THE EFFECT OF *NIGELLA SATIVA* ON THE MURINE MELANOMA CELL LINE, B16-BL6

by

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Abstract

Nigella sativa, commonly known as black seed, belongs to the botanical family of Ranunculaceae. Over recent years, there has been a growing interest in natural products including *N. sativa* due to their promising as anti-cancer effects. Several *in vitro* studies to determine the effect of *N. sativa* on the growth of the malignant melanoma cell line, B16-BL6, and non-malignant cell lines were performed. We have shown that treatment of cells with a 70% ethanol extract of *N. sativa* can significantly inhibit proliferation of both malignant and non-malignant cells. Treatment with an aqueous extract of *N. sativa* can reduce the growth of malignant cell proliferation while having a lesser effect on non-malignant cell proliferation. Ethanol extracts of *N. sativa* can induce apoptosis in treated B16-BL6 cells as confirmed using the Acridine Orange/Ethidium Bromide staining assay, Tunel assay, and Caspase activity assay. Some studies revealed that Thymoquinone is an important bioactive component of *N. sativa* and has anti-cancer effect. However, we aim to further investigate the active components of *N. sativa* using High-Performance Liquid Chromatography (HPLC) to identify the components that may be more effective in inhibiting cancer cell proliferation.

Keywords

Nigella sativa, B16-BL6 cells, Apoptosis, Caspases-2, Caspases-3, Caspases-6, Caspases-8, Caspases-9.

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This thesis is whole heartily dedicated to those who been affected by cancer. Whether they have lost battle, lost a loved one, or perhaps battling this disease right now. Remain strong and one day together we will find a cure.

Hessah Aldawd

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Abbreviations

ANOVA	Analysis of variance
AO/ EB	Acridine orange/ethidium bromide staining
ATCC	American Type Culture Collection
B16- BL6	Murine Melanoma B16- BL6
BCA	Bovine Serum Albumin
Bcl- 2	B-cell lymphoma 2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EToH	Ethanol
HBL100	Human breast
HEK293T	Human Embryonic Kidney 293 cells
HPLC	High-performance liquid chromatography
HSG	Human salivary gland cell
IL-2	Interleukin 2
LSM510	Zeiss LSM 510 Meta Confocal Microscope
MTT	Methyl thiazol tetrazolium assay
P53	tumor suppressor p53
PBS	Phosphate buffered saline

RNA	Ribonucleic acid
ROS	Reactive oxygen species
T98G	Human glioblastoma cells
TNF	Tumor necrosis factors
TUNEL	Terminal deoxynucleotidyl transferase
UVR	Ultraviolet Radiation

Chapter 1: Literature Review

1.1. Melanoma

Cancer is the leading cause of death worldwide, and in Canada it is responsible for about 30% of all deaths (Canadian Cancer Society, 2014). Among the different types of skin cancer, melanoma is the third most common and the most aggressive form with the highest mortality rate (Mandala, and Voit, 2013). In Canada, the rate of melanoma incidence has been significantly increasing every year. Based on 2013 estimates, 6000 Canadians will be diagnosed with melanoma and 1050 will die from it, with higher incidence in males than in females (Canadian Cancer Society, 2014). Melanoma has become a cancer with a major socioeconomic impact due to its relatively high incidence among young adults. Despite advances in cancer biology, malignant melanoma has a poor prognosis with a median survival of less than or equal to one year with chemotherapy (Garbe, and et al, 2011). Continuous efforts are being made to develop better and more cost effective treatments with fewer side effects that could reduce the incidence of the disease and improve the survival of cancer patients (Carocho and Ferreira, 2013). Over recent years, there has been a growing interest in natural products with anti-cancer properties as they are relatively cheap, and non-toxic with little or no side effects.

Melanoma is a well characterized cancer, and it usually starts in the skin from the malignant transformation of melanocytes – the cells that produce the pigment melanin

which gives color to the skin (Canadian Cancer Society, 2014). The transformed melanocytes proliferate uncontrollably and may begin to grow radially in the skin resulting in an early stage melanoma that is characterized by the growth of tumors along the dermo-epidermal junction (Uong and Zon, 2010). The radial growth may then be followed by vertical growth (Uong and Zon, 2010). The melanoma may invade the underlying dermis through the basement membrane and eventually metastasize (Uong and Zon, 2010).

Several genes involved in melanocyte progress have been associated with the initiation, progression and metastasis of melanoma (Uong and Zon, 2010). Genetic research studies show that defects in tumor suppressor genes, aberrant activation of receptor tyrosine kinases, mitogen activated protein kinases as well as variations and mis-expression of G-protein coupled receptors are some of the processes involved in malignant transformation of melanocytes (Wangari, et al, 2011). These genetic changes can convert melanoma from a non –invasive form into an invasive tumor that is capable of growing three-dimensionally and metastasizing (Wangari, et al, 2011).

Melanoma can affect any melanocyte-containing tissue including the skin, urinary tracts uveal cutaneous tissue, oral mucosa, and nasopharynx (Wangari, et al, 2011). There are three types of melanoma. Cutaneous melanoma is melanoma of the skin and is the most common type (Melanoma Research Foundation, 2014). Another form of melanoma that develop in any mucous membrane of the body such as the throat, nasal passages and vagina and is called mucosal melanoma (Melanoma Research Foundation, 2014). Ocular melanoma occurs in the eye and is a very unusual type (Melanoma Research Foundation, 2014).

1.2. Cell growth

Cell growth occurs through a series of highly regulated events that constitute the cell cycle. The cell cycle is divided into two preparatory phases, G1 and G2, which are required for the synthesis of cellular constituents needed to support the two functional phases, S and M phase, in order to complete cell division (Andreeff, 2013). During G1, rapid growth and metabolic activity occurs including RNA and protein synthesis (Andreeff, 2013). The cell continues to grow during S phase, and DNA is replicated. During G2 phase, the cell grows further and prepares for the M phase – Cell division. In normal cells, transition from one phase to another is regulated by checkpoints (Andreeff, 2013).

The two broad classes of genes that encode proteins which help control cell growth and proliferation are the tumor suppressor genes and the proto-oncogenes (Hyland, 2014). Tumor suppressor genes inhibit cell growth or trigger apoptosis whereas proto-oncogenes promote growth by encoding proteins that function as growth factors, growth factor receptors, nuclear transcription factors, etc (Andreeff, 2013). The proto-oncogene is usually mutated to form the oncogene which is always activated and results in unregulated cell growth and transformation (Hyland, 2014). The most frequently mutated gene in human cancer is the tumor suppressor p53 (Andreeff, 2013). Cells with mutated p53 fail to arrest in G1 and do not undergo apoptosis, resulting in uncontrolled cell growth and division leading to tumor development (Andreeff, 2013). In normal cells, the tumor suppressor genes act as braking signals during G1 to stop or slow down the cell's entry into the S phase. For example, the tumor suppressor p53 positively regulates the S phase checkpoint as shown in figure 1:1 (Ruddon, 2003). P53 is activated in

response to DNA damage, resulting in either G1 cell cycle arrest or apoptosis (Ruddon, 2003). In a similar manner, the Ras and Myc oncogenes act to inhibit the G1 and G2 phase checkpoints (Chow, 2010).

Unlike normal cells, cancer cells can proceed through these cell cycle checkpoints and can continue to replicate the damaged DNA thus accumulating mutations (Ruddon, 2003). Both copies of a tumor suppressor gene must be mutated for cancer to occur whereas only one mutated allele is sufficient to convert a proto-oncogene into an oncogene (Ruddon, 2003). Cells with mutated proto-oncogenes and tumor suppressor genes do not recognize cell signals and continue to grow and divide uncontrollably forming tumors. Genetic mutations could be the result of environmental factors or could be inherited.

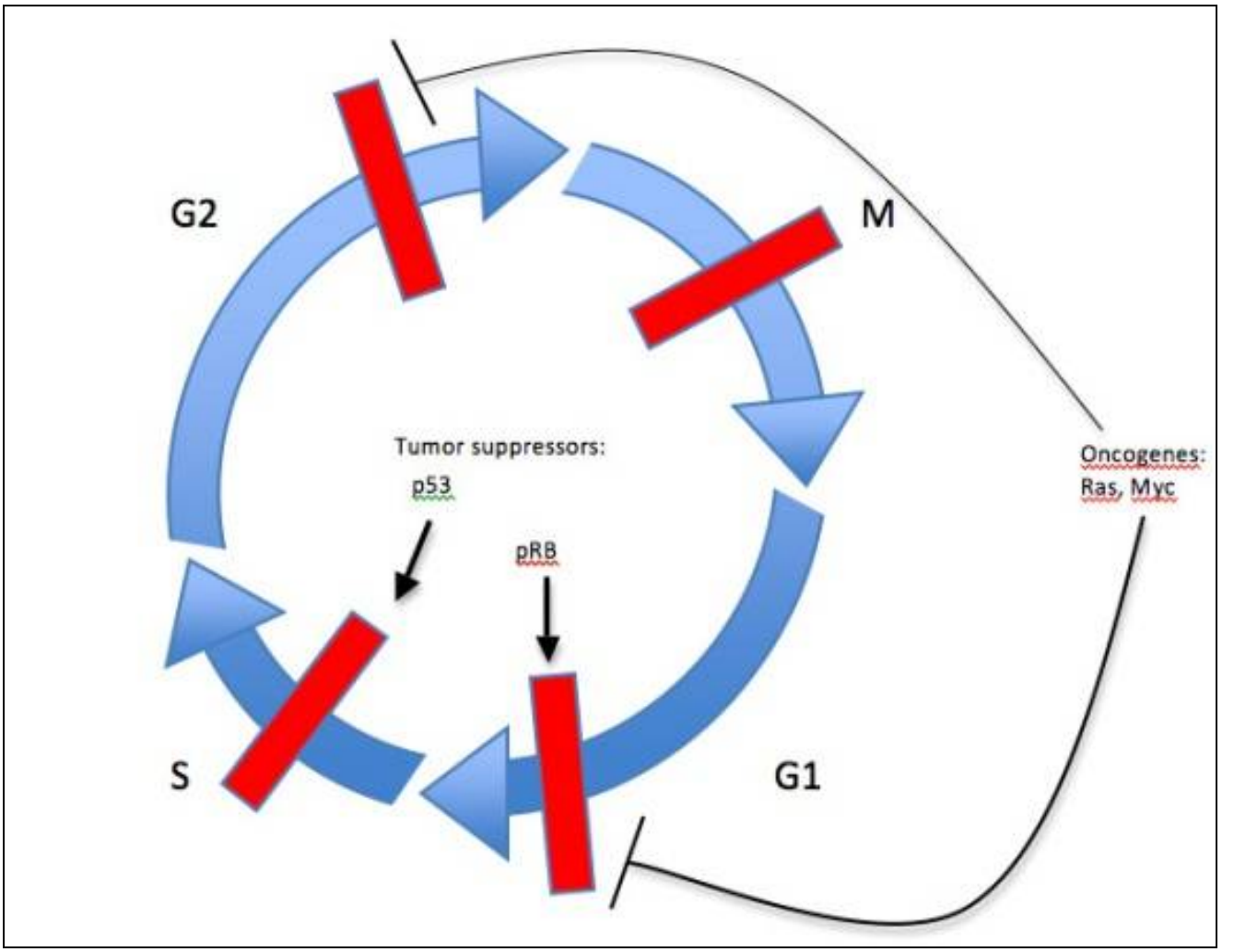


Figure 1.1. Cell cycle control by tumor suppressors and oncogenes (taken from Ruddon, 2003) <http://www.ncbi.nlm.nih.gov/books/NBK12516/>

1.3. Causes of melanoma

Melanomas like all cancers are caused by the accumulation of mutations in genes whose products directly regulate cell proliferation, apoptosis and DNA repair- (tumor

suppressor, proto-oncogenes and DNA repair genes) (Hyland, 2014).
Disruption in the balance between cell proliferation and cell death results in the loss of the ability to undergo apoptosis and/or leads to uncontrollable cell growth and cancer.

The global incidence of melanoma continues to rise. Several factors are associated with melanoma risk. However, the most important risk factors that predispose to the development of melanoma are exposure to the sun and ultraviolet radiation (UVR) (Canadian Cancer Society., 2014). UVR damages DNA through the formation of photoproducts and reactive oxygen species (ROS) (Grimaldi, et al., 2014). The risk increases with intermittent exposure and from exposure early in life (childhood and adolescence) (Armstrong, 1997). Among the three types of UVR, UVB is the most strongly associated with melanoma development. Tanning beds and sunlamps are another source of UVR and may increase the risk of melanoma (The Skin Cancer Foundation, 2014).

The risk of developing melanoma increases with the number, type, and size of common or atypical/dysplastic nevi (moles) (Mandala and Voit, 2013). Moreover, people with very large congenital melanocytic nevi are at a higher risk than those with small nevi (Canadian Cancer Society, 2014). Individuals with Familial atypical multiple mole melanoma (FAMMM) syndrome are also at a greater risk for melanoma. (Canadian Cancer Society, 2014).

Another risk factor is skin colour. The risk of melanoma is much higher in light skinned people as they have less melanin to protect them from UVR exposure (Canadian Cancer Society., 2014).

A number of gene mutations have been associated with the familial melanoma

that accounts for approximately 10% of all melanoma cases (Marzuka, et al, 2014). Mutations in these genes can result in uncontrolled cell growth and cancer. These melanoma susceptibility genes include; BRAF, p53, BAPT, and CDKN2A (cyclin-dependent kinase inhibitor 2A) (The Skin Cancer Foundation., 2014).The most common susceptibility gene is the CDKN2A locus and its mutated form shows an impaired capacity to inhibit the cyclin D1-CDK4 complex that allows for unchecked cell cycle progression (Marzuka, et al, 2014). Approximately 50% of all melanomas have oncogenic BRAF mutations, 90% of which are BRAFV600E (Box, et al, 2014). P53, the tumor suppressor gene that triggers cell death has also been shown to be inactivated in most melanomas (Box, et al, 2014). Mutations in the tumor suppressor BAP1 have also been shown to enhance the metastatic potential of certain types of melanomas (Marzuka, et al, 2014). Hence, the transformation of melanocytes into melanoma involves the interplay between some genetic factors, the tumor microenvironment, and UV exposure (Grimaldi, et al., 2014).

1.4. Apoptosis

Apoptosis is a form of programmed cell death that involves a series of genetically controlled events to eliminate old, damaged or unwanted cells from the body (Fernald and kurokawa, 2013). Apoptosis takes place via two major pathways: the extrinsic pathway, that involves death receptors; or the intrinsic pathway, that is linked to mitochondrial functions (Attoub, et al, 2013) as shown in figure 2 and figure 3. Both pathways consist of three stages: the induction phase (detection of damage signals); the

commitment phase (damage signals are integrated and modulated by pro or anti-apoptotic factors and initiator caspases which are caspase-2,-8,-9 or -10); and, the degradation phase (involves the activation of executioner caspases [e.g caspase -3 or -7] which degrade the matrix and cytoskeleton; and endonucleases which cleave DNA) (Attoub, et al, 2013).

The extrinsic pathway is initiated by extracellular ligands such as Fas and Tumor necrosis factor (TNF) binding to several types of death receptors, leading to the formation of the death-inducing signaling complex (DISC) which activates the initiator capases, caspase-8 and caspase-10 (Riedl, and Salvesen, 2007; Fernald and kurokawa, 2013). The intrinsic pathway involves the mitochondrial outer membrane permeabilization (MOMP) (Riedl, and Salvesen, 2007; Fernald, and kurokawa, 2013). MOMP triggers the release of pro-apoptotic mediators, apoptosis-inducing factor (AIF), heat-inducible serine protease HTR-A2 and cytochrome c in response to specific signals (Fernald, and kurokawa, 2013). The release of cytochrome c from the inter membrane space of the mitochondria leads to the assembly of a signaling platform, the apoptosome, which activates caspase-3/7 and leads to the cleavage of PARP (Poly ADP-ribose polymerase) and the death response (Attoub, et al, 2013; Gurung, et al, 2010; Riedl, and Salvesen, 2007). The Bcl-2 superfamily consisting of both pro- and anti-apoptotic members plays a major role in regulating apoptosis by interacting with MOMP at the mitochondria membrane (Fernald, and kurokawa, 2013).

Apoptosis is triggered in response to internal and external stimuli. Any disruption of the apoptotic process leads to abnormal cell growth and potential tumor development. Cancer cells have the ability to evade apoptosis by changing the function or expression of

anti- or pro-apoptotic proteins transcritptionally, translationally and post-translationally (Fernald, and kurokawa, 2013). Like any other cancer cell, melanoma cells also escape cell death by inactivating many sensors, regulators and effectors of the apoptotic and non-apoptotic pathways (Soengas and Lowe, 2003).

Cell death control during melanoma progression has been associated with three types of molecular changes: activation of anti-apoptotic factors; inactivation of pro-apototic effectors; and, reinforcement of survival signals (Soengas, and Lowe, 2003). Anti-apoptotic factors such as two members of the IAP family (surviving and ML-IAP) and FLIP are shown to be over expressed in invasive and metastatic melanoma (Irmler, et al, 1997; Grossman, and Altieri, 2001) Survival signaling in melanoma can be derived from the activation of the P13K/AKT/PTEN, the NF-KB and the Raf/MAPK pathways (Soengas and Lowe, 2003).

