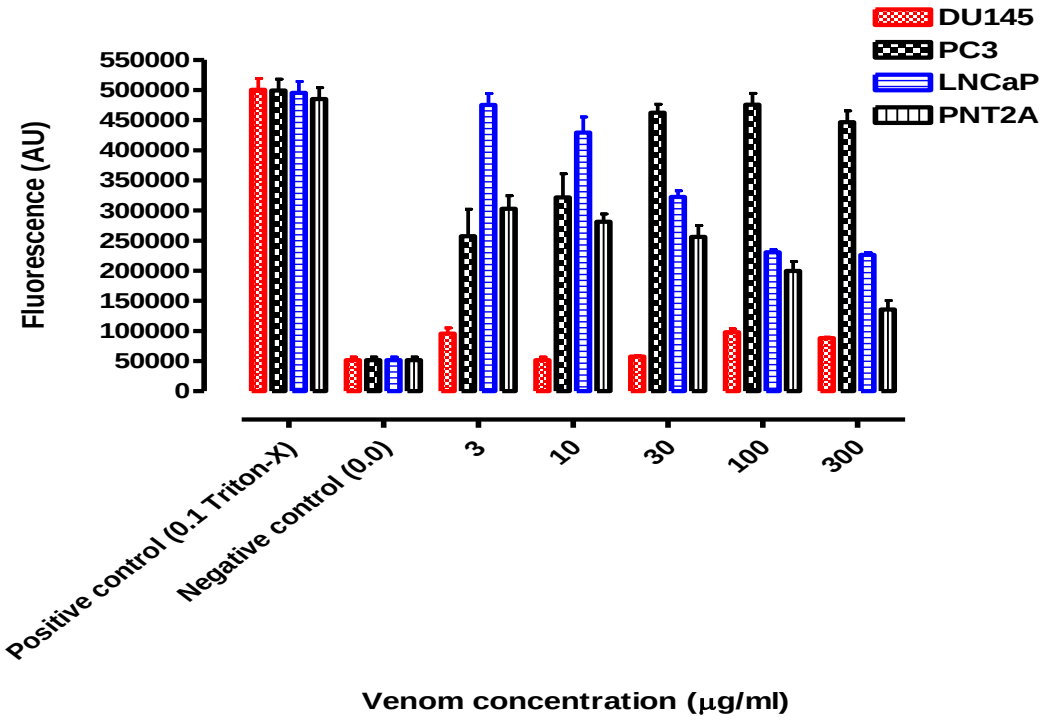



**Figure 2-12:** Changes in SYTOX green (0.5µM) intensity in cultured cell lines DU145, PC3, LNCaP and PNT2A following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. piscivorus conanti* snake venom in complete media at 37$^{o}$C. The data is represented as means ± SEM of 3 independent experiments. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 2.3.3.1 Morphological analysis

Figures 2-13; 2-14; 2-15 and 2-16 illustrate the morphological changes in the DU145, PC3, LNCaP and PNT2A cells after 24 hours of incubation in venom. Typically, the untreated cells (control) were homogeneously distributed, exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents.

However, after incubating these cell lines with *A. piscivorus conanti* venom at various concentrations (3, 10, 30, 100 and 300μg/ml) for 24 hours, various morphological abnormalities were observed. In all treated cell lines (DU145, PC3, LNCaP and PNT2A), there was evidence of clumping, rounding and loss of adherence. This observation was particularly obvious in PC3 and DU145 cell lines.

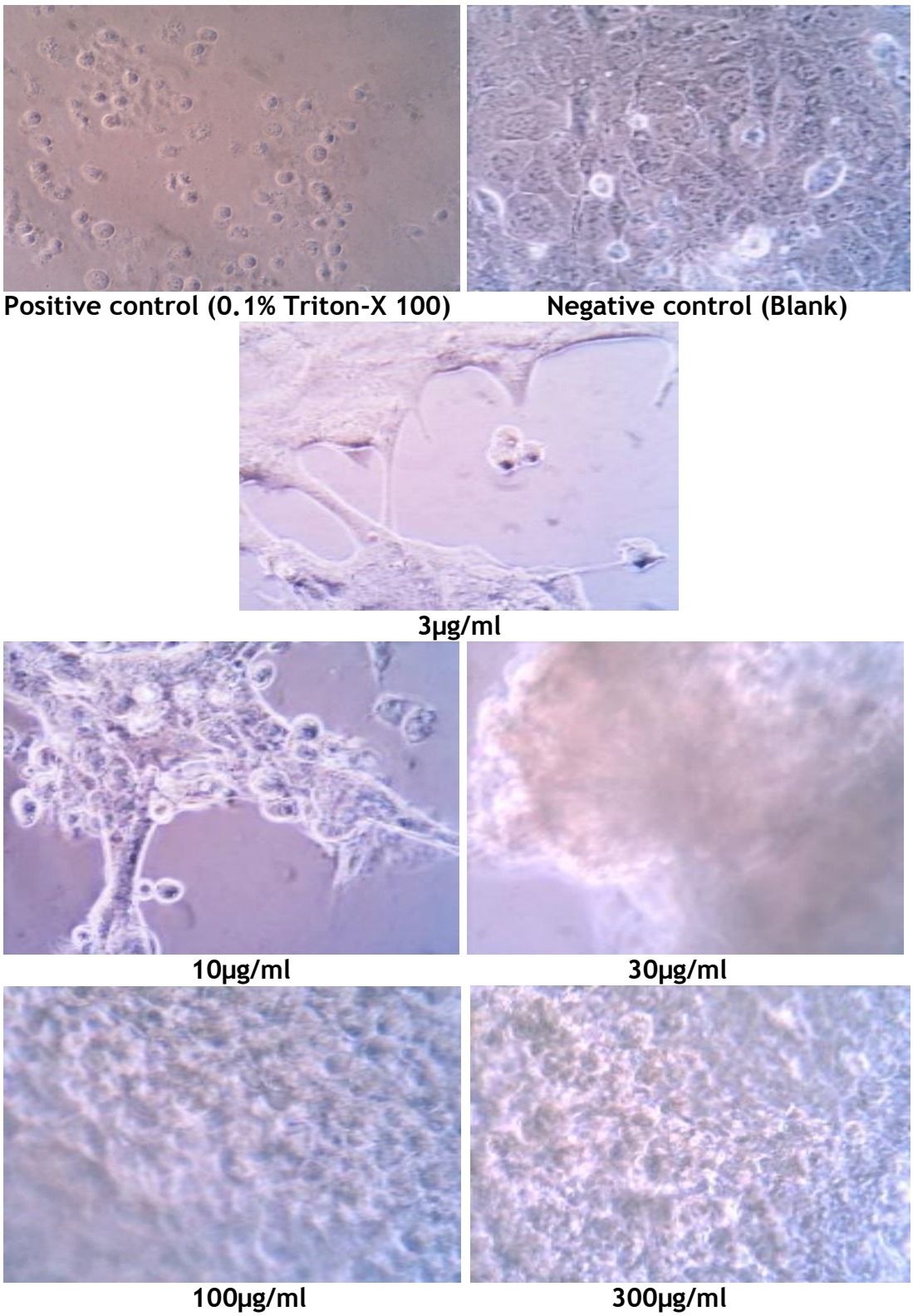



**Figure 2-13:** Light micrographs of DU145 cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. piscovorus conati* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

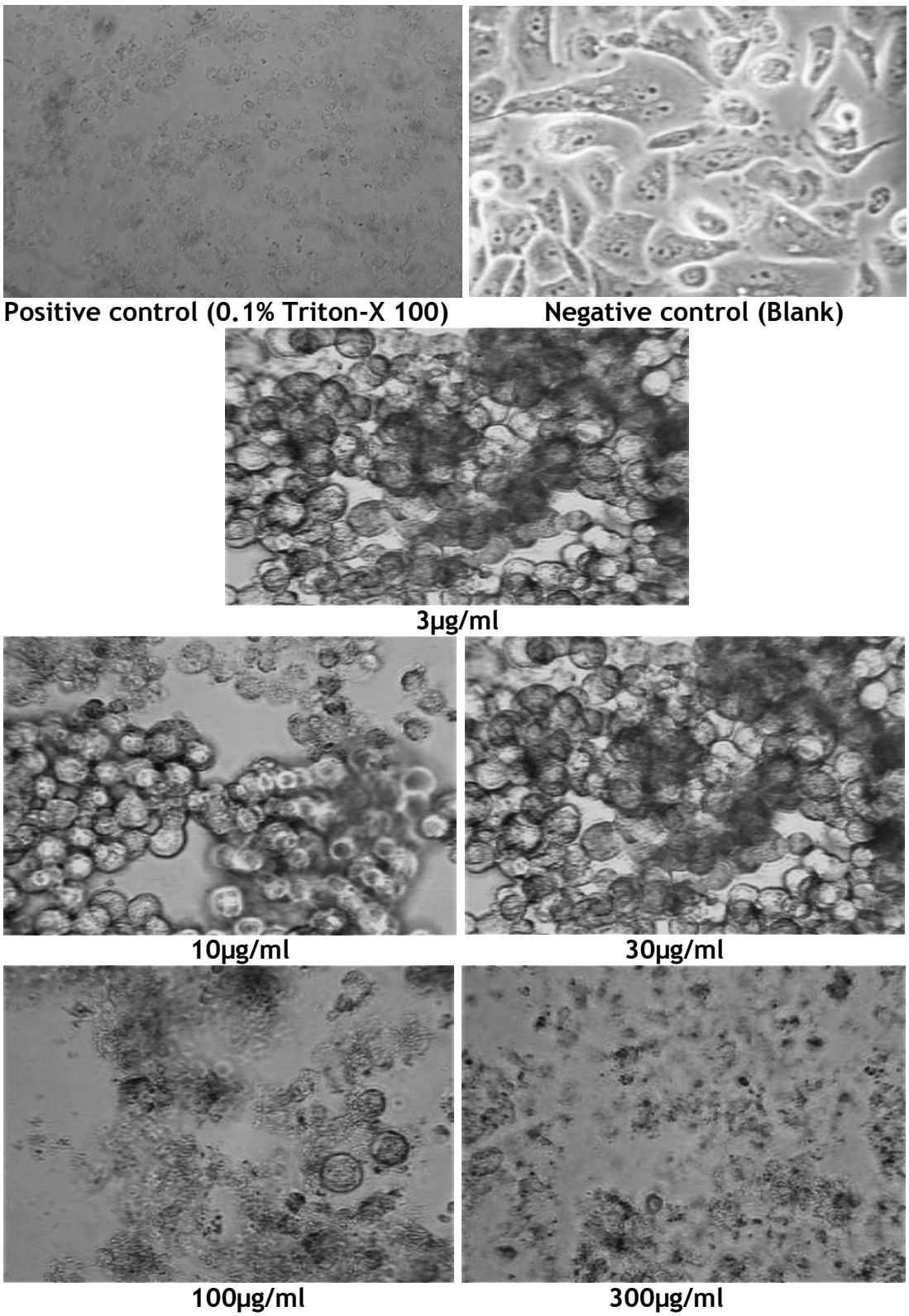



**Figure 2-14:** Light micrographs of PC3 cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. piscovorus conati* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

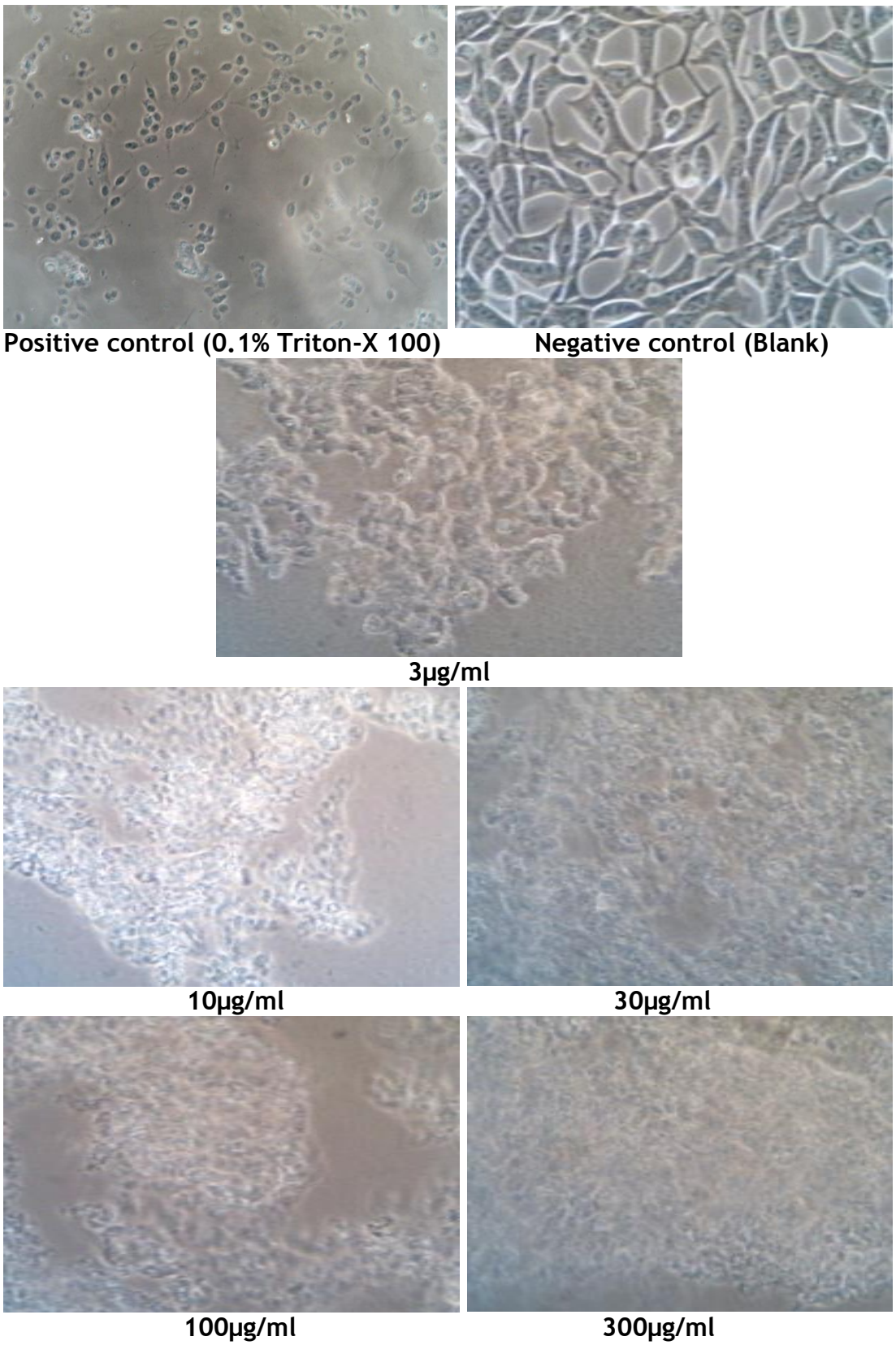
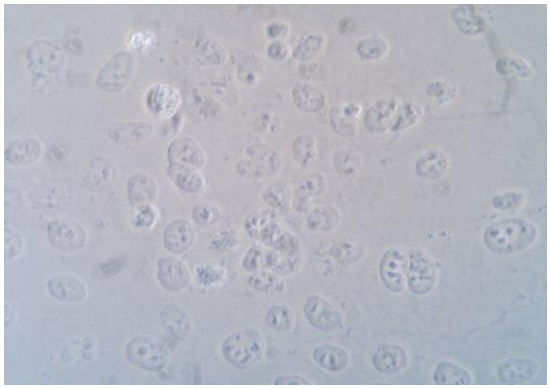
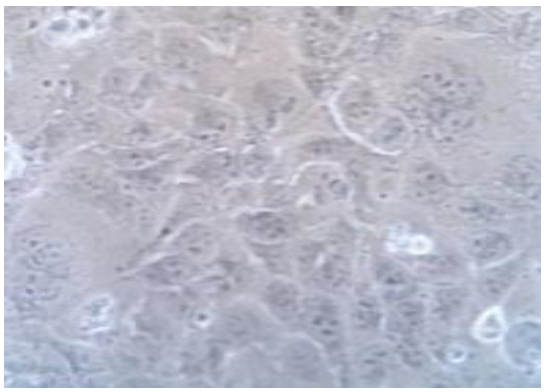


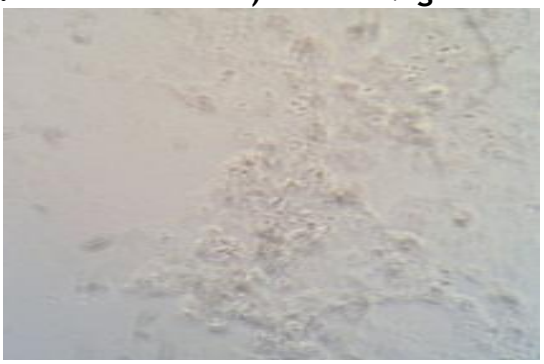

**Figure 2-15:** Light micrographs of LNCaP cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. piscovorus conati* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.



Positive control (0.1% Triton-X 100)



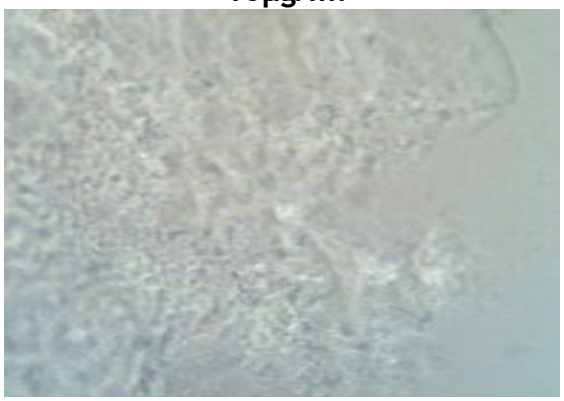
Negative control (Blank)



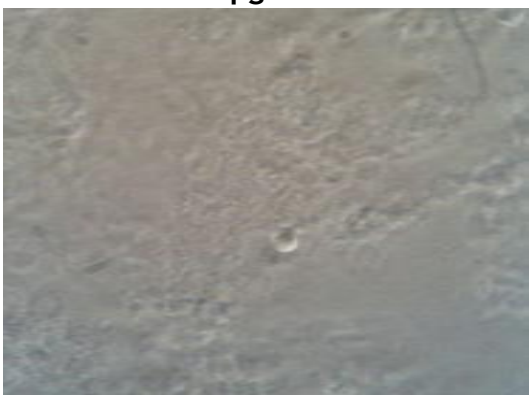
3µg/ml

10µg/ml

30µg/ml



100µg/ml



300µg/ml

**Figure 2-16:** Light micrographs of PNT2A cells following 24 hours exposure to 3, 10, 30, 100 and 300µg/ml of *A. piscovorus conati* snake venom in complete medium at 37$^{o}$C. The negative control shows a culture after 24 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### *2.3.4 Effect of Cisplatin on DU145, PC3, LNCaP, and PNT2A cell lines for 24 hours*

Cisplatin is one of the most frequently used drugs for chemotherapy of cancer that damages the cell membrane, the mitochondria, and the nucleus, not only of cancer cells, but also normal cells (Zhang, Piston et al. 2002).

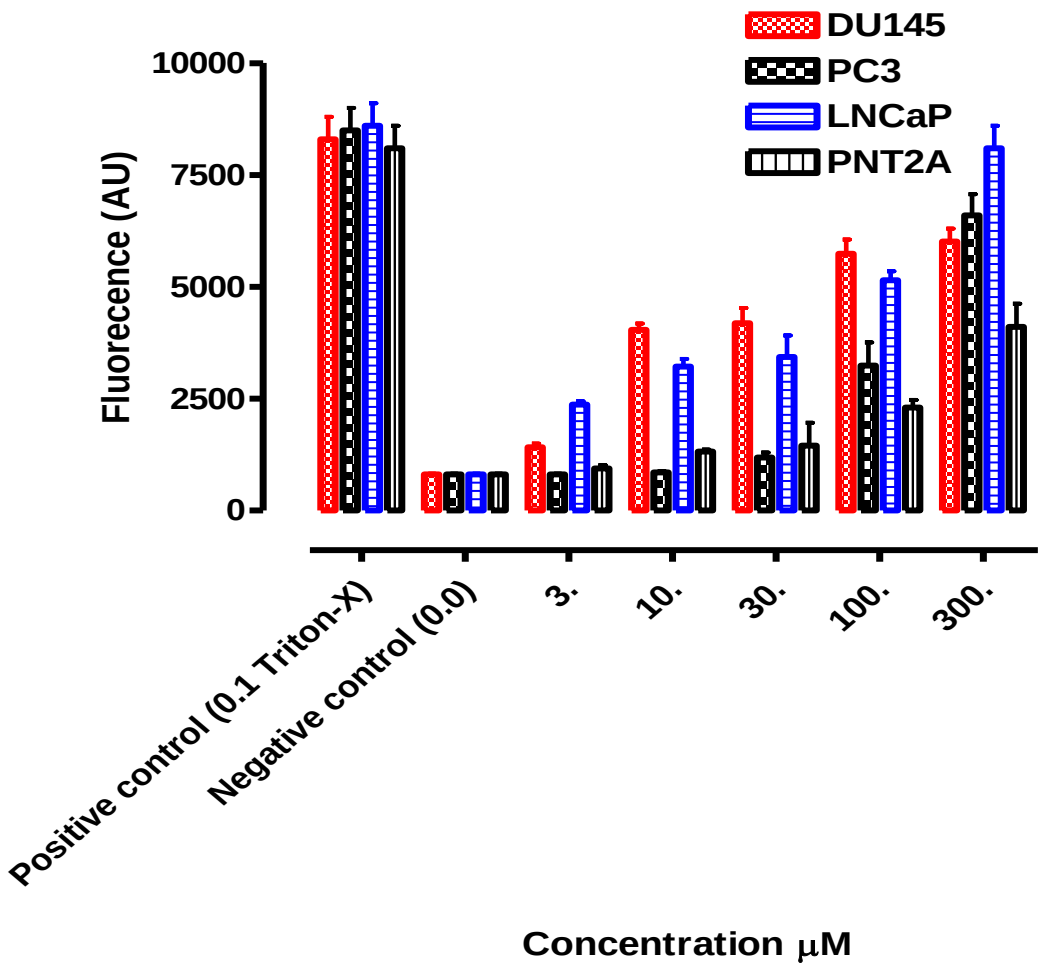



**Figure 2-17:** Changes in SYTOX green (0.5μM) intensity in cultured cell lines DU145, PC3, LNCaP and PNT2A following 24 hours exposure to 3, 10, 30, 100 and 300μM of cisplatin in complete media at 37$^{o}$C. The data is represented as means ± SEM of 3 independent experiments. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

The cytotoxic effects of cisplatin on DU145, PC3, LNCaP and PNT2A cell lines were interesting because this drug was effective in all cell lines (Figure 2-17). Increasing concentrations of cisplatin (3, 10, 30, 100 and 300μM) caused increases in SYTOX green fluorescence in the DU145 cell line. The cytotoxic effect also increased with the concentration of cisplatin in LNCaP cell line. The changes in SYTOX green fluorescence associated with PC3 cells were little bit more complex than with the DU145 and LNCaP cell lines because there were no changes observed in fluorescence at 10 and 30μM from an initial low level. The changes in fluorescence were found after 30-300μM with increasing fluorescence in PC3 cell line. The cytotoxic effects of cisplatin were found as increasing fluorescence ranging from 10-300μM concentration in PNT2A cell line while there was no effect of cisplatin at 3μM on fluorescence. Thus, overall the cisplatin showed clear cytotoxic effect against all cell lines.

#### 2.3.4.1 Morphological analysis

Morphological analysis was performed on DU145, PC3, LNCaP and PNT2A cells treated with cisplatin at 3, 10, 30, 100 and 300μM. Figures 2-18; 2-19; 2-20 and 2-21 illustrate the morphological that were observed in the cells after 24 hours of incubation. Morphological changes and several cell deaths occurred after 6 hours of incubation.

Most of the DU145 lost their characteristic appearance and coalesced to such an extent that individual cells could not be identified. Typical necrosis changes could be observed at 100 and 300μM cisplatin in almost all cell lines after incubation of 24 hours. However, formation of shrinks was evident after 24 hours of treatment with cisplatin at 30, 100 and 300μM in DU145, PC3 and LNCaP cells.

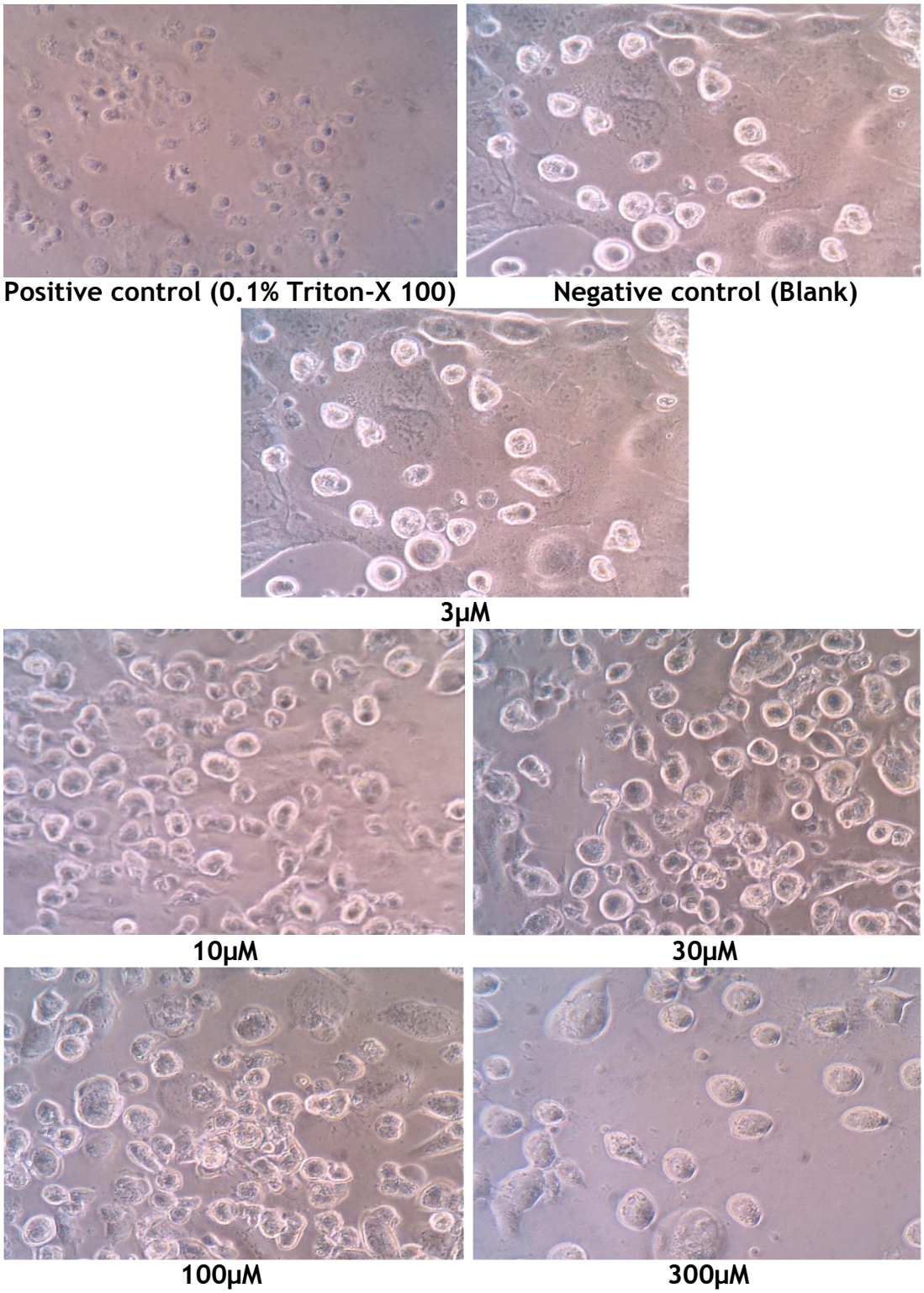



**Figure 2-18:** Light micrographs of DU145 cells following 24 hours exposure to 3, 10, 30, 100 and 300µM cisplatin in complete medium at 37$^{o}$C. The control shows a cultyre after 24 hours in complete medium but no venom. 400 X magnifications were used for micrographs using a phase-contrast microscope. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

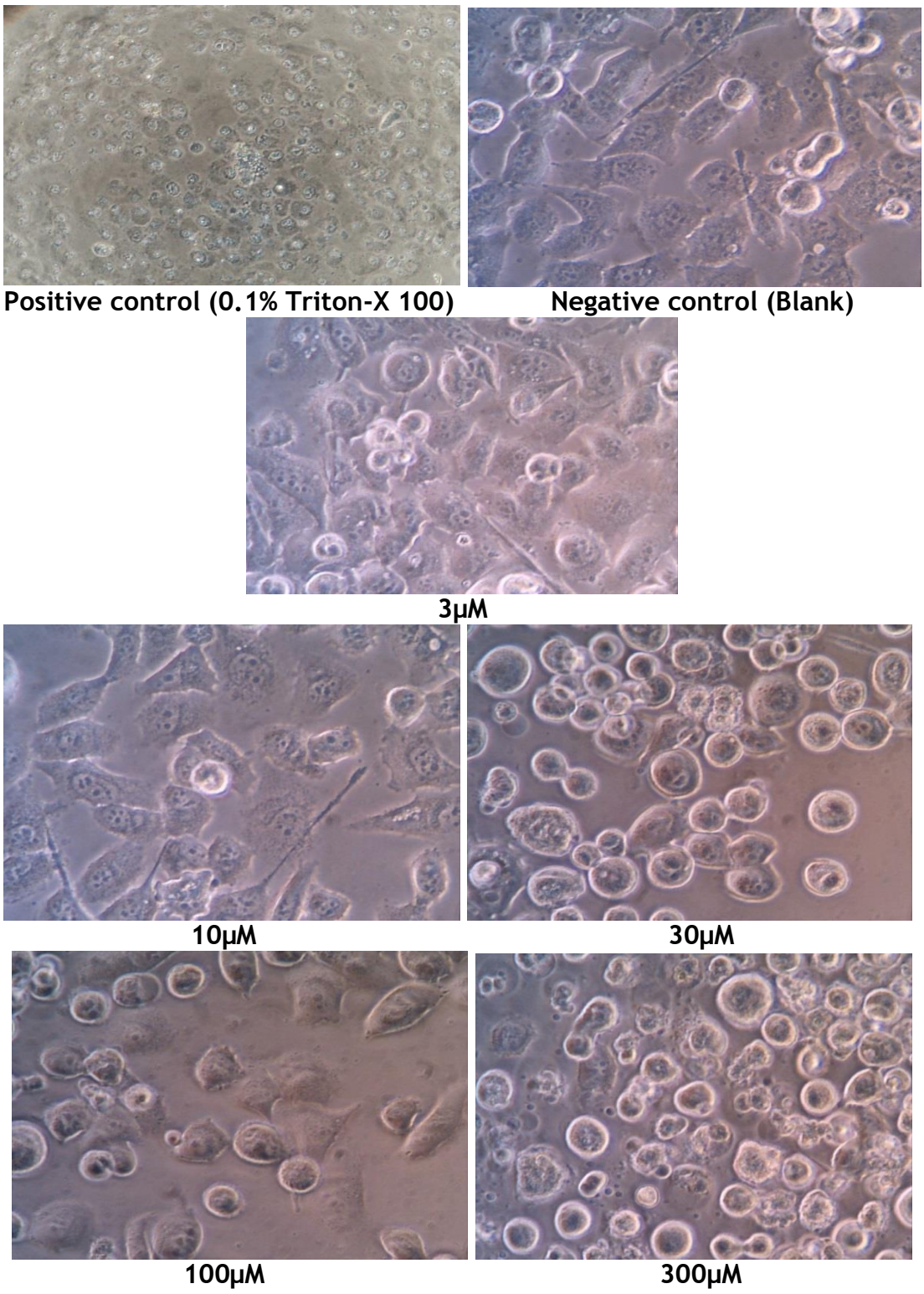



**Figure 2-19:** Light micrographs of PC3 cells following 24 hours exposure to 3, 10, 30, 100 and 300µM cisplatin in complete medium at 37$^{o}$C. The control shows a cultyre after 24 hours in complete medium but no venom. 400 X magnifications were used for micrographs using a phase-contrast microscope. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

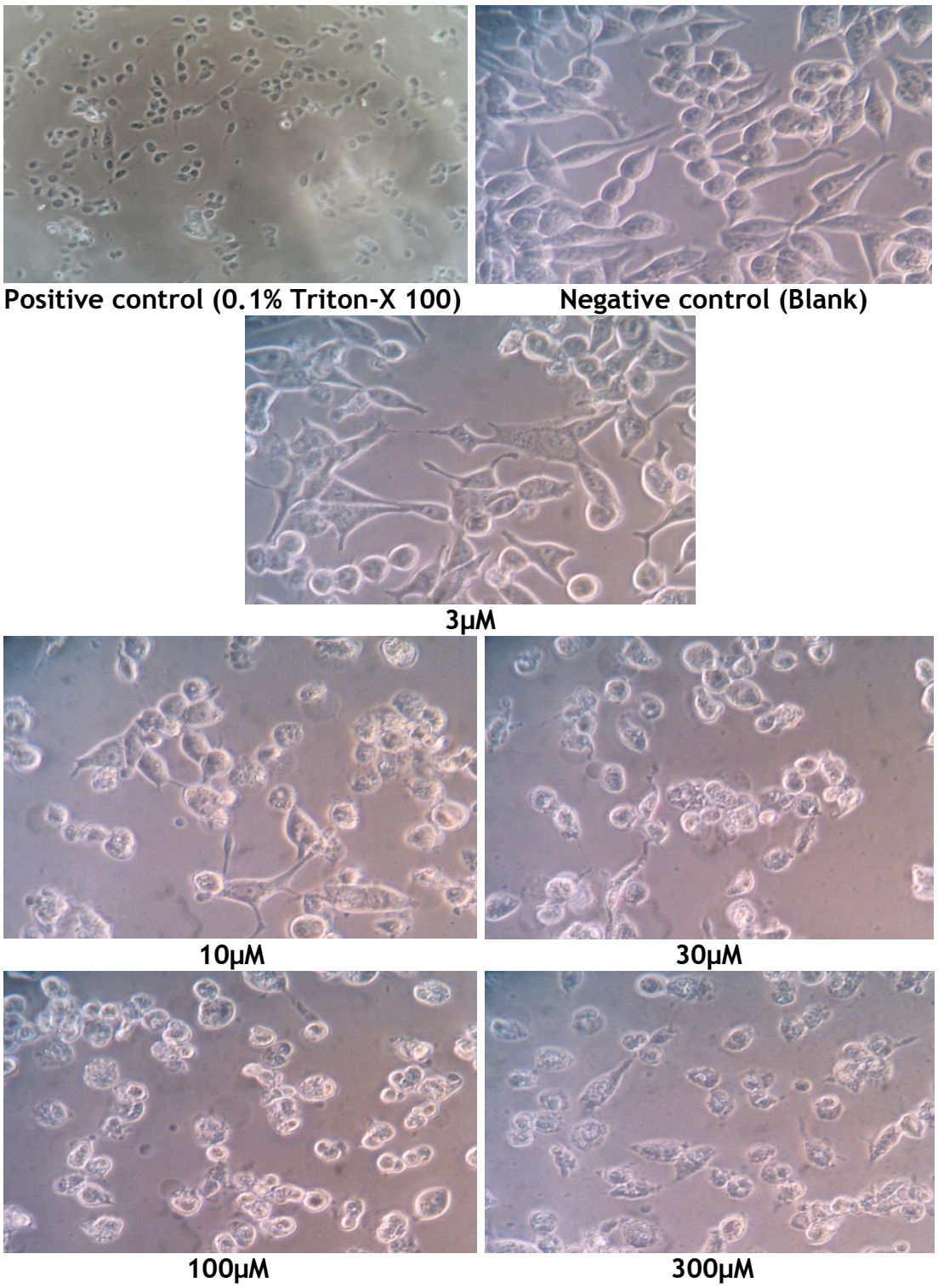



**Figure 2-20:** Light micrographs of LNCaP cells following 24 hours exposure to 3, 10, 30, 100 and 300µM cisplatin in complete medium at 37$^{o}$C. The control shows a cultyre after 24 hours in complete medium but no venom. 400 X magnifications were used for micrographs using a phase-contrast microscope. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

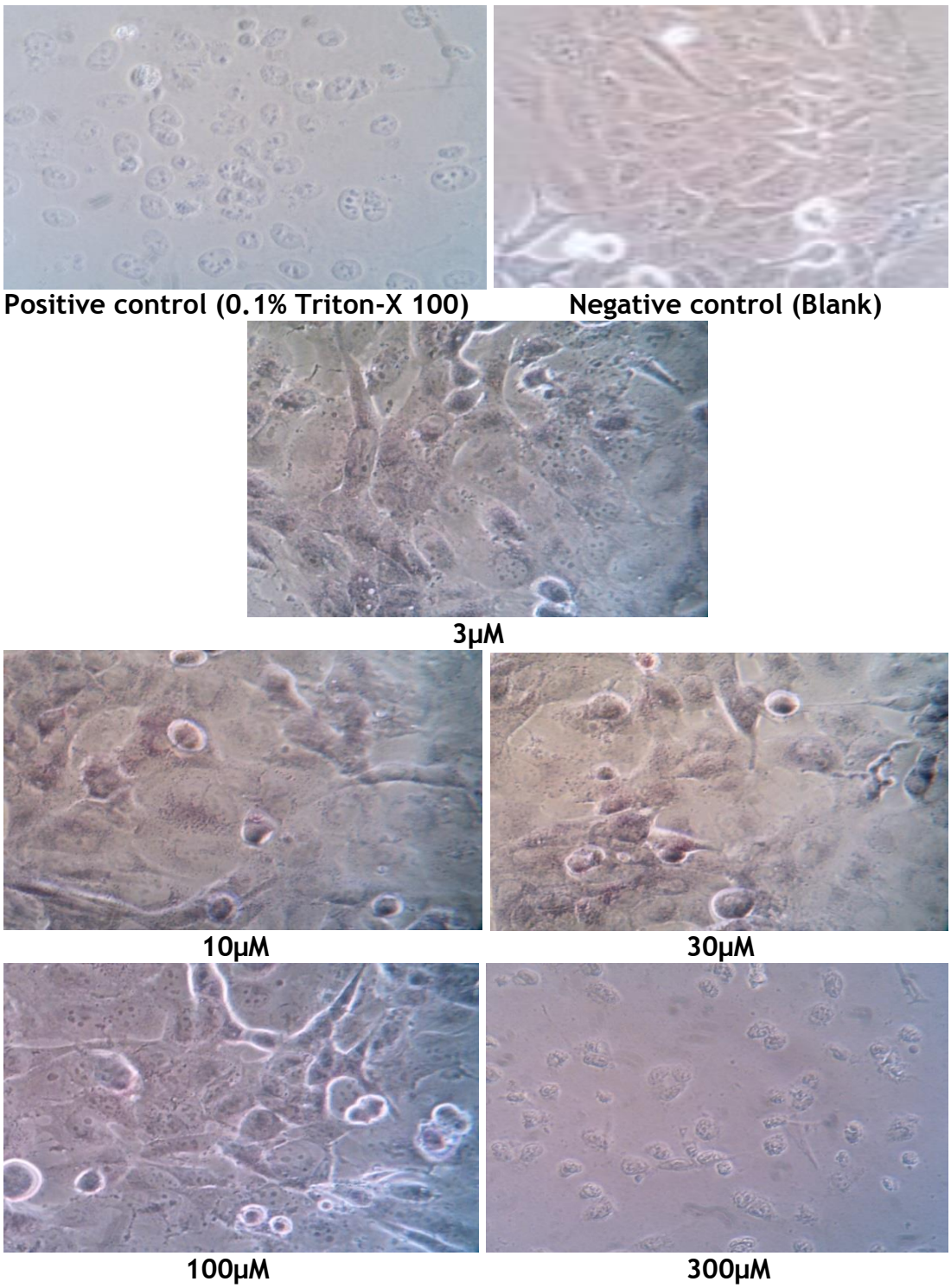



**Figure 2-21:** Light micrographs of PNT2A cells following 24 hours exposure to 3, 10, 30, 100 and 300µM cisplatin in complete medium at 37$^{o}$C. The control shows a cultyre after 24 hours in complete medium but no venom. 400 X magnifications were used for micrographs using a phase-contrast microscope. The positive control agent was 0.1% Triton-X 100 for 24 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

## 2.4 Discussion

Cell death is defined as an irreversible loss of plasma membrane integrity. Three types of cell death have been distinguished based on morphological criteria, namely apoptosis, autophagy and necrosis, in mammalian cells (Kroemer, El-Deiry et al. 2005). Apoptosis represents a major regulatory mechanism that eliminates abundant and unwanted cells during embryonic development, growth, differentiation and normal cell turnover (Golstein and Kroemer 2007). Targeting apoptosis is thought to be a potential therapeutic approach for specific and selective cancer treatment. In this study, three venoms from different snakes, *Naja pallida, Agkistrodon contortrix laticinctus*, and *Agkistrodon piscivorus conanti*, were studied for their ability to induce cell death for potential control of the cancer growth.

SYTOX Green was used as an indicator of cell death after treating four different cell lines (DU145, PC3, LNCaP and PNT2A) with different concentrations (3, 10, 30, 100 and 300μg/ml) of the three snake venoms. It was noted that all the venoms were cytotoxic against the selected cell lines in the SYTOX Green assay, and it was confirmed by light micrography with changes in cell morphology.

The cell images showed the clear morphological changes after exposure of various concentrations of snake venom on prostate cancer cell lines resulting in a cytotoxic effect and cell death. Morphological observations of cells treated for 24 hours were recorded with fluorescence cytotoxic SYTOX Green assay. *N. pallida* venom induced dead cell morphology (swelling and apoptotic shrinkage) in DU145, PC3, LNCaP and PNT2A cell lines as compared to control, while *A. contortrix laticinctus* venom caused cell rounding, clumping and losses of adhesion and completely decomposed on DU145, PC3, LNCaP and PNT2A cell line as compared to control. In the case of *A. piscivorus conanti* the results showed cells sphericity, grouped and fully decomposed at high concentrations on DU145, PC3, LNCaP and PNT2A cell lines as compared to control. There were no changes observed in control cultures and the cells were homogeneously distributed, exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents. Thus, it was demonstrated that all venoms are highly cytotoxic against different prostate cancer cell lines at various concentrations.

Some snake venoms contain toxins that are reported to induce cell death in vivo (Rahmy and Hemmaid 2000; Schaffitzel, Berger et al. 2001; Mukherjee and Maity 2002; Calderon et al. 2014) and selectively damage certain types of cells, such as muscles (Zhang and Gopalakrishnakone 1999; Rahmy and Hemmaid 2000; Chaisakul et al. 2016).

Many researchers have shown the differences in the composition of venom from taxonomically distinct snake species (Tu 1977). On the other hand, a large number of different snake venoms have also been reported to possess homologous toxins (Weinstein, Minton et al. 1985; Bober, Glenn et al. 1988). Many of these homologous toxins induce tissue damage. It is possible that the local tissue damage caused by crotaline snake venoms may be due to only a few types of chemically related toxins. Therefore, the knowledge about the action mechanism of certain toxins from one or two species may be useful in understanding similar mechanism of a wide variety of snake venom toxins with similar biological characteristics.

Cell death is currently the subject of considerable research. This interest stems, in part, from the potential of understanding and exploiting cell death programs for therapeutic purposes. Cell death has been considered to be a degenerative phenomenon (Lieberman, Baney et al. 1971). Researchers suggested that, under certain conditions, cell death might not be a passive event, but rather the result of an active process, as the overproduction of "cell suicide" instead of "cell homicide" enzymes or proteins (Farber 1982)

This investigation is the first detailed study of three snake venoms (*N. pallida, A. contortrix laticinctus,* and *A. piscivorus conanti*) on the prostate cancer cell lines DU145, PC3, LNCaP and the prostate epithelial cell line PNT2A. Results revealed that snake venom was highly effective in causing cell damage and cell death in prostate-derived cell lines. The cell death induced by *N. pallida, A. contortrix laticinctus,* and *A. piscivorus conanti* may be due a combination of apoptosis and necrosis within the same cell lines. It can be suggested that cell injury caused by the cytotoxic components of cobra venom activate a death program that leads to irreversible damage and necrotic effects on prostate cancer cell lines. This form of cell death was recognized by Schlemper *et al.,* (2000) in mesangial cells treated with anti-Thy-1.1 antibody (Schlemper, Riddell et al. 2000).

Overall, this study clearly highlights that the SYTOX Green assay confirms the cellular toxicity induced by the venom, and correlates reasonably well with the severity of cell death and membrane damage observed. Mostly, these effects were time and concentration dependent in all prostate cancer cell lines. However, in order to accurately assess relative quantification of target venom proteins, an endogenous reference protein must be identified that is suitable for cell death. Cell death induced by these venoms appears to be only partially associated with an apoptotic mechanism. To understand the complete apoptotic mechanism further in vitro biochemical analysis of lactate dehydrogenase (LDH) release was carried out as is discussed in chapter-3. In vitro release of LDH from cells provides an accurate measure of cell membrane integrity and cell death.

## 2.5 Conclusion

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. There is variety of cancer diseases, and millions of people die due to cancer throughout the world. Out of them prostate cancer is the second most common type of cancer among men after skin cancer. More than 2 million men are diagnosed with prostate cancer per year in the US. Around 27,970 men die from prostate cancer in the US each year. Prostate cancer is generally treatable with excellent survival rates, which is good news but if the prostate cancer goes undetected it, could turn into bad news quickly if it spreads.

Since cancer is one of the leading causes death worldwide and there is an urgent need to find better treatment. In recent years remarkable progress has been made towards the understanding of proposed hallmarks of cancer development and treatment. Anticancer drug developments from natural resources are ventured throughout the world. Venoms of several animal species including snake, scorpion, frog, spider etc. and their active components in the form of peptides, enzymes etc. have shown possible therapeutic potential against cancer (Calderon et al. 2014; Chisakul et al. 2016).

The search for alternative economical and natural sources (snake venoms) for cancer medicines are needed for the future in combating this dreadful disease

(prostate cancer). In the present work, three very toxic snake venoms were tested for promising effects against prostate cancer cell lines. The whole venoms in this in vitro cytotoxicity study show effects against prostate cancer cell lines but it is not clear which venom component is responsible for the cytotoxicity. Further inquiries about the effectiveness of snake venoms on cancer cell lines requires the study of shorter exposure times to get to a clearer picture of the activities of these venoms.

# CHAPTER THREE:

## TOXICITY AND MORPHOLOGICAL CHANGES INDUCED BY THREE SNAKE VENOMS ON PROSTATE CANCER CELL LINES AFTER SHORT EXPOSURE TIMES

# 3. CHAPTER THREE

## 3.1 Introduction

In continuation of the previous findings in chapter 2, confirmational studies were carried out in this chapter using a biochemical marker (LDH) of cell damage to understand the morphological changes and effectiveness of short exposures to different snake venoms on selected prostate cancer cells line. This chapter presents more details about the effects of *Naja pallida, Agkistrodon contortrix laticinctus* and *Agkistrodon piscivorus conanti* venoms on DU145, LNCaP, PC3, and PNT2A cell lines in order to gain a better understanding of their cytotoxic, apoptotic and necrotic effects. The assay of LDH release helps to identify the apoptotic and necrotic cells after a short time of exposure.

