Citrus Peel in Maintaining Cellular Quiescence of Prostate Cancer

By

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To my parents

DECLARATION

I hereby declare the work presented in this thesis is my own work and that, to the best of my knowledge original, except as acknowledged in the text, and has not been submitted for the award of any other degree at another university. Some parts of this research work have been published and a list of publications arising from this research is included in this thesis.

Balakrishnan Shammugasamy

LIST OF PUBLICATIONS

Journal Paper

B. Shammugasamy, P. Valtchev, Q. Dong and F. Dehghani, "Effect of citrus peel extracts on the cellular quiescence of prostate cancer cells" (Submitted for publication)

Conferences Presentations

B. Shammugasamy, P. Valtchev, F. Dehghani and Q. Dong, "The role of citrus peel extract in inhibiting progression and recurrence of prostate cancer", 16th International Congress on Targeted Anticancer Therapies (TAT), 2018, Paris, France (Poster presentation)

B. Shammugasamy, P. Valtchev, F. Dehghani and Q. Dong, "Potential uses of orange peel extract in inhibiting the recurrence of prostate cancer", Future Food and Health Research-Industry Engagement forum, 2017, Sydney, Australia (Oral presentation)

B. Shammugasamy, P. Valtchev, F. Dehghani and Q. Dong," The role of citrus peel extract in inhibiting progression and recurrence of prostate cancer", 2017 Postgraduate and ECR Cancer Research Symposium, 2017, Sydney, Australia (Oral presentation)

B. Shammugasamy, P. Valtchev, F. Dehghani and Q. Dong, "Potential uses of orange peel extract in inhibiting the recurrence of prostate cancer ", International Workshop on Advances in Biotechnology for Food and Medical Application, 2016, Sydney, Australia (Oral and poster presentation)

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Finally, to my family for all their love, without which any of this would matter.

Love you all endlessly.

<div align="right">Balakrishnan</div>

ABSTRACT

Cancer recurrence remains a major concern for cancer patients despite the significant advancement in cancer treatment. Cell cycle re-entry of quiescent cancer cells has been implicated as a key factor for cancer recurrence. The slow progression of prostate cancer allows a window of opportunity for intervention through diet. Therefore, the aim of this thesis was to evaluate the potential use of citrus peel extract in halting the cell cycle re-entry of quiescent prostate cancer cells.

Firstly, water extract and hexane extract of citrus peel were prepared using a maceration technique. The anticancer activity of the extracts was evaluated on prostate cancer PC-3 and LNCaP cells using *in-vitro* assays. The cell viability analysis showed that hexane extract was more effective in reducing cell viability compared to water extract in both cell lines. For cell cycle analysis, quiescent prostate cancer PC-3 and LNCAP cells were released to re-enter cell cycle in the presence of extract. Water extract completely inhibited the entry of quiescent cells from G0/G1 phase to S phase upon release from quiescence. In contrast, the hexane extract completely allowed the cells progress to S and G2/M phase. However, both extracts significantly decreased the DNA synthesis rate in PC-3 cells compared to the control when analysed using EdU incorporation assay. Hesperidin and naringin, the predominant flavonoid compounds in water extract, exhibited negligible cell cycle re-entry inhibitory effect on quiescent PC-3 cells suggesting the contribution of other bioactive compounds in the extract. More importantly, the water extract showed no toxicity when tested on the normal human fibroblast cells.

Subsequently, solvent extraction and chromatography techniques were used to identify the compounds in water extract that are responsible for the cell cycle re-entry inhibitory effect. The results showed that the water fraction exhibited the highest inhibitory effect among the fractions tested. Not only that, the inhibitory activity of fractions was lower than the water extract suggesting the compounds exhibited a greater effect in combination and the presence of a synergic effect. Using various analytical methods, citric acid was identified as one of the cell cycle re-entry inhibitor compounds present in water extract. The cell viability assay showed the IC_{50} of citric acid for prostate cancer PC-3 cells and normal epithelial prostate RWPE-1 cells were almost similar. However, citric acid significantly exhibited higher cell toxicity effect on

PC-3 cells than RWPE-1 cells at a concentration above 1 mg/mL indicating the specific toxicity of citric acid on cancer cells. Not only that, the citric acid showed no significant toxicity effect on human normal fibroblast GM3348 cells when tested on similar concentration.

As the mechanisms of cell cycle re-entry of quiescent cells remain largely unclear, a study was attempted to elucidate the protein changes by the citric acid at the cellular level. The proteomic experiment results revealed several proteins were regulated differently in the citric acid treated cells that were possible to act with the inhibition of cell cycle re-entry. The Ingenuity Pathway Analysis software predicted that citric acid possibly inhibited PC-3 cells from re-entering cell cycle mainly by suppressing PI3K/AKT and ERK/MAPK signalling pathways and activating PTEN pathway. Modulation of these signalling pathways by diet or drug has the potential to reduce the cell cycle re-entry of quiescent prostate cancer cells and preventing cancer recurrence.

The outcomes of this study have widened the applications of citrus peel extract as a chemopreventive agent for post-therapy cancer patients. The recovery and utilization of bioactive compounds from citrus peel not only reduce the waste but also will open an avenue for the development of affordable fortifying food products with potential in reducing the risk of cancer recurrence.

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1 Introduction

Cancer recurrence remains a major issue in post-therapy cancer patients. It is defined as the return of cancerous cell after a period of time (Páez, Labonte et al. 2012). The remaining cancer cells after the treatment cause cancer recurrence with a high chance of mortality (Rochat 2009, Feitelson, Arzumanyan et al. 2015). Thus, practising a healthy lifestyle with regular monitoring is vital for cancer patients to reduce the risk of cancer recurrence. Many epidemiological studies have shown a positive correlation between high intake of fruits and vegetables with a low occurrence of cancer (Block, Patterson et al. 1992, Steinmetz and Potter 1996, Key 2010, Rodriguez-Casado 2016). These findings are also in agreement with many *in-vitro* and *in-vivo* studies, which showed fruits or vegetables and their extracts possesses anticancer activity. Fruits and vegetables have the capability in preventing initiation, promotion and progression of cancer. However, the ability of fruits and vegetable in preventing cancer recurrence is still unclear and not comprehensively studied.

Citrus is consumed widely and is a source of nutrients with a broad range of health benefit including anti-cancer activity (Aggarwal and Shishodia 2006, Meeran, Ahmed et al. 2010, Mehta, Murillo et al. 2010). Epidemiological studies showed high intake of citrus and its derived products are associated with lower risk of developing various cancer including esophageal, gastric, stomach, breast, prostate and pancreas cancer (Ferrís-Tortajada, Berbel-Tornero et al. 2012, Giacosa, Barale et al. 2013, Cirmi, Ferlazzo et al. 2016, Capurso and Vendemiale 2017). The anticancer activity in citrus is contributed by a range of bioactive compounds such as flavonoids, limonoids, coumarins, phenolic acids, terpenoids, and carotenoids together with many other unknown bioactive components (Yu, Wang et al. 2005, Aggarwal and Shishodia

2006, Meeran, Ahmed et al. 2010, Mehta, Murillo et al. 2010, Zou, Xi et al. 2016). A number of *in-vitro* and *in-vivo* studies have shown the potential anticancer activity of citrus and its derived bioactive compounds in inhibiting different cancer lines by modulating various biological events leading to anti-proliferation, cell cycle arrest, apoptosis induction, anti-inflammatory, anti-angiogenesis and anti-metastatic activities (Cirmi, Ferlazzo et al. 2016, Cirmi, Maugeri et al. 2017). The citrus particularly showed a strong anticancer effect in prostate cancer by inhibiting the tumour growth completely in an *in-vivo* mouse model (Lai, Li et al. 2013b, Kim, Lee et al. 2017).

Prostate cancer is one of the leading causes of cancer-related deaths in men. About 1.1 million men were diagnosed worldwide with prostate cancer with 307,000 deaths in 2012 (Ferlay, Soerjomataram et al. 2015). Despite a significant improvement in 5-year relative survival rate up to 95% (AIHW 2017), prostate cancer recurrence remains a major problem in prostate cancer patients as up to 50% of the men will experience cancer recurrence within ten years after initial surgery (Amling, Blute et al. 2000, Hull, Rabbani et al. 2002, Roehl, Han et al. 2004). Moreover, cancer recurrence possesses high mortality threat amongst these patients.

Although the causes for cancer recurrence are still poorly elucidated, cell cycle re-entry of quiescent cancer cells has been suggested for cancer recurrence (Holmgren, O'Reilly et al. 1995). There are a complex series of molecular and biochemical signalling pathways that control the cell cycle progression. Alteration in the protein expression related to cell cycle by the bioactive compound(s) could halt the cell-cycle re-entry. Citrus and its derived bioactive compounds have been shown to arrest cell cycle progression in many cancer cell lines such as in breast, colon, prostate and lung cells (Cirmi, Ferlazzo et al. 2016). This finding shows the possibility of the bioactive compound derived from citrus to arrest the quiescent cancer cells, halt the re-entry into

the cell cycle and subsequently delay the prostate cancer recurrence. In fact, anticancer drugs act by a variety of mechanisms including by halting the cancer cells from progressing into and in the cell cycle.

Citrus is mainly consumed as juice. Citrus juice industry generates citrus peel, sacs and seed as waste products, which account for more than 50% of original fruit weight (Marín, Soler-Rivas et al. 2007). These waste products contain a high amount of different bioactive compounds than the juice itself (Miyake, Murakami et al. 1999, Wang, Chuang et al. 2008, Zhang, Wu et al. 2014). The bioactive compounds in these waste products can be used as a precursor for the production of high-valuable products with emerging applications in pharmaceutical and food industries (Schieber, Stintzing et al. 2001). In this study, we hypothesized that citrus peel may impede cell cycle re-entry of quiescent prostate cancer cells and subsequently potential in reducing the risk of cancer recurrence.

The aim of this study was to evaluate the ability of citrus peel extract in inhibiting the quiescent prostate cancer cells from re-entering cell cycle using *in-vitro* assays. To access the overall hypothesis, the experimental works were focussed on the following aims: (1) to prepare extracts that containing different spectrum of bioactive compounds from citrus peel, (2) to determine the anticancer activity of the extract including the ability in halting the cell cycle re-entry of quiescent prostate cancer cells, (3) to isolate and identify the bioactive compound(s) in extract that are responsible for the cell cycle re-entry inhibitory activity, (4) to evaluate the toxicity of extract and isolated compound(s) on normal cell lines, and (5) to identify the modulated proteins and signaling pathways at cellular level that are inhibit cell-cycle re-entry of prostate cancer cells.

2 Citrus as anticancer agent and for cancer recurrence prevention

2.1 Cancer

Cancer is the second main cause of death after cardiac-related diseases. Approximately, 14.1 million new cases in 2012 were reported worldwide and the number is predicted to increase to 20 million by 2025 (Ferlay, Soerjomataram et al. 2015). Meanwhile, the mortality rate was about 8.8 million in 2015 (Ferlay, Soerjomataram et al. 2015). Globally, the frequency of mortality by cancer is one in six and about 70% of cancer mortality happen in non-developed countries (WHO 2017).

Cancer is characterized by uncontrolled cellular proliferation resulting from the aberrant activity of various cell cycle proteins (Visconti, Della Monica et al. 2016, Otto and Sicinski 2017). The uncontrolled proliferation is highly due to the irreversible mutation of DNA that regulate cell cycle (Otto and Sicinski 2017). Physical carcinogens (e.g. ultraviolet and ionizing radiation), chemical carcinogens (e.g. asbestos, tobacco smoke and arsenic) and biological carcinogens (e.g. viruses, bacteria, or parasites) are some of the factors that cause DNA damage (WHO 2018). The mutated DNAs express irregular proteins that disturb regulatory pathways and signalling cascades. The cells have a selective advantage that allows them to multiply rapidly, acquire resistance to apoptosis, develop different cell phenotype and the ability to spread and invade other distant organs. The ability of cancer cells to break away from the primary state, travel through the bloodstream or lymphatic system to the new site and form a new tumour called as metastasis.

Cancer can occur in any part of the body as it arises from normal cells. Lung, breast, colon and prostate cancer are the top four commonly occurring cancers, which are responsible for 4 in 10 of all cancers diagnosed worldwide (Ferlay, Soerjomataram et al. 2015). Lung cancer is the most commonly diagnosed cancer worldwide for both men and women in 2012 (Figure 2.1). Meanwhile, breast cancer and prostate cancer are the second most common cancer diagnosed for women and men, respectively. A similar trend was reported in Australia for 2017, where prostate and breast cancer were the most diagnosed cancer for male and female, respectively (AIHW 2017).
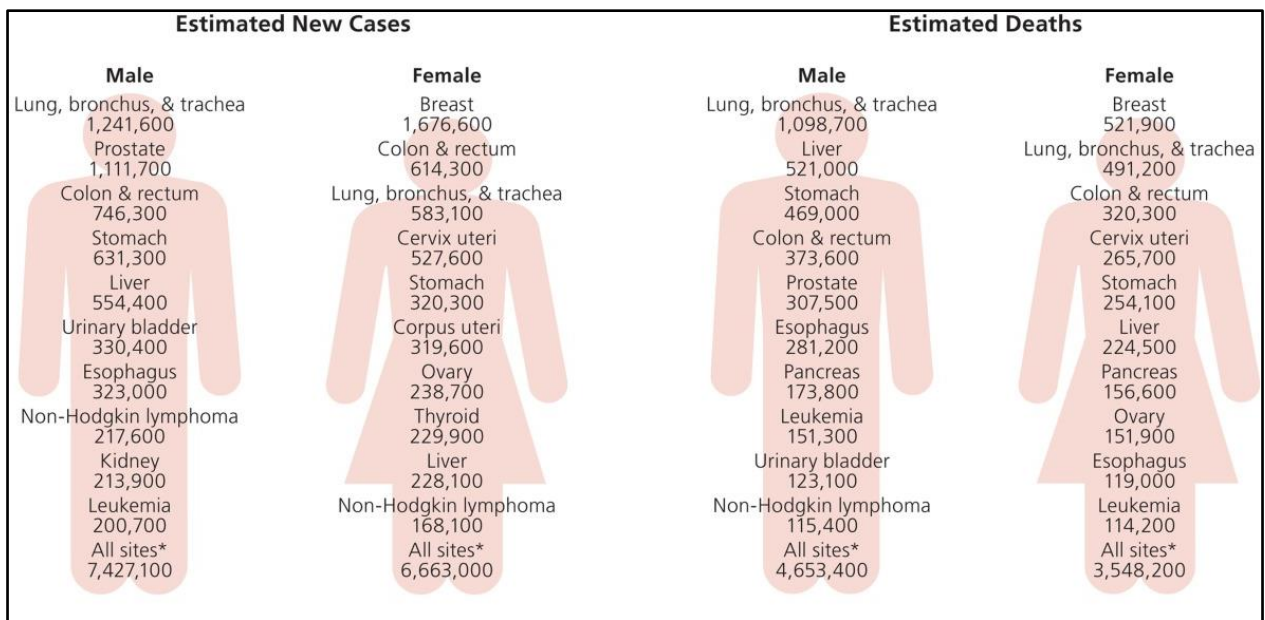
Figure 2.1: Estimated new cancer cases and deaths worldwide by sex and level of economic development (Ferlay, Soerjomataram et al. 2013).

2.1.1 Cancer recurrence

Cancer recurrence is defined as the return of cancer after a period of time when cancer couldn't be detected (Li, Rogoff et al. 2015). Cancer recurrence happens with latency periods ranging from years to decades. Current cancer treatments fail to destroy the cancer cells and the remaining residual cancer cells have the potential to grow into a new tumour. The efficacy of the treatment varies between patients as the cancer cells are extremely heterogeneous and have different sensitivity to the cancer treatments (Li, Rogoff et al. 2015). Cancer recurrence imposes high chance of mortality on cancer patients (Rochat 2009, Feitelson, Arzumanyan et al. 2015).

The causes and mechanisms for cancer recurrence are not well elucidated. One of the suggested causes for cancer recurrence is the presence of quiescent tumour cells (Holmgren, O'Reilly et al. 1995, Páez, Labonte et al. 2012), which remains after the primary intervention (Aguirre-Ghiso 2007). Some residual cancer cells enter the G0–G1 phase, develop resistance to chemotherapy and escape the immune system. The accumulated transition into quiescence through a G0–G1 arrest is postulated due to the disruption in the signalling between the cell and surrounding microenvironment (Aguirre-Ghiso 2007). However, changes in the microenvironment and any other signals could trigger the quiescent cells to re-enter the cell cycle and proliferate. This entry of the quiescent cells into the cell cycle is believed to cause cancer recurrence. The mechanisms for the transition of disseminated tumour cells that have remained quiescent into a proliferative state are still not well understood (Páez, Labonte et al. 2012). Thus, understanding the exit of tumour cells from the cell cycle and the re-entry of quiescent tumour cells to cell cycle constitutes an important point for intervention in the treatment of cancer and targets for the development of a new generation of anti-cancer drugs.

2.2 Cell cycle

The cell cycle consists of four phases known as G1 (growth phase), S (DNA synthesis), G2 (growth phase) and M (mitosis) as illustrated in Figure 2.2. Cell cycle progression starts with G1 phase where the cell will enter either from quiescence phase (G0) or M phase (Vermeulen, Van Bockstaele et al. 2003, Visconti, Della Monica et al. 2016, Otto and Sicinski 2017). At G1 phase, the cell increases its transcription and translation activity to grow and prepare the cell to synthesize DNA at later phase. At S phase, the cell duplicates its DNA content and subsequently progresses to G2 phase in preparation for cell division. While at M phase, the cell that has duplicated DNA content and organelles will start to divide to form two new daughter cells.
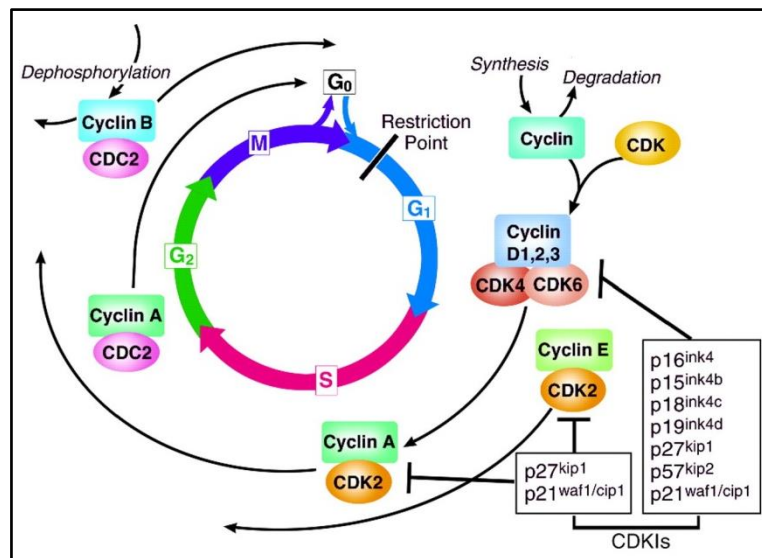
Figure 2.2: The cell cycle: phases and the regulator proteins. Progression of the cell through the cell cycle phases (G1, S, G2, and M) is promoted by cyclin-dependent kinases (CDKs), which are regulated positively by cyclins and negatively by CDK inhibitors (CDKIs) (Schwartz and Shah 2005).

2.2.1   Cell cycle checkpoints, signalling and regulatory proteins

Cell cycle progression involves a complex series of molecular and biochemical signalling pathways that either stop or progress the cells through the cell cycle. The proteins involved in each checkpoint in regulating the cell cycle mechanism are shown in Figure 2.2. The checkpoint G1/S at the end of the G1 phase will ensure the cell is free from damaged DNA. Any cell with defective DNA is arrested and eliminated thru apoptosis. The second checkpoint is at the G2/M phase. A cell with incomplete DNA due to duplication error in S phase is arrested at G2/M checkpoint (Otto and Sicinski 2017).

Progression through checkpoints is controlled by various proteins, mainly cyclin dependent kinases (CDKs) and cyclins. A CDK/cyclin complex is formed with the activation of CDK by the cyclins that are present only for short periods in the cell cycle (Vermeulen, Van Bockstaele et al. 2003, Visconti, Della Monica et al. 2016, Otto and Sicinski 2017). The activated CDK-cyclins phosphorylate numerous targets and regulate several processes that allow the release of transcription factors required for DNA replication, DNA repair, and cell cycle progression (Cobrinik 2005). The activity of Cyclin/CDKs is regulated by endogenous CDK inhibitors, which bind to this complex and halts the cell cycle progression (Otto and Sicinski 2017). Cip/Kip inhibitors such as p21, p27 and p57 inhibit all cyclin/Cdk complexes, while INK4a inhibitors such as p16INK4a p15INK4b p18INK4c p19INK4d and p19/alternative reading frame (ARF) inhibit only Cdk4 and Cdk6 (Sherr and Roberts 1999).

The entry of cell to early G1 phase is induced by different mitogenic signals such as RAS/RAF/MAPK pathways, which produces transcription factors such as MYC, AP1 and β-catenin as shown in Figure 2.3**.** These transcription factors are required for the production of

cyclin D. Three CDKs (2, 4 and 6) and their cyclins (D, E) are responsible for the progression through G1. In addition, the retinoblastoma tumour suppressor gene product (Rb) in its active state prevents the G1/S transition by binding to the E2F-DP complex. Activated CDK4/6-Cyclin D and CDK2/Cyclin E complexes phosphorylate the Rb- E2F-DP complex. This phosphorylation subsequently releases the E2F-DP for the transcription of various proteins involved in DNA replication leading to entry of cells into S phase (Malumbres and Barbacid 2001). Senescence-inducing and growth-inhibitory signals inhibit the formation of CDK/Cyclin complex (Otto and Sicinski 2017). At the S phase, cyclin A activates CDK2 and allow the progression thru the S-phase by allowing the production of enzymes and proteins required for DNA synthesis (Schwartz and Shah 2005). Levels of cyclins A and B increases at the end of S phase and in G2 phase. At the completion of G2 phase, cyclin B/CDK1 complex (also called cyclin B/cdc2) is activated by phosphorylation of cdc25c (Schwartz and Shah 2005), which allows the cells to continue mitosis. Anaphase-promoting complex (APC)/cyclosome and the degradation of cyclin B control the progression thru the mitosis (Ford and Pardee 1999). Inactivation of cyclin B/Ck1 complex allows the cells to exit M phase. The daughter cells either progress to G1 phase or exit the cell cycle and present in the G0 phase.
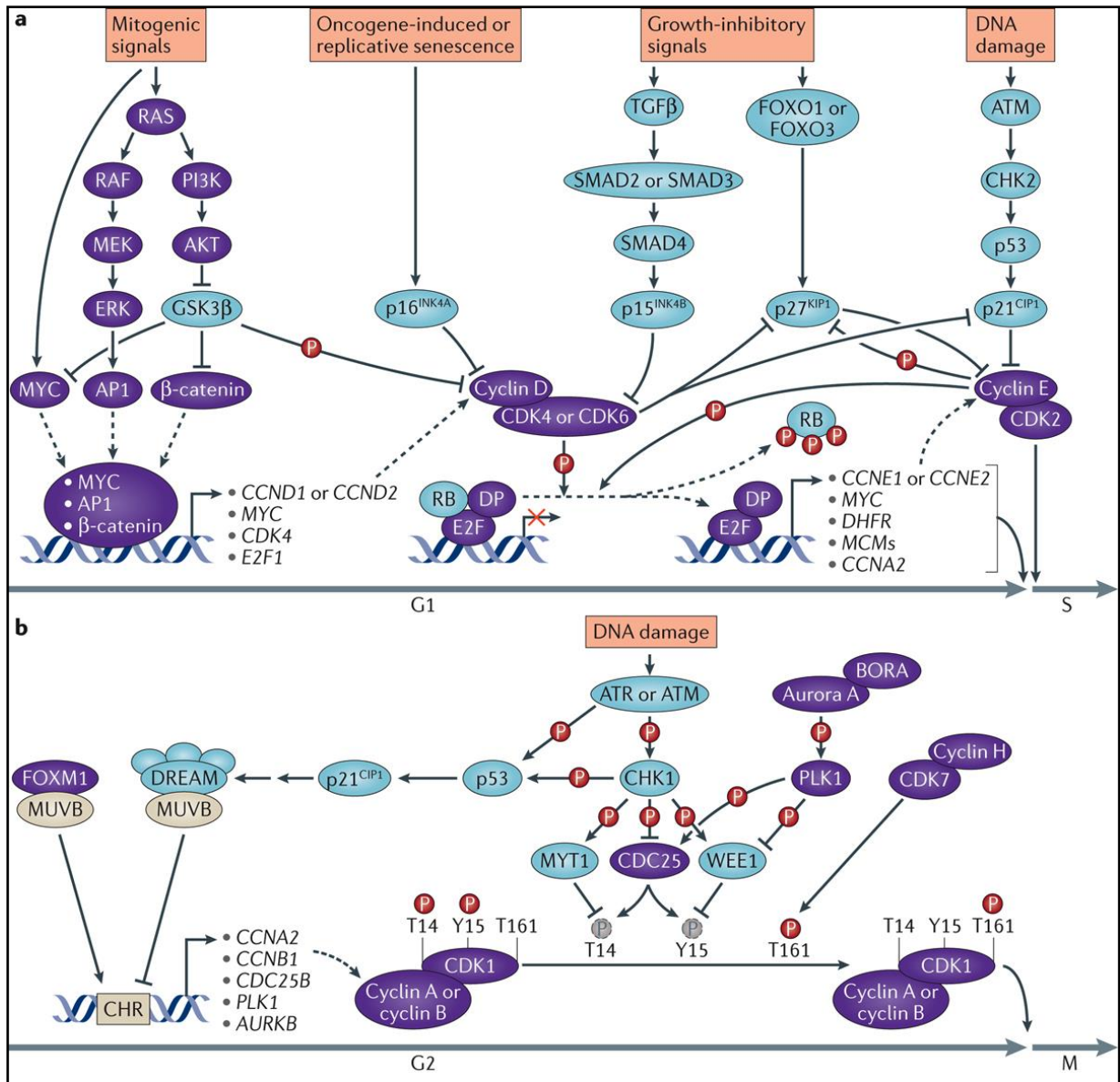
Figure 2.3: Regulation of G1–S (a) and G2–M (b). Cell cycle transitions are controlled by multiple proteins and pathways (Otto and Sicinski 2017). Purple ovals refer to proteins promoting cell cycle progression, while blues ovals refer to proteins inhibiting cell cycle transition.

2.2.2   Drugs targeting cell cycle progression

One potential strategy for cancer treatment is to use small molecules to disrupt the critical cellular machinery required for cell division. In recent years, many drugs targeting the CDKs and other cell cycle proteins were developed and tested in clinical trials to restrict the cancerous growth. Example of anticancer compounds that target the cell cycle is shown in Figure 2.4. Examples of the cell cycle drugs that have been tested in clinical phase are shown in Table 2.1. Determining and identifying the full spectrum of proteins involved in cell cycle re-entry from G0 would be useful in developing new selective drugs that target the quiescent cancer cells. As can be seen from Figure 2.4, the transition from G0/G1 to S phase involves the largest number of identified proteins, yet there is only one drug that blocks this transition, the 5-Fluouracil (5-FU) and its pro-drug, Capecitabine. However, 5-FU acts non-specifically with respect to the cell cycle and arrests the cells in G0/G1 by inhibiting the thymidylate synthase, which produces the thymidine monophosphate, the active form of thymidine required for the synthesis of DNA. Thus, the 5-FU exerts its activity non-specifically by blocking the DNA synthesis in any dividing cell which is responsible for its side effects. A new generation of drugs targeting specifically one or more proteins involved in the G0/G1-S phase transition may provide a better, more selective approach to discriminate cancer cells by exploiting conserved mutations/aberrations in these proteins.
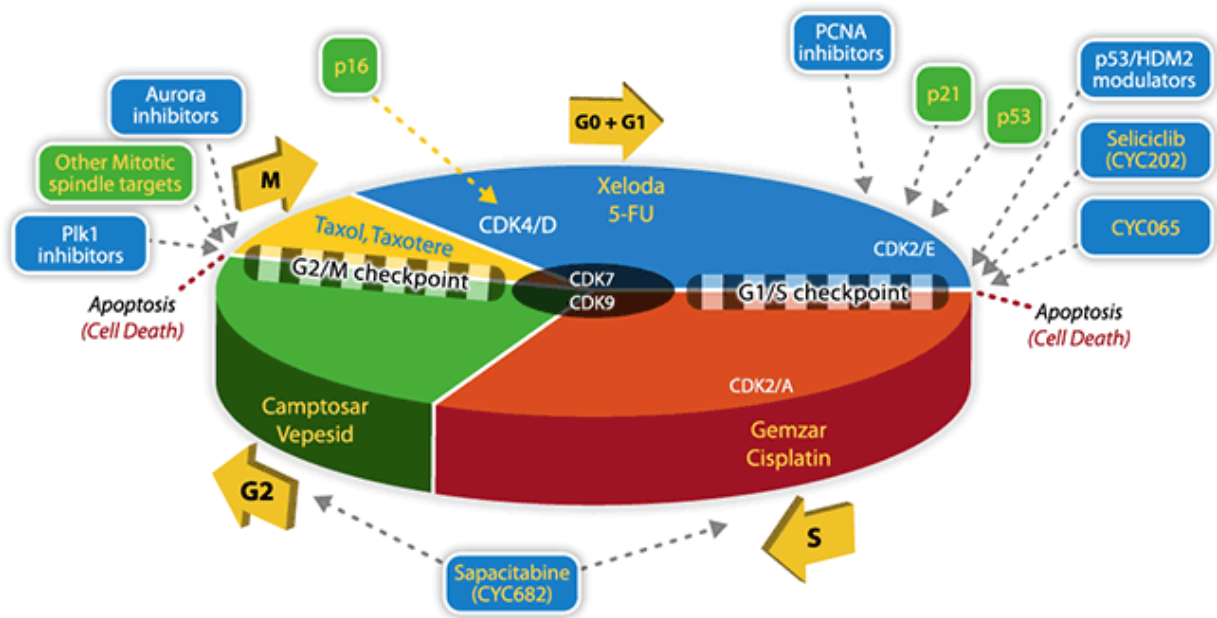
Figure 2.4: Examples of anticancer compounds that are used to inhibit cell cycle progression (Cyclacel 2018).

Table 2.1: Inhibitors of cell cycle proteins being tested in clinical development (Otto and Sicinski 2017).

Pan-CDK inhibitors
Flavopiridol*(alvocidib)
R-roscovitine‡(seliciclib)
Dinaciclib* (SCH 727965/ MK-7965)
AT7519*(AT7519M)
Milciclib‡ (PHA-848125/ PHA-848125AC)
TG02‡
CYC065‡
RGB-286638*
CDK4- and CDK6-selective inhibitors
Palbociclib‡(PD0332991)
Ribociclib‡(LEE011)
Abemaciclib‡(LY2835219)
Checkpoint kinase1 and WEE1 protein inhibitors
MK-8776* (SCH 900776)
LY2606368*(prexasertib)
AZD1775‡ (MK-1775)
GDC-0575‡ (Arry-575)
Polo-like kinase inhibitors
Rigosertib* (ON 01910.Na)
Volasertib* (BI 6727)
TKM-080301*(TKM-PLK1)
CFI-400945‡
Aurora inhibitors
Alisertib‡(MLN8237)
ENMD-2076‡
AMG 900‡

*Intravenous; ‡oral;

2.3 The potential use of bioactive compounds as cell cycle modulators

Various natural sources have shown promising anticancer activity. This has drawn the interest of various researchers to identify and evaluate the bioactive compounds responsible for the observed anticancer activity. In fact, the anticancer drugs that are purely natural or derived from natural sources made to 49% of all approved cancer therapeutics by the US Food and Drug Administration between 1940 and 2014 (Newman and Cragg 2016). Examples of prominent anticancer compounds that are being used clinically in the treatment of cancer are Camptothecin from Camptotheca acuminate and Taxol from Taxus brevifolia. (Atanasov, Waltenberger et al. 2015). In recent years, a different class of bioactive compounds were evaluated for anticancer activity. The anticancer activity of flavonoids (Wang, Wang et al. 2014, Kaur and Kaur 2015, Vue, Zhang et al. 2016), limonoids (Ozaki, Ayano et al. 1995, Miller, Taylor et al. 2000, Kim, Jayaprakasha et al. 2012), carotenoids (Nishino, Murakoshi et al. 2009), coumarins (Venugopala, Rashmi et al. 2013) and phenolic acids (De, Baltas et al. 2011, Rocha, Monteiro et al. 2012) has been intensively investigated. Some of these bioactive compounds have been shown to modulate the cell cycle of cancer cells (Singh, Dhanalakshmi et al. 2002). Therefore, identifying these active compounds and the mechanisms through which they exert their activity would be useful for the health-care purpose.

The best examples of anti-cancer drugs that target the cell cycle and have been modelled upon natural compounds are the Vinblastine and Colcemid. Vinblastine has been structurally derived from the vinka alkaloid Vincristine extracted from the rosy periwinkle (*Catharanthus roseus*) and the Colcemid is structurally related to Colhicin, extracted from the autumn crocus (*Colchicum autumnale*). Both alkaloids bind to tubulin and disrupt the mitotic spindle leading to arrest in G2/M and subsequent cell death by apoptosis. These examples provide a proof of

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concept and validate a mechanism of action that is effective against cancer. The research in this thesis has drawn motivation from these examples and aims to discover natural compounds that block the transition from the quiescent state which is more relevant to the reactivation of remaining dormant cancer cells responsible for the episodes of relapse in many types of blood cancers and carcinomas.

2.4   Citrus and cancer

Citrus belongs to the family of Rutaceae and it is one of the most common fruits consumed widely across the world. The most produced citrus fruits are sweet orange (C. *sinensis*), mandarins (C. *reticulate*), lemons (C. *limon*) and limes (C. *aurantifolia*), grapefruit (C. *paradisi*) and pommelo (C. *grandis* or C. *maxima*) (Spiegel-Roy and Goldschmidt 1996). Annual production of citrus is increasing each year and has reached about 124 million in 2016, of which oranges were the most major citrus fruit accounting for about 67 million tons (Figure 2.5) (FAO 2017).
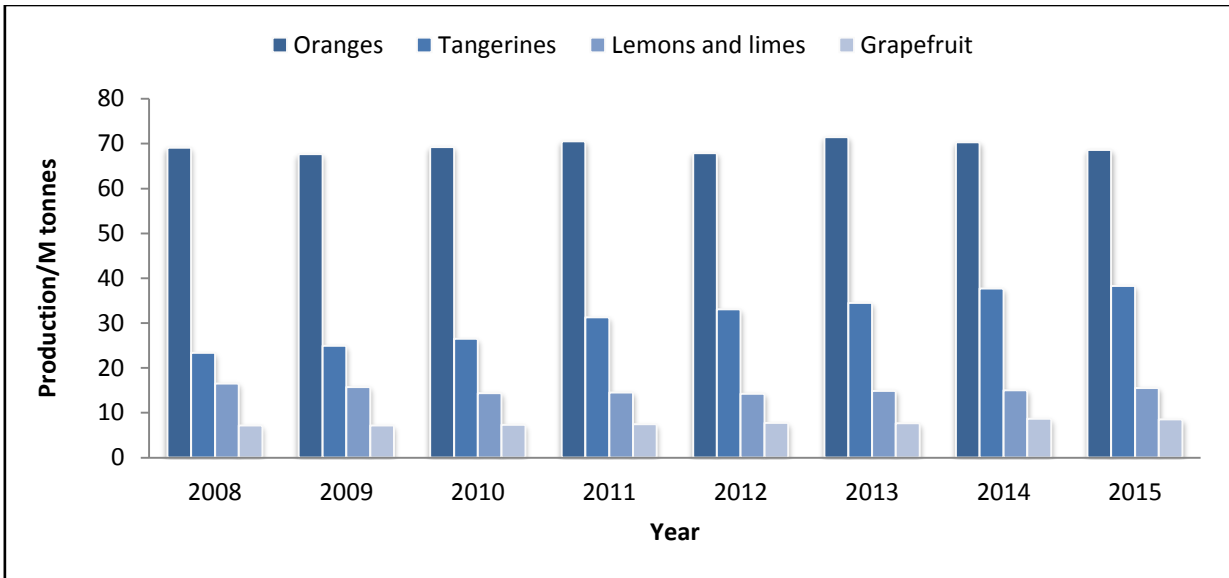
Figure 2.5: World production of citrus fruits (M tonnes) for the year 2007 to 2012 (FAO 2017).

As shown in Figure 2.6, the structure of citrus fruits includes flavedo, albedo, seed and pulp (Spiegel-Roy and Goldschmidt 1996). The edible part is the pulp that contains the juice. Citrus fruits are mainly consumed in the form of fresh fruits and in processed forms such as citrus juice. Approximately, an average of 34% of citrus fruits produced worldwide is processed to juices, although this can reach up to 96% in major citrus producing countries (Bovill 1996). In citrus fruits processing industry, a large amount of citrus by-product waste is generated and this waste is mostly underutilized. This waste could account for 50% of the fruit weight (Marín, Soler-Rivas et al. 2007). Citrus peel alone contributes nearly 40% of the total weight of the fruit (Li, Lo et al. 2006). In the United States alone, the juice industry produced about 700,000 tonnes of peel waste annually (Manthey and Grohmann 2001).

