CYTOTOXIC AND ANTIOXIDANT EFFECTS OF PHOENIX DACTYLIFERA L. (AJWA DATE EXTRACT) ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

KHUSHBOO SHAHBAZ

UNIVERSITI SAINS MALAYSIA

2020

CYTOTOXIC EFFECT OF PHOENIX DACTYLIFERA L. (AJWA DATE EXTRACT) ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

by

KHUSHBOO SHAHBAZ

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

December 2020

ACKNOWLEDGEMENT

First of all, I would like to thank Allah Almighty for His infinite blessings upon me and provided me with opportunities, resources, comfort and good health. Due to His blessings, I was able to join my research study at Universiti Science Malaysia and complete it adequately.

I am also grateful to my supervisors Dr. Jawaad Ahmed Asif, Dr. Nurul Asma Abdullah and Dr. Tang Liszen. They helped me overcome the difficulties that I encountered during my research. The guidance and aid provided were a great support and for which their help is highly appreciated. This thesis would have been certainly impossible without their support and guidance of which I am very thankful.

I am thankful to my parents and my parents-in-law for their continuous support and assistance during the research process, for which without, I would not have been able to come to my prior desired results. Their encouragements and prayers have been a major inspiration and motivation throughout the research period. I am really thankful to my husband for his continuous faith, support and patience. Whenever there was a need for advice or suggestions on my behalf, I always had the opportunity to utilize his balanced perceptions towards problems or obstacles. Also, I am grateful to my brother and sisters who were there through every thick and thin.

In addition, I offer appreciation to my friends, Dr. Khadeeja Saleem, Dr. Mahmud Mohammad Fahim, Dr. Fabiha Jasmin, Dr. Anas Imran, Dr. Yousaf Athar and Dr. Minahil Maqbool who have supported me all this time and kept me on track. They also provided me professional knowledge and guidance as per their abilities and were always there to support my work.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percentage
®	Registered
µg/ml	Microgram per milliliter
µm	Micrometer
°C	Centigrade
Aq.	Aqueous
h	Hours
g	Gram
mg	Milligram
mg/ml	Milligram per Milliliters
min	Minutes
mm	Millimeter
× g	Relative centrifugal force
OSCC	Oral Squamous Cell Carcinoma
FCM	Flow Cytometry
AIDS	Acquired Immune Deficiency Syndrome
ADF	Ajwa date flesh
ADP	Ajwa date pit
ANOVA	Analysis of variance
ATCC	American type culture collection
BAX	BCL2 associated X
BC	Before Christ
BCL	B-cell lymphoma
BQ	Betel Quid
CHO	Chinese Hamster Ovary
CIS	Carcinoma in situ
CS	Cigarette Smoking
CT-RT	Concomitant Chemo-Radiotherapy
CV	Cell viability
CytC	Cytochrome C
DISC	Death-Inducing Signaling Complex

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to Mesenchymal Transition
FAD	Flavin Adenine Dinucleotide
FADD	FAS-associated death domain
FasL	Fas Ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMN	Flavin Mononucleotide
GLOBOCAN	Global can incidence, mortality and prevalence
H2O2	Hydrogen Peroxide
HepG2	Human Liver Cell Line
HeLa	Human Cervical Cell Line
HIV	Human Immunodeficiency viruses
HN	Head and Neck
HPV	Human papilloma virus
HSC-2	Human Squamous Cell
HSD	Honestly Significant Difference
IC_{50}	50% inhibitory concentration
IGF	Insulin-like growth factor
MEM	Minimum essential medium
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCR	National Cancer Registry
NNK	Nicotine-derived nitrosamine ketone
NNN	Nitrosonornicotine
NMU	N-Nitroso-N-methylurea
NMR	Nuclear magnetic resonance
OED	Oral Epithelial Dysplasia
PBS	Phosphate buffer saline