### *3.1.1 Cell damage*

The major biological activities of $NAD^+$ and NADH can be classified into several categories: First, $NAD^+$ and NADH act as coenzymes in numerous redox reactions. However, $NAD^+$ and NADH have been long known as key co-factors in numerous dehydrogenase-mediated reactions. $NAD^+$ has been identified as the substrate for poly-ADP-ribose polymerases (PARPs), which catalyze regulatory post-translational modification of proteins, particularly those that influence chromatin structure, function as transcriptional regulators, and participate in DNA repair (Miwa and Masutani 2007; Kraus 2008).

### *3.1.2 In vitro LDH activity measurement*

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in all mammalian cells. The plasma membrane is impermeable to LDH; hence the loss of membrane's integrity can be detected by the release of LDH into the supernatant (Everse and Kaplan 1973), where its enzymatic activity can be measured. In vitro release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability irrespective of the type of cell death. LDH activity and LDH leakage are routinely used to measure the integrity of cells (Tu 1977; Chaimmatyas and Ovadia 1987; Braganca, Patel et al. 1967; Tang, Yang et al. 2004; da Silva, da Silva et al. 2002).

## 3.2 Materials and Methods

### *3.2.1 Reagents and equipment*

The same three venoms (*Naja pallida, Agkistrodon contortrix laticinctus* and *Agkistrodon piscivorus conanti*) were used for the study of LDH release in this chapter. The Thermo Scientific™ Pierce™ LDH Cytotoxicity Assay Kit was purchased from Pierce Biotechnology PO Box 117, Meridian Road Rockford, lL 61105 USA and it was used to quantify cellular cytotoxicity by a colorimetric method.

### *3.2.2 Cell culture*

The prostate cell lines DU145, PC3, LNCaP and PNT2A were used for the biochemical analysis of LDH assay against different snake venoms. The details of the culture of prostate cell lines have been explained in Chapter 2.

### *3.2.3 Lactate Dehydrogenase (LDH) assay*

LDH is a stable cytosolic enzyme which is present in a wide variety of organisms, including plants and animals. LDH is released when the cell membrane is damaged. Many cancerous cells have raised LDH levels, so LDH may be used as a tumour marker. Released LDH in culture supernatants can convert lactate to pyruvate with concomitant interconversion of NADH and $NAD^+$. Then, NADH can react with a tetrazolium salt (INT) to form a red formazan product (Figure 3-1). The absorbance of samples is measured at 490 nm by a standard 96-well plate reader (Weidmann, Brieger et al. 1995).

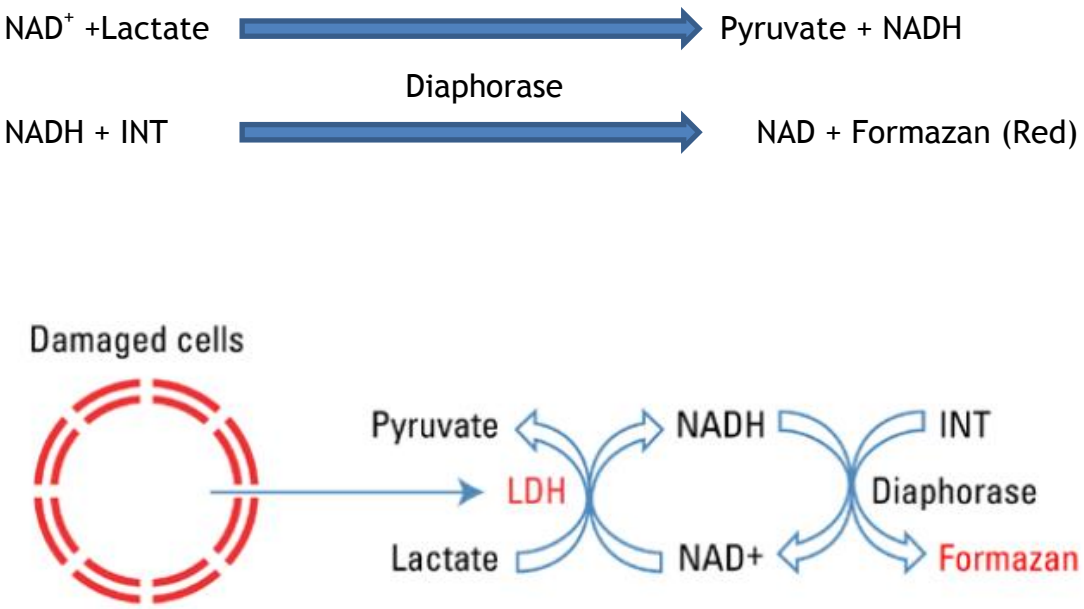



**Figure 3-1:** The chemical reaction of the LDH assay

Thus, LDH release is measured indirectly as the rate of change in absorbance at 490nm, which actually reflects the pyruvate-dependent decrease in concentration of NADH but is attributed to the activity of LDH present in the sample. NADH concentrations increase as LDH catalyzes the reaction of lactate to pyruvate with conversion of one $NAD^+$ to NADH (Roberts, Davis et al. 2001).

LDH is involved in tumour initiation and metabolism. Cancer cells rely on increased glycolysis resulting in increased lactate production instead of aerobic respiration in the mitochondria, even under oxygen-sufficient conditions (a process known as the (Warburg, 1956). This state of fermentative glycolysis is catalyzed by the A form of LDH. This mechanism allows tumorous cells to convert the majority of their glucose stores into lactate regardless of oxygen availability, shifting use of glucose metabolites from simple energy production to the promotion of accelerated cell growth and replication. For this reason, LDH A and the possibility of inhibiting its activity has been identified as a promising target in cancer treatments focused on preventing carcinogenic cells from proliferating (Figure 3-2).

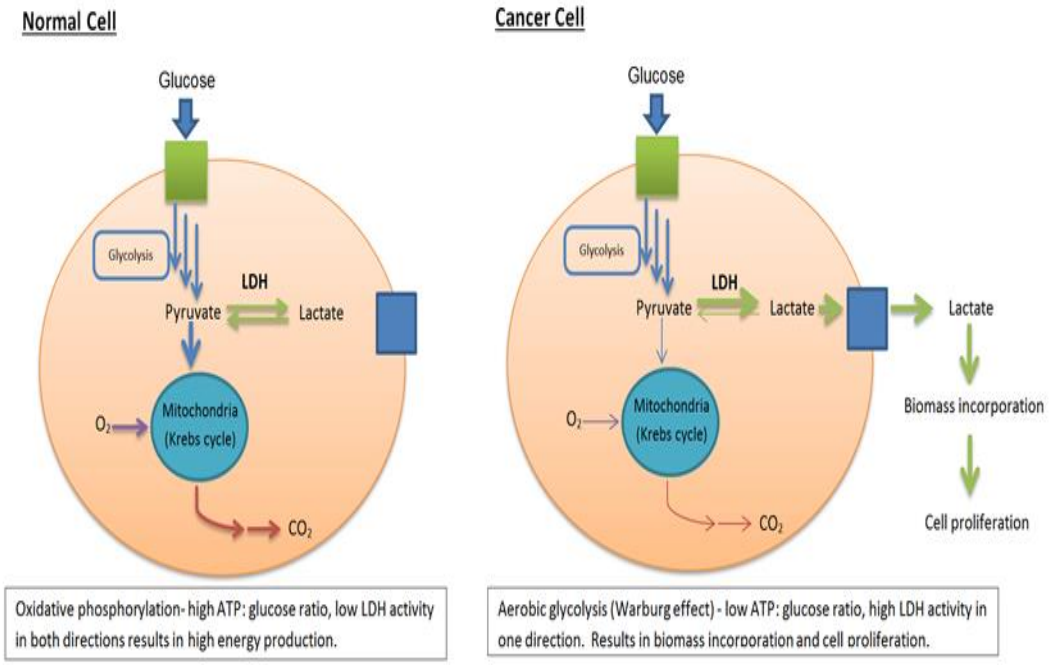
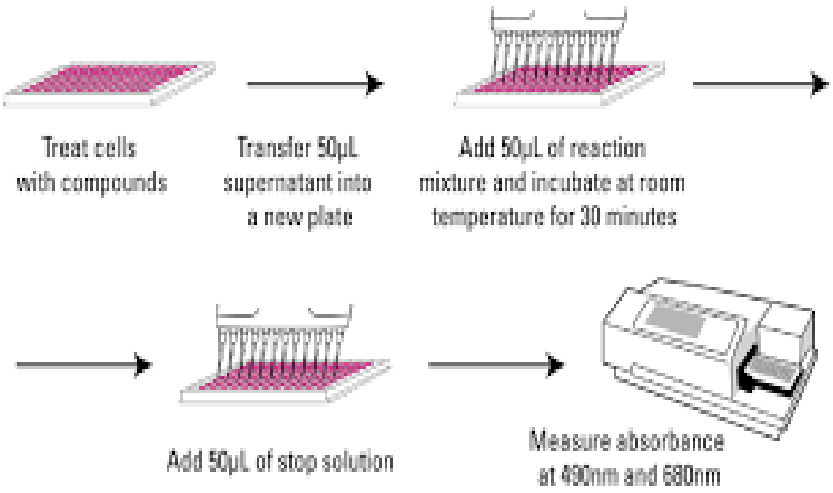



**Figure 3-2:** Comparison of LDH activity in normal and cancerous cells (Warburg, 1956).

### *3.2.4 LDH activity determination in cell lines*

All prostate cancer cell lines (DU145, PC3 and LNCaP) and the normal cell line PNT2A were seeded onto 96-well plates at around 10,000 - 40,000 cells per well and sub confluent cells were subsequently treated with each snake venom (0.1, 0.5, 1.0, 6.0 and 10.0µg/ml (*Naja pallida*, *Agkistrodon contortrix laticinctus* and *Agkistrodon piscivorus conanti*) for 2, 4 and 6 hours. After the incubation period, 15µl was taken from the medium on to a microplate well (flat bottom half well area), then 4µl INT was added to each well and the plate was covered with aluminium foil or placed in a box with an opaque material to protect from light. Plates were incubated at room temperature for 20-30 mins before being read at a wavelength of 490 nm on a plate reader (Multiskan biochomatic automatic micro plate reader). The mean absorbance/optical density for each treatment group were calculated. The % LDH release in cell line was also calculated based on the following formulae:

LDH release = (LDH activity in media) / (LDH activity in media + LDH activity in total cells) X 100% (Decker, and Lohmann-Matthes 1988).




Damage and morphological changes in the cell lines were investigated by observation with a Nikon TMS inverted phase contrast microscope and photos were taken at each evaluation time. Digital images of cells were acquired via a microscope mounted digital camera (Modicum 1000; Motic Germany), visualised using Motic Images Plus 2.0 and images stored on a laptop computer running Microsoft Windows XP.

### *3.2.5 Statistical analysis*

The data were plotted and statistical analysis was carried out using Graph Pad Prism version 4.00 for Windows, (GraphPad Software, San Diego, California, USA). Data are presented as mean ± SD and where the data was statistical significance it was represented by ($p < 0.05$).

## 3.3 Results

### *3.3.1* Effect of 2, 4 and 6 hours exposure to *Naja pallida* venom on DU145, PC3, LNCaP and PNT2A cell lines by LDH Assay

The in vitro release of LDH provides an accurate measure of cell membrane integrity and cell viability. The amounts of LDH released after exposure of cells to snake venom were concentration and time-related. This effect was significant when compared to the control value after 2, 4, and 6 hrs interval. Figure 3-3 shows the % LDH release of different concentrations (0.1, 0.5, 1.0, 6.0 and 10.0µg/ml) of *N. pallida* venom against DU145, PC3, LNCaP, and PNT2A cell lines after 2, 4 and 6 hours exposures. The % LDH release in DU145 cells was from 49-76% with *N. pallida* with different concentration (0.1, 0.5, 1.0, 6.0 and 10.0µg/ml) after 2, 4 and 6 hours. Thus clear detection of venom toxicity on the DU145 cell was found.

When the same concentrations (0.1, 0.5, 1.0, 6.0 and 10.0µg/ml) of *N. pallida* venom were applied to prostate cancer cell lines PC3 and LNCaP and to a normal cell line PNT2A, the % LDH release was 91-100%, 13-51% and 19-90 % (Figure 3-3). The effects were particularly marked in PC3 cells. With *N. pallida* venom, there appeared to be a delayed cytotoxicity. The % LDH release in control cultures without venom exposure was less than 5% at 2hr, 4hr and 6hr interval in all cell lines.

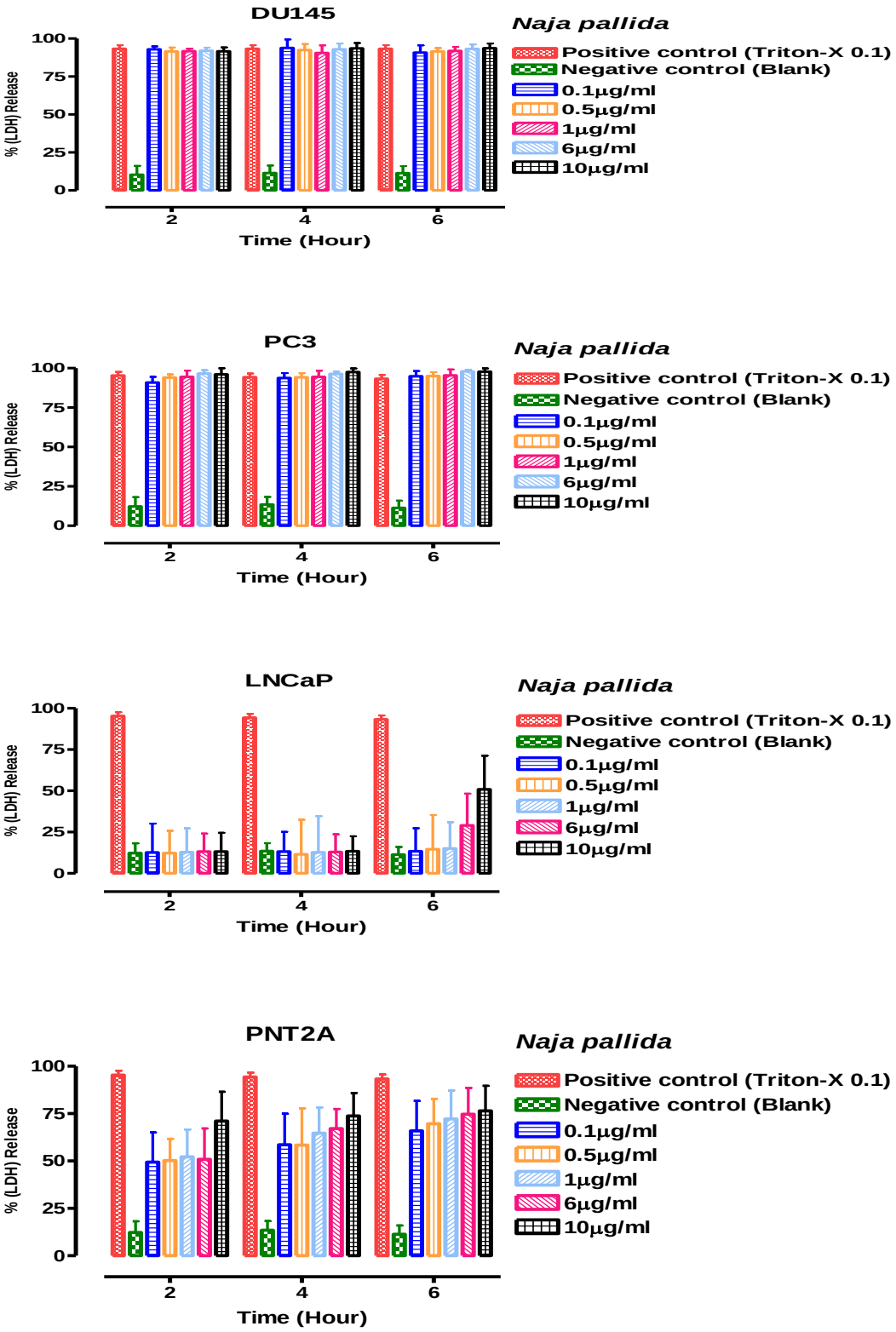



**Figure 3-3**: Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP, and PNT2A following 2, 4, and 6 hours exposure to 0.1, 0.5, 1, 6 and 10µg/ml of *Naja pallida* snake venom in complete media at 37$^{o}$C. The positive control agent was (0.1% Triton-X 100) for 2, 4 and 6 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time periods in complete medium.

#### 3.3.1.1 Morphological analysis

Untreated DU145, PC3, LNCaP and PNT2A cell lines were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 3-4, 3-5; 3-6 and 3-7). However, after incubating the cell lines with cobra venom for 2, 4 and 6 hours, the occurrence of various morphological abnormalities was observed.

At the lowest concentrations (0.1 and 0.5µg/ml), most of the cells, for example, DU145 and PC3, lost their characteristic appearance and coalesced to such an extent, that individual cells could not be identified. These cells showed a syncytium-like appearance and an increase in the density of their cellular contents. Only a few cells preserved their normal structure. In addition, some cellular swelling was observed, and areas devoid of any cultured cell appeared in-between the coalesced cells (Figures 3-4 and 3-5). The changes were even more marked in LNCaP cells (Figure 3-6).

The incubation with 1, 6 and 10µg/ml of *N. pallida* venom resulted in increased cell swelling and larger areas devoid of cells. In addition, other cells showed obvious deterioration and deformation, while some cells coalesced and lost their common structure (Figure 3-4 for DU145 cells). At the highest venom concentration tested (10µg/ml), the disappearance of normal morphological characteristics was very obvious (Figures 3-4 and 3-5) with cells showing severe shrinkage and condensation of their cellular contents on DU145 and PC3.

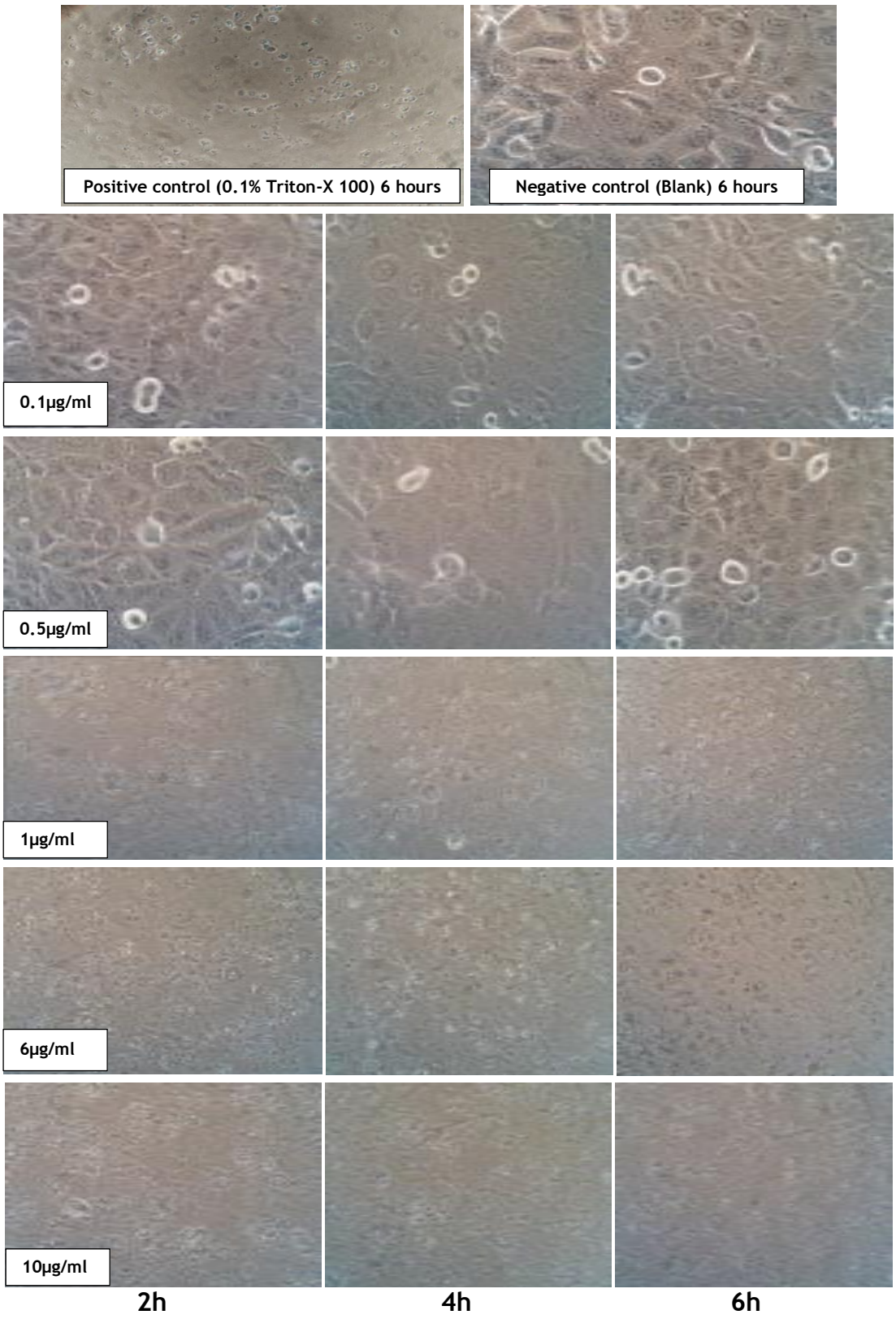



**Figure 3-4:** Light micrographs of cultured cell line DU145 following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *Naja pallida* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was (0.1% Triton-X 100) for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

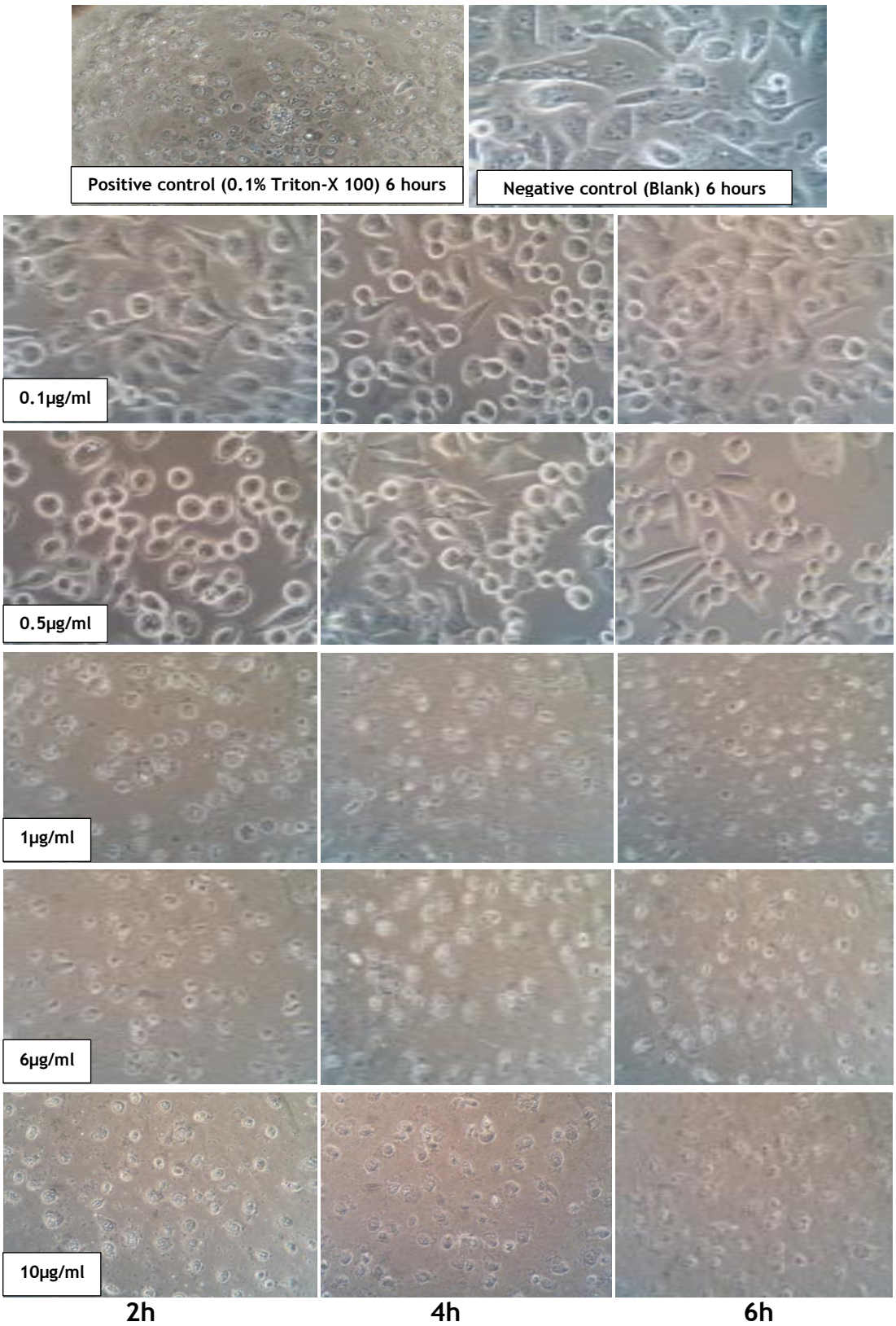



**Figure 3-5:** Light micrographs of cultured cell line PC3 following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *Naja pallida* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was (0.1% Triton-X 100) for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

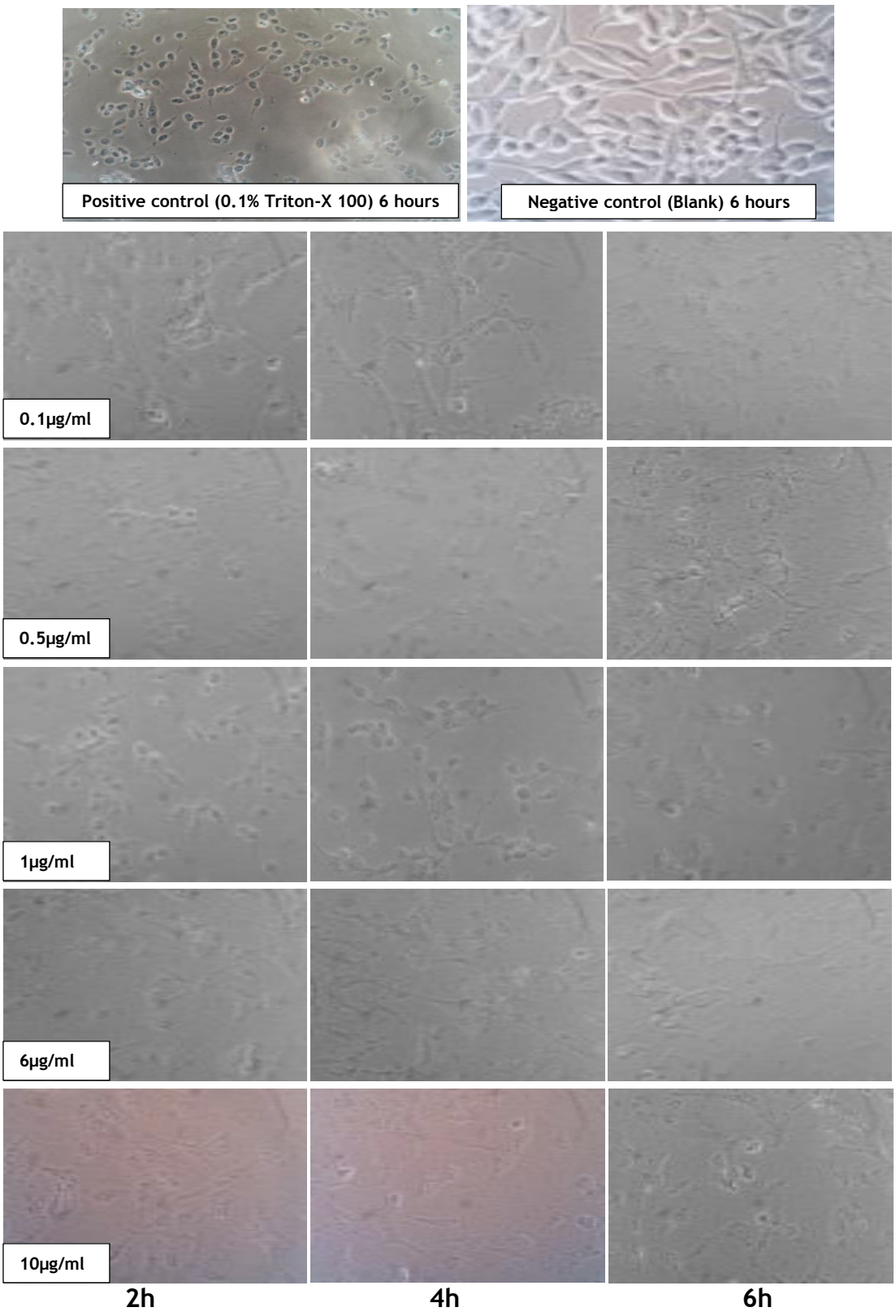



**Figure 3-6:** Light micrographs of cultured cell line LNCaP following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *Naja pallida* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was (0.1% Triton-X 100) for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

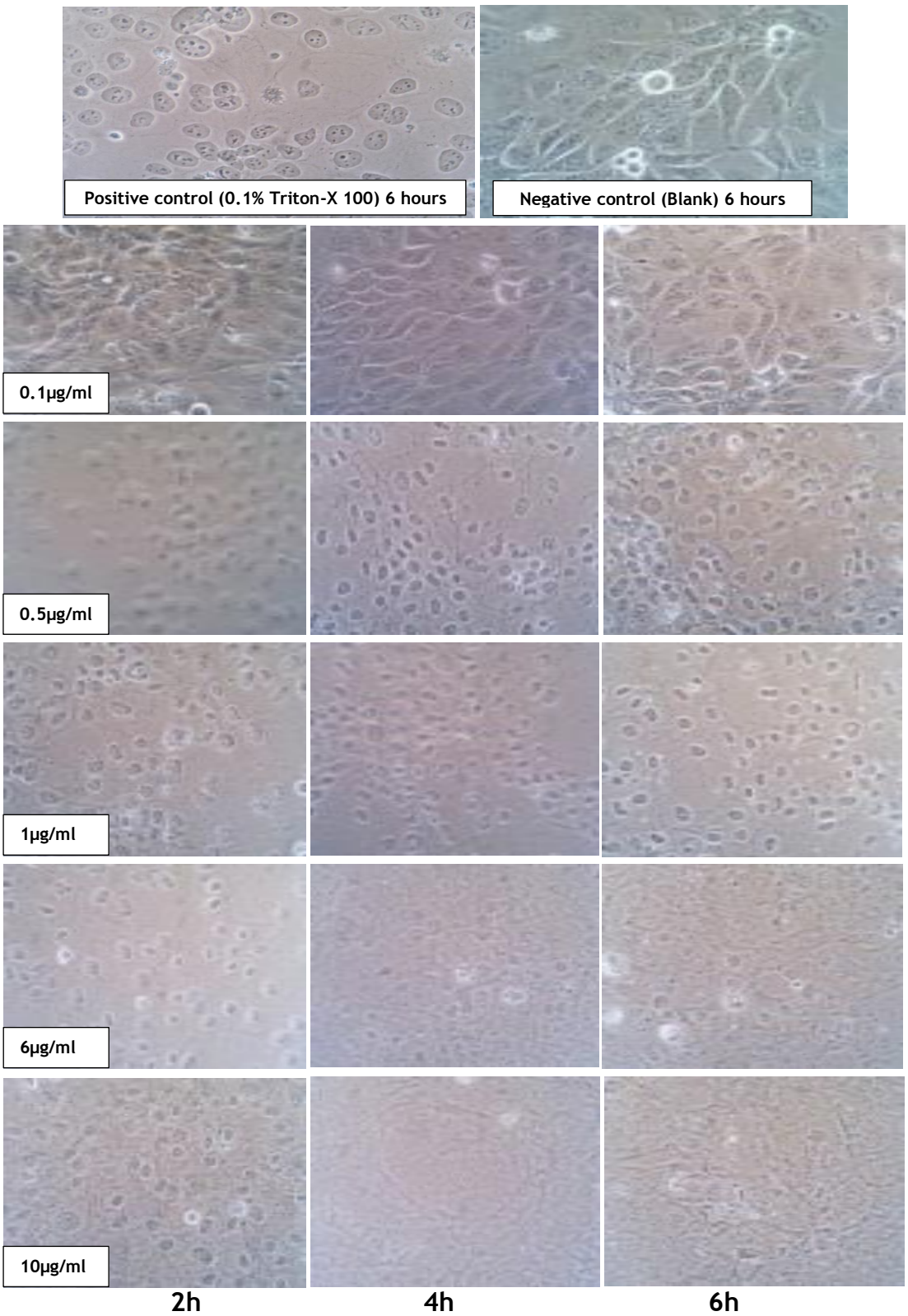



**Figure 3-7:** Light micrographs of cultured cell line PNT2A following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *Naja pallida* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was (0.1% Triton-X 100) for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### 3.3.2 Effect of *Agkistrodon contortrix laticinctus* venom on DU145, PC3, LNCaP and PNT2A cell lines for 2, 4 and 6 hours by LDH Assay

LDH assay was performed to investigate the cytotoxic effect on DU145, PC3, LNCaP and PNT2A cells treated with 0.1, 0.5, 1, 6 and 10 µg/ml of *A. contortrix laticinctus* venom for 2, 4 and 6 hours. As shown in Figure 3-8, there was a clear concentration-dependent increase in LDH released in DU145 cells and a concentration-dependent increase in LDH absorbance in PC3, LNCaP and PNT2A cells.

Figure 3-8 also shows that the amount of LDH released from the cell lines generally increased with time of incubation with *A. contortrix laticinctus* venom. This effect was particularly clear with 0.1, 0.5 and 1µg/ml on DU145 and PNT2A cells.

PC3 cells appeared to be the most sensitive to the *A. contortrix laticinctus* venom. The % LDH release in 0.1µg/ml was 43, 74 and 76 % after 2, 4 and 6 hrs incubation. The % LDH release was also high in PC3 cells at the high concentration 10µg/ml: it was 87, 94 and 100 after 2, 4 and 6 hrs. Similar effects were observed with DU145 and LNCaP cells. In the cell line PNT2A, the % LDH release was smaller as compared to other cell lines. The % LDH release was 10-37, 13-57 and 17-96% against concentrations 0.1, 0.5, 1, 6 and 10µg/ml of *A. contortrix laticinctus* venom after 2hrs, 4hrs and 6hrs (Figure 3-8). The % LDH release in control groups was less than 5% at 2hr, 4hr and 6hr interval in all cell lines.

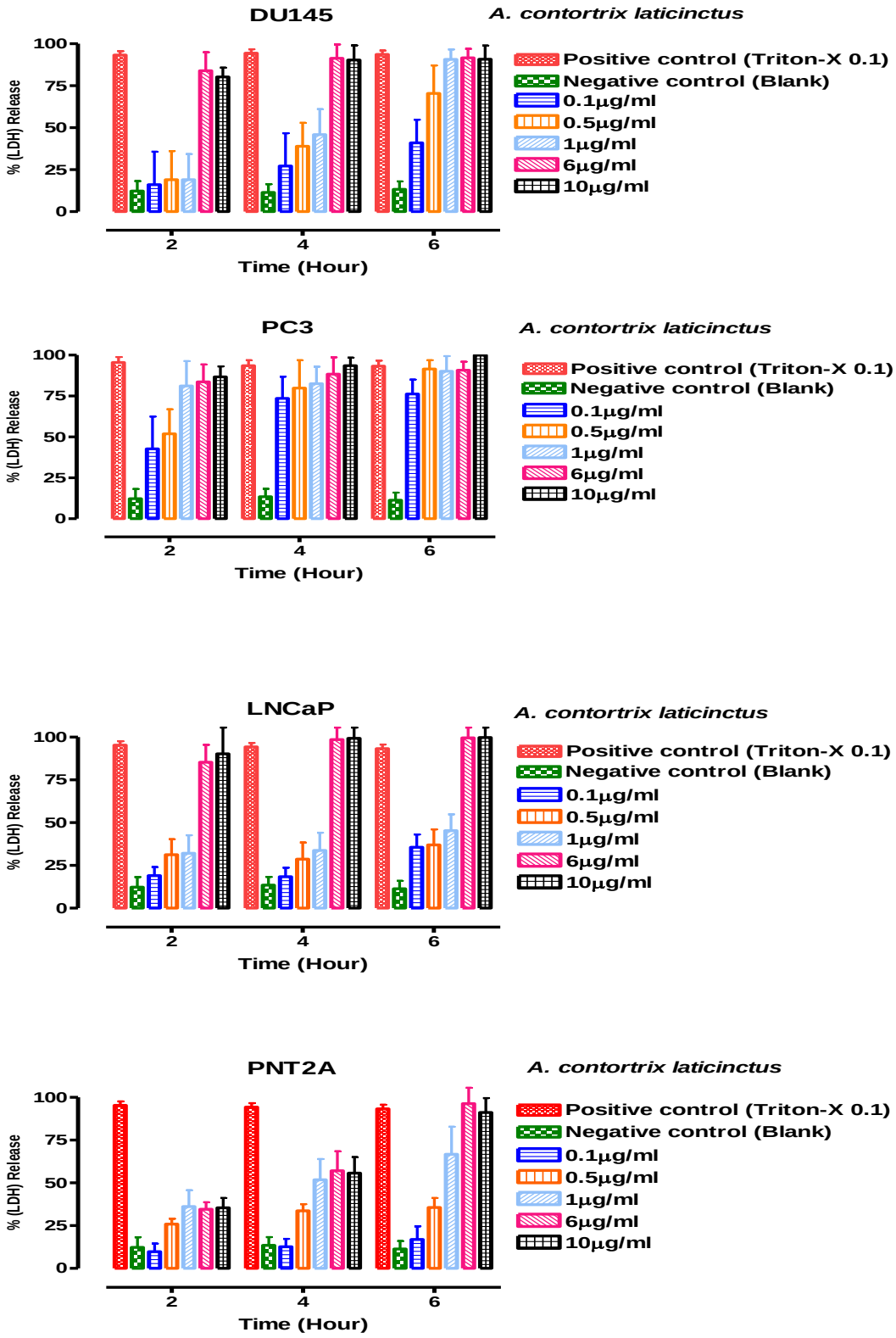



**Figure 3-8**: Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP, and PNT2A following 2, 4, and 6 hours exposure to 0.1, 0.5, 1, 6 and 10µg/ml of *A. contortrix laticinctus* snake venom in complete media at 37$^{o}$C. The positive control agent was 0.1% Triton-X 100 for 2, 4 and 6 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time periods in complete medium.

#### 3.3.2.1 Morphological analysis

Untreated DU145, PC3, LNCaP and PNT2A cell lines were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 3-9; 3-10; 3-11 and 3-12). However, after incubating the cell lines with *A. contortrix laticinctus* venom for 2, 4 and 6 hours, the occurrence of various morphological abnormalities was observed. At the highest venom concentrations of 6 and 10µg/ml, the disappearance of normal morphological characteristics was very obvious even after 2 hours exposure (Figures 3-9; 3-10; 3-11 and 3-12), with cells showing severe shrinkage and condensation of their cellular contents.

At the lower concentrations (0.1, 0.5 and 1µg/ml), with the DU145 cell line, most of the cells lost their characteristic appearance and coalesced to such an extent that individual cells could not be identified. For example, DU145 cultures showed gaps and compact cells, PC3 had spherical cells, LNCaP had gaps and cells in groups, and PNT2A cultures had decomposed cells (Figures 3-9; 3-10; 3-11 and 3-12, respectively).

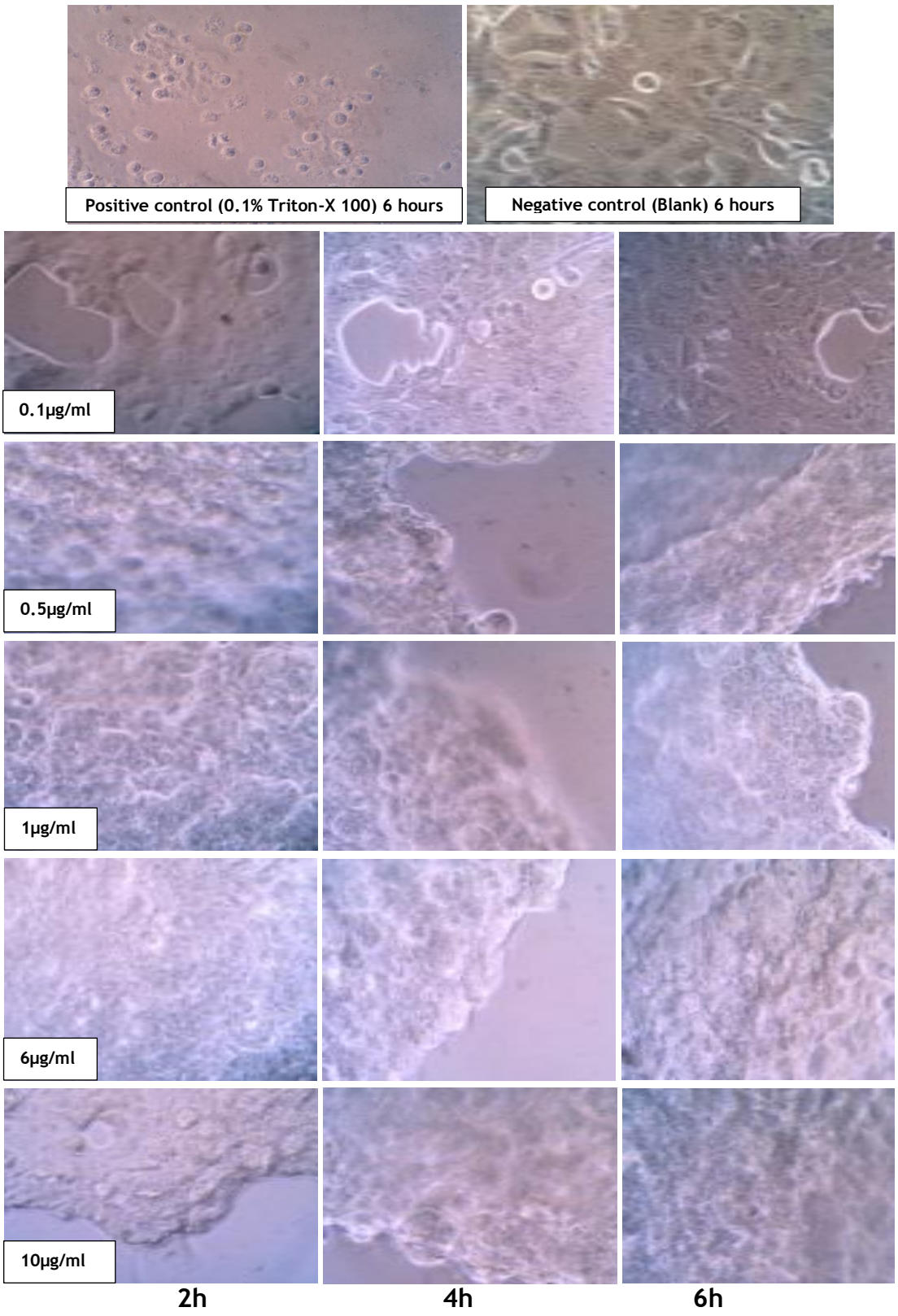



**Figure 3-9:** Light micrographs of cultured cell line DU145 following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. contortrix laticinctus* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

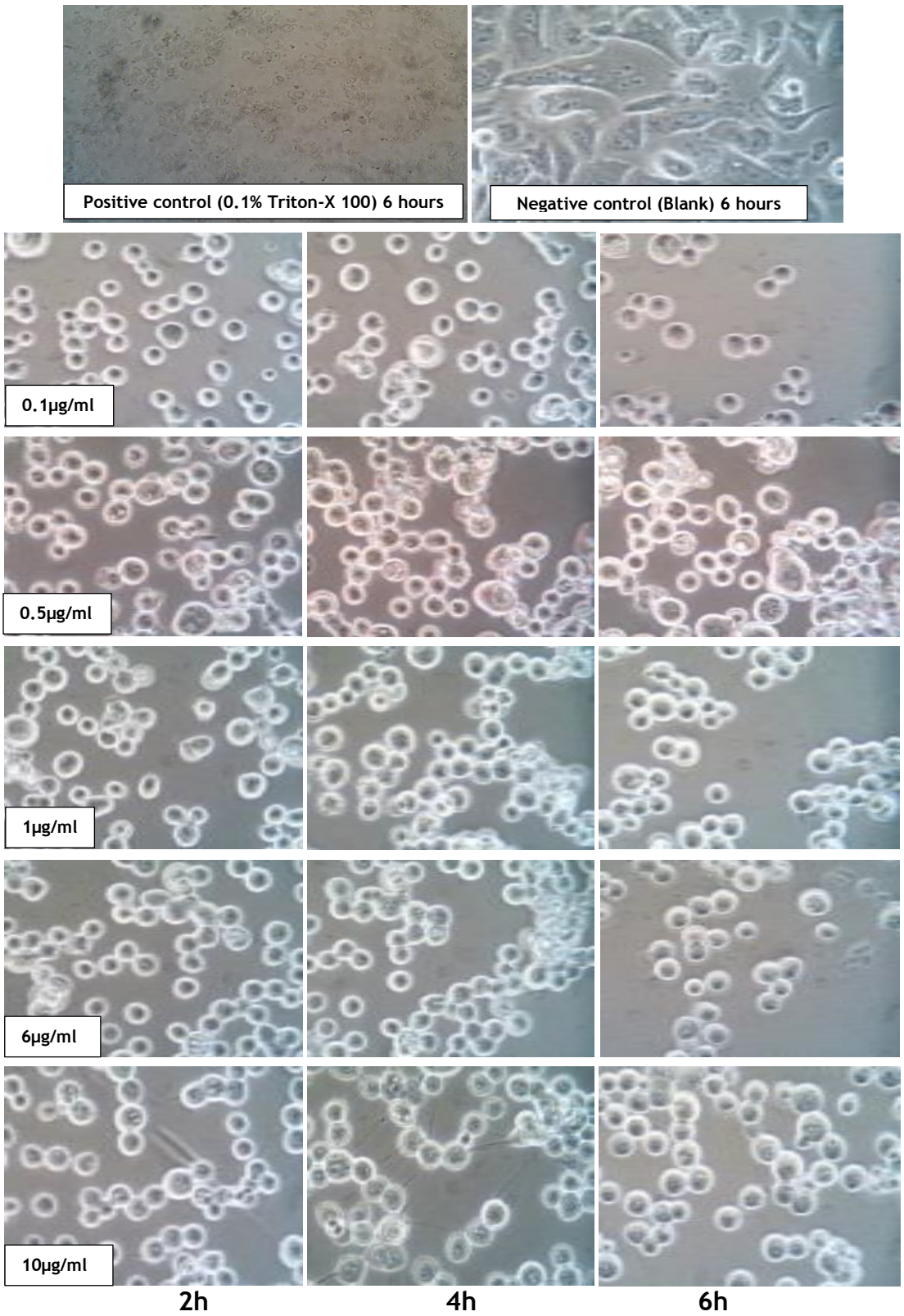



**Figure 3-10:** Light micrographs of cultured cell line PC3 following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. contortrix laticinctus* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