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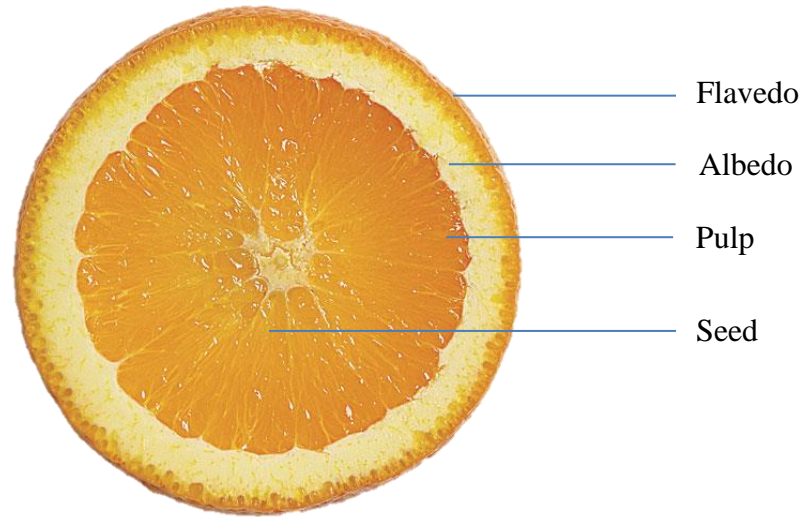
Figure 2.6: Parts of citrus fruit.

Citrus is well known for containing high amounts of vitamin C; however, recent studies showed that citrus fruits also contain significant amounts of various bioactive compounds (Silalahi 2002, Manners 2007, Tripoli, Guardia et al. 2007, Gonzalez-Molina, Dominguez-Perles et al. 2010). Citrus fruits contain different groups of bioactive compounds where some of these exerts a range of biological effects that improve human health including anti-oxidant, anti-inflammatory, anti-obesity, anti-diabetes, and cardioprotective and chemopreventive against cancer (Yu, Wang et al. 2005, Aggarwal and Shishodia 2006, Meeran, Ahmed et al. 2010, Mehta, Murillo et al. 2010, Lee, Cha et al. 2011, Aruoma, Landes et al. 2012, Chanet, Milenkovic et al. 2012, Coelho, Hermsdorff et al. 2013, Zou, Xi et al. 2016). Moreover, some of these bioactive compounds are found in higher concentration in the peel than in the juice (Peleg, Naim et al. 1991, Miyake, Murakami et al. 1999, Wang, Chuang et al. 2008, Zhang, Wu et al. 2014).

As citrus peel contains a higher amount of bioactive compounds than the other parts of the fruit, there is a growing interest and industry drive to utilize citrus peel in a more efficient way. One of the potential uses of citrus peel is as an anticancer agent. Several epidemiological studies have demonstrated an inverse association of citrus fruit consumption with cancer occurrence (Hakim, Harris et al. 2000, Li, Kuriyama et al. 2010, Ferrís-Tortajada, Berbel-Tornero et al. 2012, Giacosa, Barale et al. 2013, Cirmi, Ferlazzo et al. 2016, Capurso and Vendemiale 2017). The anticancer properties of citrus peel extract (CPE) have been evaluated in both *in-vitro* and *in-vivo* models, which revealed various anticancer activities including cell proliferation inhibitory effect in different cancer types (Cirmi, Ferlazzo et al. 2016, Cirmi, Maugeri et al. 2017). It is noteworthy that whole CPE has been shown to have higher anticancer activity than the fractionated extracts and single compounds (Mak, Wong-Leung et al. 1996, Ko, Jang et al. 2010, Zhang, Wu et al. 2014). Thus, citrus waste is potential to be turned to functional food for the cancer patients; however, sufficient investigation on the effect of citrus and its derived products on cancer still needed.

2.5   Mechanism of anticancer activity of CPE

Cancer cells differ from normal cells by their ability to proliferate without control, resistance to cell death, ability to form new blood vessels and metastasize to other parts of the body. CPEs have been shown to regulate these events through modulation of various cellular proteins as illustrated in Figure 2.7. As this study was focussed only on the anti-proliferation activity, detailed mechanism of action of CPE on different anti-proliferation proteins are described in detail on following in the coming section.

Action and mechanism of citrus peel extract

Anti-proliferation	➢ ↑ Signaling pathways : ↑ E-cadherin, ↓ β-catenin, ↓ Akt, ↓ Ras, ↓ p-GSK-3β (Ser9), ↓ ODC, ↓ Ras, ↓ EGFR, ↓ p38, ↓ MAPK and ↓ ERK ➢ ↑ Cell cycle arrest at check point (Sub G1, G2/M), ↓ Cyclins (B1, D and E), ↓CDK (CDK1), ↑CDK inhibitors (p53, p21), ↓ Other proteins (CDC25C, PCNA)
Apoptosis	➢ Intrinsic pathway: ↑ apoptotic proteins (↑ BAX, ↑ BCL-2, ↑ Bcl-xL, ↑ BAD, ↑ BID), ↑ caspases (↑ caspases-3,-7, -8, -9), ↓ apoptosis inhibitor (XIAP, cIAP1, cIAP2), ↓ other proteins (↓ DDX3X, ↓ ANP32B, ↑ PARP) ➢ Extrinsic pathway: = Receptors (= DR4, = DR5, = FAS, = FADD), = Pro-apoptotic ligands (= TRAIL, = FASL)
Anti-metastasis	➢ ↓ Metastasis enzymes (↓ MMP-2, ↓ MMP-9), ➢ Other proteins (↓ VCAM-1, ICAM-1, ↓ PLCγ-1, ↓ CXCR4, ↓ HER2/neu, ↓ p-PKC, ↑ E-cadherin, ↓ N-cadherin, ↓ Vimentin, and ↓ Fibronectin, ↓ Slug and Snail, ↓ SMAD)
Anti-inflammatory & anti-angiogenesis	➢ ↓ Inflammatory enzymes (= COX-1, ↓ COX-2, ↓ iNOS) ➢ ↓ Signaling pathways (↓ STAT3, ↓ NF-kβ, ↓ p50, ↓ p65) ➢ Other proteins (↓TNF-α, ↓ ↓ IL-1β, ↓ IL-6, ↓ IL-8) ➢ ↓ Angiogenic factor (↓ VEGF)

↑ increase; ↓ decrease; = no change

Figure 2.7: Main mechanisms through which CPE may act as anti-cancer drugs.

2.5.1 Anti-proliferation activity

Loss of controlled cell proliferation is the main attribute of cancer cells. CPEs suppress cancer cell proliferation by arresting cell cycle progression and modulating cell proliferation signalling pathways.

2.5.2 Modulation of growth signalling pathways

Down-regulation of certain oncoproteins such as epidermal growth factor receptor, Ras and Akt, which are involved in mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K) and mechanistic target of rapamycin (mTOR) signalling pathways inhibits cell proliferation and growth. CPE reduced proliferation of Hep3B cells by inhibiting the phosphorylation of PI3K and Akt that suppresses PI3K/AKT pathways while increasing the phosphorylation of ERK1/2, JNK and p38 MAPK that activates MAPK pathways (Hong, Lee et al. 2017). Suppression in the phosphorylation of Akt in U937 cells (Han, Lee et al. 2012) and mTOR by the CPE was also observed in SNU-1 cells (Moon, Kim et al. 2015). The inhibition in the proliferation signalling was also reported in the *in-vivo* model. CPE exerted suppression in the proliferating signalling through modulation of various proteins including Akt, Ras, ERK1/2 and E-cadherin in colon tumour-bearing mice (Lai, Tsai et al. 2011). These mice also showed low levels of inactive glycogen synthase kinase-3β (GSK-3β) and low accumulation of β-catenin in cell nuclei, which limits the growth signalling pathways. CPEs were also reported to reduce the ornithine decarboxylase enzyme, which controls the cell growth and proliferation through the biosynthesis and metabolism of polyamines, in several *in-vivo* studies (Lai, Tsai et al. 2011, Pan, Li et al. 2012, Lai, Li et al. 2013a).

2.5.3 Inhibition of cell cycle progression

Cell cycle progression is a highly regulated complex event and consisting of four phases, G1, S, G2 and M (Molinari 2000). Progression of cells from one phase to another is controlled through the coordinated interaction of cyclin-dependent kinases (CDKs) and their cyclin subunits to form

active complexes. The formation of the active complex is regulated by the endogenous CDK inhibitors. In normal cells, the cell cycle progression is halted when faulty DNA needs to be repaired or when cell division is not required. By halting the cell cycle progression, the growth of malignant cells can be reduced or stopped. Analysis of cell cycle distribution in CPE treated cells demonstrated that CPE caused cell cycle arrest mainly at G2/M phase (Lee, Park et al. 2012, Park, Park et al. 2012, Adina, Goenadi et al. 2014, Nagappan, Lee et al. 2016, Hong, Lee et al. 2017), although two studies showed accumulation at G1 (Moon, Kim et al. 2015, Chu, Chen et al. 2017). It was observed that CPE reduced the number of cancer cells either in G1 phase or S phase, while simultaneously increased number of cells in the G2/M phase. This difference could be due to the collective effect of multiple compounds in the CPE, the concentration of CPE, and also the different sensitivities of cancer cells to the extract.

At the G1/S transition, the cyclin-dependent kinase inhibitor (p21) silences the cyclin E/CDK2 complex and causes a G1 arrest. The p21 is directly regulated by the tumour suppressor gene p53. CPEs have been shown to up-regulate the expression of p21 and/or p53 leading to G1 arrest as observed in MCF-7 (Adina, Goenadi et al. 2014), SNU-1 (Moon, Kim et al. 2015), DU145 cells (Kim, Lee et al. 2017) and in *in-vivo* CPE treated A549 cells (Adina, Goenadi et al. 2014). Cyclin D1, which is overexpressed in many cancers, was reduced in *in-vivo* CPE treated colon cells (Lai, Tsai et al. 2011). Cyclin D1 and cyclin E were reduced in CPE treated DU145 cells (Kim, Lee et al. 2017). The proliferating cell nuclear antigen (PCNA) required for the DNA synthesis was also reduced by CPE in PC-3 tumour-bearing mice (Lai, Li et al. 2013b). It is worth to note that, since the cells at G0 and G1 are both diploid in DNA, it is not possible to separate the G0 from G1 fraction by DNA content analysis such as flow cytometry. Hence, an effect of CPE on forcing cancer cells exit from the cell cycle and/or impeding cell cycle re-entry

of G0 cells cannot be excluded. Further study is needed to verify this important mechanism. For the G2/M transition, the cell division cycle 25C (CDC25C) phosphatase activates the cell cycle controller 2 (CDC2, also named as CDK1), subsequently allowing the activated CDC2 to interact with cyclin B1 forming CDC2-cyclin B1 complex (Molinari 2000). CPEs have been shown to arrest cell cycle at G2/M by increasing the expression of p21 and decreasing the expression of cyclin B1, CDC25C, and CDC2 in A549, Hep3B and AGS cells (Lee, Park et al. 2012, Park, Park et al. 2012, Nagappan, Lee et al. 2016, Hong, Lee et al. 2017).

2.5.4 Bioactive compounds in citrus

Citrus peel contains a range of bioactive compounds such as flavonoids, limonoids, coumarins, phenolic acids, terpenoids, and carotenoids. Contents of flavonoids (Zhang, Wu et al. 2014), coumarins (Miyake, Murakami et al. 1999), carotenoids (Wang, Chuang et al. 2008) and phenolic acids (Peleg, Naim et al. 1991) were reported to be higher in the peel than the other parts of citrus fruits. The vast number of flavonoids and mixtures of their subclasses, including the flavones, flavanones, flavonols, and anthocyanidins are the main bioactive compounds in citrus peel. Flavanones being the major flavonoids are present mostly in glycoside form (hesperidin, neohesperidin, narirutin, naringin, eriocitrin and neoeriocitrin) compared to flavanone aglycones (hesperetin, naringenin and eriodictyol). Flavones are found in low amounts in citrus peel. Examples of flavone glycones are diosmin and isorhoifolin, while flavone aglycones are luteolin, apigenin, quercitrin and diosmetin. Another class of flavones known as polymethoxyflavones (PMFs) are found almost exclusively in the citrus genus (Ko, Jang et al. 2010). Examples of PMFs are nobiletin, sinensetin and tangeretin. Flavonols such as kaempferol,

quercetin, catechin, isorhamnetin and its glycoside flavonols are found in trace amount in citrus peel. Meanwhile, anthocyanidins are found only in grapefruit. The main flavonoids in CPE are nobiletin, sinensetin, neohesperidin, tangeretin, poncirin, naringin, and hesperidin (Du and Chen 2010, Lee, Park et al. 2012, Park, Park et al. 2012, Park, Park et al. 2014, Zhang, Wu et al. 2014).

It is reported that up to 62 glucoside and aglycone limonoids were found in citrus (Kim, Jayaprakasha et al. 2012). Obacunone glucoside and nomilin acid glucoside are the major limonoid glucosides in CPE (Tian, Dai et al. 2000). Coumarins are another class of bioactive compounds largely present in citrus peel. Coumarins such as 7-methoxy-8-(2-oxo-3-methylbutyl) coumarin, 5-geranyloxy-7-methoxycoumarin, auraptene, limettin and epoxyaurapten, as well the furanocoumarins such as psoralen, xanthotoxin, bergamottin and epoxybergamottin were reported in citrus peels (Miyake, Murakami et al. 1999, Lim, Moon et al. 2009, Gyawali, Jeon et al. 2012, Dugrand, Olry et al. 2013). Cinnamic acids (caffeic, p-coumaric, chlorogenic, ferulic and sinapic) and benzoic acids (protocatechuic, p-hydroxybenzoic and vanillic) are phenolic acids found in low concentration in citrus peels (Wang, Chuang et al. 2008, Xu, Chen et al. 2008). Meanwhile, carotenes (β-carotene) and xanthophylls (β-cryptoxanthin, lutein, β-citraurin, violaxanthin, (9Z)-violaxanthin and zeaxanthin) are the main carotenoids found mostly in citrus peel (Attila, Veronika et al. 2007, Wang, Chuang et al. 2008).

These bioactive compounds were found to exhibit anticancer activity at different levels by modulating various mechanisms when tested both *in-vitro* and *in-vivo* studies, suggesting the contribution of these compounds to the overall anticancer activity of CPE. Several articles have adequately reviewed the anticancer activity of these compounds (Miller, Taylor et al. 2000, Manners 2007, Nishino, Murakoshi et al. 2009, Kim, Jayaprakasha et al. 2012, Rocha, Monteiro

et al. 2012, Batra and Sharma 2013, Rawson, Ho et al. 2014, Wang, Wang et al. 2014, Kaur and Kaur 2015).

2.6 Effect of citrus peel extract on different types of cancer

CPEs have been reported to show anticancer activity in various cancer cell lines at different concentrations, which reflect the CPE composition and the cell line sensitivity. The following sections provide an overview of the *in-vitro* and *in-vivo* studies in which CPEs have demonstrated the potential of reducing the risk of cancer development and progression (Table 2.2 and Table 2.3). Detailed information about the effect of CPE on different cancers is elaborated in the following sections.

Table 2.2: *In-vitro* anticancer effect of citrus peel extract.

Variety	Compound Identification	Cell lines (IC$_{50}$, µg/mL)	Cell cycle arrest	Anti-proliferation	Pro-apoptosis	Anti-metastasis	Anti-inflammatory & anti-angiogenesis	Reference
Citrus reticulata	D	WEHI 3B (<100)						(Mak, Wong-Leung et al. 1996)
Citrus reticulata		SNU-668 (≈100)			I			(Kim, Park et al. 2005)
Citrus sinensis	D	MCF-7 (10.2-17.9)			I			(Sergeev, Ho et al. 2007)
Citrus grandis	D	U937 (60), HepG2 (31), HeLa (287), HCT-15 (87), MCF-7 (144), NCI-H460 (73), SNU-16 (90)			I*			(Lim, Moon et al. 2009)
17 citrus varieties	D	HT-29 (31-45)						(Hirata, Fujii et al. 2009)
Citrus sunki	D	HL-60 (25)	G2/M		I			(Ko, Jang et al. 2010)
Citrus aurantium	D	AGS (99)	G2/M	I	I			(Lee, Park et al. 2012)
Citrus aurantium		U937 (40-60)			I	I		(Han, Lee et al. 2012)
Citrus grandis	D	HeLa (100-200), AGS (200-400)			I			(Gyawali, Jeon et al. 2012)
Citrus aurantium	D	A549 (230)	G2/M	I	I			(Park, Park et al. 2012)
Citrus unshiu		MDA-MB-231(>200)				I		(Jin, Lee et al. 2013)
Citrus junos		HT-29 (>1200)					I	(Kim, Shin et al. 2014)
Citrus aurantifolia		MCF-7 (59)	G2/M		I			(Adina, Goenadi et al. 2014)
Citrus aurantium	D	A549			I	I		(Park, Park et al. 2014)
Citrus hassaku	D	MDA-MB-231				I		(Kim, Kim et al. 2014)
Citrus reticulata	D	HepG2 (20-40), HL-60 (25-50), MDA-MB-231 (25-50)						(Zhang, Wu et al. 2014)
Citrus paradisi, Citrus sinensis, Citrus maxima	D	Caco-2, LoVo, LoVo/ADR						(Ademosun, Oboh et al. 2015)
Citrus hassaku	D	SNU-1 (<25)	G1		I			(Moon, Kim et al. 2015)
Citrus paradesi		Kasumi-1 (2000)			I			(Wang., Lin. et al. 2015)
Citrus reticulata	D	SKOV3 (≈ 100)			I	I		(Chang, Jia et al. 2015)
Citrus platymamma	D	A549 (364)	G2/M	I	I		I	(Nagappan, Lee et al. 2016)
Citrus sphaerocarpa	D	MDA-MB-231 (>200)				I	I	(Park, Shin et al. 2016)
Citrus iyo	D	U266 (>400), K562 (200-400), DU145(>400), MDA-MB-231(>400), HepG2 (200-400), RWPE-1(>400)		I^	I^	I^	I^	(Kim, Lee et al. 2017)
Citrus platymamma	D	Hep3B (100-200), HepG2 (300-400)	G2/M	I#	I#	I#		(Hong, Lee et al. 2017)
Citrus sinensis	D	HepG2 (>500)	G1	I	I			(Chu, Chen et al. 2017)
Citrus reticulata		HCT116						(Onuma, Asai et al. 2017)

D: Determined; I: Induced, * only for U937, ^ only for DU145, # only for Hep3B

Table 2.3: *In-vivo* anticancer effect of citrus peel extract.

Sample	Animal models	Dose (route)	Duration	Effects	Reference
Citrus junos	HT-29 cells implanted mice	100 mg/kg/ daily (i.p)	4 weeks	Reduced tumor size, disease activity index and colon shortening	(Kim, Shin et al. 2014)
Citrus aurantium	A549 cells injected in mice tail vein	Twice weekly (i.p)	5 weeks	Reduced cancer metastasis	(Park, Park et al. 2014)
Citrus reticulata	Treated leukemic cells injected into mice	.	2 or 10 weeks	Reduced number of tumour cells and increased mice survival time	(Mak, Wong-Leung et al. 1996)
Citrus sinensis	AOM-induced carcinogenesis in mice	0.2 % in the diet	26 weeks	Reduced number and size of ACF, tumour burden and incidence	(Wei, Yue et al. 2003)
Citrus sinensis	Western diet inducing cancer	0.25% or 0.5% in diet	9 weeks	Reduced tumour number, multiplicity and induced apoptotic	(Fan, Kurihara et al. 2007)
Multiple citrus varieties	DMBA-induced carcinogenesis in mice	100 or 200 µL/ twice weekly (cream application)	20 weeks	Reduced epidermal thickness, number of papillomas, tumour incidence and tumour weight	(Pan, Li et al. 2012)
Multiple citrus varieties	PC-3 cells implanted mice	1 or 2 mg/kg/ five days per week (i.p) and 2 or 4 mg/kg/ five days per week (o.p)	3 weeks	Suppressed tumor size.	(Lai, Li et al. 2013b)
Multiple citrus varieties	AOM-induced carcinogenesis in mice	100 or 200 µL / five days per week (o.p)	6 weeks	Reduced number of ACF	(Lai, Li et al. 2013a)
Citrus iyo	DU145 cells implanted mice	50 or 200 mg/kg/ thrice weekly (i.p)	4 weeks	Suppressed tumour growth	(Kim, Lee et al. 2017)
Citrus sinensis	HepG2 cells implanted mice	1 or 10 mg/kg/thrice weekly in diet	3 weeks	Reduced tumour growth	(Chu, Chen et al. 2017)
Citrus sinensis	AOM-induced carcinogenesis in mice	0.01 or 0.05% in diet	4 or 18 weeks	Reduced number of ACF	(Lai, Tsai et al. 2011)

i.p: intraperitoneal injection; o.p: oral injection; ACF: aberrant crypt foci; AOM: azoxymethane; 2, 4-dimethoxybenzaldehyde

2.6.1.1 Lung cancer

Among different solvent fractions of CPE, hexane fraction was the most potent fraction when tested in lung cancer U937 cells. Subsequently, the fraction was tested on different cancer lines and NCI-H460 cell growth was strongly inhibited by the fraction (Lim, Moon et al. 2009). In A549 cells, the CPE dose-dependently inhibited cell proliferation while inducing apoptosis (Nagappan, Lee et al. 2016). Similar inhibitory effects were also observed with flavonoid-rich CPE in A549 cells (Park, Park et al. 2012). However, weak proliferation inhibitory effect was observed in normal W1-38 fibroblast cells indicating specify of the CPE. In another study, the CPE showed anti-metastatic properties by preventing the migration of A549 cells to the wounded area in the in-vitro experiment (Park, Park et al. 2014). Similar behaviour was observed in the *in-vivo* model where the CPE prevented the A549 cells in the tail vein from migrating to lungs, indicating anti-localization and anti-metastatic properties of CPE.

2.6.1.2 Breast cancer

CPEs exhibited anticancer activity in both estrogen-dependent and -independent breast cancer cells. CPE showed proliferation inhibition and apoptosis activity in estrogen receptor positive MCF-7 cells (Adina, Goenadi et al. 2014). Moreover, the combination of this CPE and doxorubicin exhibited a synergistic effect in inducing apoptosis. Thus, the use of the anticancer drug at a low dose for optimum efficacy with minimal side effects in cancer patients is possible with CPE intake. However, studies are needed in this area. Meanwhile, the potent hexane fraction of CPE showed moderate anti-proliferation activity on MCF-7 cells (IC$_{50}$: 144 µg/mL) compared to colon, lung, stomach, cervical, hepatoblastoma and lymphoma cancer lines (60-287

27

µg/mL) (Lim, Moon et al. 2009). In another study, hydroxylated PMFs rich CPE showed lower anti-proliferation and higher apoptosis activity than non-hydroxylated rich PMFs CPE (Sergeev, Ho et al. 2007). The IC_{50} values for these PMFs rich CPEs were in the range of 10.2 - 17.9 µg/mL. The difference in the IC_{50} values between these two studies could be due to PMF content as Lim, Moon et al. (2009) found the PMF content in hexane fraction was lower than in the other fractions.

Meanwhile, for hormone-independent breast cancer, CPE showed strongest anti-proliferation effect in MDA-MB-231 than in HepG2 and HL-60 cells (Zhang, Wu et al. 2014). Interestingly, when six isolated flavonoids tested individually at 20 µg/mL, only 5-demethylnobiletin showed the almost equivalent anti-proliferative effect as the CPE, while the other flavonoids showed weak or no anti-proliferative effect. In contrast, CPE obtained using supercritical carbon dioxide reduced MDA-MB-231 cells proliferation by less than 20% at 400 µg/mL (Kim, Lee et al. 2017). Limonene was the major compound in supercritical carbon dioxide CPE. However, the CPE at 100 µg/mL significantly prevented MDA-MB-231 metastasis and invasion (Kim, Kim et al. 2014). Interestingly, only one study evaluated anticancer activity of polysaccharides from citrus peel in cancer. Polysaccharides from CPE showed anti-metastatic activity in MDA-MB-231 cells with cell proliferation inhibition activity of 30% at 100 µg/mL (Park, Shin et al. 2016). Similarly, flavonoid rich CPE showed the anti-metastatic effect by significantly inhibiting MDA-MB-231 cell adhesion to HUVECs of the low dose of 10 µg/ml, although proliferation suppression of MDA-MB-231 cells was not significant at concentration up to 100 µg/ml (Jin, Lee et al. 2013). Moreover, the polysaccharides from CPE possesses anti-angiogenetic properties, as the CPE reduced significantly the tube formation in HUVEC cells without exhibiting cytotoxicity (Park, Shin et al. 2016).

2.6.1.3   Colon cancer

CPE showed prominent anticancer activity in colon cancer in both *in-vitro* and *in-vivo* studies. Hexane fraction of CPE showed strong proliferation inhibitory activity in HCT-15 cells among the other tested cancer cell lines (Lim, Moon et al. 2009). In another study, among the 17 different citrus varieties tested, only four CPEs showed strong cancer cell inhibition activity in HT-29 cells (Hirata, Fujii et al. 2009). These CPEs were fractionated using ethyl acetate and water. The ethyl acetate fractions inhibited the colon cancer cell growth with the IC_{50} values in the range of 31-45 µg/mL, which are 18-20 folds lower than that of water fractions. This finding suggests that the anticancer compounds in citrus peel targeting colon cancer cells are more soluble in ethyl acetate than water. 3,5,6,7,8,3',4'-heptamethoxyflavone was the most potent anticancer compound isolated in that study. As reported in the previous study, CPE of Citrus junos Tanaka at 1200 µg/mL showed by nearly 30% inhibition activity in HT-29 cells (Kim, Shin et al. 2014). In addition, CPE also inhibited metastasis and proteasome activities in Caco-2, LoVo and LoVo/ADR colon cancer cell lines (Ademosun, Oboh et al. 2015).

Aberrant crypt foci (ACF) is considered as a histological biomarker for colon carcinogenesis and the risk of cancer development increases with a number of incidence and multiplicity of ACFs in colon cancer tissue (Rawson, Ho et al. 2014). In one study, mice were fed with a diet containing 0.2% of CPE before carcinogen injection, during, and continuing to end of the experiment (Wei, Yue et al. 2003). The mice with CPE diet showed a reduction in the number of ACF/colon, AC/colon, colon tumours and colon tumour incidence by 34-66% compared with control. The low incidence of tumour development could be due to the highly potent PMFs. The extract contains approximately 30% of PMFs and also other monohydroxylated analogues of the PMFs. In another study, the addition of CPE at 0.25 or 0.5% to the new western-style diet reduced the

overall number of the tumor by 26-48% and overall tumour volumes by 36-63%, and also increased the number of apoptotic cells, when compared to the western-style diet alone (Fan, Kurihara et al. 2007). These data indicate that CPEs rich in PMFs can reduce tumorigenesis and enhance apoptotic activity in colon cancer cells. Meanwhile, feeding the mice with a diet containing 0.01% or 0.05% of hydroxylated PMFs for four weeks reduced the total number of ACF per colon by 40-44% compared to control (Lai, Tsai et al. 2011). When the mice were fed with hydroxylated PMFs for 20 weeks, the number of microadenomas was reduced by up to 81% compared to control. It is unclear for the factors that contribute to tumour incidence as some studies showed a reduction in the tumour formation incidence and while some studies are not. The extract contains 89.3% of hydroxylated PMFs and the intake of a small number of hydroxylated PMFs within a short period of consumption could be sufficient to exhibit their anticancer activity. Similarly, oral administration of CPE rich with PMFs, naringin and hesperidin reduced numbers of ACF and large ACF up to 40% compared to the control group in a colon tumour-bearing mice (Lai, Li et al. 2013a). It is worth mentioning that the methoxy group of PMFs could undergo auto-hydrolysis during long term storage (Qiu, Dong et al. 2010), suggesting that the anticancer activity may increase with time.

In another study, oral administration of CPE at 100 mg/kg·day improved colon shortening and disease activity index by reducing COX-2 expression, and significantly reduced the size of an HT-29 tumour in xenografts mice (Kim, Shin et al. 2014). The author suggested CPE has potential in preventing inflammation-related disease including colon cancer. Moreover, administration of dried citrus peel at a dose of 1,000 ppm in the diet reduced total ACF by 25% compared to control (Onuma, Asai et al. 2017). The antioxidant potential of CPE prevents the

colon carcinogenesis at the initiation stage as levels of reactive carbonyl species were significantly reduced in the serum of F344 rats.

As colon cancer develops from a small number of slow-growing benign polyps, it is well suited for dietary or classical, chemopreventive type of interventions and several studies have demonstrated this effect with aspirin, which is derived from salicylic acid, ubiquitously occurring in many plants. The demonstrated beneficial effect of CPEs on colon cancer justifies further investigation for its potential use as a dietary supplement with chemo-preventive activity.

2.6.1.4   Stomach cancer

CPEs demonstrated potential in inhibiting stomach cancer. CPEs suppressed proliferation by 50% in SNU-668 (Kim, Park et al. 2005), AGS (Lee, Park et al. 2012) and SNU-16 (Lim, Moon et al. 2009) at a concentration around 100 µg/mL by exerting cell cycle arrest and inducing apoptosis in *in-vitro*. The CPE had no toxicity effect to normal mouse embryo fibroblast cells (3T3-L1) when tested up to 500 µg/mL (Kim, Park et al. 2005). However, for CPE obtained using supercritical carbon dioxide extraction, the proliferation of AGS cell decreased by approximately 30% at 200 µg/mL (Gyawali, Jeon et al. 2012). On the other hand, CPE obtained with similar extraction technique induced apoptosis and inhibited SNU-1 cells with an IC_{50} value lesser than 25 µg/mL (Moon, Kim et al. 2015). The predominant compound in this CPE was auraptene, although limonene is the major compounds in CPE obtained by supercritical carbon dioxide. The auraptene could be the main anticancer compound in the CPE as it showed strongest proliferation inhibition effect on SNU-1 (IC_{50}: ≤ 7.5 µg/mL) compared to other different cancer cell lines and no toxicity was observed on HEK-293T.

2.6.1.5   Skin cancer

Only one study evaluated the anticancer activity of CPE on skin cancer. Two-stage skin carcinogenesis model was used where the mice were treated with 7,12-dimethylbenez[a]anthracene to initiate tumours, followed by repeated application of 12-O-tetradecanoylphorbol 13-acetate to promote a skin tumour. Topical application of CPE at 100 µL and 200 µL on the skin of mice suppressed epidermal thickening by 23-33% when compared with control (Pan, Li et al. 2012). Mice with topical application of 100 µL of extract also had a reduction in the number of papillomas by 25%, tumour incidence by 18%, tumour weight by 65% and a number of tumours with a diameter of above 5 mm by 33% when compared to the control group. The author suggested the anticancer properties of CPE are through down-regulation of inflammation, proliferation and angiogenesis.

2.6.1.6   Liver cancer

CPE showed proliferation inhibitory activity in HepG2 cells (Lim, Moon et al. 2009, Zhang, Wu et al. 2014). Meanwhile, among the five cancer lines tested, HepG2 cells were strongly inhibited by the supercritical carbon dioxide CPE (Kim, Lee et al. 2017). Also, the supercritical carbon dioxide CPE was more effective in inhibiting the Hep3B than that in HepG2 cells (Hong, Lee et al. 2017). The proliferation of Hep3B was dose-dependently reduced from 25 µg/mL, whereas for HepG2 it was reduced from 400 µg/mL. The CPE also induced apoptosis and cell metastasis in Hep3B cells. Chu, Chen et al. (2017) also showed the CPE at 500 µg/mL had moderate proliferation inhibition activity while inducing apoptosis in HepG2 cells. The CPE reduced the

HepG2 tumour growth when tested in the *in-vivo* model; however, the CPE did not diminish the tumour as effective as in prostate cancer-bearing mice.

2.6.1.7 Cervix cancer

CPE obtained using supercritical carbon dioxide extraction exhibited stronger anticancer activity in HeLa cells than AGS cells with IC_{50} value between 100 and 200 µg/mL (Gyawali, Jeon et al. 2012). In contrast, the hexane fraction of CPE showed the weakest inhibition activity in HeLa cells with an IC_{50} value of 286 µg/mL compared to the other cancer lines (Lim, Moon et al. 2009). Although the citrus species used in both studies were the same, the different inhibition activity was due to different extracted compounds and it clearly shows the importance of consuming the whole CPE for maximum efficacy. Meanwhile, CPE not only inhibited SKOV3 cell proliferation but also suppressed the motility of SKOV3 cells by inhibiting epithelial-to-mesenchymal transition (EMT), which is related to metastasis (Chang, Jia et al. 2015). This is in support with another finding where nobiletin and tangeretin inhibited the SKOV3 ovarian cells migration (Zhang, Wu et al. 2014).

2.6.1.8 Leukaemia

CPE has potential in reducing the risk of leukaemia, a cancer of the white blood cells. In one study, CPE showed significant proliferation inhibition in HL-60 cells at a concentration as low as 25 µg/mL (Zhang, Wu et al. 2014). The HL-60 cells exhibited strongest responsive to the six isolated flavonoids from the CPE, where the proliferation inhibition activity was significant even

at 0.8 µg/mL. Also, CPE reduced survival of Kasumi-1 cells with the IC_{50} value of 2000 µg/mL (Wang., Lin. et al. 2015). The high IC_{50} could be due to the lower sensitivity of Kasumi-1 cells or due to loss of some potent compounds in the water fraction during the fractionation. Combination of this CPE with arsenic trioxide showed a greater effect against the proliferation of Kasumi-1 cells than that of individual treatments. CPE also suppressed U937 cells proliferation by inducing apoptosis (Han, Lee et al. 2012). Supercritical carbon dioxide produced CPE exerted strong proliferation inhibition effect in K562 cells compared to the other four cancer lines (Kim, Lee et al. 2017).

In another study, diethyl ether fraction of CPE exhibited slightly stronger inhibition activity in WEHI 3B cells than the water fraction (Mak, Wong-Leung et al. 1996). The CPE also differentiated the WEHI 3B cells to macrophages and polymorphonuclear cells, which is one of the therapeutic goals in the treatment of leukaemia. As expected, crude CPE induced higher differentiation of WEHI 3B cells than the diethyl ether and water fractions. The authors suggested that methoxylated flavonoids, nobiletin and tangeretin, are the potential anti-leukemia compounds for various forms of myeloid leukaemia. The WEHI 3B cells, which were treated with diethyl ether fraction of CPE, showed slower tumour growth when inoculated in mice and the extract also prolonged the survival time of mice (Mak, Wong-Leung et al. 1996).

On the other hand, hexane fraction of CPE showed highest anti-proliferation activities via induction of apoptosis in U937 cells compared to the other six cancer lines (Lim, Moon et al. 2009). Consistent with the previous study, hexane fraction of CPE and the isolated flavonoids (nobiletin and 5-demethyltangeretin) showed greater growth inhibitory activity in HL-60 with IC_{50} value in the range of 20-25 µg/mL than the other flavonoid components which had IC_{50}s of above 100 µg/mL (Ko, Jang et al. 2010). The hexane fraction and 5-demethyltangeretin also

induced higher levels of apoptosis compared to the other tested PMFs. The author further suggested that hot water extraction is more efficient in extracting PMFs than organic solvent extraction.

The concept of minimal residual disease (MRD), or the residual load of remaining blast cells that usually survive chemotherapy and are responsible for the future remission episodes, was first derived from leukaemia. Therefore, animal models of MRD represent the ideal systems to test the effectiveness of CPEs to suppress the cell-cycle re-entry of residual blast cells.

2.6.1.9 Prostate cancer

Prostate cancer is the most common cancer in man. CPE showed a strong anticancer effect in prostate cancer by inhibiting the tumour growth in an *in-vivo* mouse model (Lai, Li et al. 2013b, Kim, Lee et al. 2017). CPE produced using supercritical carbon dioxide extraction at 400 µg/mL exerted a moderate effect in the proliferation of DU145 cells by modulating STAT3 pathway and no effect in normal prostate RWPE-1 cells (Kim, Lee et al. 2017). When the CPE was tested in mice model at a dosage of 50 and 200 mg/kg, the growth of the implanted DU145 tumour in mice was significantly reduced. The reduction is in parallel with reductions in the proteins related to proliferation and angiogenesis. Similarly, in another study, treatment with CPE by intraperitoneal injection or oral administration in PC-3 tumour-bearing mice reduced the tumour weight by 57–100% and tumour volume by 78–94% compared to control (Lai, Li et al. 2013b).

The strong anticancer activity of the extract was suggested to be due to the high concentration of PMFs and also the other compounds such as hesperidin. Remarkably, the tumour was almost

undetectable for mice treated with 2 mg/kg·day by intraperitoneal injection. However, the intraperitoneal injection at 1 mg/kg·day showed lower tumour suppression than with oral administration at 2 mg/kg/day. Both studies showed strong anticancer activity in prostate cancer cells. The intraperitoneal injection of CPE had a higher reduction of prostate tumour size than the oral administration indicating the needs to understand the bioavailability of the bioactive compounds is crucial in order to have maximum anticancer potency of CPE.

As prostate cancer is the most frequent malignant disease in men, is associated with high morbidity but develops slowly over many years and has a clear biomarker for its progression (the PSA), it is well suited for interventions based on cell-cycle inhibitors and particularly those derived from natural compounds, with good dietary representation such as compounds present in CPEs. Therefore, prostate cancer was selected as a primary focus in this study.