PBUH	Peace be Upon Him
PC3	Prostate cancer cell line
PCD	Programmed cell death
PenStrep	Penicillin-Streptomycin
PI	Propidium Iodide
PTP	Permeability Transition Pore
PS	Phosphatidylserine
R2	R-squared
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SPSS	Statistical package of social sciences software
TNM	Tumour, Node, Metastasis
TSNs	Tobacco-Specific nitrosamines
USM	Universiti Sains Malaysia
UV	Ultraviolet
v/v	volume/volume
VDAC	Voltage Dependent Anion Channels
VELscope	Visually enhanced lesion scope
WHO	World health organization
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium)
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-dipehnyltetrazolium bromide
WST-1	(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2H-tetrazolium)
XTT	T (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt)

LIST OF APPENDICES

KESAN SITOTOKSIK DAN ANTIOKSIDA PHOENIX DACTYLIFERA L. (EKSTRAK KURMA AJWA) ATAS BARISAN SEL KARSINOMA SEL SKUAMUS ORAL

ABSTRAK

Karsinoma sel skuamus oral (KSSO) adalah masalah utama kesihatan awam dunia. Prognosis bagi pesakit KSSO kekal buruk walaupun dengan kemajuan dalam modality rawatan dan teknologi diagnostic. Oleh yang demikian, terdapat keperluan yang mendesak untuk mencari satu bentuk terapi alternatif yang selamat dan efektif untuk merawat penyakit ini. Satu produk semula jadi, buah kurma Ajwa dilaporkan mempunyai kesan-kesan anti-oksidan dan anti-kanser. Namun tiada lagi kajian yang menumpu atas kesan ekstrak isi dan biji kurma Ajwa atas KSSO. Matlamat kajian ini adalah untuk mengkaji potensi sitotoksik dan apoptosis ekstrak isi kurma Ajwa (IKA) dan biji kurma Ajwa (BKA) atas barisan sel HSC-2. IKA dan BKA diesktrak dengan kaedah ekstrak pelarut menggunakan heksana, aseton dan etanol, kemudian melalui asai anti-oksidan 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2, asai 5-Diphenyltetrazolium Bromide (MTT) sebagai penilaian sitotoksik. Bagi penilaian apoptosis, kit pengesanan apoptosis Annexin-V pada 24, 48 dan 72 jam digunakan. Dalam kajian ini, ekstrak aseton IKA dan BKA mempunyai aktiviti penghapusan radikal dan anti-oksidan yang paling tinggi diikuti oleh ekstrak etanol. Manakala BKA memaparkan kesan anti-oksidan yang lebih tinggi dan signifikan daripada IKA. Dalam kajian ini, asai MTT menunjukkan ekstrak aseton IKA dan BKA adalah sitotoksik secara signifikan terhadap sel-sel HSC-2 bergantung kepada dos dan masa. Kepekatan separuh kerencatan (IC_{50}) IKA didapati adalah 8. 69 mg/ml pada 24 jam dan kerencatan pertumbuhan sel maksimum diperhatikan pada 50mg/ml. IC_{50} bagi

BKA didapati adalah 0.97 mg/ml pada 24 jam dan kerencatan pertumbuhan sel maksimum diperhatikan pada 5 mg/ml. Analisis statistik asai sitometri aliran menunjukkan bahawa rawatan dengan ekstrak IKA dan BKA mempunyai kesan apoptosis yang signifikan dan berlaku bergantung kepada dos. Ekstrak BKA menunjukkan aktiviti apoptosis yang lebih tinggi daripada ekstrak IKA. Tambahan pula, rawatan gabungan IKA dan BKA juga dilakukan ke atas sel-sel HSC-2 dan menunjukkan aktiviti apoptosis yang lebih tinggi berbanding dengan menggunakan hanya satu ekstrak. Kesimpulannya, buah kurma Ajwa menjanjikan kesan sitotoksik dengan merencat pertumbuhan dan perkembangan sel-sel KSSO dan menggalakkan kematian sel melalui kerencatan kitaran sel dan apoptosis. Selain itu, aktiviti penghapusan radikal BKA akan mempromosikan ia sebagai agen yang berpotensi untuk kajian lanjutan bagi mengasingkan kompaun anti-oksidan untuk digunakan secara gabungan denga isi buah dalam perkembangan ubat anti-kanser pada masa depan.