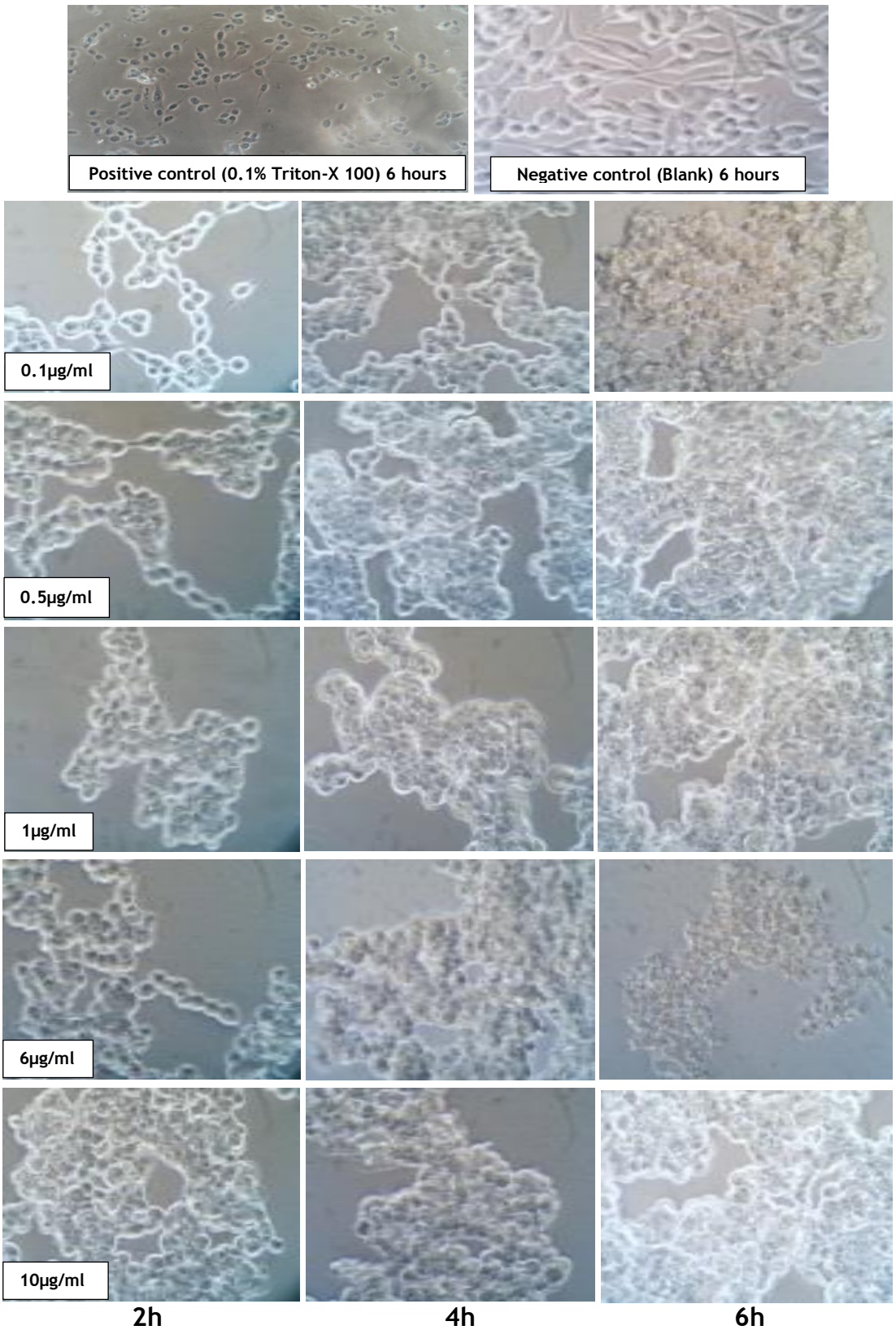



**Figure 3-11:** Light micrographs of cultured cell line LNCaP following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. contortrix laticinctus* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

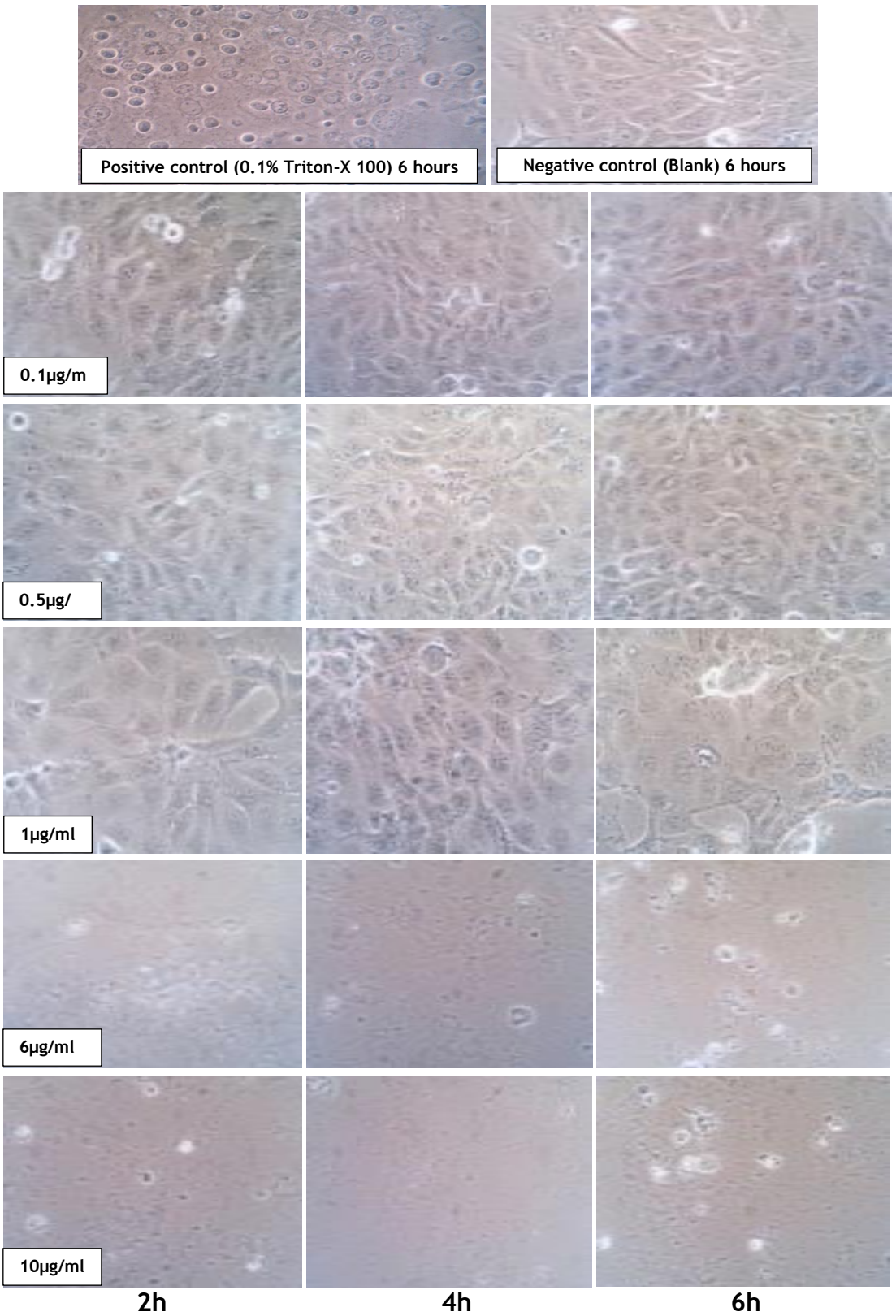



**Figure 3-12:** Light micrographs of cultured cell line LNCaP following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. contortrix laticinctus* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### 3.3.3 Effect of *Agkistrodon piscivorus conanti* venom on DU145, PC3, LNCaP and PNT2A cell lines for 2, 4 and 6 hours by LDH assay

LDH assay was performed to investigate the cytotoxic effect on DU145, PC3, LNCaP and PNT2A cells treated with 0.1, 0.5, 1, 6 and 10µg/ml of *A. piscivorus conanti* venom for 2, 4 and 6 hours. As shown in Figure 3-13, there was a clear time-dependent increase in LDH release in all cell lines but very little release overall in PC3 and LNCaP cell lines. Du145 cells were the most sensitive to this venom.

In DU145 cells, there was little release of LDH after 2hrs, while after 4hrs and 6hrs it was concentration-dependent and time-dependent. In the DU145 cells at 0.1µg/ml, 33% LDH was released after 6 hours while at 10µg/ml, 79% LDH was released observed after 6hrs. The % release of LDH was very low in PC3 cell line at 0.1, 05 and 1µg/ml after 2, 4 and 6hrs. Even at the highest concentration (10µg/ml), release was only 11%, 16 % and 21 % release after 2, 4 and 6hrs. In the LNCaP cell line, the % LDH release was similar to PC3 cell line because even at 10µg/ml release of LDH was 2%, 10% and 20% after 2, 4 and 6hrs. In PNT2A cell line, the % LDH release was found to be very low at 0.1, 0.5,1 and 6µg/ml after 2, 4 and 6hrs exposure. But at the highest concentration (10µg/ml), release was greater: 37%, 37% and 44% after 2, 4 and 6hrs respectively. The % LDH release in control group was less than 5% at 2hr, 4hr and 6hr in all cell lines.

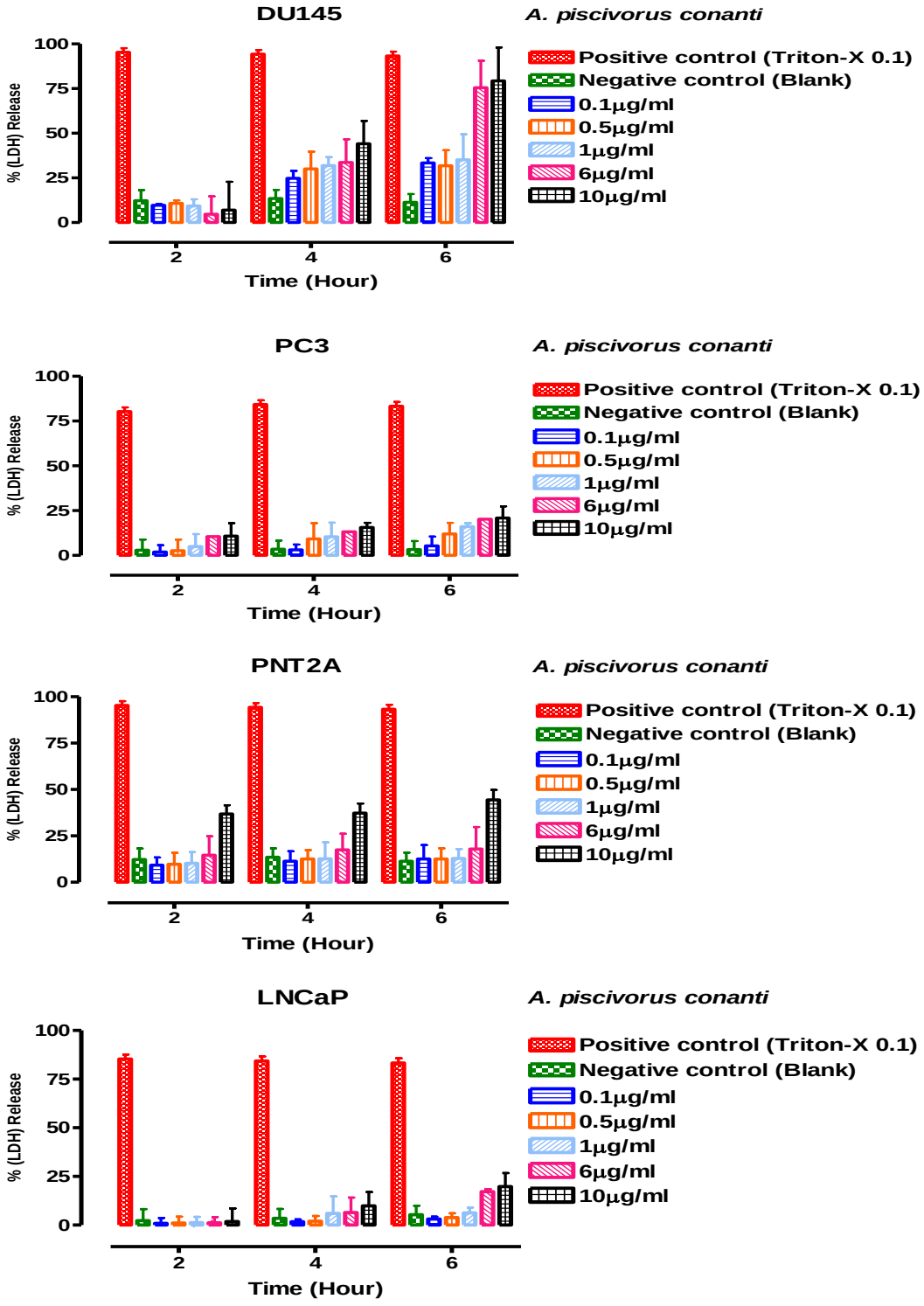



**Figure 3-13:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP, and PNT2A following 2, 4, and 6 hours exposure to 0.1, 0.5, 1, 6 and 10µg/ml of *A. piscivorus conanti* snake venom in complete media at 37°C. The positive control agent was 0.1% Triton-X 100 for 2, 4 and 6 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time periods in complete medium.

#### 3.3.3.1 Morphological analysis

Untreated DU145, PC3, LNCaP and PNT2A cell lines were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 3-14; 3-15; 3-16 and 3-17). However, after incubating the cell lines with *A. piscivorus conanti* venom for 2, 4 and 6 hours, various morphological abnormalities were observed.

At the highest venom concentrations (6 and 10µg/ml), the disappearance of normal morphological characteristics in DU145 cells was very obvious, cells showing severe shrinkage and condensation of their cellular contents after 2, 4 and 6hrs (Figure 3-14). At 0.1, 0.5 and 1µg/ml, most of the DU145 cells lost their characteristic appearance and coalesced to such an extent that individual cells could not be identified.

There were no major morphological changes in PC3 and LNCaP cell lines at 0.1, 0.5, 1 and 6µg/ml, but at 10µg/ml slight changes like shrinkage and few condensations in cellular contents were found after 6hrs only (Figure 3-15 and Figure 3-16).

In the cell line PNT2A, there were no changes found in morphological characteristic at 0.1µg/ml, 0.5µg/ml, 1µg/ml and 6µg/ml after 2, 4 and 6hrs but clear morphological changes were found with cell shrinkage and 40% condensation of their cellular contents at 10µg/ml after 2, 4 and 6hrs (Figure 3-17).

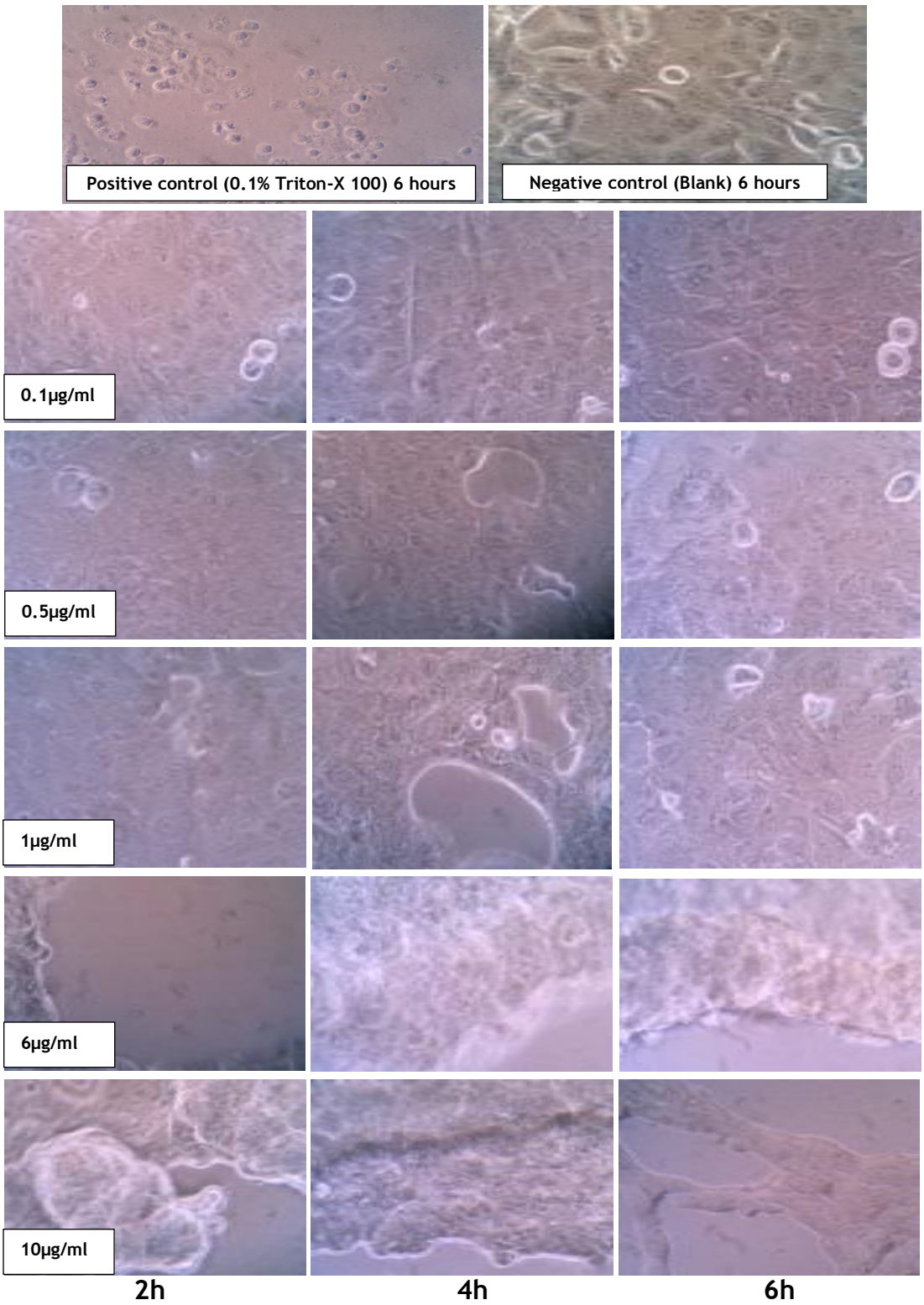



**Figure 3-14:** Light micrographs of cultured cell line DU145 following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. piscivorus conanti* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

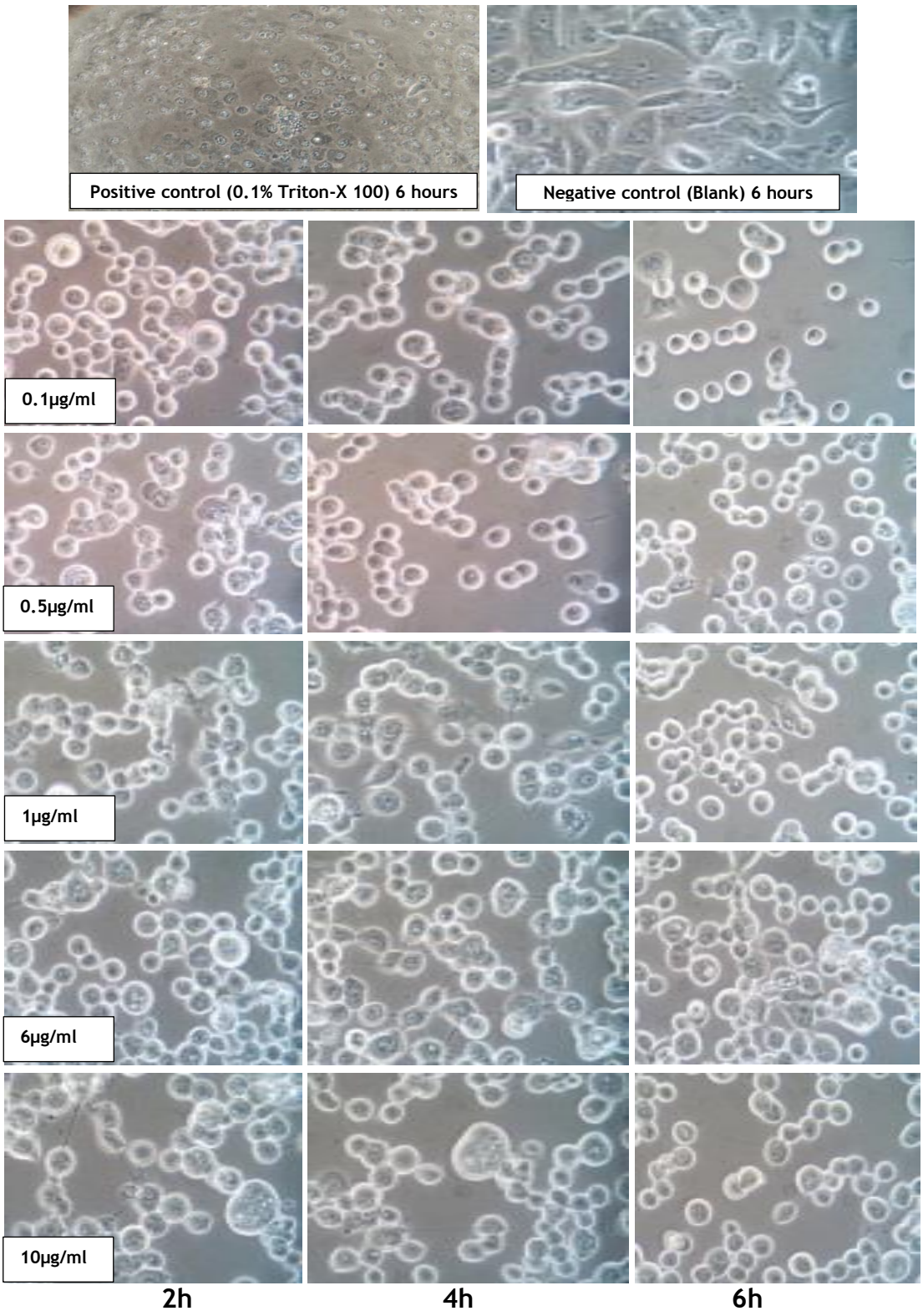



**Figure 3-15:** Light micrographs of cultured cell line PC3 following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. piscivorus conanti* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

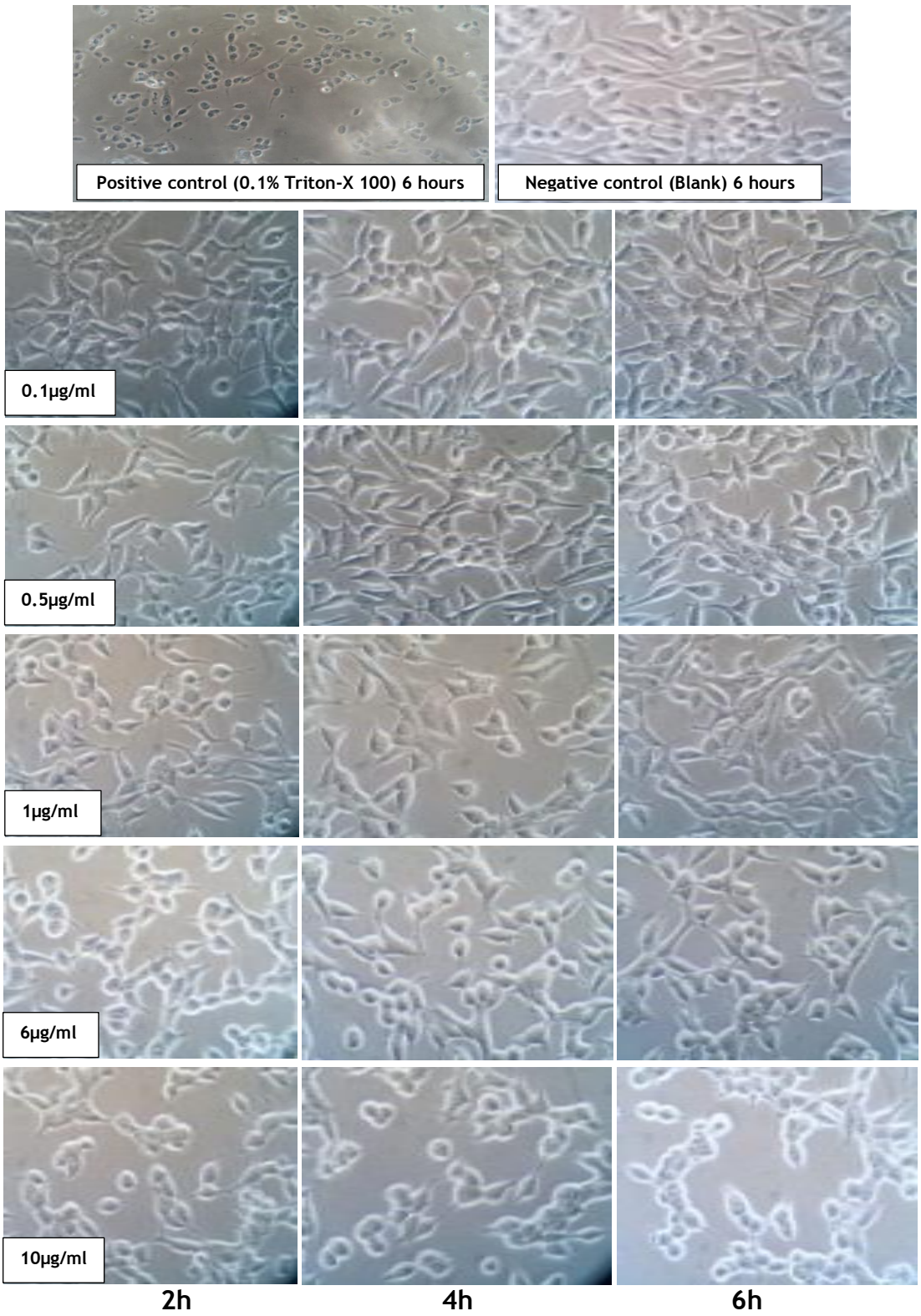



**Figure 3-16:** Light micrographs of cultured cell line LNCaP following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. piscivorus conanti* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

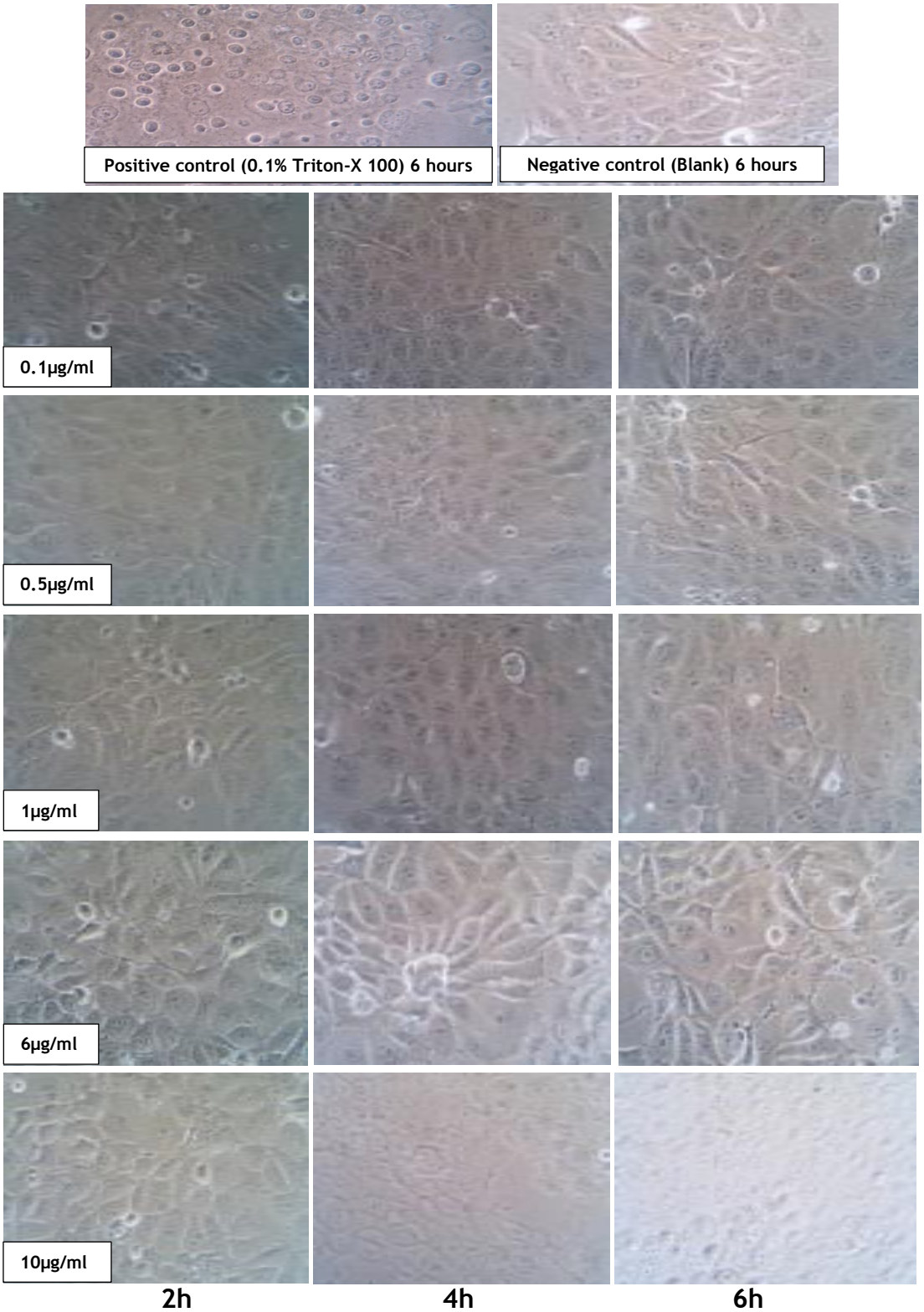



**Figure 3-17:** Light micrographs of cultured cell line PNT2A following 2, 4 and 6 hours exposure to 0.1, 0.5, 1.0, 6.0 and 10µg/ml of *A. piscivorus conanti* venom in complete media at 37$^{o}$C. The negative control shows a culture after 6 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 6 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

## 3.4 Discussion

In vitro cytotoxicity testing provides a means of ranking compounds for consideration in anti-cancer drug discovery: the historically useful and therapeutically attractive anticancer compounds exhibit effects for sustained time periods (up to 48 h or more) before demonstrating cytotoxicity (Rixe and Fojo 2007). In addition, LDH has long been used as a marker of cell death in vitro (Korzeniewski and Callewaert 1983; Decker and Lohmann-Matthes 1988).

The causes of necrotic cell death such as toxic agents or heat stress can in many cases also induce apoptotic cell death (Majno and Joris 1995; Shimizu, Eguchi et al. 1996). Often, the intensity of the insult and also the energy level of a cell decide the outcome as either apoptosis or necrosis. Low ATP concentration or impaired ATP generation pushes the mode of cell death towards necrosis (Nicotera, Leist et al. 1998; Leist and Jaattela 2001).

The highest % LDH release was observed at highest concentration (10µg/ml) of the three snake venom *N. pallida, A. contortrix laticinctus and A. piscivorus conanti* in all prostate cancer cell lines DU145, PC3, LNCaP and normal cell line PNT2A, which clearly indicated the damage of cells due to venom toxins. The cytotoxicity was remarkable and cell survival was rapidly reduced by the two highest concentrations (6 and 10µg/ml). This clearly indicated that venom directly acts on the membrane and produced lysis of organelle membranes that immediately caused cell death. The mechanism of cell death due to snake venom at high concentration is likely to be lysis of membranes, which allows the passage of ions down their concentration gradient, resulting in osmotic changes in organelles leading to necrotic cell death.

Numerous studies have indicated that cell death by apoptosis plays a crucial role in the surveillance of cancer formation and progression, and has also been implicated in regulating neuroblastoma cancer growth (Maris and Matthay 1999; Hengartner 2000). Therefore, the development of a cytotoxic agent that induces apoptosis may be important in the treatment of neuroblastoma. It has been previously reported that snake venom toxin can induce apoptosis in many cancer cell lines

(Maddika, Ande et al. 2007; Gomes, Choudhury et al. 2007; Yang, Chien et al. 2007).

The release of LDH into the culture media, which gives an accurate measure of cellular toxicity induced by the venom, correlates well with the severity of cell death and membrane damage observed in this study. Mostly, these effects were time and dose-dependent in all cell lines. Early release within 4-6 hours of significantly high percentages of LDH, especially after incubation with the highest concentrations of venom and the concomitant swelling of some treated cells, as evidenced in the morphological examination, revealed the occurrence of necrosis. It has been reported that the first step in necrosis is a reversible swelling of the cells followed by plasma membrane failure and subsequent cell lyses liberating cellular compounds (Raff 1992; Kumar 1998).

Thus, results obtained from this study indicate that these specific snake venoms cause cell death by necrosis. The apoptotic mechanism accompanied with cell necrosis was observed in some cell lines while necrosis was found in all cell lines. Although the exact mechanism of this effect was not determined, it may be due to the variations in the toxic components in each venom and the sensitivity of different cell types.

## 3.5 Conclusion

Cell deaths induced by three snake venom *N. pallida, A. contortrix laticinctus* and *A. piscivorus conanti* venom were predominantly associated with a necrotic mechanism. The contribution of the necrotic pathway may be dependent on concentration and/or time of exposure to snake venom. Overall, this study clearly highlights that *N. pallida, A. contortrix laticinctus,* and *A. piscivorus conanti* have toxicity against PC3, LNCaP and DU145 prostate cancer cells. Thus, these venoms may contain a therapeutic potential for prostate cancer therapy.

However, there is further need to explore the active constituents of the venoms and their role in cellular toxicity against prostate cancer cell lines. To determine the role of active constituents of venom on the different prostate cell lines, fractionation and isolation of venom constituents were carried out by high-

performance liquid chromatography (HPLC), ion exchange chromatography and polyacrylamide gel electrophoresis (SDS-PAGE). The details of the separation technique and fraction activity are mentioned in chapter-4.

# CHAPTER FOUR:

## FRACTIONATION OF SNAKE VENOMS AND PHOSPHOLIPASE ACTIVITY

# 4. CHAPTER FOUR

## 4.1 Introduction

Snake venoms are complex blends of different pharmacologically active constituents as well as non-toxic proteins and peptides. Snake venoms can affect different tissues that may facilitate simultaneous responses in multiple physiological systems. However, there is a challenge in developing techniques which can effectively segregate a complex mixture of proteins and peptides with dissimilar size and charge. Modern chromatographic techniques are required to isolate venom constituents. Typical approaches use low pressure chromatography gel filtration followed up by ion exchange chromatography (Stocker 1990; Bailey 1998). Further resolution of fractions from venoms is typically achieved by HPLC chromatography methods.

### *4.1.1 High Performance Liquid Chromatography (HPLC)*

Chromatography relates to the technology that performs partitioning of different components in a mixture. Tswett is considered as the pioneer who introduced chromatography through his work of segregating the pigments in green leaves using a chalk column in 1903. The term chromatography was coined to describe the coloured zones that appeared across the glass column. Chromatography mainly deals with the flow of a mobile (liquid) phase over a stationary phase (which may be a solid or a liquid). As the mobile phase passes through the stationary phase, recurring events of adsorption and desorption of the solute happen at a rate fixed by its solubility. Although this method was promoted in between late 1960s and early 1970s, it is extensively applied even today for different applications related to separation and cleansing. The application of chromatography is widely present in different scientific domains and industries such as pharmaceutical, biotechnological, environmental, polymer and food industries (Settle 1997).

The HPLC technique has been a popular choice for the study of a wide range of compounds' separation, identification and quantification over the past decade. HPLC is accomplished by injecting a small amount of liquid sample into a moving stream of liquid that goes through, under pressure, a column packed with particles of stationary phase. It relies on pumps to pass a pressurized liquid solvent containing

the sample mixture through a column filled with a solid adsorbent material (silica and polymers etc.). The components of the sample mixture are separated from each other due to their different degrees of interaction with the absorbent particles. The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. The time at which a specific analyte elutes (emerges from the column) is called its retention time (Rt) (Settle 1997).

### *4.1.2 Purification and separation of snake venom*

Ohsaka reported in 1960s that snake venom was composed of a range of protein components. Further studies have shown that the biological and pathological activities are associated with proteins and peptides in venom. In subsequent years, further biochemical studies were carried out to isolate individual protein from the venom. The venom complexity can be portrayed with relatively less difficulty using 2-D PAGE method, as shown by Fox and Serrano (2008) with fifty-five snake genera. It is noticeable that the number of proteins identified in venom is restricted to the detection level of the gel-staining methods. Low abundant venom proteins can go unidentified while applying these methods. The post-translational alteration (PTM) such as glycosylation of individual proteins can also impact on the complexity of snake venom (Earl, Birrell et al. 2006). Since a minor PTM can be identified by mass spectrometry, large-sensitivity and high-resolution mass spectrometry has been widely employed to examine snake venom (Calvete 2014).

In 2002, Fox and others successfully used 2-D PAGE along with LC/MS/MS to study the mixture of proteins of *Dispholidus typus* (Boomslang), *Crotalus atrox* (Western diamondback rattlesnake), and *Bothrops jararaca* (Jararaca) venoms (Fox, Curt et al. 2002). This analysis was credited as the first instance of comparative proteomics between *C. atrox* and *B. jararaca*, two Viperidae venoms with analogous pathologies during envenomation. Fry and others combined LC/MS mass finger printing, RP-HPLC, and Edman sequencing to study the venoms of *Acanthophis* (Death adder) and create taxonomic links among *Acanthophis* venoms (Issaq, Conrads et al. 2002; Fry, Wuster et al. 2003).

Further research investigations on venom proteomes have continued to rise since 2003. Creer, Malhotra et al. (2003) applied MALDI-TOF/MS and isoelectric focusing (IEF) to study the intraspecific changes in the *Trimeresurus stejnegeri* venom associated with genetic, ecological and geographic variations. Fox and Serrano (2008) distinguished post-translational changes applying a combination of Western blot analysis of 2-D gels, specialized staining, zymography, and LC/MS/MS. Further, FT-ICR LC/MS/MS were applied to examine proteomic affinities between the venoms of *C. atrox* and *B. jarjaraca*.

In this study, gel filtration, ion exchange chromatography, SDS-PAGE analysis, RP-HPLC and N-terminal sequencing were used for separation and purification of snake venom.

#### 4.1.2.1 Gel Filtration (GF)

Gel filtration is also called size exclusion chromatography. Molecules in solution are separated by their size and in some case molecular weight. The main application of gel-filtration chromatography is the fractionation of proteins and other water-soluble polymers. This method uses a porous matrix to segregate different proteins based on their molecular size. Different proteins have different transit times through the porous matrix. The matrix is placed in column and the division is done by passing an aqueous buffer known as the mobile phase through the column. Large protein molecules cannot enter the gel and are excluded and there is less volume required to transverse and elute sooner, while the smaller protein molecules enter the gel and they have more volume to transverse and elute later. The eluted protein portions are detected by an in-line UV monitor.

Although separation of molecules based on size was introduced in mid-1950s, the main credit for the discovery of gel filtration (GF) may be attributed to Porath and Flodin (1959) who reported the desalting of proteins and realized the potential of the technique (Lindqvist and Storgards 1955; Lathe and Ruthven 1956), Their discovery gained widespread attention with the availability of cross linked gel-filtration matrix, Sephadex in the market in 1959. This matrix is still one of the premier choices for desalting of proteins.

Gel filtration has proven to be a significant tool in determining the molecular mass distribution of polymers in aqueous solutions (Granath and Flodin 1961). The types of gel used are uncharged and static so do not bind or react with the materials being analyzed. Dextran is used as a gel, which is a homopolysaccharide of glucose residues. It is prepared with various degrees of cross-linking to control pore size. It is commercially known as Sephadex which is available as dry beads and distended on addition of water. This study adopts a gel filtration material which is called Sephadex G-75. It is mainly used to segregate proteins and peptides with molecular weights between from 3,000 to 70,000 (Nakano and Ozimek 1998).

### *4.1.3 Ion Exchange chromatography*

Ion exchange chromatography uses a charged ligand bound to the stationary phase. As the proteins pass through the column, they interact in different ways with the stationary phase depending on their charge facilitated by pH. This interaction with the stationary phase is enhanced by changing the mobile phase which increases the availability of counter ions in the protein elute (Yamamoto, Nakanishi et al. 1988). Ion exchange chromatography is a well-known method with numerous benefits that include a high throughput as well as a long lifespan of the column. In general, recovery and speed are considered as the most important parameters for an optimization process (Yamamoto, Nakanishi et al. 1988; Inamuddin and Luqman 2012).

## 4.2 Materials and Methods

### *4.2.1 Snake Venom*

*Naja pallida, A. piscivorus conanti and A. contortrix laticinctus* venom were supplied by National Natural Toxins Research Centre (Texas A&M University-Kingsville, USA) as described details in chapter two.

### *4.2.2 Cell culture*

Three prostate cancer cell lines DU145, PC3 and LNCaP, and the normal prostate cell line PNT2A were used for the study of LDH release. The cell culture methods are described in detail in chapter two.

### *4.2.3 Lactate Dehydrogenase (LDH) assay*

LDH assay of *N. pallida, A. piscivorus conanti* and *A. contortrix laticinctus* venom fractions were performed as described in chapter three.

### *4.2.4 Microscopy*

Cell were visualised using a Nikon TMS inverted phase contrast microscope. Digital images were taken at 2 hour intervals by Motic Images Plus 2.0 and acquired by computer.

### *4.2.5 Gel filtration*

The whole snake venom (freeze dried powder 500 mg) of *N. pallida, A. piscivorus conanti* and *A. contortrix laticinctus* was dissolved in 3ml of 0.1M ammonium acetate buffer (pH 6.8) and centrifuged at 10,000 RPM/g for 5 minutes at room temperature to remove the turbidity. The clear supernatant was applied on a gel chromatography column (17 mm x 1000 mm) glass column of Sephadex G-75 and equilibrated with 0.1M ammonium acetate buffer (pH 6.8) and then eluted with the same buffer. Fractions of 10ml/tube were collected at a flow rate of 18 ml/hour at $4^{o}$C. The protein fractions were detected by their absorbance at 280nm and collected in 2 ml aliquots, named as F1, F2, F3, F4 and F5. The resultant peaks were pooled into major fractions and were stored frozen (-$20^{o}$C) till further analysis of LDH and phospholipase assays.

### 4.2.6 Ion Exchange chromatography

The active fractions from snake venom (freeze dried powder) *N. pallida*, (150mg) *Agkistrodon piscivorus conanti* (50mg) and *Agkistrodon contortrix laticinctus* (20mg) venom were dissolved in 3ml of 0.1M ammonium acetate buffer (pH 6.8) and centrifuged at 10,000 RPM/g for 5 minutes at room temperature to remove any turbidity. The clear supernatant was injected on an ion exchange column (142 mm x 400 mm glass column) of CM Sephadex$^{TM}$ C-50, equilibrated with 0.1M ammonium acetate buffer (pH 6.8) and then eluted with the 1M ammonium acetate buffer (pH 6.8). The fractions were collected at a flow rate of 8ml/hour at room temperature. The protein fractions were detected by their absorbance at 280nm and collected in 1 ml aliquots, named as 1, 2, 3 and 4. The resultant peaks were pooled into major fractions and were stored frozen (-20$^{o}$C) till further analysis of LDH and phospholipase assays.

### 4.2.7 HLPC: Phenomenex Luna reverse phase column C18

C18 is the porosity of the silica particles (5µm) which is covalently bonded with an alkyl chains to provide the solid support hydrophobic stationary phase, which has a stronger affinity for hydrophobic compounds. The use of hydrophobic stationary phase can be considered the opposite or reverse, of normal phase chromatography - hence the term "reversed phase chromatography". Reversed-phase chromatography employs a polar (aqueous) mobile phase. As a result, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydrophilic molecules in the mobile phase will pass through the column and are eluted first. Hydrophobic molecules can be eluted from the column by decreasing the polarity of the mobile phase using an organic (non-polar) solvent, which reduces hydrophobic interactions. C18 is considered well suited for mapping related peptides as well as in the analysis and purification of small particles in high efficiency. Mixtures of water or aqueous buffers and organic solvents were used to elute analytes from a reversed-phase column. The solvents must be miscible with water, and the most common organic solvents used are acetonitrile, methanol, and tetrahydrofuran (THF) with the pH range of 2-8. This column can be used at a wide range of temperatures, 4-40$^{o}$C.

The isolated venom fractions from *N. pallida, A. piscivorus conanti* and *A. contortrix laticinctus* were dissolved in acetonitrile and separated by using HPLC reverse-phase chromatography equipped with a Luna 5 µ C18 Phenomenex column (250 x 4.60 mm), under linear gradient flow 0.1% to 70% (v/v) and the buffer A (0.1% trifluoroacetic acid in water) to buffer B (1% TFA in acetonitrile) at a flow rate of 1ml/min and monitored at 280 nm.

### 4.2.8 SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using approximately 10µg of snake venoms and fractions. Protein samples were run under reducing conditions on a 12% Tricine SDS-PAGE (Invitrogen) gel (Schagger and von Jagow 1987). A X Cell Sure Lock™ system (Thermo fisher Scientific) with Tricine-SDS running buffer (10×) diluted to 1× at a voltage of 200 for 60 min using a Bio-Rad Power Pac Basic. The Power Pac Basic provides constant voltage or current to instruments used in electrophoresis. The SeeBlue® Plus2 pre stained markers ranging from 4-250 kDa were used as protein standards. The SeeBlue® Plus2 Pre-Stained Standard allows easy visualization of protein molecular weight ranges during electrophoresis and evaluation of Western transfer efficiency, suitable for Tris-Glycine gels. It consists of 10 pre-stained protein bands (8 blue and 2 contrasting colours) in the range of 4-250 kDa.

### 4.2.9 Phospholipase (PLA2) Assay activity of the crude venom and fractions

Phospholipases $A_2$ ($PLA_2$) are enzymes that are commonly found in mammalian tissues as well as arachnid, insect and snake venoms. Due to the increased presence and activity of $PLA_2$ resulting from a snake or insect bite, arachidonic acid is released from the phospholipid membrane disproportionately. As a result, inflammation and pain occur at the site (Dennis 1994).

$PLA_2$ activity was measured using the assay described by Holzer and Mackessy (1996) modified for 96-well plates. The standard assay mixture contained 200$\mu$l of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20$\mu$l of

substrate (4-nitro-3-octanoyloxy-benzoic acid), 20$\mu$l of water, and 20$\mu$l of crude venom and fractions in a final volume of 220$\mu$l. After adding venom and fractions (20$\mu$g), the reading of absorbance at 37°C, was made every 2 min. The enzyme activity, expressed as the initial velocity of the reaction ($Vo$), was calculated based on the increase of absorbance after 20 min. Absorbance at 425nm was measured using a Spectra Max 340 multi well plate reader (Molecular Devices, Sunnyvale, CA, USA). The positive control purified $PLA_2$ from (Bbil-TX) *Bothriopsis bilineata smargadina* was used to measure the $PLA_2$ activity while the negative control only buffers without any enzyme.