2.7   Prostate cancer incidence

Prostate cancer is the second most common cancer diagnosed for men and the fifth leading cause of cancer death worldwide (Ferlay, Soerjomataram et al. 2015, Torre, Bray et al. 2015). About 1.1 million men were diagnosed with prostate cancer in 2012 with 307000 deaths worldwide (Ferlay, Soerjomataram et al. 2015). This number represents the 15% of all cancers diagnosed in men worldwide and the prostate cancer incidence occurred mainly in the more developed regions accounting about almost 70% of the cases (Torre, Bray et al. 2015). Prostate cancer incident rate varied by more than 25-fold in different parts of the world. Australia and New Zealand were ranked in the top of the list, while South-Central Asia was the country with the lowest prostate cancer incidence (Figure 2.8). This high incidence is mainly because of improving detection

using prostate-specific antigen (PSA). However, mortality in these regions is lower or almost equal to those with low incidence rates. Interestingly, Eastern and South-Central Asia showed the lowest incidence and mortality rates (Torre, Bray et al. 2015). In contrast, the Caribbean and Africa showed the highest mortality rates for prostate cancer.
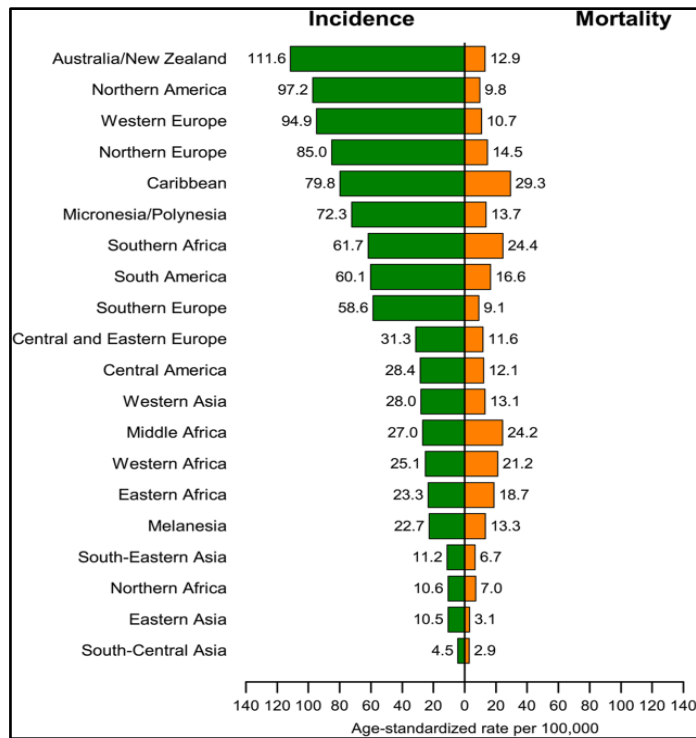
Figure 2.8: Estimated age-specific incidence and mortality rates for prostate cancer in 2012 (Torre, Bray et al. 2015).

Prostate cancer is estimated to be the most commonly diagnosed cancer for males in Australia for the year 2017 (AIHW 2017). The number of men estimated to be diagnosed with prostate cancer in Australia in 2017 was 16665 with 3452 mortality cases (AIHW 2017). The prevalence

and mortality of prostate cancer at different ages in Australia for 2017 were shown in Figure 2.9. The risk of prostate cancer increases dramatically at age 40 and above, where elderly men contribute to the high mortality rates (AIHW 2017). Meanwhile, the 5-year relative survival for prostate cancer patients in Australia increased from 58% to 95% during the year 1984–1988 and 2009–2013 (Figure 3b) (AIHW 2017).
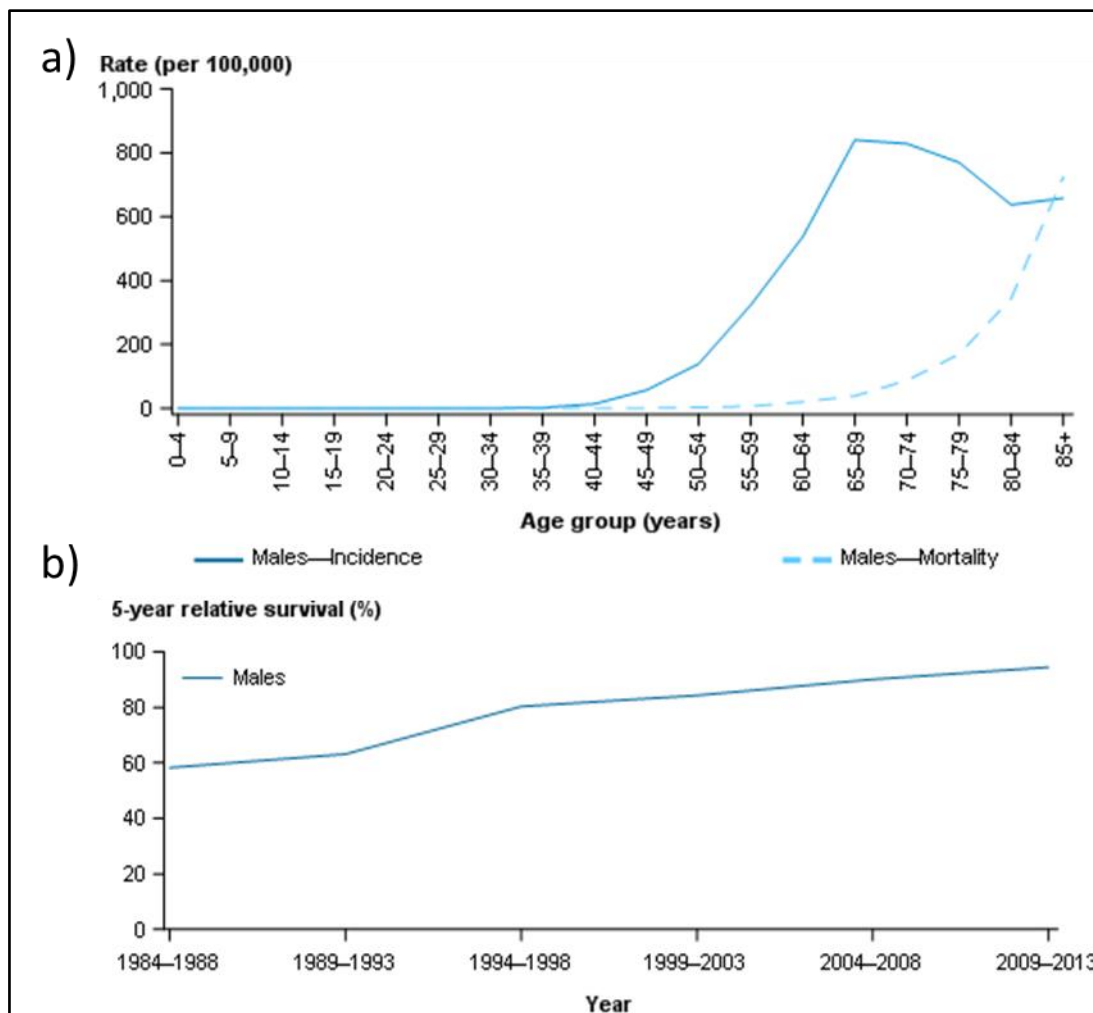
Figure 2.9: Estimated age-specific incidence and mortality rates for prostate cancer in Australia for the year 2017 (a), and 5-year relative survival from prostate cancer, 1984–1988 to 2009–2013, in Australia (b) (AIHW 2017).

The most common treatments for prostate cancer are active surveillance, surgery, radiotherapy, cryosurgery, chemotherapy and immunotherapy. Active surveillance rather than immediate radical treatment such as radical prostatectomy or radiation therapy is a preferable and reasonable approach for men with less-aggressive tumour-bearing, old, or with serious co-morbid conditions. For prostate cancer at an earlier stage, radical prostatectomy or radiation therapy is the primary curative treatment procedure. While for advanced prostate cancer, hormone therapy is preferred. At the initial stage, androgen deprivation therapy could block the androgen receptor pathway and induces tumour regression (Nieto, Finn et al. 2007). The prostate cancer becomes more severe and untreatable when it is irresponsive to androgen hormones (Feldman and Feldman 2001). Although the primary treatment could stop the prostate cancer progression, the cancer patients are more likely to experience cancer recurrence and ultimately develop metastatic disease. Meanwhile, chemotherapy is not commonly used for prostate cancer; but it is used when prostate cancer is beyond the prostate gland and hormone therapy is not responsive. These treatments frequently cause undesirable side effects to the patients (Penson, Litwin et al. , Eton and Lepore 2002).

2.7.1   Prostate cancer recurrence

Like the other types of cancer, prostate cancer is likely to recurrent in patients where approximately 35% of men experience a recurrence within 10 years following radical prostatectomy (Freedland, Humphreys et al. 2005). The prostate cancer recurrence is identified by the rise in serum prostate-specific antigen (PSA) levels, which requires immediate secondary treatment (Freedland, Humphreys et al. 2005). The rise in the PSA level is associated with severe

risk of fatality and the treatment can significantly reduce the quality of life. Prostate cancer recurrence with advanced hormone-refractory metastatic cancer was a high risk of fatality (Radhakrishnan, Miranda et al. 2010). Prostate cancer is the fifth leading cause of cancer death worldwide despite the improved 5-year relative survival rate (Bray, Ferlay et al. 2018). The median time for the cancer metastasis is six years from the time of initial prostate-specific antigen (PSA) elevation (Yamamoto, Musunuru et al. 2016), followed by a death. The long median time allows the potential use of other supplementary interventions to delay or prevent cancer recurrence.

2.7.2 Prostate cancer epidemiology and diet

Mounting evidence, both epidemiologic and laboratory studies, suggest the role of food in modulating cancer occurrence. According to a study conducted by American Institute for Cancer Research and the World Cancer Research Fund in 1997, cancer occurrence can be reduced by 30–40% with appropriate diets and lifestyle (Potter 1997, Anand, Kunnumakkara et al. 2008). Epidemiologic studies suggest that consumption of a bioactive-rich diet that includes fruits and vegetables could reduce the risk of cancer (Chan, Gann et al. 2005). In particular, risk of prostate cancer occurrence is lowered with high intake of vegetables, fruits, fish, and specific component nutrients in these foods such as tomatoes/lycopene, carotenoids, cruciferous vegetables, vitamin E, selenium, fish/marine omega-3 fatty acids, soy, isoflavones and polyphenols (Chan and Giovannucci 2001, Giovannucci 2002, Chan, Gann et al. 2005). In one cohort study, men consuming more than two servings of tomato sauce per week lowered their risk of developing prostate cancer by 20–35% (Giovannucci, Rimm et al. 2002). Moreover, a population-based

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case-control study carried out in men in western Washington state showed three or more servings of cruciferous vegetable consumption per week statistically decreases the risk of prostate cancer by 41% (Cohen, Kristal et al. 2000).

Nutritional intervention is often adapted to cancer patients to facilitate cancer therapy. Changing the diet could be favourable to cancer patients in maintaining health and improving the quality of life, and also in preventing cancer recurrence (Brown, Byers et al. 2001). It has been shown that many men after being diagnosed with prostate cancer altered their diet and lifestyle with the hope to delay prostate cancer recurrence (Brown, Byers et al. 2001). Supplementation of lycopene to the men with newly diagnosed localized prostate cancer prior to surgery decreased their PSA levels (Kucuk, Sarkar et al. 2001). Similarly, the slow progression of prostate cancer was correlated with intake of cruciferous vegetable by patients after diagnosis (Richman, Carroll et al. 2012). Chan, Holick et al. (2006) also found a moderate correlation between vegetable consumption after post-diagnostic with the risk of prostate cancer progression. Although a strong and clear association of certain foods with prostate cancer progression could not be established at this stage, most of the epidemiology studies show the ability of some foods and compounds derived from them to decrease cancer incidence and recurrence. Therefore, identifying the promising foods or bioactive compounds would be a significant deal in not only altering the risk of developing prostate cancer but also in modulating its recurrence and progression. Among many fruits and vegetables, citrus is a promising candidate with potential for developing a functional food or nutraceutical supplement for chemoprevention and management of prostate cancer.

3 Citrus peel extract in halting cell cycle re-entry of quiescent prostate cancer cells.

3.1 Introduction

Cancer is one of the leading chronic diseases globally with a high mortality rate (Torre, Bray et al. 2015). Despite significant advancement in the early detection and treatment of cancer, the current therapies still could not eradicate the risk of cancer recurrence and metastasis (Li, Rogoff et al. 2015). Cell cycle re-entry of residual quiescent cancer cells has been implicated as a major cause of cancer recurrence and metastasis (Goss and Chambers 2010, Páez, Labonte et al. 2012). Thus, arresting and preventing these quiescent cancer cells from proliferating could delay the cancer recurrence.

It has been shown that many natural bioactive compounds are able to halt the progression of cancer at different carcinogenesis phase including arresting cells at various cell cycle checkpoints (Singh, Dhanalakshmi et al. 2002). As an example, flavonoids exhibit a broad spectrum of anti-cancer activities by interfering in the initiation, promotion and progression of cancer through different mechanisms such as anti-proliferation, anti-inflammation, anti-angiogenesis, anti-metastasis, pro-apoptosis and reversal of multidrug resistance (Batra and Sharma 2013, Ravishankar, Rajora et al. 2013). The cell cycle arrest activity of flavonoids provides an opportunity in preserving quiescent cancer cells.

Citrus peel has been used traditional medicine for treating many diseases (Xu, Chen et al. 2008, Rawson, Ho et al. 2014). The results of previous *in-vitro* and *in-vivo* studies on various cancer

cell lines demonstrate that citrus peel is one of the rich sources of flavonoids and has anticancer activity (Wang, Wang et al. 2014). As no information about the ability of citrus peel in arresting quiescent cells is available, in this study we aimed to have a new approach compared with previous studies and hypothesized that flavonoid rich citrus peel could inhibit quiescent cancer cells from proliferating, therefore, can potentially reduce the risk of cancer recurrence. The outcomes of this study will enable to develop a naturally derived product from citrus peel extract (CPE) that can be used for patients after chemotherapy to reduce the risk of cancer recurrence.

3.2   Materials and methods

3.2.1   Chemicals and reagents

Hoechst 33342 (B2261), Pyronin Y (P9172), RNase A from bovine pancreas (10109142001), DMSO (D8418) and Annexin V-FITC Apoptosis Detection Kit (APOAF-20TST), hexane (270504), ethyl acetate (34858), HPLC grade acetonitrile (34851), DMSO (D8418), formic acid (5330020050) and hesperidin (PubChem CID: 174) were purchased from Sigma-Aldrich. The supplier for Narirutin (PubChem CID: 174) was Clearsynth Labs Ltd (Mumbai, India). FxCycle™ PI/RNase Staining Solution (F10797), Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (C10425), SYTOX™ AADvanced™ Dead Cell Stain (S10349), PrestoBlue™ Cell Viability Reagent (A13261) and all cell culture supplies were from Life Technologies Gibco (Darmstadt, Germany).

3.2.2 Preparation of extract

Citrus sinensis L. fruit was purchased from the local store and washed with tap water before the peel was collected. The peel was dried in a vacuum oven to remove moisture content and the dried peel was ground with a Nutribullet blender. Dried powder (20 gram) was soaked in 200 mL of a solvent such as MilliQ water or ethyl acetate: hexane (50:50, v/v) for 24 h at room temperature with continuous stirring. After 24 h, the solvent was collected, filtered using Whatman No.1 filter paper and dried using either rotary evaporator or freeze drier to remove hexane and aqueous solution, respectively. The dry residues obtained from ethyl acetate: hexane extract and water extract were dissolved in DMSO and MilliQ water, respectively. The ethyl acetate: hexane extract was labelled as hexane extract. DMSO and MilliQ water without extract were used as vehicle for control samples.

3.2.3 Cell lines and synchronization at quiescence

Bone metastasized prostate cancer cells (PC-3), lymph node metastasized prostate cancer cells (LNCaP) and human dermal fibroblast (GM3348) cells were obtained from American Type Culture Collection (Rockville, MD). GM3348 cells were cultured in complete medium containing Minimum Essential Medium (Gibco, 12571063) with 10% of fetal bovine serum (Gibco, 16000036) and 1% of Penicillin-Streptomycin (5000 U/mL). The prostate cells were cultured in RPMI 1640 (Gilco, New York) supplemented with 10% fetal calf serum (Gilco, New York) in a humidified cell incubator at 37°C in the presence of 5% CO_2. An exponential growing PC-3 and LNCaP cells were used only for cell viability test. For other experiments, quiescent cells were prepared according to the method reported by Xi, Yao et al. (2016) with small

modifications. Briefly, exponential growing PC-3 cells were seeded in T75 flask and allowed to grow in complete medium until it reaches complete confluence. Thereafter, the medium was replaced with fresh complete medium and the confluence was maintained for three days. Meanwhile, for LNCaP cells, the exponential growing cells were seeded directly to the six-well plate and allowed to grow until it reached 60–70% confluence. Then, the LNCaP cells were cultured in serum-free medium for seven days. Cell cycle re-entry was rendered by passaging the PC-3 cells at low density or by serum replenishment for LNCaP cells.

3.2.4 Cell viability assay

Exponential growing LNCaP (1×10^5 cells/well) and PC-3 (7×10^4 cells/well) cells were seeded in 96-well plates and cultured in complete medium. After 24 h, the medium was replaced with a new complete medium containing different concentrations of CPE. At the end of treatment, the medium in the well was replaced with 100 µL of complete medium containing 5% of presto blue reagent (Carlsbad, CA). Fluorescence intensity was measured after 30 minutes using microplate reader SpectraMax Plus (Molecular Devices, CA) at an excitation of 540 nm and emission of 590 nm. Blank is absorbance intensity of 100 µL of complete medium containing 5% of presto blue reagent only and control is absorbance intensity of cells exposed to the same volumes of water or DMSO applied when cells are exposed to the CPE. Inhibitory rate of cell proliferation was calculated using the following formula: Inhibitory rate (%) = (Sample-Blank) × 100% / (Control-Blank).

3.2.5 Cell cycle analysis

Quiescent LNCaP (1 × 10^5 cells/well) and PC-3 (1.5 × 105 cells/well) were cultured in complete medium containing CPE at IC$_{50}$ concentration in a six-well plate. For control cells, same volumes of water or DMSO were applied to the cells. The cells were harvested, fixed with ice-cold 70% ethanol and stored at 4°C. Prior to flow analysis, the cells were washed with phosphate buffer solution (PBS) and stained with FxCycle™ PI/RNAse Solution. DNA content was measured at excitation of 488 nm and emission of 690 nm using a FACS Canto II flow cytometer equipped with BD FACSDiva™ software (BD Biosciences). To differentiate G0 cells from G1 cells, the fixed cells were stained with 2 µg/ml of Hoechst 33258 in PBS at 37 °C for 45 min and followed by Pyronin Y at 4 µg/ml for 15 min (Xi, Yao et al. 2016). The cells were measured with flow cytometer at 350 nm Ex/450 nm Em for Hoechst 33258 and 488 nm Em/ 585 nm Em for Pyronin Y.

3.2.6 DNA synthesis assay

Approximately 1 × 10^5 cells/well of quiescent PC-3 cells were cultured in complete medium containing CPE at IC$_{50}$ concentration in a six-well plate. For control cells, same volumes of water or DMSO were applied to the cells. PC-3 cells were harvested after 24 and 48 h. The newly synthesized DNA was determined using Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit and SYTOX™ AADvanced™ Dead Cell Stain in the presence of RNase. The assay was performed according to the manufacturer's protocol. The cells were analysed with flow cytometer at an excitation of 488 nm and emission of 530 nm and 670 nm.

3.2.7 Apoptosis analysis

Apoptosis cells were determined using a FITC Annexin-V apoptosis assay according to the manufacturer's protocol. Approximately 150000 of quiescent PC-3 cells were cultured in complete medium containing CPE at IC_{50} concentration in a six-well plate. For control cells, same volumes of water or DMSO were applied to the cells. At the end of treatment, the cells were harvested, washed with PBS and suspended in 100 µL of Annexin-V binding buffer, followed by staining with of 5 µL of Annexin V FITC Conjugate and 10 µL of propidium iodide solution for 15 min in the dark. With flow cytometer, the cells were excited at 488 nm and measured the emission at 530 and 580 nm.

3.2.8 Flavonoid quantitation

The HPLC system consisted of Shimadzu DGA-20A vacuum degasser, two LC-20AD pumps, SIL-20A auto-sampler, CTO-20A column oven, SPD-M20A diode array detector and CBM-20A system controller was used for extract profiling and flavonoid quantitation. A 10 µL of extract was separated using a Phenomenex Synergi Fusion column (250 × 4.6 mm, 4 µm) at a flow rate of 1.0 mL/min with a temperature of 30°C. The mobile phase was acetonitrile and water containing 0.1% formic acid. The concentration of acetonitrile in the mobile phase was gradually increased from 20% to 30% in 10 minutes, then ramped to 70% over 5 minutes and decreased to 20% in 5 minutes. The bioactive compounds in extracts were detected at 230 nm. For flavonoid quantitation in the extract, a calibration curve constructed using standard flavonoid solution in DMSO.

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3.2.9 Statistical Analysis

IBM SPSS Statistics software version 24 was used for statistical analysis. Student's two-tailed t-test was used for comparison between two different groups and ANOVA analysis was used with Tukey multiple comparison test for multiple comparisons. All P-values <0.05 were considered statistically significant.

3.3 Results

3.3.1 Toxicity of extract on prostate cancer cells

Both CPEs obtained from extraction with water and hexane solvent were tested for cell viability on exponentially growing PC-3 and LNCaP cells for a period of 48 h. Figure 3.1 shows the images of proliferating PC-3 and LNCaP cells that were used for cell viability assay. The cell viability of extracts on PC-3 cells and LNCaP cells were shown in Figure 3.2 and Figure 3.3, respectively. Water extract in the range of 6.0-17.9 mg/mL and hexane extract in the range of 12.5-400.0 µg/mL showed cell viability inhibitory effect dose-dependently on these cancer lines. The IC_{50} (mg/mL) at 48 h for water extract and hexane extract on PC-3 cells was 13.7±0.2 and 0.156±0.016, respectively, while for LNCaP cells was 13.5±0.7 and 0.081±0.018, respectively. The IC_{50} values clearly illuminated that hexane extract was more effective than water extract in reducing viability of prostate cancer cells. This result also indicated both CPEs were efficient in reducing cell viability and impeding cell growth in both cancer lines at different concentrations.
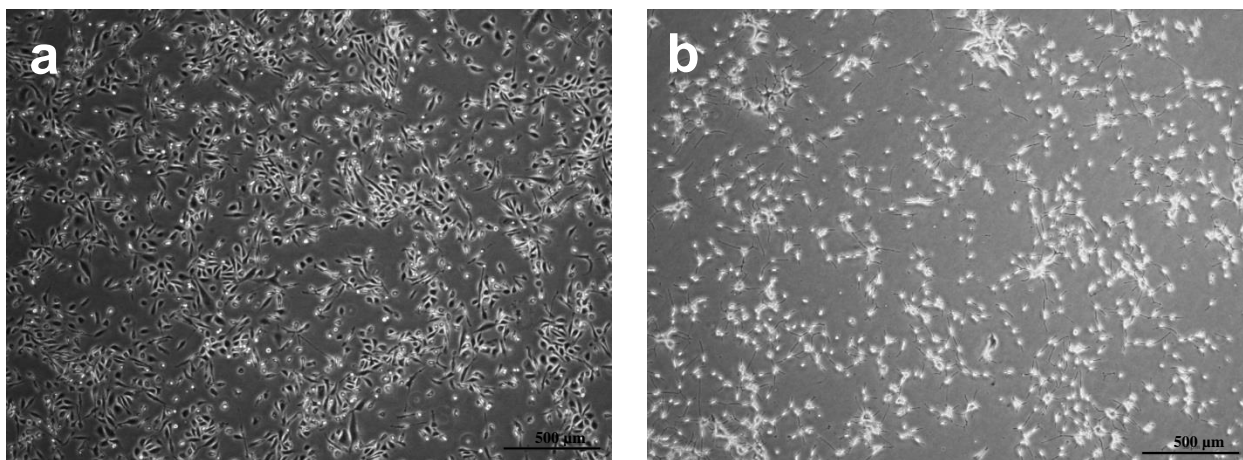
Figure 3.1: Images of proliferating PC-3 (a) and LNCaP cells (b) were assessed by Nikon Eclipse TS100 inverted microscope.

a) PC-3 cells, water extract
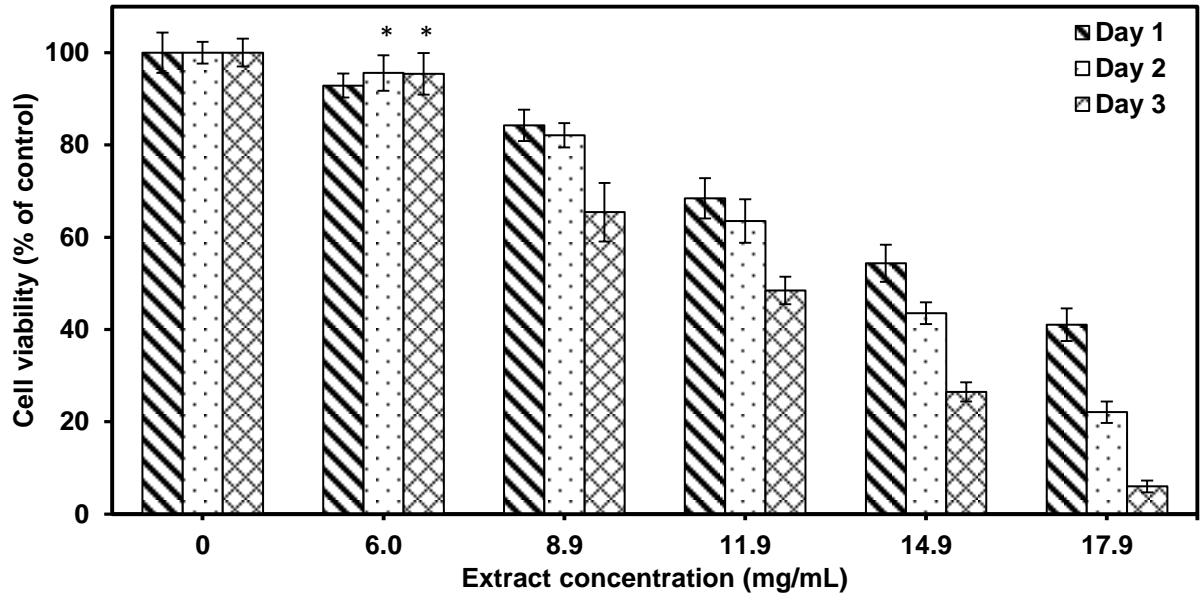
b) PC-3 cells, hexane extract
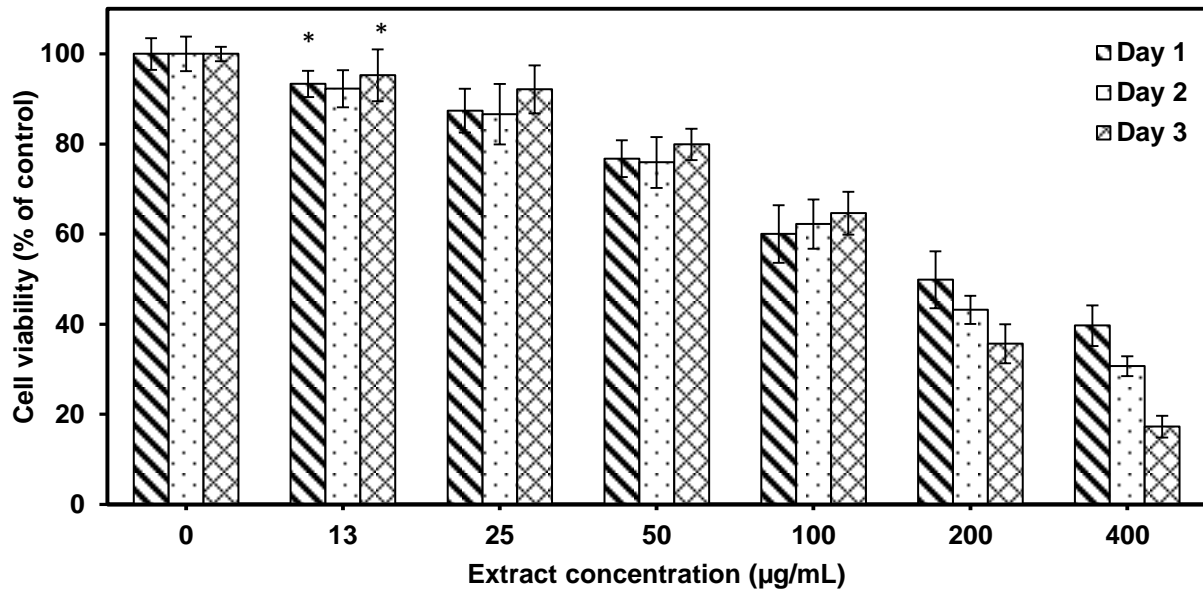
Figure 3.2: Water (a) and hexane (b) extracts decrease cell viability of PC-3 prostate cancer cells. Results are presented as the mean ± SD of three independent experiments (n=3). * indicates no significant difference compared to control at $p < 0.05$.

a) LNCaP cells, water extract
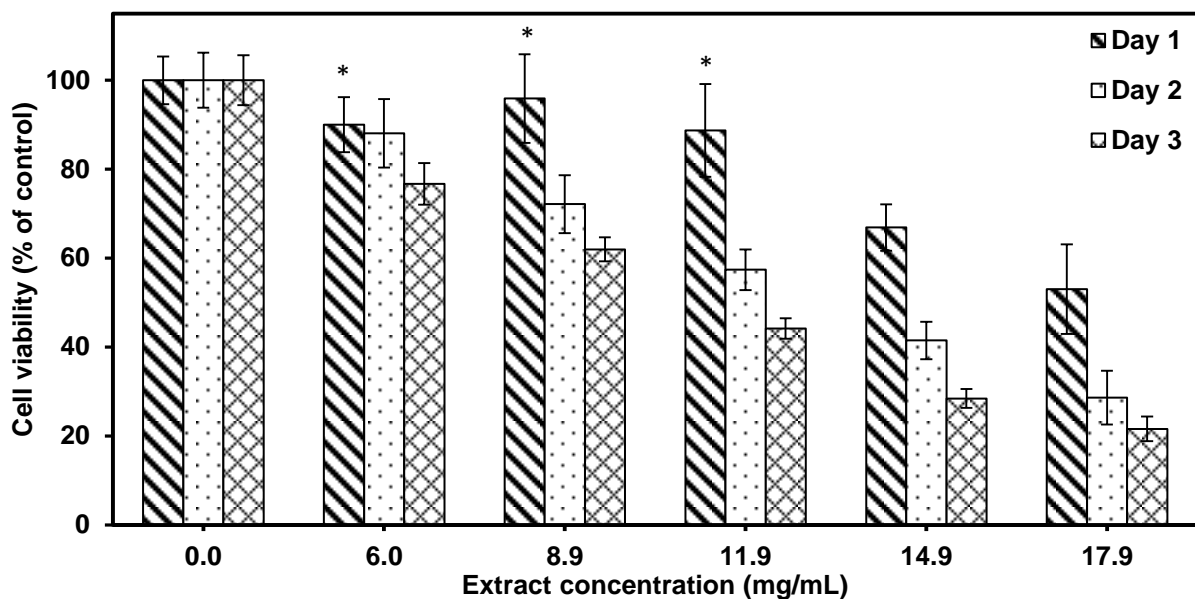
b) LNCaP cells, hexane extract
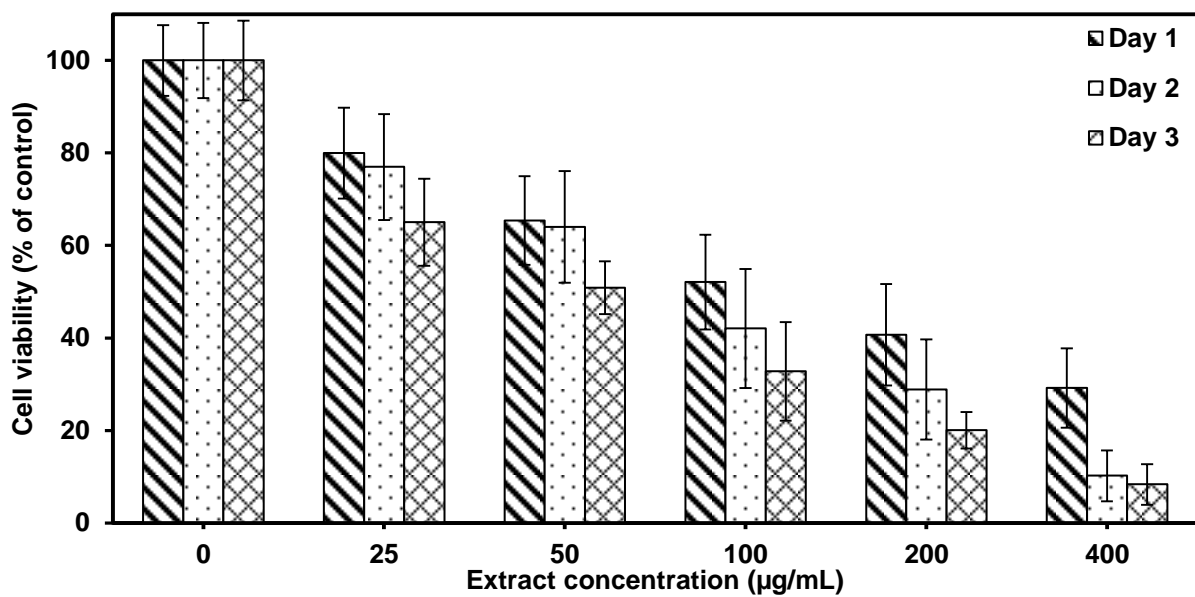
Figure 3.3: Water (a) and hexane (b) extracts decrease cell viability of LNCaP prostate cancer cells. Results are presented as the mean ± SD of three independent experiments (n=3). * indicates no significant difference compared to control at $p < 0.05$.

3.3.2 Ability of extract in halting the cell cycle re-entry of prostate cancer cells

To evaluate the ability of CPE in arresting the quiescent cells at the G0 phase, the quiescent prostate cancer cells were allowed to enter the cell cycle in the presence of CPE at IC_{50} concentration. Upon released from quiescence, the PC-3 cells were analysed at every 8 h interval for 48 h and LNCaP cells were analysed at every 24 h interval for 120 h. Figure 3.4 shows the proliferating cells before induced to quiescence and the quiescent cells that were used for the experiment. Figure 3.5 and Figure 3.6 show the PC-3 and LNCaP cells incubated in the presence of extract at different h.
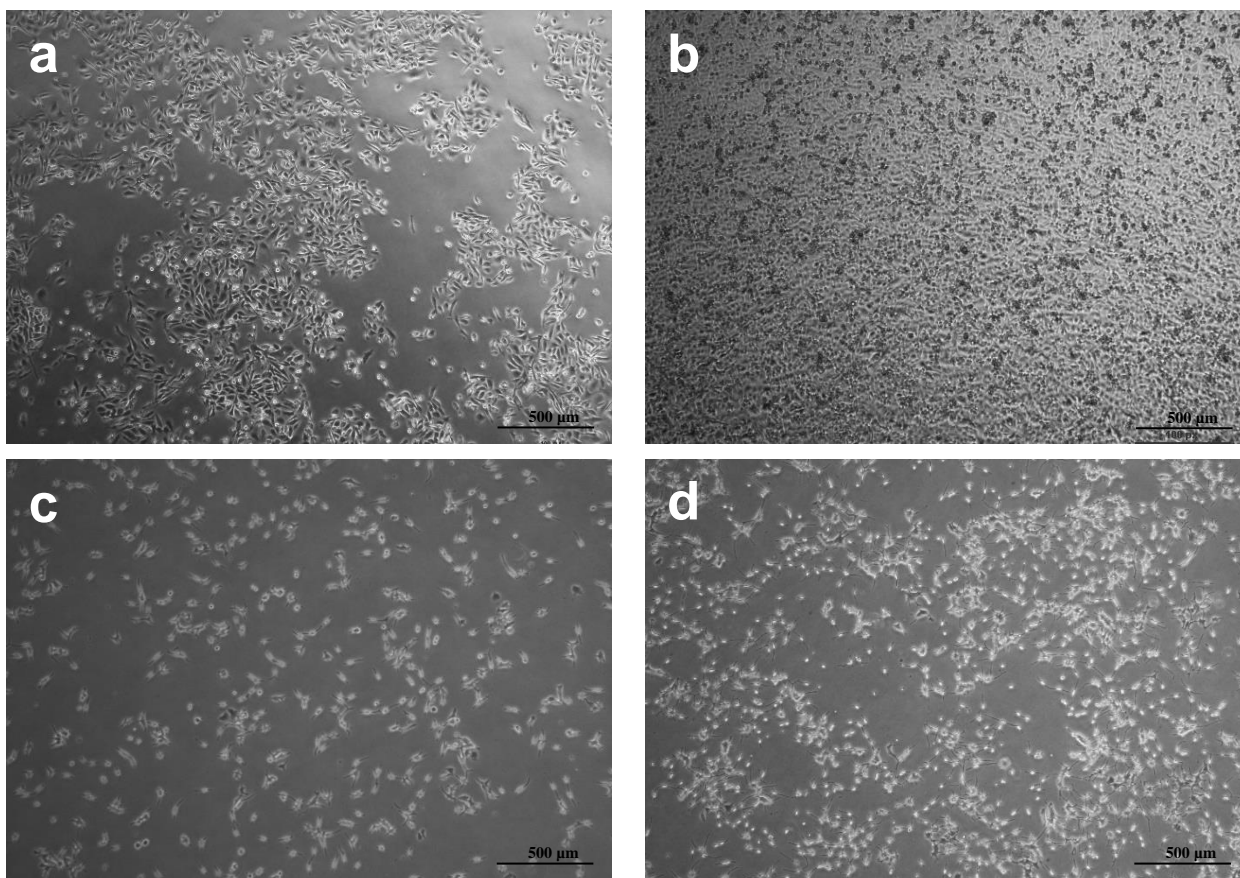
Figure 3.4: Images of proliferating PC-3 cells (a), quiescent PC-3 cells after 3 days of contact inhibition (b), proliferating LNCaP cells (c) and quiescent LNCaP cells after cultured in serum-free culture medium for 7 days (d) were assessed by Nikon Eclipse TS100 inverted microscope.