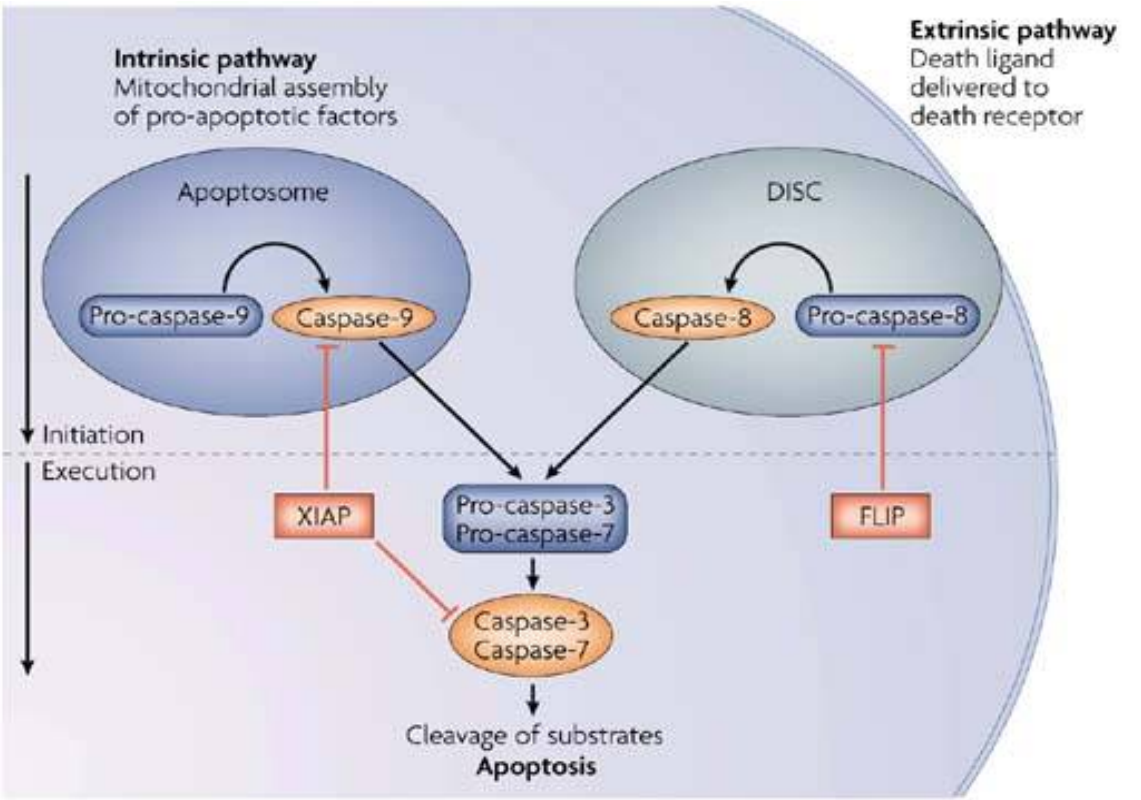


Figure 1.2. Apoptosis (taken from Riedl, and Salvesen, 2007)

<http://www.nature.com/nrm/journal/v8/n5/full/nrm2153.html>

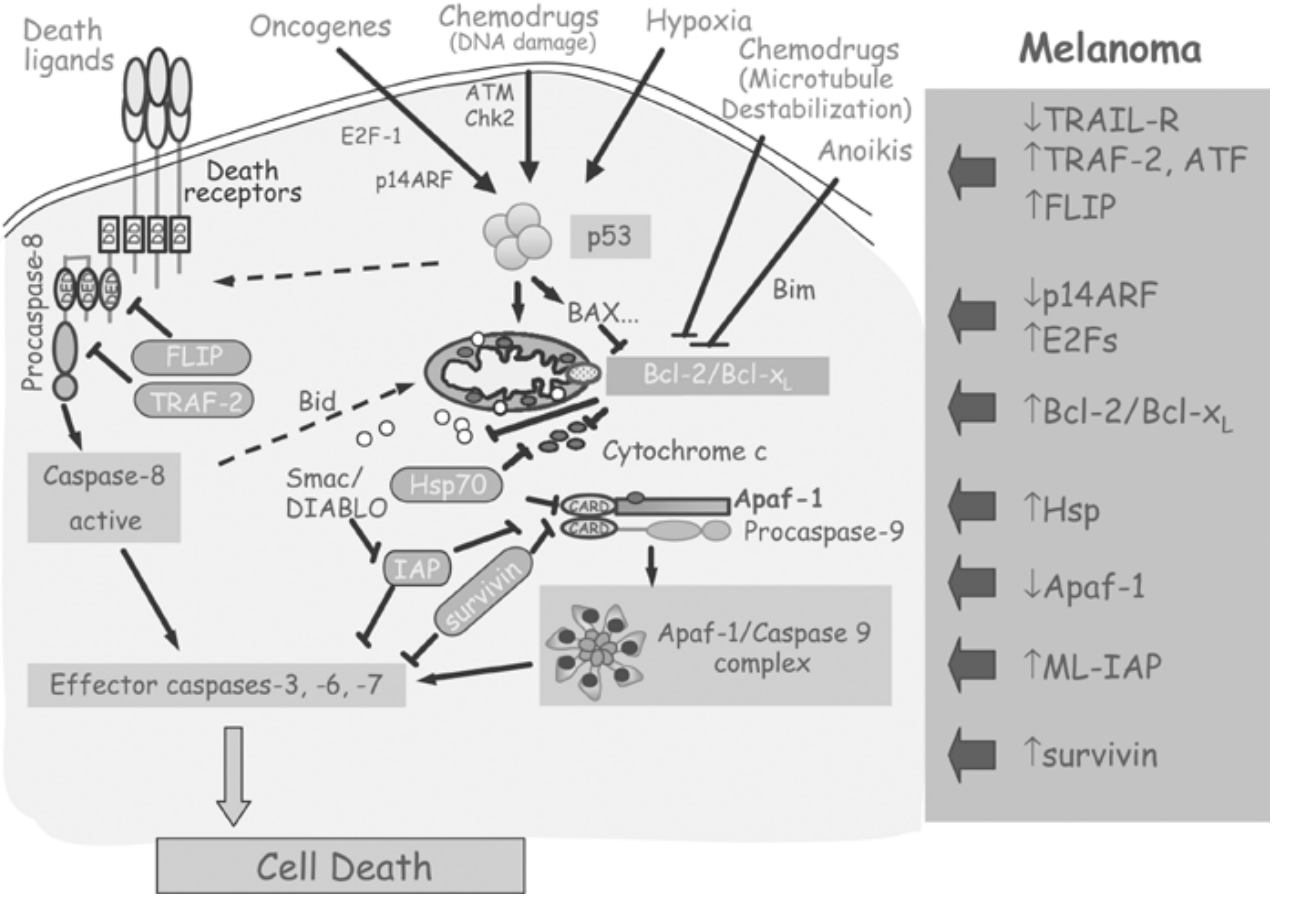


Figure 1.3. Simplified schematic of apoptotic pathways in mammalian cells:

(Death receptor-mediated) and intrinsic (mitochondrial) apoptosis (left panel). "Examples of upregulated and downregulated apoptotic factors in melanoma are indicated on the right panel" (taken from Soengas, and Lowe, 2003) <http://www.nature.com/onc/journal/v22/n20/full/1206454a.html>

1.5. Melanoma Treatment

The treatment for cancer depends on factors including the age and medical status of the patient, the extent and site of the disease, rate of disease progression, available treatments and the patients' wishes. Early Melanoma that is confined to the primary site is initially managed with surgical excision, with or without adjuvant chemotherapy and radiotherapy (Govindarajan, et al, 2003).

The three most common FDA- approved drugs that have been in use for the treatment of metastatic melanoma are dacarbazine, hydroxyurea and interleukin-2 (IL-2) even though response rates have been low and the overall survival results have been disappointing (Gogas, et al, 2013).

Dacarbazine is still considered as the standard treatment in Europe. Recent phase III clinical trials show low response rates between 6.8-13.8% and median progression-free survival between 1.5 and 2.9 months (Perrin, et al., 2014).
Currently, Hydroxyurea (HU) is used as a cancer chemotherapeutic agent which acts specifically on the S-phase of the cell cycle (Madaan, et al, 2012). HU is shown to inhibit the enzyme ribonucleoside diphosphate reductase thereby hindering the conversion of ribonucleotides to deoxyribonucleotides and as a result limiting new DNA synthesis (Madaan, et al, 2012).

IL-2 plays a central role in immune regulation as a T-cell growth factor (Gogas, et al, 2013). Administration of high doses of IL-2 has shown dose-dependent anti-tumor effects in phase II studies, although its effect has not been tested in phase III trials (Gogas, et al, 2013). Administration of IL-2 requires hospitalization due to its severe

toxic side effects (Gogas, et al., 2013). Moreover, the high costs associated with it did not allow extensive use in the community and it is not generally approved in the European countries (Gogas, et al, 2013). Surgery, chemotherapy and radiotherapy are the traditional therapies that have been shown to control local diseases in selected patients but have not had a significant impact on the survival of patients with advanced and metastatic melanoma (Wangari, et al, 2011). In fact, patients diagnosed with metastatic melanoma have a high mortality rate due to the resistance of most melanomas to chemotherapeutic drugs. Therefore, new and more effective therapies for melanoma are on-going. Immunotherapy and molecular targeted therapy hold great promise.

1.6. Immunotherapy

Immunotherapy is another form of cancer treatment that works by stimulating the body's own immune system to attack melanoma cells more effectively (American Cancer Society, 2013). It includes adoptive T cell therapy and vaccination. The main type of immunotherapy being used to treat some forms of advanced melanomas include the use of monoclonal antibodies targeted against immunosuppressive molecules such as CTLA4, PD-1 and PD-L1. One such monoclonal antibody called Ipilimumab or Yervoy is targeted against the cytotoxic T lymphocyte antigen-4 (CTLA4) which is expressed by T regulatory cells. (Wangari, et al, 2011). Ipilimumab works by blocking the CTLA-4 inhibitory signal allowing cytotoxic T lymphocytes (CTL) to destroy tumor cells (Ribas, 2012). It has been shown to increase overall survival by 2 years in patients with advanced melanoma (Weber, et al, 2009). Ipilimumab was approved in 2011 by the U.S. FDA for

the treatment of melanoma (Mandala, and Voit, 2013). Tremelimumab is another anti-CTLA 4 monoclonal antibody which is being used to treat patients with metastatic melanoma (Gogas, et al, 2013).

Another potential target molecule for immunotherapy of human melanoma is HER2. HER2 is a glycoprotein belonging to the family of epidermal growth factor receptor with intrinsic tyrosine kinase activity (Ma, et al, 2013). The use of Herceptin, a humanized monoclonal antibody has been proven to be an effective treatment in breast cancer patients with HER2 overexpression (Ma, et al, 2013). Herceptin binds to the extracellular, juxtamembrane domain of HER2. HER2 overexpression has also been demonstrated in patients with metastatic melanoma and therefore suggests the potential role of Herceptin in the treatment of melanoma (Ma, et al, 2013).

Melanoma vaccines are still being studied in clinical trials (American Cancer Sociey, 2014). However, sometimes to treat stage 3 melanomas, the Bacille Calmette-Guerin (BCG) vaccine is used by injecting it directly into tumors (American Cancer Sociey, 2013). BCG is a potent activator of the immune system which works by activating an immune response near the site of injection (American Cancer Sociey, 2013).

1.7. Molecular targeted therapy

Melanoma is usually resistant to cytotoxic chemotherapy therefore systemic therapies have been largely ineffective. In recent years, advanced understanding of the causing genetic events that cause melanoma has led to the development of new targeted drug therapies. Molecular studies show that most melanomas contain mutations,

particularly amine acid substitutions, in the serine-threonine kinase BRAF (Patol, 2013) as well as in the mitogen-activated protein kinase (MAPK) pathway (Johnson, and Sosman, 2013). Vemurafenib, Dabrafenib and Trametinib are three drugs that act against the altered BRAF gene (Canadian Cancer Society, 2014). These drugs have showed better rates of progression-free and overall survival in patients with advanced melanoma when compared to chemotherapeutic drugs including dacarbazine (Canadian Cancer Society, 2014).

Vemurafenib was approved in 2011 for the treatment of metastatic melanoma (Gogas, et al, 2013). It is a small tyrosine inhibitor that has been shown to promote complete or partial tumor regression with a median PFS (Progression Free Survival) of 7 months (Bollag, et al, 2014). Vemurafenib and Dabrafenib, the two BRAF inhibitors and Trametinib, a MEK inhibitor have all shown their effectiveness by increasing overall survival in phase III trials for patients with BRAF-mutant melanomas (Mandala, and Voit, 2013).

Additionally, imatinib is another promising targeted therapy for patients with KIT-mutant melanoma (Johnson and Sosman, 2013). Two phase II trials have been completed which demonstrate the activity of imatinib in patients with KIT mutant melanoma (Johnson and Sosman, 2013). In the first trial, 28 patients with KIT mutations were treated with imatinib and showed a durable response rate of 16%, a median progression-free survival of 12 weeks and 72% of the patients had at least temporary disease stabilization (Johnson and Sosman, 2013; Carvajal, et al, 2011). Whereas in the second trial, 43 patients with KIT aberrations showed a 23% response rate, an additional 30% of the patients showed temporary disease stabilization, and the median progression-

free survival was 3.5 months (Guo, et al, 2011; Johnson, and Sosman, 2013). Mucosal and acral melanomas often have mutations in the KIT receptor and could be treated with imatinib (Patol, 2013). The difficulty with these new treatments is that they are very responsive and are associated with significant side effects in the treated patients.

1.8. Introduction to Natural Products

The incidence of melanoma is increasing worldwide. The early stage melanoma is confined to epidermis and is curable through surgical excision (Looi, et al, 2013; Govindarajan, et al, 2003). However, the prognosis decreases with increased thickness of the lesions mainly due to the tendency of melanoma to metastasize (Govindarajan, et al, 2003). Moreover, melanomas are characterized to be highly resistant to most forms of chemotherapy which contributes to high morbidity and mortality rates in patients (Govindarajan, et al, 2003). The poor prognosis for metastatic melanoma, due to lack of effective treatments, prompts the need for compounds with greater anti-melanoma activity. Experimental studies show that many natural products and plant extracts have anticancer potential in a variety of bioassay systems and animal models (Abukhader, 2013).

A natural product (NP) is a chemical substance or group of substances produced by a living organism that are used without chemical modification or treatment. Natural Products including medicinal plants have been used for treatment and prevention of diseases for many centuries. Among the various medicinal plants, *Negilla sativa,* an oriental spice, has been widely used in herbal medicine due to its wide spectrum of

pharmacological actions (Ahmad, et al 2013). In Islamic literature, it is considered to be one of the greatest forms of healing medicine and is referred to as the miracle herb (Ahmad, et al 2013). The seeds of the plant have been used to prevent and cure disease for centuries especially in the Middle East and Southeast Asia (Gilani, et al, 2004). Recently this herb has shown encouraging potential as an anticancer agent.

1.9. *Nigella Sativa*

N. Sativa, popularly known as black seed, is an annual flowering plant that originated from southeastern Asia (Khan, et al, 2011; Ahmad, et al 2013). The plant, belongs to the botanical family *Ranunculaceae* (A), and is widely grown in different parts of the world, most commonly in Eastern Europe, the Middle East and Western Asia (Randhawa, and Alghamdi, 2011).

N. sativa grows up to 30 cm in height with linear leaves that are finely divided. The flowers are usually blue and white, without involucres (Figure 1.4). The fruits are large inflated capsules composed of 3-7 united follicles which contain the seeds. The seeds (Figure 1.5) produced are small dicotyledons, black externally with an aromatic odor and bitter taste (Ahmad, et al 2013).

Figure 1.4. *Nigella Sativa* Flower **Figure1.5. *Nigella Sativa* Seeds**

Source: http://en.wikipedia.org/wiki/File:Nsativa001Wien.jpg

http://en.wikipedia.org/wiki/File:Nigella_Sativa_Seed.jpg

The seeds are extensively used as a spice for nutritional flavorings such as in breads, soups, pickles, sauces, etc (Khan et al, 2011). It is also used in cosmetics and externally on the skin (Hajhashemi, et al, 2004). Raw seeds as well as the seed oil of N. sativa has been widely used all over the world in traditional medicine for the treatment of various illnesses including bronchitis, rheumatism, diarrhea, headache, hypertension, gastrointestinal problems and eczema (Ahmad, et al 2013; Woo, et al, 2012).

In recent years, extensive research on *N.sativa* shows that it possess a variety of phytochemical and pharmacological effects including antidiabetic, anticancer, analgesic, antimicrobial, bronchodilator, anti-inflammatory and antioxidant properties (Ahmad, et al, 2013). The chemical composition of black seeds has been characterized using a variety of analytical and spectroscopic techniques and has been shown to contain both

fixed and essential oils,15 amino acids, proteins, carbohydrates, alkaloids, saponins (Ali, and Blunden, 2003) as well as minerals including calcium, iron, sodium and potassium (Gali-Muhtasib, et al, 2006). The identified bioactive components of black seeds include thymoquinone, thymol, thymohydroquinone and dithymoquinone (Attoub, et al, 2013). However, the most important active component of black seeds is thymoquinone (TQ) found in the essential oils (Ahmad, et al., 2013).

1.10. Thymoquinone

Thymoquinone (TQ) is the major bioactive component of the volatile oils of black seed, *N. sativa* (Attoub, et al, 2013). The chemical structure of TQ is shown in (Figure 1.6).

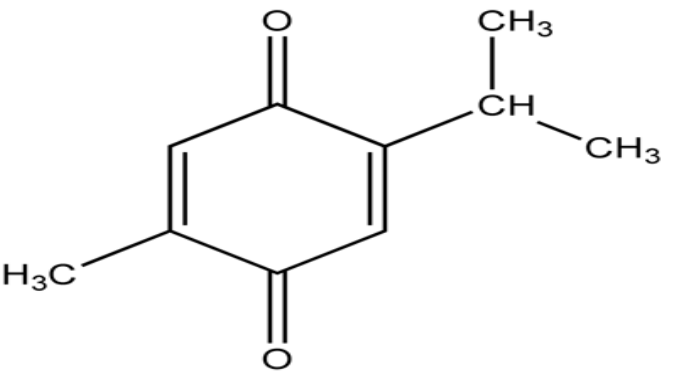


Figure 1.6. Chemical structure of thymoquinone

Cell Biology International. Portland Press limited. Web. 23 April 2014.

Source: http://www.cellbiolint.org/cbi/035/1025/cbi0351025f05.gif

TQ has been shown to exert anti-oxidant (Khan et al, 2011), anti-inflammatory and antineoplastic effects both *in vitro* and *in vivo* (Gali-Muhtasib, et al, 2006; Woo, et al, 2012). Studies show that of TQ is effective against various diseases like cancer, diabetes, asthma and kidney diseases etc (Khan, et al, 2011).

1.11. Black seed and cancer

Extensive research studies have investigated the wide spectrum of pharmacological actions of *N.sativa* including its antibacterial, antifungal, antioxidant, antidiabetic and anticancer activity (Ahmad, et al, 2013). TQ is the most abundant bioactive constituent of black seed identified so far and a major anticancer agent (Attoub, et al, 2013) with a low general toxicity (Effenberger and Schobert, 2011).

1.12. Thymoquinone's potential role as an anticancer agent

Scientific studies have revealed the potential of TQ as an anticancer agent. Evidence comes from several experimental findings that demonstrate the promising role of TQ on a variety of tumor cells including human colon, breast, brain, melanoma, pancreatic and ovarian cells (Gali-Muhtasib, et al, 2006; Gurung, et al, 2010; Rooney, and Ryan, 2005; Woo, et al, 2012).

Attoub et al (2013) examined the effect of increasing TQ concentrations on cells derived from lung (LNM35), liver (HepG2), colon (HT29), melanoma (MDA-MB-435), and breast (MDA-MB-231 and MCF-7) tumors. The results provided evidence that TQ, at non-toxic levels, showed significant inhibition of viability in these cancer cells by

inhibiting Akt phosphorylation and promoting activation of the mitochondrial signaling pro-apoptotic pathway (Attoub, et al, 2013). They further demonstrated the anticancer potential of TQ in combination with chemotherapeutic drugs such as cisplatin. TQ was shown to synergize with the DNA-damaging agent cisplatin in order to inhibit cell viability (Attoub, et al, 2013).

TQ has shown to induce apoptosis in tumor cells which is a key mechanism for the effectiveness of anti-cancer drugs. Studies show that TQ induces apoptosis using both p53-dependent and p53-independent mechanisms (Effenberger and Schobert, 2011; Gali-Muhtasib, et al, 2006; El-Mahdy, M, et al., 2005) as detected by chromatin condensation, translocation of phospatidyl serine across the plasma membrane, DNA fragmentation (Abukhader, 2013) and activation of the intrinsic apoptotic pathway (Attoub, et al., 2013). It has been found to delay tumour growth *in vivo* through cell cycle arrest in several xenograft models (El-Mahdy, et al, 2005). Moreover, several studies reported a role for TQ in "suppressing COX-2, survivin, Bcl family proteins, STAT3 phosphorylation of NF-kB, Akt activation, extracellular signal regulated kinase phosphorylation (ERK) and inhibition of telomerase activity" (Gurung, et al, 2010; Arafa, et al, 2011; Sethi, et al, 2008; Banerjee, et al, 2009; Li, 2010; Yi, et al, 2008).

A recent study by Ahmad et al, (2013) demonstrated that TQ suppresses metastasis of melanoma cells by inhibiting the NLRP3 (NACHT, LRR, and pyrin domain containing protein 3) inflammasome (Ahmad, et al, 2013). The inflammasome is a multiprotein complex which regulates caspase – 1 activation and IL-1β and IL-18 secretion when activated (Ahmad, et al, 2013). The study showed that TQ inhibits the

migration of both mouse and human melanoma cells *in vitro* and metastasis of mouse cells *in vivo*, thus suggesting a potential role for TQ as an immunotherapeutic agent in the control and prevention of metastatic melanoma (Ahmad, et al, 2013). Salomi et al (1990) showed that N. sativa extracts when applied topically in mice, inhibited two-stage initiation/promotion of skin carcinogenesis (Khan, et al, 2011). Then suggested that *N. sativa* and one of its major metabolites, TQ, may provide a good starting point for the development of an anti-melanoma therapeutic.

1.13. Thesis objectives

The first objective of this study was to test the effect of two different extracts of *N. sativa* (ethanolic, aqueous) on two malignant (murine melanoma B16-BL6, brain cancer T98G) and three non-malignant (human embryonic kidney 293T, human breast HBL 100, human salivary gland HSG) cell lines.

The second objective of this study was to determine the mechanism of action of *N. sativa* on the murine melanoma B16-BL6 cell death pathway and focus on apoptotic pathways.

Chapter 2. Materials and Methods

2.1. Drug preparation

The *N. sativa* seeds used in this study were obtained from Paris Natural Foods store of Sudbury, Canada (made by Sunn Herbal, Vancouver, BC, Canada: 150812). For the preparation of the alcoholic extract, 100mg of *N. sativa* was mixed with 1 ml of 70% ethanol. For the preparation of the aqueous extract, 100mg of *N. sativa* was mixed with 1 ml of water. Both suspensions were boiled for one hour and then filtered through 0.22 µM and stored at -80 °C until used.