### *4.2.10 Statistics analysis*

Statistical significance of results was assessed using Prism. Experimental results are expressed as mean ± S.E.M and the differences with p values of $< 0.05$ were considered statistically significant. LDH release and phospholipase ($PLA_2$) activity of the venom and fractions of experimental groups were compared with control groups, and $p < 0.05$ indicated statistical significance by analysis of variance (ANOVA) test.

## 4.3 Results

The first fractionation step with all of the snake venoms was carried out by gel filtration, using a Sephadex G-75 column. Five major fractions were obtained by gel filtration from the venom of *N. pallida* and named as F1, F2, F3, F4 and F5 (Figure 4-1). Similarly, four major fractions were isolated from *A. controtrix laticinctus* venom by gel filtration and named as F1, F2, F3 and F4 (Figure 4-19), and the six major fractions obtained from *A. piscivorus conati* venom named as F1, F2, F3, F4, F5 and F6 (Figure 4-31). LDH assay, morphological analysis and phospholipase ($PLA_2$) assay were carried out with all fractions collected from the Sephadex G-75 column.

Based on the most active fractions, further separation of fractions was carried out by using ion exchange chromatography on Sephadex CM-50 and these sub-fractions again tested on all prostate cancer cell lines to find their effectiveness. The results revealed that the most active fractions were F4 of *N. pallida* venom (Figure 4-7), F3 of *A. contortrix laticinctus* (Figure 4-25) and F4 of *A. piscivorus conanti* (Figure 4-37) based on LDH assay and morphological analysis.

The most active fractions were again sub-fractionated by Sephadex CM-50 and named as 1, 2, 3, 4 and 5 (Figures 4-7 for *N. pallida*; 4-25 for *A. contortrix laticinctus* and 4-37 for *A. piscivorus conanti*).

### *4.3.1 Gel filtration of venom of Naja pallida*

The fractionation of *N. pallida* venom (100mg) was carried out by using Sephadex G-75 column chromatography (17 mm x 1000 mm), equilibrated with 0.1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers. The five major fractions obtained by gel filtration from the venom of *N. pallida* were named as F1, F2, F3, F4 and F5 (Figure 4-1). Electrophoretic profiles of the fractions from *N. pallida* venom (10μg) were loaded onto lanes 1-5 of a 12% (w/v) polyacrylamide gel. The gels indicate quantitative differences in band intensities among the fractions.

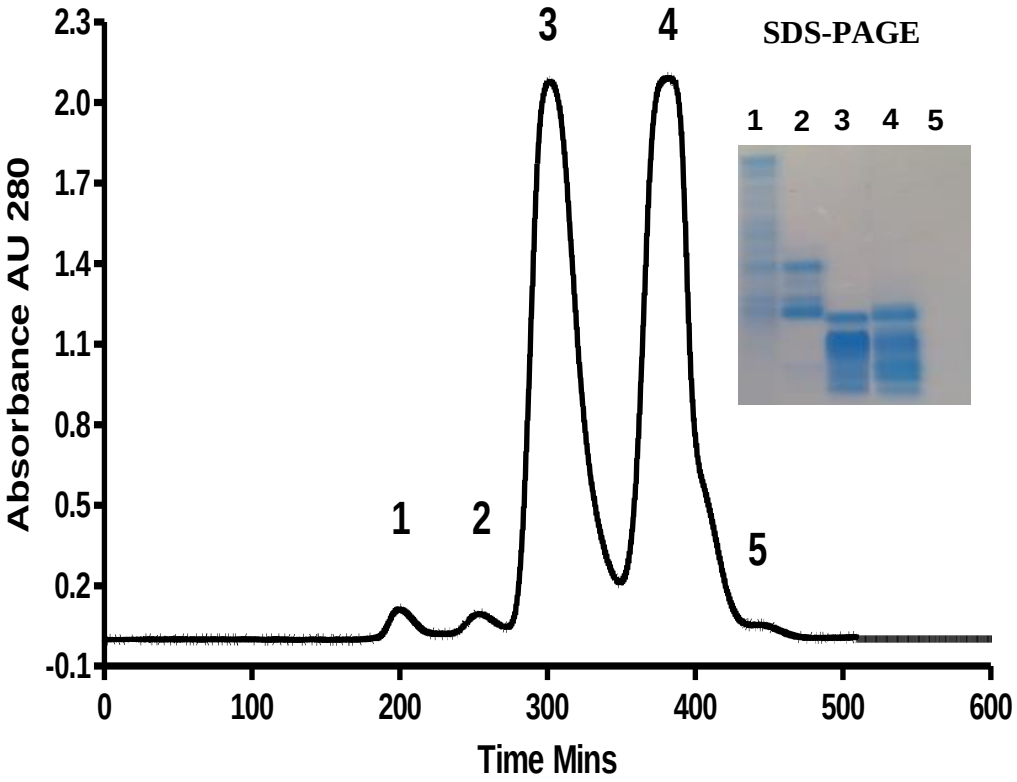



**Figure 4-1:** Fractionation of *Naja pallida* venom by Sephadex G-75 column chromatography. Venom 100 mg equilibrated with 0.1M ammonium acetate buffer (pH 6.8) applied to a column (17 mm x 1000 mm). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers. Inset: Electrophoretic profile of *Naja pallida* fractions (10µg) loaded onto lanes 1-5 of a 12% (w/v) polyacrylamide gel. More details of the SDS-PAGE are given in Figure 4-49.

#### 4.3.1.1 Effect of *Naja pallida* venom fractions on DU145, PC3, LNCaP, and PNT2A cell lines for 2 hours by LDH assay

The five fractions F1, F2, F3, F4 and F5 from *N. pallida* venom were tested for LDH release against prostate cancer cell lines DU145, PC3, and LNCaP and normal prostate cell PNT2A (Figure 4-2). The positive control (Triton-X 100) and negative control (whole medium with no venom) were also used in parallel with each test. The results showed that fraction (F1) was not effective against any prostate cell line because no LDH release was observed and it was similar to the negative control. The other fractions from *N. pallida* venom F2, F3, F4 and F5 showed effects on all cell lines at 20µg/ml after 2hrs. Fraction F2 was less effective against DU145, PC3 and PNT2A cells while it was significantly effective against LNCaP cells, where it was similar to the positive control. Fraction 3 of venom also was effective against LNCaP cell lines. Fraction F4 was the most effective and significant against all cell lines: DU145, PC3, LNCaP and PNT2A. The % LDH release was more than 90% in all cell lines except PC3 where it was 74%. The last fraction F5 was similar to F3 fraction but F5 fraction was more effective than F3 fraction against LNCaP.

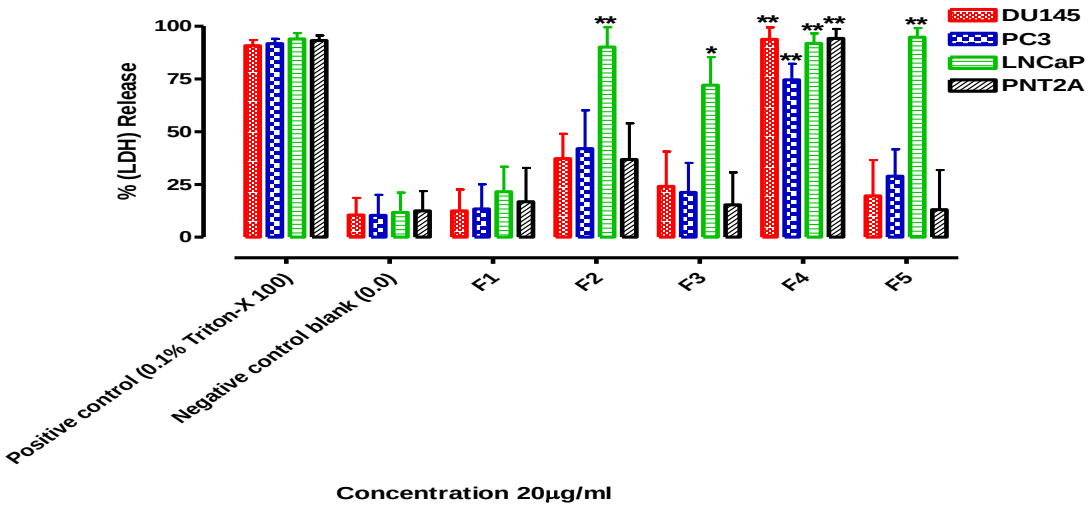



**Figure 4-2:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP and PNT2A following 2 hours exposure to 20µg/ml of *Naja pallida* fractions in complete media at 37$^{o}$C. Data represent mean ± S.E.M of 3 experiments. *p < 0.05, significantly different from untreated control groups. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.1.1.1 *Morphological analysis*

Untreated DU145, PC3, LNCaP and PNT2A cells were homogeneously distributed in the culture field, exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 4-3, 4-4, 4-5 and 4-6). However, after incubating the cell lines with *Naja pallida* venom fractions (F1, F2, F3, F4 and F5) for 2 hours, the occurrence of various morphological abnormalities was observed. The disappearance of normal morphological characteristics was very obvious with cells showing severe shrinkage and condensation of their cellular contents. Fraction 4 (F4) was the most active with completely decomposed cells so that individual cells could not be identified.

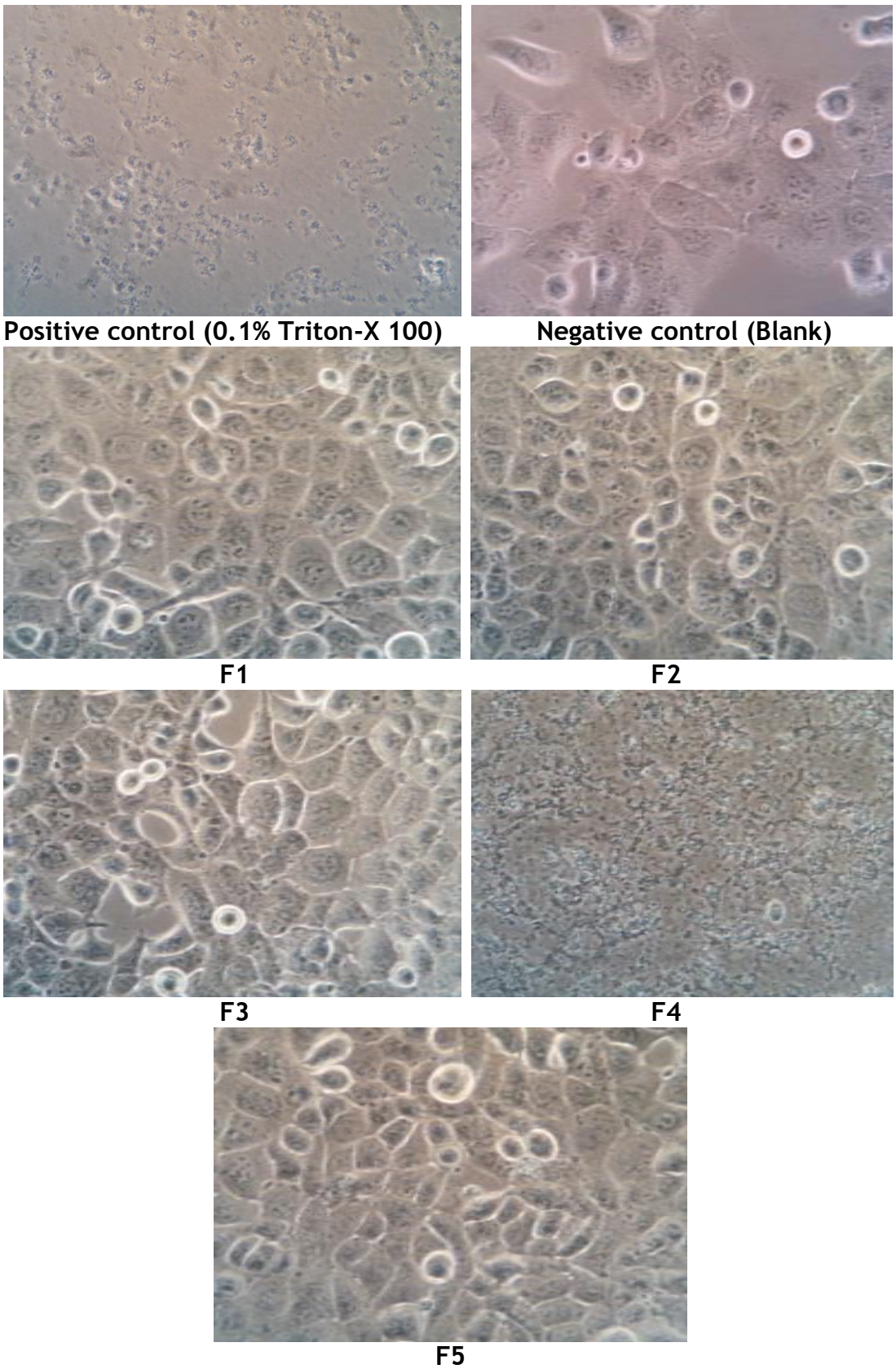



**Figure 4-3:** Light micrographs of DU145 cells following 2 hours exposure to 20µg/ml of *Naja pallida* venom fraction in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

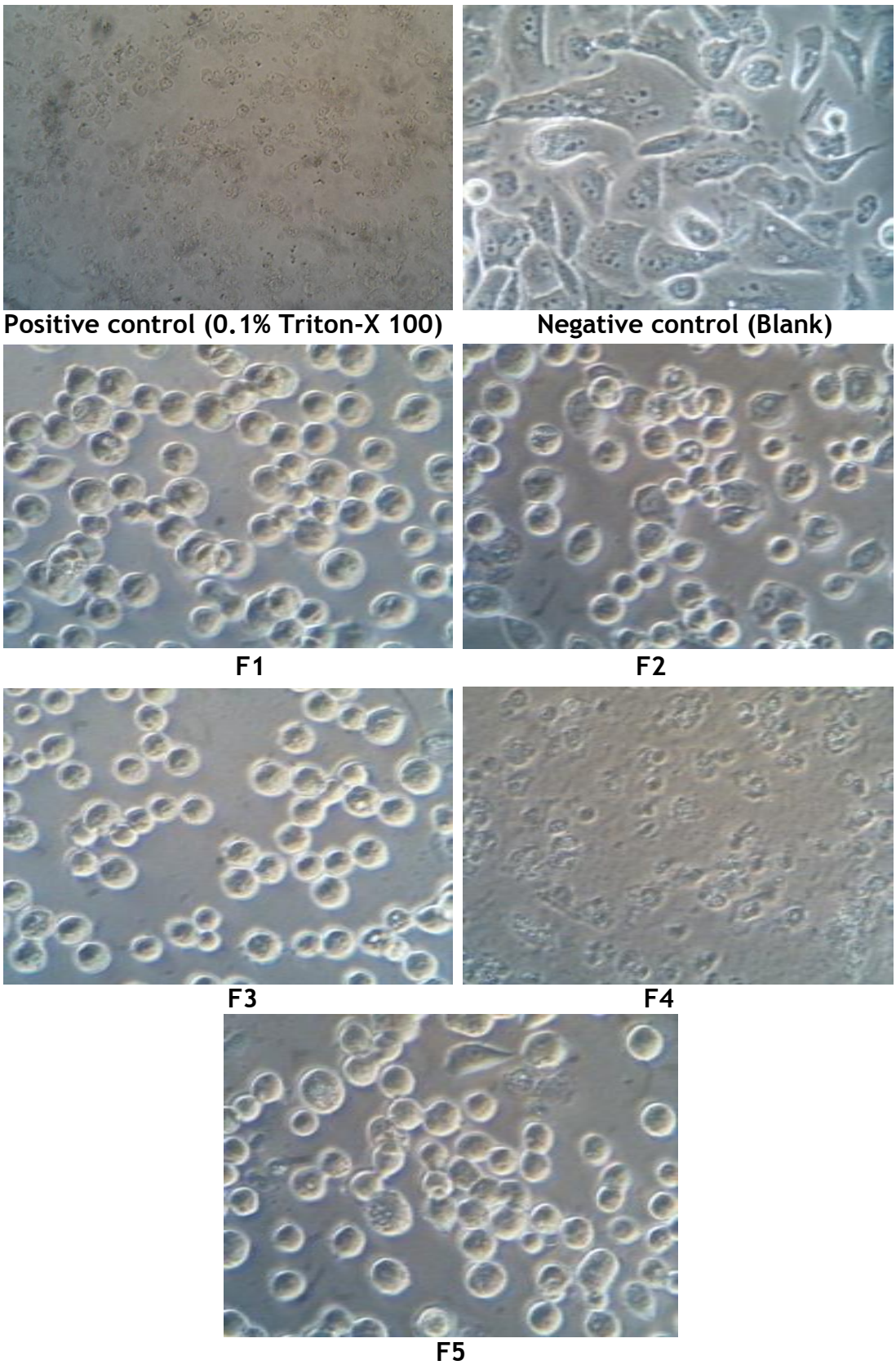



**Figure 4-4:** Light micrographs of PC3 cells following 2 hours exposure to 20µg/ml of *Naja pallida* venom fraction in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

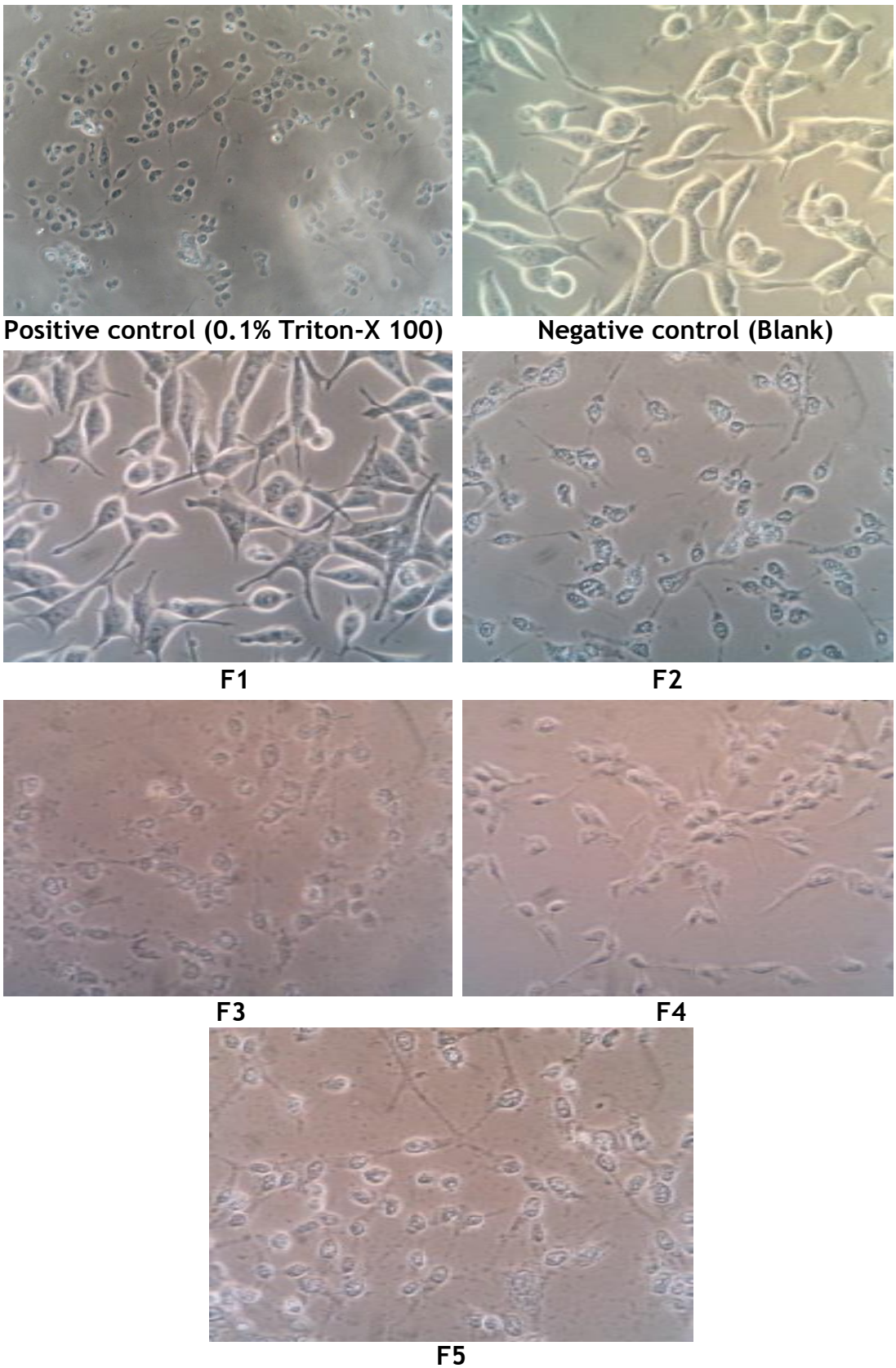



**Figure 4-5:** Light micrographs of LNCaP cells following 2 hours exposure to 20µg/ml of *Naja pallida* venom fraction in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

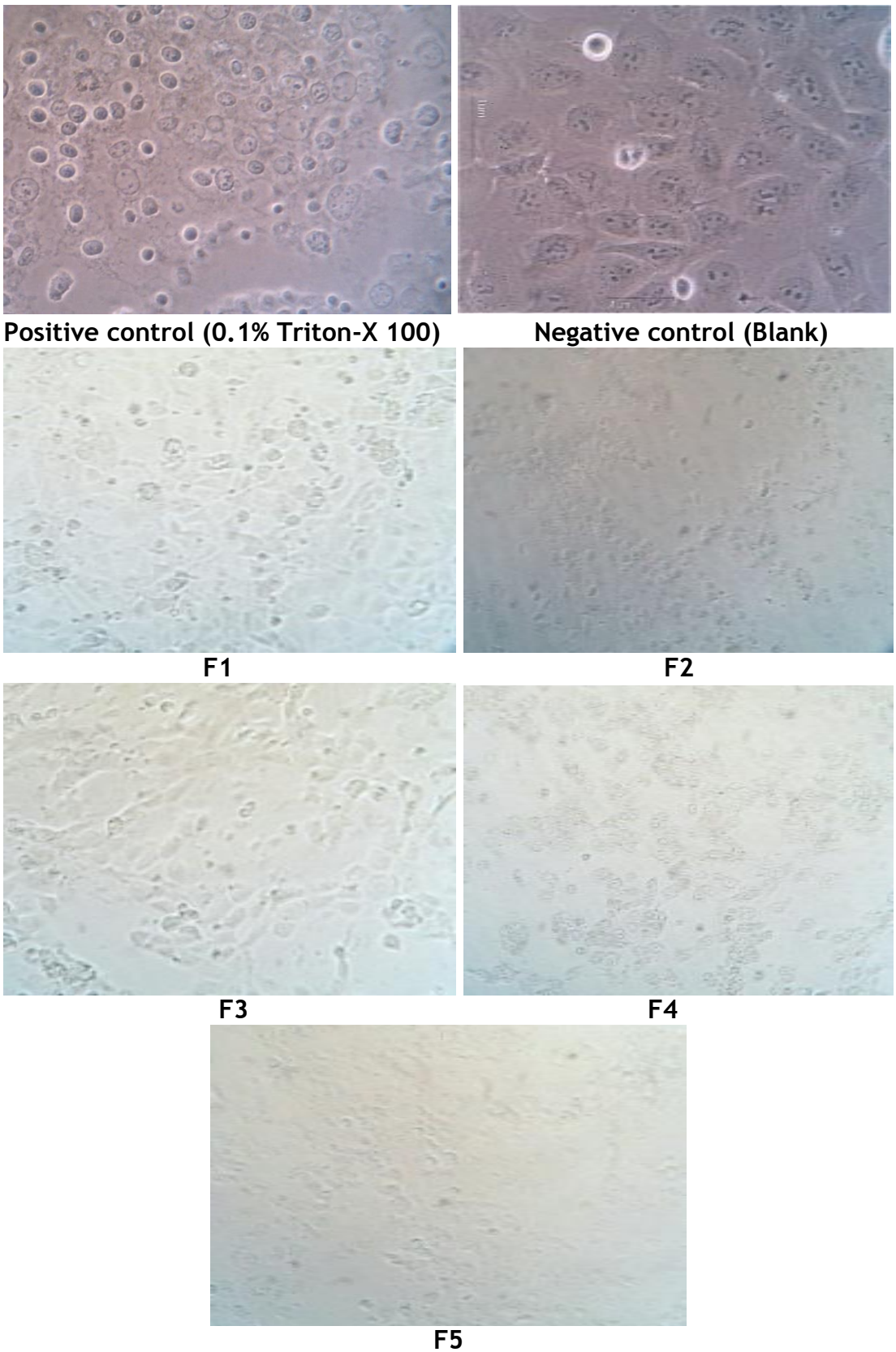



**Figure 4-6:** Light micrographs of PNT2A cells following 2 hours exposure to 20µg/ml of *Naja pallida* venom fraction in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### 4.3.1.2 Ion Exchange separation of *Naja pallida* venom fraction 4

Based on the above LDH assays and morphological analysis, fraction 4 (F4) was found to be the most effective fraction against prostate cancer cell lines. Therefore, F4 was further fractionated by ion exchange chromatography using a Sephadex C-50 column. This yielded four sub-fractions, named P4F1, P4F2, P4F3 and P4F4. Peak 3 in the chromatogram indicated a high content of protein while peak 4 showed much less amount of protein content at 280nm absorbance. Peaks 1 and 2 did not show any considerable protein at 280nm absorbance (Figure 4-7). These sub-fractions were freeze dried for further analysis but the two sub-fractions 1 and 2 did not freeze dry due to the low content of protein. The subfractions 3 and 4, named P4F3 and P4F4, were used for further cytotoxic analysis against all prostate cell lines.

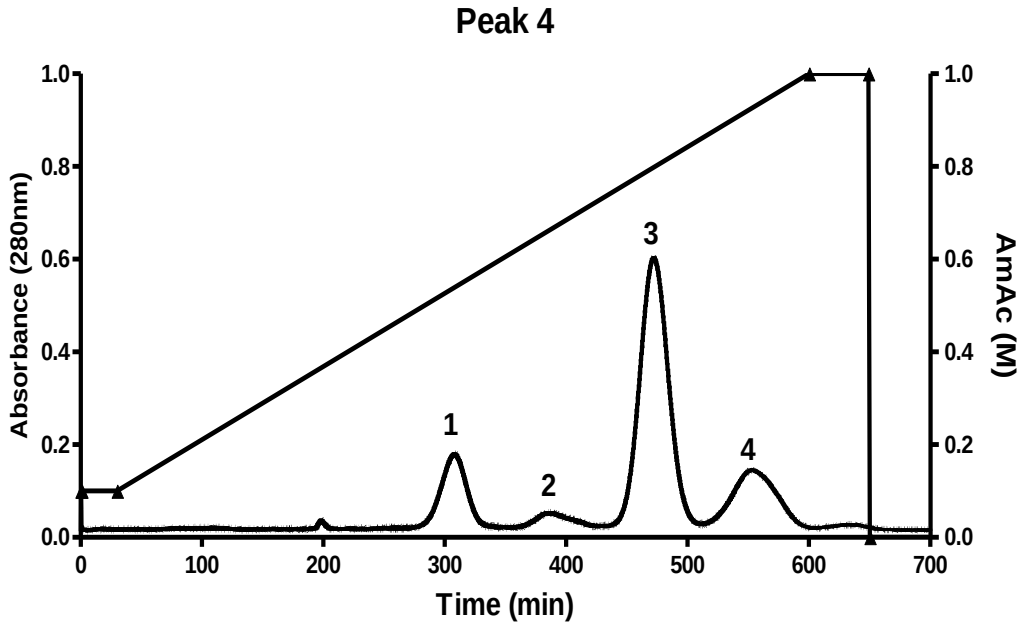



**Figure 4-7:** Fractionation of *Naja pallida* venom fraction 4 by Sephadex C-50 ion exchange chromatography. Fraction 4 (150 mg) applied to a 142mm x 400mm column, equilibrated with 0.1 and eluted to 1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers.

#### 4.3.1.2.1 Effect of sub-fractionated *Naja pallida* venom on DU145, PC3, LNCaP and PNT2A cell lines for two hours

LDH assay was performed to investigate the cytotoxic effect of sub-fractions on the different cell lines DU145, PC3, LNCaP and PNT2A. The sub-fractions 3 and 4 (named as P4F3 and P4F4) were used at 5 and 10µg/ml for 2hours. As shown in Figure 4-8, *N. pallida* sub-fractions P4F3 and P4F4 clearly induced significant increase of LDH release in DU145 and PC3 cell lines. This increment was more than 70% in DU145 cells while it was more than 50% in PC3 cell lines. The effects on LNCaP and PNT2A cells were smaller (less than 40%) and not statistically significant as compared to control.

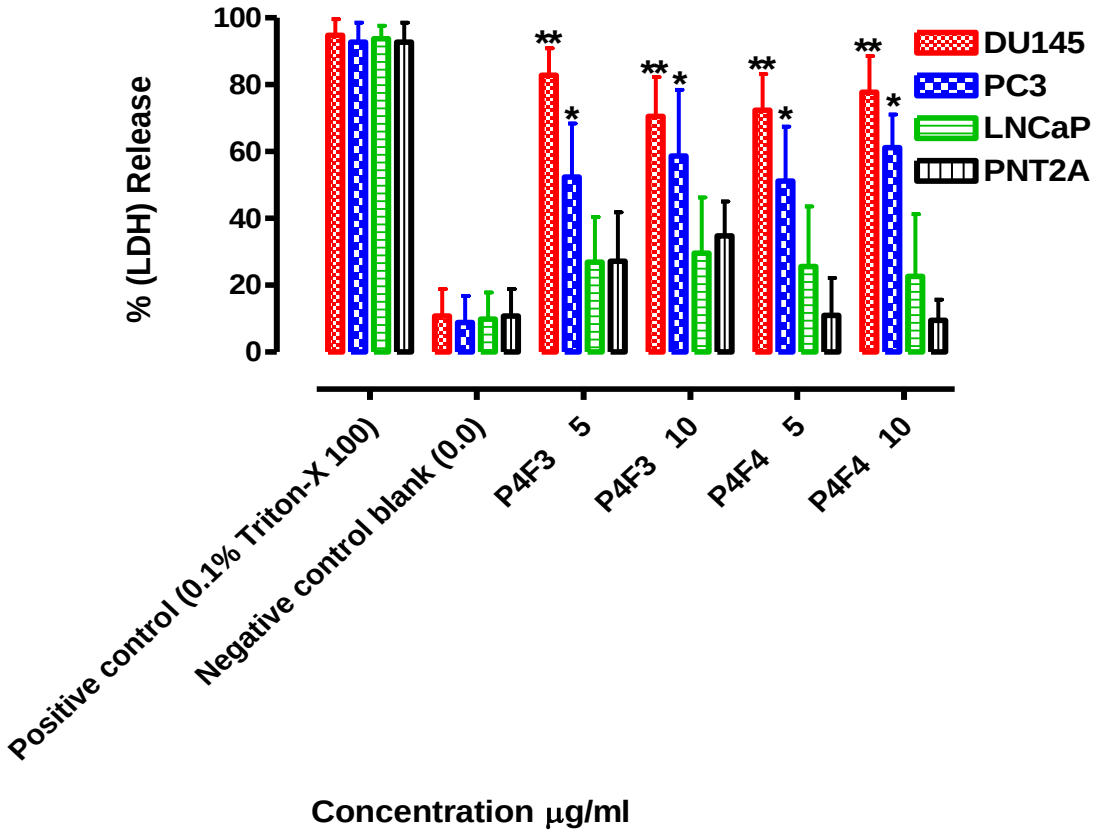



**Figure 4-8:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP, and PNT2A following 2 hours exposure to 5 and 10µg/ml of sub-fractions P4F3 and P4F4 from *Naja pallida* snake venom in complete media at 37$^{o}$C. Data represent mean ± S.E.M. of three experiments.*$p < 0.05$, significantly different from untreated control group. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.1.2.1.1 Morphological analysis

Morphological alterations induced by *N. pallida* venom sub-fractions were also tested in DU145, PC3, LNCaP and PNT2A cell lines. Untreated cells were thin and elongated with two tapering ends (Figure 4-9, 4-10, 4-11 and 4-12). Incubation with 5 and 10µg/ml of *N. pallida* sub-fractions P4F3 and P4F4 venom showed that PC3 cells lost their common elongated shape and appeared in a form of numerous roughly rounded cells of variable size (Figure 4-10). Areas devoid of cells were also recorded with DU145. After the incubation with 5 and 10µg/ml of the *N. pallida* sub-fractions, the DU145 cells fragmented. Few rounded cells and some areas devoid of cells were also noticed (Figure 4-9). In line with the results from the LDH release assay, much less morphological damage was observed in LNCaP and PNT2A cells exposed to sub-fractions 3 and 4 (Figure 4-11 and 4-12).

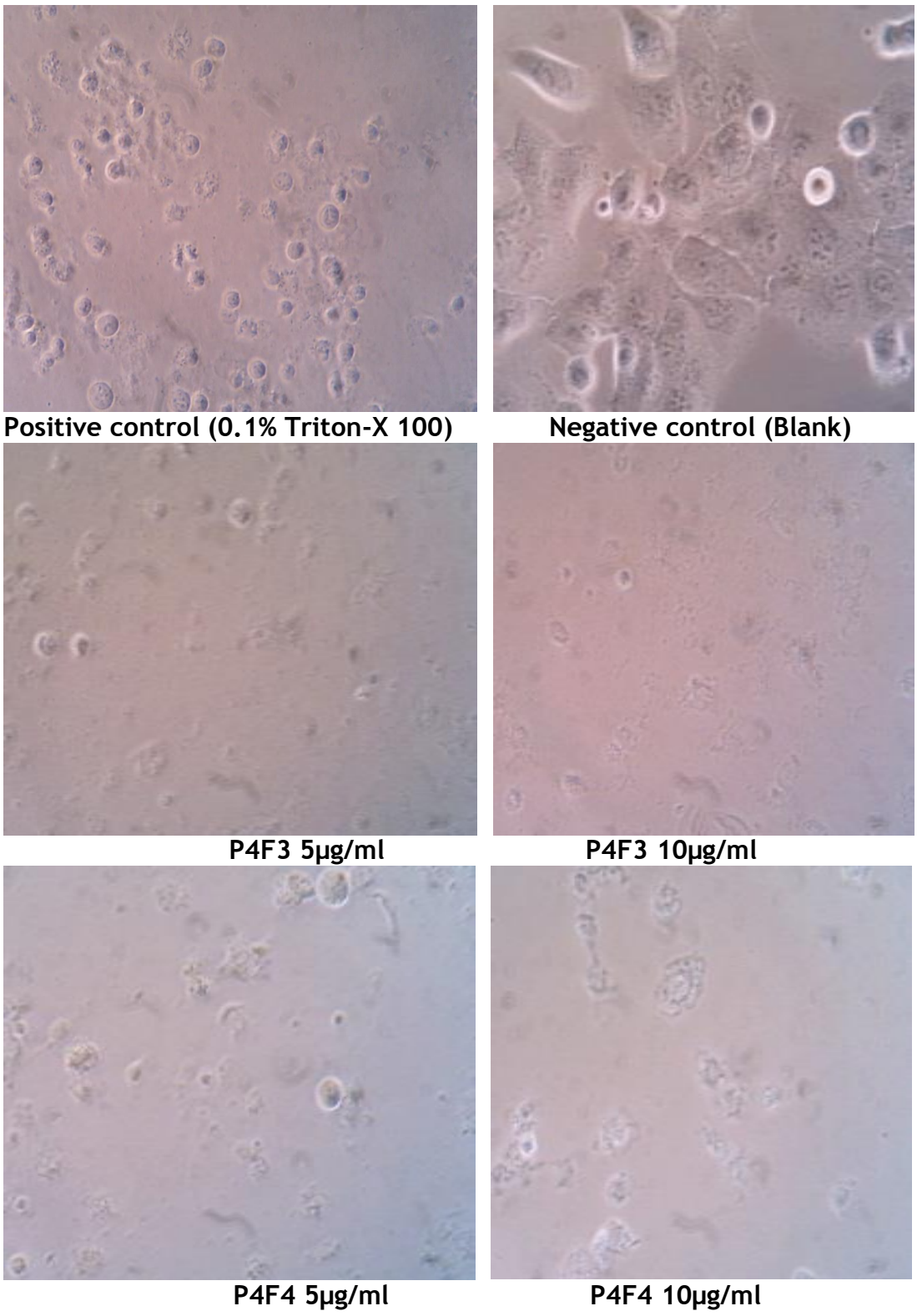



**Figure 4-9:** Light micrographs of cultured cell line DU145 following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fraction P4F3 and P4F4 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

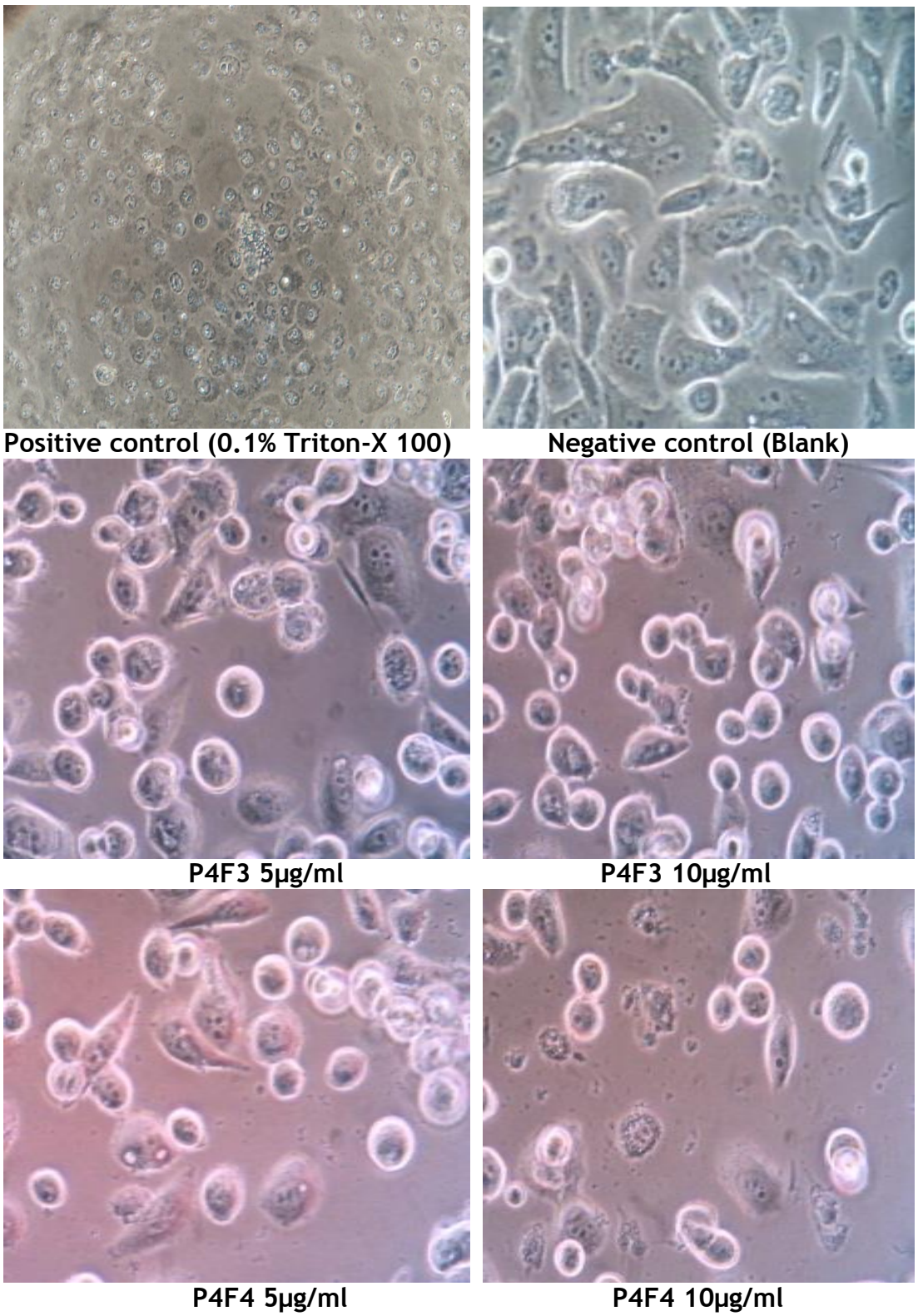



**Figure 4-10:** Light micrographs of cultured cell line PC3 following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fraction P4F3 and P4F4 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

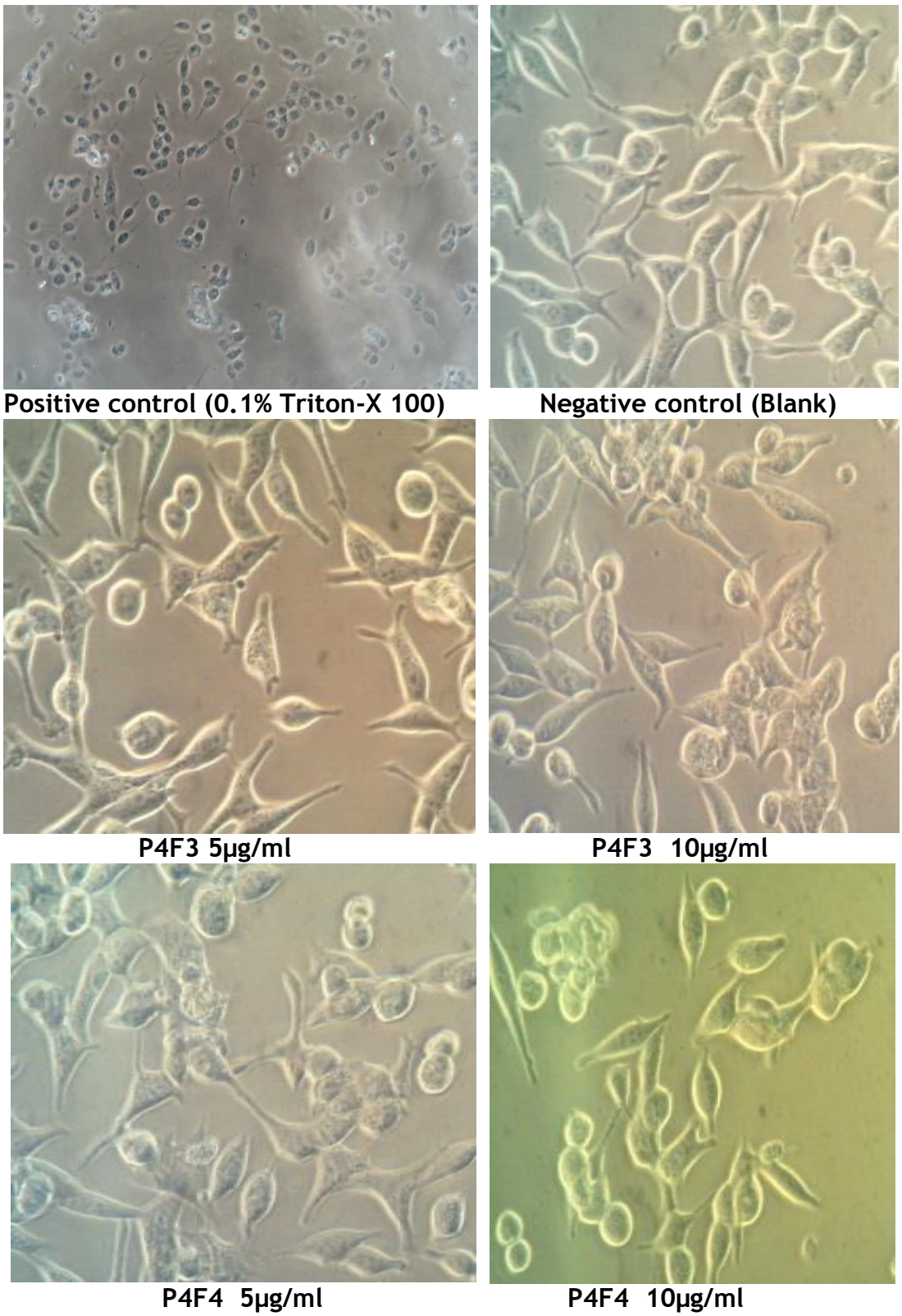



**Figure 4-11:** Light micrographs of cultured cell line LNCaP following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fraction P4F3 and P4F4 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

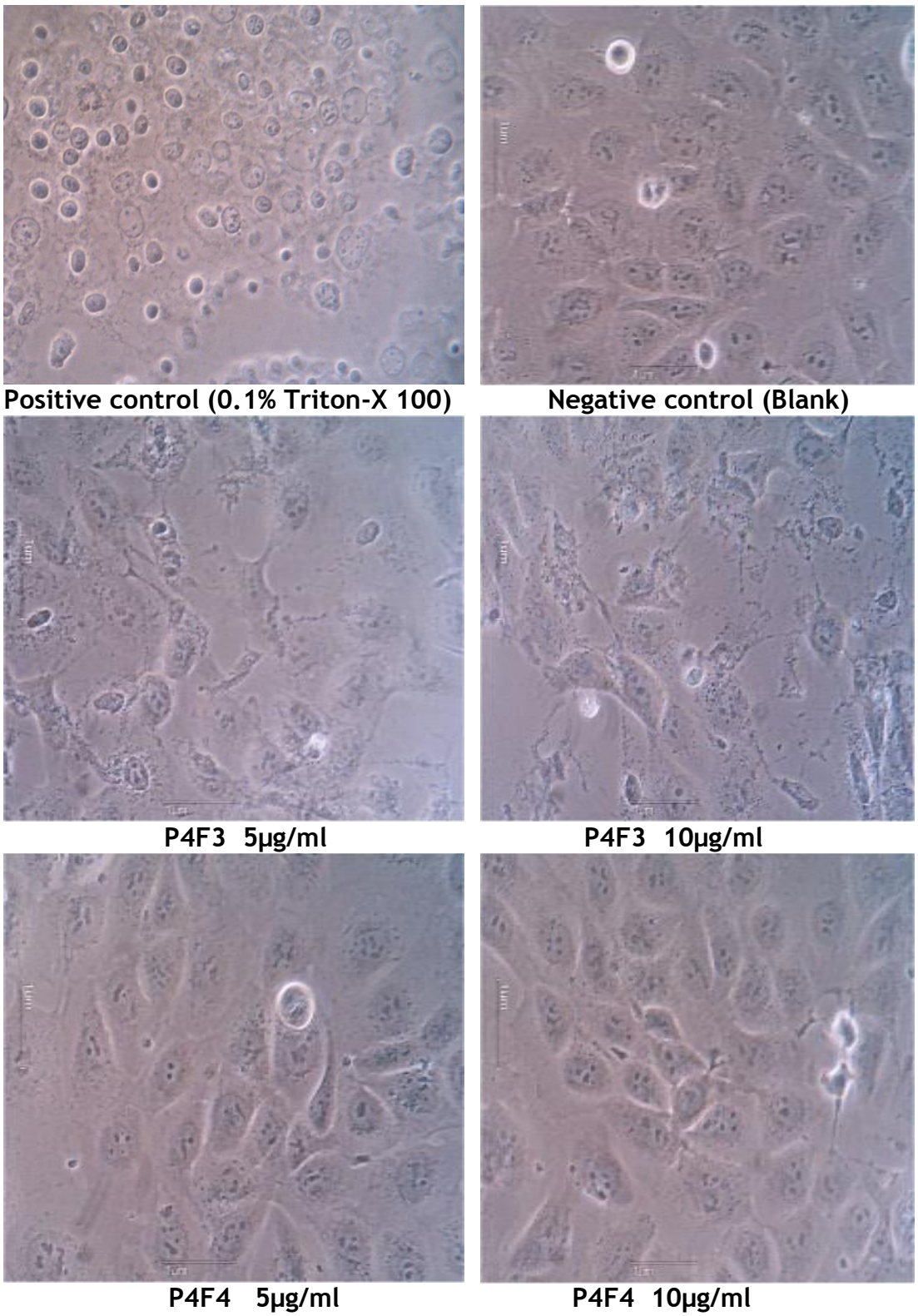



**Figure 4-12:** Light micrographs of cultured cell line PNT2A following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fraction P4F3 and P4F4 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

#### 4.3.1.3 HPLC Separation of *Naja pallida* venom sub-fractions

The two most active sub-fractions of *N. pallida* venom fraction 4, namely sub-fraction 3 (P4F3) and sub-fraction 4 (P4F4), were further separated by HPLC. The reverse phase Phenomenex column Luna 5µ C18 (250 × 4.60 mm) was used. The sub-fraction 3 (P4F3) gave two major peaks at 280nm absorbance and both peaks were collected and named as P4F3-1 and P4F3-2 (Figure 4-13). These purified fractions were again tested for LDH release and morphological changes to find out their effectiveness against cell lines DU145, PC3, LNCaP and PNT2A. These purified fractions were also used for sequencing analysis (Chapter 5).

