CYTOTOXICITY OF *CLINACANTHUS NUTANS* METHANOL-WATER EXTRACT ON HUH-7 CELL LINE

By

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LIST OF ABBREVIATIONS

CN	*Clinacanthus nutans*
COX-2	Cyclooxygenase-2
GC-MS	Gas chromatography-mass spectrometry
HCC	Hepatocellular carcinoma
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
PGE_2	Prostaglandin E_2
SRB	Sulphorhodamine B
TCA	Trichloroacetic acid
WHO	World Health Organization

ABSTRAK

Sitotoksisiti ekstrak *Clinacanthus nutans* Metanol-Air Terhadap Sel-sel Huh-7

Terdapat banyak kes-kes kanser dilaporkan setiap tahun dan setiap hari, ia semakin meningkat. Ramai orang mencari herba-herba semulajadi yang mempunyai keupayaan untuk merawat kanser dan tidak bergantung kepada rawatan yang biasa digunakan untuk kanser, yang iaitu kemoterapi dan radioterapi. Salah satu tumbuhan herba yang menjadi terkenal ialah *Clinacanthus nutans* atau dipanggil Belalai gajah oleh rakyat tempatan Malaysia. Objektif kajian ini adalah untuk menentukan kesan sitotosik ekstrak metanol air tumbuhan ini terhadap sel-sel Huh-7, karsinoma selular hepato mantap yang diambil daripada hati seorang lelaki Jepun berusia 57 tahun. Sel-sel yang diperolehi daripada JCRB, Jepun dikulturkan terlebih dahulu mengikut protocol sebelum dibenihkan ke dalam telaga plat-96. Kemudian, ekstrak metanol-air dimasukkan ke dalam setiap telaga yang dibijikan dan dirawat selama 24 jam, 48 jam dan 72 jam dengan kepekatan 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg dan 10 mg. Penentuan kematian sel-sel diukur dengan menggunakan penyerapan gelombang melalui pengunaan reagen MTT dan SRB. Keputusannya, ekstrak ini mempamerkan sifat-sifat menghambat pertumbuhan sel tetapi tidak berkesan pada dos 4 mg selepas dirawat 24 jam tetapi ada perubahan pada 72 jam. Ekstrak ini menunjukan kesan yang positif pada dos 10 mg selepas dirawat selama 24 jam. Ekstrak *Clinacanthus nutans* metanol-air mempunyai sifat-sifat menghambat pertumbuhan sel Huh-7 tetapi memerlukan dos yang tinggi.

ABSTRACT

Cytotoxicity of *Clinacanthus nutans* Methanol-Water Extract on Huh-7 Cell Line

There are many of cancer cases reported yearly and it has been increasing as day passes by. Many sought out the capabilities of natural herbs instead of relying on the common treatment currently being used for cancer which are chemotherapy and radiotherapy. One of the popular plants being used currently is *Clinacanthus nutans* or commonly called 'belalai gajah' by local Malaysians. The objective of this research is to determine the cytotoxicity effects of the methanol-water extract of this plant on Huh-7 cell lines, a well differentiated and well-established hepato cellular carcinoma taken from the liver of a 57 year old Japanese male. The cells obtained from JCRB, Japan is cultured first according to protocol before seeding into 96-well plates. Then, the prepared methanol-water extract is then added into each well which was seeded and is treated for time point 24 hours, 48 hours and 72 hours with concentrations of 1 mg, 2 mg, 3 mg, 4 mg, 5mg, 6 mg, 7 mg, 8 mg, 9 mg and 10 mg. The cell viability determination is measured by wavelength absorbance through the use of MTT and SRB reagent. The results showed that the extract does exhibit cell proliferation inhibiting properties but is not effective at 4 mg dose after 24 hours and at 72 hours, there is some significant inhibition. However, it is very effective at 10 mg dose after 24 hours as the percentage of cell viability is very low. The methanol-water extract of *Clinacanthus nutans* does shows cell proliferation inhibiting properties however only applicable at higher doses in Huh-7 cell line.

CHAPTER 1

INTRODUCTION

1.1 Introduction

In 2012, there is an estimated number of 14.1 million new cases of cancer and 8.2 million deaths from cancer occurred worldwide. The most frequently diagnosed cancers and the main cause of death in both men and women are lung and breast cancer. Liver cancer too, is a common cause of death from cancer with a recorded cases of 788 000 number of deaths in 2015 (WHO, 2017). Hepatic cancer occurs most of the time as hepatocellular carcinoma (HCC) and is responsible for an estimated 75% of all main liver cancer which is the reason for over 600 000 deaths every year (Jemal et al., 2011). When hepatocellular carcinoma is diagnosed, it is usually at the stage of metastasis or locally advanced stage. Currently, the treatment for HCC are surgery, radiation therapy and chemotherapy however, there is no effective therapy to treat advanced HCC eventhough many advances in treatment had been made (Zhuo et al., 2015). Although the most common treatment for HCC is chemotherapy, it still have limitations due to its severe side effects (Zhuo et al., 2015).

As an alternative, many people sought the help of traditional herbs to aid in the treatment of cancer and many have claimed of its effectiveness in treating and curing cancer. One of the most famous traditional herb being used commonly by many is the plant *Clinacanthus nutans*, from the family Acanthaceae. Frequently known as the Sabah Snake Grass or 'belalai gajah' by Malaysian locals, this plant is used traditionally for inflammation treatment and various viral infection treatment (Yong et al., 2013). In addition, previous work has been done onto this plant for its phytochemical and biological constituents as well and many compounds were isolated from the plant. The chloroform

extract of this plant has the highest potential in scavenging free radicals and growth inhibition of some cultured cell lines (Yong et al., 2013). Most studies of this plant extract were done in methanol, ethanol, aqueous and chloroform however, the benefits of the plant in methanol water solution has yet to be investigated.

1.2 Objective

General objective

The main objective of this study is to investigate the anti-proliferative effects of *Clinacanthus nutans* methanol-water extract on Huh-7 cell lines.

Specific objective

1. To determine the cytotoxic effect of *Clinacanthus nutans* methanol-water extract on the Huh-7 cell line.

To analyse the phytochemical contents of *Clinacanthus nutans* methanol-water extract by using gas chromatography-mass spectrometry.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans*

Clinacanthus nutans from the family Acanthaceae, is a renowned traditional herbal plant in the Asia region. Also referred to as the Sabah Snake Grass in Malaysia or Belalai gajah, the freshly taken leaves are either boiled with water and drank as herbal tea or used for rash treatment, snake bites and skin lesions caused by herpes simplex virus, for fever and diuretics treatment as well (Aslam, Ahmad, & MAMAT, 2015). As treatment for skin rashes, snake bites and insect bites, lesions caused by herpes simplex virus (HSV) and the varicella-zoster virus (VZV) in Thailand, the alcoholic extract of the fresh leaves is used and applied externally (Aslam et al., 2015).

The leave extracts were reportedly to possess analgesic and anti-inflammatory properties (Satayavivad, Bunyaoraphatsara, Kitisiripornkul, & Tanasomwang, 1996) and antiviral activities to viruses such as varicella-zoster virus (Thawaranantha et al., 1992) and herpes simplex virus type-2 (C Jayavasu, Balachandra, Sangkitporn, Bunjob, & Chavalittumrong, 1992; Chuinradee Jayavasu, Dechatiwongse, & Balachandra, 1992). Isolated from the plant in a phytochemical study is a list of flavonoids, steroids, triterpenoids, cerebrosides, glycoglycero-lipids, glycerides and sulphur-containing glycosides (Tu et al., 2014). The *C. nutans* ethanol extract showed anti-dengue virus, anti-inflammatory and immune-modulating activity in a bioactive screening (Tu et al., 2014).