2.2 Tissue culture

The cell lines that were tested with *N. sativa* included the B16-BL6 (murine melanoma), T98G (brain cancer), HBL 100 (human breast), 293T (human embryonic kidney) and HSG (human salivary gland). Cell lines were obtained from the American Type Culture Collection, (Manassas, VA). All of these cells were maintained in Dulbecco's Modified Essential Medium (DMEM, Hyclone, Logan, UT) which was supplemented with 10% fetal bovine serum (Hyclone), 100 µg/ml streptomycin, and 100 µ/ml penicillin (Invitroogen, Burlington, ON) at 37°C in a humidified atmosphere in a 5% CO_2 incubator.

2.3. MTT assay (Methyl Tetrazolium Blue)

To assess the effect of the *N. sativa* extracts on the growth of different malignant and non-malignant cell lines, the MTT assay was performed. The reduction of MTT to an insoluble formazan purple salt can be quantified by measuring the absorbance of light at 540nm. Since reduction can only occur in living cells, the color intensity is an important measure of the viability of cells. Approximately, $2x10^3$ cells/well were added a 96-well plate and incubated at 37°C with 5% CO_2 for 24 hours. The cells were treated with various concentrations (0.05, 0.1, 0.5, 1, and 2 %) of the *N. sativa* extracts. The viability of the cells determined each day for five days. 10 µl of MTT was added to each well at a final concentration of 0.4 µg/ml and incubated for 4 hours. The media was removed and 100 µl of dimethyl sulfoxide (DMSO) was pipetted into each well to solubilize the formazan crystals. The absorbance was then measured at 540 nm using a (SpectraMax 340 PC 389) plate reader. The data for cell growth at each concentration was statistically analyzed by performing ANOVA using Graph Pad Prism Software.

2.4. Morphological analysis

Morphological changes in cells treated with various doses (0.05, 0.1, 0.5, 1, and 2 %) of both ethanolic and aqueous extracts of *N. sativa* were documented using an inverted phase contrast Axiovert 100 microscope Northern Eclipse software.

2.5. Cell Staining assay (Acridine Orange/Ethidium Bromide)

Acridine orange and ethidium bromide staining was performed to visualize changes in cellular morphology associated with apoptosis. B16-BL6 cells were plated on glass cover slips and incubated overnight at 37°C with 5% CO_2. The cells were then treated with the indicated *N. sativa* preparations for different time points (24, 48, and 72). Camptothecin was used as a positive control for apoptosis. The cells were stained with 10 µg/ml of acridine orange (Sigma-Aldrich) and 10 µg/ml of ethidium bromide (Sigma-Aldrich) at 37°C for 15 minutes. The coverslips were rinsed in PBS, and mounted onto a glass slide. The fluorescence was viewed using an LSM 510 fluorescence microscope.

2.6. TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling)

The tunel assay was performed to detect for DNA fragmentation that results from apoptosis (Roche, Laval, QB). B16-BL6 cells were plated onto glass cover slips, incubated for 24 hours at 37°C with 5% CO_2 and then treated with media containing the indicated *N. sativa* preparations for three different time points (0, 48 and 72 hours). Camptothecin was used as a positive control for apoptosis. The cells were washed with PBS and then fixed in 1 ml of 10% formaldehyde. 1 ml of 10% formaldehyde was added to each well and incubated at 4°C for 5 minutes. The fixative solution was removed, the cells washed twice with PBS and then permeabilized using 1% triton X- 100 in PBS permeabilisation solution incubated for 3 minutes at 4°C and then washed twice with PBS. 50 µl of the Tunel reaction mixture was added to each well and incubated for 1

hour. Cells were viewed using a Zeiss LSM5 fluorescence microscope and images were recorded.

2.7. Caspase activity assay (caspase colorimetric protease assay sampler kit)

The caspase activity assay was performed to determine the increased enzymatic activity of caspases-2, -3, -6, -8, -9 in apoptotic cells by a colorimetric reaction (Apotarget Caspase Colometric Protease Assay Sampler Kit, Invitrogen Corporation). B16-BL6 cells cultured on 150x20 cm plate were treated with the indicated *N. sativa* preparations for 24 hours. Cells corresponding to 3-5 x 10^6 cells/sample were lysed in 50 µl of chilled Cell Lysis Buffer at 4 °C, incubated on ice for 10 minutes, and then centrifuged at 10,000 x g for 1 minute. The supernatants were transferred to a new tube and incubated on ice. The supernate were diluted to a concentration of 50-200 µg protein per 50 µL Cell Lysis Buffer (1- 4 mg/mL). To each sample, 50 µL of 2x Reaction Buffer (containing 10 mM DTT) was added. Then, 5 µL of the 4 mM colorimetric substrate (200 µM final concentration) was added to each sample and incubated at 37 °C for 1 - 2 hours. The (96-well) plate was read on a (SpectraMax 340 PC 389) plate reader using 405 nm wavelength. The fold-increase in caspases-2, -3, -6, -8, -9 activity were determined by comparison with uninduced control. Camptothecin was used as a positive control treatment for caspase activation.

2.8. HPLC (High-performance liquid chromatography)

20 gm of *N. sativa* powder extract was suspended and boiled for one hour in 200 ml of 70% ethanol. The mixture was filtered through 1 mm whatman filter paper and the filtrate was frozen at -80 ºC and dried using a lyophilizer. For some experiments the *N. sativa* extract was fractionated over an ethanol-water gradient. For these experiments, the *N. sativa* extract was mixed in 20 gm poly (vinylpolypyrrolidone) matrix (Sigma Chemical, St Louis, MO) and 200 ml water. The mixture was poured into a column and eluted with different concentration of ethanol to fractionate it into different fractions. It was eluted with 200 ml each of water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 100% ethanol. The fractions were freeze dried and the resulting powder weighed. Resuspended extracts were characterized using HPLC on a Breeze 2 chromatography system (Waters Inc, Toronto, ON). Each fraction of *N. sativa* was separated using a Sunfire C18 column 3.5 µm resin 4.6x100 mm. The solvents (A) 60 volumes 10 mM phosphate buffer, pH 6.6, 20 volumes acetonitrile, and 20 volumes methanol, and (B) 30 volumes 10 mM phosphate buffer, pH 6.6, 35 volumes acetonitrile, and 25 volumes methanol, were used. To determine the alkaloid composition of these fraction, solvents were pumped through the column at 1 ml/min. Over a time period of 40 min, the solvents were mixed using a liner gradient starting with 100% buffer A and finishing with 100% B. Then, for 10 min, 100% buffer B was pumped through column. Lastly, for 5 min, a gradient starting with 100% B and finishing with 100% was pumped through the column. The *N. sativa* components were identified at a wavelength of 245 nm. The identified peaks were compared to the (Thymoquinone) standard (Sigma).

2.9. Statistical analysis

In this study, the data were presented as mean ± standard deviation of three independent experiments performed in triplicate and were analysed by ANOVA using Graph Pad Prism Software. In the caspase activity assay, the data were presented from two experiments.

Chapter 3. Results

3.1. Cell viability

The anti-proliferative effect of *N. sativa* against malignant (B16-BL6, TG8G) cell lines and non-malignant (293T, HSG, HBL100) cell lines was determined using the MTT assay. Treatment with the ethanolic extract of *N. sativa* at a high concentration (2%) showed a significant ($p<0.05$) inhibition of 90% cell growth in the malignant cell lines B16-BL6 and T98G compared to other concentrations (0.05%, 0.5%) and untreated cells (Figures 3.1,.3.3). Treatment with the aqueous extract of *N. sativa* at a high concentration (2%) showed a significant ($p<0.05$) decrease in the number of B16-BL6 and T98G cells by ~50% (Figures 3.2, 3.4) compared to other concentrations (0.05%, 0.5%) and untreated cells.

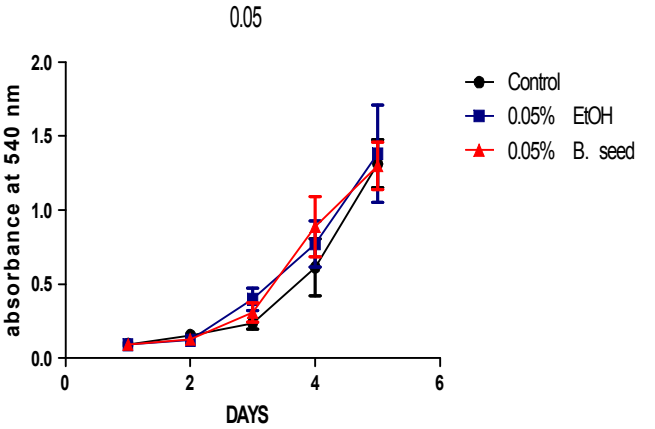
Treatment with the ethanolic extract of *N. sativa* at a high concentration (2%) on non-malignant cell lines (HEK 293T, HSG, HBL 100) completely inhibited cell growth compared to other concentrations (0.05%, 0.5%) and untreated cells (Figures 3.5, 3.7, 3.9). Treatment with the aqueous extract of *N. sativa* at all concentrations (0.05, 0.5, and 2 %) on non-malignant cell lines (HEK 293T, HSG, HBL 100) caused an increase of cell growth over time (Figures 3.6, 3.8, 3.10).

In all experiments, the ethanolic extract of *N. sativa* was more effective in inhibiting cell growth than was the aqueous extract of *N. sativa*.

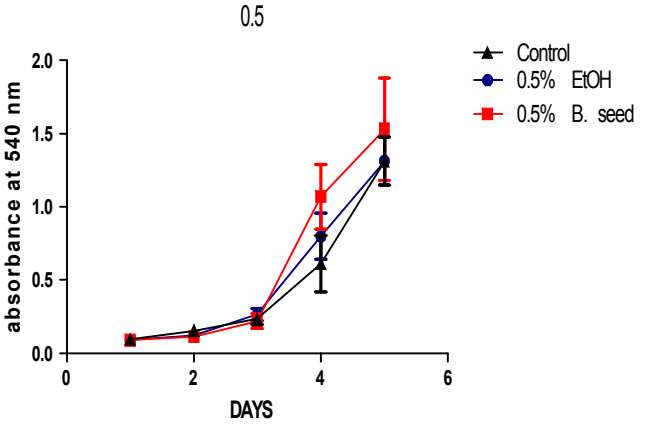
Figure 3.1. Effect of the *N. sativa* ethanolic extract on B16-BL6 cell proliferation.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT (Thiazolyl Blue Tetrazolium Bromide) assay. B16-BL6 cells were exposed to increasing concentrations of 0.05% panel A, 0.5% panel B%, and 2% panel C of *N. sativa* ethanolic extract and cultured for five days. Optical density at 540 nm was determined each day and the average ± standard deviation for 8 values was determined. Graphs show the average optical density for each day of treatment for one of at least 3 independent experiments.

A


0.05
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
DAYS
Control
0.05% EtOH
0.05% B. seed

B


0.5
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
DAYS
Control
0.5% EtOH
0.5% B. seed

C

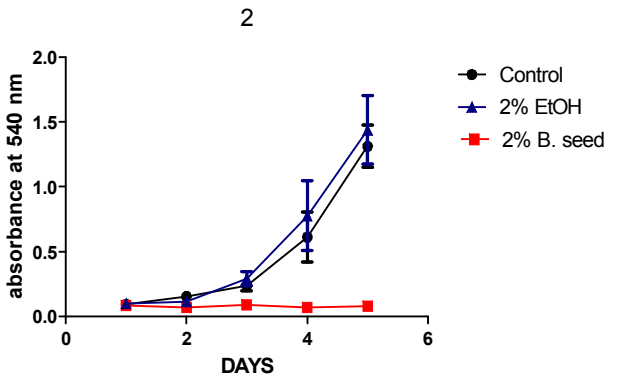
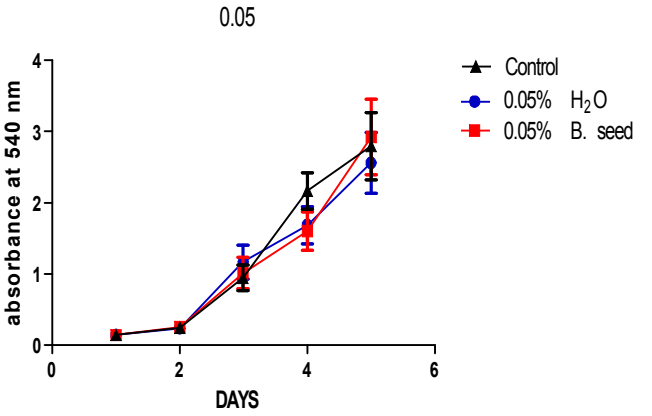

2
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
DAYS
Control
2% EtOH
2% B. seed

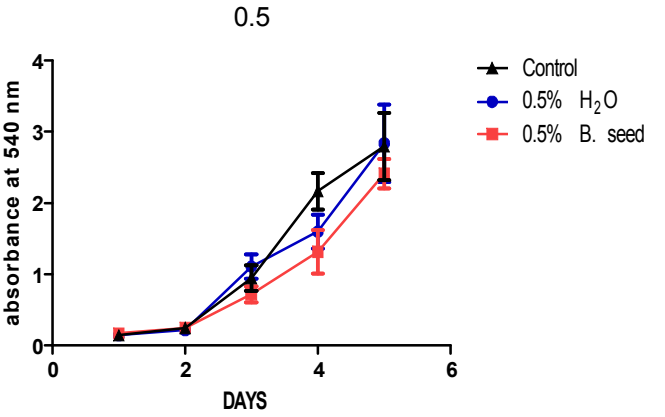
Figure 3.2. Effect of the *N. sativa* aqueous extract on B16-BL6 cell proliferation.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. B16-BL6 cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* aqueous extract and cultured for five days.

A


0.05
absorbance at 540 nm
DAYS
Control
0.05% H2O
0.05% B. seed

B


0.5
absorbance at 540 nm
DAYS
Control
0.5% H2O
0.5% B. seed

C

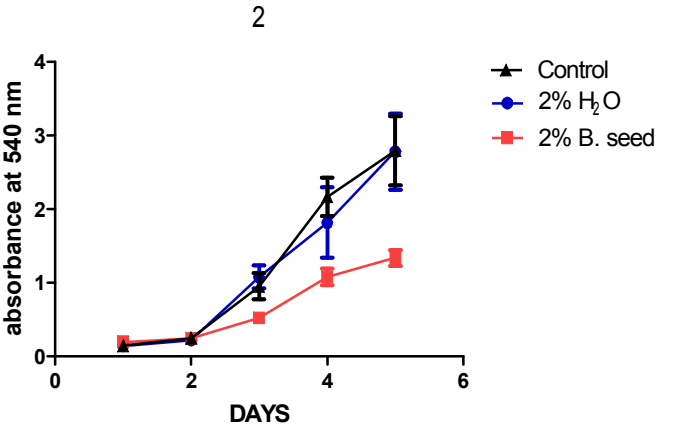
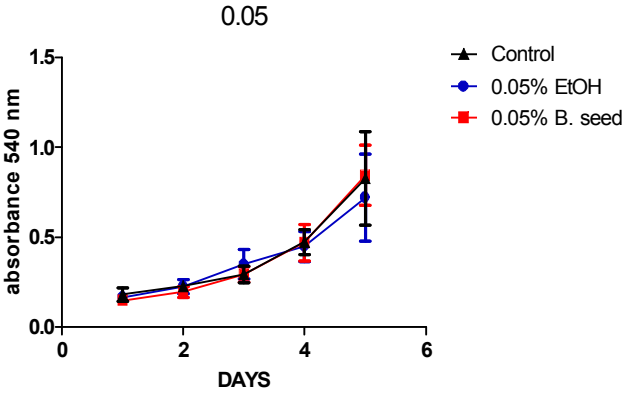

2
absorbance at 540 nm
DAYS
Control
2% H2O
2% B. seed

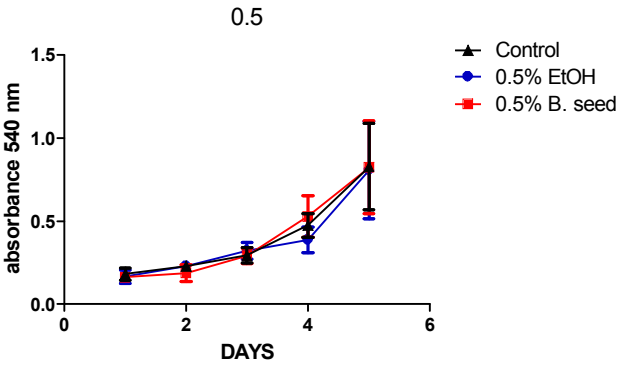
Figure 3.3. Effect of the *N. sativa* ethanolic extract on T98G cell proliferation.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. T98G cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* ethanolic extract and cultured for five days.

A


0.05
absorbance 540 nm
DAYS
Control
0.05% EtOH
0.05% B. seed

B


0.5
absorbance 540 nm
DAYS
Control
0.5% EtOH
0.5% B. seed

C

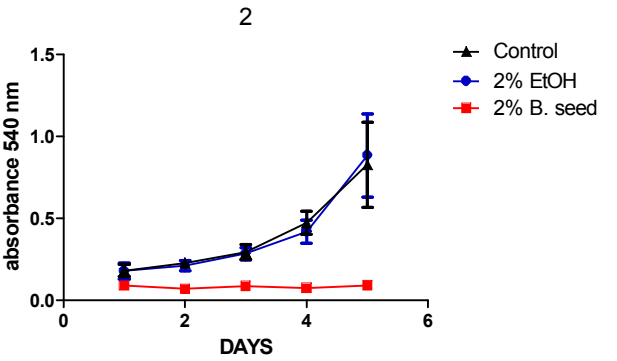
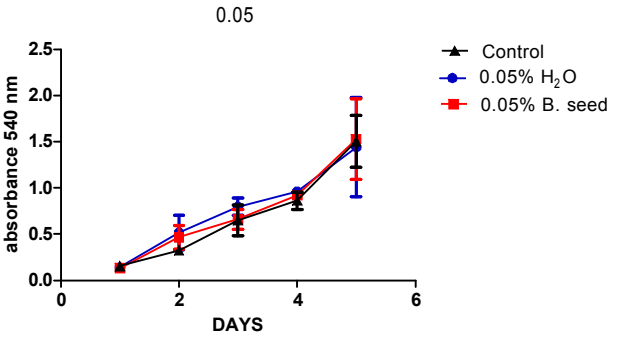

2
absorbance 540 nm
DAYS
Control
2% EtOH
2% B. seed

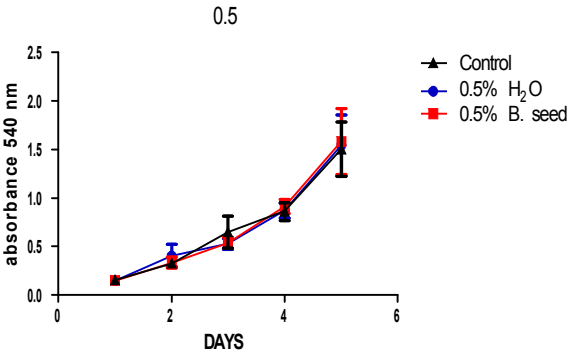
Figure 3.4. Effect of the *N. sativa* aqueous extract on T98G cell proliferation.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. T98G cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* aqueous extract and cultured for five days

A


0.05
absorbance 540 nm
DAYS
Control
0.05% H2O
0.05% B. seed

B


0.5
absorbance 540 nm
DAYS
Control
0.5% H2O
0.5% B. seed

C

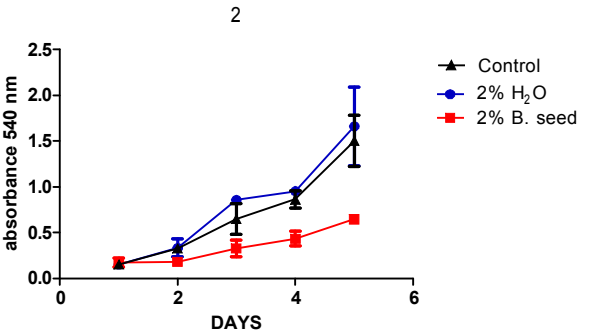
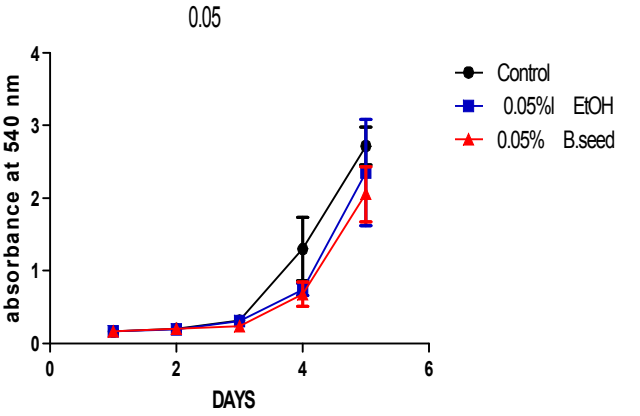

2
absorbance 540 nm
DAYS
Control
2% H2O
2% B. seed

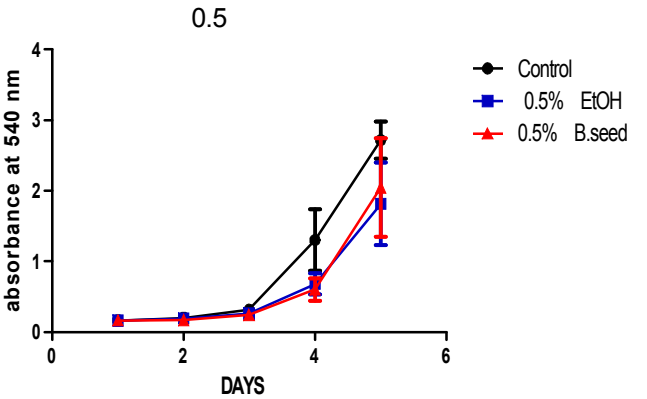
Figure 3.5. Effect of the *N. sativa* ethanolic extract on the proliferation of non-malignant HEK 293T cells.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. HEK 293T cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* ethanolic extract and cultured for five days.