	Water extract		Hexane extract	
	Control	Treated	Control	Treated
8 h				
16 h				
24 h				
32 h				
40 h				
48 h				
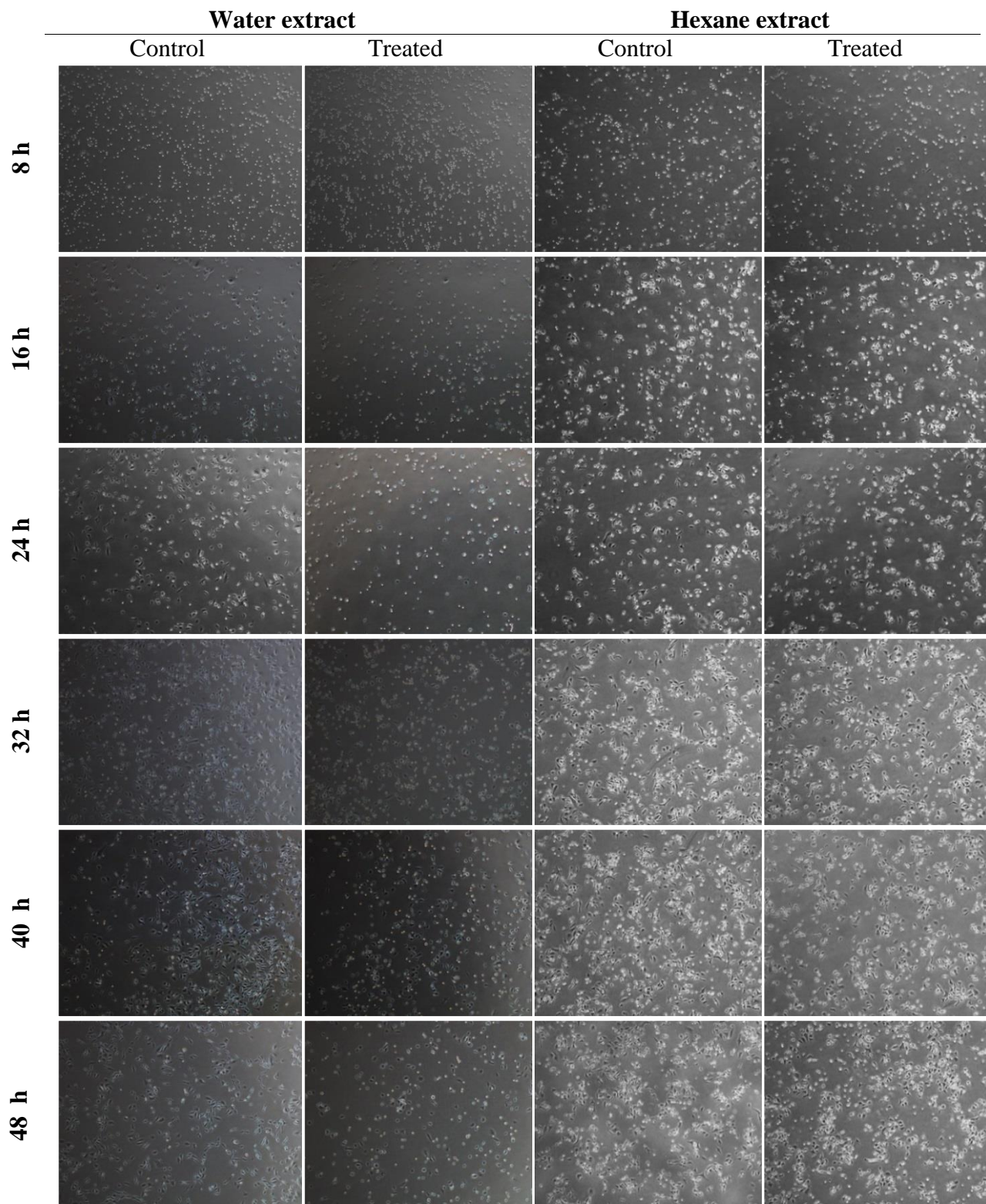
Figure 3.5: Images of quiescent PC-3 cells grown in water or hexane extract at different hours upon released from quiescence were taken by Nikon Eclipse TS100 inverted microscope.

	Water extract		**Hexane extract**	
	Control	Treated	Control	Treated
24 h				
48 h				
72 h				
96 h				
108 h				
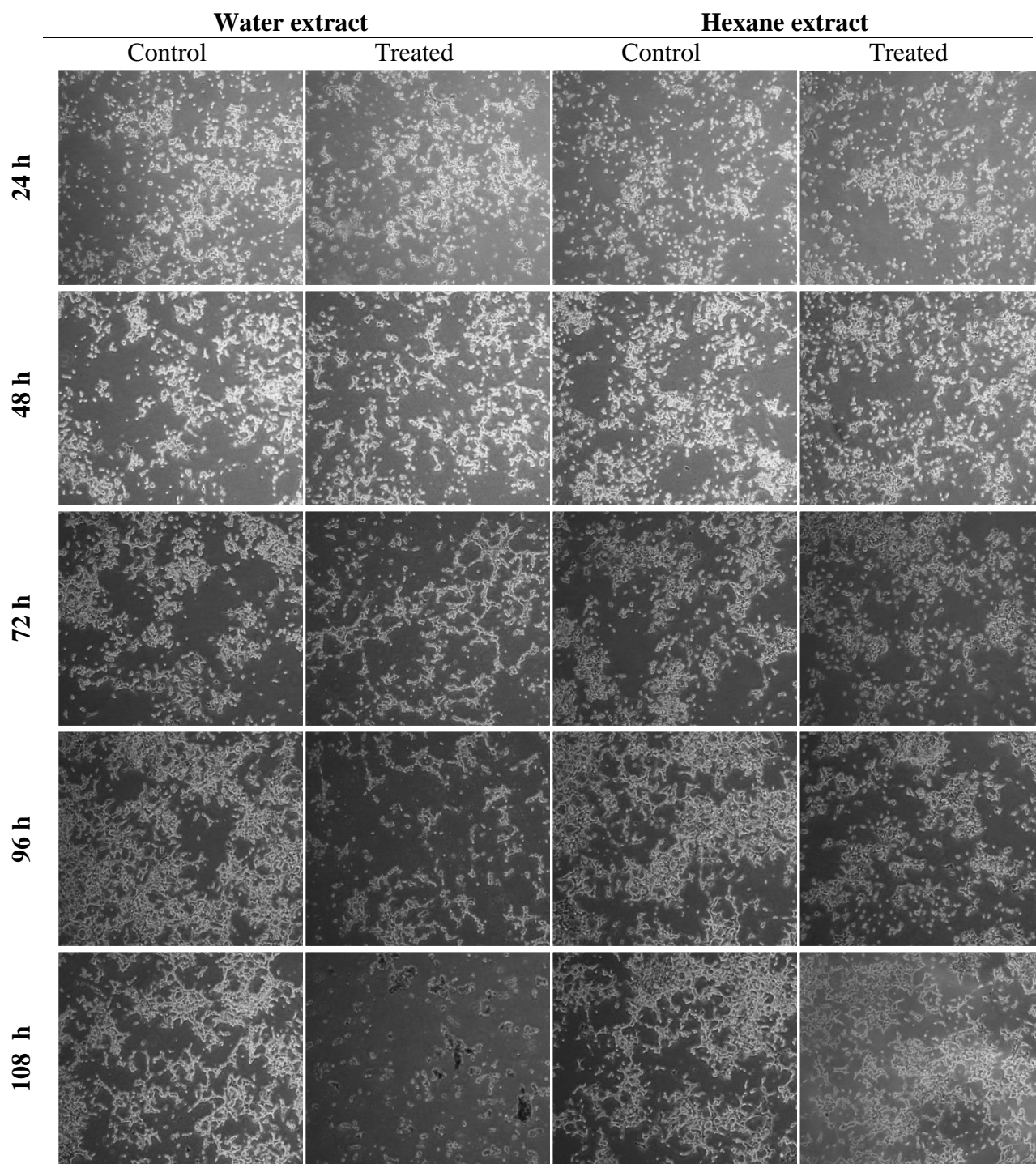
Figure 3.6: Images of quiescent LNCaP cells incubated in water or hexane extract at different hours upon released from quiescence were assessed by Nikon Eclipse TS100 inverted microscope.

Cell cycle profile of these cells was analysed by measuring propidium iodide-stained DNA with flow cytometry. As shown in Figure 3.7 and Figure 3.8, when the proliferating PC-3 and LNCaP cells were forced to enter quiescence, the number of cells in G0/G1 phase was increased, while S and G2/M phases were decreased. Compared to proliferating cells, both quiescent PC-3 and LNCaP cells differed in the number of cells at the G0/G1 phase by 24% and 17%, respectively. Upon released from quiescence, the cells re-entered the cell cycle and a reduction in the proportion of cells in the G0/G1 cells and increased of S and G2/M in control PC-3 and LNCaP cells. PC-3 cells were re-entered cell cycle more rapidly than LNCaP cells, after nearly 16 h following release from quiescence. The difference between PC-3 cell treated with water extract and control at 48 h in term of the number of cells at the G0/G1 phase was 18%, whereas for hexane extract was 2%. For LNCaP cells, the difference in the number of cells at G0/G1 at 120 h was 12% for water extract, while for hexane extract was 3%. This significant difference in the number of cells at the G0/G1 phase clearly indicated the effectiveness of water extract in halting cell cycle re-entry of PC-3 and LNCaP cells ($p < 0.05$). In contrast, the hexane extract exhibited negligible cell cycle inhibitory activity in both cell lines.

The propidium iodide staining method indicated the CPE arrested the cells at G0/G1 and it was unclear whether the cells progressed from G0 to G1. It has been reported that G0 cells contain a lower amount of RNA than G1 cells due to the low gene transcription (Gao, Ouyang et al. 2004). Thus, a double staining method using Hoechst 33258 for DNA content and Pyronin Y for RNA content was used to differentiate the G0 and G1 cells for samples at 24 and 48 h. As shown in Figure 3.9, quiescent PC-3 and LNCaP cells have higher percentages of G0 cells than their respective proliferating cells, which was in agreement with a previous study (Xi, Yao et al. 2016). A reduction in the percentage of G0 cells upon release from quiescence was observed in

control PC-3 and LNCaP cells. During cell cycle re-entry, the PC-3 and LNCaP cells treated with hexane extract did not show any difference with their respective control cells. A reduction in the G0 population by 21% (vs. 22% for control) for PC-3 cells at 48 h, whereas by 15% (vs. 14% for control) for LNCaP cells at 96 h were observed for hexane extract upon released from quiescence phase. However, no significant reduction in the G0 population was observed in both PC-3 and LNCaP cells treated with water extract with their control ($p < 0.05$). The G0 population was reduced by 1% for water extract treated PC-3 cells at 48 h. In contrast, water extract treated LNCaP cells showed a higher amount of G0 cells by 8% than the quiescent cells. These results suggested that water extract is capable in inhibiting cell cycle re-entry of quiescent prostate cancer cells by preserving the cells in G0 phase.
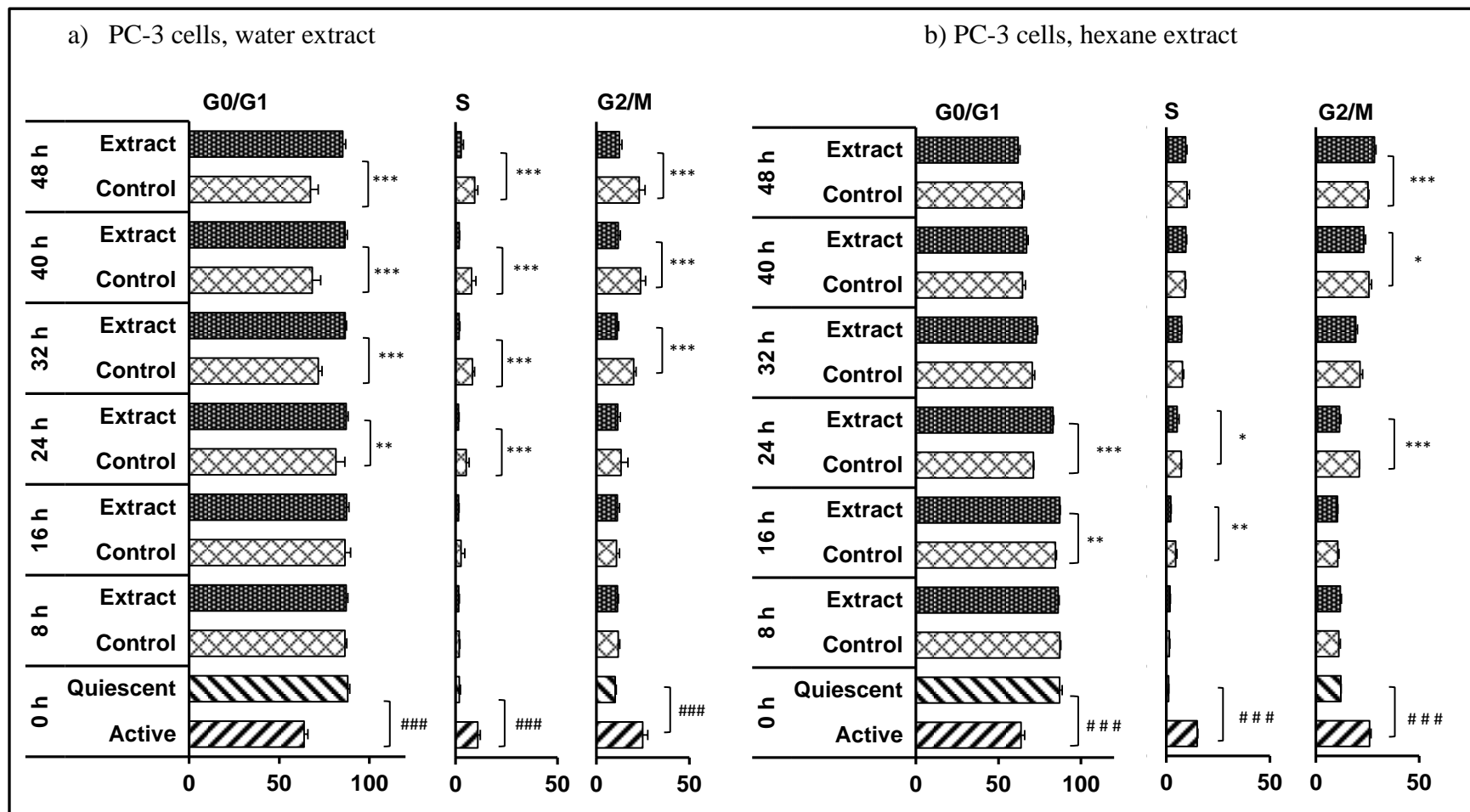
Figure 3.7: Cell cycle profile for PC-3 cells treated with hexane (a) and water (b) extract upon release from quiescence. Active (control cells: non-quiescent cells). Quiescent (quiescent cells: PC-3 after contact inhibition for 3 days). Data are expressed as the mean±S.D of three independent experiments compared with non-quiescent controls (Control; [###]$P < 0.001$) or water or DMSO vehicle control cells at each time point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
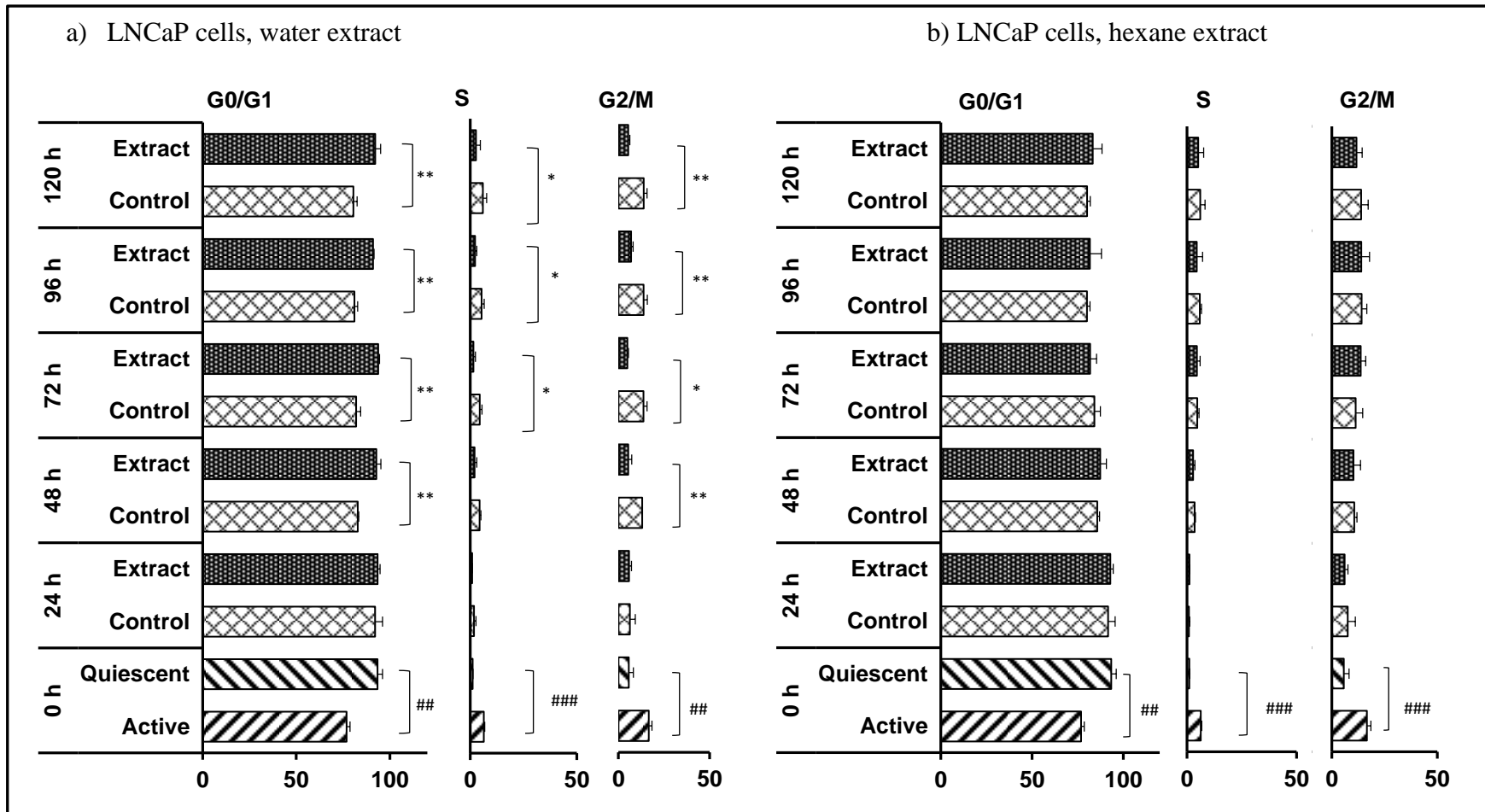
Figure 3.8: Cell cycle profile for LNCaP cells treated with hexane (c) and water (d) extract upon release from quiescence. Active (control cells: non-quiescent cells). Quiescent (quiescent cells: LNCaP after serum withdrawal for 7 days). Data are expressed as the mean±S.D of three independent experiments compared with non-quiescent controls (Control; [##]$P < 0.01$, [###]$P < 0.001$) or water or DMSO vehicle control cells at each time point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

a) PC-3, water extract

b) PC-3, hexane extract

c) LNCaP, water extract

d) LNCaP, hexane extract
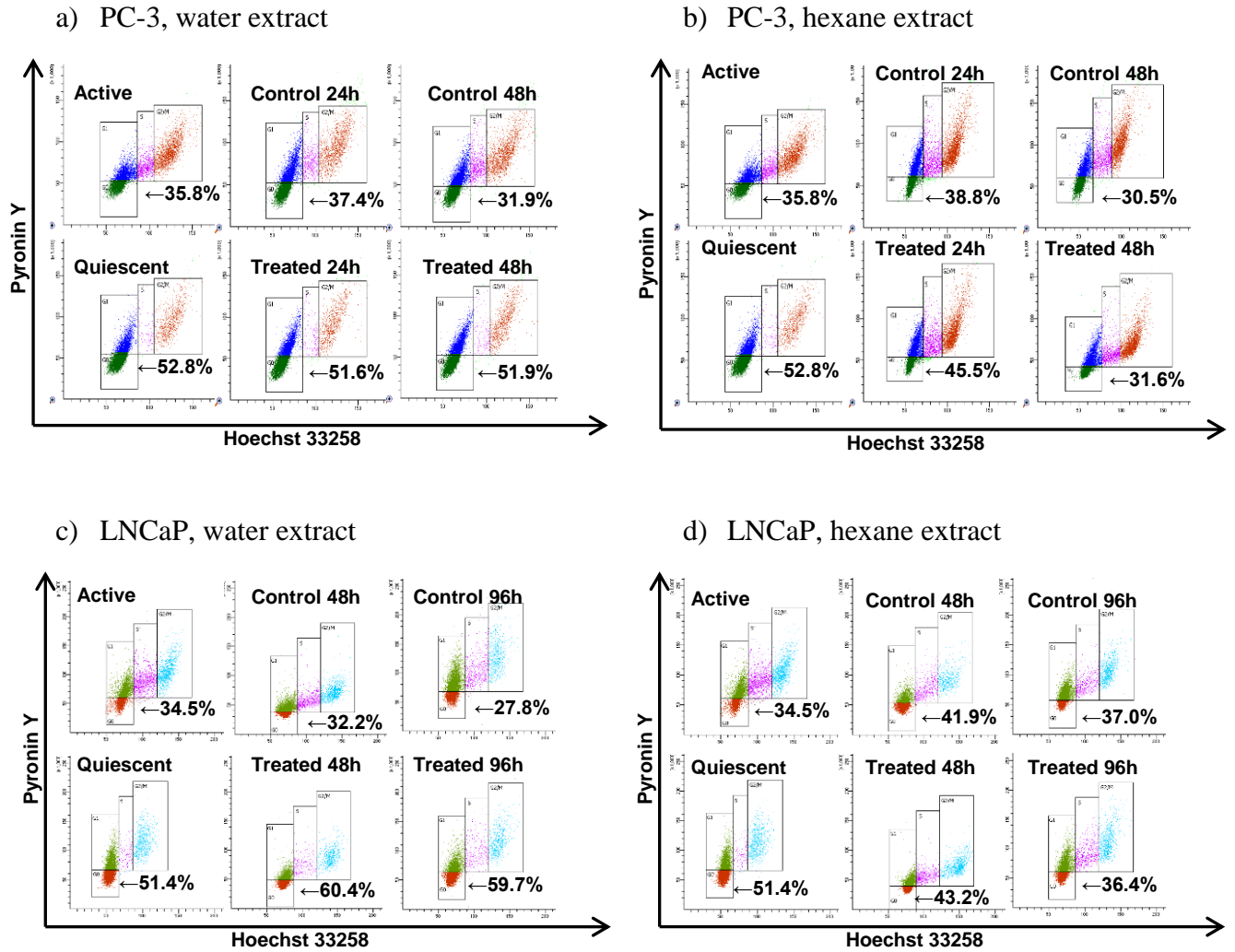
Figure 3.9: Representative flow cytometer images showing proportion of G0 cells in proliferating, quiescent, and during cell cycle re-entry with CPE at IC_{50} or control at indicated h for PC-3 cells treated with water (a) and hexane (b) extract, while for LNCaP cells treated with water (c) and hexane (d) extract. Proliferating (control cells: non-quiescent cells). Quiescent (quiescent cells: LNCaP after serum withdrawal for 7 days or PC-3 after contact inhibition for 3 days).

3.3.3 Effect of extract on DNA synthesis rate of prostate cancer cells

Effect of CPE at IC_{50} concentration on DNA synthesis rate of quiescent PC-3 cells upon released from quiescence was measured using Click-iT EdU incorporation assay. The DNA synthesis rate was directly proportional to the amount of EdU incorporated by the cells. The result in Figure 3.10 shows the percentage of incorporated EdU in PC-3 cell treated with CPE and control at 24 and 48 h. Percentage of cells with EdU marker for control PC-3 cells that were either treated with water or DMSO was in the range of 16.5 to 18.6%, whereas for CPEs was in the range of 1.7 to 7.0%. Hexane extract showed a significant reduction in cell proliferation as indicated by the lower EdU incorporation by 58 and 71% compared to control at 24 and 48 h, respectively ($p < 0.05$). Whereas for the water extract-treated cells, the EdU incorporation was lower by 89% and 86% than control at 24 and 48 h, respectively ($p < 0.05$). Results from the EdU incorporation assay illustrated that both CPEs significantly decreased cell proliferation in PC-3 cells with the water extract possesses a stronger proliferation inhibitory effect than hexane extract. Representative flow cytometry plots of cells at S phase with incorporated EdU marker are shown in Figure 3.11.
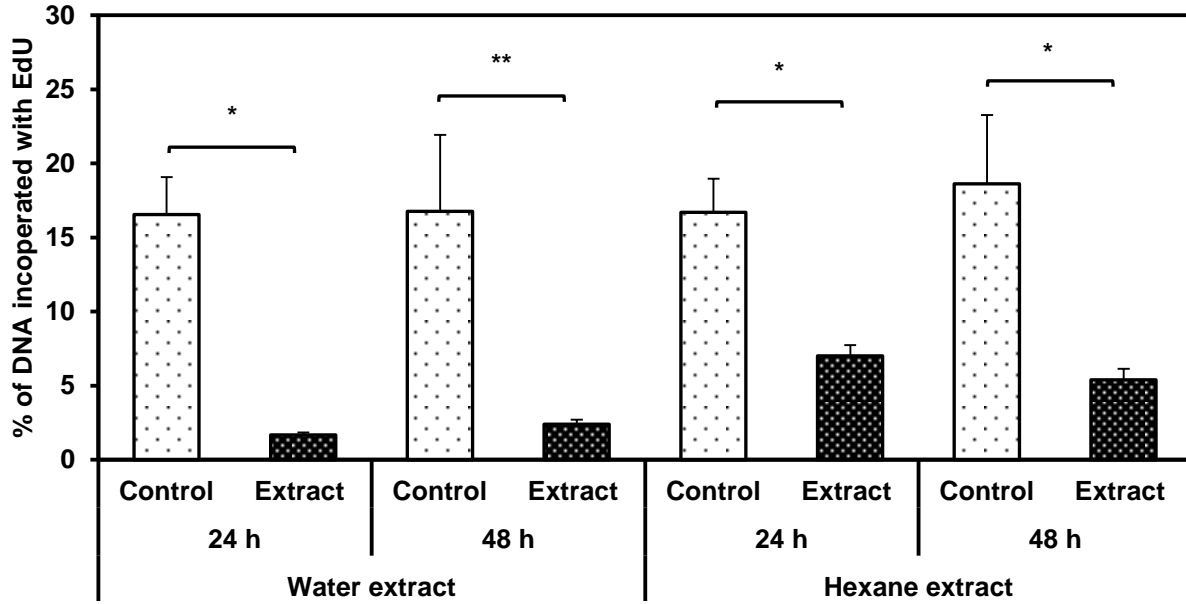
Figure 3.10: Percentage of EdU incorporation on PC-3 cells cultured with water (a) and hexane (b) extract at 24 and 48 h upon release from quiescence. Data are expressed as the mean±S.D of three independent experiments (b). *$P < 0.05$, **$P < 0.01$ vs. control.
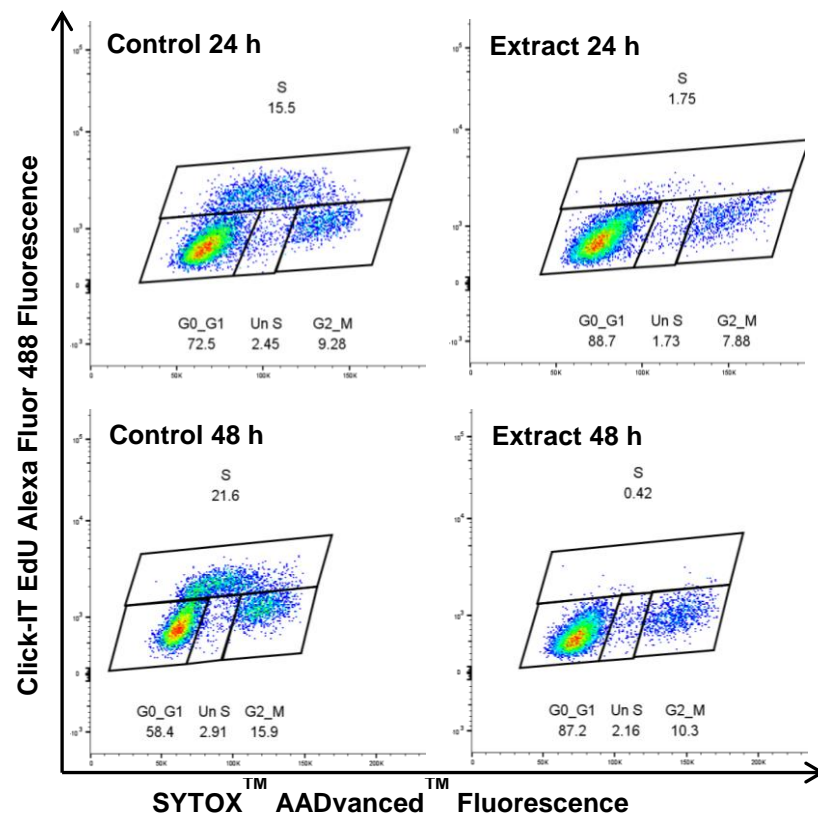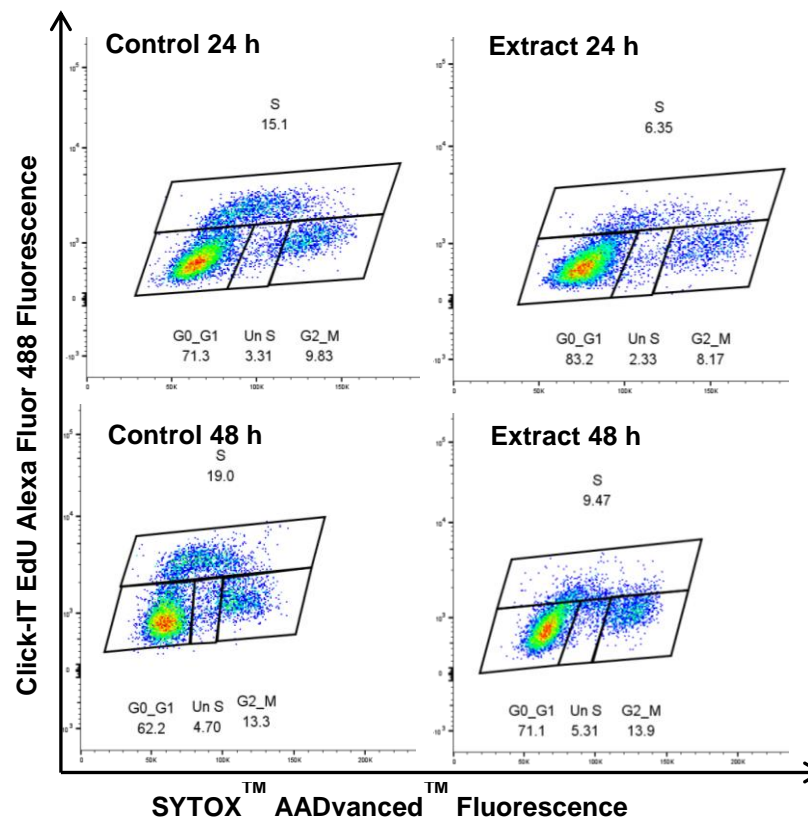
Figure 3.11: Representative flow cytometry plots for the PC-3 cells with EdU incorporation cultured with water extract (a) and hexane (b) extract at 24 and 48 h upon release from quiescence.

3.3.4 Apoptosis-inducing effect by extract on prostate cancer cells

CPEs ability in inducing apoptosis on quiescent PC-3 cells upon released from quiescence was determined using FITC Annexin-V apoptosis assay. Numbers of viable, necrotic, early apoptotic, and apoptotic cells at 24 and 48 h were shown in Figure 3.12. A significant difference in the number of apoptotic cells between control and hexane treated cells at 24 and 48 h ($p < 0.05$) was observed, which suggesting apoptosis-inducing effect of hexane extract on PC-3 cells. The difference in the number of apoptotic cells was nearly two-fold. In contrast, the apoptotic effect was not observed in cells treated with water extract. Moreover, compared to control a negligible variation in the number of viable, necrosis and early apoptosis cells were observed for the cell treated with water extract. Similarly, the hexane extract showed no difference for early apoptosis on PC-3 cells and it slightly increased the number of necrosis cells at 24 and 48 h.
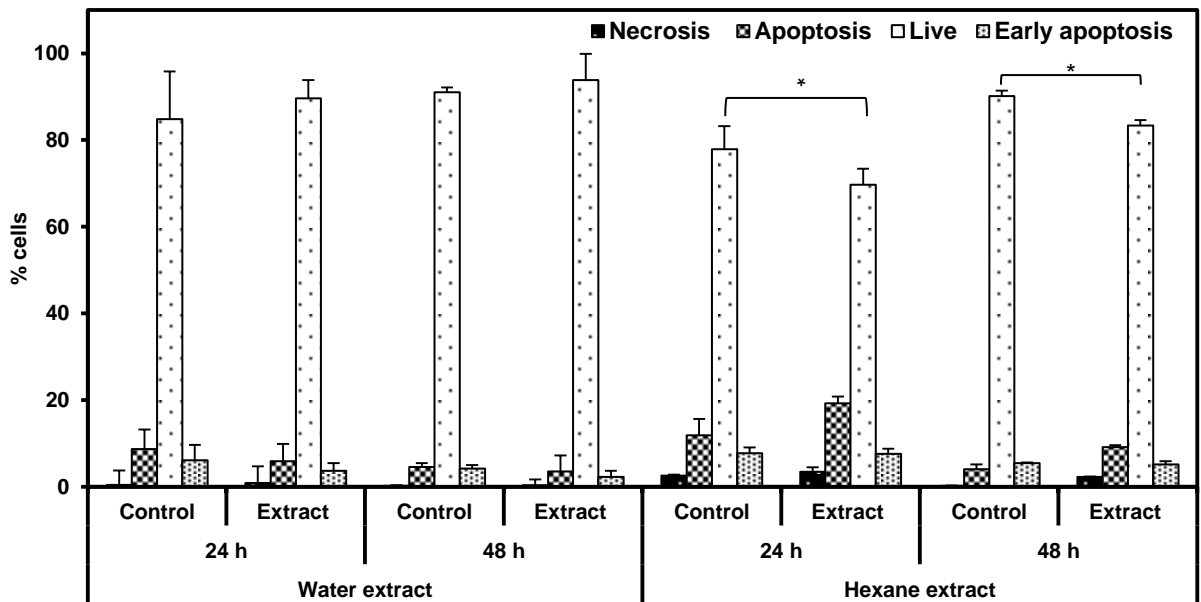
Figure 3.12: Percentages of viable, necrotic, early apoptotic, and apoptotic cells in quiescent PC-3 population exposed to extracts at 24 and 48 h. Data are expressed as the mean±S.D. of three independent experiments. * indicates a significant difference from control, $p < 0.05$.

3.3.5 Extract profiling and flavonoid quantitation

The two CPEs obtained using different extraction solvents were expected to have variation in the bioactive compound content. The composition of extracted bioactive compounds in hexane and water extracts was profiled using reverse-phase HPLC. The two CPEs used in this study have different compound profiles as shown in Figure 3.13. Water extract contains more peaks at a higher intensity than the hexane extract. This high number of peaks indicates the water solvent extracts a range of various water-soluble compounds from the citrus peel. As expected, hexane extract contained more non-polar compounds as observed at 15 to 17 minute, which could be the polymethoxyflavones. Two major peaks in water extract were identified to be narirutin and hesperidin using standard samples. Concentrations of narirutin and hesperidin in water extract were 400 and 514 µg/mL, respectively as shown in Table 3.1.
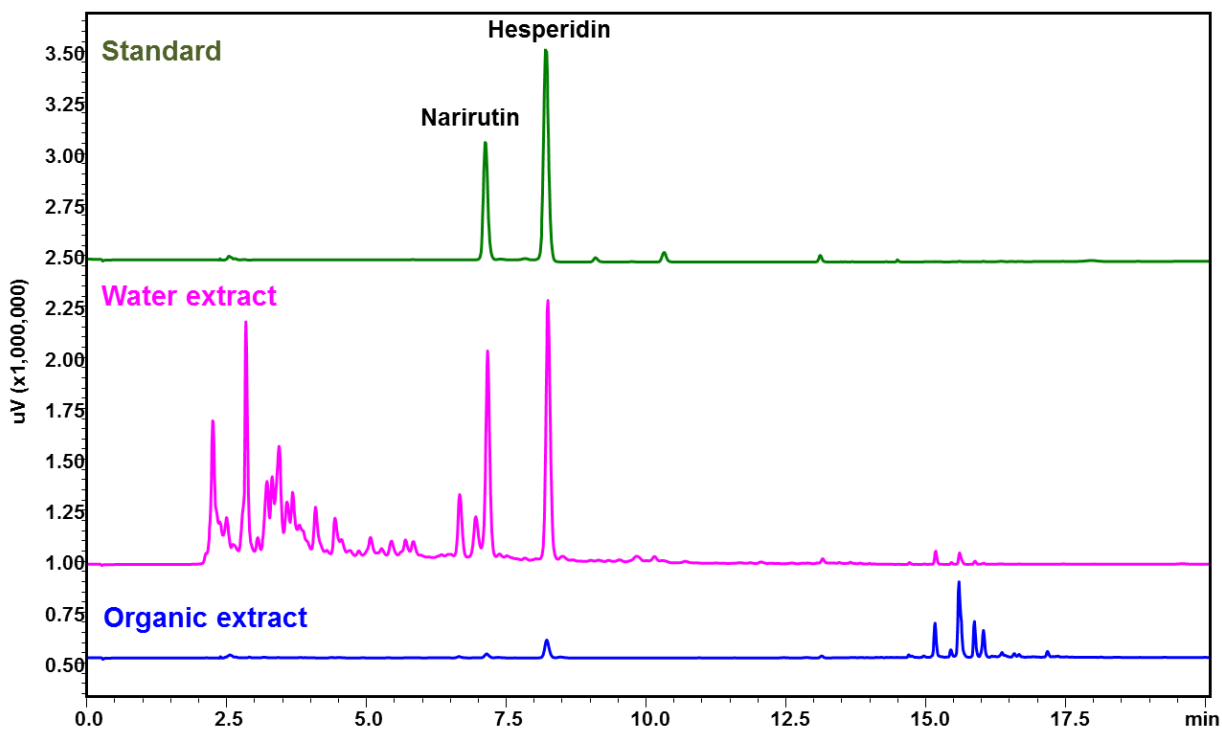
Figure 3.13: HPLC chromatogram at UV 230 nm of standards, water extract and hexane extract.