Sub-fraction 4 (P4F4) gave three major peaks at 280nm absorbance and these peaks were collected, named as P4F4-1, P4F4-2 and P4F4-3 (Figure 4-13) and used for further analysis. The LDH assay was carried out of major purified fraction named P4F3-2, P4F4-1 and P4F4-2. These purified fractions (P4F3-1, P4F3-2, P4F4-1, P4F4-2 and P4F4-3) also used for sequencing analysis. The details of sequencing analysis are given in Chapter 5.

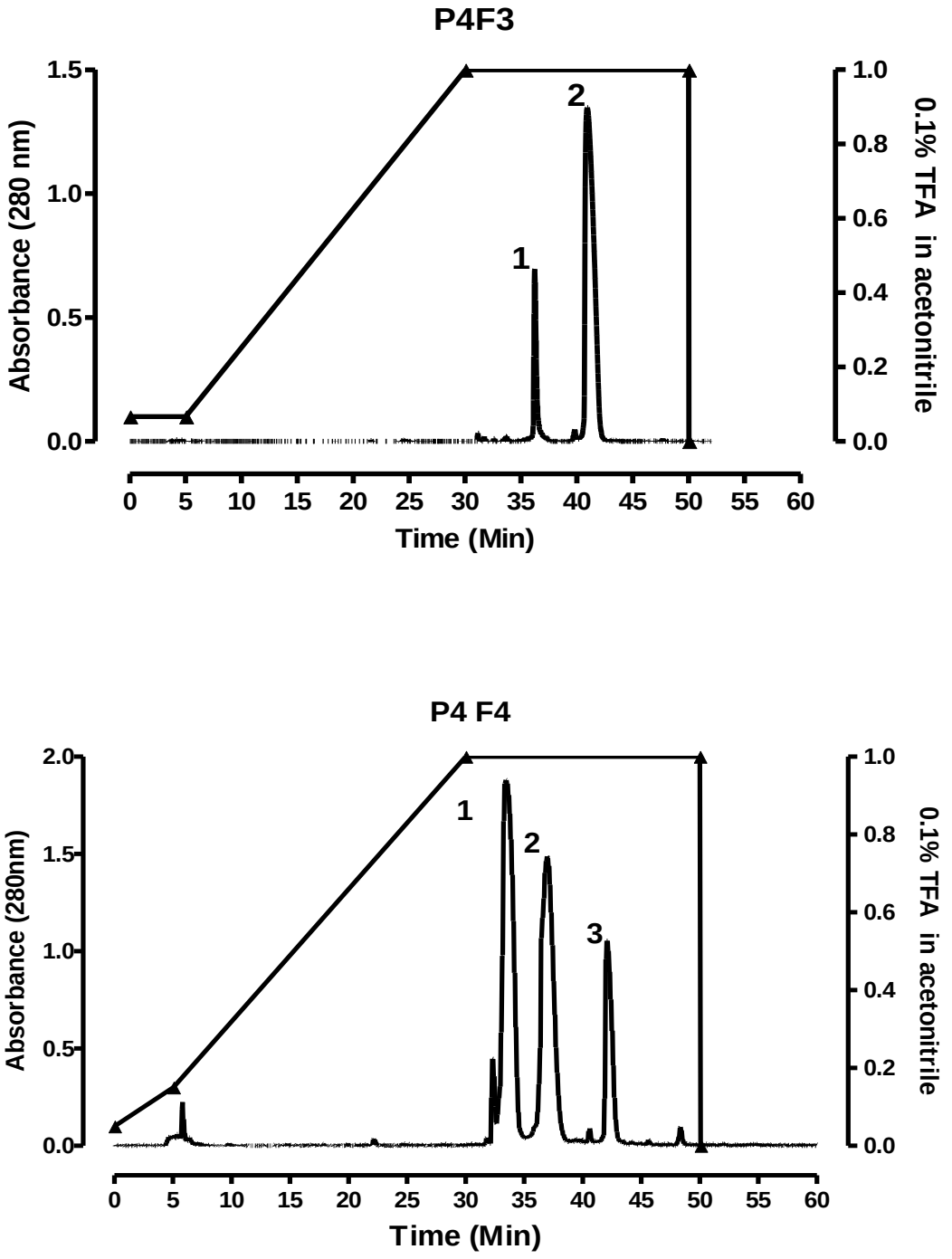



**Figure 4-13:** Purification *Naja pallida* sub-fractions 3 (P4F3) and 4 (P4F4) by reverse-phase HPLC. A Luna 5 µ C18 Phenomenex column (250 × 4.60 mm), under linear gradient flow 0.1% (v/v) and the buffer (0.1%trifluoroacetic acid in water) and (0.1% TFA in acetonitrile) at a flow rate of 1ml/min and monitored at 280nm.

#### 4.3.1.3.1 Effect of *Naja pallida* venom sub-fractions isolated from HPLC on DU145, PC3, LNCaP and PNT2A cell lines for 2 hours by LDH assay

The major purified peaks P4F3-2, P4F4-1 and P4F4-2 were selected for LDH assay to investigate cytotoxic effects on the different cell lines, DU145, PC3, LNCaP and PNT2A. The cells were treated with 5 and 10µg/ml fractions P4F3-2, P4F4-1 and P4F4-2 for 2 hours. As shown in Figure 4-14, fractions P4F3-2, P4F4-1 and P4F4-2 of *N. pallida* were very active against PC3 cells at both concentrations. LDH release was more than 50% and this increment was significant as compared.

LDH release was only 5% in PC3 cells. LDH release was significantly increased in LNCaP cells each of the three fractions at 5µg/ml and 10µg/ml, except P4F4-1at 5µg/ml. There was no significant increment LDH release in the other two cell lines DU145 and PNT2A, being equivalent to negative control values (Figure 4-14).

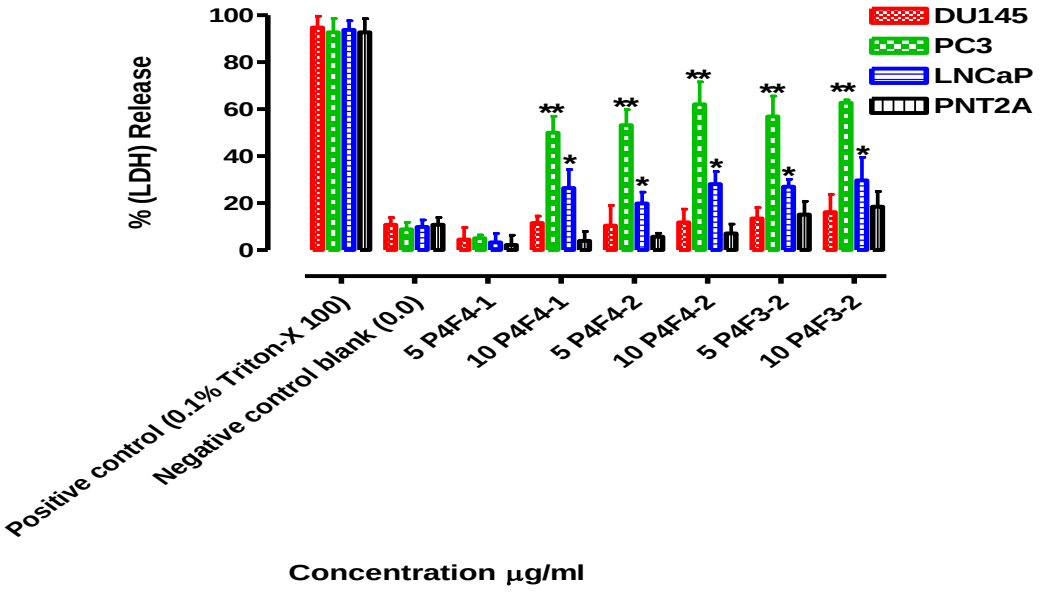



**Figure 4-14:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP and PNT2A following 2 hours exposure to 5 and 10µg/ml of sub-fractions from HPLC separation of *Naja pallida* snake venom peak 4, fractions 3 and 4 incubation in complete media at 37$^{o}$C. Data represent mean ± S.E.M. of 3 experiments.*$p < 0.05$, significantly different from untreated control group. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.1.3.1.1 Morphological analysis

Morphological alterations induced by *N. pallida* sub-fractions P4F3-2, P4F4-1 and P4F4-2 were also recorded in DU145, PC3, LNCaP and PNT2A cell lines. Untreated cells were thin and elongated with two tapering ends (Figures 4-15 to 4-18). Incubation with 5 and 10µg/ml of P4F3-2, P4F4-1 and P4F4-2 showed that DU145 cells did not completely lose their common elongated shape although numerous roughly rounded cells of variable size appeared (Figure 4-15). Clear morphological changes in PC3 cells were seen after the incubation with 5 and 10µg/ml of P4F3-2, P4F4-1 and P4F4-2 (Figure 4-16). Similarly, LNCaP cells showed morphological changes with clear shrinkage with P4F3-2 at 10µg/ml and P4F4-2 at 10µg/ml (Figure 4-17). Finally, PNT2A cultures showed spherical and shrinkage cells after P4F3-2 at 5µg/ml and 10µg/ml (Figure 4-18). Little change was seen with P4F4-1 and P4F4-2.

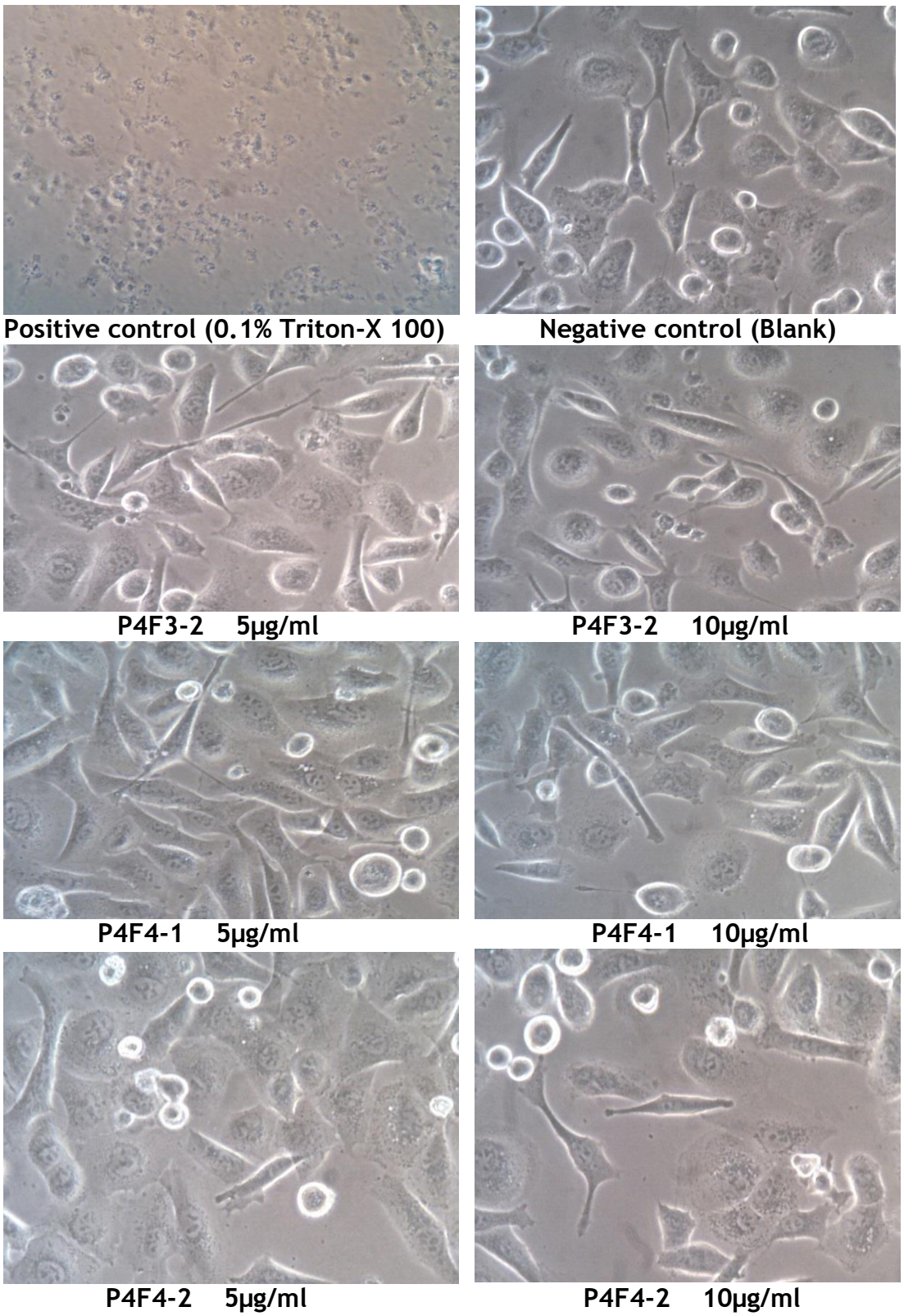



**Figure 4-15:** Light micrographs of cultured cell line DU145 following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fractions P4F3-2, P4F4-1 and P4F4-2 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

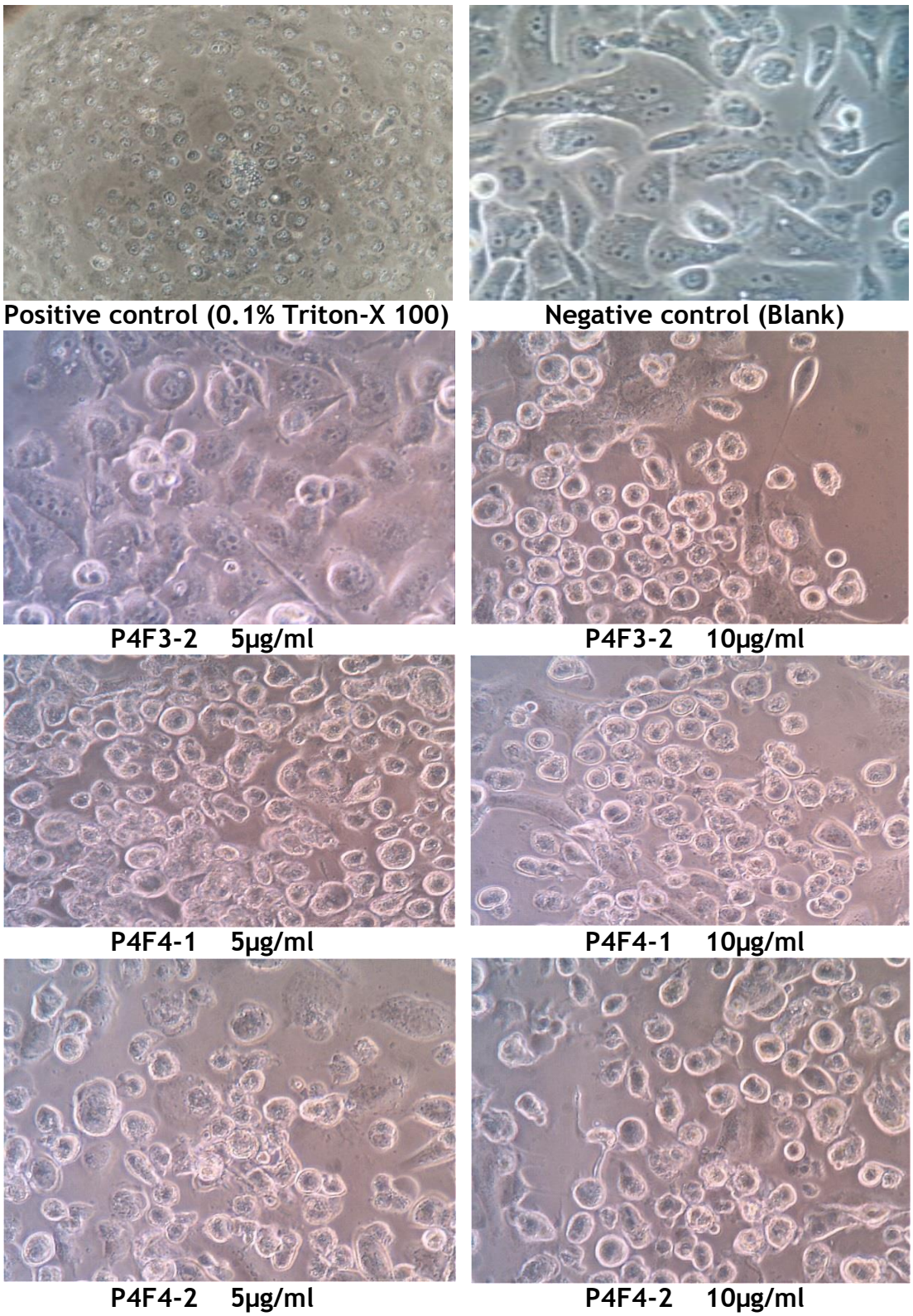



**Figure 4-16:** Light micrographs of cultured cell line PC3 following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fractions P4F3-2, P4F4-1 and P4F4-2 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

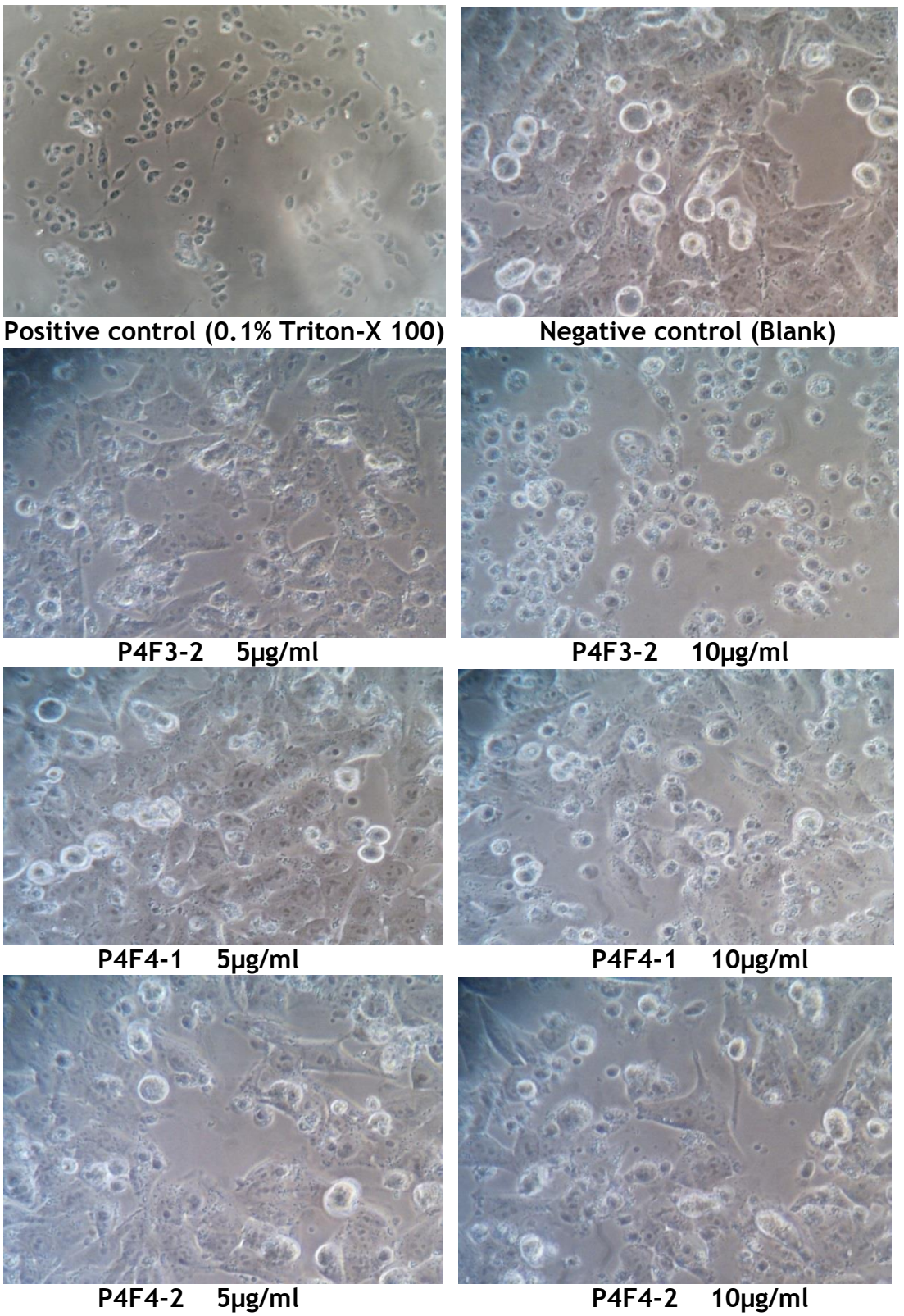



**Figure 4-17:** Light micrographs of cultured cell line LNCaP following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fractions P4F3-2, P4F4-1 and P4F4-2 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

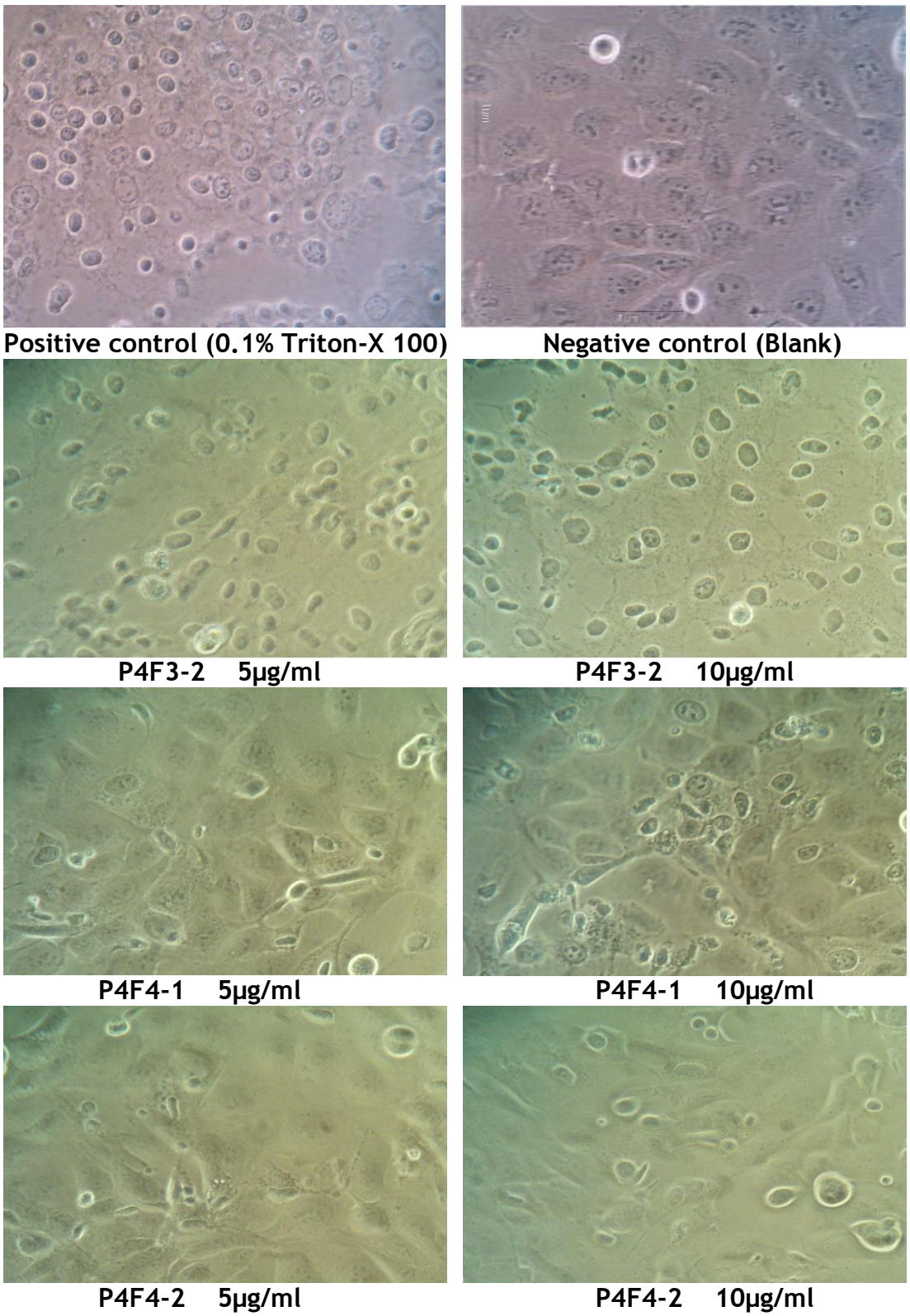



**Figure 4-18:** Light micrographs of cultured cell line PNT2A following 2 hours exposure to 5 and 10µg/ml of *Naja pallida* sub-fractions P4F3-2, P4F4-1 and P4F4-2 in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### *4.3.2* Gel filtration of venom of *Agkistrodon contortrix laticinctus*

Gel filtration was carried out on *A. contortrix laticinctus* venom to fractionate the active constituents of the venom. Venom (500mg) was applied to a Sephadex G-75 column (17 mm x 1000 mm), equilibrated with 0.1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers 1-4 (Figure 4-19). The four major fractions (F1, F2, F3, and F4) were freeze-dried before testing on the prostate cell lines.

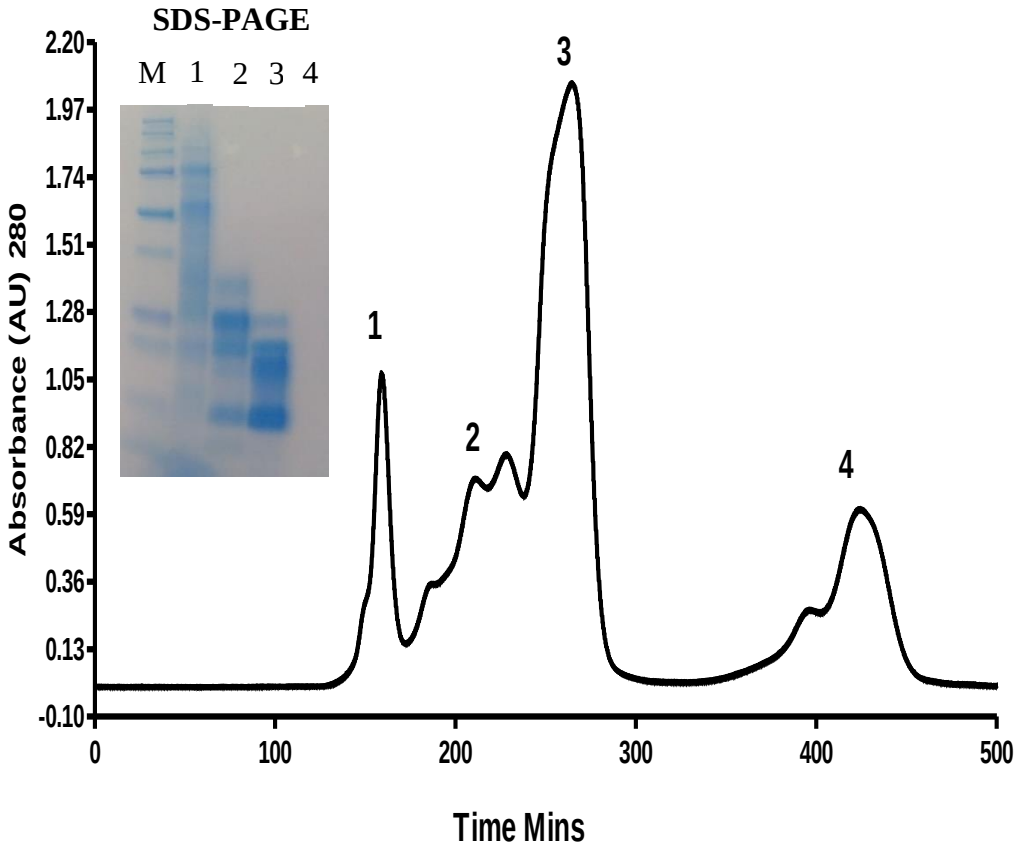



**Figure 4-19:** Fractionation of *A. contortrix laticinctus* venom by Sephadex G-75 column chromatography 500 mg venom in 17 mm x 1000 mm, equilibrated with 0.1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers. Inset: Electrophoretic profile of *A. contortrix laticinctus* fractions. Molecular weight markers (0.4µg) were loaded in lane M and venom fractions (10µg) were loaded onto lanes 1-4 of a 12% (w/v) polyacrylamide gel. More details of the SDS-PAGE are given in Figure 4-49.

#### 4.3.2.1 Effect of *A. contortrix laticinctus* venom fractions on DU145, PC3, LNCaP and PNT2A cell lines for 2 hours by LDH assay

The four fractions F1, F2, F3 and F4 from *A. contortrix laticinctus* venom were tested at 20μg/ml for LDH release from prostate cancer cell lines DU145, PC3 and LNCaP and normal prostate cell line PNT2A (Figure 4-20). Positive and negative controls were also used in parallel with each test. The results showed that fraction F1 was not effective against DU145, LNCaP and PNT2A cells because little LDH release was observed, but it was slightly effective against PC3 cells (% LDH release was 37%), although this was not significant as compared to positive control. Fractions F2 and F3 were effective against DU145, PC3 and LNCaP cells but they were not effective against the normal cell line PNT2A. With F2, the % LDH release was 29%, 39% and 43% observed in DU145, PC3 and LNCaP cell lines respectively, while it was 10% LDH in PNT2A cell line which was less than even negative control. With F3, the % release was 82% (PC3), 69% (LNCaP) and 42% (DU145), while it was 9.5% in PNT2A cells. F4 had no significant effect against any cell line (Figure 4-20).

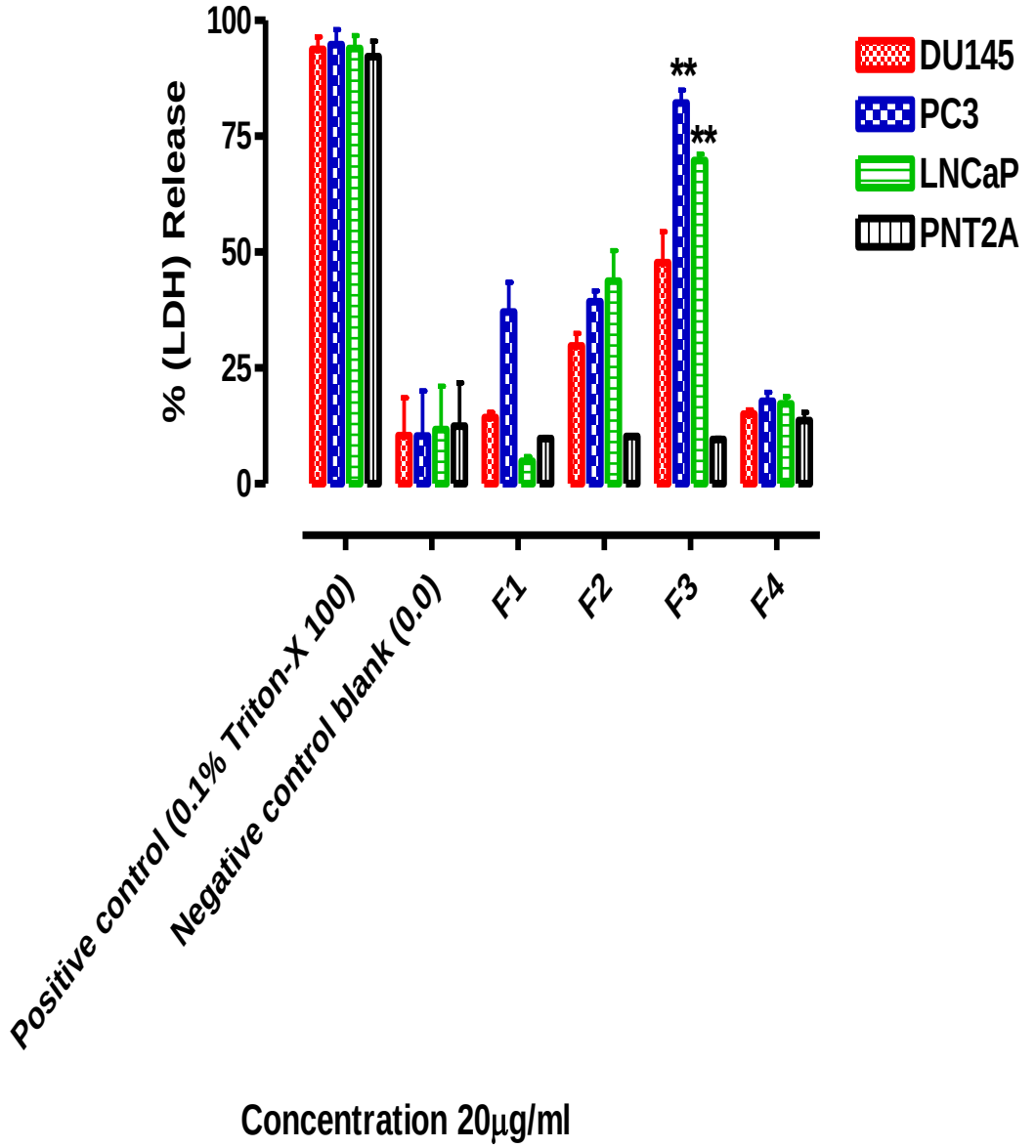



**Figure 4-20:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP and PNT2A following 2 hours exposure to (20µg/ml of sub-fractions) *A. contortrix laticinctus* snake venom in complete media at 37$^{o}$C. Data represent mean ± S.E.M. of three experiments.*p < 0.05, significantly different from untreated control group. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.2.1.1 *Morphological analysis*

Untreated DU145, PC3, LNCaP and PNT2A cell lines were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 4-21; 4-22; 4-23 and 4-24). However, after incubating these cell lines with *A. contortrix laticinctus* venom fractions for 2 hours, the occurrence of various morphological abnormalities was observed. The disappearance of normal morphological characteristics was very obvious with F1, F2 and F3 (but not with F4) applied to DU145, PC3 and LNCaP cells (Figures 4-21; 4-22 and 4-23) with cells showing severe shrinkage and condensation of their cellular contents. Most of the cells lost their characteristic appearance and coalesced to such an extent that individual cells could not be identified. Effects on PNT2A cells were much less obvious (Figure 4-24).

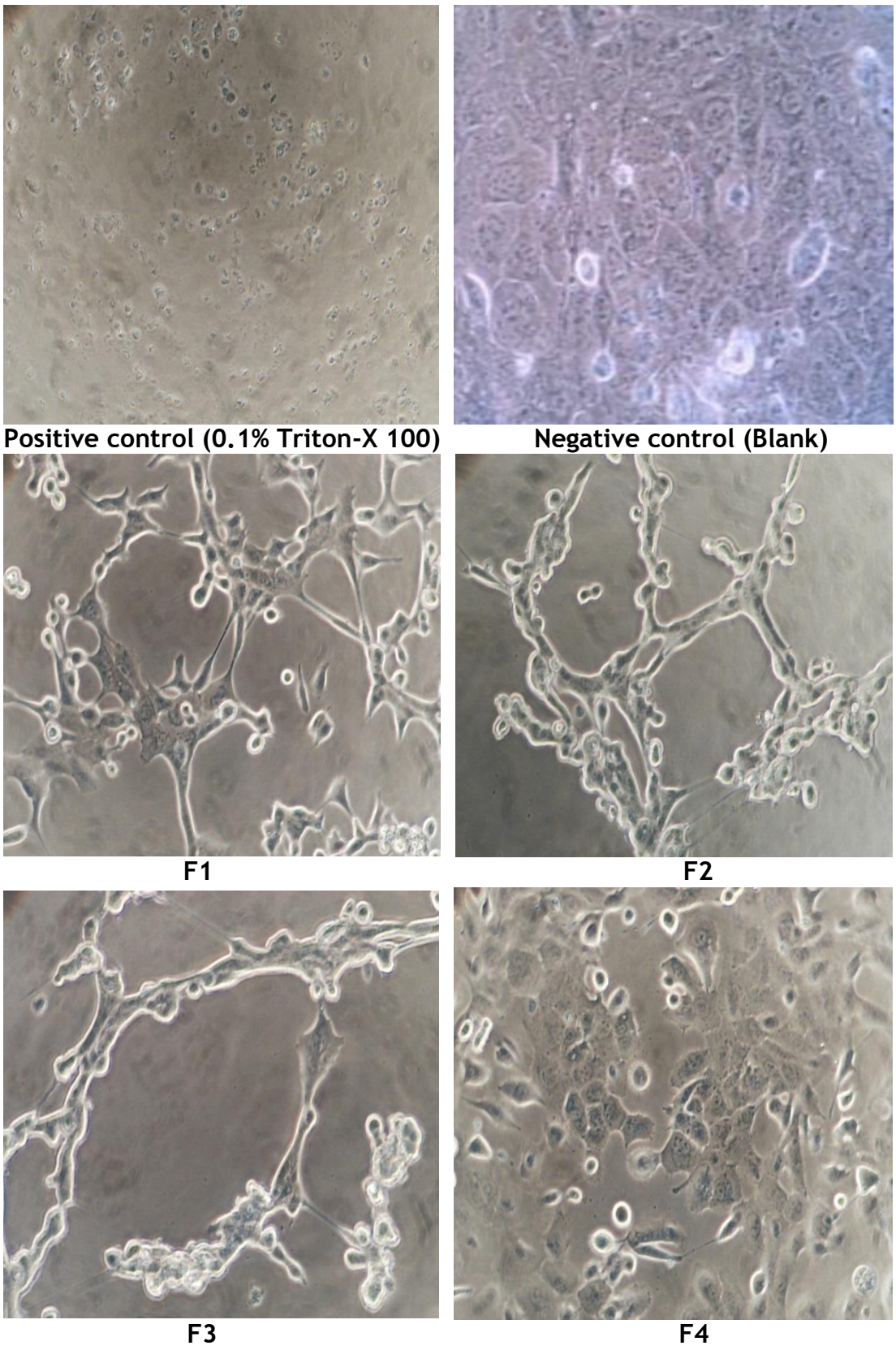



**Figure 4-21:** Light micrographs of cultured cell line DU145 following 2 hours exposure to 20µg/ml of *A. contortrix laticinctus* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

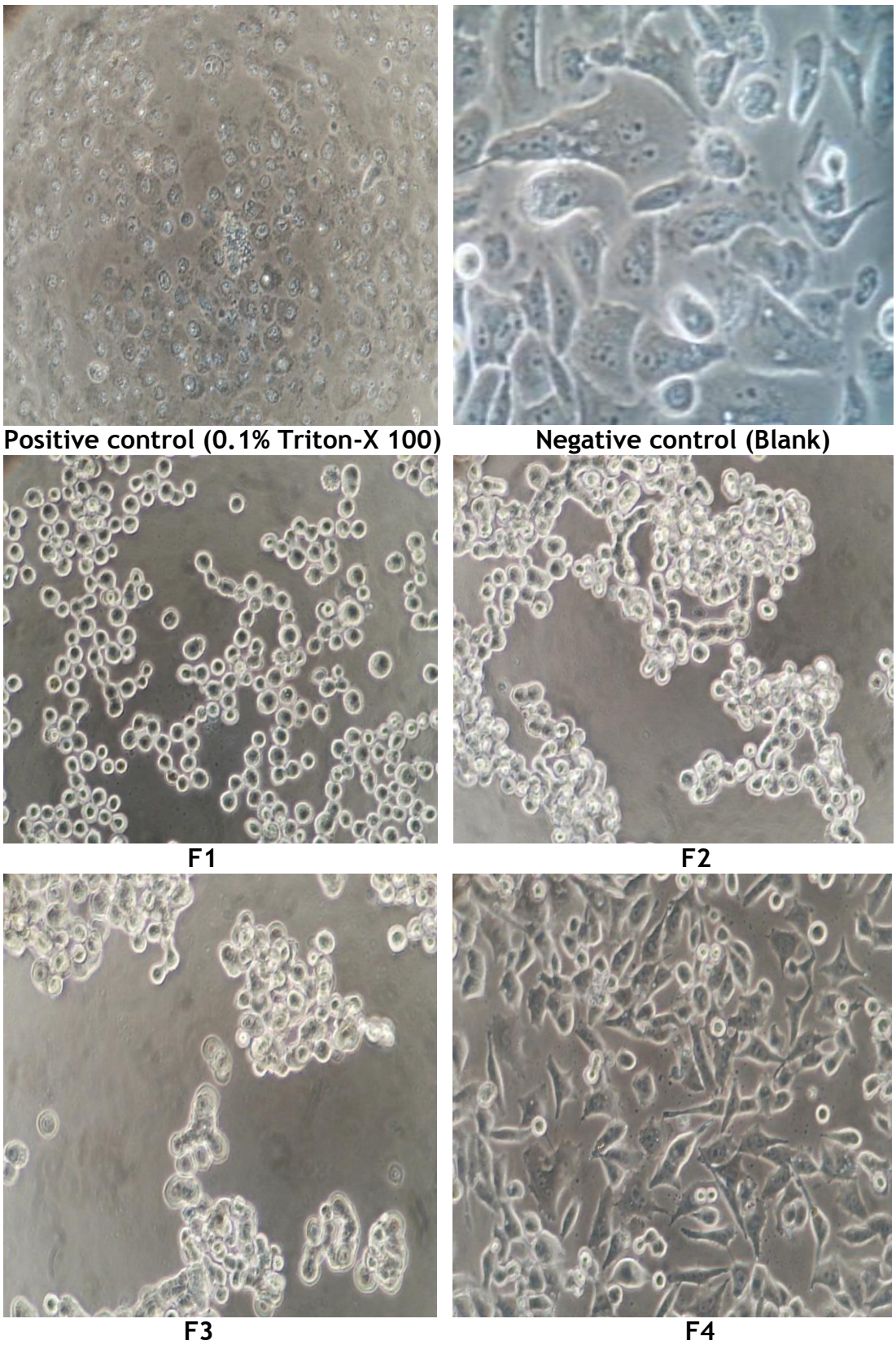



**Figure 4-22:** Light micrographs of cultured cell line PC3 following 2 hours exposure to 20µg/ml of *A. contortrix laticinctus* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

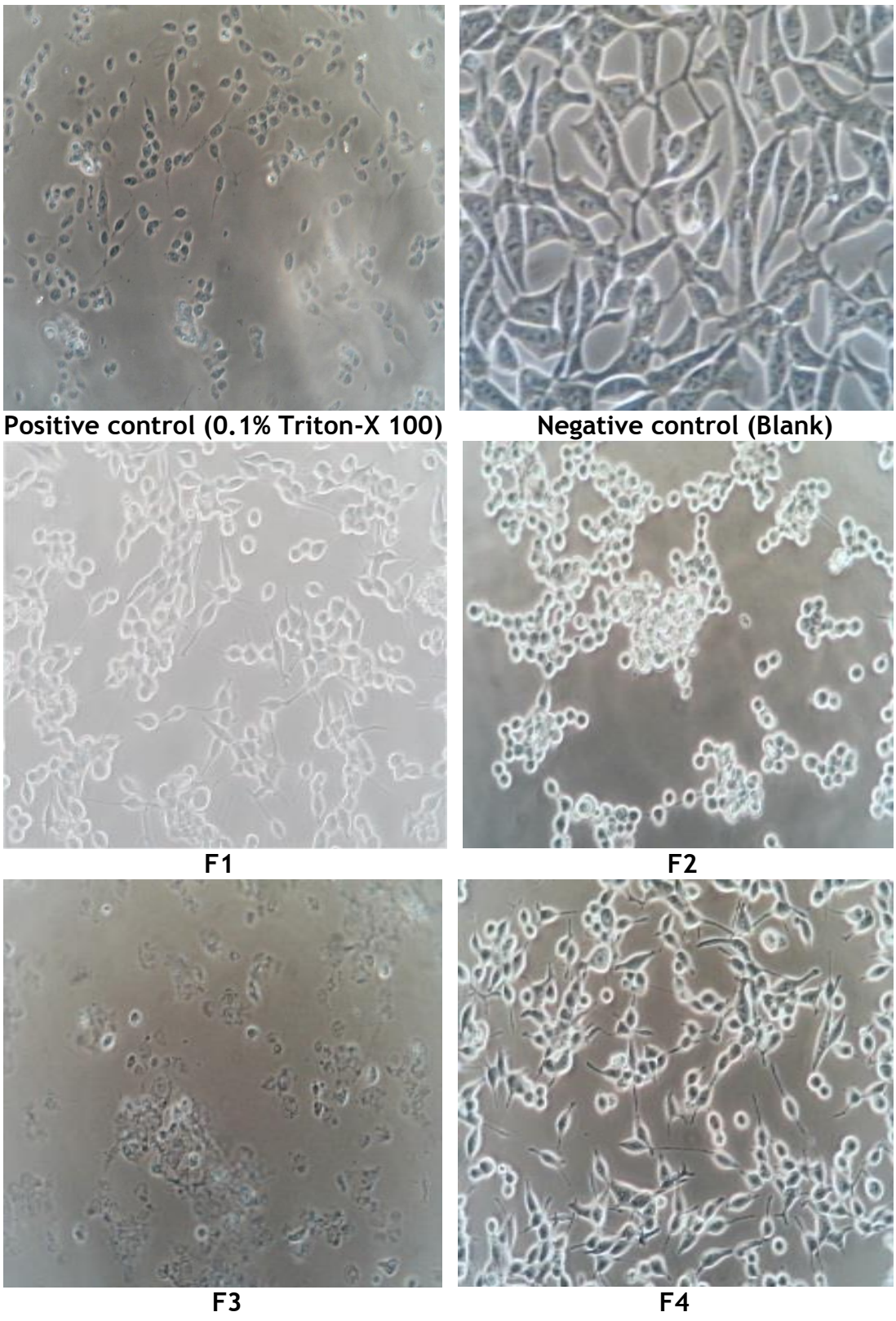



**Figure 4-23:** Light micrographs of cultured cell line LNCaP following 2 hours exposure to 20µg/ml of *A. contortrix laticinctus* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

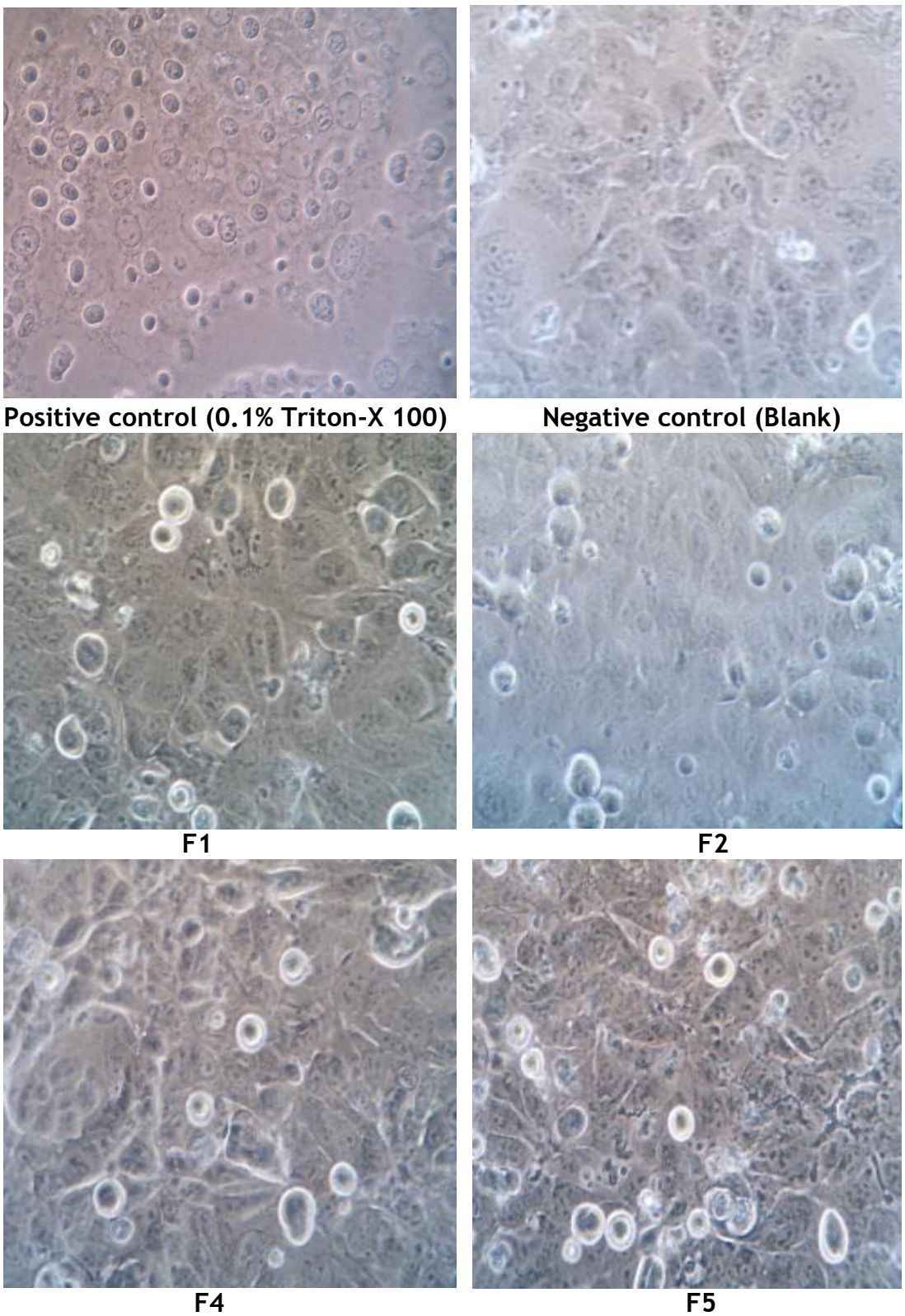



**Figure 4-24:** Light micrographs of cultured cell line PNT2A following 2 hours exposure to 20µg/ml of *A. contortrix laticinctus* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### 4.3.2.2 Ion Exchange separation of *Agkistrodon contortrix laticinctus* fraction 3

The fractionation of *A. contortrix laticinctus* fraction 3 on Sephadex C-50 column allowed the purification of two major fractions, named fractions 3 and 4 (Figure 4-25). Fractions 1 and 2 did not show any powder after freeze-drying. Fractions 3 and 4 were tested with the prostate cell lines.