CYTOTOXIC EFFECT OF PHOENIX DACTYLIFERA L. (AJWA DATE EXTRACT) ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

ABSTRACT

Oral squamous cell carcinoma (OSCC) is a major public health problem globally. The prognosis for patients with OSCC remains poor despite the advancement in treatment modalities and diagnostic technology. Therefore, there is an urgent need to explore natural products that effectively cure this disease. Ajwa date fruit, was reported to have strong anti-oxidant and anti-cancer properties. However, no study has focused on Ajwa date flesh and pits extract effect on OSCC yet. The aim of the current study is to investigate the cytotoxic and apoptotic potential of Ajwa date flesh (ADF) and Ajwa date pit (ADP) extract on HSC-2 cell line. ADF and ADP were extracted with solvent extraction method using hexane, acetone and ethanol, which were then subjected to antioxidant assay by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay for cytotoxicity assessment. For apoptosis assessment, Annexin-V apoptosis detection kit for 24, 48 and 72 hours was used. In this study, acetone extracts of ADF and ADP had the highest radical scavenging and antioxidant activities followed by the ethanolic extracts. Whereas, ADP appeared to have significantly higher antioxidant effects than the ADF. In this study, the MTT assay demonstrated that acetone extracts of ADF and ADP were significantly cytotoxic against HSC-2 cells in a dose and time-dependent manner. The half inhibitory concentration (IC_{50}) of ADF was found to be 8.69 mg/ml at 24h and the maximum cell growth inhibition was observed at 50 mg/ml. The IC_{50} for the ADP was found to be 0.97 mg/ml at 24h and the maximum cell growth

inhibition was observed at 5 mg/ml. Statistical analysis of the flowcytometry assay showed that the treatment with ADF and ADP extracts had significant apoptotic effect which occurred in a dose-dependent manner. At treatment with higher doses of ADP and ADF extract HSC-2 cells were seen in late apoptotic stage. ADP extract demonstrated higher apoptotic activity than ADF extract. In addition, combined treatment of ADF and ADP were also performed on HSC-2 cells which demonstrated higher apoptotic activity when compared to the single extract. In conclusion, Ajwa date fruit has promising cytotoxic effect by inhibiting growth and proliferation of OSCC cells and inducing cell death by apoptosis. Furthermore, high radical scavenging activity of ADP will promote it as a potential candidate for further research to isolate the antioxidant compounds and to be used in combination with the fruit flesh in the development of anti-cancer drug in future.

CHAPTER 1

INTRODUCTION

1.1 Background

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in humans globally with an estimated 53,260 new cases and a mortality of 10,750 deaths annually, with twice as high incidence in male than in female. OSCC accounts for more than 90% of all oral malignancies occurring in oral cavity (Yakob *et al.*, 2014). However, incidence rate decreased by 2% in blacks and an increase of 1% has been seen among whites, irrespective of genders (Society, 2020). The highest rates are found in developing countries such as South Asia, Pacific region, Latin America and in parts of Central and Eastern Europe (Petersen, 2003). The incidence of the disease is influenced by several factors such as use of tobacco, betel quid chewing, alcohol drinking, infection with HPV, genetics, radiation, unhealthy diet and physical inactivity (Kalavrezos and Scully, 2015; Popkin, 2007). 25% of oral cancers are associated with tobacco intake (smoking/chewing), 7-19% associated with alcohol consumption, 10-15% associated with micronutrients insufficiency and more than 50% with betel quid/pan chewing (Petti, 2009).

The prognosis for patients with oral squamous cell carcinoma remains poor in spite of advances in diagnostic technology. The basic and most important diagnostic tools are the visual examination of the oral cavity and biopsy of the lesion. Further tests can be performed to find out the cause, type or extent of the cancer which may include personal and family medical history, cancer profiling tests, and scans. Some of the latest additional tools are light-based oral screening device such as VELscope, specific salivary testing for macromolecules, mRNA's and DNA transcripts (Nagler,

2009). However, clinicians are still faced with the challenge to diagnose oral cancer in its early stages.