Table 3.1: Concentration of flavonoids in extracts

Sample	µg/mL	
	Narirutin	**Hesperidin**
Water	400.00 ± 6.57 (50)	513.74 ± 5.43 (64)
Hexane	7.29 ± 0.02 (1.5)	12.14 ± 0.15 (2.5)

n=3, mean ± std,
Value in bracket denotes the calculated concentration of the compound in extract exposed to the cells at IC50 value.

3.3.6 Effect of hesperidin and narirutin on cell cycle of prostate cancer

Hesperidin and narirutin were the prominent compounds in water extract and it was presumed that these compounds could contribute to the observed cell cycle inhibitory activity. The quiescent PC-3 cells were allowed to enter cell cycle in the presence of hesperidin or narirutin in the range of 18.75-300 µg/mL. An increase in the numbers of cells in S and G2/M phases was observed when the quiescent PC-3 cells were released in the presence of hesperidin or narirutin as depicted in Figure 3.14. The quiescent cells entered cell cycle upon release from quiescence and it indicated that these flavonoids were not directly responsible for the cell cycle re-entry inhibitory activity. Hesperidin at 300 µg/mL killed almost all of the PC-3 cells and only about half of these cells survived at 150 µg/mL. However, this observation was not noted for narirutin.

a) Hesperidin
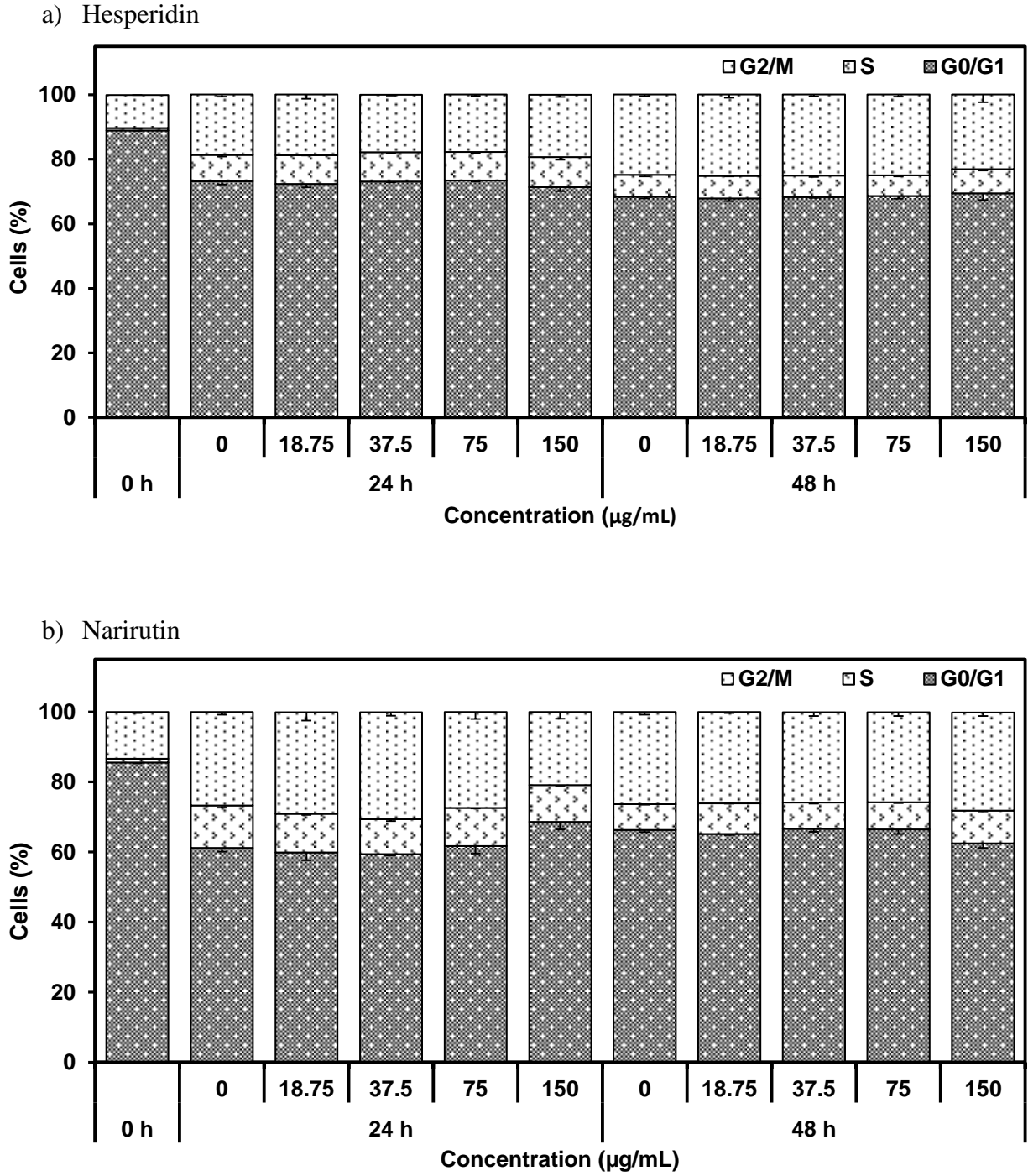
b) Narirutin

Figure 3.14: Cell cycle profile of PC-3 cells treated with hesperidin (a) and narirutin (b) at different concentrations (μg/mL) upon release from quiescence. Data are expressed as the mean±S.D of one representative experiment.

3.3.7 Influence of pH of water extract on the cell cycle inhibitory effect

Addition of citrus peel extract to the cell culture medium lowered the pH of the cell culture medium. Thus, effect of pH of water extract on the PC-3 cell cycle re-entry inhibitory activity was determined by comparing the cell cycle of the cells cultured in culture medium containing extract (as it is) with cells cultured in neutralized (pH 7.4) culture medium containing extract using sodium hydroxide. The cells were cultured at IC_{50} of water extract for 24 h. There was no increment in the numbers of cells in S and G2/M phases for cell cultured at pH 6.4 and 7.4 (Figure 3.15). Number of PC-3 cells in each phase at pH 6.4 and 7.4 after 24 h incubation was not significantly different to each other ($p < 0.05$), indicating the effect was induced by the compounds in the extract. The cells were tolerable to reduced pH at the IC_{50} of water extract.
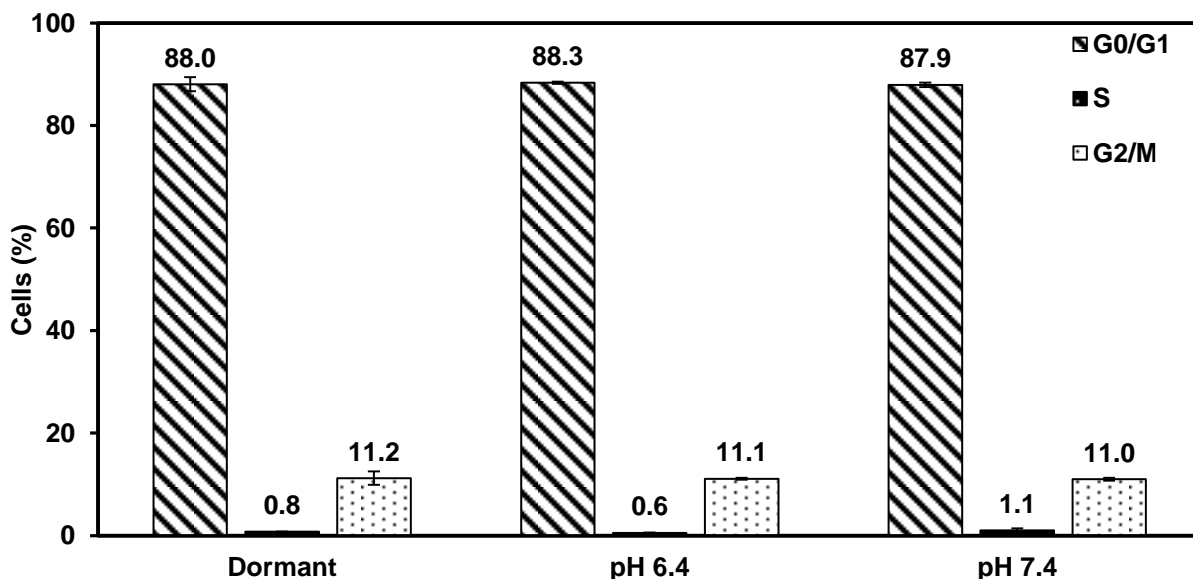
Figure 3.15: Cell cycle profile of PC-3 cells treated with water extract at different pH upon release from quiescence. Data are expressed as the mean±S.D of one representative experiment (n=3).

3.3.8 Water extract toxicity on the fibroblast cells

Water extract at different concentrations up to 23.8 mg/mL was evaluated on exponentially growing normal GM3348 fibroblast cells for the cell viability. Percentage of live cells after exposure to water extract at different concentrations for 24, 48 and 72 h was measured with PrestoBlue® cell viability assay. Figure 3.16 (a) shows the effect of water extract at different concentrations on the cell viability of fibroblast cells. Number of survived cells after cultured in extract dose-dependently decreased and also with an increase in incubation duration. Water extract significantly inhibited viability of fibroblast cells at concentration above 17.9 mg/mL at 24 h and 11.9 mg/mL at 48 h compared to control ($p < 0.05$). It is also noted that increment in the concentration of extract reduced the pH of culture medium. Thus, to eliminate the effect of pH of culture medium on the cell viability of cells, the extract in culture medium was neutralized with sodium hydroxide to pH around 7.4. The extract at neutral pH did not significantly inhibit the fibroblast cells up to 23.8 mg/mL at 24 h. However, a significant inhibitory effect was observed for concentration above 17.9 mg/mL at 48 h (Figure 3.16 (b)). The pH of culture medium has a significant effect on the fibroblast cell viability and this result concluded that the fibroblast cells are sensitive to the pH.

a) GM3348 cell, water extract without neutralization step
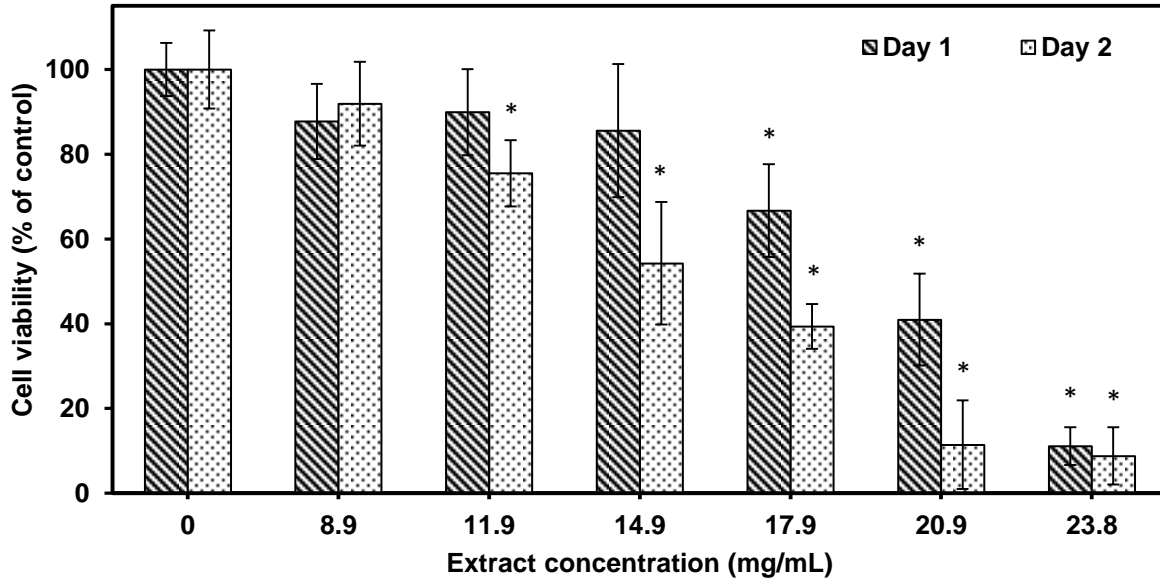
b) GM3348 cell, water extract with neutralization step
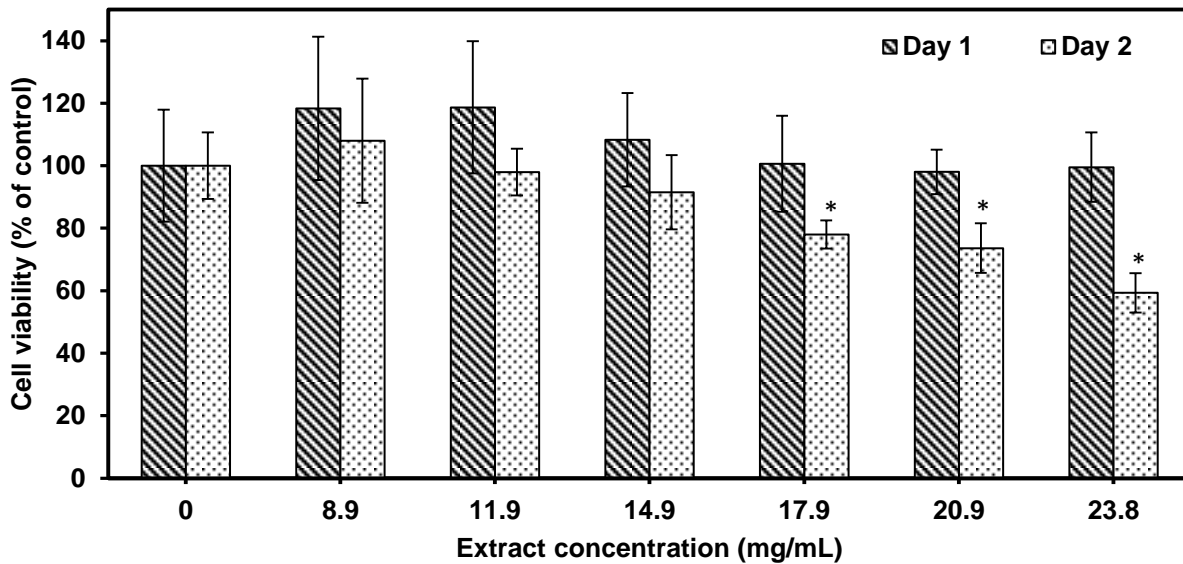
Figure 3.16: Water extract without neutralization step (a) and with neutralization step (b) decrease cell viability of GM3348 fibroblast cells. Results are presented as the mean ± SD of two independent experiments (n=6). * indicates significant difference than control (0 mg/mL) at $p <$ 0.05.

3.3.9 Changes in cell cycle profile of proliferating prostate cancer cells by water extract

The water extract was also tested on the actively proliferating PC-3 cells to investigate the phase of the cell cycle that the water extract could arrest. The active cells were exposed to extract at different concentrations up to 72 h. As illustrated in Figure 3.17, the cells cultured without extract showed the number of cells increased in G1 phase and decreased in G2/M with the increase in incubation time. However, a reduction in the increment in the number of cells at the G1 phase was observed for cell cultured with extract at a concentration of 6 mg/mL. Meanwhile, for cells cultured in the extract at 8.9, 11.9 and 14.9 mg/mL, the number of cells at the G1 phase was almost similar or slightly reduced. The cells cultured with extract at 17.9 mg/mL showed the lowest amount of cells at the G1 phase compared to the other samples. No change in the number of cells at any cell cycle phases was observed for cells cultured at this concentration. Not only that, the number of cells at G2/M was increased with the increase of concentration and incubation period. It can be concluded that the water extract reduced the progression of cells from G0/G11 phase to S phase.
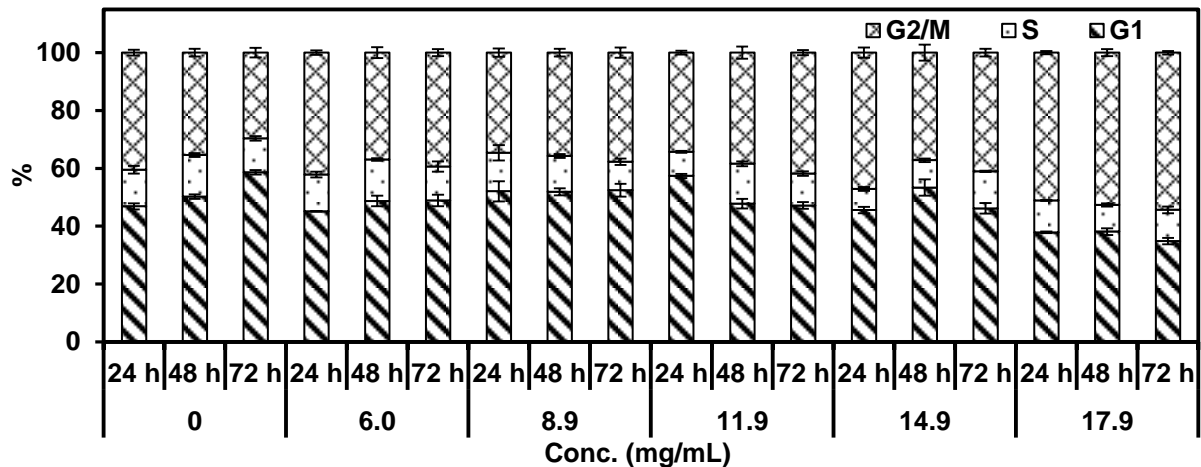
Figure 3.17: Cell cycle profile for actively proliferating PC-3 cells treated with water. Data are expressed as the mean±S.D of one technical experiment (n=3).

3.4   Discussion

In this study, two different solvents, water and hexane, were used to extract a different range of bioactive compounds from the citrus peel. Both CPEs dose-dependently reduced cell viability of proliferating PC-3 and LNCaP cells. Hexane extract showed stronger cell viability inhibitory effect than the water extract on these cells. This result is in agreement with previous studies where the hexane fraction showed stronger cell viability inhibitory effect than water fraction on different cancer cells such as U937 leukaemia and HT-29 colon cells (Hirata, Fujii et al. 2009, Lim, Moon et al. 2009). The difference in the IC_{50} values between two CPEs shows the variation in the composition of extract. The high IC_{50} values of water extract was due to the ability of the water solvent that extracts various types of hydrophilic compounds such as sugars, polysaccharides and proteins.

The ability of extract to prevent cell cycle re-entry was tested by allowing the quiescent cells to re-enter cell cycle in the presence of extract. It is interesting to note that only water extract was able to prevent the PC-3 and LNCaP cells from entering S phase from G0/G1 phase upon released from quiescence. Induction of cell cycle re-entry upon release from quiescence involves a rapid up-regulation of mRNA for various proteins, which can be measured with RNA content. Water extract maintains the cells in quiescence as a higher amount of cells in G0 phase was found for treated than the control. The low incorporation of EdU in water extract-treated cells further verifies that the PC-3 cells were in quiescence and did not proliferate. These results also revealed that the hexane extract suppressed the proliferation of PC-3 cells and failed to prevent the from re-entering the cell cycle upon released from quiescence. This finding is in agreement with our results with cell viability and cell cycle analysis.

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It is reported that CPEs and the bioactive compounds derived from the extracts exhibit apoptosis in many cancer lines including colon, leukaemia, lung and breast (Cirmi, Ferlazzo et al. 2016). In this study, the water extract showed no apoptotic effect on quiescent PC-3 cells in contrast to the observed mild apoptotic effect by the hexane extract on quiescent PC-3 cells. Most likely increasing the extract concentration could lead to significant apoptosis effect. The cell cycle and apoptosis results demonstrated that the extract reduced the cell viability mainly by suppressing cell proliferation rather than inducing apoptosis. Moreover, this finding also suggested that the hexane extract contains apoptosis-inducing compounds in higher amount or the compounds are more toxic than those present in water extract. It was hypothesised that the LNCaP cells could have a similar response to the EdU incorporation and apoptosis to both extracts. It was also noted that LNCaP cells treated with water extract had high amount dying cells, which probably due to the apoptosis.

The above results show that the water extract is effective than hexane extract in preventing the quiescent prostate cancer cells from re-entering the cell cycle. It was presumed that the presence of high amount of hesperidin and narirutin in water extract was responsible for the observed cell cycle re-entry inhibitory activity. When tested on quiescent PC-3 cells, these two flavonoids showed no effect in halting cell cycle re-entry upon released from quiescence. These flavonoids also did not arrest the quiescent PC-3 cells at G2/M phases; although, flavonoids are known for G2/M cell cycle arrest (Ren, Qiao et al. 2003). In one study, PC-3 cells exhibited negligible proliferation inhibition response to the hesperidin at 61 μg/mL (Lee, Wilson et al. 2010). In this study, a significant amount of dead cells was observed for PC-3 cells treated with hesperidin at 150 μg/mL. Moreover, the hesperidin at 300 μg/mL killed completely the PC-3 cells whereas narirutin showed no toxicity effect, indicating a significant role of the methoxy group at B

aromatic ring in hesperidin structure in inducing strong toxicity effect. As reported previously a small difference in the flavonoid structure could have a significant anticancer effect (Manthey and Guthrie 2002, Qiu, Dong et al. 2010).

This result shed light on the presence of other bioactive compounds in the extract that play a key role in cell cycle re-entry inhibitory activity. Most likely the synergic effect of the combination of different compounds such as flavonoids led to observing bioactivity for keeping the cancer cell in the dormant phase. It was suggested that single compound may not show the similar effect as the crude extract or combination of few compounds as the single compound may not behave the same way as the compound in whole foods (Liu 2004). This concept has encouraged the use of complex mixtures of bioactive substances such as crude extract rather than isolated individual compound for maximum biological activity. In this study, the water extract showed low or no toxicity on normal fibroblast cells compared to prostate cancer cells, which indicates the selectivity of the extract in targeting cancer cells. Moreover, CPE has been reported to have no toxicity effect in normal RWPE-1 cells (Kim, Lee et al. 2017) and the *in-vivo* experiments showed no adverse effect on the organs of mice treated with CPE (Lai, Li et al. 2013b, Kim, Lee et al. 2017). This study provided information that water extract of citrus peel that is rich in bioactive compounds could be a good source in preventing quiescent cancer cell from entering the cell cycle. Moreover, the citrus peel that is currently treated as landfill will be utilized in a better way by converting it into food supplements and nutraceutical products.

3.5   Conclusion

In conclusion, this study demonstrated that water extract from citrus peel was effective in preventing quiescent prostate cancer cells from re-entering cell cycle when tested on an established *in-vitro* model of prostate cancer quiescent cells. Not only that, the water extract was also exhibited stronger proliferation inhibitory effect on prostate cancer cells than normal fibroblast cells. Even though more studies are required to identify the potent bioactive compound to verify this finding, this study provided an initial finding to encourage the use of whole citrus peel extract for post-therapy prostate cancer patients.

4 A new cell cycle inhibitor, citric acid, from water extract of citrus peel.

4.1 Introduction

Cell cycle is a vital mechanism of a cell in controlling cell division and progression. A cell is progressed in a highly coordinated event through four distinct phases (G0/G1, S, G2 and M) of the cell cycle (Schwartz and Shah 2005, Otto and Sicinski 2017). Dysregulation of this process could lead to a continuous cell cycle division, which is regarded as a fundamental character for cancer (Schwartz and Shah 2005, Otto and Sicinski 2017). Therefore, modulation of cell cycle proteins and signalling pathways to halt the cell cycle progression is a promising strategy against cancer (Vermeulen, Van Bockstaele et al. 2003, Schwartz and Shah 2005).

Flavopiridol, Palbociclib and Volasertib are some of the developed new anticancer drugs that specifically target the cell cycle modulator proteins. However, many modern chemotherapy drugs show low efficacy and non-specific toxicity in targeting cancer cells, while are resulting in some undesirable side health effects. These limitations have encouraged the search for anticancer compounds from natural sources (Newman and Cragg 2016). A review of the anticancer drugs showed about 49% of small molecule-based anticancer drugs that are either natural products or directly derived therefrom were approved by the US Food and Drug Administration to the market between 1940 and 2014 (Newman and Cragg 2016). It clearly indicates that nature is a rich source for novel drug discovery.

Citrus contains considerable amounts of biologically active compounds such as flavonoids, limonoids, coumarins, phenolic acids, terpenoids, and carotenoids (Yu, Wang et al. 2005, Zou, Xi et al. 2016), along with many other unknown bioactive components. The various biological effects exhibited by citrus are mainly due to the activity of either individual or interaction of various bioactive compounds present in citrus (Liu 2004). Citrus extracts have been reported to arrest various lines of cancer cells at G0/G1 or G2/M phases (Cirmi, Ferlazzo et al. 2016). In the previous study, citrus peel extract (CPE) was shown to exhibit anticancer activity by inhibiting cell cycle re-entry on quiescent prostate cancer. It was hypothesized from that study that one or more compounds in CPE could contribute to the observed cell cycle re-entry inhibitory activity. To the best of our knowledge, no information on the active components associated with cell cycle re-entry inhibitory activity in citrus is available. Given the rich nature of citrus, the isolation and identification of bioactive compounds from citrus have a high practical value. Thus, the aim of this chapter was to isolate and identify the potent compounds with cell cycle re-entry inhibitory activity from citrus peel.

4.2   Materials and methods

4.2.1   Chemicals and reagents

HPLC grade acetonitrile (34851), hexane (270504), ethyl acetate (34858), DMSO (D8418), trifluoroacetic acid (T6508) and were obtained from Sigma-Aldrich. PrestoBlue™ Cell Viability Reagent (A13261), FxCycle™ PI/RNase Staining Solution (F10797), and all cell culture supplies were from Life Technologies Gibco (Darmstadt, Germany).

4.2.2 Preparation of extract

The water extract was prepared as described in Section 3.2.2.

4.2.3 Solvent-solvent fractionation

Water extract of citrus peel was fractionated using conventional solvent-solvent extraction technique. A 10 mL of water extract was extracted serially with hexane (10 mL × 2) and ethyl acetate (10 mL × 2). The hexane, ethyl acetate and water residue fractions were dried in a vacuum oven until complete dryness. The dried extracts were dissolved with 10 mL of phosphate buffer solution to achieve the initial concentration. The water extract and the fractions obtained from water extract were tested on the PC-3 cells.

4.2.4 Preparative HPLC

Preparative LC separation was carried out on a Waters 600 controller equipped with Waters 600 binary pump, Waters 2487 UV detector and Waters Fraction Collectors III. The separation was carried out on a Waters Sunfire C18 OBD (19 mm × 150 mm, 5 μm) at a flow rate of 7 mL/min with a mobile phase of water and acetonitrile containing 0.1% trifluoroacetic acid. The concentration of acetonitrile in water was maintained at 0% for 1 min and then increased gradient to 100% over 60 min before returned to 0% in 5 minutes. For single compound isolation, the gradient of acetonitrile from 0% to 100% was extended over 2 h. The absorbance was detected at 230 nm. Collected fractions were dried under reduced pressure, dissolved in water to the original concentration.

4.2.5 Cell culture

Prostate cancer (PC-3) and human dermal fibroblast (GM3348) cells were cultured as described in Section 3.2.3. Meanwhile, normal prostate epithelial (RWPE-1) cells obtained from the American Type Culture Collection (Rockville, MD). RWPE-1 cells were cultured in the complete medium of keratinocyte serum-free medium (Gibco, 17005042). The cells were grown in a humidified cell incubator at 37°C in the presence of 5% CO_2.

4.2.6 Cell viability assay

Exponential growing PC-3 (7000 cells/well) cells, RWPE-1 (6000 cells) and GM3348 (2500 cells/well) were seeded in 96-well plates in complete medium. After 24 h, the medium was replaced with a new complete medium containing different concentrations of extract/fraction/citric acid. The pH of culture medium containing citric acid was neutralized with sodium hydroxide to pH 7.4 before adding to the cells. The cell viability was measured as described in section 3.2.4.

4.2.7 Cell cycle analysis

Cell cycle analysis was performed as described in section 3.2.5.

4.2.8 Structure elucidation

The isolated compound was identified by comparing the spectral data of mass spectrometry, nuclear magnetic resonance (NMR), and Fourier transform infrared (FTIR) with the commercially available standard. For mass spectrometry, Bruker amaZon SL quadrupole ion trap mass spectrometer equipment with atmospheric pressure chemical ionization (APCI) ion source was used. Mass range of m/z of 150–1000 was used for a full scan of the mass spectra in negative ion mode. The 1H-NMR spectra of the compound in D_2O was recorded in a Bruker Avance III 600 MHz spectrometer operating at 600 MHz. The chemical shifts are expressed in δ (parts per million) and the coupling constants (J) in Hz. IR spectra were recorded with a Nicolet 6700 FTIR spectrometer in the range 400–4000 cm^{-1} from the average of 64 scans.

4.2.9 Statistical Analysis

IBM SPSS Statistics software version 24 was used for statistical analysis. Student's two-tailed t-test was used for comparison between two different groups and ANOVA analysis was used with Tukey multiple comparison test for multiple comparisons. All P-values <0.05 were considered statistically significant.

4.3 Results

4.3.1 Effect of different fractions of water extract on cell cycle re-entry inhibition

Different fractions of water extract obtained using the solvent-solvent extraction method was tested on quiescent PC-3 cells. These quiescent cells were induced to re-enter cell cycle by re-plating at low density with or without extract. As shown in Figure 4.1, the control cells re-entered cell cycle upon release from quiescence where the number of cells at G0/G1 phase was reduced from 87% to 71% at 24 h and 66% at 48 h. In contrast, the PC-3 cells treated with water extract did not show any significant reduction at 24 h and reduced to 79% at 48 h ($p < 0.05$). Meanwhile, water fraction showed the strongest cell cycle re-entry inhibitory activity among the other fractions at the first 24 h. Number of cells at G0/G1 for water fraction at 24 h was 86%, whereas for other fractions were between 73-75%. At 48 h, cell cycle re-entry inhibitory activity was not observed for all fractions. Numbers of cells at G0/G1 phase for cells treated with different fractions were in the range of 66-71%, which was insignificant compared to control (65.9%), $p < 0.05$. A reduction in the number of cells at G0/G1 was accompanied with an increase in the number of cells at S and G2/M phase when the quiescent cells entering the cell cycle. A similar observation was noted for all samples except the water extract and water fraction at 24 h. As the water fraction showed the highest cell cycle re-entry inhibitory activity, the compound(s) that are responsible for the observed biological were predicted to be hydrophilic compounds.
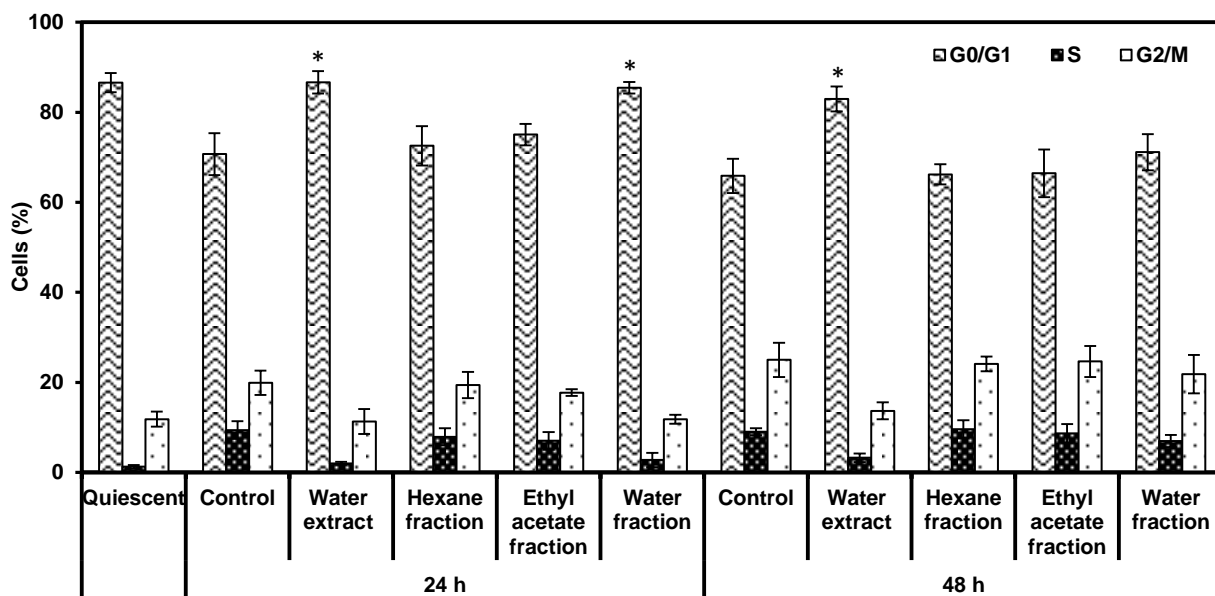
Figure 4.1: Cell cycle profile of PC-3 cells treated with extract and solvent-solvent extraction fractions of water extract upon release from quiescence. Control cells were exposed to same amount of water as the water extract and the fraction treated cells. Quiescent cells are PC-3 cells after contact inhibition for 3 days. Data are expressed as the mean±S.D of three independent experiments. *indicates a significant difference compared to control, $p < 0.05$.

4.3.2　Extract fractionation using preparative high-pressure liquid chromatography

Since water fraction showed the highest cell cycle re-entry inhibitory activity, the extract was subjected for separation using a preparative HPLC system equipped with a C18 column. The polar compound will elute out earlier from column than non-polar compound because the non-polar compounds have a stronger chemical interaction with the material of the column. The HPLC chromatogram of water extract indicated that the extract contained a broad range of compound ranging from polar to non-polar compounds (Figure 4.2). The water extract contained

many major peaks indicating by the broad and tall peaks such as at 18 min and 24 min. The peaks at 36-40 min are more likely to be polymethoxyflavones based on the reported literature. The extract were fractionated into three fractions based on time where elute between 0-14 min represented fraction 1, elute between min 14-28 represented fraction 2 and elute between 28-42 min represented fraction 3.
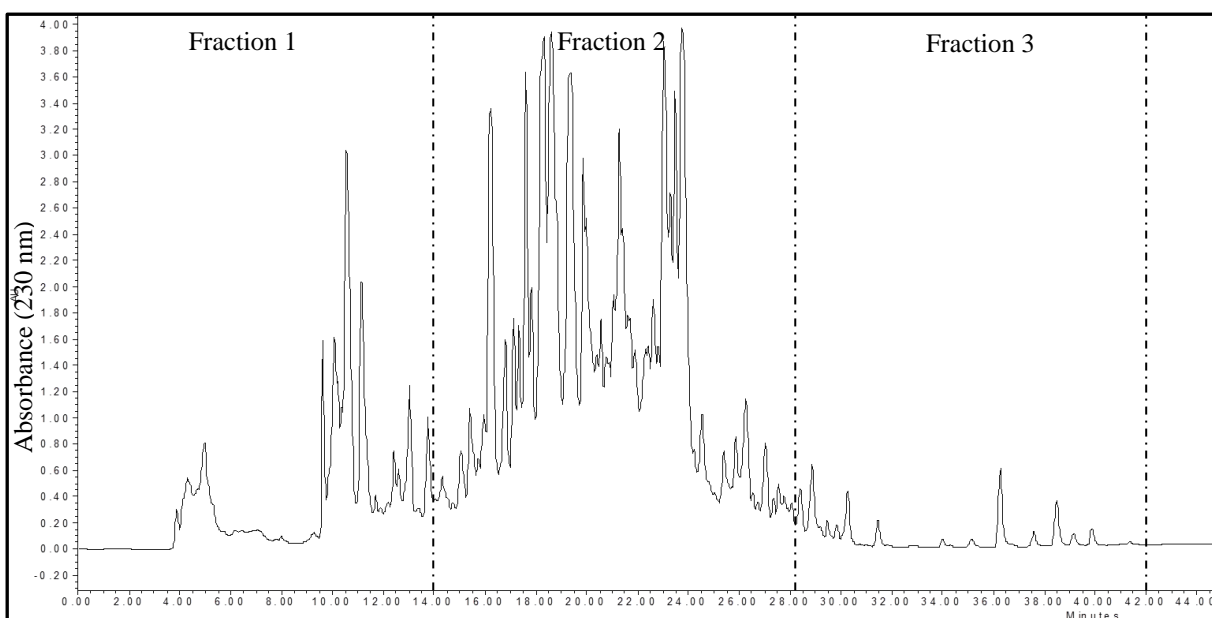
Figure 4.2: Fractionation of water extract using a Sunfire OBS C18 HPLC preparative column at an UV wavelength of 230 nm. The fraction was collected at 14 minutes interval as indicated in the figure using fraction collector.