The chemical compositions (Sakdarat, Shuyprom, Ayudhya, Waterman, & Karagianis, 2006) and bioactive constituents (Sakdarat, Shuyprom, Pientong, Ekalaksananan, &

Thongchai, 2009) of *clinacanthus nutans* has been extensively studied and six known *C*-glycosyl flavones which were isolated from the plant are vitexin, isovitexin, shaftoside, isomollupentin, 7-*O*-*β*-glucopyranoside, orientin, isoorientin (Teshima et al., 1997), five sulphur-containing glycosides (Teshima et al., 1998), lupeol, *β*-sitosterol (PIMCHIT Dampawan, Huntrakul, Reutrakul, Raston, & White, 1977), stigmasterol (P Dampawan, 1976), botulin (Lin, Li, & Yu, 1983), two glycoglycerolipids (Satakhun, 2001) and a mixture of nine cerebroside and a monoacylmonogalatosylglycerol (Tuntiwachwuttikul, Pootaeng-on, Phansa, & Taylor, 2004).

In addition, from the leaves of hexane and chloroform extract of *clinacanthus nutans* Lindau leaves, 13-hydroxy-(13-S)-phaeophytin b, pupurin-18-phytyl ester, phaeophorbide were isolated (Ayudhya et al., 2001), isolation of trigalactosyl and digalactosyl diglycerides from the leave extract which possesses anti-herpes simplex virus effect (Janwitayanuchit et al., 2003), 132-hydroxy-(132-S)-chlorophyll b, 132-hydroxy-(132-R)-chlorophyll-b, 132-hydroxy-(132-S)-phaeophytin-b, 132-hydroxy-(132-R)-phaeophytin-b, 132-hydroxy-(132-S)-phaeophytin-a, 132-hydroxy-(132-R)-phaeophytin-a, 132-hydroxy-(132-R)phaeophytin, purpurin-18-phytyl ester and phaeophorbide-a in the hexane and chloroform leave extract of *clinacanthus nutans* (Sakdarat et al., 2006; Sakdarat et al., 2009).

In the aerial parts of the *clinacanthus nutans* plant ethanol extract, four new compounds which are clinamides A, clinamides B, clinamides C and 2-*cis*-entadamide A respectively and three known compounds which are entadamide A, entadamide C and *trans*-3-methylsulfinyl-2-propenol were isolated (Tu et al., 2014).

2.2 Huh-7 Cell Line

Huh-7 cell line obtained from a liver tumour in a 57- year old Japanese male back in the year 1982 is a well-established and a well differentiated hepatocyte-derived cellular carcinoma cell line (Krelle, Okoli, & Mendz, 2013). Unlike other established human hepatoma cell lines with differentiated behaviours of the liver, Huh-7 can be proliferated in a medium which is chemically defined and contains minute amounts of selenium instead of serum.

Other liver cell lines such as Huh-1, Huh-4, Huh-6, Hep3B, PLC/PRF/5 requires the addition of serum. In addition, Huh-7 was found to be responsible for secretion of mitogen hepatoma-derived growth factor which promotes cell growth in the absence of growth factors which are found in a serum. It has a high permissibility towards Hepatitis C Virus which makes this cell a suitable model in the HCV replication studies (Steinmann & Pietschmann, 2013).

2.3 Cell Death

Cell death occurs when the cells are confronted with a process which is reversible at first before it became irreversible. The Nomenclature Committee on Cell Death (NCCD) came out with a proposal that in the event a cell is dead, one of the few criteria which is to be met is loss of plasma membrane integrity, defined by dye incorporation, complete fragmentation of the cell, including its nucleus into discrete bodies which are normally known as ‘apoptotic bodies’ and or its corpse have been engulfed by an adjacent cell *in vivo*. Cell death is classified based in its morphological characteristics, enzymological criteria, functional aspects or immunological characteristics. Morphological characteristics which can be observed are such as apoptotic, autophagic, necrotic or associated with mitosis. Cell death caused by functional aspects could be either programmed or accidental and physiological or pathological (Kroemer et al., 2009).

2.4 Apoptosis

Apoptosis is defined as cell death based on specific morphological aspects and is not a synonym for programmed cell death or activation of caspase (Kerr, Wyllie, & Currie, 1972; Kroemer et al., 2009). In the event of cell apoptosis, it can be seen that the cell became more rounded, the pseudopods retract, cellular volume is reduced (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorrhexis), and either the presence of some classical or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing and being engulfed by another resident phagocyte. Therefore, apoptosis is specially applied to cell death events which occurs from several manifestation of the stated features.

Apoptosis specifically affects single cells which are scattered and histologically manifested by formation of small, roughly spherical or cytoplasmic fragments in ovoid shape which contain pyknoyic remnants of nuclei. Under the observation of electron microscope, the structural changes of apoptosis occurs in two discrete stages which are formation of apoptotic bodies firstly and secondly, phagocytosis and degradation by other cells. Apoptotic bodies formed involves the marked condensation of both the nucleus and cytoplasm, nuclear fragmentation and protuberance separations which are formed on the surface of the cell. This will in turn, produce many membrane bounded, compact and cell remnants of various sizes which are well preserved. A fully developed apoptotic body will show organelles closely packed together but still intact both chemically and structurally (Kerr et al., 1972). Apoptotic body often occurs in clusters in the intercellular space and the contents are dependent on the cellular constituents which happened to be present in the cytoplasmic swelling. Small bodies occasionally composed of nearly condensed nuclear chromatin while others consists of only cytoplasmic elements. With the help of a light microscope, only large members of the cluster of apoptotic bodies in the intercellular space can be observed. Those which are smaller in size tend to disperse from their origin site and is often seen in the spaces between the parenchymal and

sinusoid-lining cells if their site of origin is in the liver or adrenal cortex. If the site of origin is from the glandular and muscosal epithelium and the renal tubules are normally shed into the lumen. Only a few will enter the blood vessels.

The condensation of apoptotic bodies is presumely a result from the extrusion of water. However, the specific mechanism involved is unknown. Rough estimation of the degree of condensation suggests that there might be formation of small membrane-bounded fragments without new synthesis of plasma membrane. Many tissues were studied and it was shown that majority of the apoptotic bodies is found within the cytoplasm of intact cells due to them being rapidly phagocytosed because of the properties of their surface membrane. Therefore, the role of apoptosis is suitable in regards to tissue homeostasis as it results in extensive deletion of cells with little disruption to tissues. The remains of fragmented cells affected are immediately disposed of intact cells located nearby. Inflammation is absent as is induced by coagulative necrosis. In addition, as a result of possible cell defaecation, lysosomal residual bodies disappear (Kerr et al., 1972).

There is little knowledge on the factors which cause the initiation of apoptosis or the nature of the cellular mechanisms which are activated before morphological characteristic changes appear. However, it is shown that in certain cases of apoptosis, it is a permanent programmed event which is determined by *intrinsic "clocks"* which is specific for the type of cell involved.

2.5 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay

One of the large group of heterocyclic organic compounds responsible for the formation of highly colored and most of the time, insoluble formazans after a reduction process are tetrazolium salts. They were first prepared back in 1894 and has been used widely as an indicator for both biological redox systems and viability tests (Altman, 1976). One of the most often used method to measure the cell proliferation activity and cytotoxicity is the

reduction of the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a monotetrazolium salt (Liu, Peterson, Kimura, & Schubert, 1997; Mosmann, 1983). It was introduced back in 1983 by Mosmann in order to assess the potentiality of novel antitumour agents. The assay was developed to evaluate the effects of lymphokines which are proliferative, mitogen stimulation and complement-mediated lysis (Mosmann, 1983). The assay was then further developed broadly to provide fast and competent method for screening and to determine the mitochondria impairment by possible cytotoxic novel antitumour agents (Alley et al., 1988).