The common treatment modalities currently used for the treatment of OSCC are surgery, radiation therapy and chemotherapy. More advanced and promising treatments, such as targeted therapies, immunotherapies, and anti-cancer vaccines have been introduced for few selective cancer types, but are still in early stage, patient selective, expensive and are not being used as a routine treatment (Liu, 2014). Despite the advancement in research and therapy, no improvement in the survival rate has been observed in recent years(Rivera, 2015). Normally, the 5-year survival rate is 50% but if detected in early stages, it is increased to 60-80% (Inagi et al., 2002; Yakob et al., 2014; Zini et al., 2010). Such treatments, however, come with adverse side effects and reduced quality of life and high mortality rates. Therefore, there is an urgent need to find an effective natural therapy to cure this disease. In this context, novel natural and herbal products which are less cytotoxic to normal cells and more effective against tumour cells, need to be discovered, for the successful outcome of cancer treatment.

Date palm (Phoenix Dactylifera L.) from the Family of Arecaceae is an important plant of desert regions cultivated worldwide, majorly in Middle East, North Africa, Pakistan, India, Southern Europe and South America. Date palm is important economically due to its great nutritional and phytochemical composition. Ajwa date is one of the most popular varieties of date palm belonging to the city of Al Madinah Al Munawwara in the Kingdom of Saudi Arabia. Ajwa date flesh is soft covered with a thin black skin which has black lining and the seed is hard and elongated with a vertical groove. Ajwa date has been around for ages and is of great research value due to its medical properties. Ajwa dates have high nutritional value compared to other date

varieties because Ajwa dates are rich in carbohydrates, minerals, proteins, vitamins and dietary fibres. Previous studies had investigated its phytochemical compositions which include phenols, flavonoids, sterols and carotenoids, which has anti-inflammatory, anti-oxidant, hepatoprotective, anti-mutagenic and anti-cancerous potentials (Baliga *et al.*, 2011; El-Far *et al.*, 2016). These biologically active compounds are also responsible for the free radical scavenging activity (Vayalil, 2002). Furthermore, in a previous study, Ajwa date extract did not show any toxic effect on a normal cell line Vero, therefore it can be considered as a safe natural product that can be further investigated against oral squamous cell cancer.(Siddiqui *et al.*, 2019)

The date pits are usually discarded while eating or used for animal feed but recently, studies have shown Ajwa date pits to be medically-beneficial as the pits are also enriched with certain phenolic and flavonoid compounds which have many beneficial effects due to the strong anti- oxidant and anti-inflammatory properties(Adeosun et al., 2016). In Islam, Ajwa dates has a remarkable status as narrated in Hadith by Sahih Al-Bukhari: The Prophet (Peach be upon him) said "Whoever eats seven Ajwa dates every morning will not be harmed by poison or witchcraft all that day until night comes"(Khalid et al., 2016).

In a previous study, Ajwa date showed strong anti-cancer effects in human prostate cancer cell line (PC3) (Mirza et al., 2018). In another study, date palm pits were evaluated for their anti-genotoxic activity against DNA damaged rats which were induced with N-Nitroso-N-methylurea as a carcinogen. It was observed that the presence of minerals and phytochemicals were responsible for anti-cancerous activity (Diab and Aboul-Ela, 2012). Therefore, Ajwa date fruit has promising cancer-preventive properties.