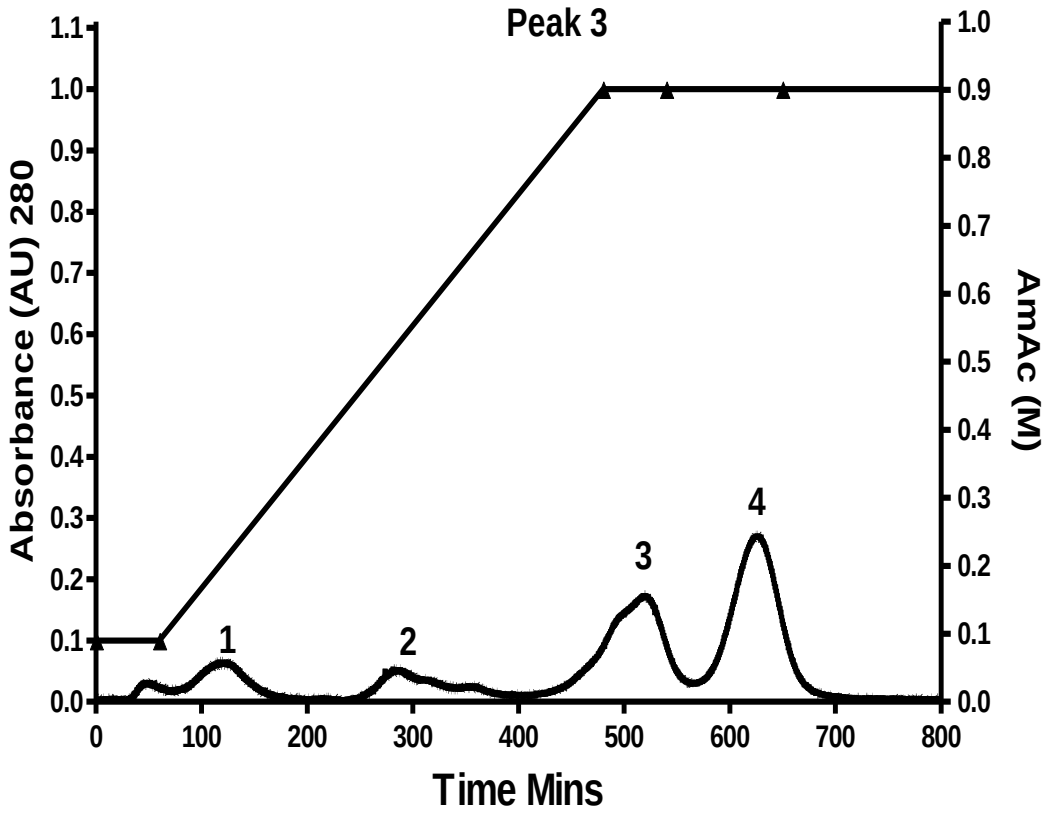



**Figure 4-25:** Fractionation of *A. contortrix laticinctus* venom fraction 3 by Sephadex C-50 ion exchange chromatography. Venom (20mg) was applied to a 142mm X 400mm column and eluted with 0.1 to 1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers.

#### 4.3.2.2.1 Effect of *Agkistrodon contortrix laticinctus* sub-fractions on DU145, PC3, LNCaP and PNT2A cell lines for two hours by LDH assay

LDH assays were performed to investigate cytotoxic effects on the different cell lines (DU145, PC3, LNCaP and PNT2A). The cells were treated with 5 and 10µg/ml of sub-fractions 3 and 4 of *A. contortrix laticinctus* peak 3 for 2 hours (Figure 4-26). As shown in Figure 4-26, these sub-fractions did not cause an increase in LDH release from any of the cell lines.

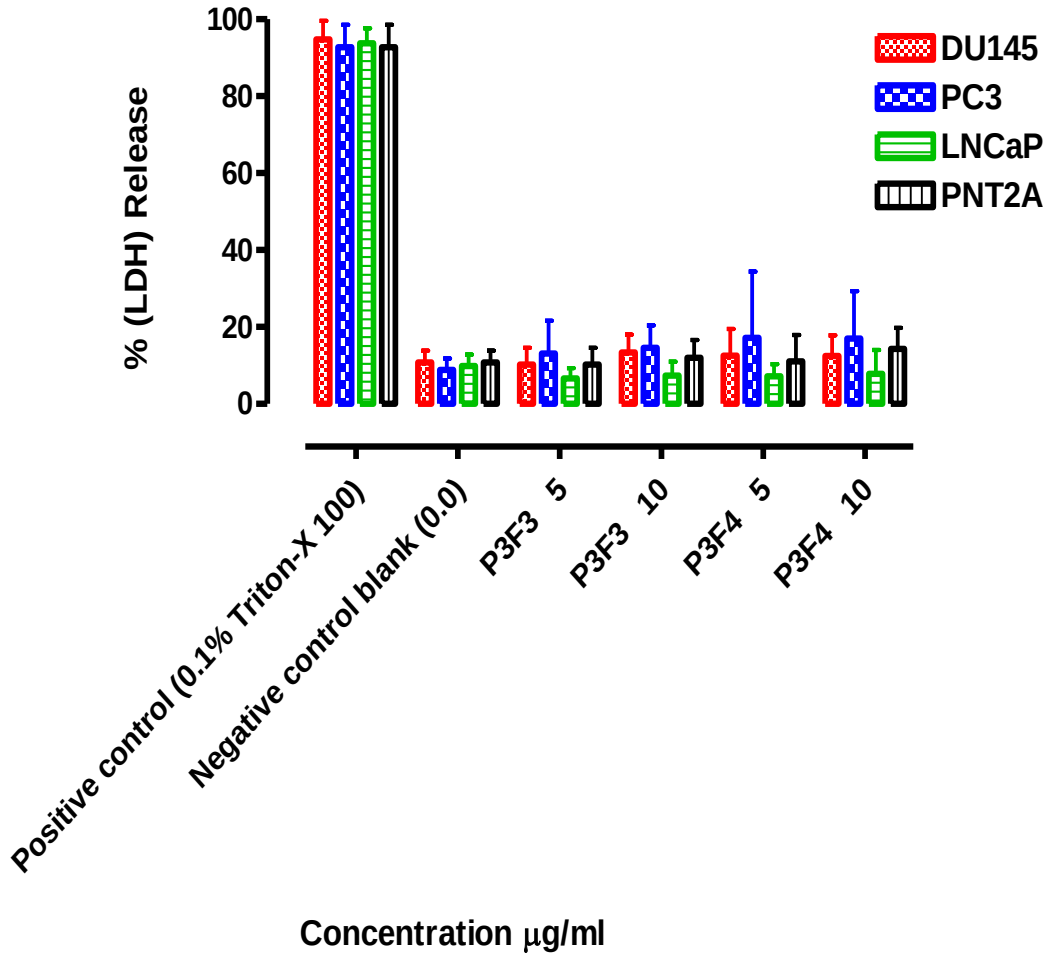



**Figure 4-26:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP and PNT2A following 2 hours exposure to 5 and 10µg/ml of sub-fractions from peak 3 from *A. contortrix laticinctus* snake venom in complete media at 37$^{o}$C. Data represent mean ± S.E.M. of three experiments. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.2.2.1.1 Morphological analysis

Untreated DU145, PC3, LNCaP and PNT2A cells were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 4-27; 4-28; 4-29 and 4-30). However, after incubating the cell lines with *A. piscivorus conanti* sub-fractions venom for 2 hours, slight morphological abnormalities were observed. At 5 and 10µg/ml, the disappearance of normal morphological characteristics was obvious for most cell lines. For example, DU145 cultures had gaps and compacted cells at 5 and 10µg/ml (Figure 4-27), and PC3 cells changed to spherical shapes at 5 and 10µg/ml (Figure 4-28). However, LNCaP and PNT2A were not so clearly changed in appearance (Figures 4-29 and 4-30). The LDH experiment showed no effects of these fractions while the fractions slightly changed the morphology, possibly through effects on cytoskeletal elements.

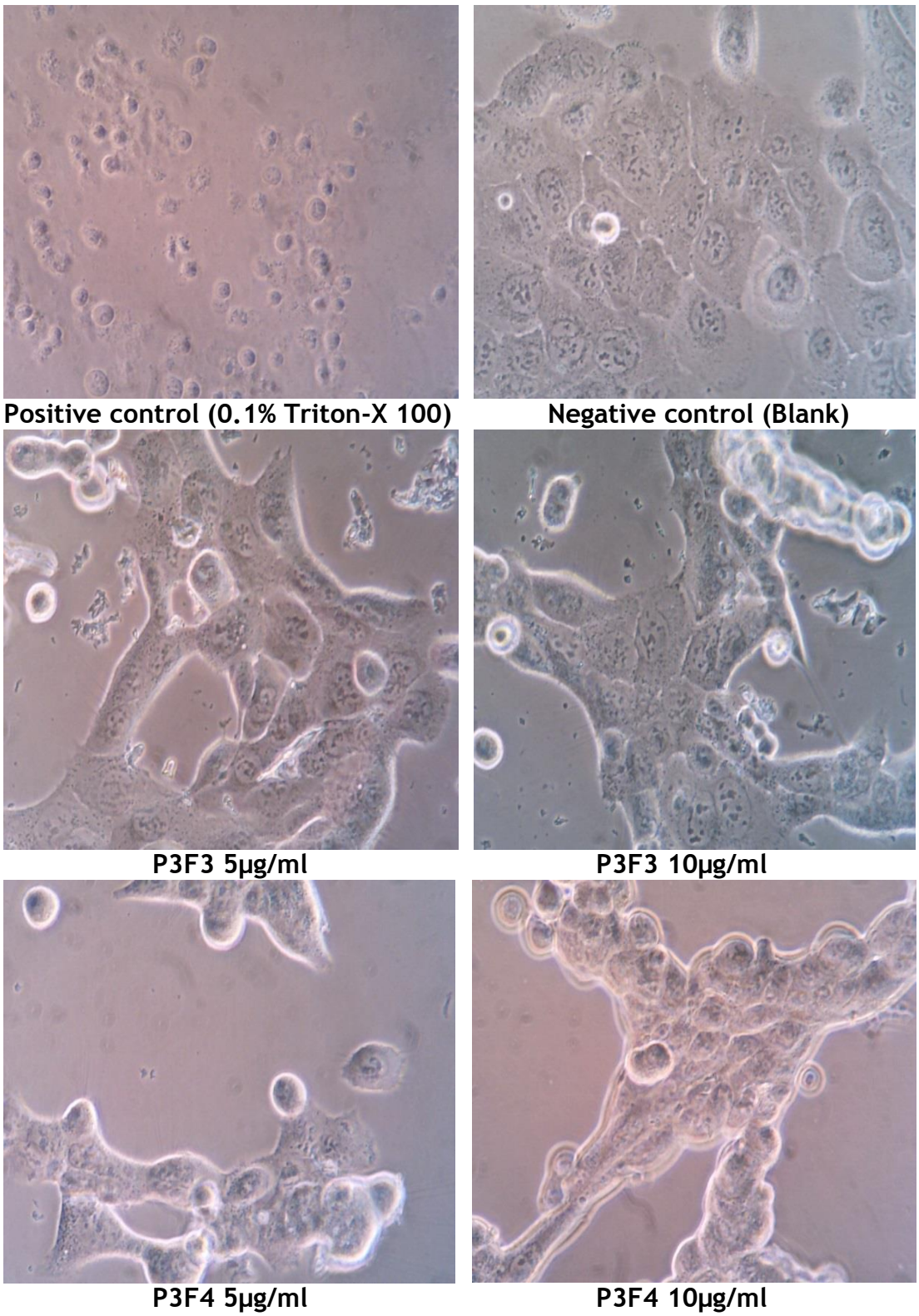



**Figure 4-27:** Light micrographs of cultured cell line DU145 following 2 hours exposure to 5 and 10µg/ml of *A. contortrix laticinctus* sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

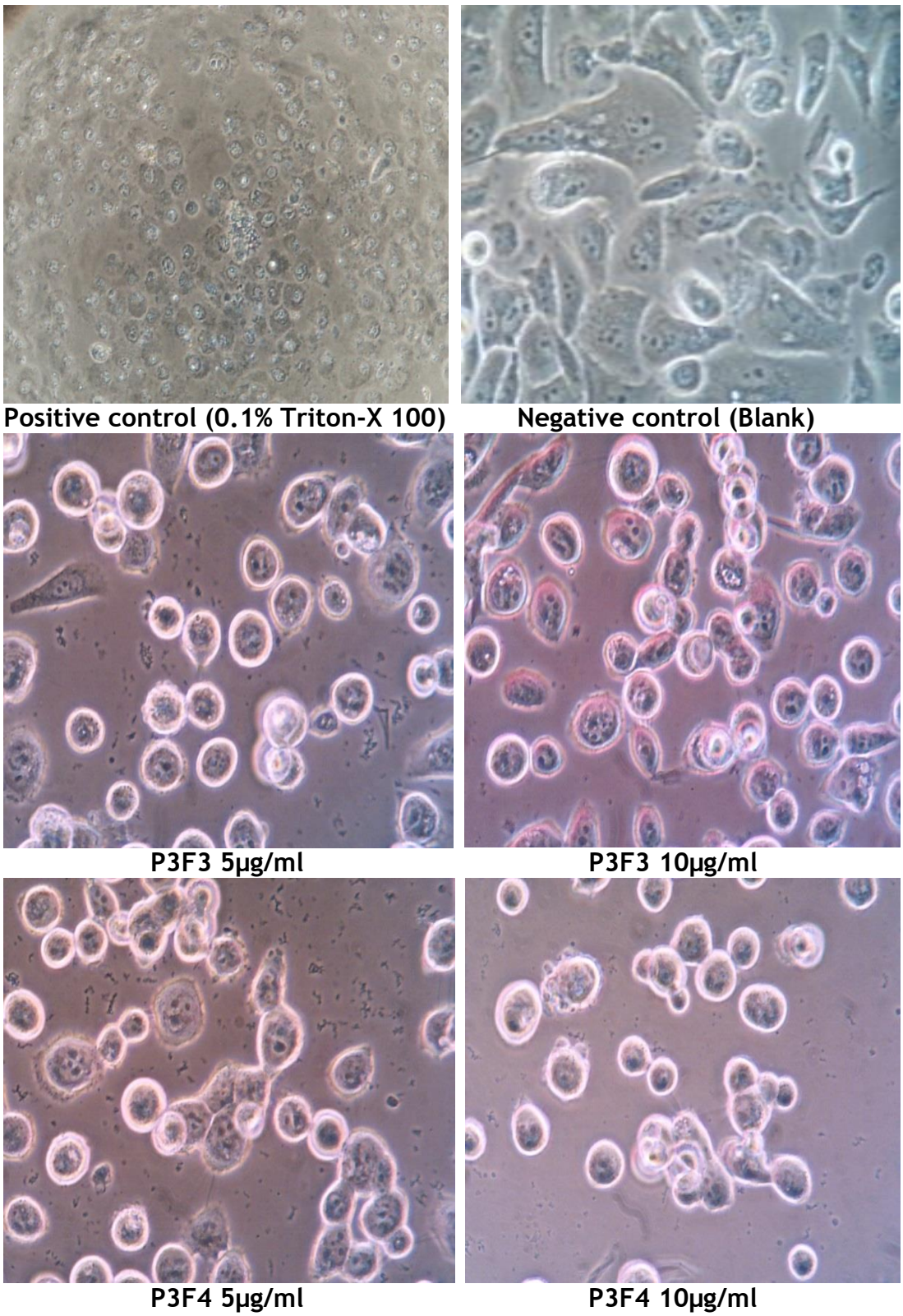



**Figure 4-28:** Light micrographs of cultured cell line PC3 following 2 hours exposure to 5 and 10µg/ml of *A. contortrix laticinctus* sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

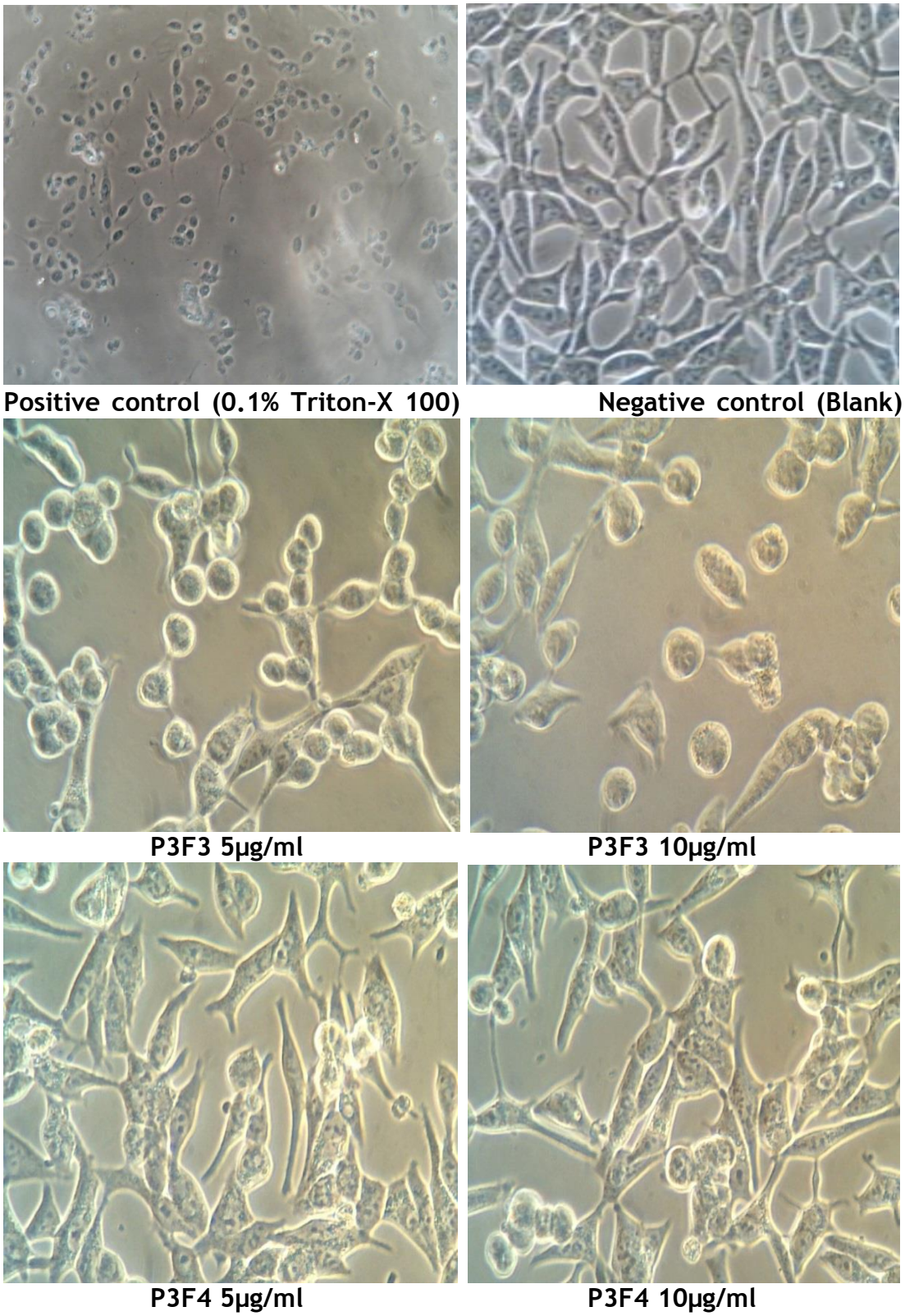



**Figure 4-29:** Light micrographs of cultured cell line LNCaP following 2 hours exposure to 5 and 10µg/ml of *A. contortrix laticinctus* sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

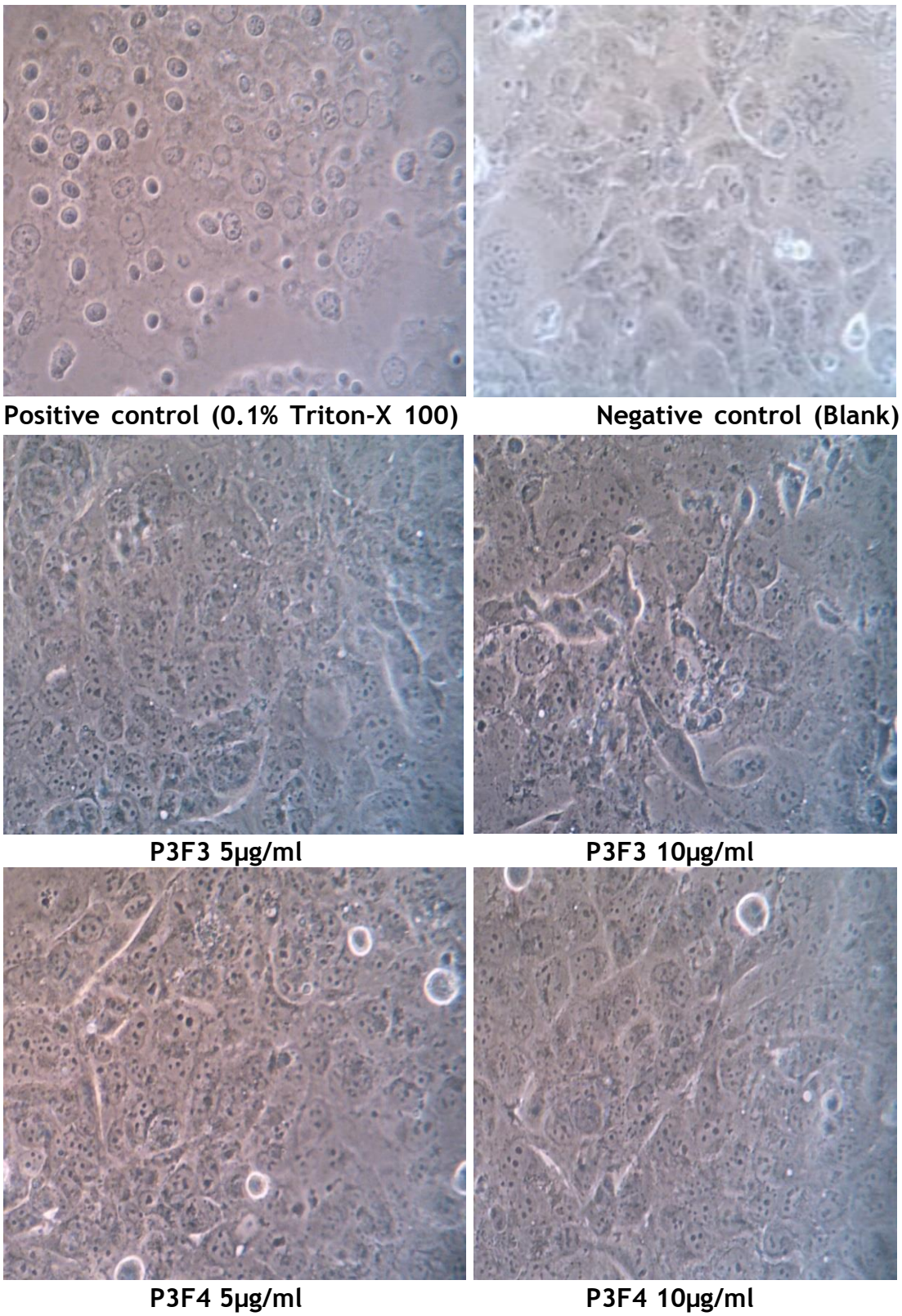



**Figure 4-30:** Light micrographs of cultured cell line PNT2A following 2 hours exposure to 5 and 10µg/ml of *A. contortrix laticinctus* sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### *4.3.3* Gel filtration of venom of *Agkistrodon piscivorus conanti*

Gel filtration of *A. piscivorus conanti* venom (100 mg) was carried out by using a Sephadex G-75 column (17 mm x 1000 mm), equilibrated with 0.1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers 1-6 (F1, F2, F3, F4, F5 and F6), as clearly indicated in Figure 4-31. These fractions were freeze-dried before testing on the cell lines. Fractions 1 to 5 gave visible freeze dried powder but fraction F6 did not freeze dry due to lack of protein. Therefore, five fractions were used for testing on cell lines to find out the effectiveness.

Electrophoretic profiles of the *A. piscivorus conanti* peaks were also established. Venom fractions (10µg) were loaded onto lanes 1-5 of a 12% (w/v) polyacrylamide gel. The details of electrophoretic profile are given later in this chapter (see Figure 4-49).

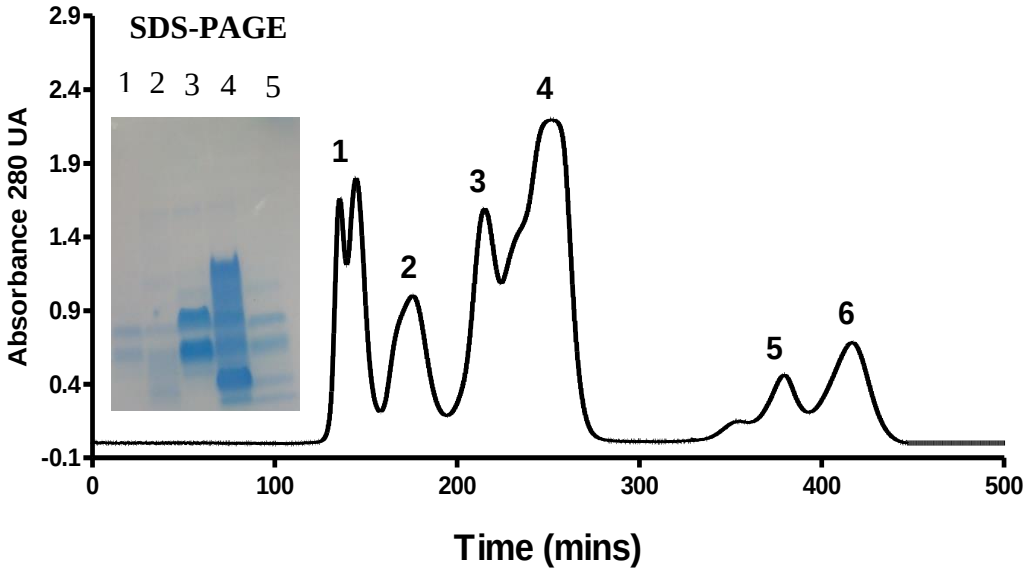



**Figure 4-31:** Fractionation of *Agkistrodon piscivorus conanti* venom by Sephadex G-75 column chromatography. Venom (100 mg) was applied to the column (17 mm x 1000 mm), equilibrated with 0.1M ammonium acetate buffer (pH 6.8). The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers. Inset: Electrophoretic profile of venom peaks (10µg) were loaded onto lanes 1-5 of a 12% (w/v) polyacrylamide gel. More details of the SDS-PAGE are given in Figure 4-49.

#### 4.3.3.1 Effect of *Agkistrodon piscivorus conanti* venom fractions on DU145, PC3, LNCaP and PNT2A cell lines for 2 hours by LDH assay

The five fractions F1, F2, F3, F4 and F5 from *A. piscivorus conanti* venom were tested at 20μg/ml for LDH release against prostate cancer cell lines DU145, PC3, LNCaP and normal prostate cell PNT2A (Figure 4-32). Positive and negative controls were also used in parallel with each test. The results showed that fraction F1 was not effective against DU145, LNCaP and PNT2A cells but it was slightly effective against PC3 cells (LDH release was 41% which was significant as compared to the negative control). F2 was not significantly effective against any cell line (approximately 20% LDH release). F3 was active against PC3 and LNCaP cells, but it was not effective against DU145 and PNT2A cells. The LDH release was 81% and 45% in LNCaP and PC3 cell line respectively, while LDH release of 27% and 23 % was observed in DU145 and PNT2A cells respectively. Fraction F4 induced LDH release in PC3 and LNCaP cells (73% and 70% release respectively), while it there was only 15% and 29% LDH release in DU145 and PNT2A cells respectively. Fraction F5 had no significant effects on any of the cell lines.

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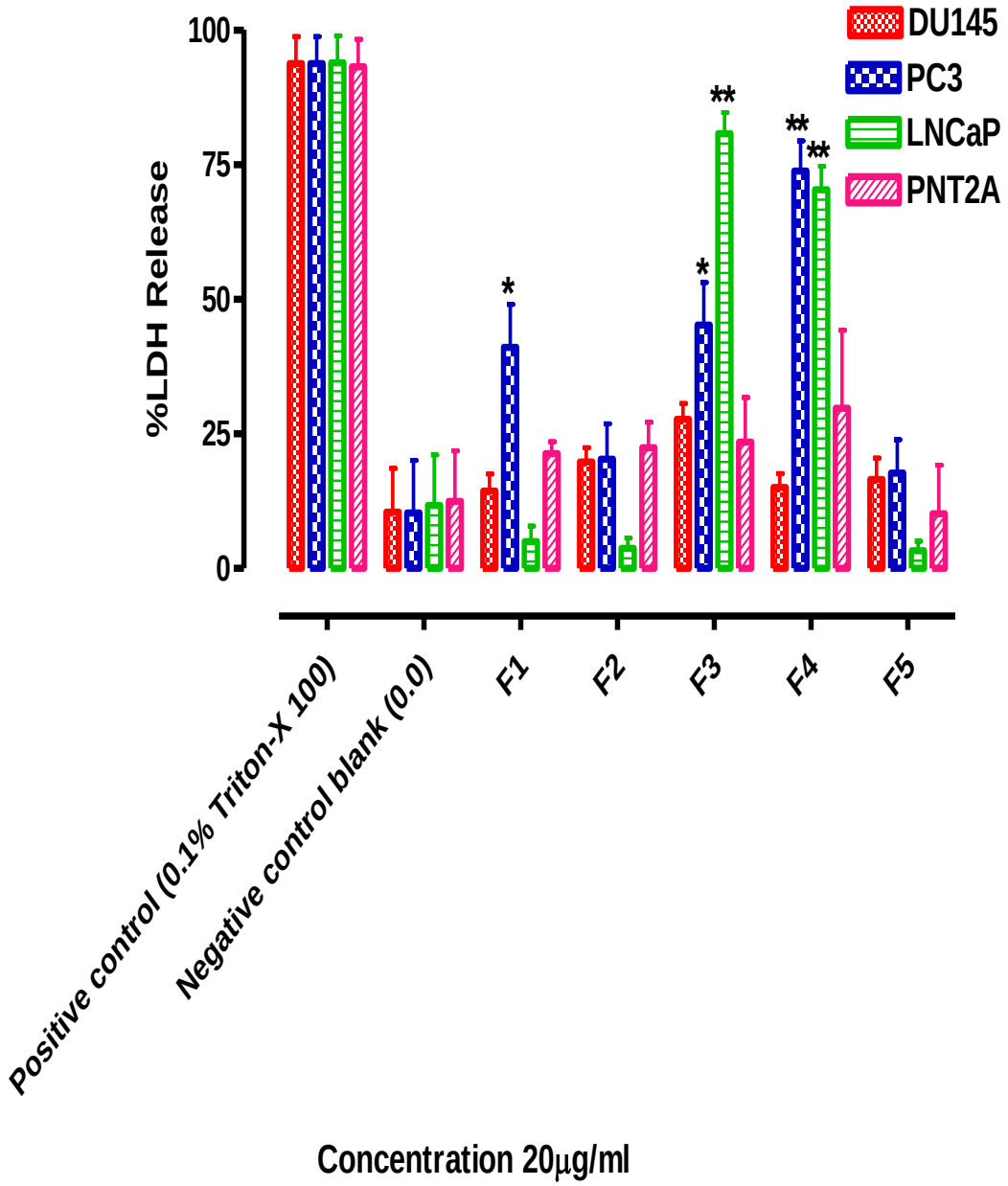


**Figure 4-32:** Changes in LDH release in cultured cell lines DU145, PC3, LNCaP and PNT2A following 2 hours exposure to 20µg/ml of *A. piscivorus conanti* fractions in complete media at 37$^{o}$C. Data represent mean ± S.E.M of three experiments.*p < 0.05 significantly different from untreated control groups. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.3.1.1 *Morphological analysis*

Untreated DU145, PC3, LNCaP and PNT2A cells were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 4-33; 4-34; 4-35 and 4-36). However, after incubating cell lines with *A. piscivorus conanti* fractions for 2 hours, various morphological abnormalities were observed. In DU145 cell cultures, disruptions to the cell monolayers were seen after exposure to F3 and F4 (Figure 4-33). With PC3 cells, the changes were mainly a rounding up of cells, and these were found with F1, F2, F3, F4 and, to a smaller extent, F5 (Figure 4-34). With LNCaP cells, exposure to the fractions resulted in a partial rounding up of the cells (Figure 4-35). With PNT2A cells, there were little or no obvious morphological changes after any of the fractions (Figure 4-36).

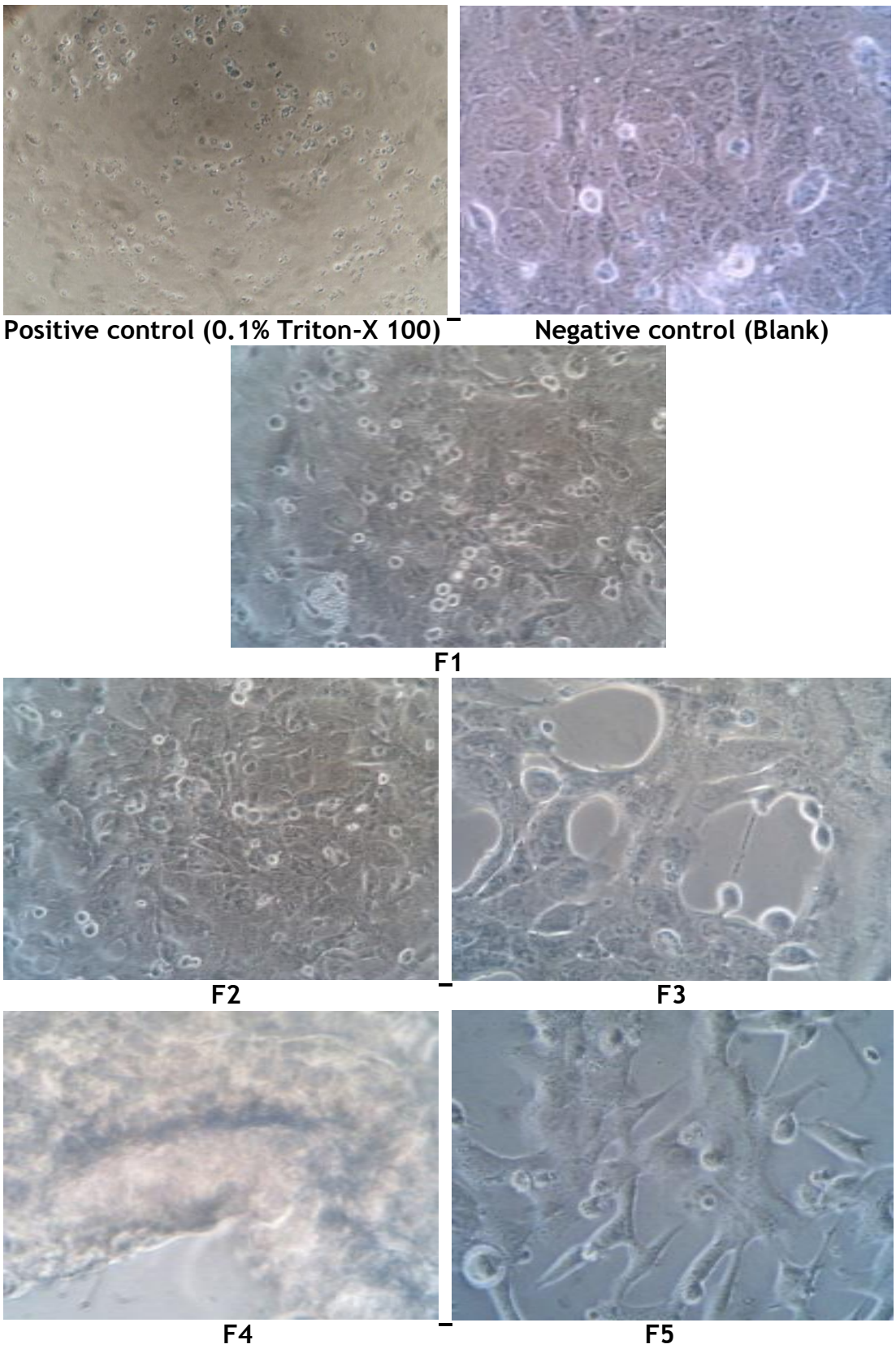


**Figure 4-33:** Light micrographs of cultured cell line DU145 following 2 hours exposure to 20µg/ml of *A. piscivorus conanti* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

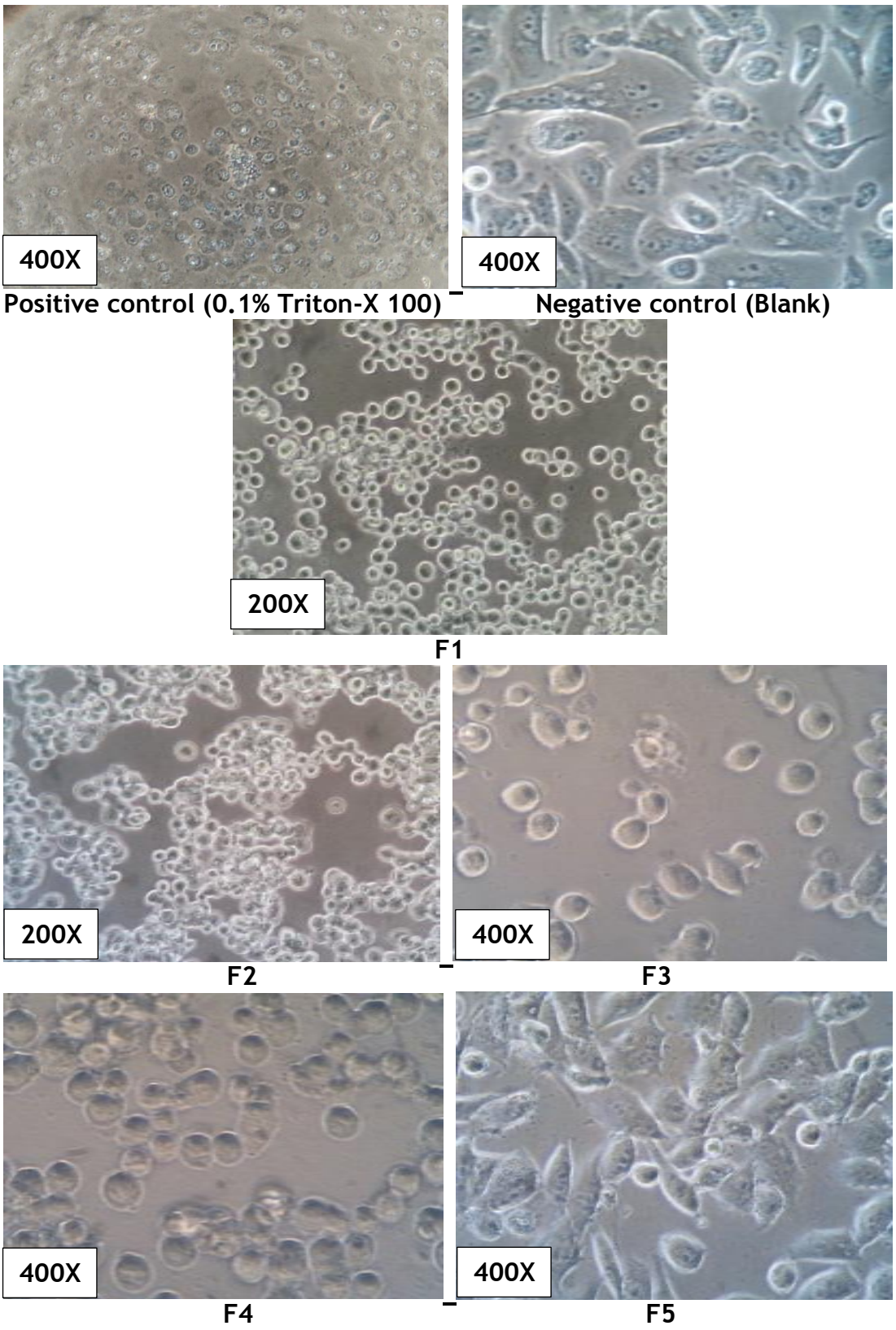


**Figure 4-34:** Light micrographs of cultured cell line PC3 following 2 hours exposure to 20µg/ml of *A. piscivorus conanti* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 200 X and 400 X magnifications were used for micrographs using a phase-contrast microscope.

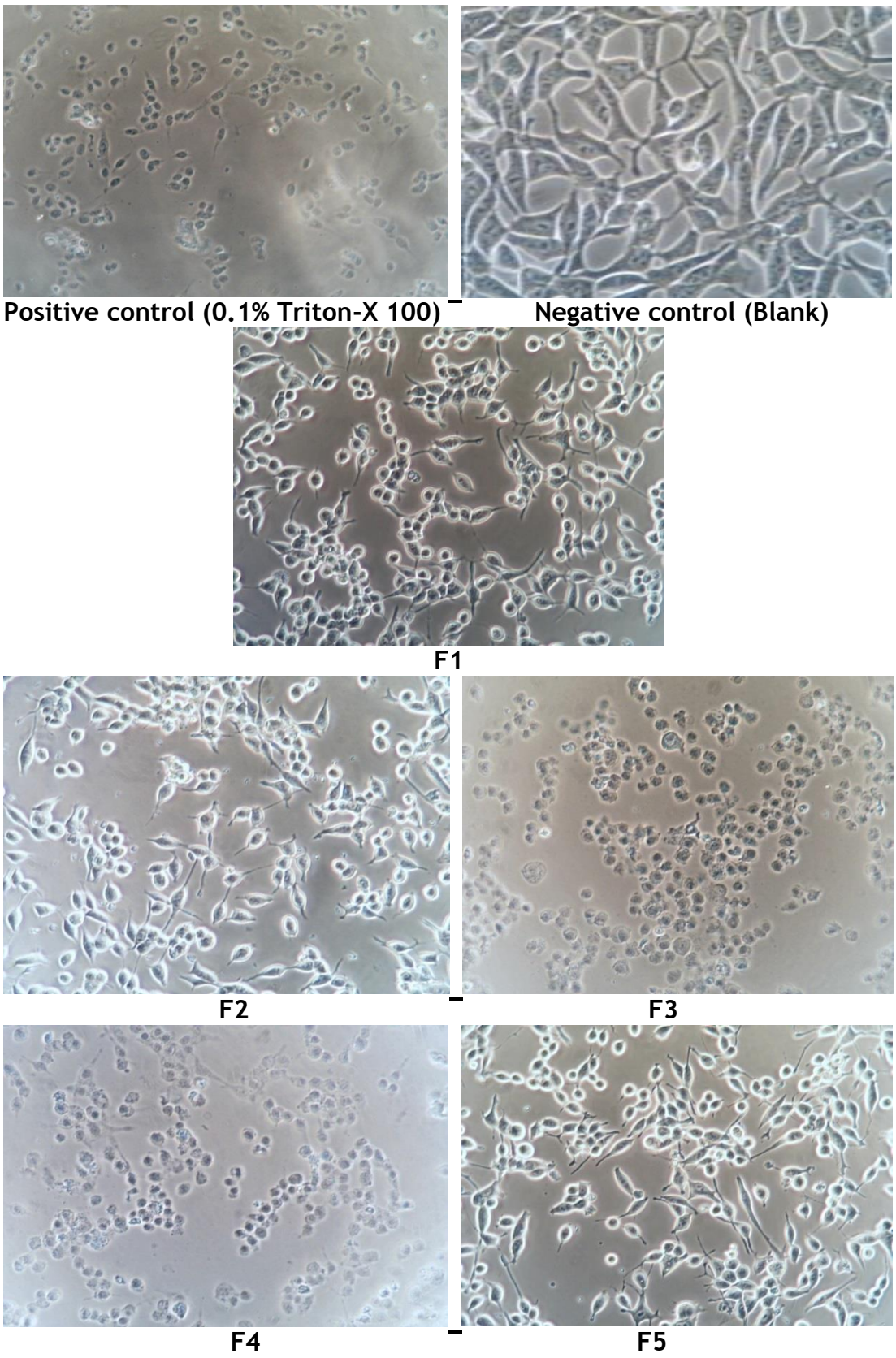


**Figure 4-35:** Light micrographs of cultured cell line LNCaP following 2 hours exposure to 20µg/ml of *A. piscivorus conanti* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 200 X magnifications were used for micrographs using a phase-contrast microscope.