In addition, it has been used in evaluating the cytotoxicity of some soluble synthetic polymers which are thought to be drug carriers (Sgouras & Duncan, 1990). A wide assumption on MTT is that the active mitochondria in living cells reduced the salt. By isolating and using the mitochondria obtained from the brains of rats and B12 cells, it was found that malate, glutamate and succinate supported the reduction of the MTT salt. On the contrary, the data which is shown in this study does not show to support the limitations of the role of mitochondria in the MTT reduction by the intact cells (Liu et al., 1997).

Although there is evidence that in viable cells, the MTT reduces the active mitochondria, it is missing a concrete proof on the relationship between mitochondria with MTT as there are numerous nonmitochondrial dehydrogenases or flavin oxidases that reduce MTT as well (Altman, 1976). Thus, without adequate perception of the site and the enzymatic system which is affected in the reduction of MTT, it is complicated to clarify the disparities between MTT assay with other methods to measure cell growth and viability (Berridge & Tan, 1993). The MTT formazan accumulates intracellularly in a granular form with a perinuclear localization which is predominant. Also, as a result of prolonged incubation with MTT, there is a formation of formazan crystals with needle-like characteristics on the cell surface (Shearman, Hawtin, & Tailor, 1995). Findings by Liu et al. (1997) showed that at the external part of the mitochondria in B12 cells, cellular MTT reduction can occur.

Due to the many sites in a cell which is capable of MTT reduction, it was predicted that the cellular reduction of MTT will have a morphologically diffused appearance however, it was shown otherwise that the blue or purple MTT formazan is deposited intracellularly in a granular form (Liu et al., 1997; Shearman et al., 1995). In time, the formazan granules will become bigger and darker and slowly, it will get closer to the plasma membrane and then, needle-like formazan crystals will appear on the cell surface. After a certain period of time, the intracellular formazan granules will slowly decrease or disappear. Needle-like formazan crystals formation at the cell surface is exceptionally heterogeneous among the cells. To obtain 98-99% of cells surface to form formazan crystals, at least 3 hours of MTT reduction is recommended (Liu et al., 1997; Shearman et al., 1995). Upon detailed examination and observation on the formation of the needle-like formazan crystals on the cell surface, it was shown that the needle-like formazan crystals are formed from the release of intracellular formazan granules and not by the MTT reduction at the plasma membrane (Liu et al., 1997).

2.6 Sulforhodamine B (SRB) Assay

Sulforhodamine B or also known as SRB is an inexpensive, rapid and sensitive method developed by Skehan et al., (1990) to measure the cellular protein content of cultures adhered or suspended in 96-well microliter plates. The assay is dependent on the capability of SRB to be able to bind to the protein components of the cells which are fixed on the cultured plate. It is a bright-pink aminohexane dye with two sulfonic groups which are able to bind to basic amino-acid residues under mild acidic conditions. Firstly, the cultures of the cells are fixed with trichloroacetic acid and is stained for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) which is dissolved in 1% acetic acid. After the 30 minute period, the dye which is not bounded to the proteins are washed four times with 1% acetic acid and then 10mM unbuffered Tris base [tris (hydroxymethyl)aminomethane] is used onto the protein-bound dye. This is to enable determination of the optical density

via microtiter plate reader (Skehan). In the experiment conducted, 21 histological dyes in measuring cell density and drug cytotoxicity is first compared. Out of the 21 dyes used, 13 provided adequate basis for cytotoxity determination. Then, an optimized protocol is then developed to obtain the best seven dyes.

Among all the dyes used, SRB have shown better results as it has a strong intensity in the staining of the assay and it can detect densities as low as 1000 – 2000 cells per well and at cell densities of 5000 per well, it has a signal-to-noise ratio of 4.83. The sensitivity level is as good as other fluorescent dyes when compared and is by far greater in power to other protein-staining methods which uses conventional visible dyes (Vichai & Kirtikara, 2006). In addition, the SRB method was proven to be suitable as after the fixation of the cells by TCA and stained by SRB, the cell monolayers which are dried can be stored and the color extracted from the cells stained by SRB is stable as well. This assay has been then widely used in testing of drug toxicity towards both cancerous and non-cancerous cell lines. When testing cancer cell sensitivity towards radiation and interactions between chemotherapy and radiotherapy, the SRB method is shown to achieve desirable results when testing *in vitro*.

CHAPTER 3

METHODOLOGY

3.1 Plant extract preparation

Fresh leaves of *Clinacanthus nutans* were obtained from Tasek Gelugor, Pulau Pinang. The leaves were first cleaned with tap water and then with distilled water. The leaves were stored at -80ºC until further use. For the extraction process, 100 g of fresh leaves were grinded with 400 ml methanol and 100 ml distilled water. Then, it is soaked in methanol water solution in the ratio of 4:1 for 24 hours. After 24 hours, the solution with the leaves are filtered, the solution discarded and the filtered leaves are soaked in a new solution of methanol water for another 24 hours. This process is repeated the following day as well. After the third day, the leaves are filtered from the solution and put in a rotary evaporator to obtain the separate compound and the extract compound. The separate compound is thrown away while the extract compound is kept. The extract compound is then freeze dried to remove the water to obtain the crude extract.

3.2 Cell culture of Huh7 cells

Huh7 cell lines were obtained from JCRB Cell Bank, Japan. The cells were cultured in Dulbecco's Modified Eagle Medium containing low glucose, supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin. The cells were grown inside an incubator at 37ºC supplied with 5% CO_2 condition in a T-25 flask. Subculture of the cells were done once the cells achieved cell confluency of 80%-90% to a T-75 flask. The cells were subculture again once it reached confluency of 80%-90% or seeded to 96 well plates for antiproliferative and cell viability studies.

3.3 Cell viability and anti-proliferative studies by using MTT assay

Cells are seeded into 96-well plates with cell density of 5 x 10^3 per well in triplicates and is left inside the incubator overnight (Zhuo et al., 2015). The cells were treated with *C. nutans* methanol-water extract into respective wells with concentrations 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg and 10 mg. The cells were incubated at time points 24 hours, 48 hours and 72 hours respectively. After 24 hours of treatment, 10µL MTT solution of 5mg/mL were added to each well and is wrapped in aluminium foil to prevent light access to the solution and is incubated for 3 hours. After 3 hours, the plate is taken out from the incubator and the media in each plate is removed before addition of 100µL of DMSO. The reading is obtained from the microplate reader with optical density measured at 570 nm. The MTT assay process was repeated for time points 48 hours and 72 hours.

3.4 Cell viability and anti-proliferative studies by using SRB assay

Cells are seeded into 96-well plates with cell density of 5 x 10^3 per well in triplicates and is incubated overnight (Zhuo et al., 2015). The cells were then treated with *C. nutans* methanol-water extract into respective wells with concentrations 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg and 10 mg. The cells were then incubated at time points 24 hours, 48 hours and 72 hours respectively. After 24 hours of treatment, 50µL of 50% cold TCA is added to the cells and incubated for 30 minutes at room temperature for 30 minutes. The plate is then washed five times with distilled water and dried. The cells were then stained with 100 µL of 0.4% SRB in 1% acetic acid for 30 minutes at room temperature and then rinsed four times with acetic acid. The plate was dried again before adding 100 µL of 10mM Tris buffer and shaken for 5 minutes. The reading is then obtained by using a microplate reader to read the absorbance at 540 nm.