The fractions collected using preparative HPLC were tested for cell cycle re-entry inhibitory activity on quiescent PC-3 cells and the result is illustrated in Figure 4.3. Interestingly, all the fractions showed strong cell cycle re-entry inhibitory activity as the number of cells at G0/G1 did

not decrease for the first 24 h compared to quiescent cells ($p < 0.05$). However, the inhibitory activity of the fraction reduced for the next 24 h, especially for fraction 2 and 3. At 48 h, only fraction 1 showed strong significant inhibitory activity compared to control ($p < 0.05$) and this indicate that the potent compound presents in this elution region. The other two fractions still showed higher activity compared to control even at 48 h, indicating the fractions contained the cell cycle inhibitory compounds. However, due to the concentration factor, the fraction showed mild biological activity.
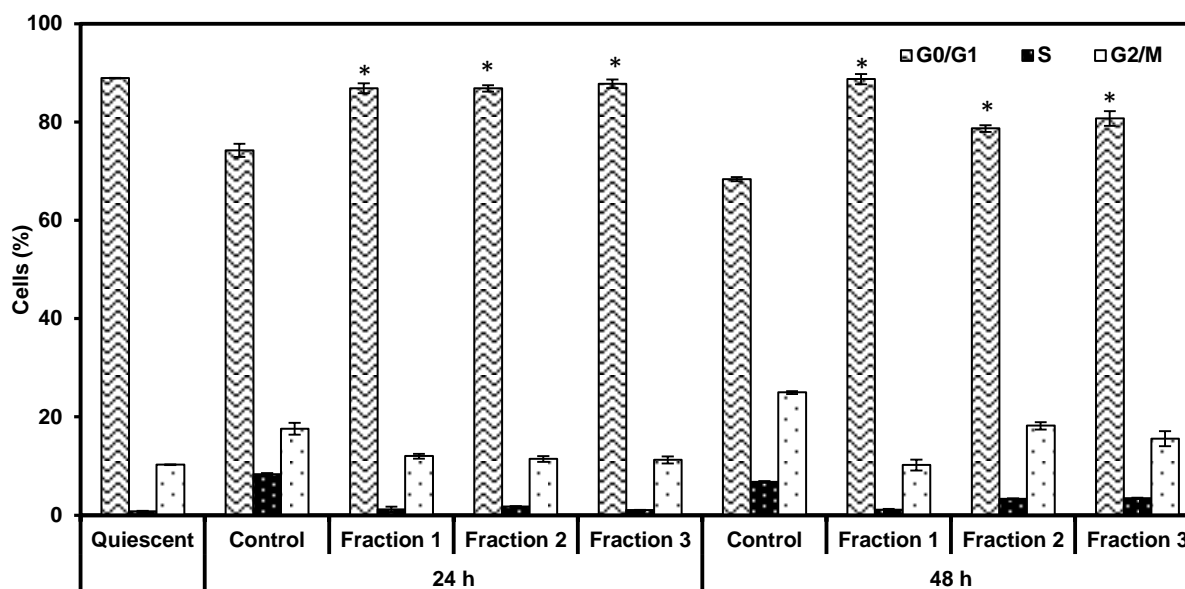
Figure 4.3: Cell cycle profile of PC-3 cells treated with fractions of the extract obtained using preparative HPLC system upon release from quiescence. Quiescent cells are PC-3 cells after contact inhibition for 3 days. Data are expressed as the mean±S.D of representative one experiment. *indicates a significant difference compared to control, $p < 0.05$.

The fraction 1 that showed the highest inhibition was further fractionated with 2 minutes interval to identify the compounds (Figure 4.4). The process yielded six fractions and the fraction between min 10-12 showed the highest cell cycle re-entry inhibitory activity at 24 h and 48 h. It was also observed that at 48 h, the inhibitory effect of the fraction is not as strong as at 24 h. Fraction collected between min 10-12 contained five compounds and the compounds were collected and tested individually.
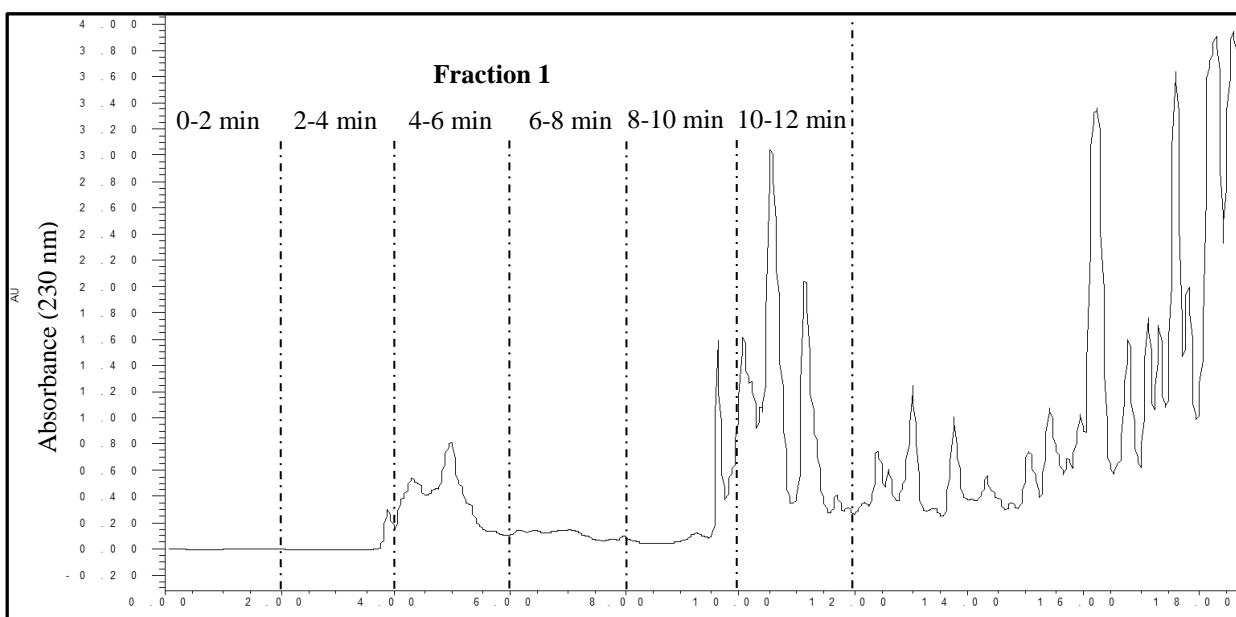
Figure 4.4: Fractionation of fraction 1 using a Sunfire OBS C18 HPLC preparative column at an UV wavelength of 230 nm. The fraction was collected at every 2 minutes interval as indicated in the figure using fraction collector.

Only one compound out of five compounds in potent fraction was found to contribute to the inhibitory effect. As shown in Figure 4.5, the compound showed a significant inhibitory effect when tested on the quiescent PC-3 cells up to 48 h ($p < 0.05$). Number of cells at G0/G1 reduced from 87% to 85% at 24 h and to 83% at 48 h.
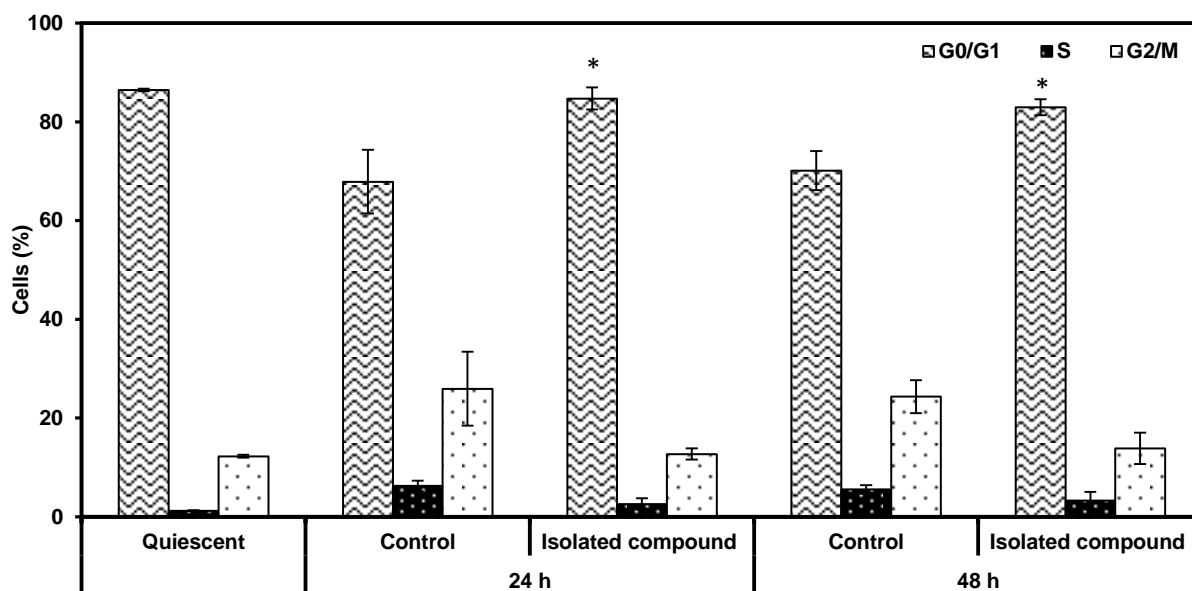
Figure 4.5: Cell cycle profile of PC-3 cells treated with the isolated compound upon release from quiescence. Quiescent cells are PC-3 cells after contact inhibition for 3 days. Data are expressed as the mean±S.D of three independent experiments. *is used to indicate the significant difference compared to control, $p < 0.05$.

4.3.3 Compound identification with analytical methods

The isolated compound was subjected to mass spectrometry to determine molecular mass and compound structure. The compound has a molecular mass of 192 because the [M⁻] was 190.76

(Figure 4.6). The low molecular mass indicated that the compound was a small compound. However, the UV spectrum showed the compound was undetectable at 280 nm, thus eliminated the presence of benzene ring. To identify the functional group on the molecule, the compound was analysed with FTIR. The FTIR result showed the compound contained –OH and C=O functional groups (Figure 4.7). This is parallel with high solubility of the compound in water, low retention in C18 column and the no UV detection at 280 nm. Based on retention time and UV spectrum, citric acid standard showed the possible match. Figure 4.8 shows the 1H-NMR (600 MHz, CD3OD) of citric acid standard and isolated compound, δH: 6.17 (1H, d, J = 1.4 Hz, H-6), 6.38 (1H, s, H-8), 8.07 (1H, d, J = 8.7 Hz, H-2), 6.89 (1H, d, J = 8.7 Hz, H-3), 6.89 (1H, d, J = 8.7 Hz, H-5), 8.07(1H, d, J = 8.7 Hz, H-6). The NMR data is in agreement with the citric acid standard and the reported literature values. The structure of the isolated compound was identified as a citric acid (PubChem CID: 311): $C_6H_8O_7$.
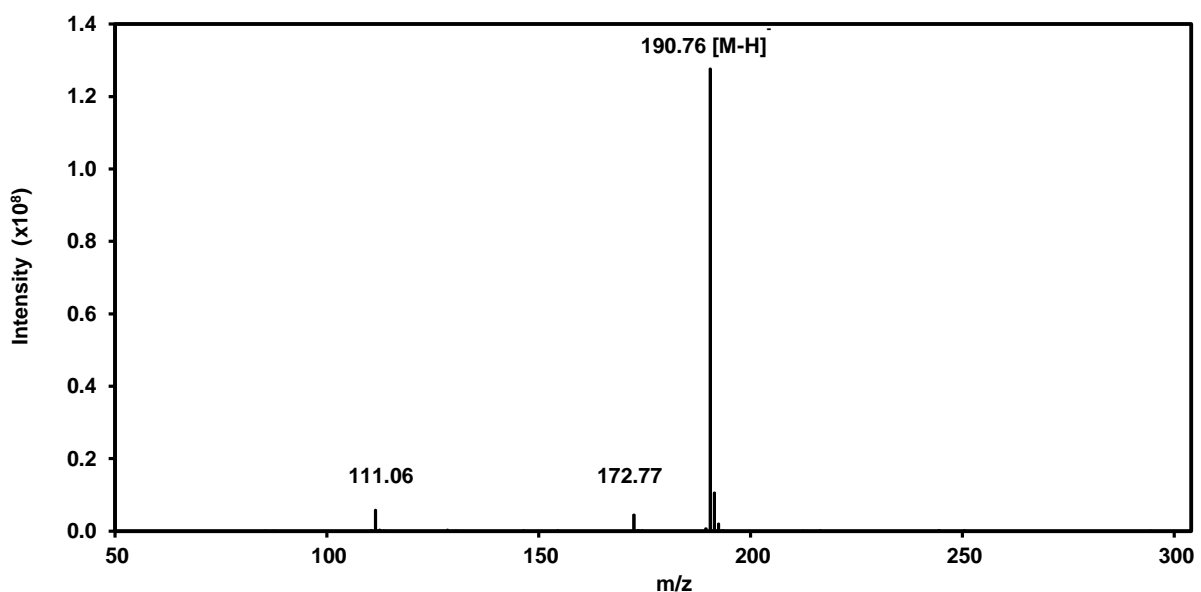
Figure 4.6: APCI-MS spectra of the isolated compound.
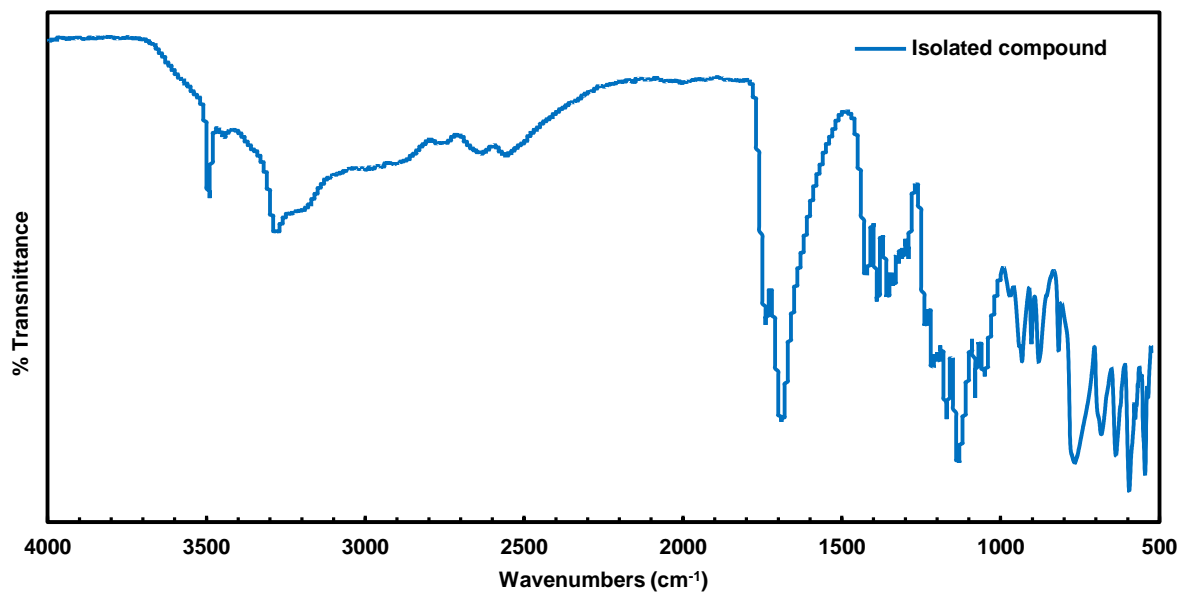
Figure 4.7: FTIR spectrum of the isolated compound.
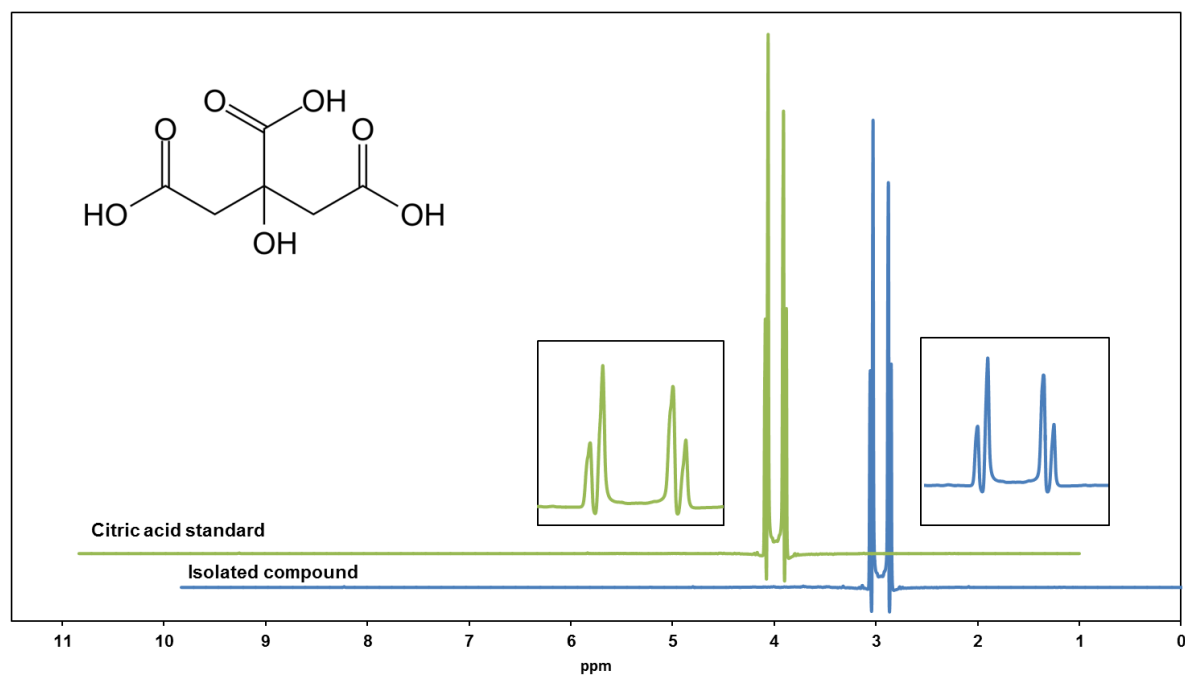
Figure 4.8: 1H-NMR spectrum in D_2O of the isolated compound and citric acid standard.

4.3.4 Citric acid toxicity on cancer and non-cancer cells

Citric acid dose-dependently inhibited cell viability of PC-3 cells as the cell viability decreased with the increase of citric acid concentration when tested in the range of 0.125-1.25 mg/mL (Figure 4.9). Significant toxicity effect was observed at a concentration above 0.5 mg/mL with stronger cell viability inhibitory effect over prolonged exposure to citric acid. Cell culture medium containing citric acid standard was neutralized to pH 7.4 with sodium hydroxide to remove the effect of pH on the cell viability.
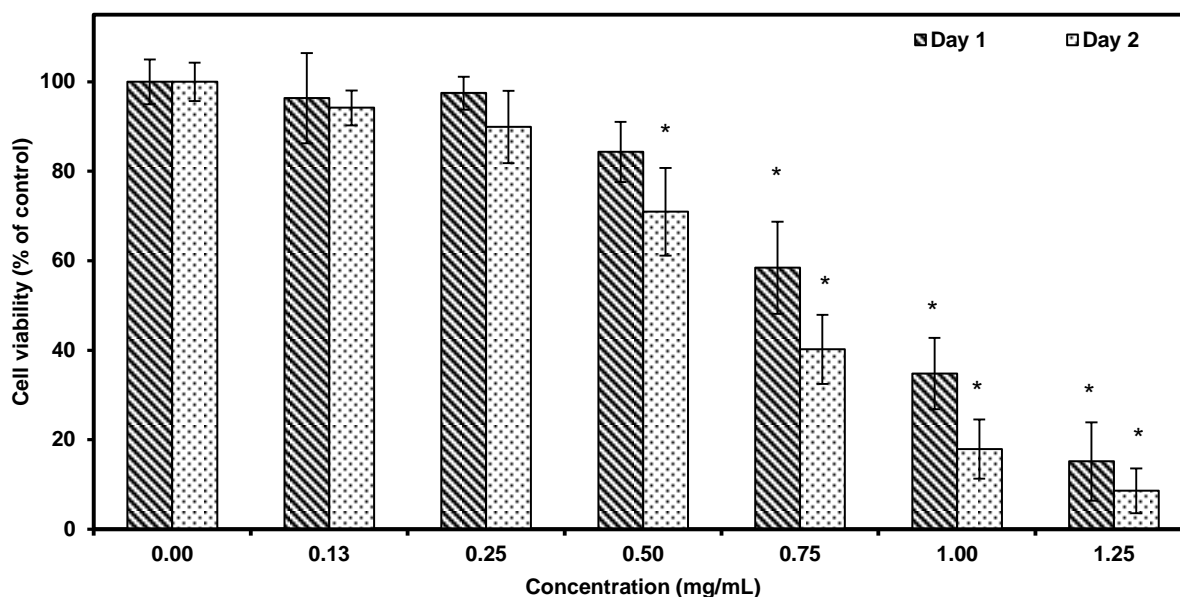
Figure 4.9: Cell viability effect of the citric acid standard on the PC-3 cells. Data are expressed as the mean±S.D of three independent experiments. * indicates a significant difference compared to control, $p < 0.05$.

Citric acid was also tested on the normal epithelial prostate (RWPE-1) cells for the cell viability effect. Citric acid showed cell viability inhibitory effect on the RWPE-1 cells as the number of cells decreased with the increase of citric acid concentration and the incubation duration. Citric acid with the concentration above 0.5 mg/mL in the medium was significantly inhibited cell growth compared to control. However, it can be seen that the effect of citric acid on the viability of RWPE-1 cells reduced at a concentration above 1 mg/mL. The observed cell viability effect was due to the citric acid standard and not from the pH of culture medium as the culture medium was neutralized to pH 7.4 after the addition of citric acid standard.
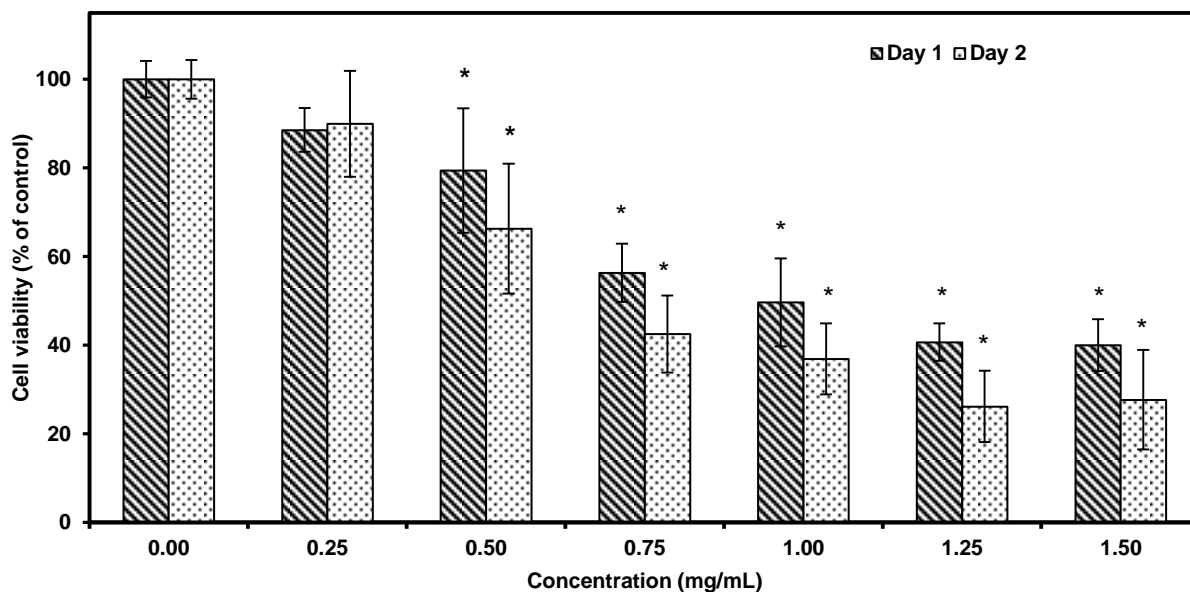
Figure 4.10: Cell viability effect of the citric acid standard on the RWPE-1 cells. Data are expressed as the mean±S.D of three independent experiments. * indicates significant difference compared to control, $p < 0.05$.

The comparison of the cell viability of PC-3 and RWPE-1 after 48 h of incubation with citric acid is shown in Figure 4.11. No significant difference in cell viability between both cells was observed at a concentration between 0-0.75 mg/mL ($p < 0.05$). However, citric acid significantly exhibited higher cell viability inhibitory effect on PC-3 cells than RWPE-1 cells at a concentration above 1 mg/mL ($p < 0.05$). The cell viability inhibitory effect at a concentration above 1 mg/mL on PC-3 cells by citric acid was more than one fold stronger compared on RWPE-1 cells.
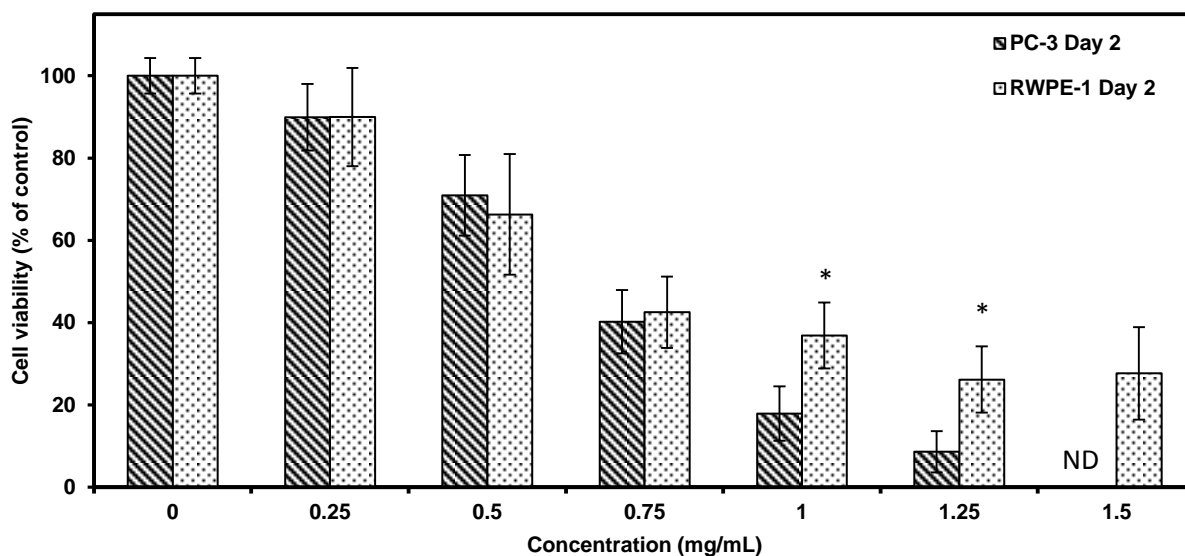
Figure 4.11: Cell viability effect of citric acid standard on the PC-3 and RWPE-1 cells. Data are expressed as the mean±S.D of three independent experiments. * indicates a significant difference compared to PC-3 cells at the same concentration, $p < 0.05$. ND, not determined.

Interestingly, citric acid in the range of 0.125-1.25 mg/mL showed an insignificant effect on the cell viability of human normal fibroblast GM3348 cells (Figure 4.12).
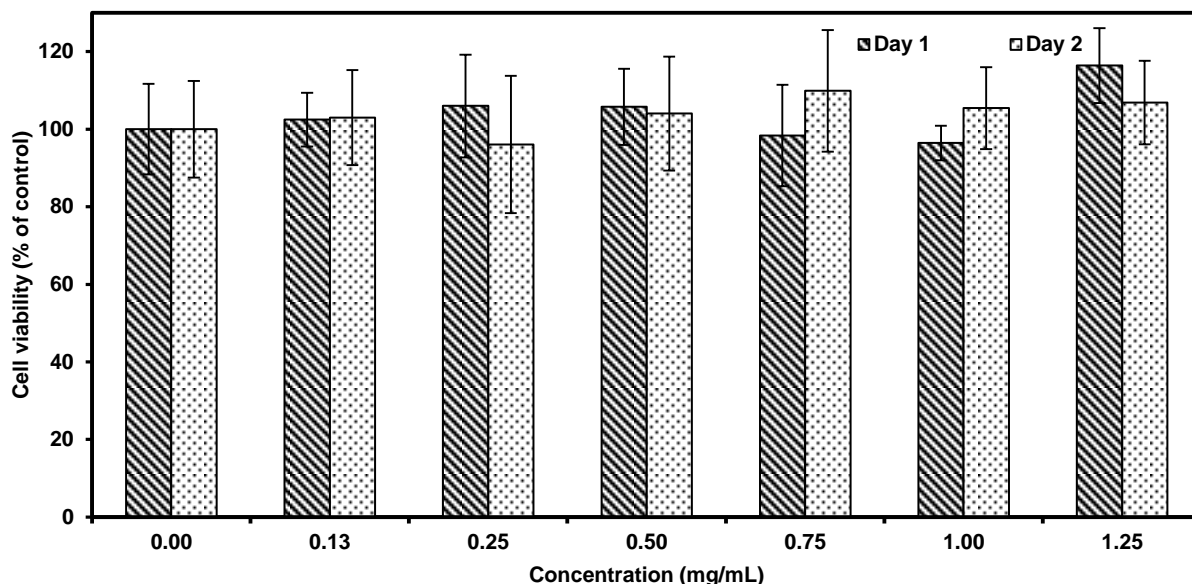
Figure 4.12: Cell viability effect of the citric acid standard on the human fibroblast normal GM3348 cells. Data are expressed as the mean±S.D of three independent experiments.

4.4 Discussion

In the previous chapter, water extract of citrus peel was shown to exhibit strong cell cycle re-entry inhibitory effect on quiescent PC-3 and LNCaP cells. As identifying the potent compound could be meaningful in different aspects, assay-guided isolation of potent compounds was performed. Solvent-solvent extraction that is commonly used for compound isolation was used at the initial stage in this study. The result of solvent-solvent extraction indicated that the potent compounds in the extract could be hydrophilic as the water fraction showed the highest

inhibitory activity compared to other fractions. No suitable extraction solvent was available to extract the hydrophilic compounds from water fraction.

Thus, reversed-phase HPLC was used to separate the compounds. The water extract was fractionated to three different fractions to eliminate a large number of fraction analysis and also to evaluate the potential loss of anticancer activity due to the synergic effect. Only one fraction showed a strong effect in inhibiting cell cycle re-entry up to 48 h, while the other two fractions exhibited a mild effect. It can be concluded that these fractions still possessed the activity; however, it was not strong to stop the cells from entering the S phase, which could be due to the concentration or synergic factor. These fractions were prepared to the initial concentration similar to water extract, thus the inhibitory effect of the extract was not strong to stop the cancer cells from re-entering into the cell cycle. It is possible for these fractions to exhibit the activity with the increase of the concentration. Further fractionation of the strongest fraction led to the isolation of one compound that was responsible for the observed cell cycle re-entry inhibitory activity. The compound was identified as citric acid.

Citric acid is a weak tricarboxylic acid found majorly in citrus fruits. Citric acid also presents in various produced foods because citric acid is used as food additives. An adult takes approximately 4 g of citric acid daily via various sources including foods (Goldberg, Grass et al. 1989). It is reported the plasma citrate levels is approximately about 19.2 μg/mL and the level is affected by the oral citrate load (Goldberg, Grass et al. 1989). Due to the pH of blood, citric acid exists mainly in citrate in blood and urine (Mycielska, Patel et al. 2009).

Citrate has been suggested to play a role in cancer where low concentration of citrate in cancer cells is believed to promote cancer aggressiveness (Mycielska, Patel et al. 2009, Giskeødegård,

Bertilsson et al. 2013, Philippe and Hubert 2016). A metabolic profiling of prostate cancer tissue samples revealed citrate concentration was twice lower in high-grade cancer tissue than in low-grade cancer tissue (Giskeødegård, Bertilsson et al. 2013). Not only that, the intracellular citrate in PC-3 prostate cancer cells were about 40% lower than the than normal PNT2-C2 epithelial cells (Mycielska, Broke-Smith et al. 2006). It was also reported that the concentration of citrate in peripheral zone tissue of the normal prostate was about 12000–14000 nmols/gram, which is relatively higher than the concentration of citrate on other tissues (250–450 nmols/gram) and blood plasma (100–200 nmols/gram)(Costello and Franklin 2006).

In this study, citric acid significantly reduced cell viability of prostate cancer cells dose-dependently at a concentration above 250 µg/mL. Previous *in-vitro* studies have shown that citric acid and citrate inhibited proliferation of different cancer lines such as A549, MCF-7, BxPC3, B16F10, WM983B, EC109, MSTO-211H, SKOV3, IGROV1-R10, BGC-823 and SGC-7901 cells at different sensitivity when tested in the concentration up to 6 mg/mL (Zhang, Varin et al. 2009, Lu, Zhang et al. 2011, Lincet, Kafara et al. 2013, Ying, Chen et al. 2013, Chen, Lv et al. 2017, Ren, Seth et al. 2017). Tumour growth reduction by citrate was also observed in A549 lung cancer *in-vivo* mice model (Ren, Seth et al. 2017). In this study, citric acid showed selectivity in targeting cancer cells as citric acid showed no cell viability effect on the GM3348 cells, while a stronger effect on PC-3 than on the RWPE-1 cells. PC-3 and RWPE-1 cells showed different sensitivity to citric acid treatment at a concentration above 1 mg/mL as significant different viability effect between these cells were observed. In one study, citrate concentration up to 1.54 mg/mL retained more than 75% of normal RWPE-1 prostate cells after 48 h of incubation (Hong, Choi et al. 2012). Citrate was also reported to have no effect on the endocytosis, lateral motility and adhesion of normal prostate epithelial (PNT2-C2) cells in contrast to PC-3M

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prostate cancer cells (Mycielska, Broke-Smith et al. 2006). However, in another study, citrate showed no selectivity on normal and cancer lung cells at similar tested concentrations (Ren, Seth et al. 2017). This indicates that citric acid, like the other compounds, has different toxicity level on cells. It is important to mention that the culture medium was neutralized to pH 7.4 after the addition of citric acid in this study to ensure the pH of solution does not affect the cell growth.

In the present study, citric acid prevented the quiescent PC-3 cells from entering S phase by arresting the cells at the G0/G1 phase. For proliferating cells, it was shown citric acid-induced cell cycle arrest at the G2/M phase and S phase on HaCaT cells (Ying, Chen et al. 2013), while sodium citrate arrested the gastric cancer cells at G2/M (Wang, Zhang et al. 2016). In another study, temporary growth retardation was observed on lung cancer MSTO-211H cells when the cells were exposed to citrate (Zhang, Varin et al. 2009). Not only that, administration of cisplatin at the end of citrate exposure caused massive apoptosis with no any cell growth recovery of MSTO-211H even after 14 days, which were not observed with the treatment of cisplatin or citrate alone (Zhang, Varin et al. 2009). Strong cytotoxicity was also observed in SKOV3 and IGROV1-R10 ovarian cells that were treated with ABT-737 compound after exposure to citrate for 24 h (Lincet, Kafara et al. 2013). In another study, the combination administration of citrate and cisplatin reduced tumour growth in lung cancer *in-vivo* model (Ren, Seth et al. 2017). These findings suggest the ability of citric acid to enhancing the sensitivity and efficiency of chemotherapy drugs.

Citrate also was shown to induce apoptosis in different cancer including nerve, lung, esophageal, breast, skin, pancreas and ovary cancer (Zhang, Varin et al. 2009, Kruspig, Nilchian et al. 2012, Lincet, Kafara et al. 2013, Chen, Lv et al. 2017, Ren, Seth et al. 2017). However, no apoptosis was observed in this study when the PC-3 cells were treated with the citric acid up to 48 h. Sub-

G1 was not found in the result of flow cytometry (data not shown), which is similar to the previous finding using water extract of citrus peel on the quiescent prostate cancer cells.

Citrate was also reported to play a significant role in glycolysis and the tricarboxylic acid cycle as citrate is one of the main intermediates of the tricarboxylic acid cycle (Mycielska, Patel et al. 2009). It was found the citrate suppressed glycolysis and the tricarboxylic acid cycle *in-vitro* and *in-vivo* on A549 tumour cells (Ren, Seth et al. 2017). Similarly, citrate was shown to decrease glycolysis on *in-vitro* and *in-vivo* gastric cancer cells (Wang, Zhang et al. 2016). The decreases in the glycolysis and the tricarboxylic acid cycle were reported due to the inhibition of phosphofructokinase enzyme (Kruspig, Nilchian et al. 2012, Lincet, Kafara et al. 2013). Sodium citrate significantly reduced selectively the phosphofructokinase-1 on the human gastric cancer cell line (SGC-7901) (Wang, Zhang et al. 2016). In another study, citrate suppressed phosphofructokinase in Ras tumours only and not in Neu driven tumours (Ren, Seth et al. 2017). Citrate is regarded as an inhibitor of phosphofructokinase enzyme (Norberg and Siesjo 1975) and citric acid could be an anticancer agent.

When looking at the citrate intake safety, no significant toxicity to the internal organs of the animals treated with citrate was observed (Ren, Seth et al. 2017). The animals that were given citrate (total dose 8 g per day) by gavage or drinking water had citrate concentration at approximately 3 mM in plasma, which was roughly eight times than those noted in non-citrate treated animals (Ren, Seth et al. 2017). Not only that, two patients that were given citrate orally showed no health side effect and was well-tolerated (Halabe 2009, Bucay 2011). As surgery and radiotherapy are not preferred on elderly men as it tends to worsen health, functional food supplements could be an alternative option in halting risk of progression and recurrence of prostate cancer. Citrus peel extract that contains citric acid together with various bioactive

compounds has been shown to induce cell cycle re-entry inhibitory effect on quiescent prostate cancer cells. Combination of various bioactive compounds together with citric acid in citrus peel extract could mimic the synergic effect observed when a drug is administered in the presence of another drug or compound during cancer treatment.

4.5   Conclusion

In this study, citric acid was identified as one of the potent compounds in citrus peel extract that are responsible for the cell cycle re-entry inhibitory effect on prostate cancer cells. Citric acid showed toxic selectivity by targeting cancer cells than normal prostate cells. A synergic effect was observed where the fractions showed a reduction in the strength of cell cycle inhibitory effect compared to water extract. Intake of citrus peel extract as a whole is suggested for optimum health-promoting effect.

5 Proteomic analysis for the identification of cell cycle re-entry inhibitory proteins in quiescent prostate cancer cells.

5.1 Introduction

Cancer cells are characterized by their uncontrolled proliferation; however, some of these cells cease proliferating and enter a quiescent state. These quiescent cells can remain dormant for a long period and eventually may result in cancer recurrence (Yeh and Ramaswamy 2015). The quiescent cells can re-enter the cell cycle upon the presence of growth-factor signalling stimulations (Olejniczak, La Rocca et al. 2013). Thus, understanding the re-entry of quiescent cells into the cell cycle is vital for the effective treatment of malignant cancers.