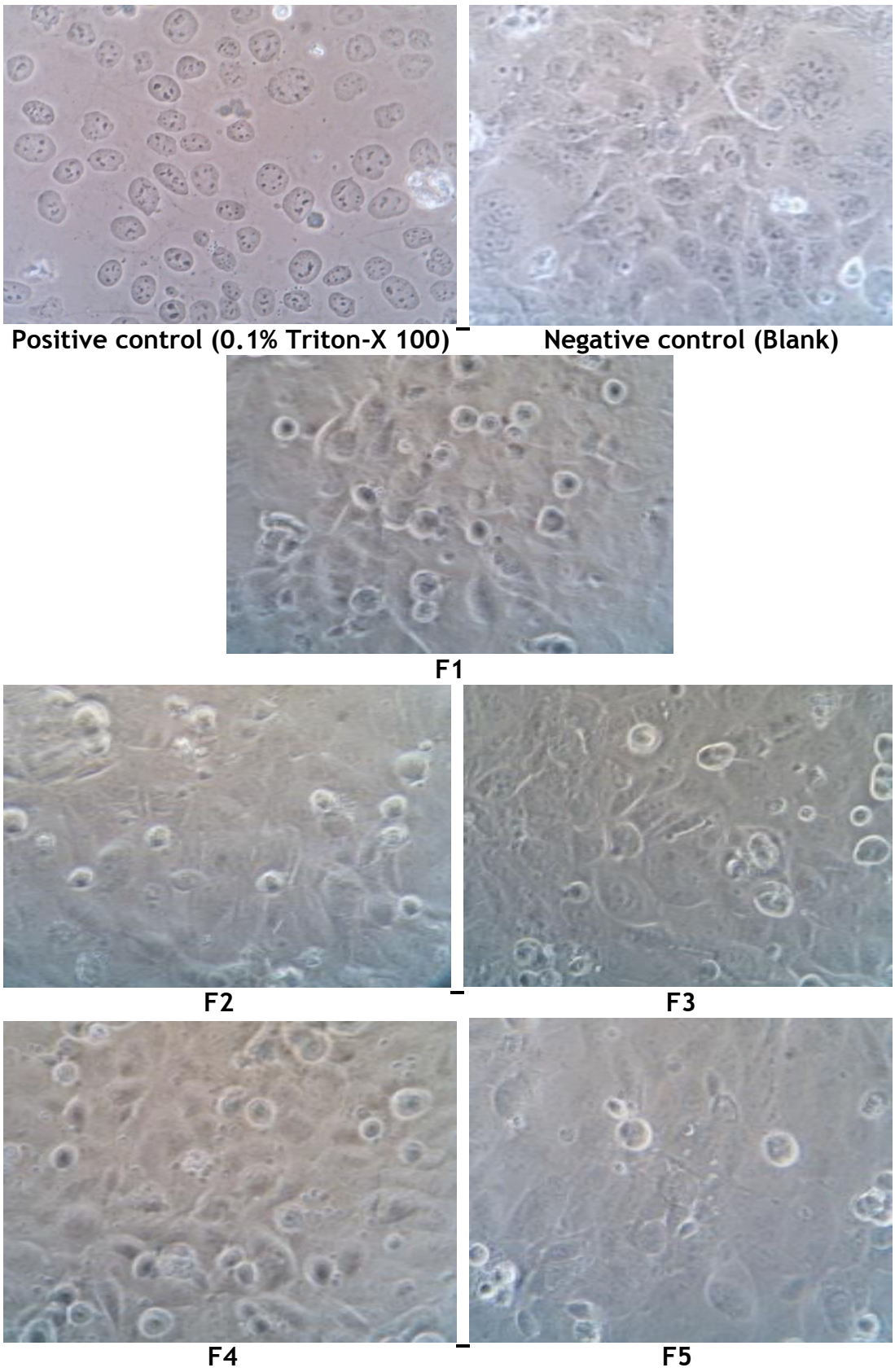


**Figure 4-36:** Light micrographs of cultured cell line PNT2A following 2 hours exposure to 20µg/ml of *A. piscivorus conanti* fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

### 4.3.3.2 Ion Exchange separation of *Agkistrodon piscivorus conanti* peak 4

Based on the above LDH assays and morphological analysis, peak four (P4) was found to be the most effective fraction. It was further fractionated by ion exchange chromatography on a Sephadex C-50 column yielding five sub-fractions, designated 1 to 5 (Figure 4-37). There appears to be considerable amounts of protein in sub-fractions 4 and 5 while the other fractions 1, 2 and 3 did not show very much protein. Sub-fractions 4 and 5 were named as P4F4 and P4F5 and these fractions were used for cytotoxic analysis against the prostate cell lines.

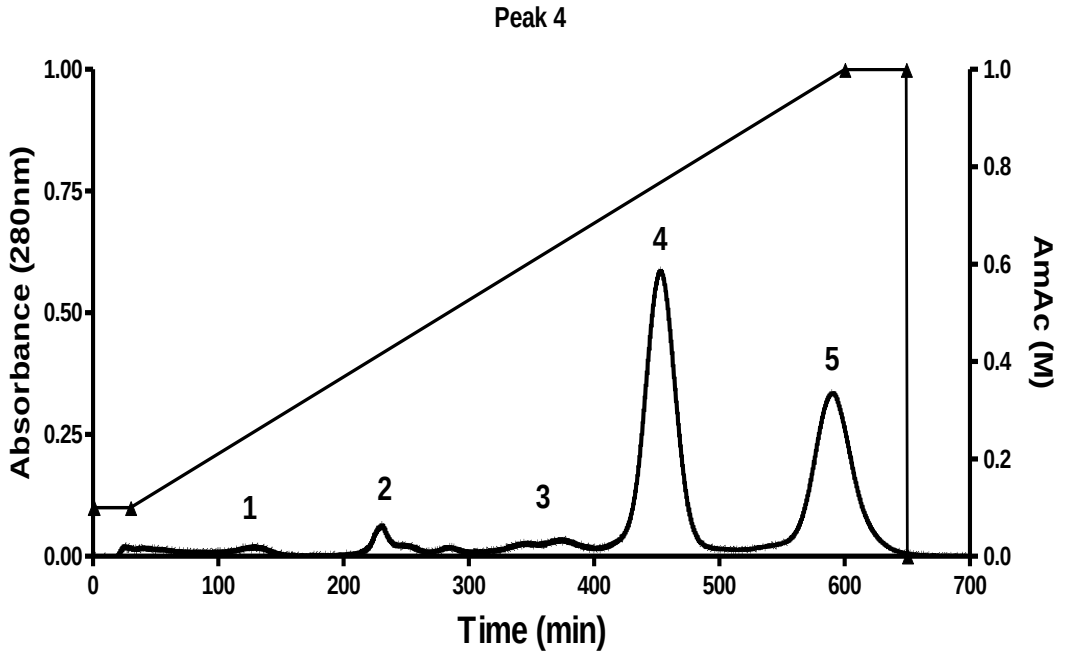


**Figure 4-37:** Fractionation of *A. piscivorus conanti* venom by Sephadex C-50 ion exchange chromatography. Peak 4 (50mg) in 0.1M ammonium acetate buffer was added to the column (142mm x 400mm, equilibrated with 0.1 M ammonium acetate buffer, pH 6.8), and eluted with a linear gradient of 0.1 to 1.0M ammonium acetate buffer. The eluent was monitored at 280 nm and the fractions pooled as indicated by the numbers.

#### 4.3.3.2.1 Effect of *Agkistrodon piscivorus conanti* venom sub-fractions on DU145, PC3, LNCaP and PNT2A cell lines for 2 hours by LDH assay

The LDH assay was performed to investigate cytotoxic effects on the different cell lines DU145, PC3, LNCaP and PNT2A. The cells were treated with 5 and 10µg/ml of sub-fraction 4 (P4F4) and sub-fraction 5 (P4F5) of *A. piscivorus conanti* venom for 2 hours. As shown in Figure 4-38, P4F4 and P4F5 did not cause much increase in LDH release in any of the cell lines except PC3 with P4F5. The sub-fraction P4F5 induced 38% and 45% LDH release in PC3 cells at 5µg/ml and 10µg/ml respectively. This increase in % LDH release was significant as compared to the negative control. The other cell lines, DU145, LNCaP and PNT2A, showed less than 20% increase in % LDH release at 5µg/ml and 10µg/ml after 2hours of exposure and such increments were not significant (Figure 4-38).

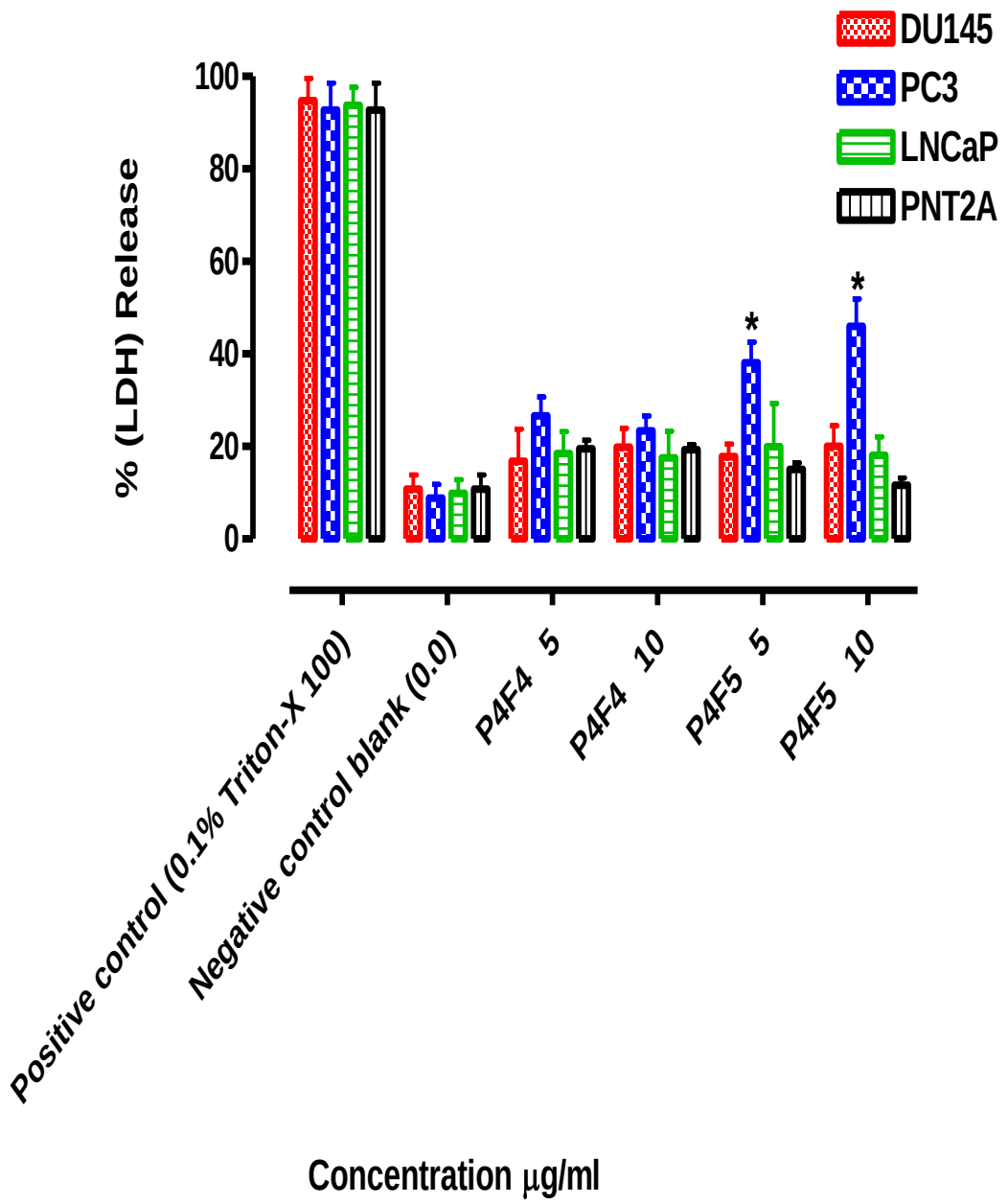


**Figure 4-38:** Changes in % LDH release in cultured cell lines DU145, PC3, LNCaP and PNT2A following 2 hours exposure to 5 and 10µg/ml of sub-fractions from *A. piscivorus conanti* snake venom in complete media at 37$^{o}$C. Data represent mean ± S.E.M of three experiments.*$p < 0.05$, significantly different from untreated control group. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure (as used with the snake venoms). The negative control measurements were made with cells maintained in the same time period in complete medium.

#### 4.3.3.2.1.1 Morphological analysis

Untreated DU145, PC3, LNCaP and PNT2A cell lines were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogeneous or slightly granulated cellular contents (Figures 4-39; 4-40; 4-41 and 4-42). However, after incubating cell lines with *A. piscivorus conanti* sub-fractions for 2 hours, the occurrence of various morphological abnormalities was observed. At 5 and 10µg/ml, the disappearance of normal morphological characteristics was very obvious (Figures 4-39; 4-40; 4-41 and 4-42) with many cells showing rounded appearance. This was particularly apparent with PC3 cells (Figure 4-40).

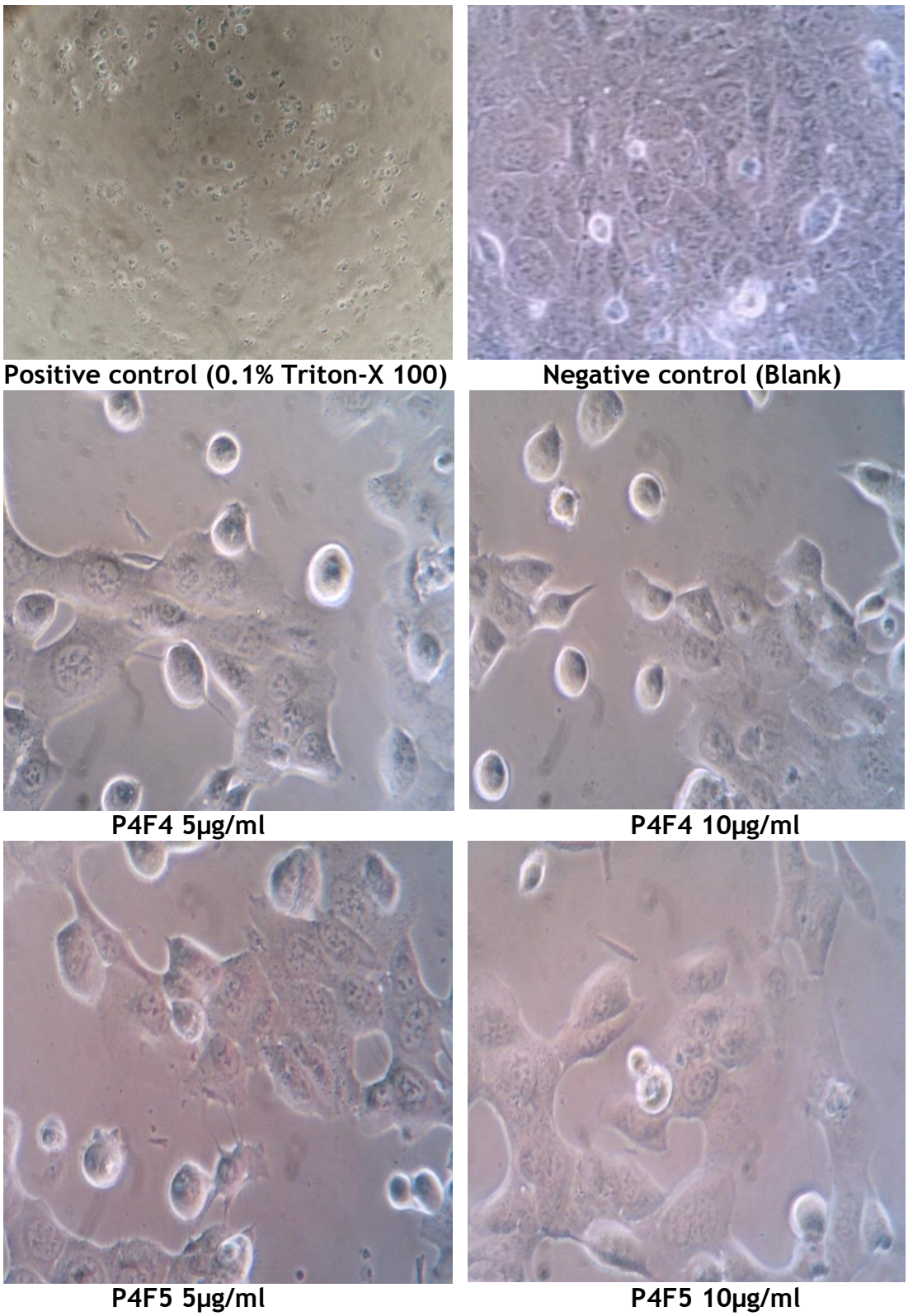


**Figure 4-39:** Light micrographs of DU145 cells following 2 hours exposure to 5 and 10µg/ml of *A. piscivorus conanti* P4F4 and P4F5 sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

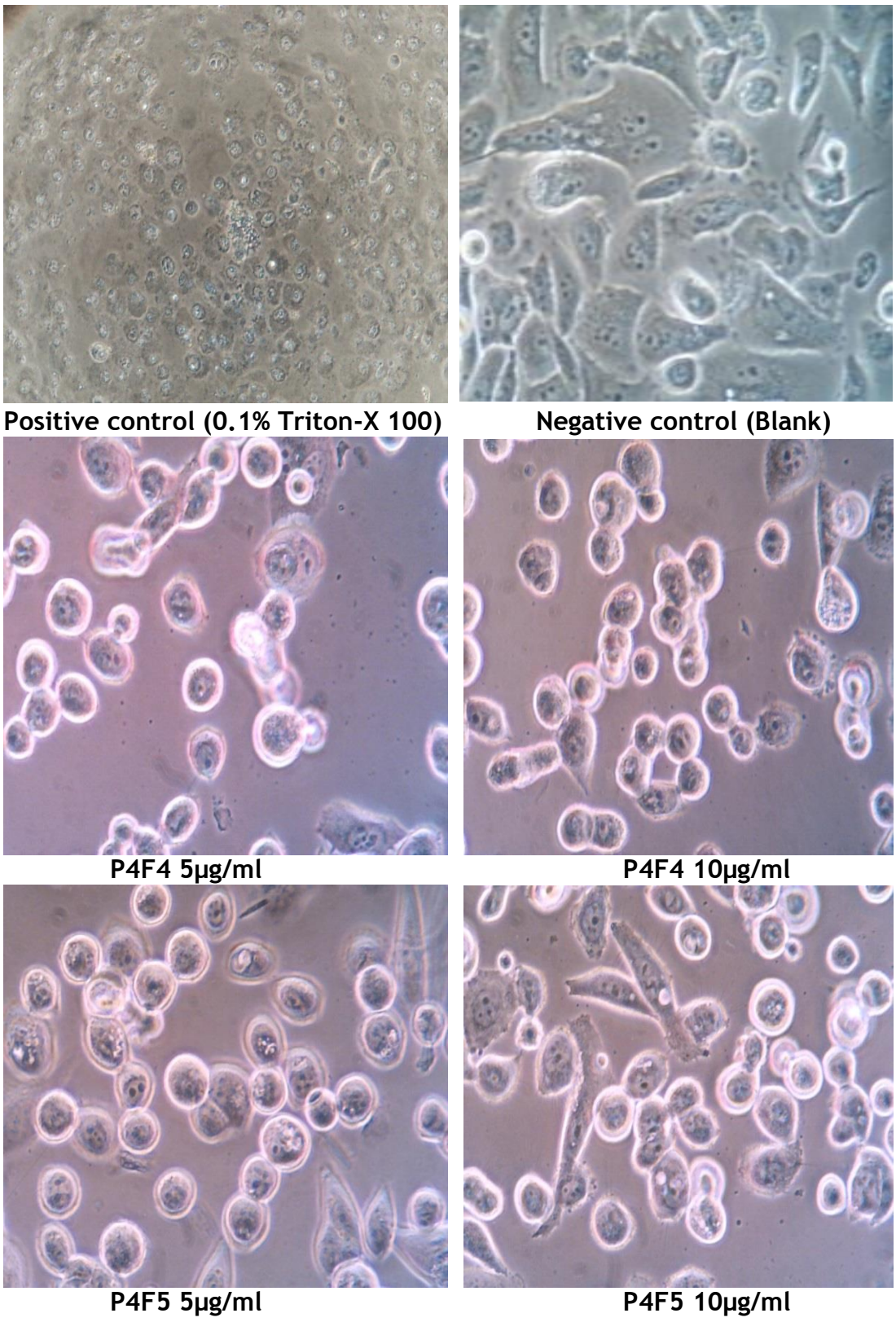


**Figure 4-40:** Light micrographs of PC3 cells following 2 hours exposure to 5 and 10µg/ml of *A. piscivorus conanti* P4F4 and P4F5 sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

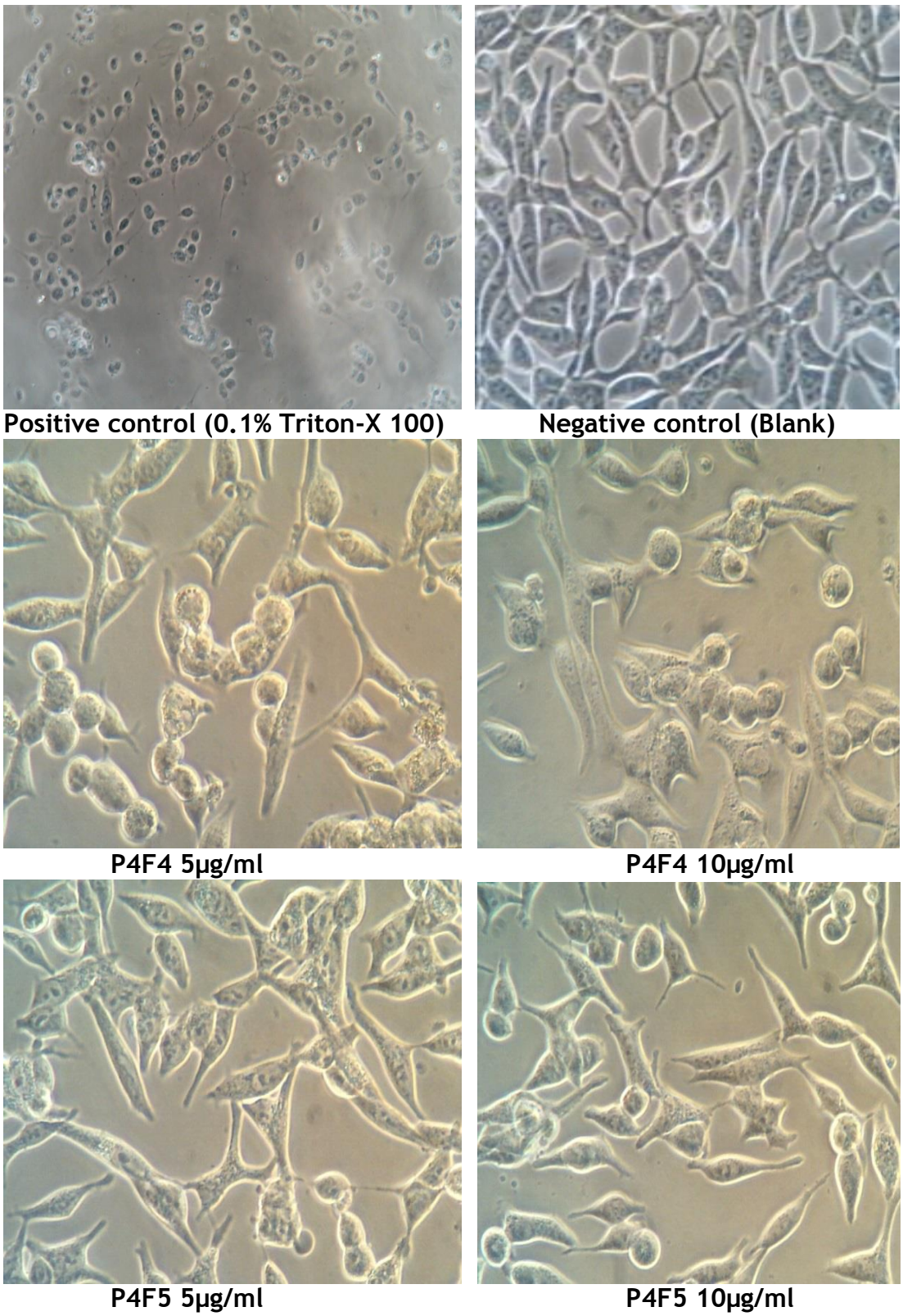


**Figure 4-41:** Light micrographs of LNCaP cells following 2 hours exposure to 5 and 10µg/ml of *A. piscivorus conanti* P4F4 and P4F5 sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

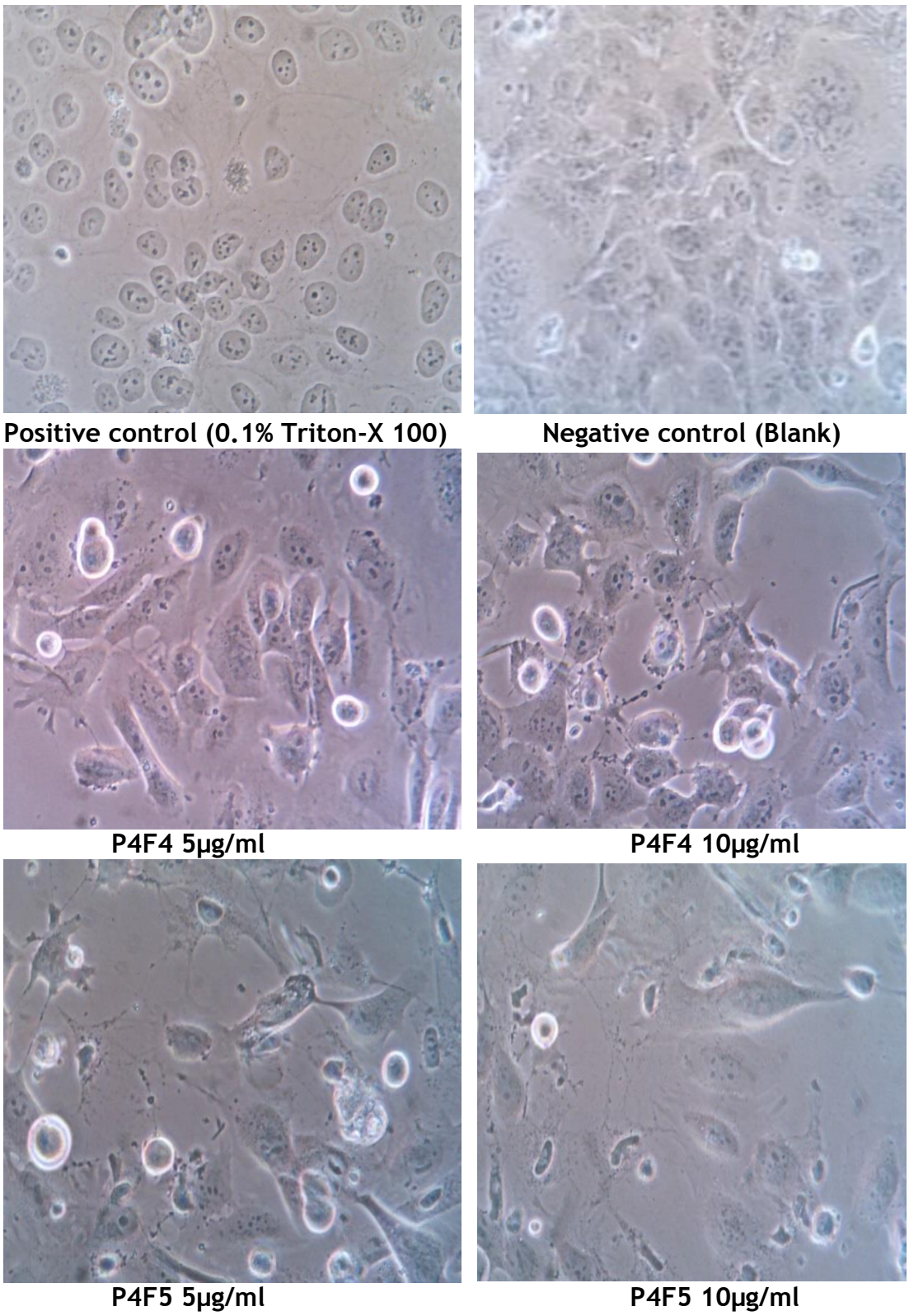


**Figure 4-42:** Light micrographs of PNT2A cells following 2 hours exposure to 5 and 10µg/ml of *A. piscivorus conanti* P4F4 and P4F5 sub-fractions in complete media at 37$^{o}$C. The negative control shows a culture after 2 hours in complete medium but no venom. The positive control agent was 0.1% Triton-X 100 for 2 hours exposure. 400 X magnifications were used for micrographs using a phase-contrast microscope.

#### 4.3.3.3 HPLC separation of *Agkistrodon piscivorus conanti* sub-fractions 4 and 5

The two most abundant sub-fractions of *A. piscivorus conanti* venom were fraction 4 (P4F4) and fraction 5 (P4F5) and these were separated by HPLC methods. The reverse phase Phenomenex column Luna 5µ C18 (250 × 4.60 mm) was used to separate the sub-fractions. The sub-fraction 4 (P4F4) and sub-fraction 5 (P4F5) appeared as single major peaks at 280nm absorbance and they were collected and named as P4F4-1 and P4F5-1 respectively (Figure 4-43). These purified fractions P4F4-1 and P4F5-1 did not freeze dry due to lack of protein. Therefore, these purified fractions did not continue to LDH assay and sequencing analysis.

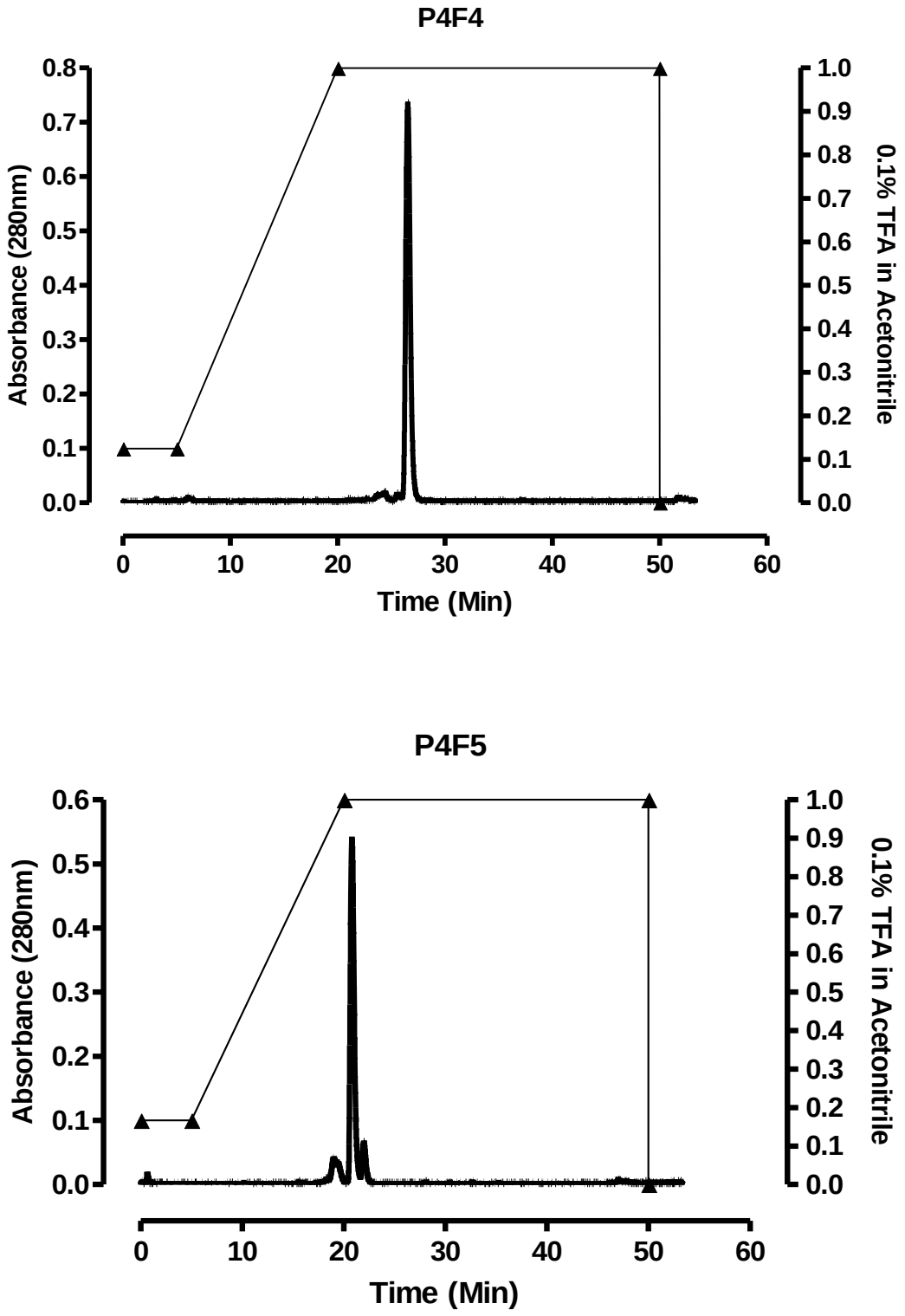


**Figure 4-43:** Analysis of *A. piscivorus conanti* venom sub-fractions P4F4 and P4F5 by reverse-phase HPLC. A Luna 5µ C18 Phenomenex column (250 × 4.60 mm), under linear gradient flow 0.1% and the buffer (0.1% trifluoroacetic acid in water) and (0.1% TFA in acetonitrile) at a flow rate of 1ml/min and monitored at 280 nm.

### 4.3.4 Phospholipase activity of whole *Naja pallida* venom and its fractions

Whole venom and four different fractions (F1, F2, F3 and F4) isolated by gel filtration were examined after 20 minutes incubation in the phospholipase $A_2$ assay (Figure 4-44). Whole venom showed substantial phospholipase $A_2$ activity with 10 nmols/min, while the fractions of this venom had less than 5 nmols/min. The fraction F4 had more phospholipase activity than other fractions.

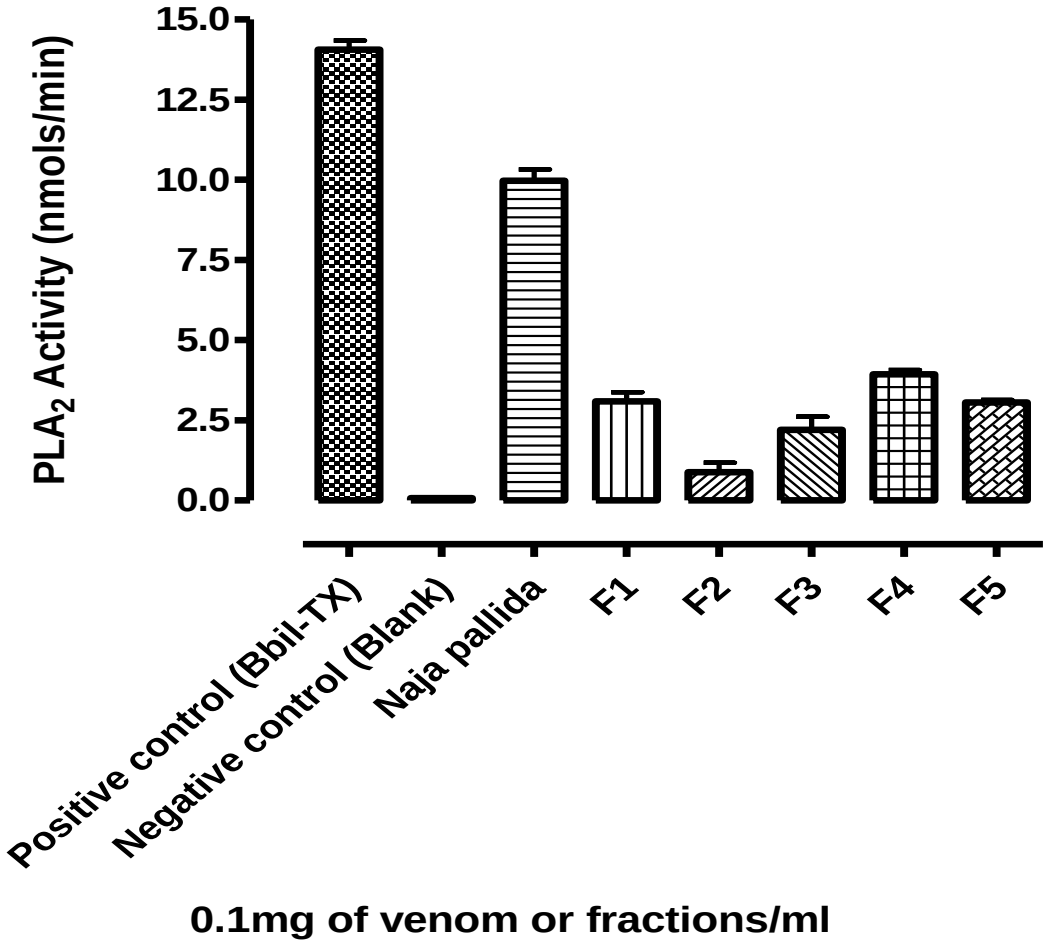


**Figure 4-44:** $PLA_2$ enzymatic activity of *Naja pallida* whole venom and its fractions after 20 minutes. Data represent mean ± S.E.M. of three experiments. The positive control was purified $PLA_2$ from (Bbil-TX) *Bothriopsis bilineata smargadina* venom; the negative control was complete buffer solution with no $PLA_2$.

#### 4.3.4.1 Phospholipase activity of *Naja pallida* fraction 4 (F4) and its sub-fractions P4F3 and P4F4

Phospholipase activity of *N. pallida* fraction 4 (F4) and its active sub-fractions 3 and 4 (P4F3 and P4F4) was assayed by using 0.1mg/ml fractions. The $PLA_2$ activity of sub-fraction P4F3 was more than that of fraction 4 (F4) and sub-fraction P4F4 (Figure 4-45).

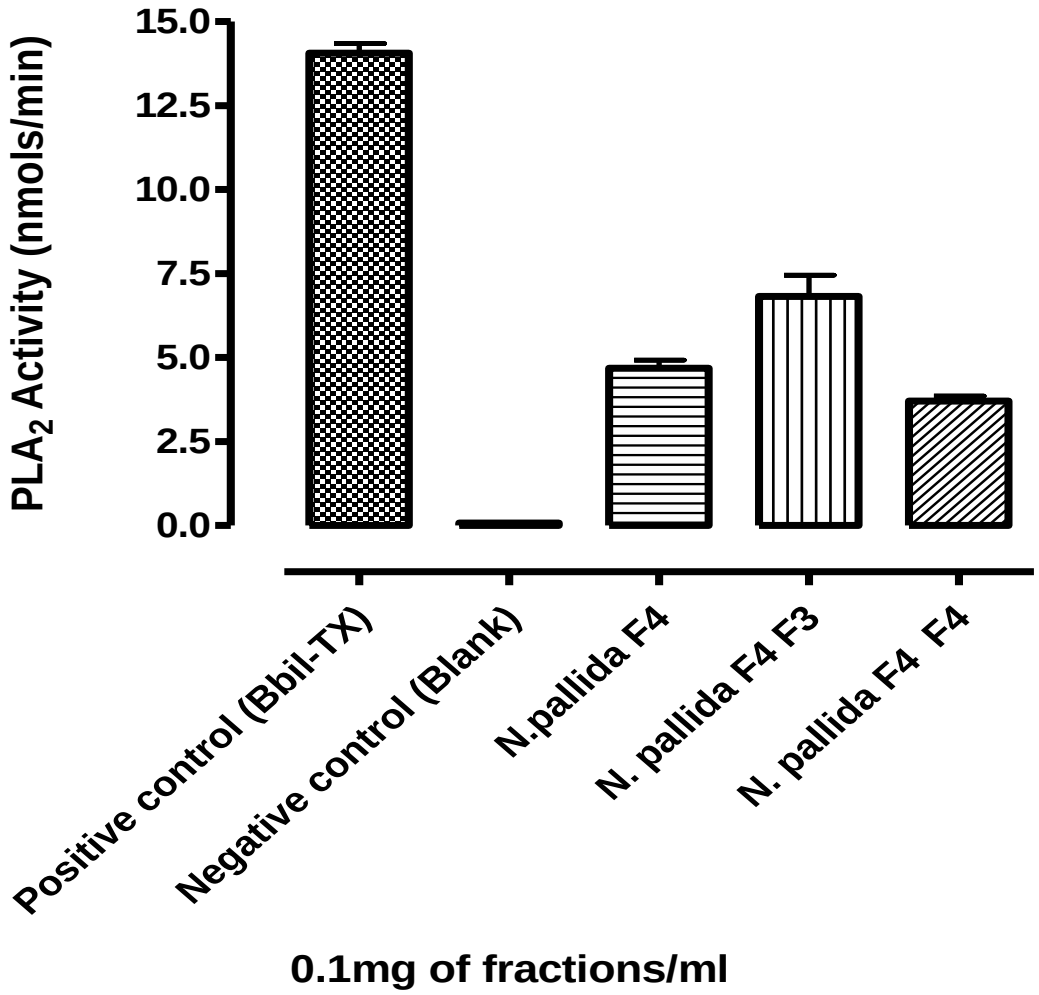


**Figure 4-45:** $PLA_2$ enzymatic activity of fraction F4 and its sub-fractions from *Naja pallida* venom after 20 minutes. Data represent mean ± S.E.M. of three experiments. The positive control was purified $PLA_2$ from (Bbil-TX) *Bothriopsis bilineata smargadina* venom; the negative control was complete buffer solution with no $PLA_2$.

### *4.3.5* Phospholipase activity of whole *Agkistrodon contortrix laticinctus* venom and its fractions

Whole venom of *A. contortrix laticinctus* and its gel filtration fractions were tested for phospholipase activity. Results indicated more activity of $PLA_2$ was found in whole venom than in fractions, although fraction 3 (F3) and fraction 4 (F4) were almost equal to whole venom (Figure 4-46). The sub-fractions from ion exchange chromatography were not tested because of insufficient quantities.

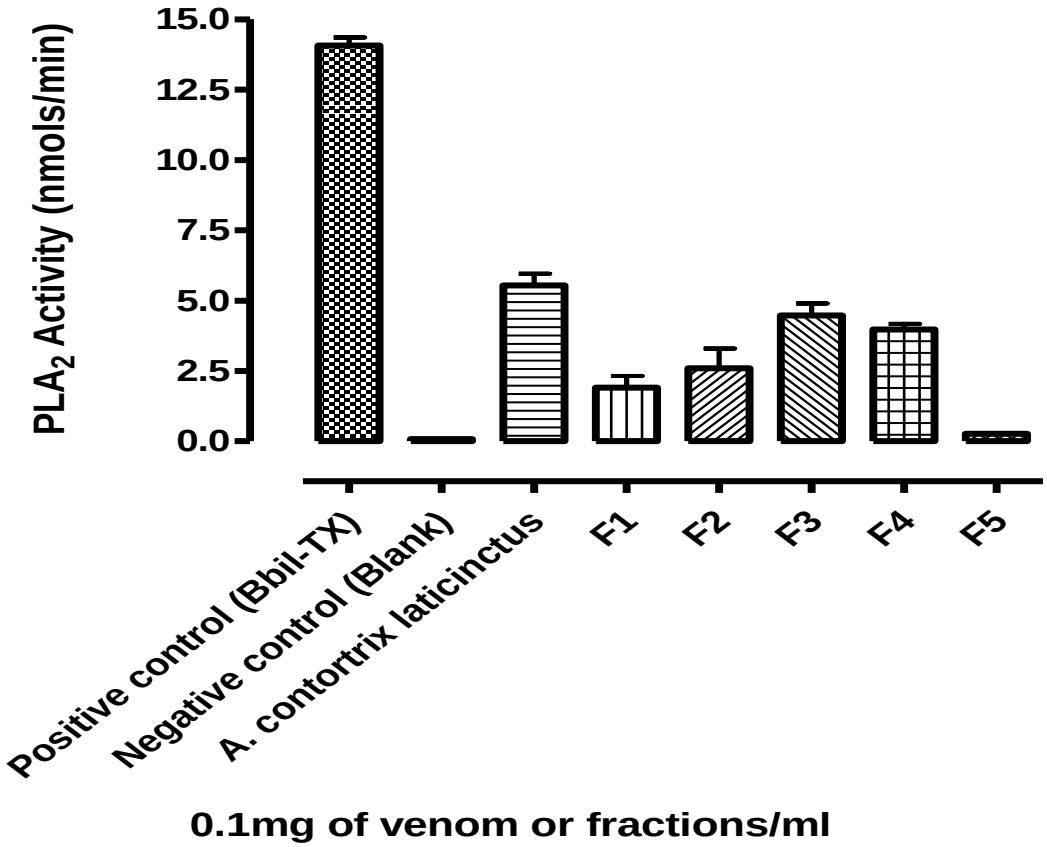


**Figure 4-46:** $PLA_2$ enzymatic activity of *A. contortrix laticinctus* of whole venom and its fractions after 20 minutes. Data represent mean ± S.E.M. of three experiments. The positive control was purified $PLA_2$ from (Bbil-TX) *Bothriopsis bilineata smargadina* venom; the negative control was complete buffer solution with no $PLA_2$.