3.5 Gas Chromatography- Mass Spectrometry (GC-MS) Analysis of *Clinacanthus nutans* Methanol-Water extract

Analysis on the methanol-water extract of CN was done by using gas chromatography-mass spectrometry (GCMS) to determine the phyto-chemical constituents of the methanol water extract. The oven initial temperature was set to 70ºC with an initial time of 2.00 minutes and to a maximum temperature of 325ºC. The equilibrium time is 0.50 minutes. The injector is set to split mode with initial temperature of 280ºC and the injection volume is 1.0 µL. The final temperature of the oven is set to 280ºC and final time of 20.00 minutes. The total run time is 32.50 minutes. The carrier gas used is helium. The mass spectra is obtained and the compounds are identified by comparing their mass spectra with the National Institute of Standards and Technology (NIST) library data.

CHAPTER 4

4.1 RESULTS

4.1.1 Cell culture of Huh7

Huh7 cells obtained from JCRB Cell Bank, Japan were cultured in low glucose Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin Streptomycin. Cultured cells were incubated at 37ºC with 5% carbon dioxide. Figure 1.1 shows the morphological characteristic of the cells at first passage under 4x magnification. Figure 1.2 shows the cells after two days of incubation under magnification of 4x. Figure 1.3 shows the morphological characteristic of the cell at 10x magnification, after two days of incubation. Figure 1.4 shows the morphological characteristics of the cell under 20x magnification.

Figure 1.1: Morphological characteristic of Huh7 cells one day after culture under magnification of 4x.

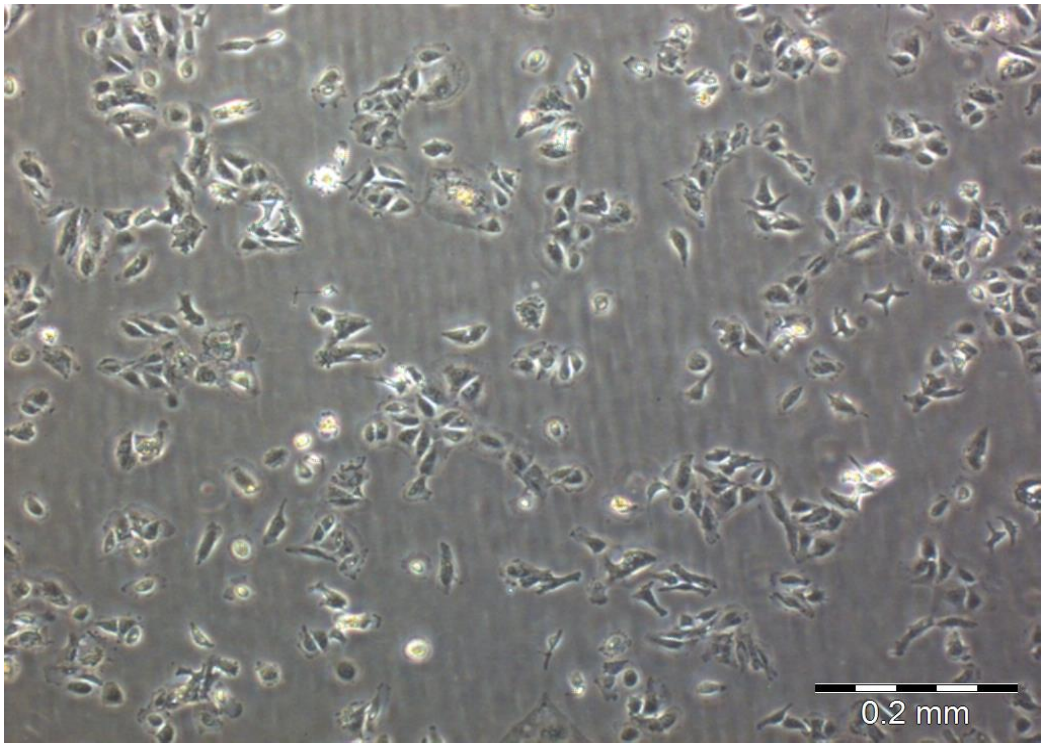


Figure 1.2: Morphological characteristics of Huh7 cells after one day after culture under 10x magnification.

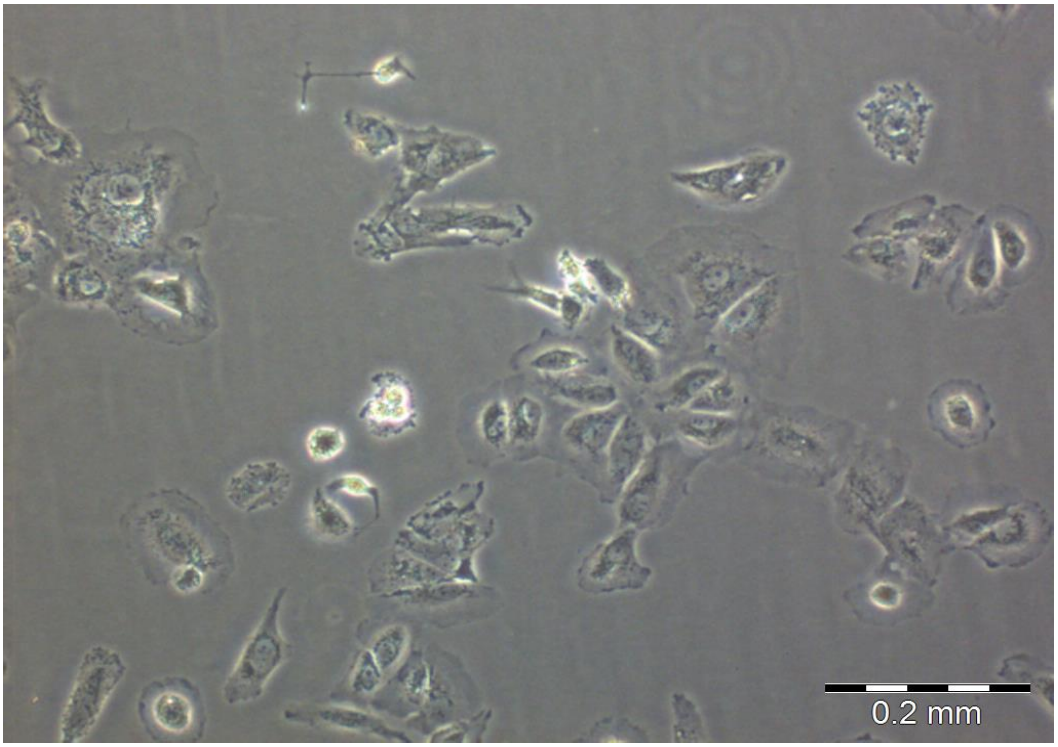
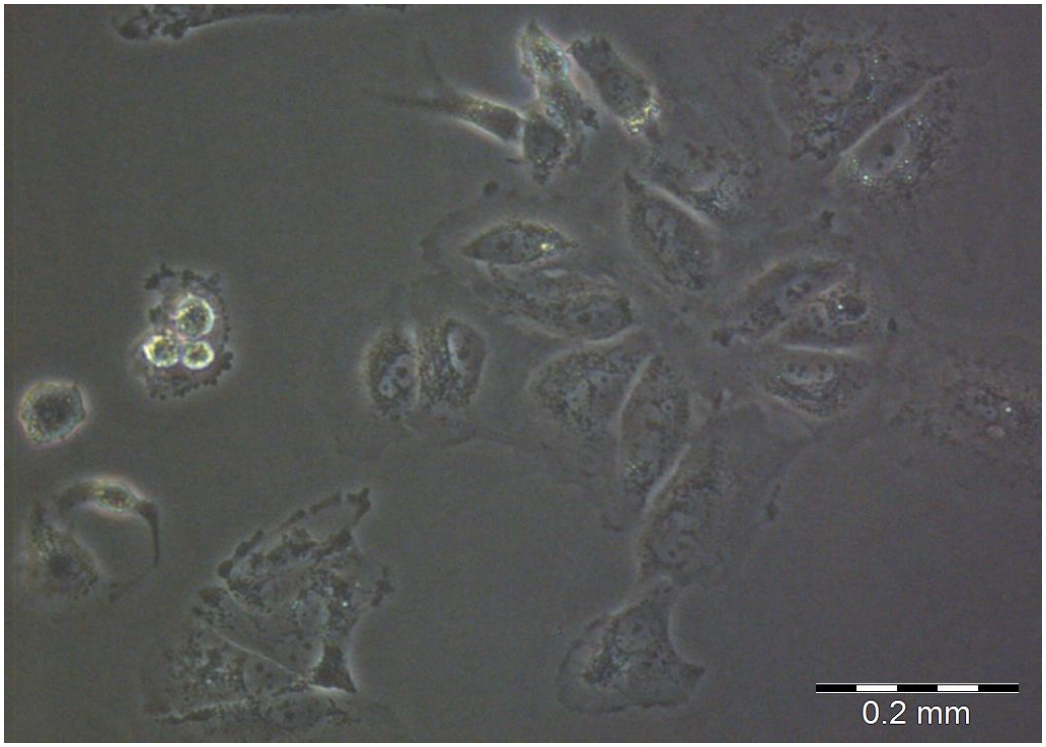