A


0.05
absorbance at 540 nm
DAYS
Control
0.05%l EtOH
0.05% B.seed

B


0.5
absorbance at 540 nm
DAYS
Control
0.5% EtOH
0.5% B.seed

C

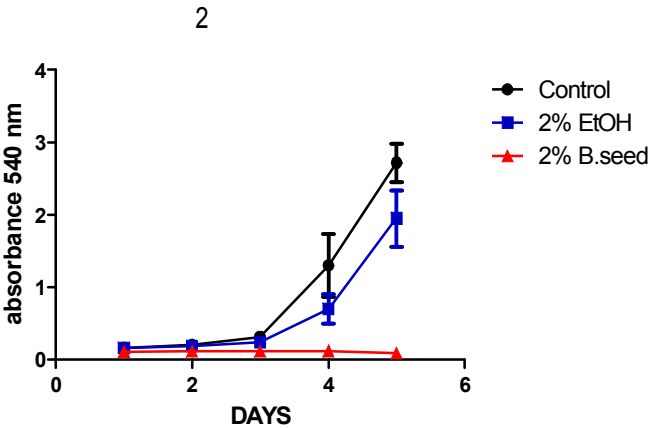
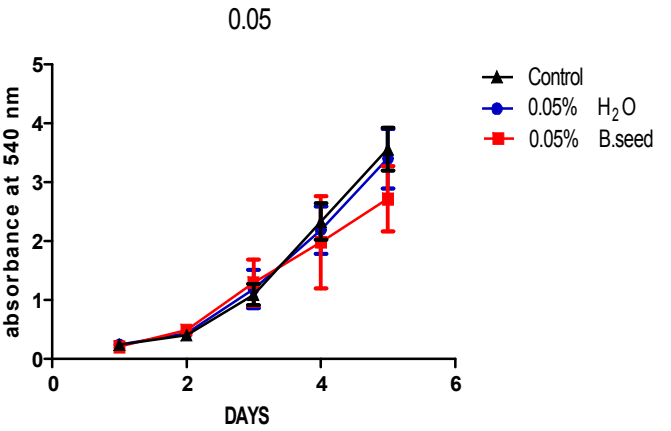

2
absorbance 540 nm
DAYS
Control
2% EtOH
2% B.seed

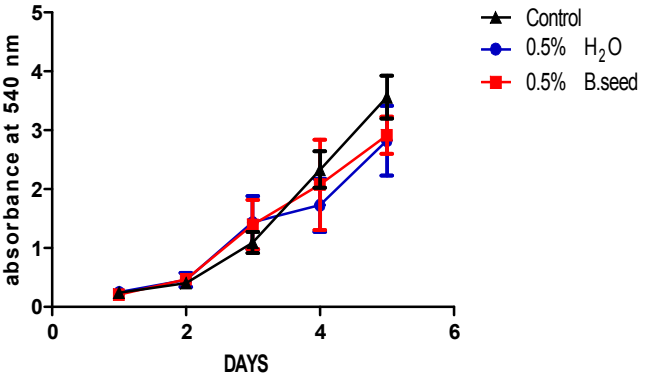
Figure 3.6. Effect of *N. sativa* aqueous extract on the proliferation of non-malignant HEK 293T cells.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. HEK 293T cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* aqueous extract and cultured for five days.

A


0.05
absorbance at 540 nm
DAYS
Control
0.05% H2O
0.05% B.seed

B


0.5
absorbance at 540 nm
DAYS
Control
0.5% H2O
0.5% B.seed

C

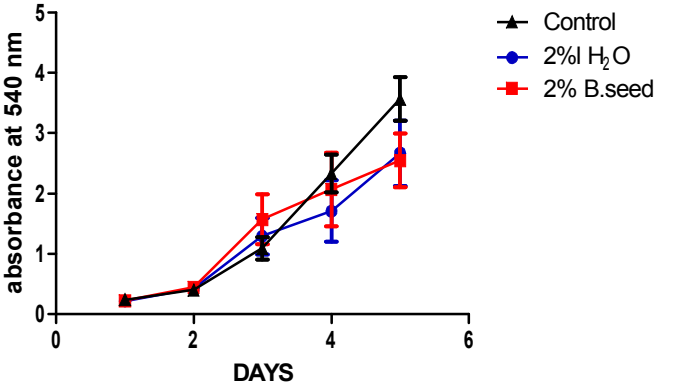
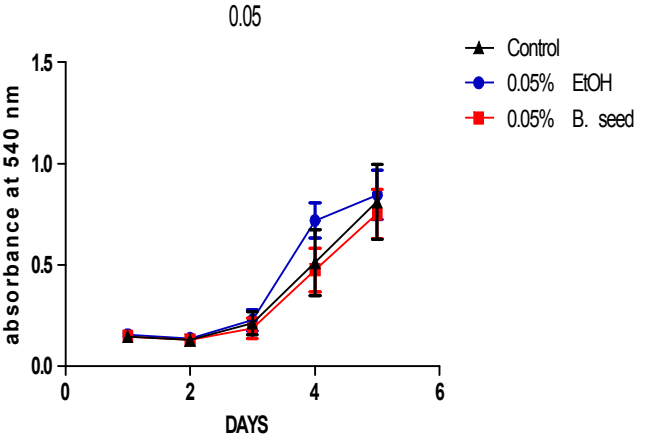

2
absorbance at 540 nm
DAYS
Control
2%l H2O
2% B.seed

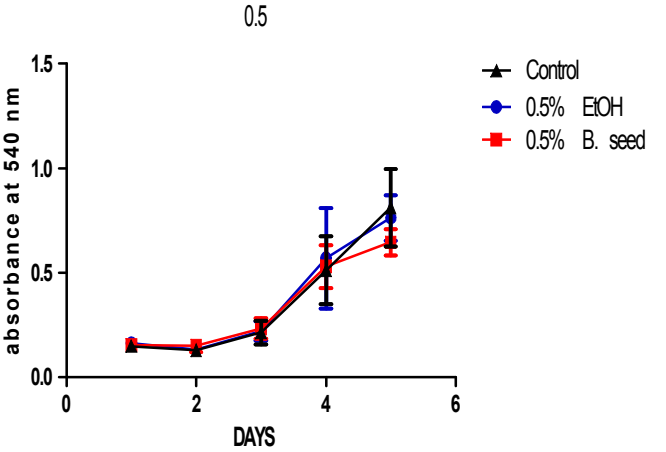
Figure 3.7. Effect of the *N. sativa* ethanolic extract on the proliferation of non-malignant HSG cells.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. HSG cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* ethanolic extract and cultured for five days.

A


0.05
Control
0.05% EtOH
0.05% B. seed
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
DAYS

B


0.5
Control
0.5% EtOH
0.5% B. seed
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
DAYS

C

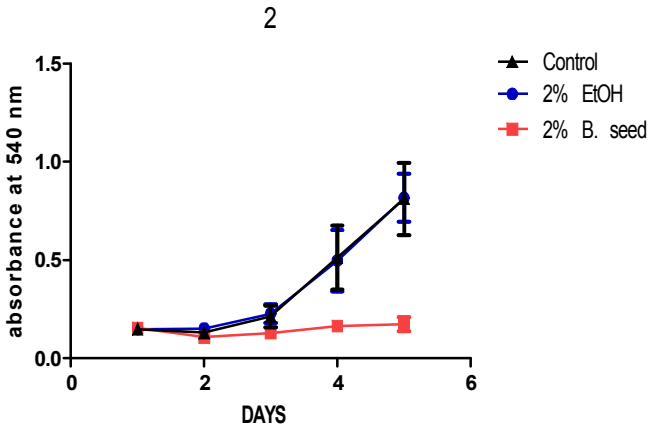
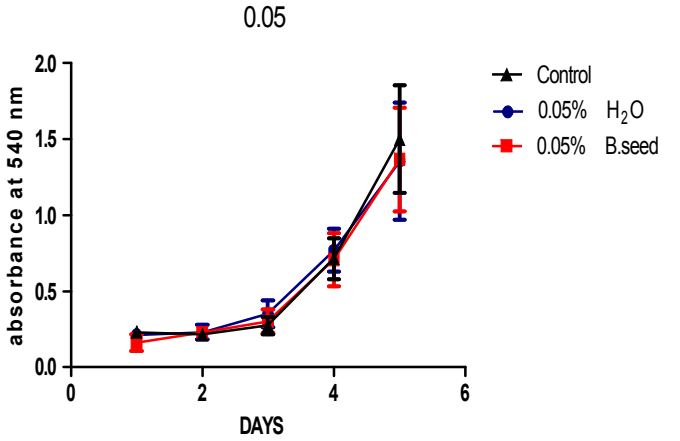

2
Control
2% EtOH
2% B. seed
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
DAYS

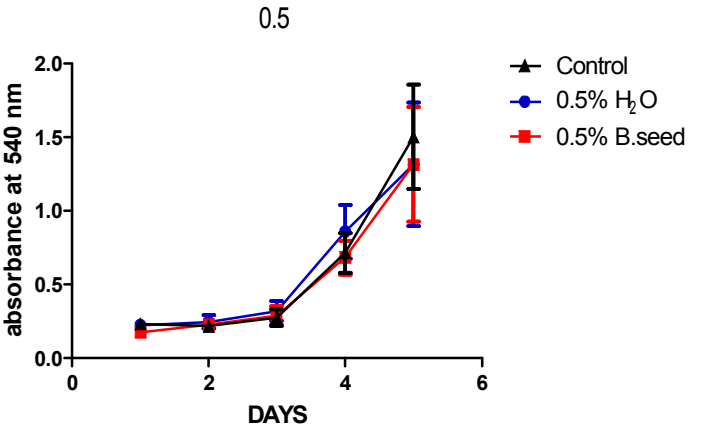
Figure 3.8. Effect of the *N. sativa* aqueous extract on the proliferation of non-malignant HSG cells.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. HSG cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* aqueous extract and cultured for five days.

A


0.05
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
DAYS
Control
0.05% H2O
0.05% B.seed

B


0.5
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
DAYS
Control
0.5% H2O
0.5% B.seed

C

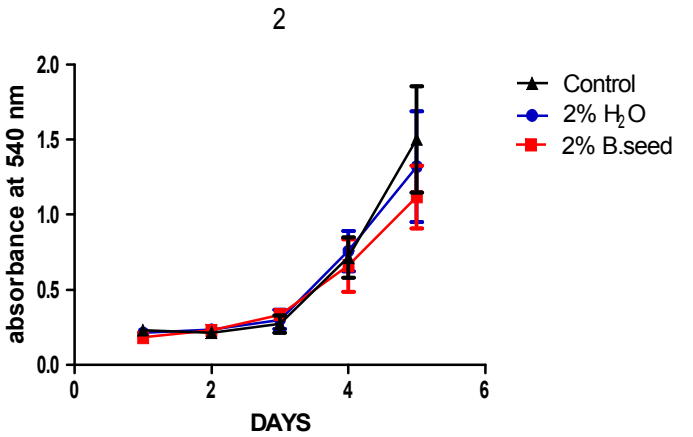
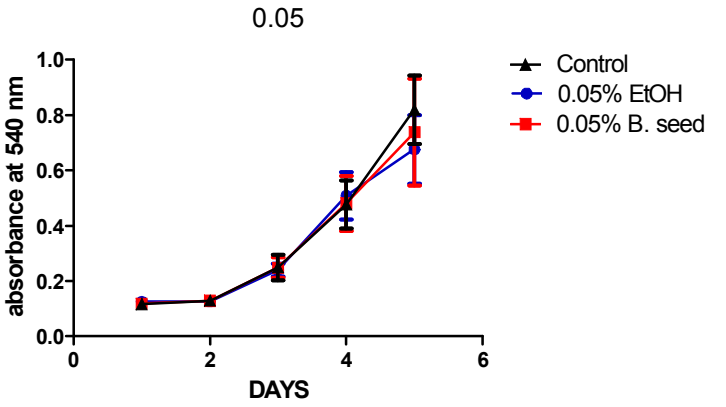

2
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
DAYS
Control
2% H2O
2% B.seed

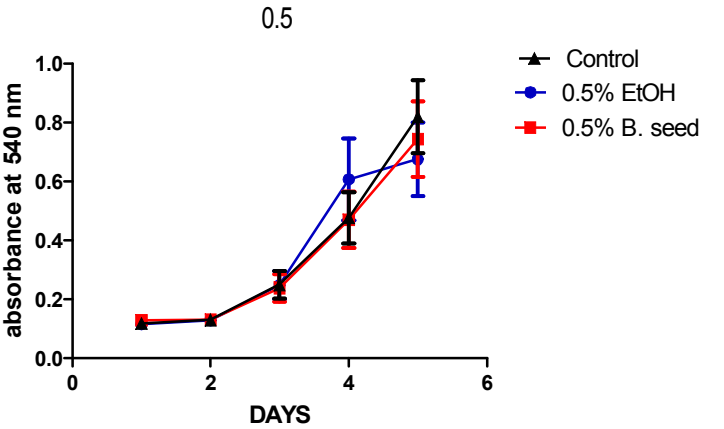
Figure 3.9. Effect of the *N. sativa* ethanolic extract on the proliferation of non-malignant HBL100 cells.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. HBL100 cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* ethanolic extract and cultured for five days.

A


0.05
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
DAYS
Control
0.05% EtOH
0.05% B. seed

B


0.5
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
DAYS
Control
0.5% EtOH
0.5% B. seed

C

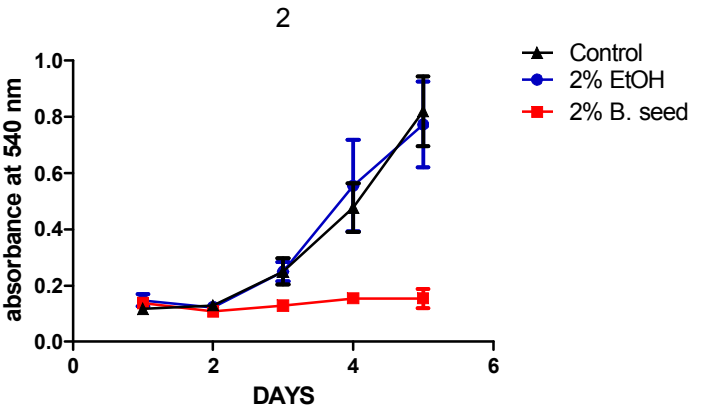
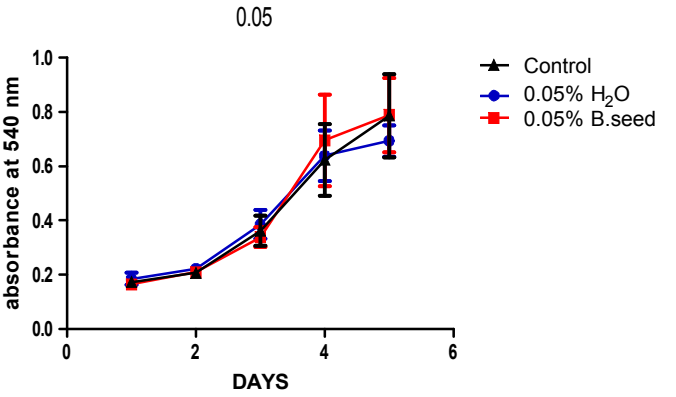

2
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
DAYS
Control
2% EtOH
2% B. seed

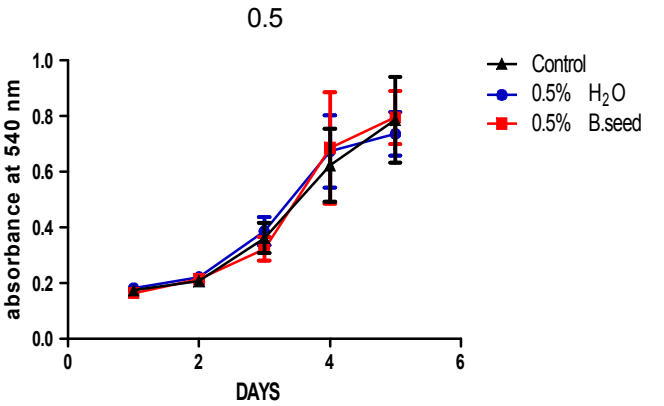
Figure 3.10. Effect of *N. sativa* aqueous extract on the proliferation of non-malignant HBL100 cells.

The effect on cell proliferation in response to treatment with *N. sativa* was assessed using the MTT assay. HBL100 cells were exposed to increasing concentrations 0.05% (panel A), 0.5% (panel B), and 2% (panel C) of *N. sativa* aqueous extract and cultured for five days.

A


0.05
absorbance at 540 nm
DAYS
Control
0.05% H2O
0.05% B.seed

B


0.5
absorbance at 540 nm
DAYS
Control
0.5% H2O
0.5% B.seed

C

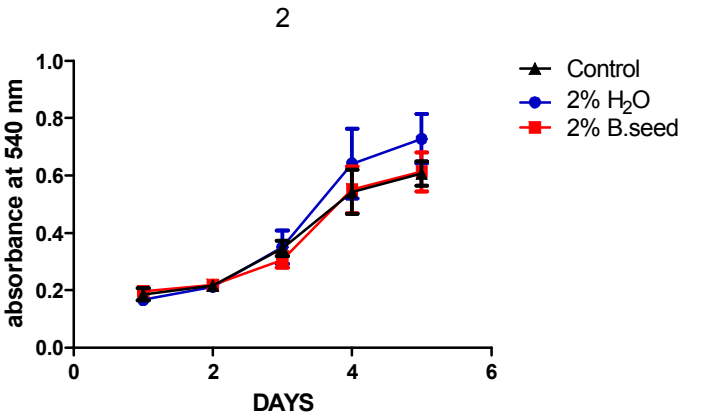
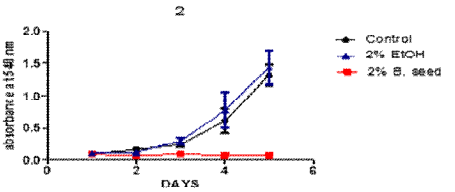
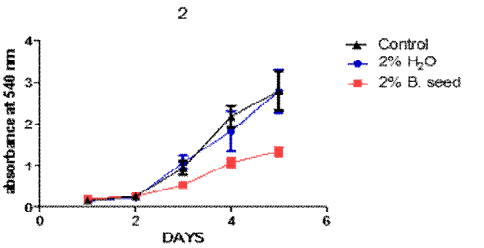

2
absorbance at 540 nm
DAYS
Control
2% H2O
2% B.seed

Figure 3.11. Comparison of the effect *N. sativa* extracts on malignant and non-malignant cell lines.