1.2 Problem Statement

Oral cancer is a dangerous and debilitating disease in many parts of the world. The risk of being afflicted by oral cancer can be reduced by changing certain modifiable lifestyle factors e.g. tobacco and alcohol consumption (Warnakulasuriya, 2009a). Due to the delayed detection of oral cancer, the prognosis is poor. And the standard care of treatment like surgery, chemotherapy and radiotherapy, leave the patient with poor quality of life, high rate of cancer recurrence with drug resistance and only a 50% of five years survival rate (García-Martín *et al.*, 2019). Therefore, there is an urgent need to treat the oral cancer with less invasive treatment method, as well as to prevent the occurrence and to eradicate oral cancer for the betterment of future generation. For this reason, natural products should be explored to discover new treatments for the cure of cancers. In the previous literature, different solvents have been used to extract different bioactive components according to their research needs, therefore three solvent have been selected in the present study to extract the maximum amount of polyphenols and flavonoids. To this date, and to our best knowledge, no study has been conducted to oversee the cytotoxic and apoptotic effects of Ajwa date flesh and pit on Human Oral Squamous Cell Carcinoma cell line (HSC-2).

1.3 Justification of Study

Oral cancer is not at top of the list for mortality rate but still has great burden on the society. It is very important clinically to find the best treatment modalities, which provide the patient with best quality of life, therefore naturally occurring compounds are of great research interest as surgical option alone or in combination with radiotherapy and chemotherapy does not improve the 5-year survival rate significantly. For the prevention of oral cancers, it is important for the people to

include antioxidant foods in their diet. The anticancer effects of Ajwa date flesh (ADF) and Ajwa date pits (ADP) on human oral cancers remain unexplored. Therefore, the present study will provide an insight to explore the antioxidant potential of Ajwa date flesh and pits, it will also help us understand the possible anticancer effects of Ajwa date flesh and pits on the OSCC cell line. HSC- 2 cell line was selected for this study. This cell line was established from the oral tissues of squamous cell carcinoma, whereas HSC-3 cell line derived from the tumor of metastatic lymph nodes originated from tongue squamous cell carcinoma and HSC-4 cell line originated from the squamous cell carcinoma of tongue. In order to study the anticancerous effect of Ajwa date on the whole oral cavity, instead of just tongue or the metastatic lymph nodes of tongue, HSC-2 cell line was selected. There is no literature available proving the anti-cancerous properties of ADF and ADP on OSCC, providing me with a great opportunity to attempt to evaluate its likely cytotoxic effects and apoptotic effects on OSCC cells in vitro.

1.4 Objectives

1.4.1 General

The aim of this study is to determine the antioxidant activity of the different extracts of Ajwa date flesh (ADF) and Ajwa date pit (ADP) and to carry out in vitro assessment of the cytotoxic and apoptotic effect of Ajwa date flesh and pits on OSCC cell line.

1.4.2 Specific

1) To evaluate the antioxidant activity of ADF and ADP extracts with three different solvents i.e., N-hexane, aq. ethanol and aq. acetone using DPPH antioxidant assay.

2) To determine the cytotoxic potential of ADF and ADP extract on the OSCC cell line by using Methyl-Thiazole Tetrazolium (MTT) assay.

3) To study the pro apoptotic effect of ADF and ADP extract on the OSCC cell line by flow cytometry.

1.4.3 Research Questions

1) Which solvent (n-hexane, acetone, ethanol) extract the highest number of antioxidants from ADP and ADF?

2) Does the ADF and ADP extracts cause significant decrease in the cell viability of OSCC cell line?

3) Does the ADF and ADP extracts cause significant apoptotic effect in OSCC cell line?

1.4.4 Null Hypothesis

ADF and ADP extract cause no cytotoxic and apoptotic effects on OSCC cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Oral Squamous Cell Carcinoma (OSCC)

In 2020, it was estimated that 53,260 new cases of oral cancer and pharynx will be reported in United States and 10,750 people will die from this cancer with higher occurrence in men than women. (Society, 2020)

Cancers arising from lips, tongue, mouth, larynx, oropharynx, nasopharynx and hypopharynx are collectively known as head and neck (HN) cancer. Globally it is the 6th most common cancer (Warnakulasuriya, 2009c). It has been documented that 90% of these cancers are squamous cell carcinoma (SCC)(Pai and Westra, 2009). Oral squamous cell carcinoma also have similar risk factors as the rest of head and neck cancers (Warnakulasuriya, 2009c).