Figure 1.3: Morphological characteristics of Huh7 cells after one day culture under 20x magnification.



4.1.2 Anti-proliferative studies

Figure 2.1: Morphology of Huh-7 cells after seeding and before treatment under 4x magnification.

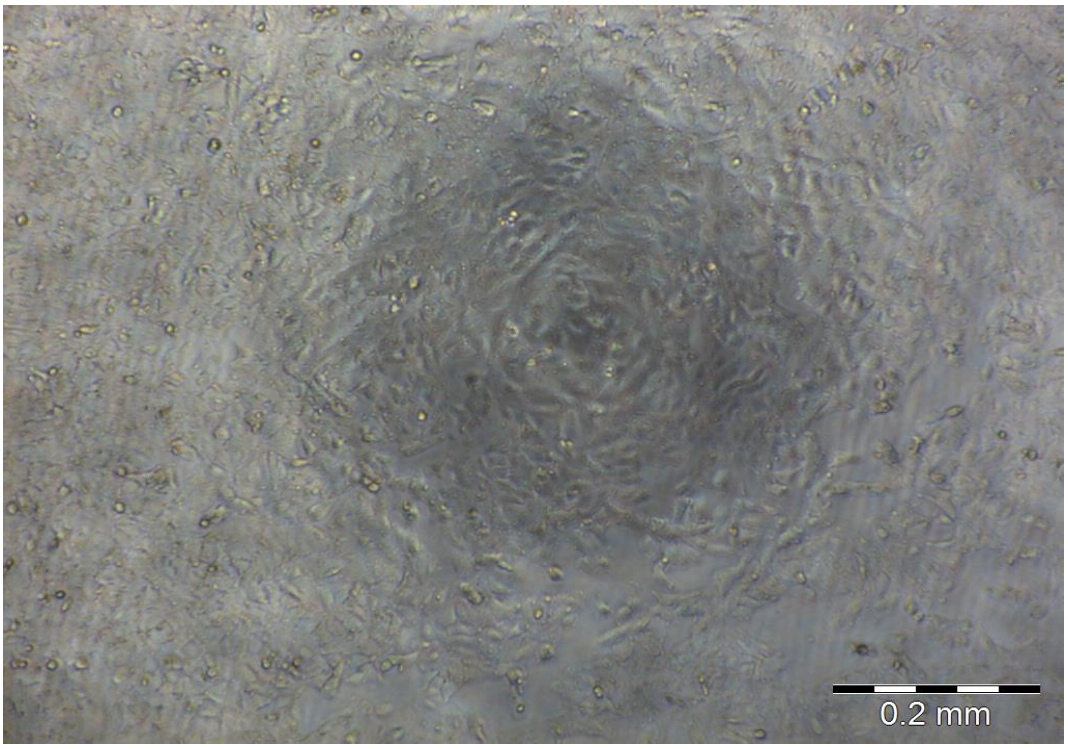


Figure 2.2: Morphology of Huh-7 cells after being treated 10 mg of *C. nutans* methanol-water extract under 4x magnification.

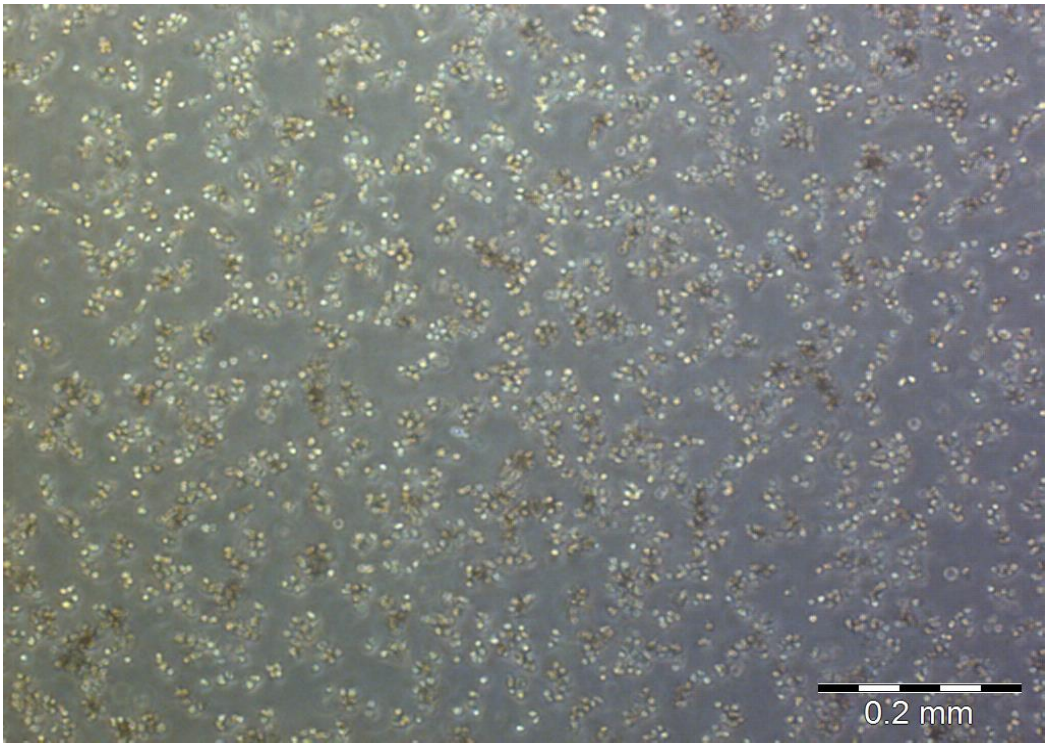


Figure 2.3: Morphology of Huh-7 cells after being treated 10 mg of *C. nutans* methanol-water extract under 20x magnification.