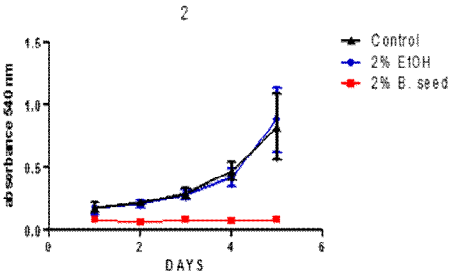
The effect on cell proliferation in response to treatment with the high dose (2%) of *N. sativa* extract (both ethanol and aqueous) for B16-BL6, T98G, HEK, HSG, and HBL100 was assessed using the MTT assay, and all of the cell lines were cultured for five days.

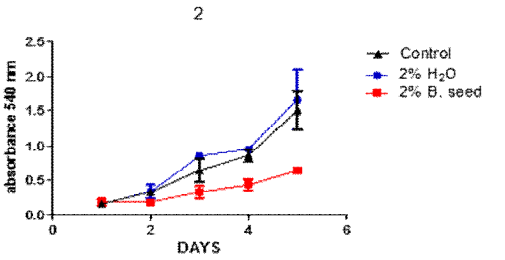
B16-BL6



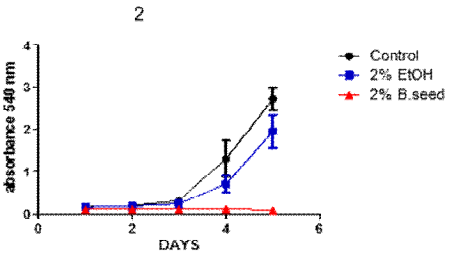


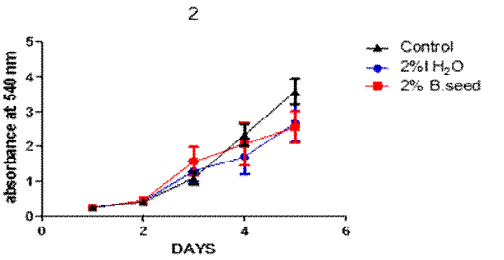
T98G



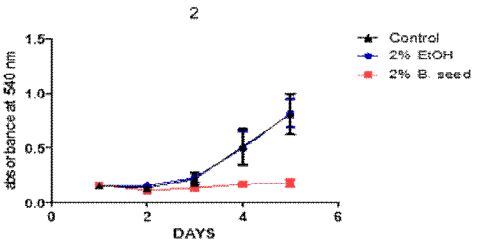


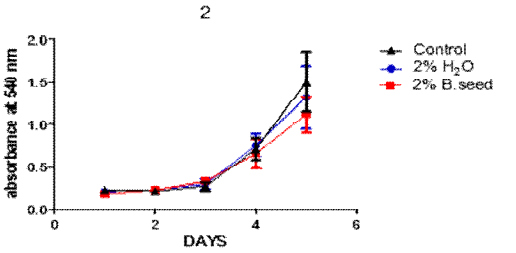
HEK



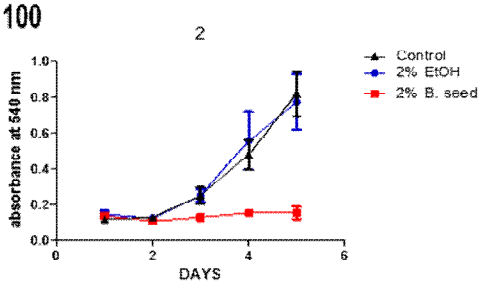


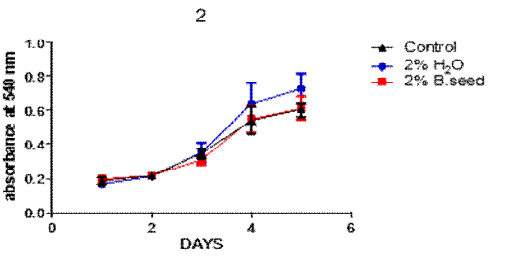
HSG





HBL 100



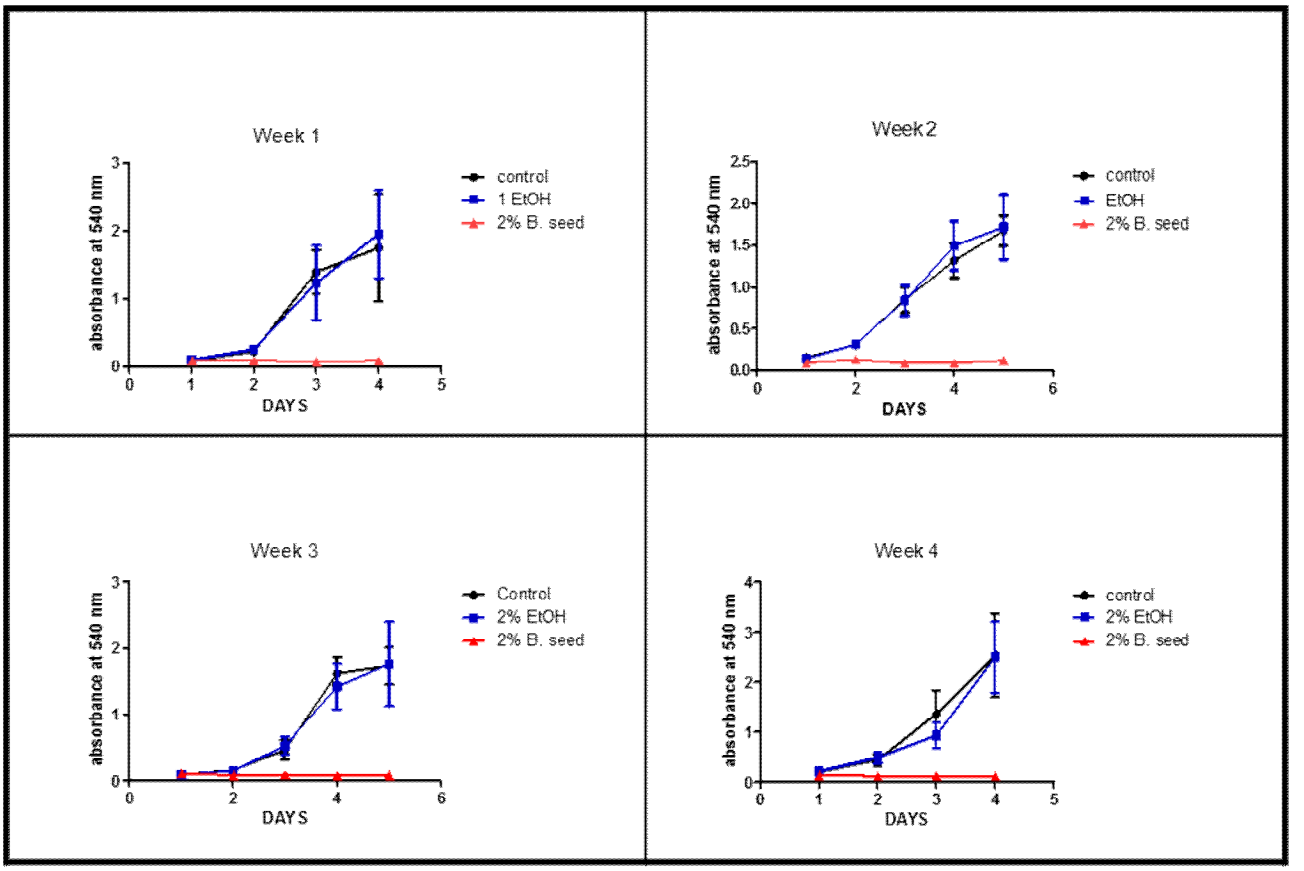


3.2. Stability of the *N. sativa* ethanolic extract

The ethanolic extract of *N. sativa* was stored at 4 ℃ for four weeks to test the stability of the active compounds in the *N. sativa*. The effect of the ethanolic extract of *N. sativa* on the growth of B16-BL6 cells was tested each week. The growth assays showed very similar results for each week indicating that the *N. sativa* ethanolic extract was stable and maintained the ability to inhibit cell growth (Figure 3.12)

Figure 3.12. The stability of the *N. sativa* ethanolic extract (4 weeks).

For four weeks, the effect on cell proliferation in response to treatment with *N. sativa* ethanolic extract was assessed using the MTT assay. B16-BL6 cells were exposed to the high dose (2%) of *N. sativa* ethanolic extract and cultured for several days.


Week 1
absorbance at 540 nm
DAYS
control
1 EtOH
2% B. seed
Week 2
absorbance at 540 nm
DAYS
control
EtOH
2% B. seed
Week 3
absorbance at 540 nm
DAYS
Control
2% EtOH
2% B. seed
Week 4
absorbance at 540 nm
DAYS
control
2% EtOH
2% B. seed

3.3. Morphological changes in malignant (B16-BL6) cell line after treatment with *N. sativa*.

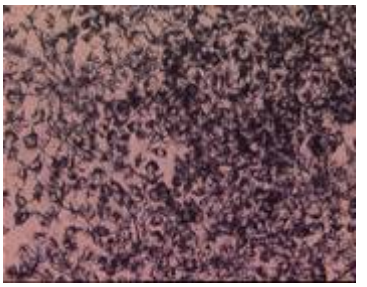
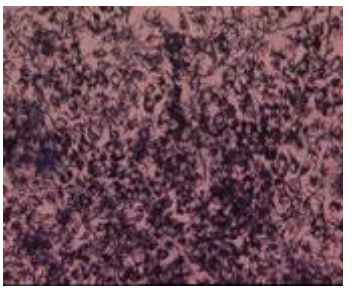
The morphology of B16-BL6 cells treated with *N. sativa* was examined to determine any effects of treatment on cell morphology. B16-BL6 cells were treated with low (0.05%) and high (2%) concentrations of *N. sativa* for 72 h at 37°C. B16-BL6 cells that were treated with a high dose (2%) of the *N. sativa* ethanolic extract showed visible damage to the cells and a decrease in cell number (Figure 3.13 A). There was no morphological difference in the B16-BL6 cells that were treated with a low dose (0.05%) of the *N. sativa* ethanolic extract compared to the control cells (Figure 3.13 A). The cells appeared to be similar in number and in shape.

B16-BL6 cells were also treated with a high dose (2%) of the *N. sativa* aqueous extract which was able to reduce number of cells significantly compared to the control cells although not as much as cells treated with the high dose ethanol extract (Figure 3.13 B). There was no morphological difference in B16-BL6 cells that were treated with a low dose (0.05 %) of the *N. sativa* aqueous extract compared to the control cells (Figure 3.13 B).

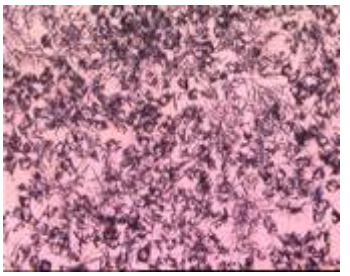
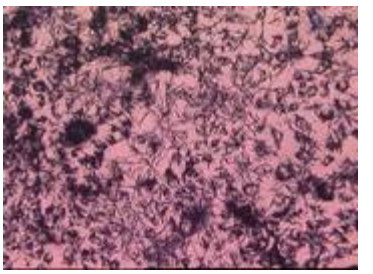
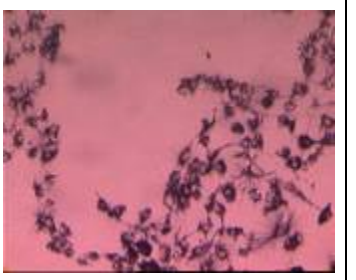
Figure 3.13. Morphological changes in B16-BL6 cells exposed to *N. sativa* for 72 h.

B16-BL6 cells were treated with the ethanolic and aqueous extracts of *N. sativa* at various concentrations (0.05% and 2%) and incubated for 72 h at 37°C. Before the pictures were taken, 10 µl of MTT was added to the treated cells at a final concentration of 0.4 µg/ml and incubated for 4 hours. Experiments were performed in triplicate. Figure **A** is the ethanolic extract of *N. sativa*. Figure **B** is the aqueous extract of *N. sativa*.

A

Control	0.05%	2%
		

B

Control	0.05%	2%
		

3.4 Morphological changes in non-malignant (239T) cell line after treatment with *N. sativa*.

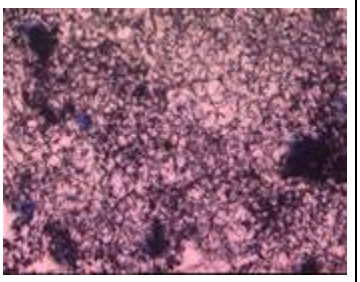
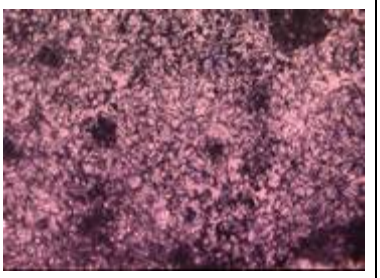
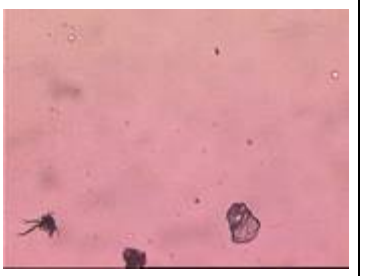
The morphology of 239T cells treated with *N. sativa* was examined to determine any effects of treatment on cell morphology. 239T cells were treated with a low (0.05%) and a high (2%) concentrations of *N. sativa* for 72 h at 37°C. 239T cells that were treated with a high dose (2%) of the *N. sativa* ethanolic extract showed visible damage to the cells and decrease in cell number (Figure 3.14 A). There was no morphological difference in 239T cells treated with a low dose (0.05 %) of the *N. sativa* ethanol extract compared to the control cells (Figure 3.14 A).

239T cells treated with a high dose (2%) of the *N. sativa* aqueous extract showed an increase in the cell number and no change in the morphology of the 239T cells (Figure 3.14 B). There was no morphological difference in 239T cells that were treated with a low dose (0.05%) of the *N. sativa* aqueous extract compared to the control cells (Figure 3.14 B).

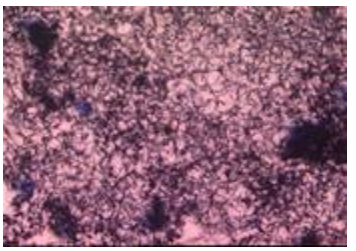
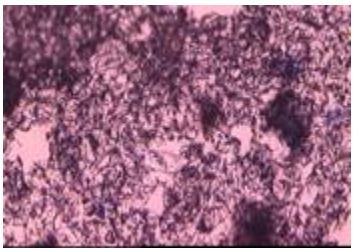
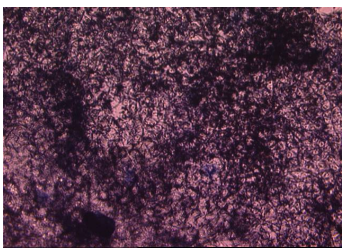
Figure 3.14. Morphological changes in 239T cells exposed to various concentrations of *N. sativa* for 72 h.

239T cells were treated with ethanolic and aqueous extracts of *N. sativa* at various concentrations (0.05% and 2%) and incubated for 72 h at 37°C. Before the pictures were taken, 10 µl of MTT was added to the treated cells at a final concentration of 0.4 µg/ml and incubated for 4 hours. Experiments were performed in triplicate. Figure **A** is the ethanolic extract of *N. sativa*. Figure **B** is the aqueous extract of *N. sativa*.

A

Control	0.05%	2%
		

B

Control	0.05%	2%
		

3.5. Induction of Apoptosis

B16-BL6 cells were stained with acridine orange and ethidium bromide to observe the nuclear changes and the formation of apoptotic bodies that are characteristic of apoptosis. B16-BL6 cells were exposed to different (0.05% and 2%) concentrations of *N. sativa* for 72 h at 37°C. B16-BL6 cells that were treated with a high dose (2%) of *N. sativa* ethanolic extract showed a significant change in cell morphology; membrane blebbing and complete destruction of nuclear structure (Figure 3.15). There were small morphological changes in B16-BL6 cells treated with a low dose (0.05%) of *N. sativa* ethanolic extract compared to the control cells (Figure 3.16). B16-BL6 cells that were treated with a high dose (2%) of *N. sativa* aqueous extract contain bright green dots in the nuclei as a result of nuclear fragmentation and chromatin condensation (Figure 3.15). There were no morphological changes in B16-BL6 cells treated with a low dose (0.05 %) of *N. sativa* aqueous extract compared to the untreated control cells (Figure 3.16).

B16-BL6 cells were stained with the TUNEL reaction mixture to detect DNA fragmentation which occurs during the late stage of apoptosis. B16-BL6 cells were exposed to different (0.05% and 2%) concentrations of *N. sativa* for 72 h at 37°C. B16-BL6 cells that were treated with a high dose (2%) of *N. sativa* ethanolic extract showed a significant increase in TUNEL staining, which indicated apoptosis, compared to a low dose (0.05%) and the untreated control cells (Figure 3.17, 3.18). B16-BL6 cells treated with a high dose (2%) of *N. sativa* aqueous extract showed a small increase in TUNEL staining compared to the control cells (Figure 3.17).

Figure 3.15. Treatment with *N. sativa* for 72 h induced apoptosis in B16-BL6 cells using acridine orange/ ethidium bromide staining: Inlayed view of treated cells.

B16-BL6 cells were treated with the ethanolic and aqueous extracts of *N. sativa* at various concentrations (0.05% and 2%). Camptothecin was used as a positive control for apoptosis. After 72 h of incubation at 37°C, apoptotic cells were detected by acridine orange (green) and of ethidium bromide (red) staining. Pictures were viewed under a LSM 510 fluorescence microscope, and experiments were done in triplicate.

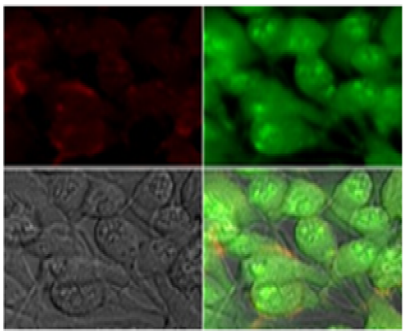
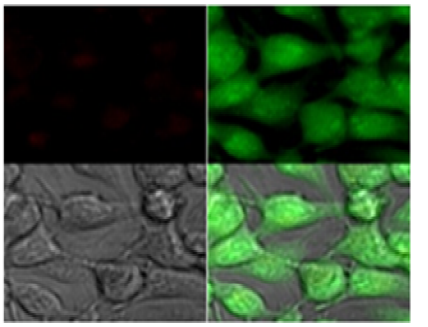
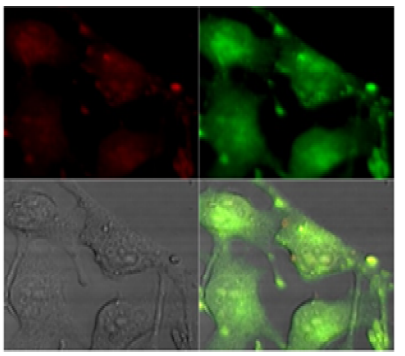
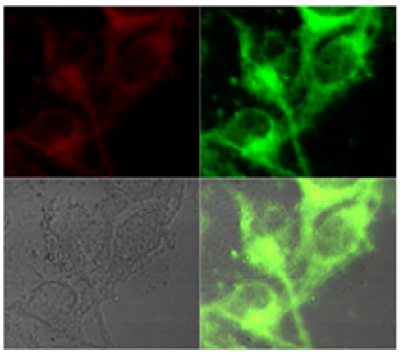
Positive control	Negative control
	
2% H_2O	2% EtOH
	

Figure 3.16. Treatment with various concentrations of *N. sativa* for (24, 48, and 72 h) induced apoptosis in B16-BL6 cells using acridine orange/ ethidium bromide staining: inlayed view of treated cells.

B16-BL6 cells were treated with the ethanolic and aqueous extracts of *N. sativa* at various concentrations (0.05% and 2%). Camptothecin was used as a positive control for apoptosis. After different time points (24, 48, and 72 h) of incubation at 37°C, apoptotic cells were detected by acridine orange (green) and of ethidium bromide (red) staining. Pictures were viewed under a LSM 510 fluorescence microscope, and experiments were done in triplicate.