The proteins involved in the cell cycle process have been identified and the mechanisms for regulating the transition of cells from quiescence to the proliferative state were elucidated in several studies (Malumbres and Barbacid 2001, Wang and Lin 2013, Yeh and Ramaswamy 2015). Key factors proteins involved in cell cycle progression are cyclin-dependent kinase (CDK), CDK inhibitors and cyclin (Otto and Sicinski 2017). Modulation of several proteins such as the DREAM complex, DYRK1B, NR2F1, Axl, Tyro3, and uPAR was also associated with regulation of cell quiescence. Although, various proteins have been implicated in cell cycle regulation, the precise mechanism of cancer relapse and cell cycle re-entry of quiescent cells is complex and unknown.

Citric acid or citrate has shown anticancer activity on multiple cancerous cell lines such as breast, pancreas, lung, and esophageal cancer cells (Chen, Lv et al. 2017, Mycielska, Dettmer-Wilde et al. 2018). Previous studies have also reported that citrate and citric acid arrest gastric and ovarian cancer cells at G2/M phase (Lincet, Kafara et al. 2013, Wang, Zhang et al. 2016), and mesothelioma cells at G0/G1 phase (Zhang, Varin et al. 2009). In this study, it was found that citric acid and citrate from citrus inhibited cell cycle re-entry of quiescent PC-3 cells. Although various mechanisms have purported for the anticancer activity of citric acid, the precise mode of action of citric acid in cell cycle re-entry inhibition has not yet been identified.

Understanding the relation between cancer quiescence and metabolism may lead to the identification of novel biomarkers and the development of an active compound for the prevention of cancer. Therefore, in this chapter, it was aimed to determine the inhibition mechanism of citric acid on quiescent PC-3 cells. Stable-isotope labelling by amino acids in cell culture (SILAC) was used to compare the protein expression during the cell cycle re-entry of citric acid treated quiescent cells with the control. Subsequently, computational analysis was performed to identify critical protein differences between the treated and untreated cell populations.

5.2   Materials and methods

5.2.1   Chemicals and reagents

Trypsin Protease MS Grade (90057), RPMI 1640 SILAC Protein Quantitation Kit (A33971), Qubit Protein Assay (Q33211), FxCycle™ PI/RNase Staining Solution (F10797), trypsin

(90057), dithiothreitol (R0861) were obtained from Life technologies. Urea (U4883), thiourea (T8656), iodoacetamide (A3221), triethylammonium bicarbonate buffer (T7408), trifluoroacetic acid (T6508), formic acid (5330020050), MS grade acetonitrile (1000292500) were obtained from Sigma.

5.2.2 Cell culture and SILAC labelling

Bone metastasized prostate cancer cells (PC-3) obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI-1640 SILAC culture media supplemented with $^{12}C_6$-(LIGHT) or $^{13}C_6$ (HEAVY)-L-lysine RPMI containing 10% of dialyzed fetal bovine serum in a humidified cell incubator at 37°C in the presence of 5% CO_2. The medium of both populations was replaced every two days and the cells were passaged routinely when the confluency reached 80–90%. The percentage of incorporation efficacy was determined before the cells were used for the experiment and it was typically more than 99%. The SILAC labelled PC-3 cells in a T25 flask were forced to enter quiescence by contact inhibition method as described in Section 3.2.3 (Xi, Yao et al. 2016). Briefly, after three days of contact inhibition, the HEAVY labelled cells were trypsinized and seeded at low density in T25 flask together HEAVY culture medium containing citric acid at a concentration of 700 µg/mL (neutralized with sodium hydroxide to pH 7.4). Meanwhile, the LIGHT labelled cells were trypsinized and seeded at low density in T25 flask together LIGHT culture medium. At the end of the treatment, the cells were trypsinized, washed with PBS and the dry pellet was stored at -80°C prior to sample preparation for mass spectrometry. Three different independent biological experiments were prepared for MS analysis. About 10% of each sample was fixed with 70% ethanol and subjected for cell cycle

analysis with a flow cytometer.

5.2.3 Protein quantitation using Qubit method

The Qubit protein assay was conducted according to the manufacturer's protocol. Briefly, the Qubit working solution was prepared by diluting the Qubit® Protein Reagent 1:200 in Qubit® Protein Buffer. The protein standards (10 µL) or the samples (1-20 µL) were mixed with Qubit working solution to a final volume of 200 µL. The solution was incubated for 15 minutes at room temperature and the fluorescence of the dye was measured using the Qubit 2.0 fluorometer. Protein concentration was calculated using the formula: concentration of the sample in µg/mL = the value is given by the fluorometer * (200/volume of the sample used).

5.2.4 Sample preparation for mass spectrometry

The cell pellet was dissolved in 200 µL of lysis buffer containing 100 mM Tris Buffer pH 7.6, 6M urea and 2M thiourea. The cells were homogenized with two pulses of 20 seconds using a tip-probe sonicator. The homogenate was centrifuged at 16,000 g for 10 mins at 4 °C and the supernatant was collected. One mL of -20°C cooled acetone was added to the collected supernatant and the solution was stored at -20°C overnight to precipitate the proteins. The solution was centrifuged at 16,000 g for 10 min at 4 °C and the supernatant was removed. The protein pellet was dissolved in 80% acetone, centrifuged and the pellet was dried with a nitrogen stream. The dried protein pellet was dissolved in 50 µL lysis buffer and total protein in the solution was quantified using the Qubit Protein Assay Kit. A 50 µg of protein from LIGHT and

HEAVY were combined and made up to 50 µL with lysis buffer. The proteins were reduced in 10 mM of dithiothreitol at room temperature for 30 min and alkylated in 25 mM of iodoacetamide in the dark at room temperature for 30 min. The excess iodoacetamide was reduced with 20 mM of dithiothreitol at room temperature for 30 min. The solution was diluted with 50 mM triethylammonium bicarbonate buffer in the ratio of 1: 6 and digested with trypsin at a ratio of 1:20 (trypsin/protein, w/w) for 16 h at 37°C. The digested solution was acidified with trifluoroacetic acid to a final concentration of 1% (v/v). The digested solution was centrifuged at 16,000 g for 10 mins at 4°C and the peptides in the supernatant were loaded on Millipore peptide concentrator C18 ZipTip (ZTC18S096). The cartridge was washed three times with 0.1% of trifluoroacetic acid and the peptides were eluted with 60% of acetonitrile. The cleaned peptide samples were resuspended in 3% (v/v) acetonitrile and 0.1% (v/v) formic acid for LC-MS/MS analysis (1 µg/injection).

5.2.5 LC-ESI-MS/MS

LC-MS/MS analysis was carried out on a nanoflow Ultimate 3000 µHPLC (Dionex Corp, Sunnyvale, CA, USA) coupled online to a Q-Exactive™ Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a nanospray ionization ion source in positive mode. Peptides were separated using an in-house packed C18-reversed phase column (75 µm × 45 cm) with a linear gradient of 5–35% Buffer B over 90 min at 250 nL/min at 60°C (Buffer A = 0.1% (v/v) formic acid; Buffer B = 80% (v/v) acetonitrile, 0.1% (v/v) formic acid). An MS1 scan was acquired from 350–1550 (70000 resolution, $3e^{6}$ automatic gain control, 100 ms injection time). Twenty most intense precursor ions from a full MS1 scan were selected in each cycle for MS/MS

data-dependent acquisition with higher energy collision dissociation (HCD) fragmentation and detection in the Orbitrap (17500 resolution, $1e^5$ automatic gain control, 60 ms injection time, 30 eV normalized collision energy, 1.2 m/z isolation window, $1.7e^5$ precursor intensity threshold; minimum charge state of 2+; dynamic exclusion of 90 s).

5.2.6 Analysis of mass spectrometric data

The raw data were processed with Proteome Discoverer 2.2 (Thermo Fisher Scientific, Bremen, DE). MS/MS spectra were searched using SEQUEST HT algorithm against the reviewed UniProt human database containing 20,373 entries (June, 2018). MS mass tolerance was set to 10 ppm and MS/MS mass tolerance was set to 0.05 Da. Identical peptides from the different samples co-elute as pairs of peaks and distinguishable by the mass difference between the heavy and light isotope labels. The peptides were identified with up to two missed cleavages for tryptic peptides with oxidation (M), acetylation of the N-terminus and SILAC modifications of lysine (K) as variable modifications, while carbamidomethylation (C) was used as a fixed modification. The peptides and proteins were validated for false discovery rates (FDR) using percolator algorithm Node and protein FDR validator node with a q-value threshold of 0.01. Only proteins that identified with high confidence, belong to master protein and contain at least one unique peptide were selected for further analysis. Ambiguous proteins were manually removed by comparing the absence/presence in three different biological replicates.

5.2.7 Bioinformatics analysis

Protein-protein interactions and networks were evaluated using STRING v10 software (https://string-db.org/) with the confidence level of more than 0.4. Gene ontology annotation was obtained using PANTHER tool (http://www.pantherdb.org/). Ingenuity® Pathway Analysis (http://www.ingenuity.com/) was used to search the relevant pathways of these identified proteins.

5.3 Results

5.3.1 Cell cycle analysis of the prostate cancer cells

Contact-inhibited prostate cancer PC-3 cells were released from quiescence phase by passaging the cells at low density in the presence of citric acid (neutralized with sodium hydroxide to pH of 7.4) at IC_{50} concentration (0.7 mg/mL) for treated (HEAVY) cells or in phosphate buffer solution for control (LIGHT) cells. The IC_{50} of citric acid for PC-3 cells were determined in the previous chapter. The cells after 16 and 24 h were collected and analysed for the DNA content using BD FACS Canto II flow cytometer. Figure 5.1 shows an image of PC-3 cells that were incubated with citric acid at 16 and 24 h upon released from quiescence phase. Furthermore, the result of the cell cycle of PC-3 cells before and after release from quiescence phase in the presence of citric acid at 16 and 24 h is depicted in Figure 5.2. It was observed that when the cells were released from quiescence, the number of cells at the S phase for control sample at 16 and 24 h was increased compared to the quiescent cells. Meanwhile, a number of cells at the G2/M phase for control sample at 16 h was almost unchanged and an increased from 11.8% to 18.8% at 24 h

was observed. This effect was proportional to the decrease in the number of cells at the G1 phase for the control. However, for treated cells, no changes in the number of cells in each phase were observed at 16 and 24 h compared to quiescent cells. These cell cycle re-entry inhibition results are in broad agreement with the results from Chapter 4.

Control at 16 h	Treated at 16 h
Control at 24 h	Treated at 24 h
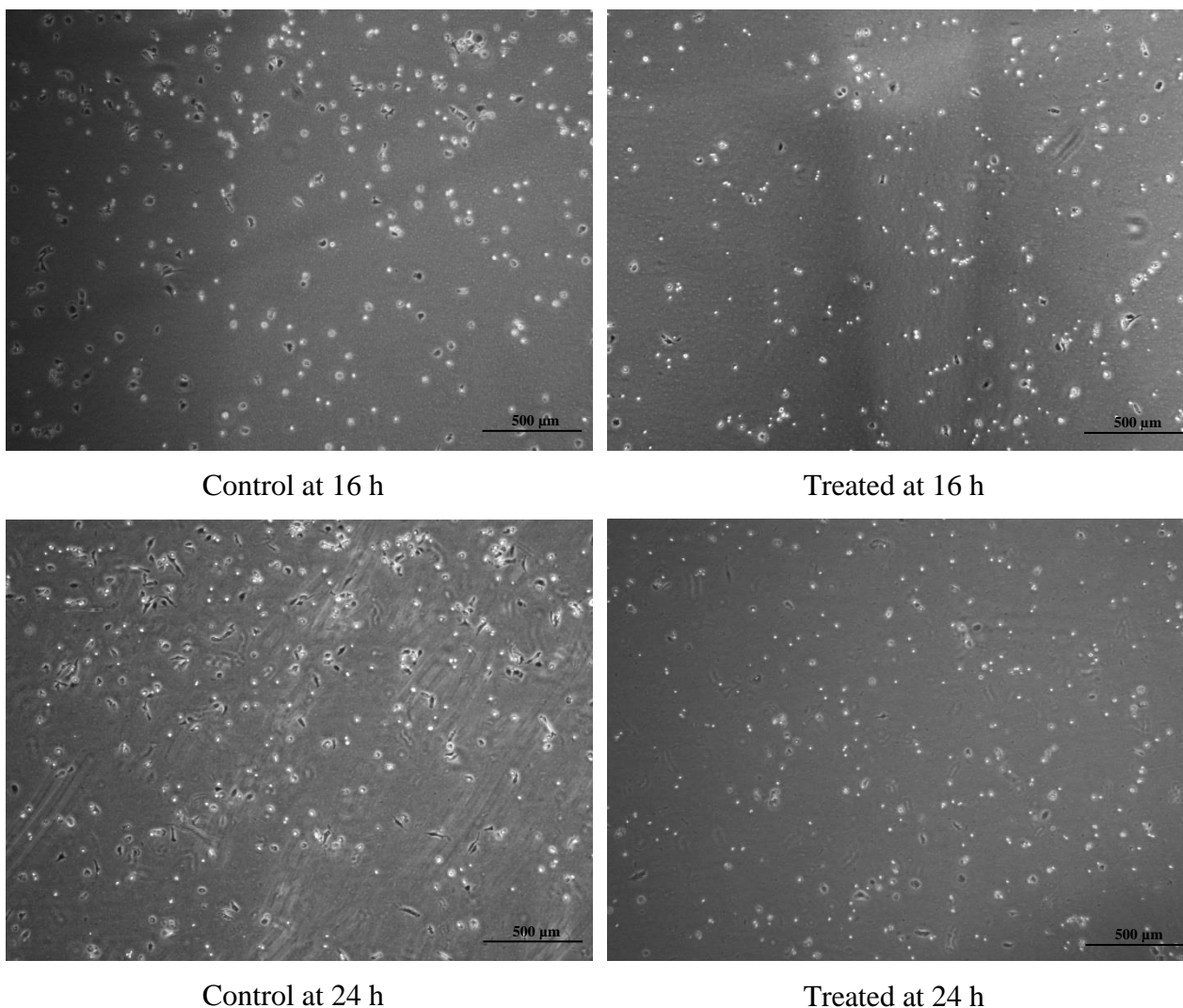
Figure 5.1: Image of PC-3 cells treated with citric acid standard (pH 7.4) at 16 and 24 h upon release from quiescence were assessed by Nikon Eclipse TS100 inverted microscope.

	Quiescent	Control	Treated	Control	Treated
	0 h	16 h		24 h	
▨ G0/G1	87.8	86.05	86.75	77.6	86.65
▦ S	1.25	2.2	0.65	3.7	0.9
☐ G2/M	10.95	11.85	11.3	18.8	12.4
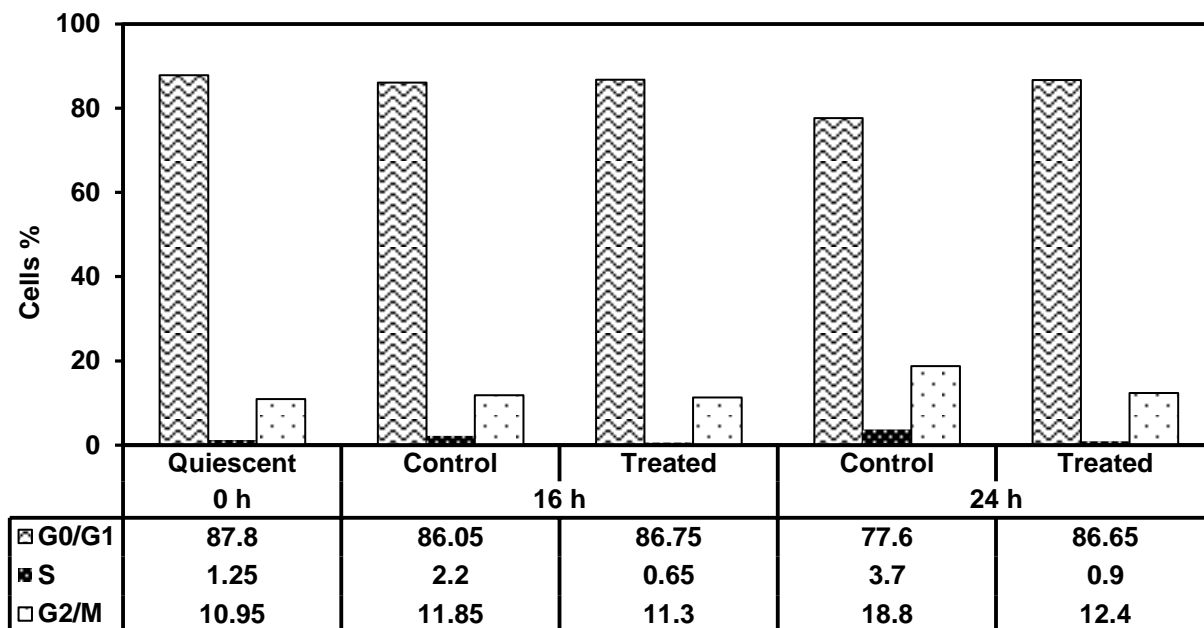
Figure 5.2: Cell cycle profile of PC-3 cells treated with citric acid standard upon release from quiescence. Quiescent cells are PC-3 cells after contact inhibition for 3 days. Data are expressed as the mean of two independent biological experiments (n=2).

5.3.2   Proteomic analysis of the prostate cancer cells

A quantitative proteomic analysis was conducted based on a SILAC labelling as illustrated in Figure 5.3 to identify the changes in the protein expression during cell cycle re-entry of quiescent prostate cancer cells (PC-3). Three biological samples were analysed by LC-MS/MS that included control and citric acid treated PC-3 cells.
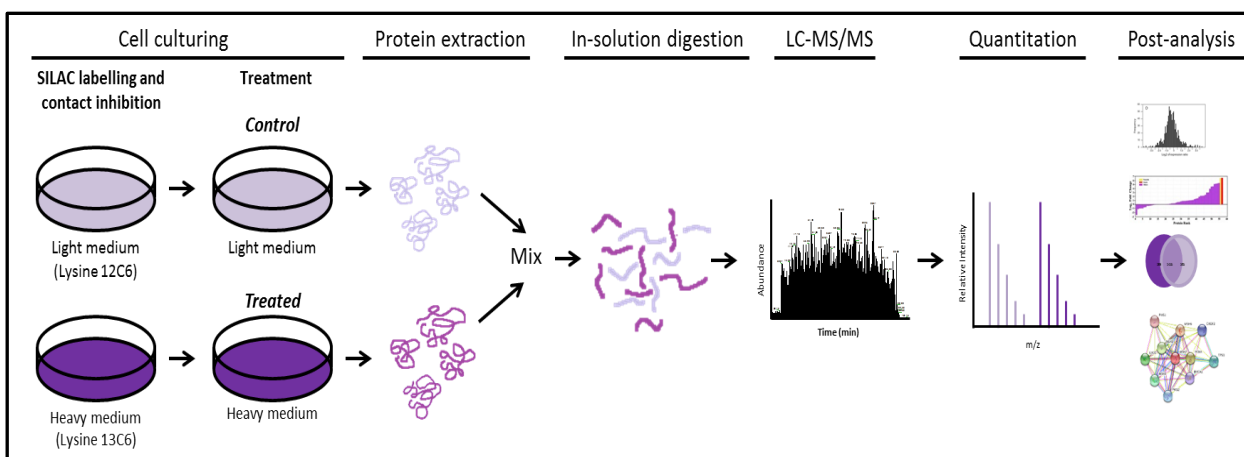
Figure 5.3: Schematic representation of steps undertake for SILAC labelling and proteomic analysis. The LIGHT and HEAVY SILAC labelled proliferating PC-3 cells were contact inhibited for three days before passaged at low density in the presence of phosphate buffer solution (control) and citric acid standard (treated). At 16 and 24 h, total cell lysate proteins were collected, mixed and digested with trypsin before analysed with liquid chromatography based tandem mass spectrometry (LC-MS/MS). The ratio of HEAVY vs. LIGHT intensity of peptide MS spectrum was used for the fold change calculation. The ratios were subjected to post-analysis interpretation.

5.3.3   Identification of differentially expressed proteins by SILAC labelled proteomics

Peptides obtained from the digested proteins of PC-3 cells were analysed using LC-MS/MS. Figure 5.4 shows the total ion chromatogram for samples at 0, 16 and 24 h. The chromatograms were almost similar in intensity and elution pattern. The raw spectral data were interpreted using Proteome Discoverer 2.2 by searching against reviewed Homo sapiens UniProt database at 1% FDR at both peptide and protein levels. The samples of MS/MS spectra of a peptide fragmented from the FN3KRP protein are presented in Figure 5.5 (a). In the MS/MS analysis, b and y ions of

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various peptides were detected and matched in Proteome discoverer software analysis. A SILAC quantitation method was used to determine the abundance of protein expression in control and treated PC-3 cells. Figure 5.5 (b) shows the intensity of LIGHT and HEAVY labelled doubly-charged peptide for the FN3KRP protein with m/z value differ by 3 Da.
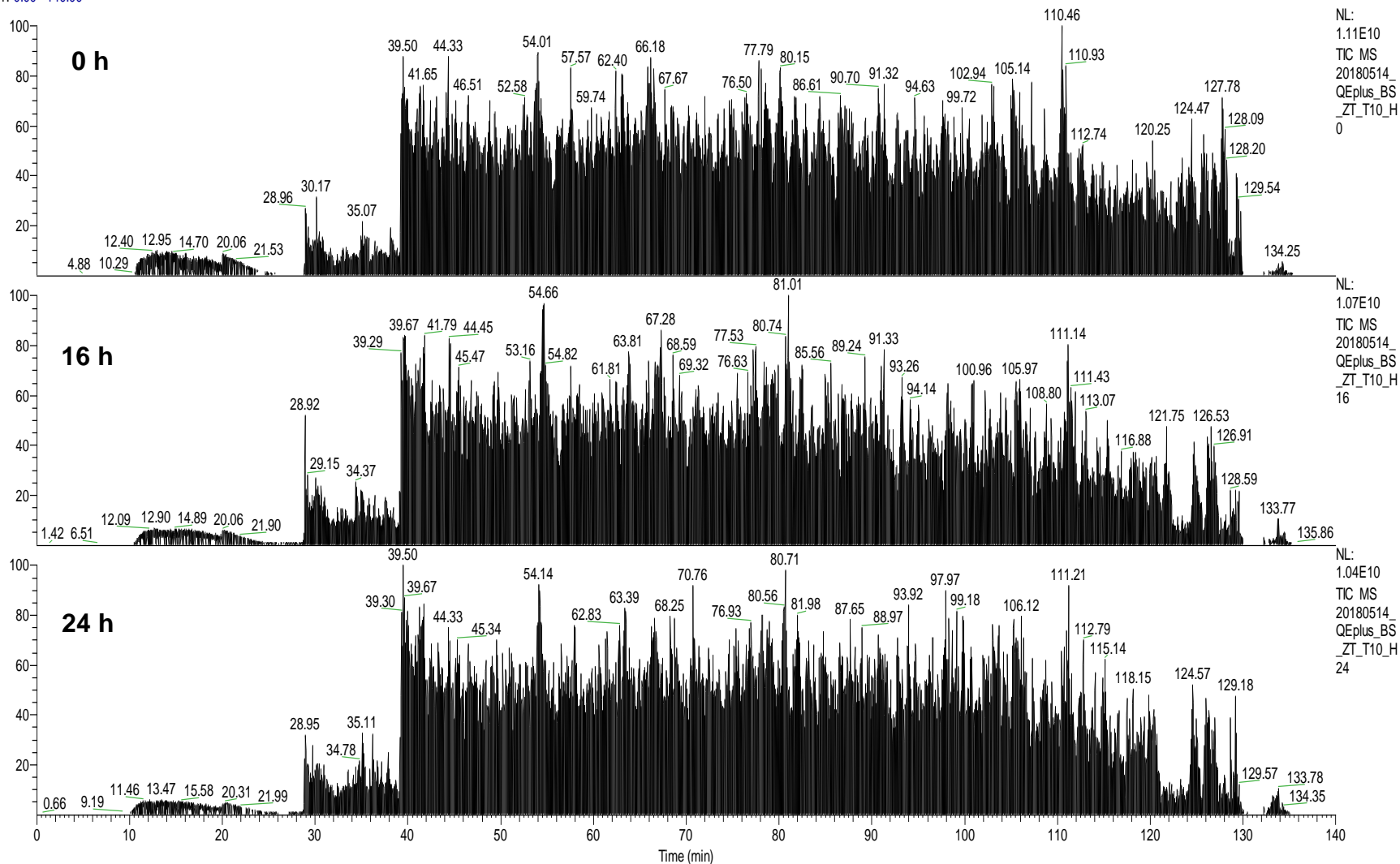
Figure 5.4: Examples of total ion chromatogram of digested proteins of whole prostate cancer PC-3 cells at a different time.
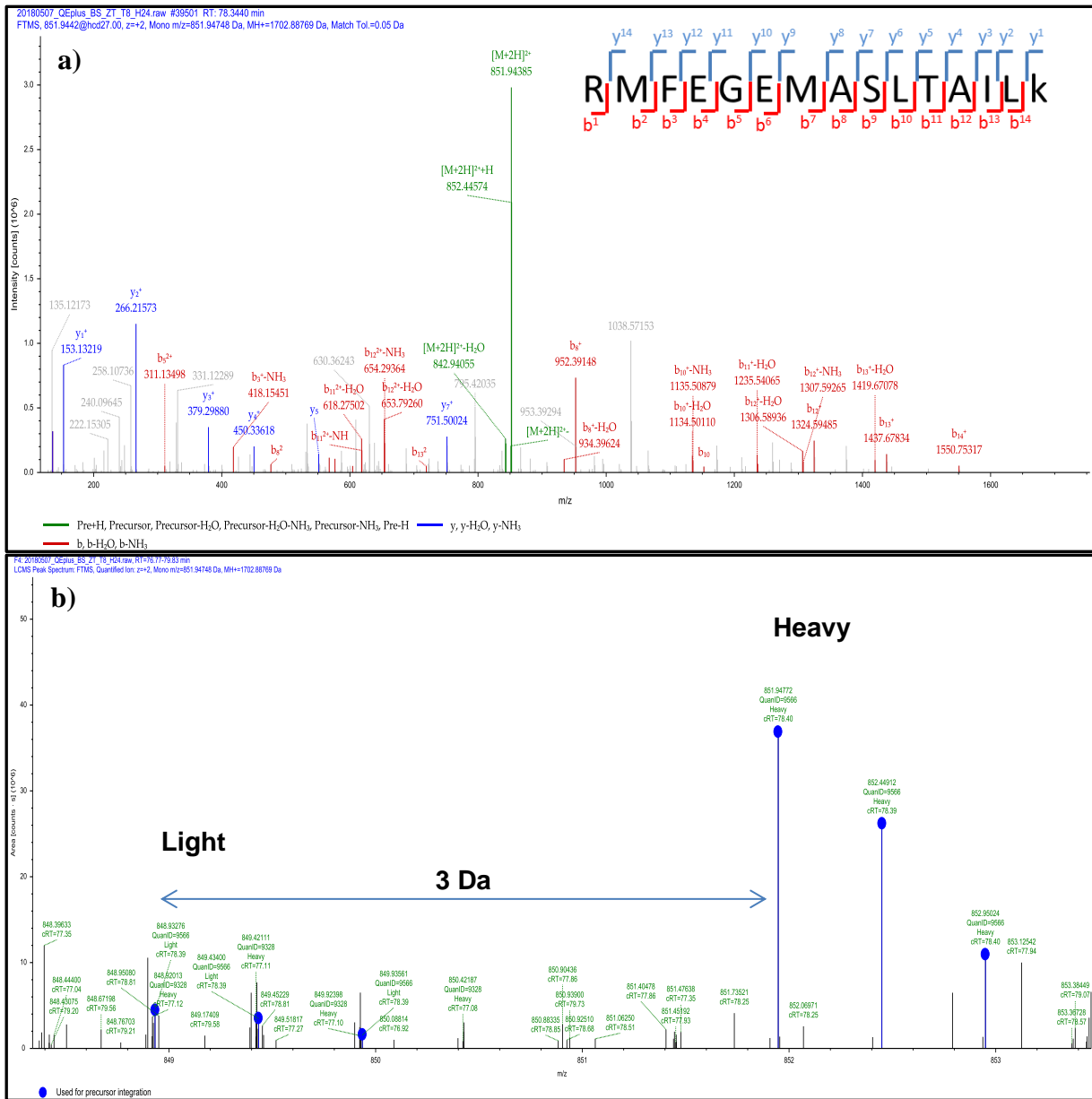
Figure 5.5: MS/MS spectra of RMFEGEMASLTAILk peptide from FN3KRP protein (a) and MS spectra for light (control) and heavy (treated) labelled lysine corresponding to the RMFEGEMASLTAILk doubly-charged peptide ion separated by the specific 3 Da delta mass (b).

5.3.4 Differentially expressed proteins in cell cycle re-entry of PC-3 cells

The processed raw spectral data revealed an average of about 3000 proteins in each sample. The protein lists were filtered using the following parameters: protein FDR confidence (high), abundance LIGHT and HEAVY (any value), unique peptides (equal or more than 1), master protein (master), and contaminant (not). For each sample, nearly 1400 proteins were quantified with high confidence and their log2 (HEAVY/LIGHT) ratio are shown in Figure 5.6. The log2 ratio distribution is shown in Figure 5.6 and most of the proteins were within one-fold log2. There were more proteins with the larger ratio at 24 h compared to 0 and 16 h as indicated by the distribution plots. The high number of proteins within one-fold log2 shows that the proteins from control and treated samples were mixed at an equal amount. The proteins that were differentially expressed by at-least one-fold and found at least once from three biological experiments at 16 h and 24 h are listed in Table 5.1 and Table 5.2.

Number of differentially expressed proteins at least by one-fold at 16 and 24 h were 68 proteins and 62 proteins, respectively. Among these proteins, 17 proteins were found at both 16 and 24 h (Figure 5.6), which were ALDH3A1, S100A2, SYNGR2, PFKP, SUCLA2, MAPK1, SUMO1, KPNA2, NHP2, AHSG, IGF2BP3, PCBP3, LUC7L2, NAA10, FN3KRP, ACADM and HBA1. Among the proteins listed in Figure 5.6, S100A2, H3F3A, CDK1, NEDD8, MCM4, JPT1, AKT1, IDE and LMO7 are associated with cell cycle and cell cycle-related signalling.
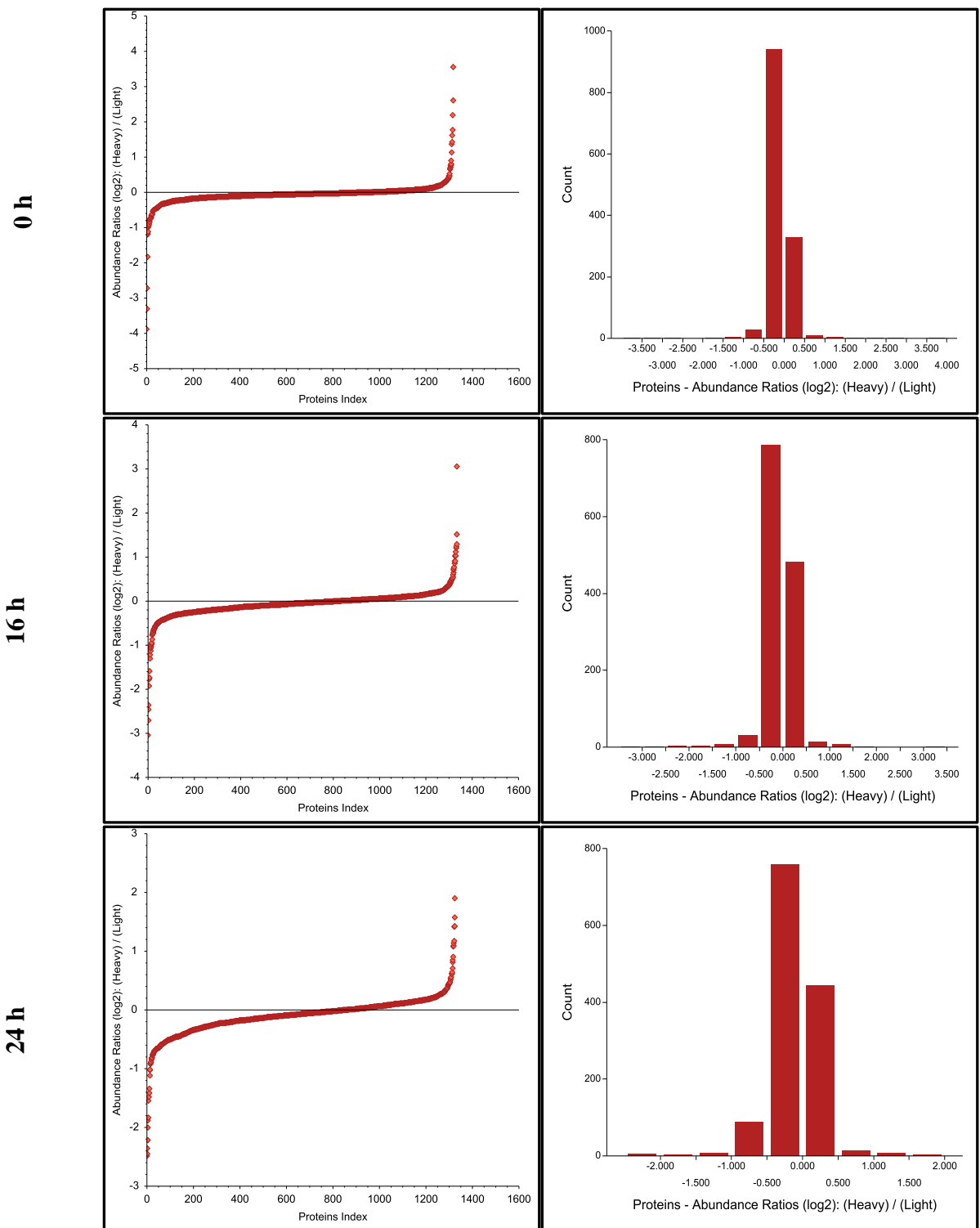
Figure 5.6: Plots (right) and histogram graph (left) of normalized log2 SILAC ratios against high confidence identified proteins sorted by their ratios in ascending order at 16 and 24 h for one biological experiment.

Table 5.1: List of proteins that were differentially expressed at least one-fold at 16 h in PC-3 cells.

Protein accession	GENE name	Protein name	Coverage [%]	Unique Peptides	log2(H/L)
P02765	AHSG	Alpha-2-HS-glycoprotein	7	3	-3.90
P29034	S100A2	Protein S100-A2	28	4	-3.31
P84243	H3F3A	Histone H3.3	65	2	-3.27
P69905	HBA1	Hemoglobin subunit alpha	35	5	-3.06
O60684	KPNA6	Importin subunit alpha-7	14	2	-2.98
P27694	RPA1	Replication protein A 70 kDa DNA-binding subunit	6	3	-2.71
O60220	TIMM8A	Mitochondrial import inner membrane translocase subunit Tim8 A	27	2	-2.56
P40616	ARL1	ADP-ribosylation factor-like protein 1	17	2	-2.46
Q9NNW7	TXNRD2	Thioredoxin reductase 2, mitochondrial	10	2	-2.37
Q9BZZ5	API5	Apoptosis inhibitor 5	7	4	-1.93
Q8N1G4	LRRC47	Leucine-rich repeat-containing protein 47	15	7	-1.86
P61006	RAB8A	Ras-related protein Rab-8A	28	2	-1.79
Q01813	PFKP	ATP-dependent 6-phosphofructokinase, platelet type	16	5	-1.79
Q9P2R7	SUCLA2	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	25	7	-1.74
O75844	ZMPSTE24	CAAX prenyl protease 1 homolog	8	2	-1.70
O60888	CUTA	Protein CutA	16	2	-1.60
P62495	ETF1	Eukaryotic peptide chain release factor subunit 1	7	3	-1.55
P42704	LRPPRC	Leucine-rich PPR motif-containing protein, mitochondrial	44	48	-1.51
P35237	SERPINB6	Serpin B6	43	13	-1.45
O43237	DYNC1LI2	Cytoplasmic dynein 1 light intermediate chain 2	9	1	-1.40
P61160	ACTR2	Actin-related protein 2	37	1	-1.34
P11310	ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	19	6	-1.31
P33991	MCM4	DNA replication licensing factor MCM4	6	3	-1.30
O43760	SYNGR2	Synaptogyrin-2	8	2	-1.27
P51397	DAP	Death-associated protein 1	35	2	-1.24
O14786	NRP1	Neuropilin-1	9	5	-1.22
Q9UK76	JPT1	Jupiter microtubule-associated homolog 1	26	2	-1.22^
P53634	CTSC	Dipeptidyl peptidase 1	16	5	-1.21
P31749	AKT1	RAC-alpha serine/threonine-protein kinase	4	1	-1.16
Q9Y3A5	SBDS	Ribosome maturation protein SBDS	11	3	-1.16
P52292	KPNA2	Importin subunit alpha-1	10.5	3.5	-1.15^
P20962	PTMS	Parathymosin	23	2	-1.14
Q14554	PDIA5	Protein disulfide-isomerase A5	5	1	-1.14
P13796	LCP1	Plastin-2	33	13	-1.11
P21980	TGM2	Protein-glutamine gamma-glutamyltransferase 2	41	19	-1.10
P62861	FAU	40S ribosomal protein S30	19	2	-1.09
Q6DD88	ATL3	Atlastin-3	20	7	-1.08
P62136	PPP1CA	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	52	2	-1.07

Protein accession	GENE name	Protein name	Coverage [%]	Unique Peptides	log2(H/L)
P12110	COL6A2	Collagen alpha-2(VI) chain	3	3	-1.06
Q9H3P7	ACBD3	Golgi resident protein GCP60	5	2	-1.06
P41227	NAA10	N-alpha-acetyltransferase 10	19	3	-1.04
P62854	RPS26	40S ribosomal protein S26	40	1	-1.04
P35222	CTNNB1	Catenin beta-1	13	7	-1.04
P62244	RPS15A	40S ribosomal protein S15a	44	7	-1.03
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	40	2	1.02
P14735	IDE	Insulin-degrading enzyme	2	2	1.02
Q9NX24	NHP2	H/ACA ribonucleoprotein complex subunit 2	13	1	1.02
P28482	MAPK1	Mitogen-activated protein kinase 1	34	7	1.03
O14602	EIF1AY	Eukaryotic translation initiation factor 1A, Y-chromosomal	15	0	1.08
P16402	HIST1H1D	Histone H1.3	17	8	1.09
P00414	MT-CO3	Cytochrome c oxidase subunit 3	5	1	1.12
O95834	EML2	Echinoderm microtubule-associated protein-like 2	7	3	1.20
P08174	CD55	Complement decay-accelerating factor	4	2	1.24
P31153	MAT2A	S-adenosylmethionine synthase isoform type-2	30	2	1.25
P35914	HMGCL	Hydroxymethylglutaryl-CoA lyase, mitochondrial	16.5	3	1.25^
Q96I24	FUBP3	Far upstream element-binding protein 3	24	10	1.29
Q15843	NEDD8	NEDD8	41	3	1.35
P30838	ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	11	3	1.51
Q14257	RCN2	Reticulocalbin-2	6	1	1.54
O00244	ATOX1	Copper transport protein ATOX1	47	3	1.56
O14548	COX7A2L	Cytochrome c oxidase subunit 7A-related protein, mitochondrial	29	1	1.63
P57721	PCBP3	Poly(rC)-binding protein 3	18	1	1.66
Q99627	COPS8	COP9 signalosome complex subunit 8	30	4	2.16
O00425	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3	4	1	2.88
Q9Y383	LUC7L2	Putative RNA-binding protein Luc7-like 2	11	5	3.06
P52907	CAPZA1	F-actin-capping protein subunit alpha-1	69	8	3.13
P63165	SUMO1	Small ubiquitin-related modifier 1	28	1	4.33
Q9HA64	FN3KRP	Ketosamine-3-kinase	16	3	5.10

^ Found in two samples, log2 H/L means the ratio of treated to control

Table 5.2: List of proteins that were differentially expressed at least one-fold at 24 h in PC-3 cells.