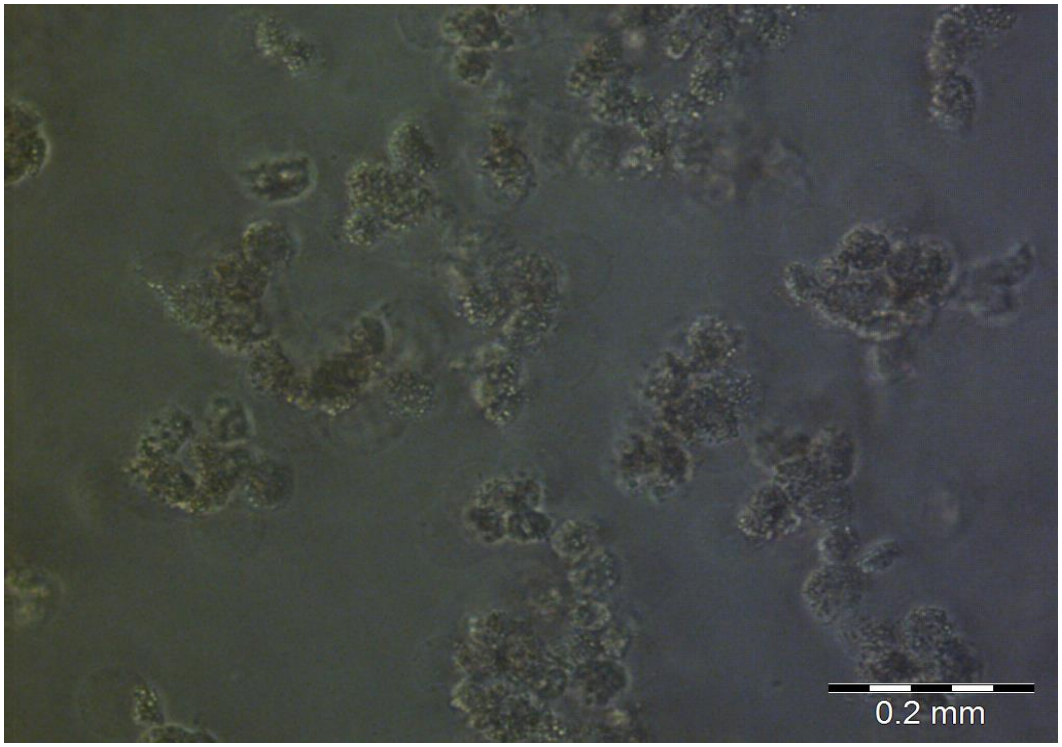


Figure 2.4: Morphology of Huh-7 cells after being treated 5 mg of *C. nutans* methanol-water extract under 10x magnification.

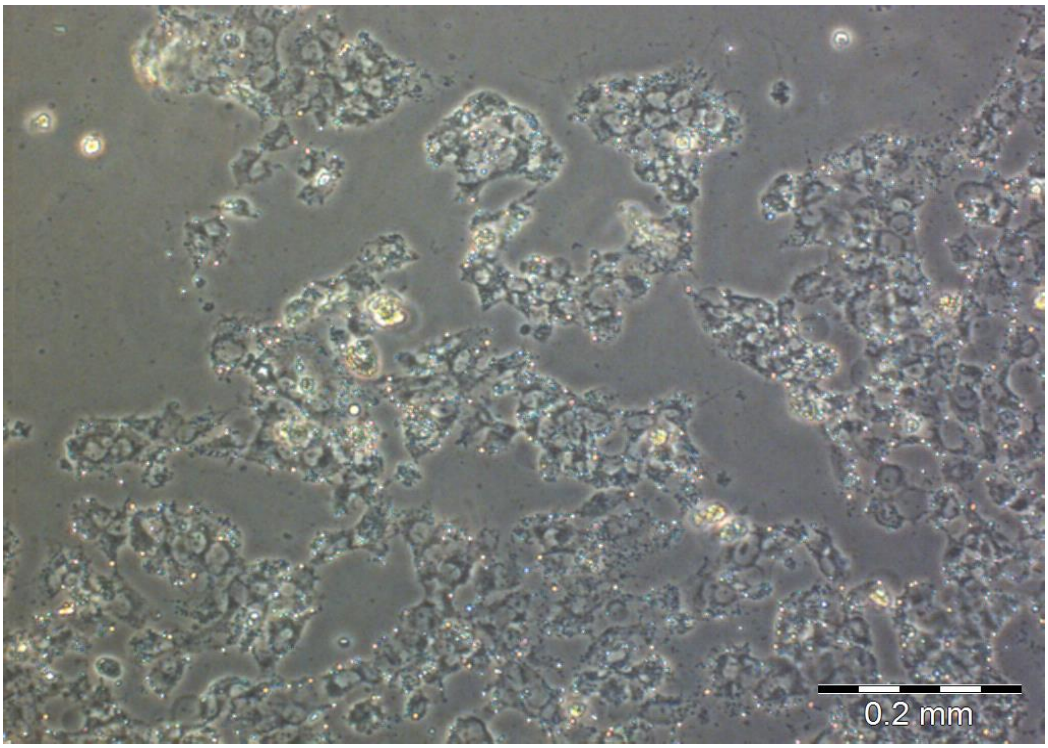
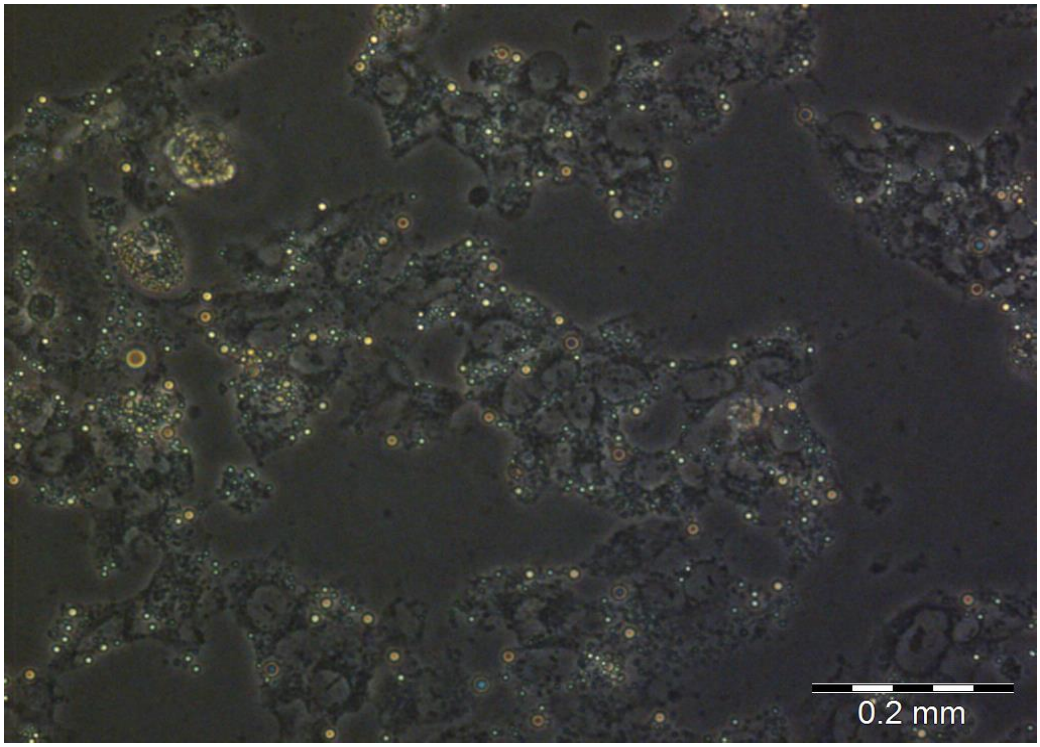


Figure 2.5: Morphology of Huh-7 cells after being treated 5 mg of *C. nutans* methanol-water extract under 20x magnification.