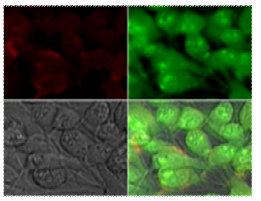
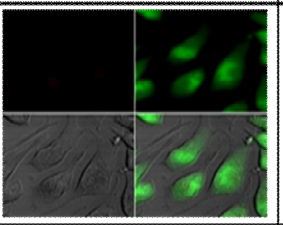
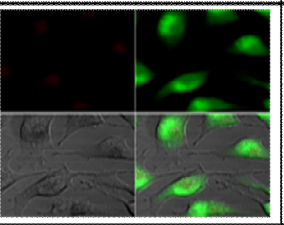
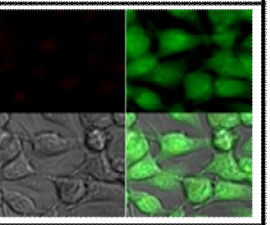
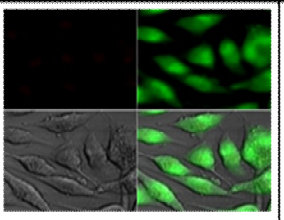
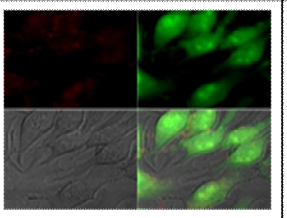
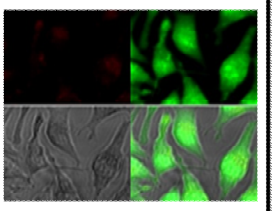
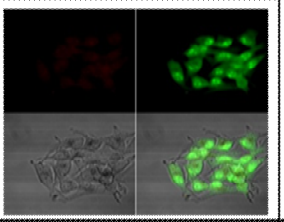
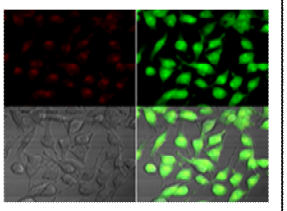
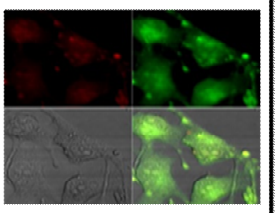
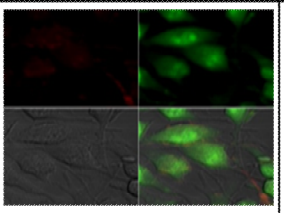
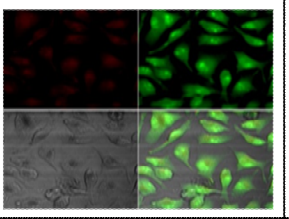
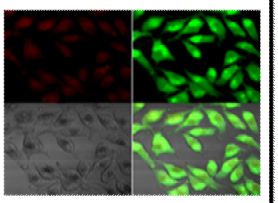
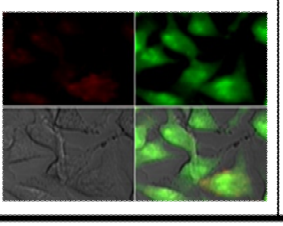
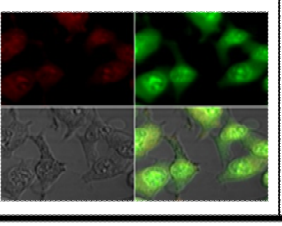
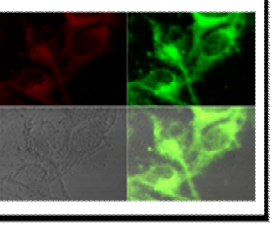
Positive control	24h	48h	72h
			
Negative control			
0.05% H_2O			
2% H_2O			
0.05% EtOH			
2% EtOH			

Figure 3.17. Treatment with *N. sativa* for 72 h induced apoptosis in B16-BL6 cells using the TUNEL assay: Inlayed view of treated cells.

B16-BL6 cells were treated with the ethanolic and aqueous extracts of *N. sativa* at various concentrations (0.05% and 2%) of *N. sativa*. Camptothecin was used as a positive control for apoptosis. After 72 h of incubation at 37°C, apoptotic cells were detected by TUNEL assay. Pictures were viewed under a LSM 510 fluorescence microscope, and experiments were done in triplicate.

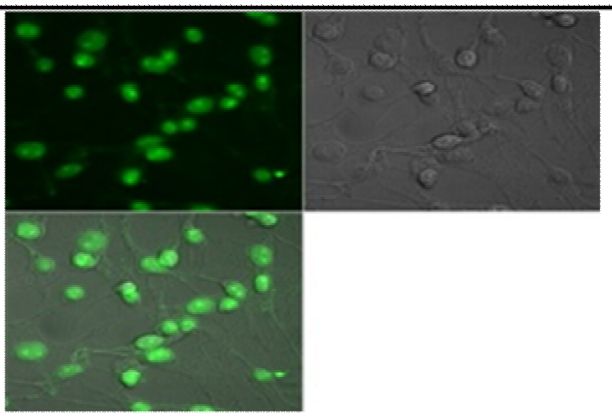
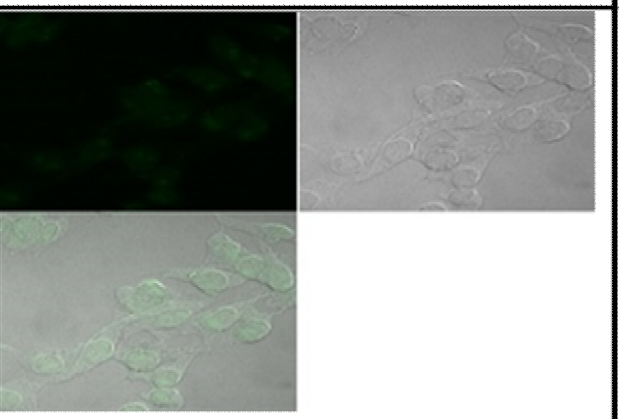
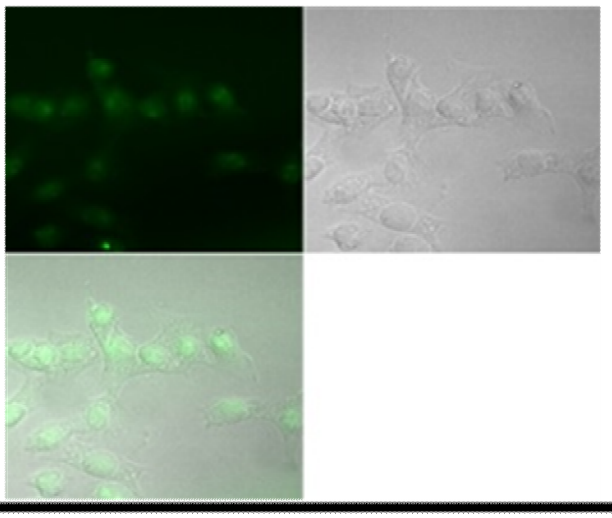
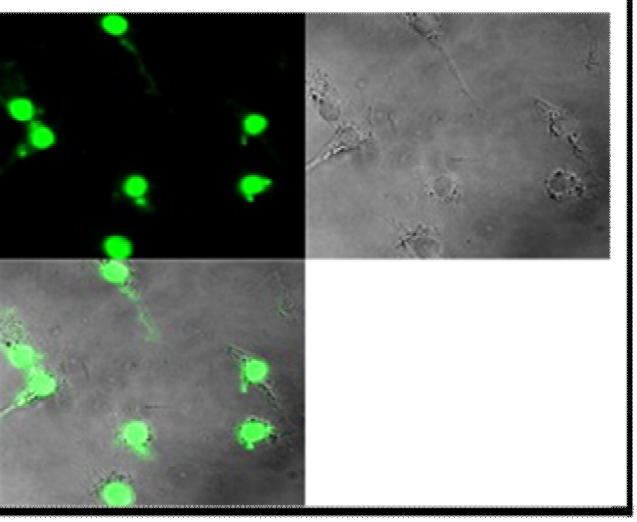
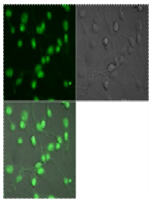
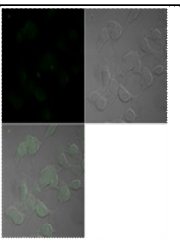
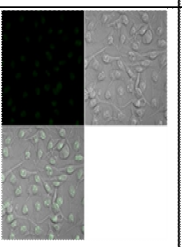
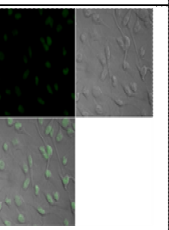
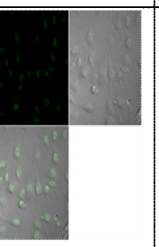
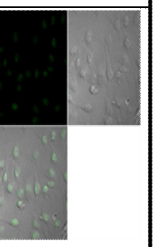
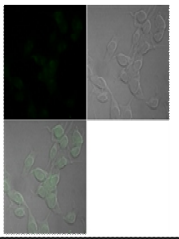
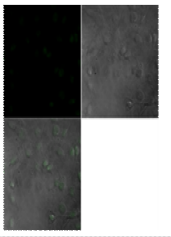
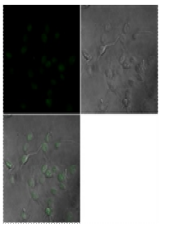
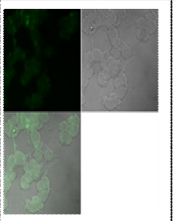
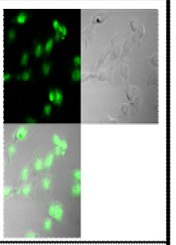
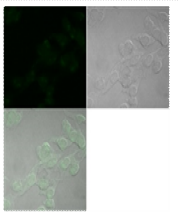
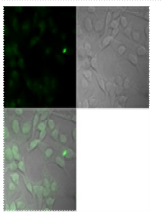
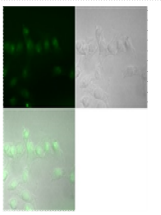
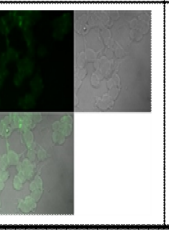
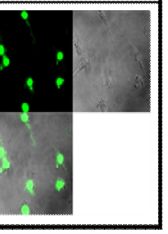
Positive control	Negative control
	
2% H_2O	2% EtOH
	

Figure 3.18. Treatment with various concentrations of *N. sativa* for (24, 48, and 72h) induced apoptosis in B16-BL6 cells using the TUNEL assay.

B16-BL6 cells were treated with the ethanolic and aqueous extracts of *N. sativa* at various concentrations (0.05% and 2%) of *N. sativa*. Camptothecin was used as a positive control for apoptosis. After (24, 48, and 72 h) of incubation at 37°C, apoptotic cells were detected by TUNEL assay. Pictures were viewed under a LSM 510 fluorescence microscope, and experiments were done in triplicate.

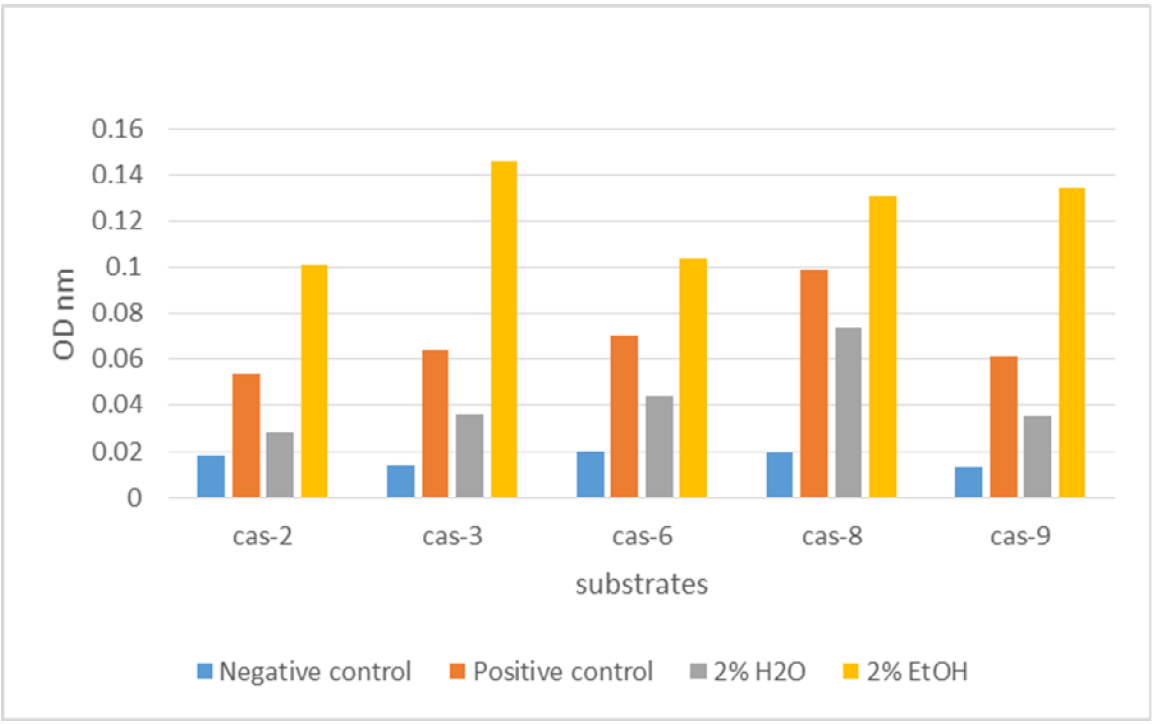
	Positive control	Negative control	0.05% H_2O	2% H_2O	0.05% EtOH	2% EtOH
						
0h						
48h						
72h						

3.6. Effect of *N. sativa* on the expression of caspase -2, -3, -6, -8 and -9 in B16-BL6 cells

Caspase activity assay was used to determine whether treatment with *N. sativa* caused apoptosis. This assay uses spectroscopy to detect of the chromophore *p*-nitroaniline (*p*-NA) after cleavage from the labeled peptide substrate VDVAD (for caspase-2), DEVD (for caspase-3), VEID (for caspase-6), IETD (for caspase-8), or LEHD (for caspase-9). B16-BL6 cells were treated with ethanolic and aqueous extracts of *N. sativa* at a high concentration (2%) for 24 h at 37°C. For each essay, $3x10^6$ cells were lysed in 50 µl of lysine buffer and incubated in ice 10 minutes. The B16-BL6 cell lysate were incubated with the individual substrates at 37°C for 2 h, and the caspase activities were determined by measuring the changes in absorbance at 405 nm using a plate reader. B16-BL6 cells that were treated with the high concentration of the ethanolic extract of *N. sativa* showed a significant increase in caspase -2, -3, -6, -8, and -9. Caspase -3 activity was the highest compared to the control cells (Figure 3.19). B16-BL6 cells were treated with the high concentration of the aqueous extract of *N. sativa* showed a smaller increase in caspase -2, -3, -6, -8, and -9. Caspase -8 activity was the highest compared to the control cells (Figure 3.19). Experiments were performed in duplicate assays.

Figure 3.19. Effect of *N. sativa* on the expression of caspase -2, -3, -6, -8 and -9 in B16-BL6 cells.

Caspase activities were determined by colorimetric assays using caspase-2, -3, -6, -8 and -9 activation kits (Life technology). B16-BL6 were treated with high dose (2%) ethanolic or aqueous extracts of *N. sativa* for 24 h. Camptothecin was used as a positive control for apoptosis.The caspase activities were determined by measuring changes in absorbance at 405 nm using a (SpectraMax 340 PC 389) plate reader.

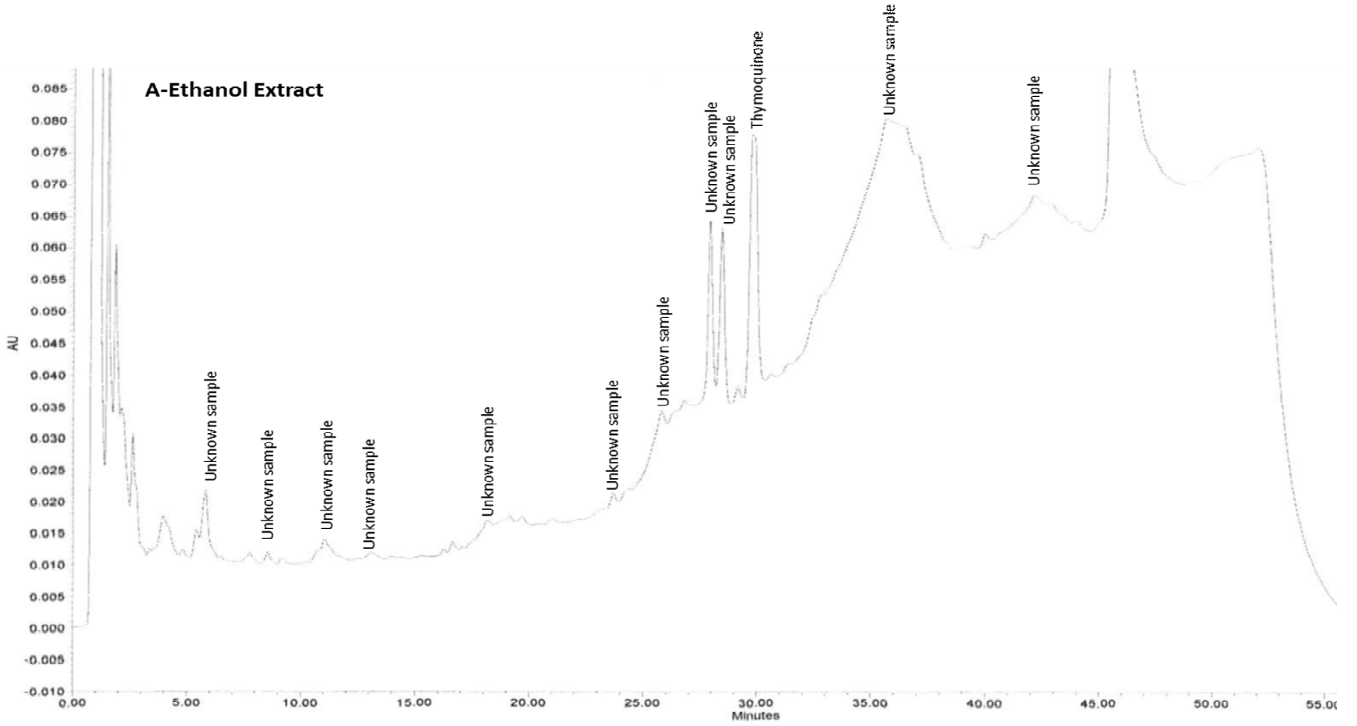

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD nm
cas-2
cas-3
cas-6
cas-8
cas-9
substrates
Negative control
Positive control
2% H2O
2% EtOH

3.7. HPLC

High performance liquid chromatography was performed to separate the components of *N. sativa* extracts and to indicate the presence of any unknown compound(s) that could be responsible for inhibiting cancer cell proliferation. Ethanol extract has more peaks than water extract. *N. sativa* extracts were fractionated into various ethanol fraction starting with the aqueous fraction, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 100% ethanol subjected to HPLC as described in section 3.8. By comparing the spectra and retention times of standards, these fractions were characterized for the presence of any peaks. Number of peaks change for different fractions (100% ethanol fraction has very low levels of alkaloids).

Figure 3.20. HPLC analysis of *N. sativa* extracts (water, ethanol)

N. sativa extracts were analyzed by using a high performance liquid chromatography method. Each extract (water, ethanol) of *N. sativa* was subjected to HPLC and peaks identified using a wavelength of 245 nm. Thymoquinone was used as a known compound standard to identify the peaks of the chromatogram in *N. sativa* extracts. Chromatogram **A** is ethanol extract, and chromatogram **B** is water extract.