### *4.3.6* Phospholipase activity of whole *Agkistrodon piscivorus conanti* venom and its fractions

Phospholipase activity of whole venom and the different fractions (F1, F2, F3, F4 and F5) obtained from gel filtration was determined using the 4-nitro-3-octanoyloxy-benzoic acid substrate after 20 minutes. Results shown in Figure 4-47 indicate that F1 and F5 have no $PLA_2$ activity while the whole venom and fractions F2, F3 and F4 showed good $PLA_2$ activity. Fraction 3 (F3) and fraction 4 (F4) were more prominent in $PLA_2$ activity than crude venom.

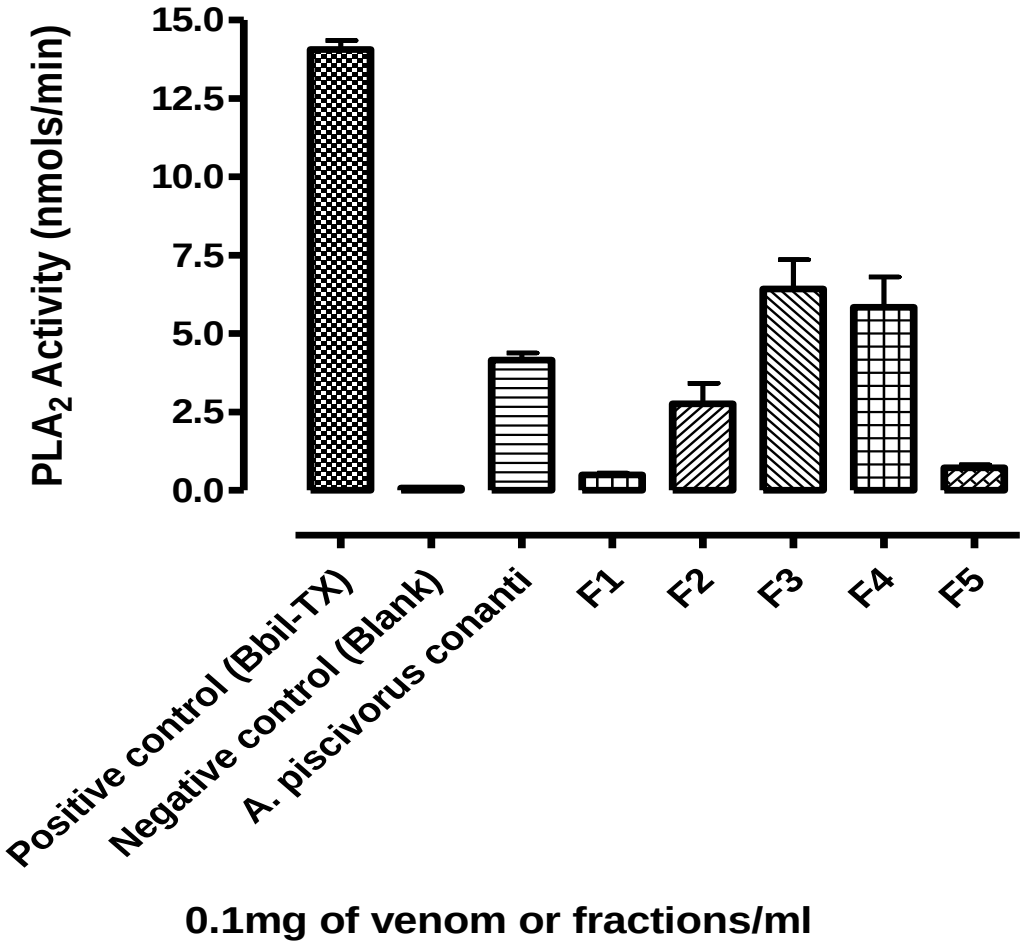


**Figure 4-47:** $PLA_2$ enzymatic activity of whole *A. piscivorus conanti* venom and its fractions after 20 minutes. Data represent mean ± S.E.M. of three experiments. The positive control was purified $PLA_2$ from (Bbil-TX) *Bothriopsis bilineata smargadina* venom; the negative control was complete buffer solution with no $PLA_2$.

### 4.3.6.1 Phospholipase activity of *Agkistrodon piscivorus conanti* fraction 4 and its sub-fractions

Phospholipase activity of the highly active fraction 4 (F4) and its three sub-fractions 3, 4 and 5 (named P4F3, P4F4 and P4F5) obtained from ion exchange chromatography was assessed using the 4-nitro-3-octanoyloxy-benzoic acid substrate after 20 minutes. Results shown in Figure 4-48 that sub-fraction 4 (P4F4) had more $PLA_2$ activity than the fraction 4 (F4) and sub-fractions 3 and 5 (P4F3 and P4F5). Therefore, most of the $PLA_2$ activity in fraction 4 appeared to be localised in its sub-fraction 4 (P4F4) (Figure 4-48).

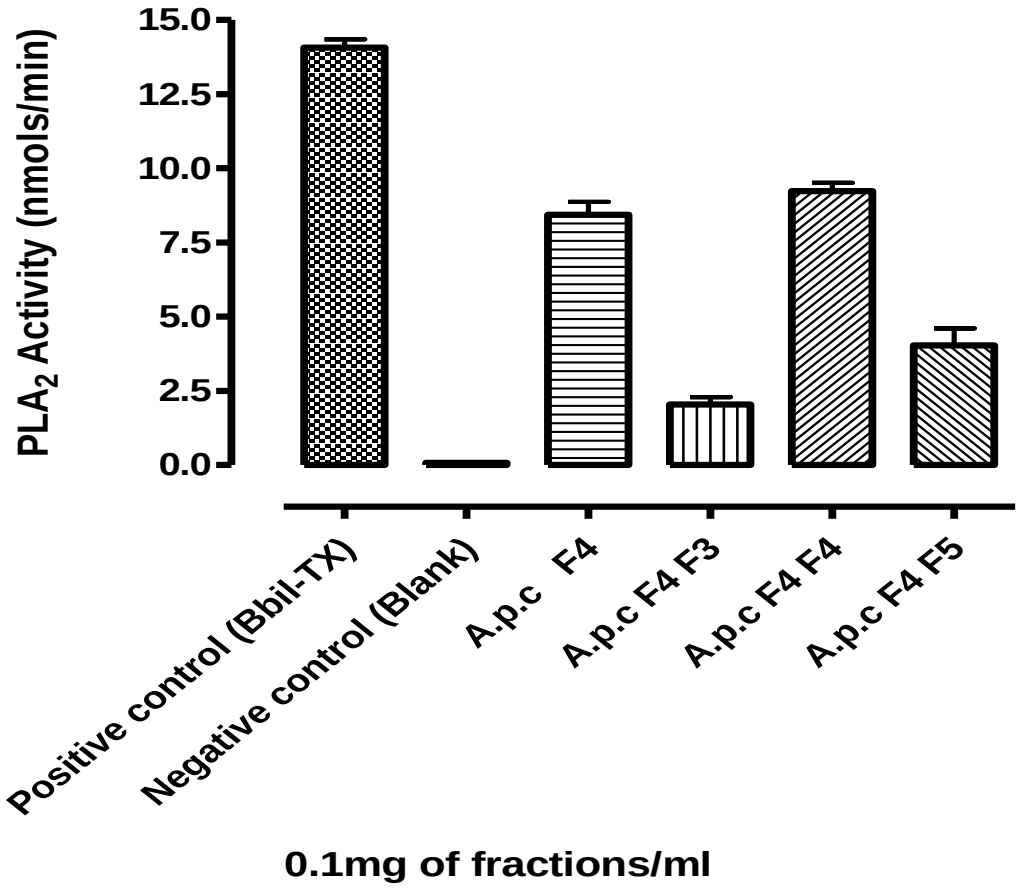


**Figure 4-48:** $PLA_2$ enzymatic activity in *A. piscivorus conanti* fraction 4 and its sub-fractions after 20 minutes. Data represent mean ± S.E.M. of three experiments. The positive control was purified $PLA_2$ from (Bbil-TX) *Bothriopsis bilineata smargadina* venom; the negative control was complete buffer solution with no $PLA_2$.

### *4.3.7 SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis*

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was carried out with the three snake venoms (*N. pallida, A. contortrix laticinctus and A. piscivorus conanti*) to visualise proteins ranging from ~10kDa to ~250kDa. The SDS-PAGE analysis was carried out parallel to gel filtration of the individual snake venoms. The electrophoretic profiles indicated that the venom protein molecular weights were in between ~10kDa to ~250kDa and there was no similarity to each other. The main differences were found quantitatively in the region of ~10kDa to ~37kDa rather than qualitatively. The profiles of the gel filtration fractions from the three snake venoms, *N. pallida*, *A. contortrix laticinctus* and *A. piscivorus conanti* on SDS-PAGE electrophoresis are shown in Figure 4-49.

A. *Naja pallida*

Electrophoretic profiles of *N. pallida* gel filtration fractions 1-5 are shown in the middle panel of Figure 4-49. F1-F5 show quantitative differences in band intensities among the fractions. There were strongly staining bands at ~35kDa in fraction 2 (F2), ~20kDa in fraction 3 (F3) and ~25kDa in fraction 4 (F4) (Figure 4-49). Fraction 1 appeared to have some staining at higher molecular weights, while fraction 5 showed little staining for protein.

B. *A. contortrix laticinctus*

Molecular weight markers (0.4µg) were loaded in the lane marked M and venom fractions (10µg) were loaded onto lanes marked F1-F4 (left-hand panel of Figure 4-49). The active fractions F2 and F3 of *A. contortrix laticinctus* showed strongly staining bands at ~25kDa and ~20kDa respectively (Figure 4-49).

C. *A. piscivorus conanti*

The gel filtration fractions of *A. piscivorus conanti* venom were loaded as F1 to F5 in the right-hand panel of Figure 4-49. The loading of venom gel filtration fraction was as follow, Lane 1: fraction 1 (10µg). Lane 2: fraction 2 (10µg). Lane 3: fraction 3 (10µg). Lane 4: fraction 4 (10µg). Lane 5: fraction 5 (10µg). All fractions

of *A. piscivorus conanti* (but particularly F3 and F4) showed strongly staining bands at ~30kDa and below (Figure 4-49).

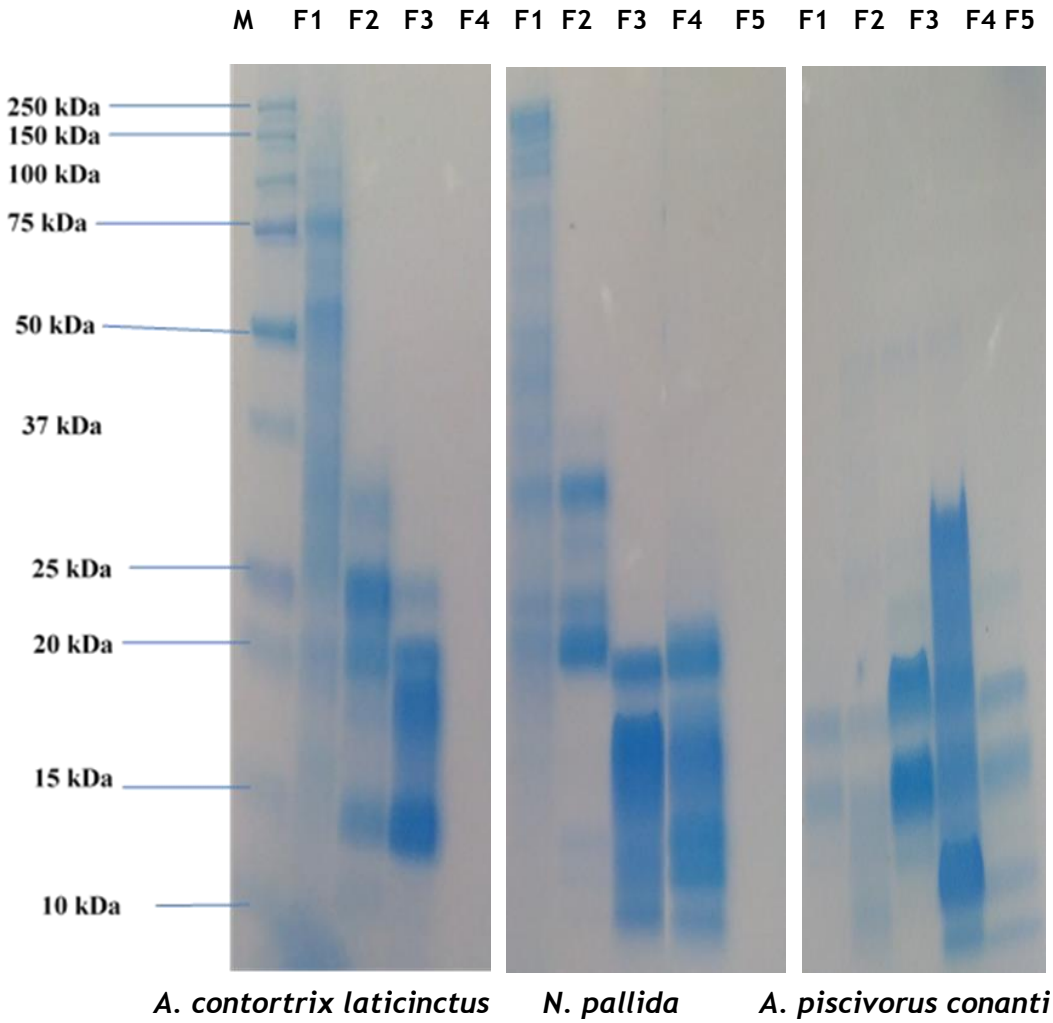


**Figure 4-49:** Gel electrophoresis (SDS-PAGE) of gel filtration fractions from the three snake venoms.

## 4.4 Discussion

Three snake venoms *N. pallida, A. contortrix laticinctus* and *A. piscivorus conanti,* were fractionated by sequentially using gel filtration, ion exchange chromatography and HPLC to find out the active cytotoxic constituents against prostate cancer cell lines. After each filtration technique, the fractions were collected and tested by the LDH release assay as well as by morphological inspection in all cell lines: DU145, PC3, LNCaP and PNT2A. The phospholipase $A_2$ activity of whole venoms and the fractions and sub-fractions was also determined.

The initial fractionation of *N. pallida* venom by gel filtration gave five fractions. All fractions (F1, F2, F3, F4 and F5) were effective against almost all cell lines except for fraction F1 (Figure 4-2). Overall, the most active fraction was F4, which induced more than 95% LDH release in all cell lines. This high release of LDH is presumably due to cell damage, as confirmed by morphological analysis of the cell lines (Figures 4-3, 4-4, 4-5 and 4-6).

After the success of fraction 4, it was further fractionated by ion exchange chromatography and four sub-fractions were collected and tested for LDH release and by morphological analysis. Results revealed that sub-fraction P4F3 and P4F4 caused significant LDH release of the cell lines (DU145, PC3, PC3, LNCaP and PNT2A) (Figure 4-8). The effectiveness on LDH assays was also confirmed by morphological changes in cell lines (Figures 4-9, 4-10, 4-11 and 4-12).

Finally, the sub-fraction (P4F3 and P4F4) were further purified by reverse phase HPLC and purified fractions were collected and named P4F3-1, P4F3-2 from P4F3 while two other purified fractions P4F4-1 and P4F4-2 were isolated from P4F4 (Figure 4-13). The major purified fractions P4F3-2, P4F4-1 and P4F4-2 were used for LDH assay. Results revealed that the purified fractions P4F3-2, P4F4-1 and P4F4-2 of *N. pallida* were very active against with PC3 and LNCaP cell lines. There was no significant LDH release observed in the other two cell lines DU145 and PNT2A (Figure 4-14). This LDH increment was confirmed by the morphological changes induced by P4F3-2, P4F4-1 and P4F4-2 on prostate cancer cell lines (Figures 4-15 to 4-18).

Phospholipase activity of whole *N. pallida* venom and its gel filtration fractions (F1, F2, F3 and F4) was measured. Whole venom showed powerful $PLA_2$, as did fractions F3 and F4, while fractions F1 and F2 showed less $PLA_2$ activity (Figure 4-44). The most active fraction and its sub-fractions 3 and 4 (named P4F3 and F4F4) were similarly assayed. The activity of $PLA_2$ of sub-fraction P4F3 were more as compared to fraction 4 (F4) and sub-fraction P4F4 (Figure 4-45).

The venom of *A. contortrix laticinctus* and its fractions were studied similarly to *N. pallida* venom. The results with *A. contortrix laticinctus* venom gel filtration fractions showed that fractions F2 and F3 were effective against DU145, PC3 and LNCaP cells while these fractions were less effective against the PNT2A cell line. Fraction F3 showed excellent LDH release in PC3 cells, followed by LNCaP and DU145 cells, the % release being 82%, 69% and 42% respectively, while it was only 9.5% in PNT2A cells (Figure 4-20). The effectiveness of fraction 3 (F3) was also seen and confirmed by morphological analysis of prostate cancer cell lines DU145, PC3 and LNCaP (Figure 4-21; Figure 4-22 and Figure 4-23). Fractions F1 and F4 had little or no effect on the LDH release from the cells (Figure 4-20).

The most active fraction (F3) of *A. contortrix laticinctus* venom was further fractionated by ion exchange chromatography and four sub-fractions were collected (Figure 4-25). The major sub-fractions were selected for the cytotoxicity studies. Results did not show any significant LDH release in any prostate cell line (Figure 4-26) with sub-fractions P3F3 and P3F4 did not showed any significant LDH release as compared to whole venom and fraction F3. These sub-fractions were not found to cause marked morphological changes (Figures 4-27; 4-28; 4-29 and 4-30). Therefore, these sub-fractions P4F3 and P4F4 were not further purified by HPLC and protein sequencing was not carried out.

The whole venom of *A. contortrix laticinctus* and its fractions were also tested for phospholipase activity. Results indicated more activity of $PLA_2$ was found in whole venom as compared to fractions, but fraction 3 (F3) and fraction 4 (F4) were almost equal in activity to venom (Figure 4-46).

The third venom in the study was *A. piscivorus conanti* which was also fractionated by gel filtration. The five of the six fractions obtained (Figure 4-31)

were tested with the LDH assay against prostate cancer cell lines DU145, PC3, and LNCaP and normal prostate cells PNT2A. Fraction F1 was effective against PC3 cells, while F2 was not effective against any cell line. F3 and F4 were both effective against DU145 and PC3 cells; and F5 was not effective against any cell line (Figure 4-32). Overall, fraction 4 (F4) was considered to be the most active fraction as confirmed by morphological changes in the cell lines (Figures 4-33; 4-34; 4-35 and 4-36). The normal prostate cell line PNT2A was little affected by any of the fractions.

Fraction 4 (F4) of *A. piscivorus conanti* was further fractionated by ion exchange chromatography and five sub-fractions were collected (Figure 4-37). The sub-fraction P4F4 had little or no activity, but P4F5 showed activity in the LDH release assay and 45% LDH release in PC3 cells only. There was no significant effect on other prostate cell lines, DU145, LNCaP and PNT2A (Figure 4-38). P4F4 and P4F5 also showed morphological changes in PC3 cells (Figure 4-40). These fractions P4F4 and P4F5 were checked for purity by HPLC and single and pure peaks were found in each sub-fraction (Figure 4-43), but these sub-fractions were not used for protein sequencing due to the low yield.

Phospholipase $A_2$ activity of whole venom *A. piscivorus conanti* and its five different gel filtration fractions (F1, F2, F3, F4 and F5) was determined (Figure 4-47). Fraction 3 (F3) and fraction 4 (F4) were more prominent in $PLA_2$ activity than crude venom. Further phospholipase activity determinations were carried out on fraction 4 (F4) and its three sub-fractions 3, 4 and 5 (P4F3, P4F4 and P4F5) obtained from ion exchange chromatography. Sub-fraction 4 (P4F4) was higher in $PLA_2$ activity than fraction 4 (F4). Sub-fractions 3 and 5 (P4F5) were less active in $PLA_2$ assays than F4.

Different anticancer molecules of various molecular weights have been reported in snake venoms. The anticancer molecules of less than 15 kDa have been reported (Shipolini, Kissonerghis et al. 1975). The high molecular weight components reported are sL-amino-acid oxidases (LAAO) (Du and Clemetson 2002; Fox 2013; Lee, Hudgens et al. 2013). Different classes of peptides have their own specificity for different types of cancer cell lines. There are several reports showing that snake venoms or some of the components have an impact on cancer cells: for

example, *Walterinnesia aegyptia* venom was active against the human cell lines RPMI8226 and U266 (Badr, Al-Sadoon et al. 2012) and there are nanoparticle-sustained delivery of snake venom against two types of cancer cells MDA-MB-231 and PC3 (Badr, Al-Sadoon et al. 2013) and King cobra (*Ophiophagus hannah*) venom LAAO inhibited PC3 cells (Lee, Fung et al. 2014). PC3, LNCaP and K-562 were treated with fractions of *Vipera lebetina* venom that reduced the viability of PC3 cells but had no significant effect on the viability of LNCaP and K-562 cells (Samel, Trummal et al. 2012).

In the present work, the three venoms and isolated fractions of *N. pallida*, *A. contortrix laticinctus* and *A. piscivorus conanti* showed strong cytotoxic effects on prostate-derived cell lines, DU145, PC3, LNCaP and PNT2A. Activity was detected in low, medium and high molecular weight fractions. Cell lines were inhibited by most of the venom fractions and a few of the sub-fractions, but rarely by HPLC-purified fraction *N. pallida* venom, although its fractions were found to be more effective than those from *A. contortrix laticinctus* and *A. piscivorus conanti* venoms. Therefore, further study, including protein sequencing, was carried out only with *N. pallida* fractions (as described in chapter 5) while the rest of the snake venoms were not studied further.

Since many snake venoms contain $PLA_2$ enzymes that exhibit a wide variety of pharmacological effects, the venoms in this work were tested for $PLA_2$ activity. In addition to their direct effects, some $PLA_2$ enzymes in snake venoms (particularly cobra venoms) potentiate the activity of directly acting cytotoxins (Hodges et al. 1987). Phillips (1970) reported about direct haemolytic factor isolated from the snake *Naja naja* that was different to $PLA_2$ and independently showed direct activity (Condrea, Devries et al. 1964). In the present research, $PLA_2$ activity was found in whole venom and some fractions only, while sub-fractions did not show any activity. This may contribute to some of the effects found in the prostate cell lines.

## 4.5 Conclusion

In this chapter, fractions from the venoms of *N. pallida; A. contortrix laticinctus* and *A. piscivorus conanti* were screened for their ability to induce cytotoxicity and necrosis in four human cell lines (DU145, PC3, LNCaP and PNT2A). Throughout this study, cytotoxicity and cell viability assays have been used extensively to screen cell death. Although these assays did not distinguish between apoptosis and necrosis, they were used to quantitatively determine inhibition of cell growth and proliferation. This study demonstrates the successful streamlining of the screening process of snake venoms with potential anticancer activity by eliminating poor candidates on the basis of cytotoxic criterion that takes into consideration effective concentration and selectivity for cancer cells over normal cells.

The results of this study have demonstrated that snake venoms may contain components with potentially selective effects on prostate cancer cells. It is also clear from the results that purified fractions are needed to obtain selective activity.

Finally, to achieve the main goal of the project, that is the isolation and characterization of peptides with selective effects on prostate cancer cells, further studies (including protein sequencing) were carried out only with the most active samples, namely *N. pallida* venom and its fractions. These experiments are described in chapter-5.

# CHAPTER FIVE:

## SEQUENCE ANALYSIS OF *NAJA PALLIDA* VENOM PROTEINS

# 5. CHAPTER FIVE

## 5.1 Introduction

It is well-known that snake venoms are rich in many proteins and peptides that include cardiotoxins, myotoxins, phospholipase $A_2$ as major components. Such proteins and peptides of snake venom play an important role in cytotoxicity. Discovering the structures and functions of proteins in snake venom is an important tool for understanding cellular mechanisms, and many enable the targeting of specific metabolic pathways.

Protein sequencing is a technique to determine the amino acid sequence of a protein. There are two major direct methods of protein sequencing which are mass spectrometry and the Edman degradation reaction. A protein sequencer is a machine that is used to determine the sequence of amino acids in a protein. They work by tagging and removing one amino acid at a time, which is analysed and identified. This is done repeatedly for the whole polypeptide, until the whole sequence is established.

Determining which amino acid forms the N-terminus of a peptide chain is useful. A generalised method for N-terminal amino acid analysis is the reaction of peptide with reagent, hydrolysis of protein and determination of amino acid by chromatography and comparison with standard. There are many different reagents which can be used to label terminal amino acids. They all react with amine groups and will therefore also bind to amine groups in the side chains of amino acids such as lysine - for this reason it is necessary to be careful in interpreting chromatograms to ensure that the right spot is chosen. Two of the more common reagents are Sanger's reagent (1-fluoro-2, 4-dinitrobenzene) and dansyl derivatives such as dansyl chloride. Phenylisothiocyanate, the reagent for the Edman degradation, can also be used.

In the preceding chapter 4, I described the purification of *N. pallida* venom and identified two low molecular weight proteins that were also characterized biologically and pharmacologically. It is known that low molecular weight components (in the range of 6-7kDa) in cobra venoms can include cytotoxins and cardiotoxins. Based on many reports, more than 50 snake venom cytotoxins and

cardiotoxins with different primary sequences have been identified (Dufton and Hider 1988).

### *5.1.1 Cytotoxins*

Three-finger toxins (3FTx) constitute an important super-family of snake venom proteins. All have three beta-stranded loops resembling three fingers, emerging from a globular core and stabilised by four conserved disulfide bridges. They are diverse in their biological functions, and include neurotoxins, cardiotoxins (or cytotoxins), fasciculins, platelet-aggregation inhibitors and specific ion-channel blockers (Osipov, Astapova et al. 2004; Mackessy 2010; Kini and Doley 2010).

For many years, there have been many publications about the amino acid sequences of snake venom cytotoxins (Hayashi, Takechi et al. 1971; Inoue, Okumura et al. 1989; Shafqat, Zaidi et al. 1990; Guo, Wang et al. 1993; Osipov, Astapova et al. 2004; Panda Subhamay and Chandra Goutam 2013; Munawar, Trusch et al. 2014). Cytotoxins exhibit cytotoxic activities against many types of cells, for example, bladder cancer cells and human leukaemia cells K562 (Yang, Chien et al. 2007), and HepG2 human liver cells (Chen, Lv et al. 2009), and breast cancer cells (Lin, Su et al. 2010).

The cardiotoxins have ability to induce systolic cardiac arrest in the rodent while their potent cytolytic effects on a wide variety of cells in vitro explains their designation as cytotoxins or cytolysins (Hodges, Agbaji et al. 1987; Rees, Samama et al. 1987).

Cytotoxins can cause a lot of necrotic tissue damage after envenoming by cobras of the *Naja* genus (Weinstein, Minton et al. 1985). The study carried out by Ownby, Fletcher et al. (1993) demonstrated that intramuscular injection of these toxins in mice induces overt myonecrosis (Ownby, Fletcher et al. 1993). In addition to cytotoxins, some phospholipases $A_2$ found in *Naja* venoms are cytolytic (Chwetzoff, Tsunasawa et al. 1989), and they can act synergistically with cardiotoxins and therefore may contribute to the necrotic effects of the venoms.

In three-dimensional structures, cardiotoxins adopt a three-fingered loop-folding topology dominated by beta sheet (Bilwes, Rees et al. 1994; Jayaraman, Kumar et al. 2000), but individual toxins vary in details, including the extent of secondary structure and the positions of invariant side chains. Some studies suggested that cardiotoxins may damage cells by their ability to interact with lipid on the cell membrane (Forouhar, Huang et al. 2003; Kao, Lin et al. 2009; Kao, Wu et al. 2009). Batenburg, Bougis et al. (1985) found that *Naja mossambica* cytotoxins were able to induce leakage of cardiolipin vesicles.

There are many studies which show that cobra venom cytotoxins can damage different cancerous cell lines: for example, cancer cells T24 (Chien, Yang et al. 2004), colon adenocarcinoma cells Colo205 (Tsai, Yang et al. 2006), human leukaemia cells K562 (Yang, Chien et al. 2007) SK-N-SH (Chen, Moriya et al. 2009), leukaemia HL-60 cells (Chien, Yang et al. 2008), HepG2 human liver cells (Chen, Lv et al. 2009), cells of oral squamous cancer Ca9-22 (Chien, Chang et al. 2010), and breast cancer MCF-7 human cells and MDA-MB-231 cells (Chiu, Lin et al. 2009) It was also worthwhile to note that cytotoxin CTXIII had a low toxicity to normal cell lines in the laboratory, for example, peripheral blood mononuclear cells (Su, Su et al. 2003), and T-lymphocytes (StevensTruss, Messer et al. 1996), and human embryonic kidney HEK-293 cells (Chiu, Lin et al. 2009). This clearly indicates that some cytotoxins can be selectively effective against various cancer cell lines.

Recently, the mechanisms proposed to be involved in the inhibition of cell growth by cobra venom cytotoxins include the arrest of the cell cycle (Yang, Chien et al. 2007; Chen, Lv et al. 2009) with the participation of each of the paths reticulum- and mitochondria- dependent endoplasmic, inhibition of the protein in humans that is named c-Jun by the JUN gene kinase N-terminals (Yang, Chien et al. 2007) and NFkB (Chiu, Lin et al. 2009), and down-regulation of JAK2 / PI3 K-mediated activation signals (Lin, Su et al. 2010).

### *5.1.2 N-terminal Sequence Analysis*

The isolation of snake venom fractions P4F3-1, P4F3-2, P4F4-1, P4F4-2 and P4F4-3 was carried out from RP-HPLC by using Luna 5μ C18 Phenomenex column

(250 x 4.60mm) at Strathclyde University, UK. The N-terminal sequence analysis of the first 15 amino acids of these fractions was carried out in the Jožef Stefan Institute, Slovenia at the Department of Molecular and Biomedical Sciences.

### *5.1.3 BLAST Analysis of the N-terminal Sequence*

BLAST (Basic Local Alignment Search Tool) is one of the most widely used bioinformatics programs for sequence searching. The program compares the nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. The identity of the partially sequenced peptides was specified by comparison with other known sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## 5.2 Materials and Methods

### 5.2.1 Sequence analysis of purified fractions

The purified fractions P4F3-1, P4F3-2, P4F4-1, P4F4-2 and P4F4-3 of *N. pallida* venom were dissolved in 20% acetonitrile (v/v) / 0.1% trifluoroacetic acid (v/v) in highly purified water, and 50 pmols of each sample were used for the analysis. Sequence analysis was performed using a Procise® Protein Sequencing System 494A (PE Applied Biosystems). The Procise® Protein Sequencing System is composed of four integrated modules: i) the Procise Protein Sequencer, ii) the Model 140C Microgradient Delivery System, iii) the variable-wavelength UV detector, and iv) a Microsoft Windows NT-based computer equipped with Procise control software and SequencePro software. The Procise system sequentially cleaves N-terminal amino acids from protein/peptide chains and analyzes the resulting phenylthiohydantoin (PTH)-amino acid residues. PTH amino acid derivatives were prepared using Edman chemistry and Pulsed-liquid method. The analysis of PTH-derivatives was performed on RP C18 Spheri-5 (5μm, 220 x 2.1 mm) column (Brownlee) of 140C HPLC system (PE Applied Biosystems). Reagents and solvents were used in high quality sequencing grade and suitability of PE Applied Biosystems. The protein sequencing analysis was carried out under supervision of Prof. Dr. Igor Križaj (Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia).

## 5.3 Results

### 5.3.1 Sequence analysis of Naja pallida peptides

The N-terminal sequencing of purified fractions P4F3-1, P4F3-2, P4F4-1, P4F4-2 and P4F4-3 of *N. pallida* was carried out as described above. Results revealed that fraction P4F3-1 showed two protein sequences NLYQFKNM and LKXNQLIP which were in 5:1 relative abundance. The protein sequence NLYQFKNM was 10 pmols and the protein sequence LKXNQLIP was 2 pmols. These protein sequences NLYQFKNM and LKXNQLIP were homologous to phopholipase 2 ($PLA_2$) and three finger toxin (3FTX). Thus both proteins $PLA_2$ and 3FTX were found in fraction P4F3-1 (Table 5-1).

The N-terminal sequencing of fraction P4F3-2 indicated a single protein sequence LKXNQLIPPFWKTXP which was identical to the previously characterised three-finger cardiotoxin gamma (3FCTγ originally thought to be from *N. nigricollis* but likely to be from *N. pallida*). This fraction was pure because it showed single protein sequence (Table 5-1).

Fraction P4F4-1 showed the single N-terminal sequence LKXNKLIPIAYKTXPEGKNLXYK and this sequence was homologous to three finger toxin cytotoxin 4 (3FCT4 previously isolated from *N. mossambica*).

Fraction P4F4-2 showed four protein sequences: LKXNQLIPPFWKTXP, LKXNKLIPIAYKTXP, RQXTQQKPPFYMN--, and NLYQFKNMIHXTV--. These sequences were homologous to three finger toxin cardiotoxin gamma (3FCTγ), three finger toxin cytotoxin 4 (3FTC4), three finger toxin (3FTX) and phospholipase $_2$ ($PLA_2$) respectively. The four peptides were found in the ratio of 5:5:1:1 (Table 5-1).

Finally, fraction P4F4-3 indicated a single N-terminal sequence of TDRCCFVHDC which was identical to three finger toxin (3FTX) (Table 5-1).

BLAST (Basic Local Alignment Search Tool) analysis of purified protein sequences of P4F3-2, P4F4-1 and P4F4-3 was used for comparison and matched with cytotoxins/ cardiotoxins in the *Naja* genus. *Naja* is a genus of venomous elapid

snakes known as cobras. *Naja* are the most widespread and the most widely recognized as cobras. Various species occur in regions throughout Africa, Southwest Asia, South Asia, and Southeast Asia. The purified fractions of protein sequences were matched and compared with other Naja protein sequences like CLBP *Naja naja naja*, Cm-CLPB *Naja naja*, Cm-CLPB *Naja naja*, Cm-CLPB *Naja naja sia atra*, Cm-cyt I *Naja naja*, Cm-cyt II *Naja naja*, Cm-cyt III *Naja naja*, Cm-cyt IV *Naja naja*, CTX1 *Naja mossambica*, CTX2 *Naja mossambica*, CTX3 *Naja mossambica*, CTX1 *Naja pallida*, CX1 *Naja kauothia*, CX2 *Naja nivea*, CX3 *Naja kauothia*, CX3 *Naja nivea*, CX3 *Naja melanoleuca*, CX4 *Naja haja annulifera, Naja nigricollis* (Table 5-2).

Some *Naja nigricollis, Naja mossambica, Naja nivea* cytotoxins were matched with BLAST score of 43.9 for the best three finger toxin (3FTX), three-finger cardiotoxin gamma (3FCTγ), three finger toxin cytotoxin 4 (3FTC4) and other Naja matched for phopholipase 2 ($PLA_2$) but fractions P4F3-1 and P4F4-2 were found to contain mixtures of proteins (Table 5-2).

**Table 5-1:** Protein sequence analysis of *Naja pallida* purified fractions

| Purified Fractions | Protein Sequence | Abundance Ratio | Matched peptides | BLAST source with code |
|---|---|---|---|---|
| **P4F3-1** | 1 2 3 4 5 6 7 8<br>N L Y Q F K N M L<br>K X N Q L I P | 10pmols<br>2pmols<br>(5:1) | Phospholipase2 ($PLA_2$)<br>Three finger toxin(3FTX) | |
| ***P4F3-2** | 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5<br>L K X N Q L I P P F W K T X P | Nil | Three finger cardiotoxin gamma (3FCTγ) | *Naja nigricollis*/ *Naja pallida* (P01468) |
| ****P4F4-1** | 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 20 1 2 3<br>L K X N K L I P I A Y K T X P E G K N L X Y K | Nil | Three finger cytotoxin 4 (3FCT4) | *Naja mossambica*<br><br>(P01452) |
| **P4F4-2** | 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5<br>L K X N Q L I P P F W K T X P<br>L K X N K L I P I A Y K T X P<br>R Q X T Q Q K P P F Y M N - -<br>N L Y Q F K N M I H X T V - - | 100pmols<br>100 pmols<br>20 pmols<br>20 pmols<br>(5:5:1:1) | Three finger cardiotoxin gamma (3FCTγ)<br>Three finger cytotoxin 4 (3FCT4)<br>Three finger toxin(3FTX)<br>Phospholipase2 ($PLA_2$) | |
| *****P4F4-3** | 1 2 3 4 5 6 7 8<br>L K X N Q L I P | Nil | Three finger toxin (3FTX) | |

**(*)(**) and (***) Indicated the matched profile of protein sequences are single protein.**

**Table 5-2:** BLAST search of amino acid sequences of cytotoxins/cardiotoxins from the Elapidae snake family.

| **Majority of Elapidae venom protein sequence →** | **LKCNXLIPPF 10 ↓** | **WKTCPAGKNL 20 ↓** | **CYKMTMVXAP 30 ↓** | **KVPVKXGCID 40 ↓** | **VCPKNSLLXK 50 ↓** | **YVCCNTDKCN 60 ↓** |
|---|---|---|---|---|---|---|
| **P4F3-2 (*) *Naja pallida*** | **LKXNQLIPPF** | **WKTXP** | | | | |
| **P4F4-1 (**) *Naja pallida*** | **LKXNKLIPIA** | **YKTXPEGKNL** | **XYK** | | | |
| **P4F4-3 (***) *Naja pallida*** | **LKXNQLIP** | | | | | |
| ***Naja pallida* CX 1** | LKCNQLIPPF | WKTCPKGKNL | CYKMTMRAAP | MVPVKRGCID | VCPKSSLLIK | YMCCNTDKCN |
| **CLBP Naja *naja naja*** | LKCHNTQLPF | IYKTCPEGKN | LCFKATLKKF | PLKFPVKRGC | NCPKNSALLK | YVCCSTDKCN |
| **Cm-CLPB *Naja naja*** | LKCHNTQLPF | IWKTCPEGKN | LCFKATLKKF | PLKIPIKAGC | NCPKNSALLK | YVCCSTDKCN |
| **Cm-CLPB *Naja naja*** | LKCHNTQLPF | IYKTCPEGKN | LCFKATLKKF | PLKFPVKAGC | NCPKNSALLK | YVCCSTDKCN |
| **Cm-CLPB *Naja naja*** | LKCHNTQLPF | IYKTCPEGKN | LCFKATLKKF | PLKFPVKAGC | NCPKNSALLK | YVCCSTDKCN |
| **Cm-cyt I *Naja naja*** | LKCNKLIPIA | SKTCPAGKNL | CYKMFMMSDL | TIPVKAGCID | VCPKNSLLVK | YVCCNTDACN |
| **Cm-cyt II *Naja naja*** | LKCNKLIPLA | WKTCPAGKAL | CYKMFMVAAP | KVPVKAGCID | ACPKNSLLVK | YVCCNTDACN |
| **Cm-cyt III *Naja naja*** | LKCNKLIPLA | YKTCPAGKNL | CYKMFMVSNK | TVPVKAGCID | ACPKNSLLVK | YVCCNTDACN |
| **Cm-cyt IV *Naja naja*** | LKCNKLVPLF | YKTCPAGLNL | CYKMFMVATP | KVPVKAGCID | VCPKSSLLVK | YVCCNTDACN |
| **CTX1 *Naja mossambica*** | LKCNQLIPPF | WKTCPKGKNL | CYKMTMRAAP | MVPVKRGCID | VCPKSSLLIK | YMCCNTNKCN |
| **CTX2 *Naja mossambica*** | LKCNQLIPPF | WKTCPKGKNL | CYKMTMRAAP | MVPVKRGCID | VCPKSSLLIK | YMCCNTNKCN |
| **CTX3 *Naja mossambica*** | LKCNRLIPPF | WKTCPEGKNL | CYKMTMRLAP | KVPVKRGCID | VCPKSSLLIK | YMCCNTNKCN |
| **CTX1 *Naja pallida*** | LKCNKLIPLA | YKTCPAGKNL | CYKMYMVSNK | TVPVKAGCID | VCPKNSLVLK | YDCCNTDACN |
| **CX 1 *Naja kauothia*** | LKCNKLVPLF | YKTCPAGKNL | CYKMFMVSNK | TVPVKRGCID | VCPKNSLVLK | YVCCNTDRCN |
| **CX 2 *Naja nivea*** | LKCNQLIPPF | WLTCPLGLNL | CYLMTMRAAP | MVPVLAGCID | VCPLSSLLIK | YMCCNTDLCN |
| **CX 3 *Naja kauothia*** | LKCNKLIPLA | YKTCPAGKNL | CYKMFMVSNK | TVPVKRGCID | ACPKNSLLVK | YVCCNTDACN |
| **CX 3 *Naja nivea*** | LKCNQLIPPF | WKTCPKGKNL | CYNMYMVSTS | TVPVKRGCID | VCPKNSALVK | YVCCNTDRCN |
| **CX 3 *Naja melanoleuca*** | LKCNRLIPPF | WKTCPEGKNL | CYKMTMRLAP | KVPVKRGCID | VCPKSSLLIK | YMCCNTNKCN |
| **CX 4 *Naja haja annulifera*** | LKCNKLIPPF | WKTCPKGKNL | CYKMYMVSTL | TVPVKRGCID | VCPKNSALVK | YVCCNTNKCN |
| ***Naja nigricollis*** | LKCNQLIPPF | WLTCPLGLNL | CYLMTMRAAP | MVPVLAGCID | VCPLSSLLIK | YMCCNTDLCN |
| ***Cytotoxin isolated in the present work from *Naja pallida*** | | | | | | |

## 5.4 Discussion

As described earlier in the Introduction, snake venoms are complex mixtures of many enzymatic and non-enzymatic proteins, as well as small peptides. Several major venom protein superfamilies, including three-finger toxins, phospholipases $A_2$ ($PLA_2$), serine proteinases, metalloproteinases, proteinase inhibitors and lectins, are found in almost all snake venoms, from front-fanged viperids (vipers and pit vipers) and elapids (cobras, mambas, sea snakes, etc.). However, these proteins vary in abundance and functionality between species. In order to assess the mechanisms underlying the biological effects of venoms, it is generally necessary to isolate individual components and study them separately. This is equally relevant when looking for agents with therapeutic potential from venoms.

The main objective of the work in this thesis was to identify possible anti-cancer activity in three snake venoms and to isolate active components. All three venoms studied had cytotoxic effects on the three prostate cancer-derived cell lines, but they were similarly active against the normal prostate cell line. After several fractionation steps, some samples from the venom of red spitting cobra *N. pallida* appeared to be selective on the cancer cell lines. Therefore, these purified fractions were studied in more detail (in chapter-3 and chapter-4). In chapter-5, the focus was to identify the peptides by N-terminal sequencing procedure with BLAST search tools.

The N-terminal sequence of fractions P4F3-1, P4F3-2, P4F4-1, P4F4-2 and P4F4-3 of *N. pallida* were clearly homologous to known proteins such as three finger toxin (3FTX), phopholipase $A_2$ ($PLA_2$), three finger cardiotoxin gamma (3FCTγ), and three finger toxin cytotoxin 4 (3FTC4). Thus, these protein sequences were matched with a family of Elapidae polypeptide cytotoxins/ cardiotoxins. As shown in Table 5-1, the cytotoxins isolated in the present work matched the cytotoxin/cardiotoxin like basic protein (CLBP) (Shipolini, Kissonerghis et al. 1975; Inoue, Ohkura et al. 1987; Inoue, Okumura et al. 1989; Shafqat, Zaidi et al. 1990), with other cytotoxins in other types of snakes. Petras et al. (2011) made a closer comparison of the venom proteins and showed that the major toxin (cytotoxin) is

made up of similar molecules in the five African spitting *Naja* venoms. They clearly reported that all of the venoms contain similar 3FTx similar toxins isolated in *N. mossambica* (N-terminal sequence LKCNKLIPIAYKTCP, and N-terminal sequence LKCNQLIPPFWKTCP), and *N. katiensis* (N-terminal sequence LKCNRLIPPFWKTCP). The venoms of *N. nigricollis*, *N. katiensis*, and *N. pallida* also contain a 3FTx that possesses N-terminal (XQCTQQKPPFYMNCP) similar with *N. atra* Q9W6W6. In addition, each venom from *N. pallida*, *N. katiensis*, and *N. nigricollis*, two *N. nubiae*, or three *N. mossambica* contain homologous $PLA_2$ molecules, which share N-terminal sequences and very similar molecular masses (Petras, Sanz et al. 2011).

In the present work, two cytotoxins, P4F3-2 and P4F4-1, isolated from the venom of the red spitting cobra *Naja pallida* were found to be selectively effective against two prostate cancer cell lines. Such specificity was also reported with other cell lines by Wu, Ming et al. (2013) for the cytotoxin P4 of the venom of *Naja atra* (on MCF-7, P388, K562, and H22 and on human cell line 16HBE).

CTXIII from the Taiwan cobra *Naja atra* atra was found to exhibit a variety of bioactivities with anti-cancer potential. For example, CTXIII inhibits the cellular proliferation and induces apoptosis of various cancer cells, including breast cancer (Chiu, Lin et al. 2009), leukaemia cells (Yang, Chien et al. 2007), colorectal cancer (Tsai, Yang et al. 2006), and oral cancer (Chien, Lin et al. 2009; Chien, Chang et al. 2010).The *Naja naja atra* CTX III was reported to have cytotoxic activity with cell lines including OSCC, Ca9-22, SAS, and CAL27, through cell cycle arrest at the S-phase arrest and induction of apoptosis (Yen, Liang et al. 2013). In addition, CTXIII has been shown to inhibit MDA-MB-231 cell metastasis (Lin, Chien et al. 2012). Another study showed the effects of *Naja naja atra* cardiotoxins 3 (CTX3) and *Naja nigricollis* toxin γ was associated with their membrane-damaging activity (Kao, Lin et al. 2012). However, until now, little was known about the effect of CTXIII or other cardiotoxins on prostate cancer cell lines.

## 5.5 Conclusion

Although the toxins isolated from *Naja pallida* venom in this study are likely to be identical to cytotoxins found earlier in other cobra venoms, the toxins P4F3-2 (3FTX) and P4F4-1 (3FCTγ) were shown for the first time to be selectively cytotoxic on prostate cancer cell lines in vitro, while sparing a normal prostate cell line. On this basis, the two peptides could be potential leads for anti-cancer agents. Of course, there are many hurdles to be overcome before the true potential of these compounds will be established. There needs to be further testing on a wider range of cancer and normal cells in vitro, followed by tests using relevant animal models of cancer, as well as more general toxicity testing.

There is further need to draw attention of researcher to continue the future work which is suggested below:

## 5.6 Future work

A. Further need to investigate the side effects of these cytotoxins on different body organs, and to understand the toxicity mechanism.

B. To investigate the mechanism of anticancer action and specificity of the anticancer peptides.

C. The detailed study of anti-cancer drug delivery system of cytotoxins.

# CHAPTER SIX:

## REFERENCES

# 6. CHAPTER SIX

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