Anti-proliferative studies were done on the cell lines and the cell viability was determined by the absorbance of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and absorbance of sulforhodamine B (SRB) assay and the results are shown in Table 3 and Table 4. The results of the treated cells were recorded at three time points which are 24 hours, 48 hours and 72 hours. The lowest concentration used is 1 mg and the highest is 10 mg.

Table 1.1, 1.2 and 1.3 shows the average cell viability after 24, 48 and 72 hour treatment of *C. nutans* methanol-water extract respectively at ten concentrations (1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg and 10 mg) by using MTT assay.

Table 2.1, 2.2 and 2.3 shows the average cell viability after 24, 48 and 72 hours of treatment with *C. nutans* methanol-water extract respectively at ten concentrations (1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8, mg, 9 mg and 10 mg) by using SRB assay.

Based on the results shown, the treatment for 24 hours and 72 hours both have IC_{50} value of 5 mg. However, for the treatment with 48 hours, the IC_{50} was not well established as the viability of the cells did was not more than 50%. However, the trend line shows that there is a steady decrease in the cell viability after treatment with the *C.nutans* methanol-water extract at three time points which are 24 hours, 48 hours and 72 hours from MTT results. However, from the SRB results, there are signs of cell death however due to the color intensity of the extract at higher concentrations, the reading is affected as SRB assay relies heavily on the optical absorbance.

Figure 3.1: Effect of *C.nutans* methanol-water extract on the Huh-7 cell line at increasing concentrations at three different time points via MTT assay

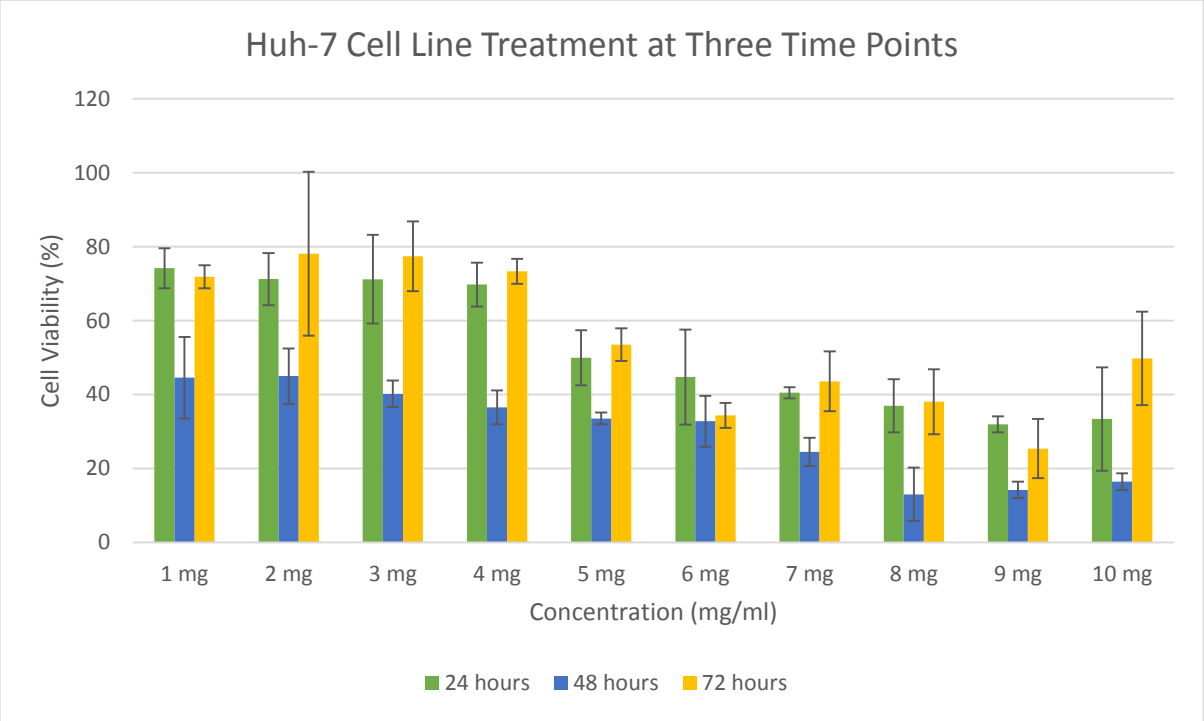


Figure 3.2: Effect of *C.nutans* methanol-water extract on the Huh-7 cell line at increasing concentrations at three different time points via SRB assay

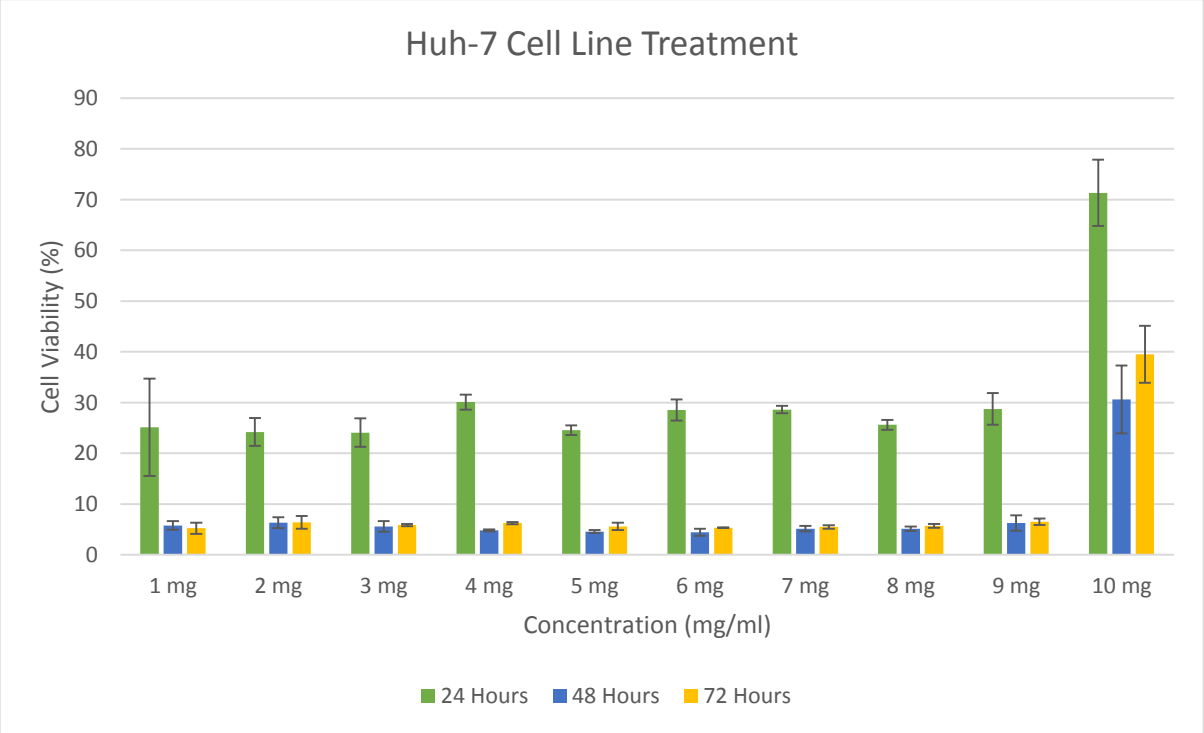


Figure 4.1: Huh-7 cell line treatment at 24 hours with different concentrations via MTT assay