A-Ethanol Extract
AU
Minutes
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample
Unknown sample

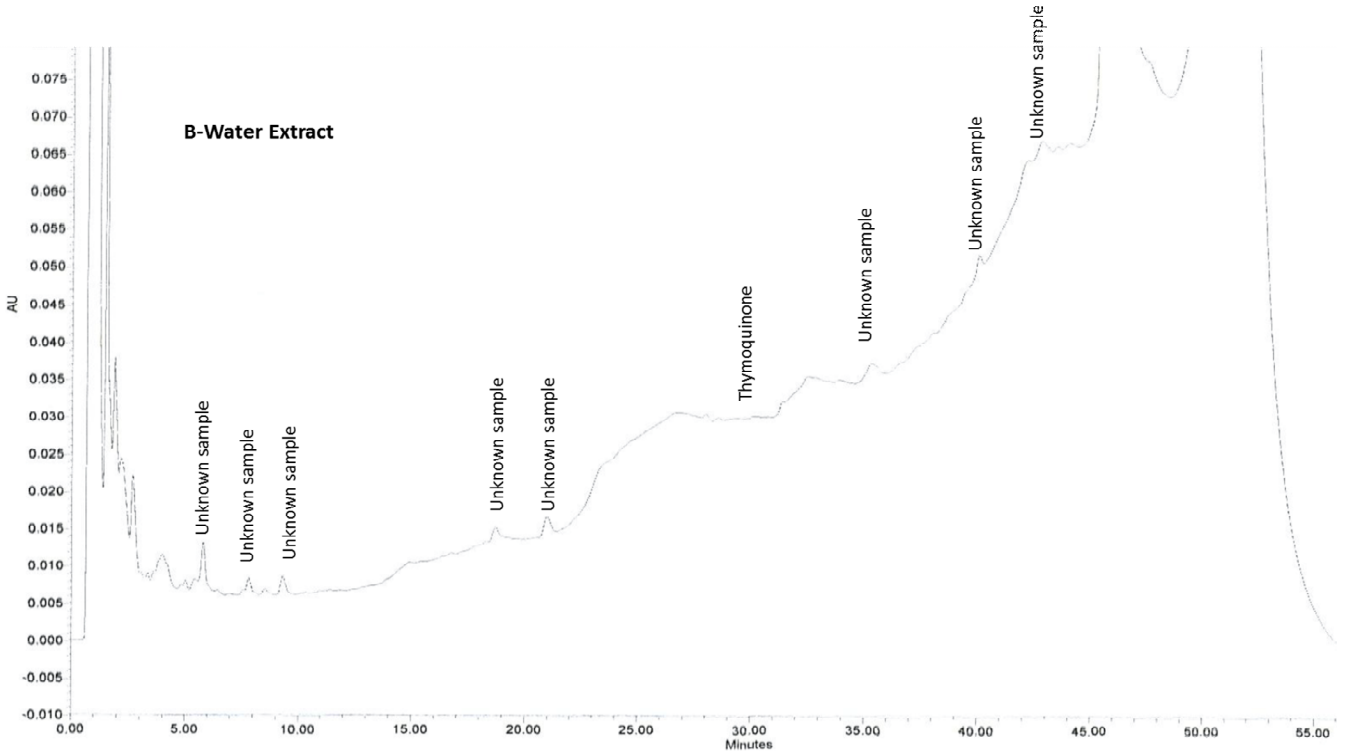
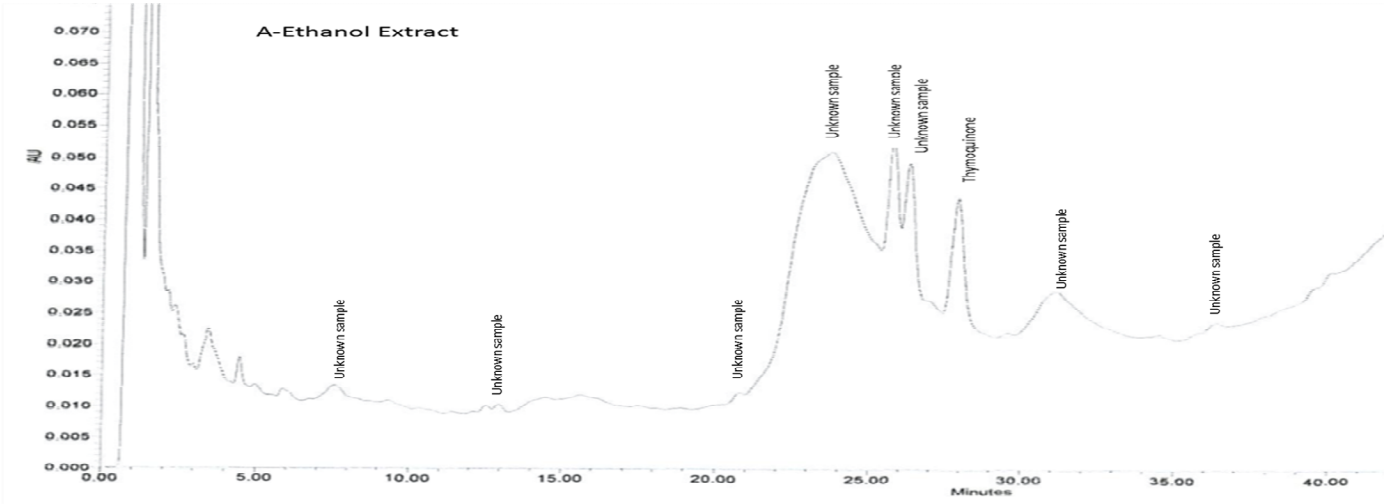

B-Water Extract
AU
Minutes
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample
Unknown sample
Unknown sample

Figure 3.21. HPLC analysis of *N. sativa* extract (20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, 100% ethanol)

N. sativa extracts were analyzed using high performance liquid chromatography. The *N. sativa* extract was fractionated using an ethanol series and, each fraction (20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, 100% ethanol) of *N. sativa* was subjected to HPLC and peaks using a wavelength of 245 nm. Thymoquinone was used as a known compound standard to identify the peaks of the chromatogram of *N. sativa* extracts. Chromatogram **A** is ethanol extract, **B** is water fraction, **C** is 20% ethanol fraction, **D** is 40% ethanol fraction, **E** is 60% ethanol fraction, **F** is 80% ethanol fraction, and **G** is 100% ethanol fraction.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AU
Minutes
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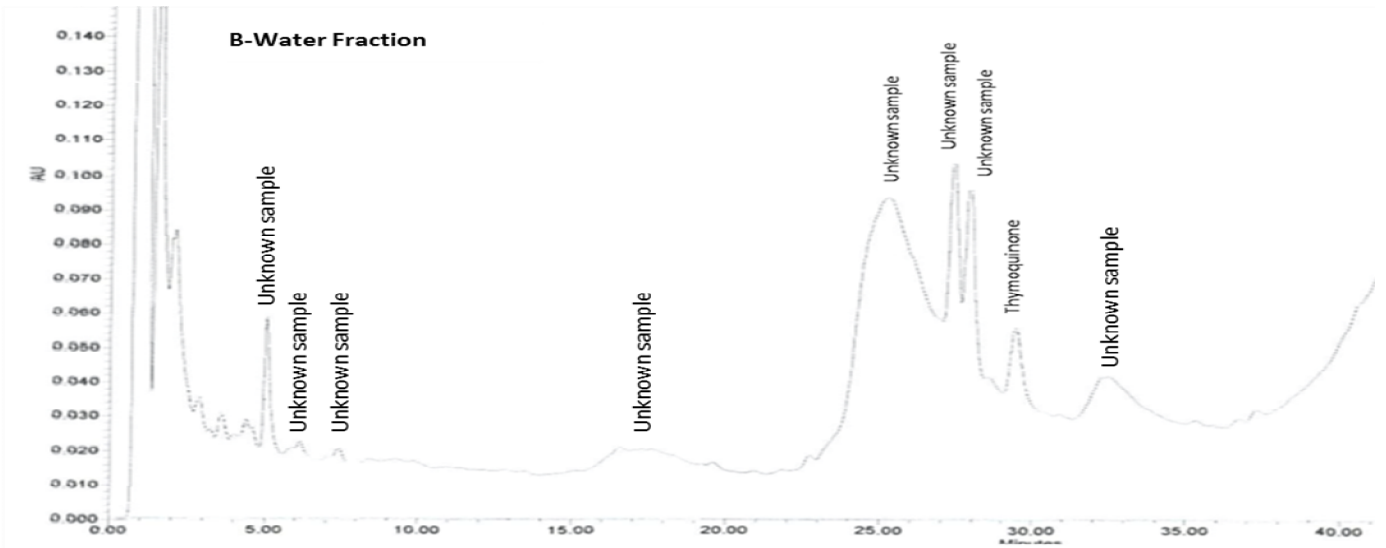
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample
Unknown sample


B-Water Fraction
AU
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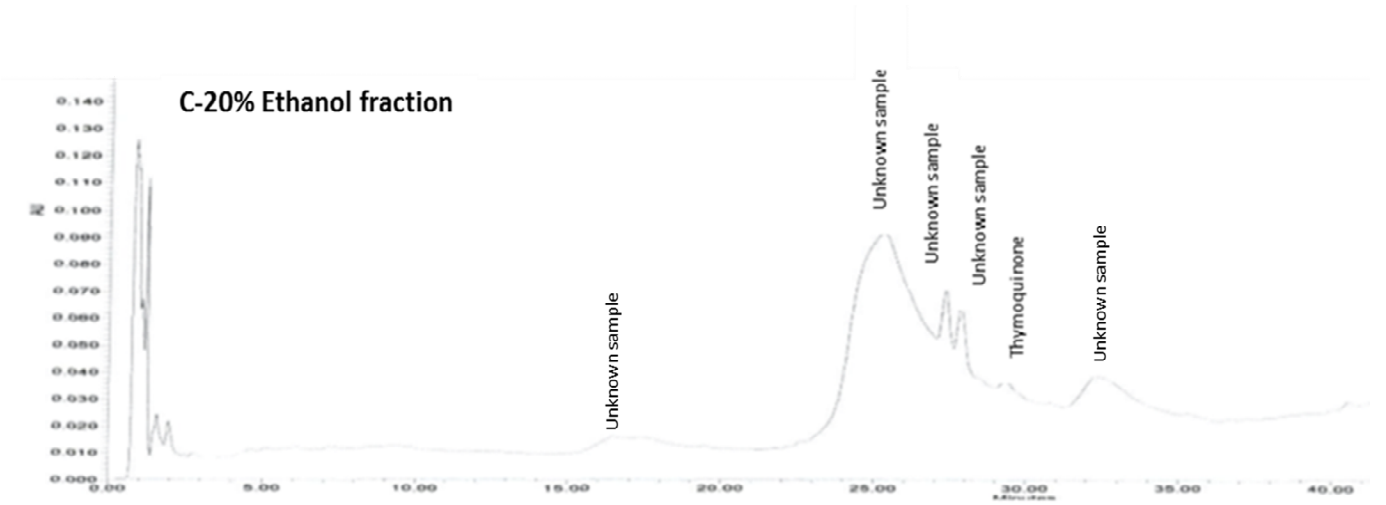
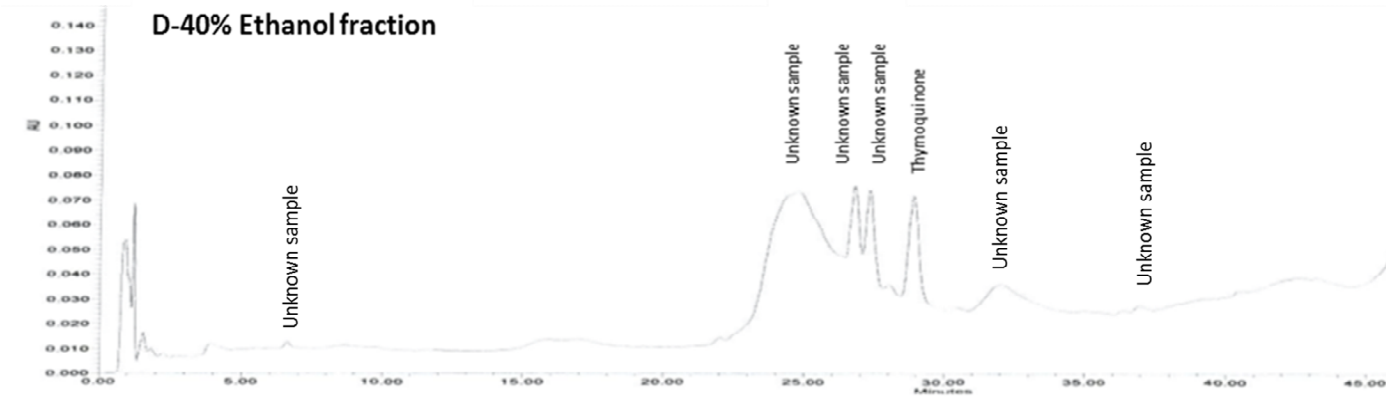
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample


C-20% Ethanol fraction
AU
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample
Unknown sample


D-40% Ethanol fraction
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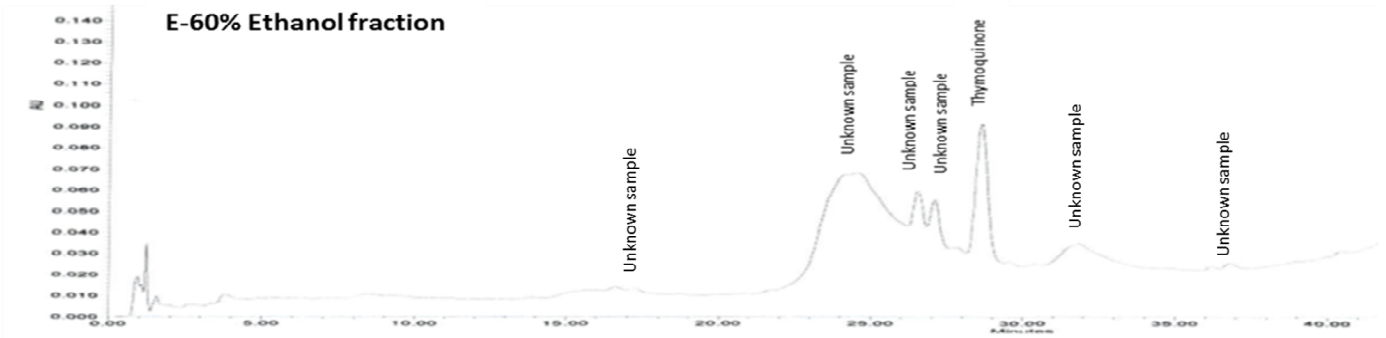
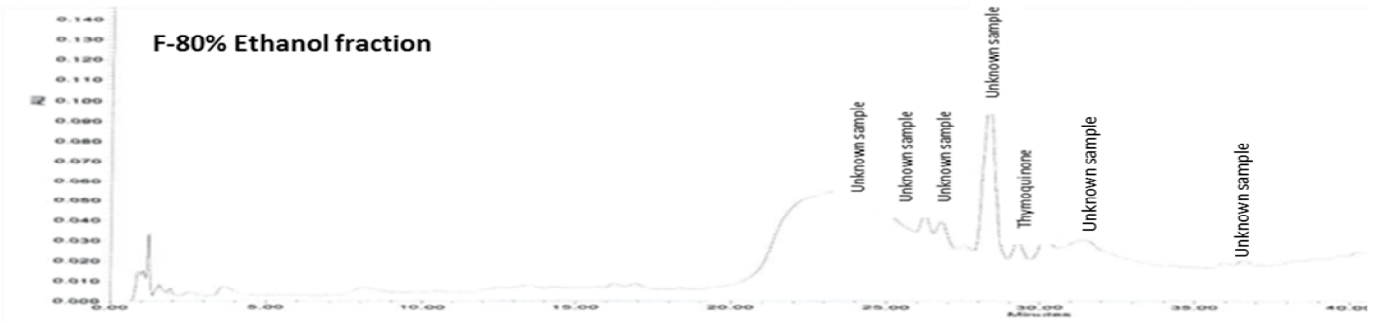
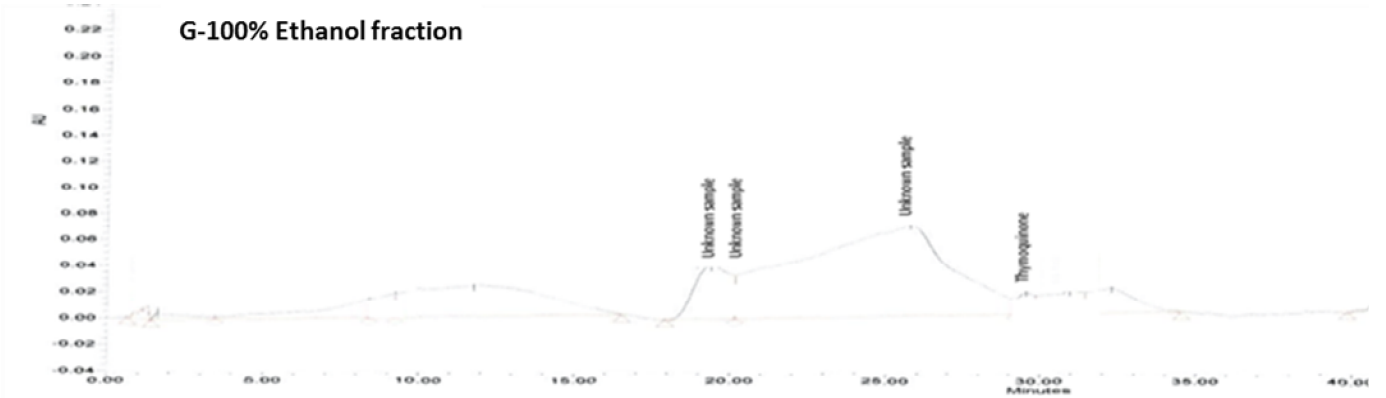
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample
Unknown sample


E-60% Ethanol fraction
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unknown sample
Unknown sample


F-80% Ethanol fraction
Unknown sample
Unknown sample
Unknown sample
Unknown sample
Thymoquinone
Unkrown sample
Unknown sample

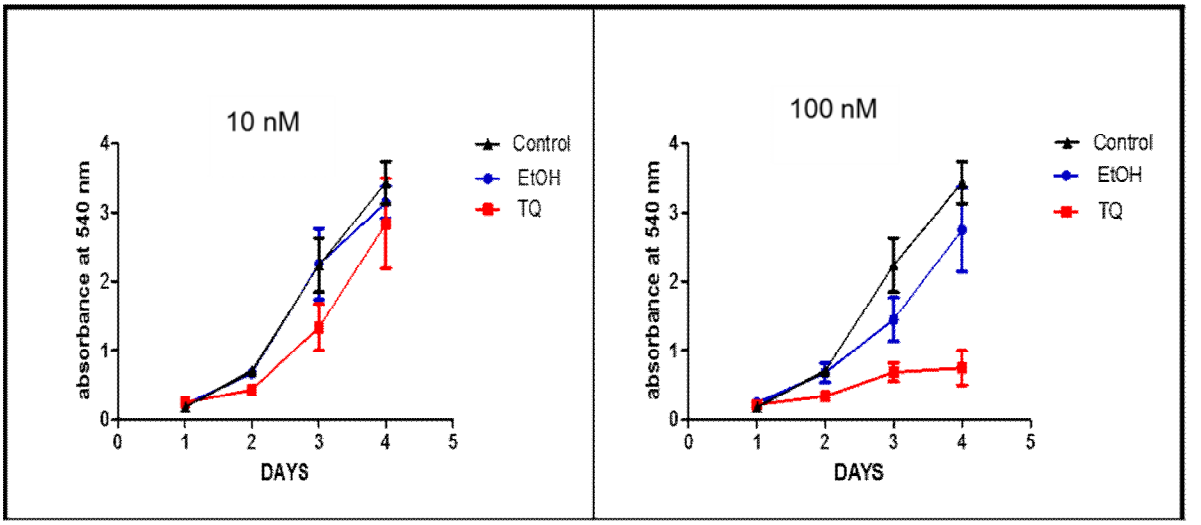

G-100% Ethanol fraction
Unknown sample
Unknown sample
Unknown sample
Thymoquinone

3.8. Individual components of *N. sativa*

The anti-proliferative effect of Thymoquinone (Sigma) against the aggressive murine melanoma cell line B16-BL6 was determined using the MTT assay. The result showed significant ($p<0.05$) cell inhibition in response to treatment with the high (100 nM) concentration of Thymoquinone compared to the low (10 nM) concentration (Figure 3.22).