Accession	GENE name	Protein name	Coverage [%]	Unique Peptides	log2(H/L)
P02765	AHSG	Alpha-2-HS-glycoprotein	7	3	-3.47^
Q32MZ4	LRRFIP1	Leucine-rich repeat flightless-interacting protein 1	2	1	-3.47
P69905	HBA1	Hemoglobin subunit alpha	35	4	-2.49
Q9NYY8	FASTKD2	FAST kinase domain-containing protein 2, mitochondrial	2	1	-2.45
Q9P2R7	SUCLA2	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	16	5	-2.40^
P04183	TK1	Thymidine kinase, cytosolic	6	1	-2.36
P06493	CDK1	Cyclin-dependent kinase 1	21	6	-2.22
Q01813	PFKP	ATP-dependent 6-phosphofructokinase, platelet type	15	6	-2.11
Q9UHD1	CHORDC1	Cysteine and histidine-rich domain-containing protein 1	5	1	-2.01
Q9Y5B9	SUPT16H	FACT complex subunit SPT16	5	5	-1.93
P41227	NAA10	N-alpha-acetyltransferase 10	14	2	-1.88
A6NHR9	SMCHD1	Structural maintenance of chromosomes flexible hinge domain-containing protein 1	1	2	-1.65
Q13409	DYNC1I2	Cytoplasmic dynein 1 intermediate chain 2	13	4	-1.60
P23921	RRM1	Ribonucleoside-diphosphate reductase large subunit	2	1	-1.55
P52292	KPNA2	Importin subunit alpha-1	11	4	-1.52^
O75494	SRSF10	Serine/arginine-rich splicing factor 10	19	3	-1.49
P63220	RPS21	40S ribosomal protein S21 (Small ribosomal subunit protein eS21)	65	5	-1.49
O15460	P4HA2	Prolyl 4-hydroxylase subunit alpha-2	6	2	-1.42
P29034	S100A2	Protein S100-A2	27	3	-1.41
O43760	SYNGR2	Synaptogyrin-2	8	2	-1.38
P29218	IMPA1	Inositol monophosphatase 1	30	6	-1.35
P82673	MRPS35	28S ribosomal protein S35, mitochondrial	8	1	-1.34
Q05682	CALD1	Caldesmon	4	2	-1.33
P15924	DSP	Desmoplakin	1	2	-1.33
P67936	TPM4	Tropomyosin alpha-4 chain	31	1	-1.23^
P11310	ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	22	7	-1.19
O15355	PPM1G	Protein phosphatase 1G	5	2	-1.16
Q08211	DHX9	ATP-dependent RNA helicase A	19	21	-1.12
Q96FW1	OTUB1	Ubiquitin thioesterase OTUB1	29	6	-1.12
O95340	PAPSS2	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2	4	2	-1.10
Q96KP4	CNDP2	Cytosolic non-specific dipeptidase	6	2	-1.08
O75533	SF3B1	Splicing factor 3B subunit 1	7	4	-1.08
P17844	DDX5	Probable ATP-dependent RNA helicase DDX5	40	13	-1.05
P28288	ABCD3	ATP-binding cassette sub-family D	9	5	-1.03

Accession	GENE name	Protein name	Coverage [%]	Unique Peptides	log2(H/L)
		member 3			
P61289	PSME3	Proteasome activator complex subunit 3	23	5	1.00
P31483	TIA1	Nucleolysin TIA-1 isoform p40	6	2	1.00
O00116	AGPS	Alkyldihydroxyacetonephosphate synthase, peroxisomal	12	6	1.07
Q9UBS4	DNAJB11	DnaJ homolog subfamily B member 11	21	5	1.08
O60841	EIF5B	Eukaryotic translation initiation factor 5B	5	5	1.13
Q15287	RNPS1	RNA-binding protein with serine-rich domain 1	8	3	1.17
P57721	PCBP3	Poly(rC)-binding protein 3	19	1	1.21
Q9H444	CHMP4B	Charged multivesicular body protein 4b	19	5	1.31
Q8WWI1	LMO7	LIM domain only protein 7	10	14	1.38
P63165	SUMO1	Small ubiquitin-related modifier 1	36	4	1.40
P30838	ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	8	2	1.41
P28482	MAPK1	Mitogen-activated protein kinase 1	34	5	1.42
Q08722	CD47	Leukocyte surface antigen CD47	3	1	1.45
P61026	RAB10	Ras-related protein Rab-10	24	3	1.57
O00425	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3	5	1	1.58
P20591	MX1	Interferon-induced GTP-binding protein Mx1	7	3	1.62
P62314	SNRPD1	Small nuclear ribonucleoprotein Sm D1	20	2	1.64
Q9NX24	NHP2	H/ACA ribonucleoprotein complex subunit 2	13	1	1.87
O60869	EDF1	Endothelial differentiation-related factor 1	27	3	1.88
Q9NR50	EIF2B3	Translation initiation factor eIF-2B subunit gamma	7	3	1.89
Q96CM8	ACSF2	Acyl-CoA synthetase family member 2, mitochondrial	11	5	2.08
Q8TD19	NEK9	Serine/threonine-protein kinase Nek9	1	1	2.18
Q13464	ROCK1	Rho-associated protein kinase 1	2	1	2.36
Q6UW68	TMEM205	Transmembrane protein 205	17	2	2.76
Q9HA64	FN3KRP	Ketosamine-3-kinase	11	2.5	3.26^
Q08378	GOLGA3	Golgin subfamily A member 3	3	3	3.42
Q9Y383	LUC7L2	Putative RNA-binding protein Luc7-like 2	13	5	3.68
Q13596	SNX1	Sorting nexin-1	6	2	6.56

^ Found in two samples

log2 H/L means the ratio of treated to control

16 hours

24 hours

ATOX1 EML2 SBDS DAP
TGM2 LCP1 AKT1 CUTA
ATL3 SERPINB6 COX7A2L
IDE FUBP3 HIST1H1D
TXNRD2 RPS26 JPT1 CD55
MCM4 API5 FAU EIF1AY
MAT2A ACBD3 H3F3A
RPA1 COL6A2 RCN2
COPS8 CTNNB1 MT-CO3
ETF1 KPNA6 HMGA1 ARL1
LRPPRC RPS15A PTMS
DYNC1LI2 LRRC47
TIMM8A NEDD8 ACTR2
PPP1CA CAPZA1 NRP1
RAB8A PDIA5 HMGCL
ZMPSTE24 CTSC

ALDH3A1
S100A2
SYNGR2
PFKP
SUCLA2
MAPK1
SUMO1
KPNA2
NHP2
AHSG
IGF2BP3
PCBP3
LUC7L2
NAA10
FN3KRP
ACADM
HBA1

RAB10 TPM4 CDK1 ROCK1
EIF2B3 ABCD3 PAPSS2
RNPS1 TK1 CD47
TMEM205 LMO7 FASTKD2
PPM1G AGPS LRRFIP1
CHMP4B OTUB1 PSME3
P4HA2 MRPS35 MX1
CHORDC1 DDX5 TIA1
GOLGA3 CNDP2 DSP EIF5B
SF3B1 NEK9 SUPT16H
SRSF10 RPS21 RRM1 SNX1
DNAJB11 CALD1 SNRPD1
DYNC1I2 SMCHD1 DHX9
EDF1 IMPA1 ACSF2

Figure 5.7: Venn-diagram showing the differently expressed proteins at least one-fold at 16 h and 24 h.

5.3.5 Bioinformatics analysis

To identify the biological effect induced by the citric acid on the cell cycle re-entry inhibitory effect, the analyses were focused on the regulated pathways rather than the fold change of the protein expression.

5.3.6 Protein-protein interaction network

STRING database was used to identify the protein-protein interactions and associations of co-expressed or co-regulated proteins among the differentially expressed proteins. STRING predicts these interactions based on neighbourhood, gene fusion products, homology and similarity of co-expression patterning, experiments, databases, and text mining.

The predicted protein-protein interactions of significantly expressed proteins at 16 and 24 h are shown in Figure 5.8. A large proportion of proteins did not show any link at the chosen confidence level (STRING score=0.4) at 16 and 24 h. A strong potential interaction was found involving NEDD8, SUMO1, FAU, NHP2, RPS23, RPS15A, ETF1 and EIF1AY proteins at 16 h, while SRSF100, DDX5, RNPS1, SNRPD1, SEF3B1, DHX9, RPS21, NHP2 and EIF5B were predicted at 24 h. The two clusters found at 16 and 24 h were involved in protein synthesis and cell growth.

AKT1 and CDK1 were revealed as the major hub proteins that tentatively interacted with many proteins at 16 h and 24 h, respectively. AKT1 is involved in many processes such as metabolism, proliferation, cell survival, growth and angiogenesis. CDK1 promotes the G2-M transition and also regulates the G1 progression and G1-S transition (The UniProt Consortium 2017).

Meanwhile, no biological processes were predicted at 16 h by the STRING database. In contrast, cellular metabolic process, mRNA splicing, cellular macromolecule metabolic process, macromolecule metabolic process and regulation of mRNA splicing were the predicted biological processes at 24 h.

A number of notable protein-protein interactions were observed at 16 h and 24 h time points. At 16 h, an inhibiting interaction was observed between MAPK1 and AKT1, and a post-translational modification was observed between AKT1 and CTNNB1. Meanwhile, at 24 h, two post-translational modifications between CDK1 and NEK9, MAPK1, CALD1 and TK1, while another one with MAPK1 with ROCK1 and CALD1 were observed.
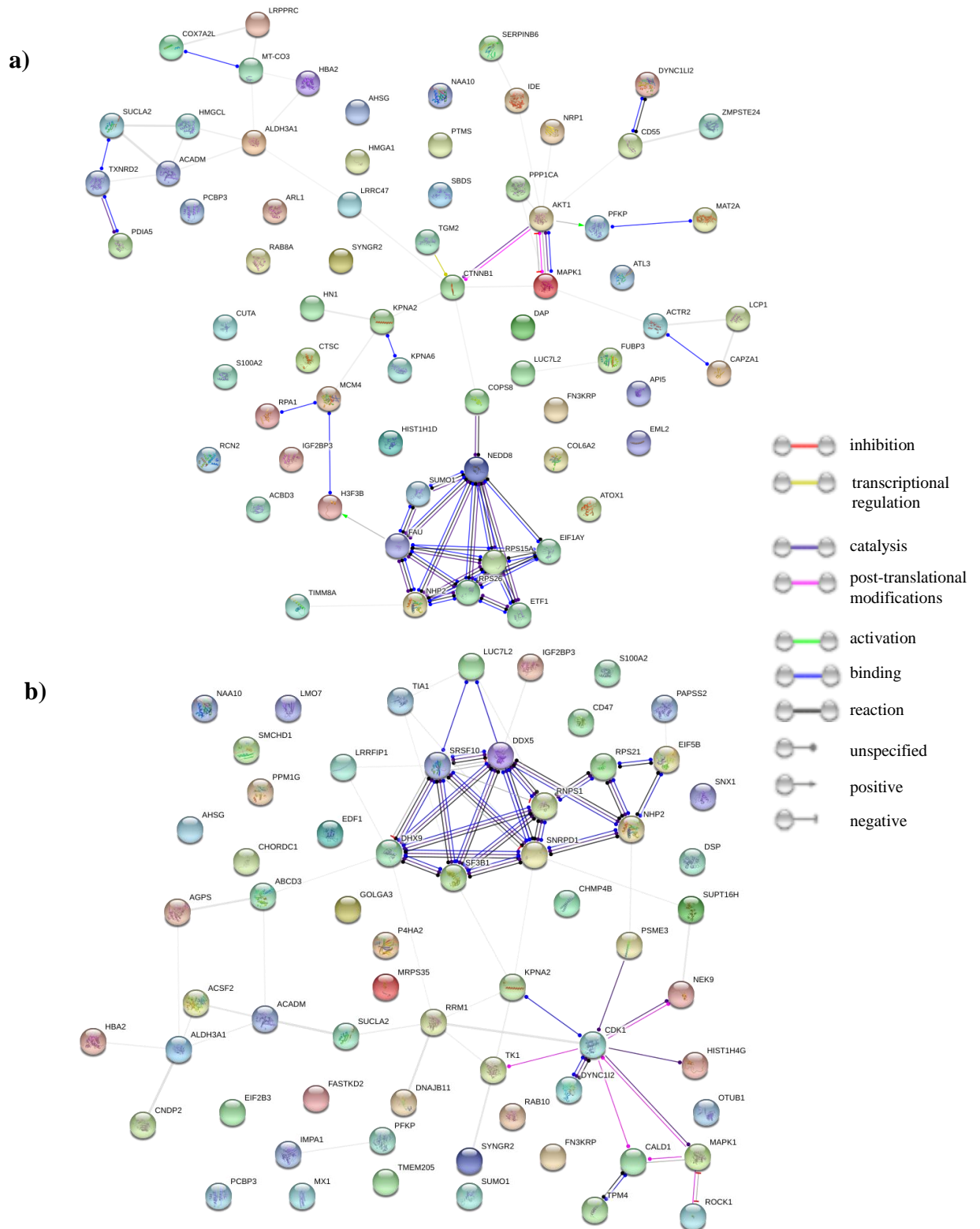
Figure 5.8: Predicted protein-protein interactions for significantly regulated proteins in citric acid treated PC-3 cells by STRING at 16 (a) and 24 (b) h.

5.3.7 Classification of Differentially Expressed Proteins

Gene ontology (GO) analysis using PANTHER was used to identify the biological processes significantly affected by citric acid treatment in PC-3 cells. The identified proteins were mainly involved in cellular processes, metabolic processes, and cellular component organization at 16 and 24 h as shown in Figure 5.9. The number of proteins involved in response to stimulus and development process decreased at 24 h compared to 16 h, while the other biological processes were almost similar. A cellular process involves any process that is carried out at the cellular level including cell communication among the cells. Meanwhile, metabolic processes involve the chemical reactions and pathways, including anabolism and catabolism, by which living organisms transform chemical substances including macromolecular processes such as DNA repair and replication, and protein synthesis and degradation.
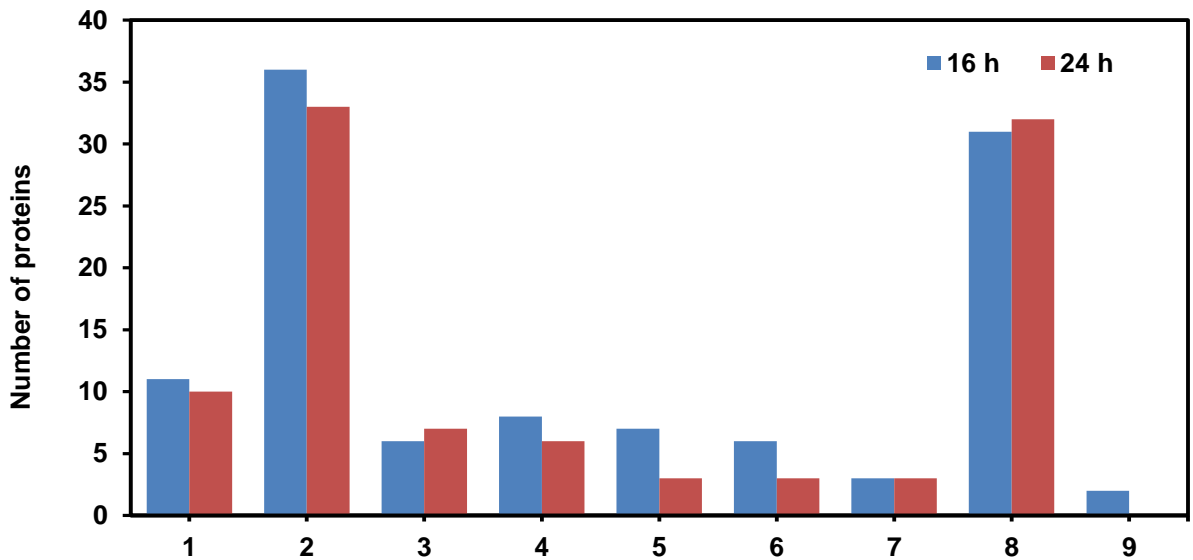
Figure 5.9: Gene ontology of regulated proteins classified according to biological processes. 1: cellular component organization or biogenesis, 2: cellular process, 3: localization, 4: biological regulation, 5: response to the stimulus, 6: developmental process, 7: multicellular organismal process, 8: metabolic process and 9: immune system process.

5.3.8 Identification of regulated canonical pathways

Ingenuity Pathway Analysis (IPA) was used to identify canonical pathways from the identified proteins in this study. Canonical pathways are the established pathways at the cellular level based on the collective evidence from various experimental findings. IPA predicts the possible pathways that could occur in the cells based on the identified proteins with their respective fold changes. It was found that for most of the quantified proteins, the log2 (H/L) ratio was below ± 1. Therefore, IPA was used to obtain a holistic view of changes involved in the cell cycle re-entry process, focusing on all identified proteins with log2 (>±0.2). These data are shown in Figure 5.10.

Theoretically, the 0 h samples are expected to have an activation score near 0 and coded with white colour as the ratios of heavy and light proteins were supposedly near to 1. However, some of the predicted canonical pathways of samples at 0 h have activation score not equal to 0 due to the presence of several proteins with abundance (H/L) ratio not equal to 1. The orange coloured box indicates the signalling pathway is activated, while the blue coloured box indicates the signalling pathway is suppressed in the sample. The intensity of the colour is dependent on the number of proteins identified and the magnitude of fold changes in the signalling pathway.

IPA results suggest that citric acid may exert its cell cycle inhibitory effect in PC-3 cells via multiple canonical pathways. The prominent pathway was EIF2 signalling pathway, which was suppressed in citric acid treated cells. mTOR signalling, ERK/MAPK signalling, PI3k/AKT signalling, VEGF signalling, and regulation of eIF4 and p70S6K were suppressed in citric acid treated cells. Meanwhile, RhoGDI signalling, PTEN signalling, AMPK signalling and HIPPO signalling were activated in citric acid treated cells. Although sumoylation was activated at 16

and 24 h, it was less activated compared to 0 h. This observation is in accordance with the predicted molecular changes. As the cells enter the cell cycle, they require various cell cycle activation signalling pathways and formation of a range of metabolic compounds for cell growth.

Figure 5.11 shows the protein identified in the citric acid treated PC-3 cells for the ERK/MAPK and PI3k/AKT canonical pathways. The expressions of these proteins were suppressed in citric acid treated PC-3 cells.
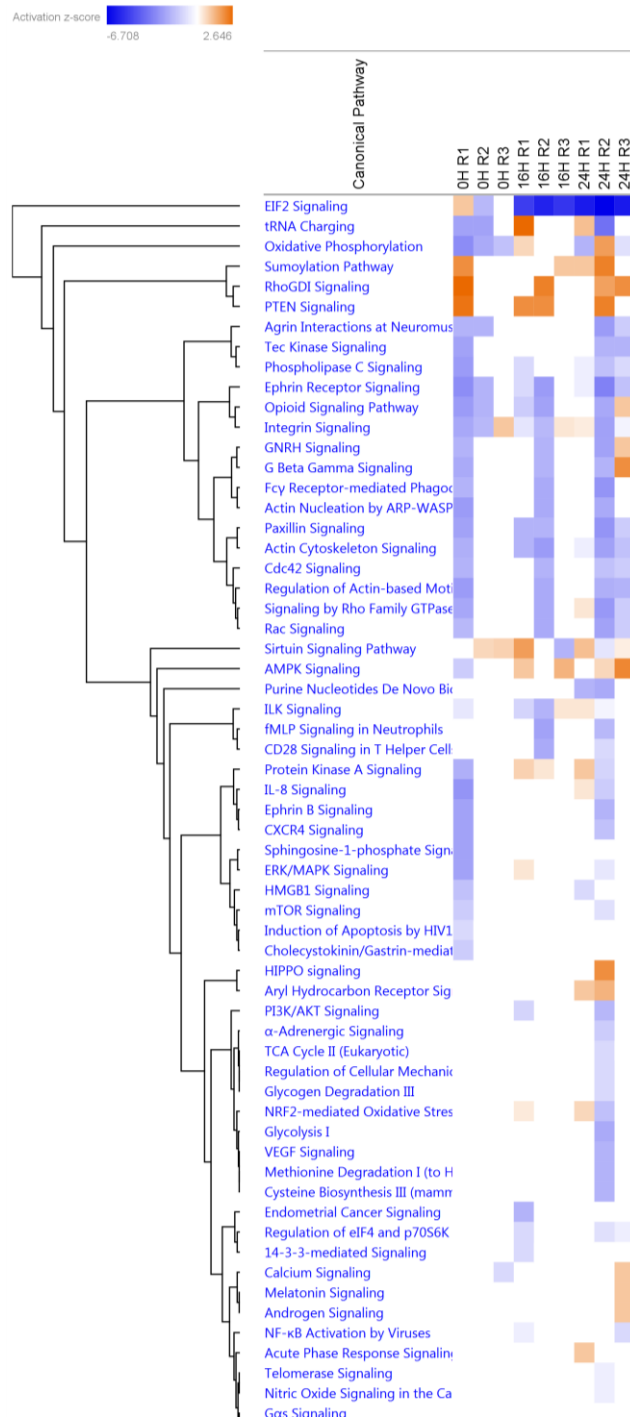
Figure 5.10: Regulated canonical pathways in cell cycle inhibited PC-3 cells were predicted by IPA analysis. Proteins are grouped by Canonical Pathways prioritized by hierarchical clustering, and z-scores are shown as heat maps. Greater intensity in orange colour indicates higher activation, while greater intensity in blue colour indicates higher suppression.
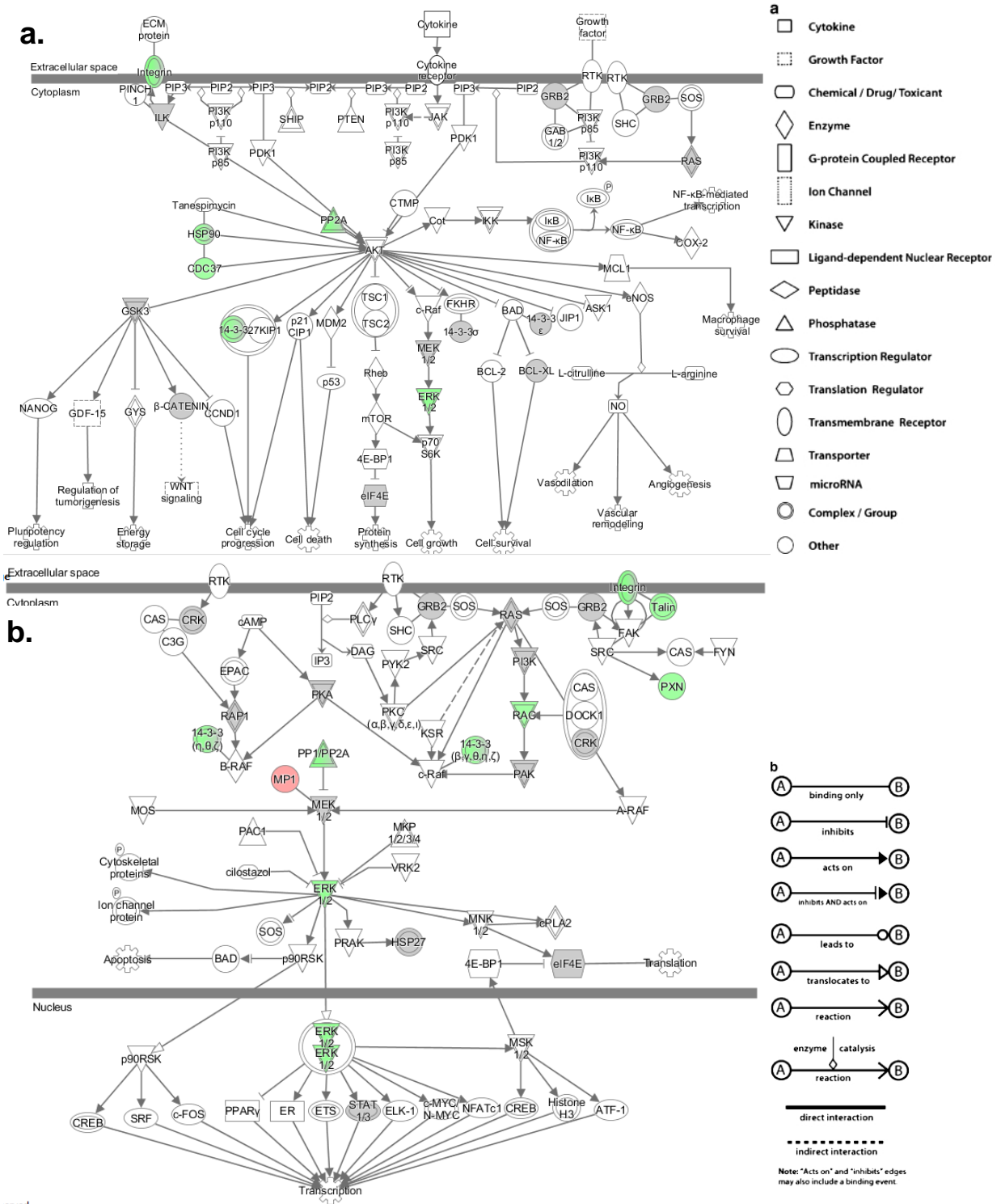
Figure 5.11: Proteins found in PI3K/AKT (a) and ERK/MAPK (b) canonical pathways predicted by IPA. Proteins labelled with grey means found in experiment, red means up-regulated and green means down-regulated in citric acid treated PC-3 cells.

5.4   Discussion

It has been reported that citric acid or citrate exhibited anticancer activity by affecting cancer cell metabolism, inducing tumour cell differentiation, activating the immune response and apoptosis *in-vitro* in multiple cell types (Ren, Seth et al. 2017, Mycielska, Dettmer-Wilde et al. 2018). However, there is little or no information available for cell cycle re-entry inhibitory effect of citric acid on cancer cells. This study was sought to identify the difference in the protein expression between the citric acid treated cells and control using a SILAC-based proteomic approach. The experimental result highlighted the differences in gene ontology as well as specific candidate factors that play a role for the citric acid response in inhibiting quiescence PC-3 cells from re-entering the cell cycle.

The PI3K/AKT and ERK/MAPK signalling pathways are known to be key regulators of cancer cell dormancy (Yeh and Ramaswamy 2015) and both were predicted to be modulated by citric acid in this study. AKT and MAPK1 were identified at 16 h, implying the significance of both pathways in inducing the cells into the cell cycle. It has been shown that the activation of PI3K/AKT signalling pathway is negatively controlled by PTEN (Georgescu 2010). In the present study, the IPA result also showed that PTEN signalling was activated in citric acid treated PC-3 cells in parallel with suppression of ERK/MAPK and PI3K/AKT signalling. Moreover, these findings align with a prior report that showed citrate inhibited IGF-1R, phosphorylated AKT, activated PTEN and increased expression of p-eIF2a on proliferating cancer cells (Mycielska, Dettmer-Wilde et al. 2018).

Moreover, the PI3K/AKT and ERK/MAPK signalling pathways are also highly responsive to mitogenic factors, which can similarly lead to the metabolic activation of quiescent cells, and

their re-entry into the cell cycle (Otto and Sicinski 2017). A study suggested that the metabolic activity of the cells determines whether the cell needs to proliferate or exit the cell cycle (Duan and Pagano 2011). In the present study, succinate—CoA ligase and ATP-dependent 6-phosphofructokinase were the two proteins down-regulated in citric acid treated PC-3 cells and these proteins are involved in cellular metabolism. Citrate was reported previously to suppress glycolysis and the tricarboxylic acid cycle both *in-vitro* and *in-vivo* cancer cells by inhibiting phosphofructokinase enzyme (Ren, Seth et al. 2017). It was hypothesised that high citric acid in the culture medium could inhibit cell cycle re-entry of quiescence PC-3 cells by suppressing the glycolysis and the tricarboxylic acid cycle. This is parallel with findings of a study where an axolotl oocyte extract negatively regulated the cellular metabolism of breast cancer cells in order to maintain the quiescency (Saad, Alberio et al. 2018). Cancer cells use glycolysis as a primary way to produce energy, and suppression of glycolysis could inhibit cancer cell growth and induce apoptosis (Vander Heiden, Cantley et al. 2009).

The regulated proteins were sorted by fold change to identify proteins that could maintain the cells in quiescence. Among the significantly regulated proteins identified in this study, CDK1, S100A2, JPT1, AKT1, NEDD8, MAPK1 and LMO7 were associated with cell cycle and growth signalling. Knockdown of JPT1 was shown to suppress growth rate of PC-3 cells by modulating negatively AKT-mediated GSK3B signalling and also resulted in prolongation of the G1 phase in the cell cycle (Varisli, Gonen-Korkmaz et al. 2011). Meanwhile, S100A2 was found in a higher level in normal prostate cells compared to null in prostate cancer cells, indicating the key role of loss of this protein during the progression of prostate cancer in humans (Gupta, Hussain et al. 2003). SNX1 was another overexpressed protein in citric acid treated PC-3 cells and this protein was reported as a tumour suppressor in gastric cancer (Zhan, Zhang et al. 2018).

Verification of these proteins, for example by knocking out the protein in the cells, could reveal the possible cell cycle re-entry inhibitory activity.

Although this study was limited with the identification of proteins involved in the re-entry of quiescence PC-3 cells, it still shed light on the high likely mechanisms that occurred in the citric acid treated cells population. Moreover, this study also provided a screened list of proteins involved in the cell cycle re-entry of quiescent PC-3 cells that can be validated in future studies. In the current study, the cells were labelled with heavy lysine and the experimental results showed that a large fraction of identified peptides had no lysine amino acid on the peptide chains. By labelling the proteins with both heavy lysine and heavy arginine, a number of proteins with high accuracy and confidence can be increased.

5.5   Conclusion

Suppressing cancer cell progression by modulating metabolic pathways is a current emerging area of cancer research and it has been proven effective in cancer treatment. Citric acid is one of the non-toxic and promising compounds for targeting cancer during metabolism. This study showed that the citric acid most likely inhibited quiescent PC-3 cells from re-entering cell cycle by suppressing PI3K/AKT and ERK/MAPK signalling pathways and activating PTEN signalling pathway. Modulations of these signalling pathways by any compounds or proteins are able to prevent the re-entry of quiescent cancer cells and thus potential to reduce the risk of cancer recurrence.

6 Conclusions and recommendations

6.1 Major findings and significance of results

Quiescent cancer cells have been regarded as one of the factors for cancer recurrence. Diet enriched with anticancer bioactive compounds could be a great strategy for cancer recurrence intervention by halting the cell cycle re-entry of quiescent cancer cells. Citrus peel has been shown to have anticancer activity on different cancer lines on both *in-vitro* and *in-vivo* models in the last two decades; however, the citrus peel inhibitory effect on cell cycle re-entry of quiescent cells was not reported previously. Thus, the effect of citrus peel extract and it constitutes on cell cycle re-entry of quiescent prostate cancer (PC-3 and LNCaP) cells was evaluated using an established *in-vitro* model of prostate cancer quiescent cells.

This PhD thesis integrates three objectives dedicated to evaluating the possibility to use citrus peel in preventing prostate cancer recurrence. The first objective was focused on the evaluation of citrus peel extract in inhibiting the cell cycle re-entry of quiescent prostate cancer. Thus, a new experimental design to mimic the cancer recurrence was used in this study. For extract preparation, two different solvents were used, water and hexane: ethyl acetate solvent, to extract a range of bioactive compounds from citrus peel. Although both water and hexane extract reduced cell viability of prostate cancer cells, only water extract was capable in preventing the cells from re-entering the cell cycle. Water extract strongly inhibited the quiescent cancer cells that were mainly in the G0/G1 phase from entering cell cycle or S phase without causing apoptosis. Not only that, the extract showed selectivity in targeting cancer cells as lower toxicity was observed on normal human fibroblast cells compared to prostate cancer cells. The inability of hesperidin and naringin to inhibit the cell cycle re-entry of quiescent prostate cells suggested

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that the major compounds present in the extract are not necessarily responsible for the observed effect and highlights the importance of non-predominant compounds in the extract.

As identifying the responsible compounds for the observed biological effect could be meaningful in different aspects, the second objective was focused on isolation and identification of bioactive compounds from the water extract. Flavonoids are the major compounds in citrus and it has been shown that these compounds arrest a variety of different cancer lines at G0/G1 or G2/M phases. However, in this study, it was shown that naringin and hesperidin were not responsible for the observed cell cycle inhibitory effect. Thus, chromatography technique was used to screen the potent compounds from a range of natural compounds present in the extract. Citric acid was identified as one of the compounds in the water extract of citrus peel that was responsible for the observed cell cycle re-entry inhibitory effect. Moreover, citric acid showed toxic selectivity on targeting cancer cells than normal prostate cells. It was noticed that the strength of biological activity reduced when the extract was fractionated, which indicates the synergic effect of various compounds. These results further suggested that citric acid being the potent cell cycle re-entry inhibitor in the extract and exhibits greater cell cycle re-entry inhibitory effect in the presence of other bioactive compounds. Moreover, cancer recurrence does not occur via one mechanism, thus the presence of multiple compounds as a whole extract is required for modulation of multiple mechanisms related to cancer recurrence prevention.

With the identification of the compound responsible for the cell cycle inhibitory effect, the third aim was to determine the possible mechanisms involved at the cellular level that trigger the quiescent cells to re-enter the cell cycle. Knowledge on the transition of quiescent cells to proliferating cells by entering into cell cycle is crucial for cancer treatment. Thus, stable isotope labelling using amino acids in cell culture-based proteomic experiment coupled with mass

spectrometric was used to determine the changes in the protein expression in the PC-3 cells when the cells were released from the quiescence. The results showed the citrate most likely inhibited PC-3 cells from entering cell cycle mainly by suppressing PI3K/AKT and ERK/MAPK signalling pathways and activating PTEN pathway through regulation of a number of key functional proteins.

In conclusion, this study showed that citrus peel extract could arrest the quiescent prostate cancer and thus potential in preventing cancer recurrence. Moreover, citric acid was identified as a promising compound with low toxicity and a powerful inhibitory effect on the cell cycle re-entry of quiescent prostate cancer cells. This low toxicity and selectivity of citric acid justify further investigations for the potential of citric acid as a compound with chemo-preventive and therapeutic. Citrus peel extract that contains a range of bioactive compounds including citric acid can be converted into high-value nutraceutical products with the aim to prevent recurrence of prostate cancer in patients.

6.2   Future work

The outcomes of this study broaden the understanding of the use of citrus peel for prostate cancer recurrence prevention. However, more studies are needed for this research. Although citric acid was identified as a potent anticancer compound with cell cycle re-entry inhibitory activity in this study, there are potential for the presence of other compounds in the extract with a similar biological effect. The other bioactive compounds were not able to identify due to the low concentration of the compound in the extract. It is possible for these compounds to be identified if the concentration of fraction or extract is increased.

Hexane extract showed a very strong cell toxicity effect at a low concentration on the prostate cancer cells in this study. It is predicted that few compounds could contribute to this toxicity. It is also observed that the hexane extract contained very non-polar compounds, which are difficult to be eluted out from the C_{18} HPLC column. It will be interesting to identify these compounds and their likely effect on the cancer cells. Not only that, the non-polar compounds in citrus peel extract are insoluble in water, therefore it may be interesting to look at the bioavailability and ways to increase their absorption into the body.

Moreover, with the identified potential pathways that initiate the cell cycle re-entry, it is important to validate the protein to ensure the accuracy of acquired data. It is also recommended to conduct an *in-vivo* study using mice model to confirm the potency of these active compounds and strengthen the hypothesis of this study. It is still unclear whether the intake of synthetic citric acid will exhibit similar cell cycle inhibitory effect as the citrus peel extract. An *in-vivo* experiment to compare the difference between citric acid and citrus peel extract containing citric acid can reveal the benefits of the natural extract. Moreover, a citrus peel powder or solution can be prepared by having the potent compounds and its stability and efficacy should be evaluated. It is crucial to evaluate the stability of extract or powder at different conditions as degradation of the certain bioactive compound will reduce the biological effect.

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