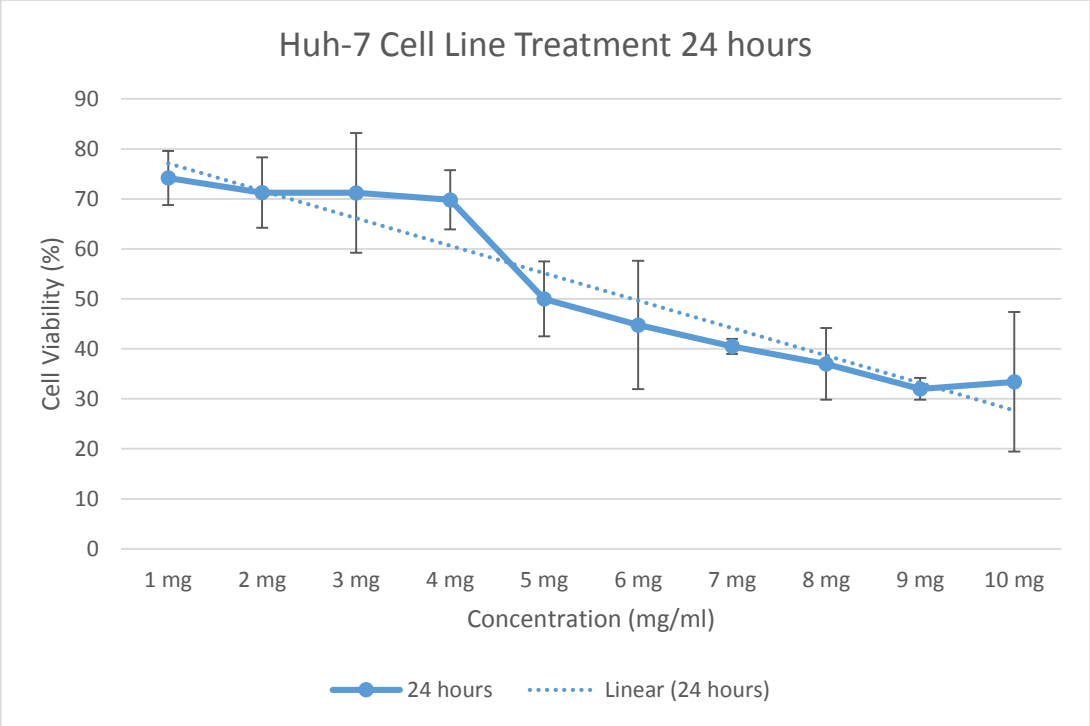


Figure 4.2: Huh-7 cell line treatment at 48 hours with different concentrations via MTT assay

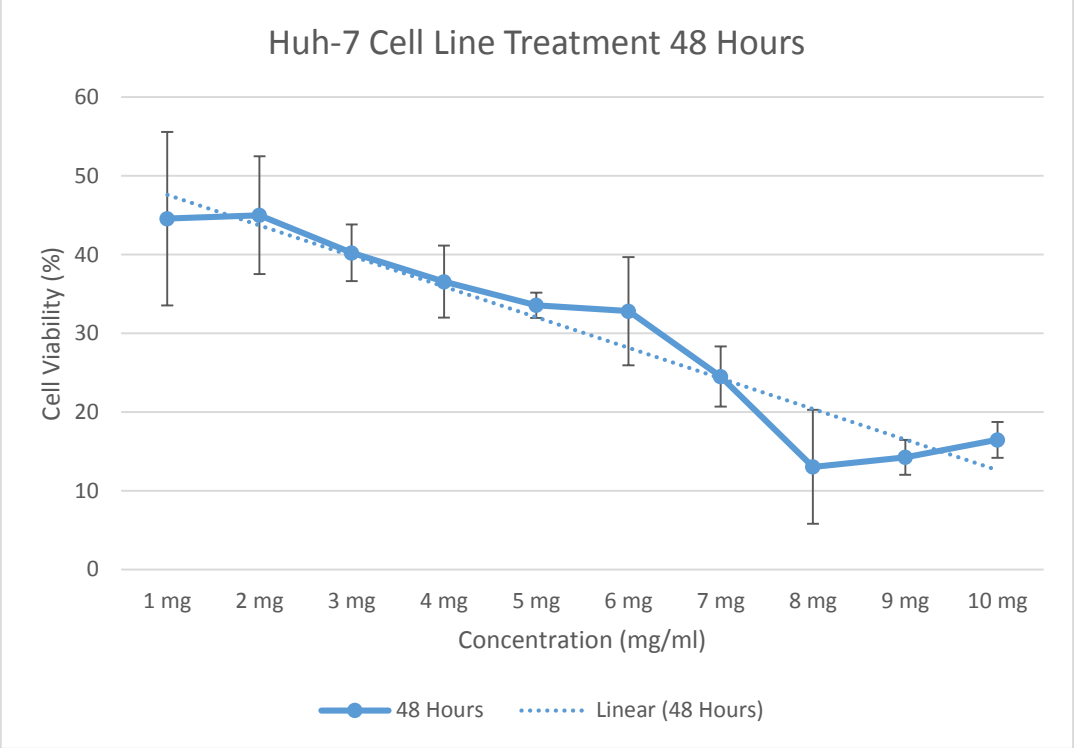


Figure 4.3: Huh-7 cell line treatment at 72 hours with different concentrations via MTT assay

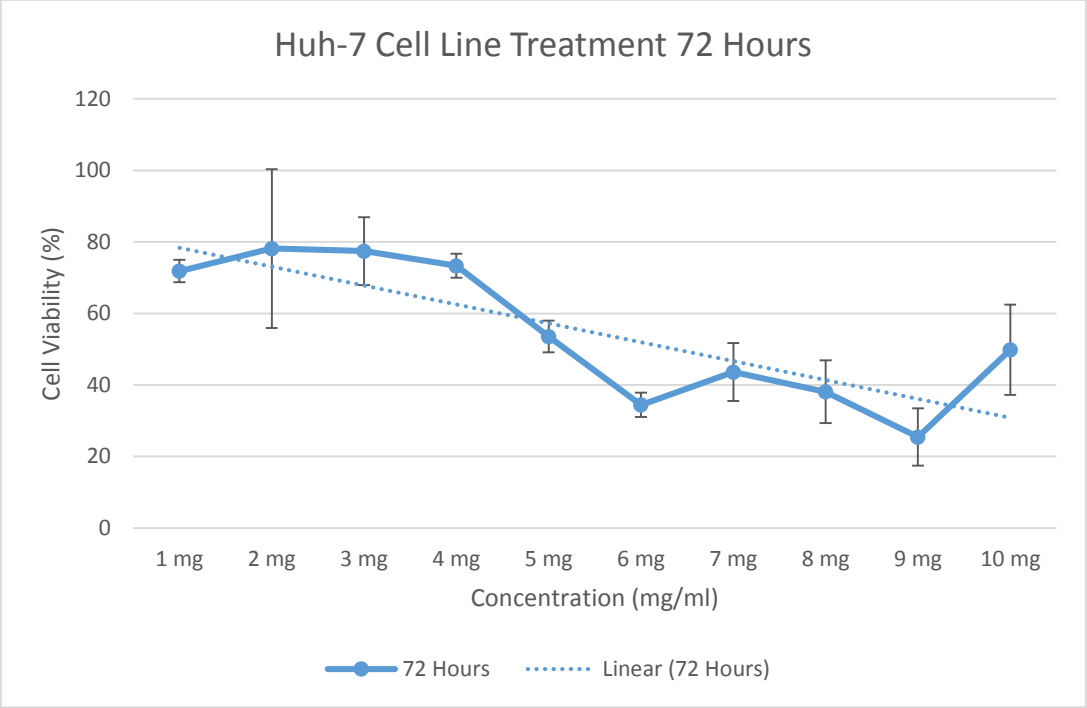


Figure 5.1: Huh-7 cell line treatment at 24 hours with different concentrations via SRB assay