Figure 3.22. Effect of the Thymoquinone ethanolic extract on B16-BL6 cell proliferation.

The effect on cell proliferation in response to treatment with Thymoquinone was assessed using the MTT assay. B16-BL6 cells were exposed to a low dose (10 nM) and a high dose (100 nM) of Thymoquinone and cultured for four days.


10 nM
absorbance at 540 nm
DAYS
Control
EtOH
TQ
100 nM
absorbance at 540 nm
DAYS
Control
EtOH
TQ

Chapter 4. Discussion

Melanoma is one of the diseases which is considered to be resistant to cytotoxic agents causing a high rate of deaths (Looi, et al, 2013). For that reason, finding different sources of anticancer agents has become crucial to identify cytotoxic agents that have activity agents on melanoma (Looi, et al, 2013). Natural products are gaining more interest not just because of their anticancer effects, but also of their abundance (Gali-Muhtasib, et al, 2006).

Recently, one important studied plant was *N. sativa* (Gali-Muhtasib, et al, 2006). It has been used in some parts of Asia as a source of spice and food preservatives, as well as for its ability to prevent many diseases for thousands of years (Santoso, et al, 119). Many reports from different laboratories have shown indications of *N. sativa* health benefits, such as *in vitro* antioxidant and anticancer activities (Dilshad, et al, 2012; Randhawa & Alghamdi, 2011; Ahmad, et al, 2013). It has been shown that a methanol extract of *N. sativa* was able to induce *in vitro* cytotoxicity in the human prostate cancer (PC3) cell line, and it has been also shown that the ethanolic extract and chloroform fraction of seed extracts of *N. sativa* induced apoptosis in HeLa cells (Hasan, et al, 2013; Ayman, 2012).

This study was designed to investigate the effect of *N. sativa* on malignant and non-malignant cell lines. It was shown that the 70% extracted ethanol of *N. sativa* had a cytotoxic effect and was able to inhibit the growth of malignant and non-malignant cell lines. In contrast, *N. sativa* extracted with water had a reduced ability to reduce the cell

growth of malignant cell lines and could cause an increase in cell growth for some of non-malignant cell lines. The ethanolic extract of *N. sativa* had the greatest anti-proliferative activity, and that may be because the active compounds of *N. sativa* were more soluble in 70% ethanol than the aqueous extract. These data support the idea of other studies that there is an effective anti-cancer components in *N. sativa*.

4.1. Anti-proliferative activity of *N. sativa*

It has been reported in some studies that *N. sativa* extract has cytotoxic effects which can have a huge influence on several types of cancer cell lines (Al-Sheddi, et al, 2014; Hasan, et al, 2013; Ayman, 2012). Al-Sheddi's study found that there was a strong cytotoxic effect by using both *N. sativa* seed and its oil extract to treat a human lung cancer cell line (A-549), and they both significantly reduced cell growth of A-549 cells ($p<0.001$) (Al-Sheddi, et al., 2014).

The current study used the thiazolyl blue tetrazolium bromide (MTT) assay to determine the cell viability of various malignant and non-malignant cell lines after treatment with *N. sativa*. Since the MTT assay measures an activity of living cells, its uptake and change to soluble MTT into purple formazan products is a measure of cells number. (Arafa, et al, 2011). Our MTT assays were carried out over several days which allowed a measure of changing cell numbers, on cell growth.

The results showed that treatment with the highest dose (2%) of the *N. sativa* ethanolic extract inhibited the cell growth in both the malignant (B16-BL6, T98G) and non-malignant (293T, HSG, and HBL 100) cell lines. However, treatment with the highest dose (2%) of the *N. sativa* aqueous extract showed lower levels of inhibition in

cell growth in all tested malignant cell lines. On the other hand, all non-malignant cell lines were able to continue proliferating when treated with the aqueous extract of *N. sativa* at all concentrations (0.05, 0.1, 0.5, 1 and 2 %). This suggests that the ethanolic and aqueous extracts contained some different compounds and that the ethanolic extract contained a potent cytotoxic agents.

In Al-Sheddi's study, after 24h of treatment with *N. sativa* seed extract at 0.25 mg/ml and higher doses, the typical cell morphology and cell adhesion capacity of the human lung cancer (A-549) cells were changed (Al-Sheddi, et al, 2014). Moreover, treatment with N. sativa seed oil at 0.1 mg/ml had the ability to start reducing the shape and cell adhesion capacity of the human lung cancer (A-549) cells (Al-Sheddi, et al, 2014).

In our study, the morphology of cells treated with *N. sativa* was examined to determine any effects of treatment on cell morphology. After 72 h of treatment of B16-BL6 cells with the highest dose (2%) of *N. sativa* ethanolic extract there was obvious damage to the cells as shown by changes in cell shape and a decrease in cell number. Whereas treatment of malignant cell with the highest dose (2%) of *N. sativa* aqueous extract was able to reduce the number of cells and started to change the typical shape of the cells. From these results, it was clearly indicated that *N. sativa* ethanolic extract has more cytotoxic effects on cell growth than *N. sativa* aqueous extract. Therefore, *N. sativa* ethanolic extract has potent anticancer chemicals which induce apoptosis that were not found in the aqueous extract.

Our *in vitro* results for the murine melanoma cell line B16-BL6, showed that treatment with low doses of *N. sativa* resulted in inhibition of cell growth ranging from 5% to 30% fewer cells. B16-BL6 cells treated with the high dose of *N. sativa* for 24 h showed a huge inhibition of cell growth greater than 50%. From these results, we suggest that *N. sativa* seeds have effective anticancer components causing the death of cancer cells.

4.2. *N. sativa* and the Induction of Apoptosis

Anticancer drugs derived from natural products can have different mechanisms of action, althugh the mechanism is based on their ability to induce apoptosis (Shafi, et al., 2009). Apoptosis is an active physiological mechanism that cause obvious morphological changes to the cells, such as DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinking, and apoptotic body formation (Shafi, et al, 2009). Several studies have found that natural products are able to regulate apoptotic pathways (Daniel, et al, 2006), and from our results it is proposed that *N. sativa* is able to induce apoptosis in B16-BL6 cells.

In the present study, we investigated if murine melanoma B16-BL6 cells treated with *N. sativa* extracts used apoptosis as its mode of action to decrease cell growth. Apoptosis was investigated by examining the morphological features of cells such as nuclear chromatin condensation and DNA fragmentation. TUNEL assays and acridine orange/ethidium bromide staining assays were used to determine whether the cytotoxicity

activity of *N. sativa* is due to the induction of apoptosis. B16-BL6 cells treated with the highest dose of the ethanolic or aqueous extracts of *N. sativa* were stained with acridine orange/ethidium bromide. Acridine orange is a fluorescent dye which stains DNA in both dead and live cells green, and ethidium bromide is a sensitive dye which stains DNA in cells that have lost membrane integrity red.

We have shown that B16-BL6 cells treated with the *N. sativa* ethanolic extract induced DNA fragmentation after 72 h, whereas B16-BL6 cells treated with the *N. sativa* aqueous extract for 72 h was not as effective as the *N. sativa* ethanolic extract.

Moreover, to identify DNA fragmentation which happens during the late stage of apoptosis, a TUNEL assay was performed. The results showed that B16-BL6 cells treated with both extracts of *N. sativa* at 0 h did not cause DNA fragmentation. On the other hand, B16-BL6 cells were highly apoptotic after 72 h of treatment due to DNA fragmentation. Cells treated with the high dose of the *N. sativa* ethanolic extract showed higher levels of DNA fragmentation than cells treated with the aqueous extract. From these last two experiments, it is indicated that the *N. sativa* ethanolic extract has a stronger activity more to induce apoptosis and cause DNA fragmentation than the *N. sativa* aqueous extract.

Recent studies using the TUNEL assay demonstrated that the methanolic extract of *N. sativa* seeds, in dose-dependent manner, induced DNA fragmentation caused by apoptosis in human cervical cancer cells and MDA-MB-231 cancer cell line (Hasan, et al, 2013; Dilshad, et al, 2012).

In addition, apoptosis depends on a group of intracellular proteases that have cysteine in their active sites, called caspases (Alberts, et al, 2008). Caspases play an important role in the apoptosis mechanism because many of the morphological and biochemical changes are associated with caspases-dependent degradation (Alberts, et al, 2008). Caspases exist in the cells as inactive pro-caspases which are activated by proteolytic cleavage to generate the active proteases (Alberts, et al, 2008).

To confirm that B16-BL6 cells were undergoing apoptosis in response to treatment with *N. sativa,* a caspase activity assay was performed. In both extracts of *N. sativa,* B16-BL6 cells showed increased activity for caspase -2, -3, -6, -8, and -9. Treatment with the high dose of *N. sativa* ethanolic extract for 24 h showed a huge increase in all caspase activity, and caspase -3 activity was the highest compared to the control cells. In contrast, treatment with the high dose of *N. sativa* aqueous extract for 24 h showed a smaller increase in all caspase activity, and caspase -8 activity was the highest compared to the control cells. *N. sativa* extracts activated both the extrinsic and intrinsic pathways. This could be caused by the presence of several compounds in the mixture which could activate both pathways.

We have presented evidence showing that the ethanolic extract of *N. sativa* was able to be more effective in inducing apoptosis than the aqueous extract. Hasan' study suggested that *N. sativa* activates intrinsic and extrinsic death pathway by increased activity of caspase-3, 8, and 9 in human cervical cancer cells (Hasan, et al, 2013). *N. sativa* extracts contain TQ which is known to cause cell death, probably through

apoptosis. Interestingly, TQ is more soluble in ethanol and would be expected to be a higher concentration in the ethanolic extract compared to the aqueous extract.

4.3. Characterization of *N. sativa*

A high performance liquid chromatography method was used to identify the phytochemical components in *N. sativa*. In recent studies, many known bioactive components were isolated from *N. sativa* by HPLC include, thymol, thymohydroquinone, thymoquinone, and dithymoquinone (Muzaffar, 2013). However, many studies reported that thymoquinone is the major active constituent of *N. sativa* (Muzaffar, 2013; Gali-Muhtasib, et al, 2006). Experimental studies have also revealed the chemotherapeutic potential of thymoquinone for anti-cancer activity in combination with clinically used drugs like doxorubicin, cisplatin and isofosfamide in certain cancer cell lines including the 518A2 melanoma (Effenberger and Schobert, 2011). It was observed that when 518A2 melanoma cells were treated with equimolar mixtures of doxorubicin and TQ, TQ caused an increase in reactive oxygen species (ROS) leading to growth inhibition (Effenberger and Schobert, 2011).

The purpose of fractionating *N. sativa* is to characterize the components that may have anti-cancer effects. In our study, we use TQ as a known compound standard to detect the peaks in the chromatogram that will help to indicate whether the active compound from *N. sativa* is a known compound or an unknown compound. The results showed that (ethanol extract, 40% ethanol fraction, and 60% ethanol fraction) have unknown compounds and a high level of TQ. Other extracts (aqueous extract, 20%

ethanol fraction, 80% ethanol fraction) have also unknown compounds but a low level of TQ.

Lastly, from our results we suggested that the anti-cancer activity of *N. sativa* is not only due to TQ, but there may be other anti-cancer agents which need to be further investigated in the future. The water extract which does not contain TQ also has anti-proliferation activity suggesting other components are involved in regulating growth of cell culture.

4.4 Conclusion

For many years, *N. sativa* is has been one of the well-known natural products that has been used ability to prevent many diseases as well as cancer.

In our study, we found that the 70% ethanol extract of *N. sativa* was shown to have cytotoxic effects on different types of malignant (B16-BL6, T98G) and three non-malignant (293T, HBL 100, HSG) cell lines. However, the aqueous extract of *N. sativa* was able to reduce the cell viability only of malignant cell lines. The available data in this study showed that the ethanol extract of *N. sativa* has cytotoxic activity against murine melanoma B16-BL6 cells by inducing apoptosis**.** The results from Acridine Orange/Ethidium Bromide staining assay, TUNEL assay, and caspase activity assay led us to conclude that the mechanism of death for B16-BL6 cells in response to the ethanol extract of *N. sativa* treatment was cellular apoptosis. In the future, before the development of a promising anti-cancer drug for cancer treatment from *N. sativa*, extra research will be required to identify and characterize the unknown components of *N. sativa*.

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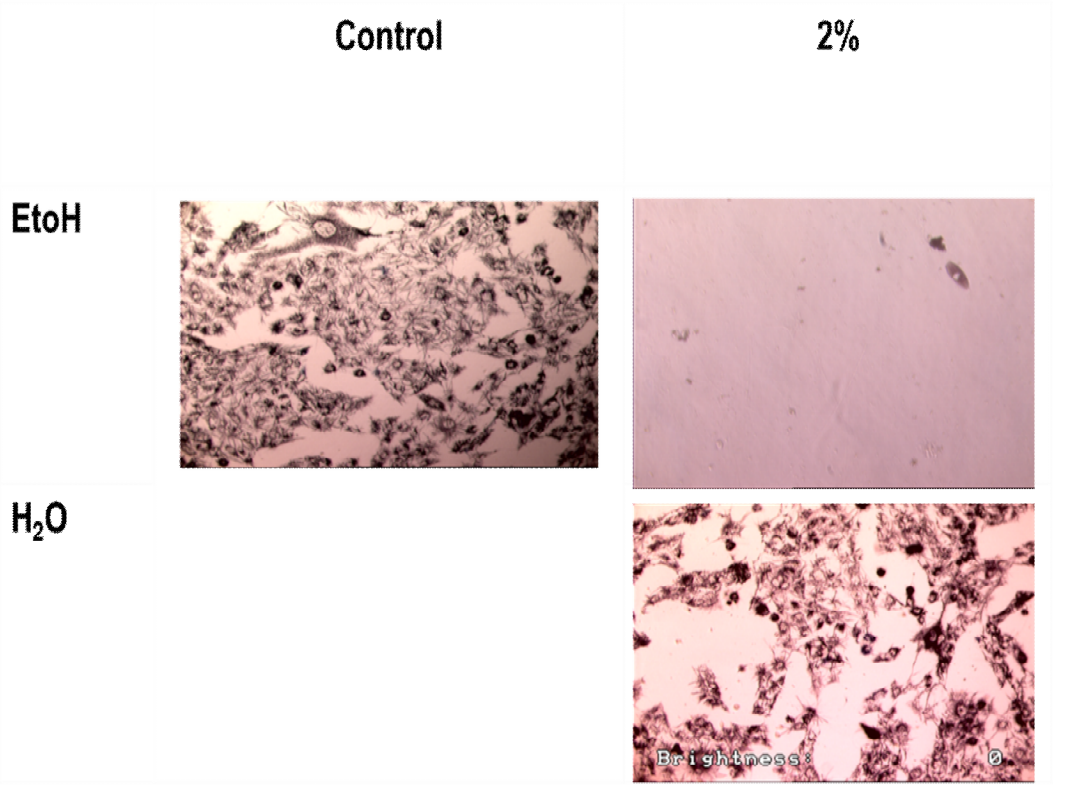
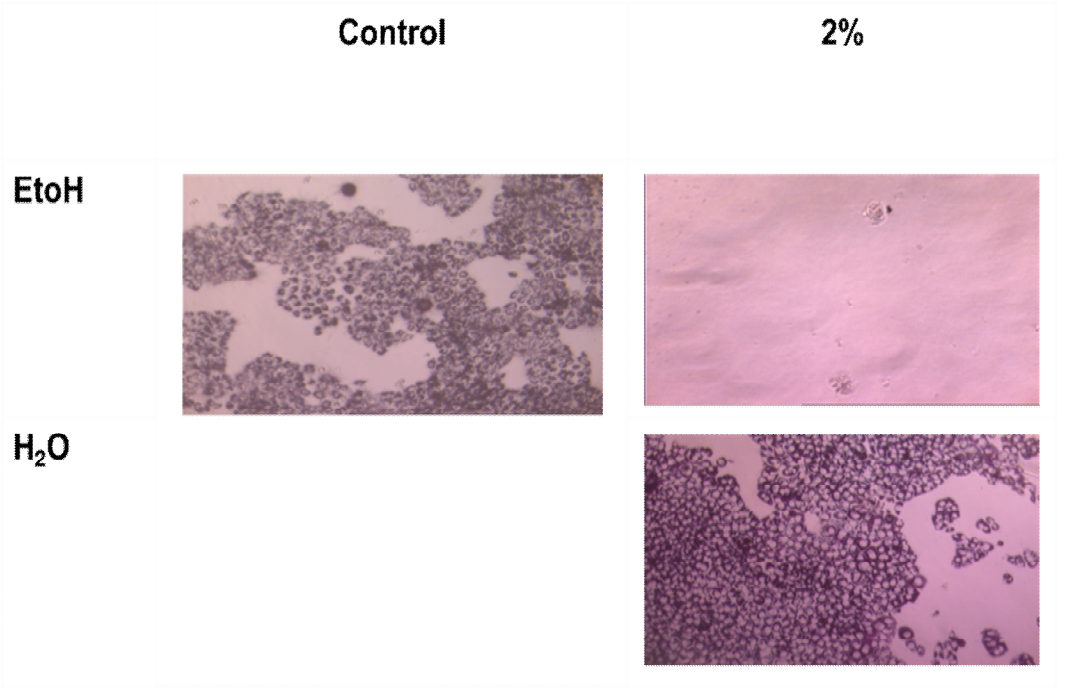
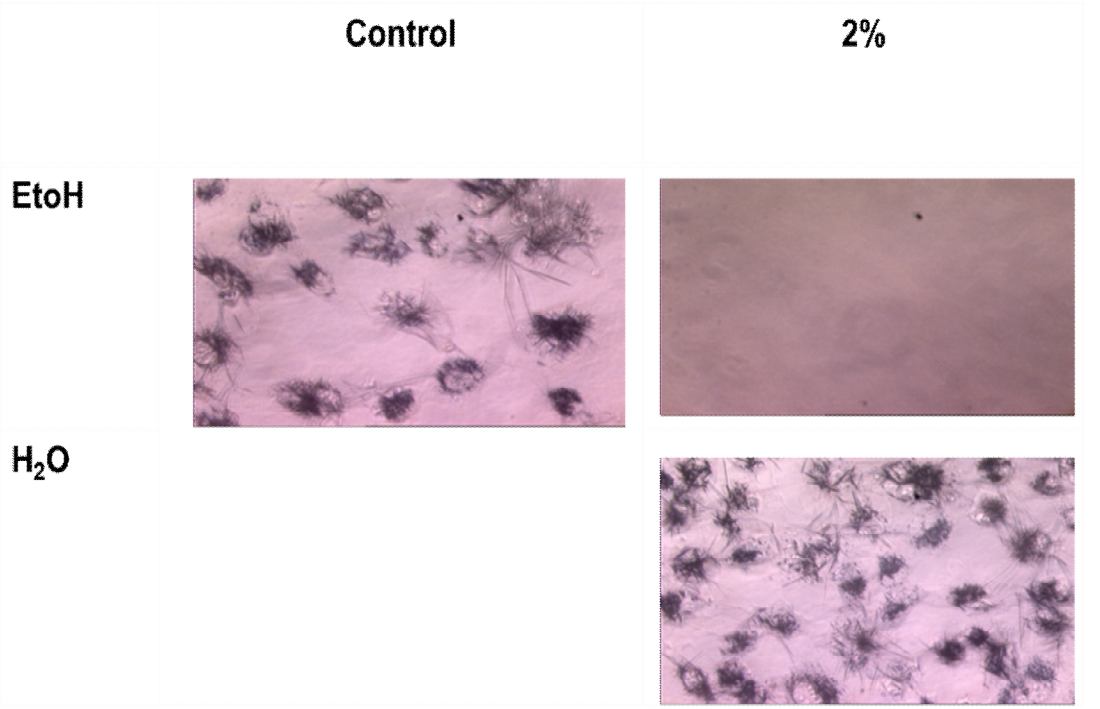
Appendix



Morphological changes in T98G cells exposed to various concentrations of *N. sativa* with ethanolic extract or aqueous extract for 72 h.



Morphological changes in HSG cells exposed to various concentrations of *N. sativa* with ethanolic extract or aqueous extract for 72 h.



Morphological changes in HBL 100 cells exposed to various concentrations of *N. sativa* with ethanolic extract or aqueous extract for 72 h.