The routine care for the prevention of oral cancer is the screening of high-risk areas of oral cavity such as posterior and lateroventral part of tongue, floor of oral cavity, and tonsillar pillar in high risk populated areas of world. Another concerning fact about OSCC is its high recurrence rate after initial treatment (Chen *et al.*, 2013)

Worldwide, it was noticed that 25% oral cancers were caused by the tobacco-based products like cigarettes, cigars as well as smokeless tobacco like chewing and/or dipping tobacco. Alcohol consumption contributes 7-19%, micronutrients deficiency contributes 10-15% and the contribution of betel quid chewing is more than 50% (Petti, 2009).

In cases of lip and intra-oral cancer, several changing patterns were observed. In last 30 years, the occurrence of lip cancer has decreased whereas the cases of tongue cancer have increased in males, especially in young people. In females, minor increase was observed due to the usage of tobacco and alcohol (Scully and Bagan, 2009).

Oral cancer cells proliferate due to the alteration in the protective mechanism against the unchecked and faulty growth of damaged cells. Oral cancer is a leading form of cancer in developing countries including South Asia, Pacific regions, Latin America and in parts of central and eastern Europe (Petersen, 2003). The incidence rate is twice as high in men compared to women. From 2007 to 2016 the incidence of OSCC has decreased by 2% in black men and women but increased by 1% in white men and women. The reason for increase is the association with Human Papillomavirus (HPV) as a risk factor (Society, 2020).

In Malaysia, oral cancer was not enlisted in top 10 common cancers since last two decades, But according to the latest report of the National Cancer Registry (NCR), oral cancer is now ranked eighth most common cancers in both males and females.(Ghani *et al.*, 2019)

Even though the incidence and occurrence of oral cancer is less than 1% as compared to other lethal types, but the burden of the disease cannot be ignored, on the patients' families and the entire Malaysia as whole (Ghani *et al.*, 2013).

According to the 5 years report of Hospital Universiti Sains Malaysia (Hospital USM) in Kelantan, the survival percentage from oral cancer was 18%, with an average of 9 months survival. Several factors have influenced the survival of the patients like gender, TNM stage, tumour site, histological type, age and treatments. OSCC was mostly seen at buccogingival mucosa site in Indian females due to the betel quid chewing habit, whereas in Chinese male it presented at the tongue/floor of mouth due to smoking or drinking habits (Razak *et al.*, 2010).

Males with primary lung and prostate cancer and females with breast and lung cancer often present with secondary head and neck tumours. In US, head and neck cancer ranked second with the highest percentage of primary cancer cases i.e., 15%.

Oral cavity and pharynx tumours entirely spread through lymphatics unlike most other solid cancers. Globally, the occurrence of oral cancer percentage in males is high as compared to females where it depends on age of individual. According to a report of 2013, the occurrence of oral cancer is ranked eleventh among all prevailing cancer sites. One third of all the cancer cases of the world occur in the in Indian subcontinent. In India, the incidence of oral cancer is higher in male than females while in Pakistan this incidence is higher in females. The lowest percentage has been found in Eastern Asia and Western Africa, and very few cases are found in North Africa (García-Martín *et al.*, 2019).

2.1.1 Etiology (Major risk factors and emerging risk factors)

Different etiological factors reflect different patterns of oral cancer prevalence in different geographical regions. In West, where cigarette smoking and alcohol consumption are common risk factors; betel quid and smoking are the primary risk factors in South-East-Asian countries. (Kumar *et al.*, 2016b)

Use of tobacco and alcohol synergistically increase the risk factors to thirty folds. These situations are mainly observed in people who drink and smoke massively (Society, 2020).

Cancer growth may be started as a combined result of genetic mutations and unhealthy social activities. Deficiency of nutrients, use of tobacco and alcohol, betel quid chewing were major causative factors that promoted head and neck cancer. But the consumption of tobacco was the leading factor responsible for millions of deaths (Gupta *et al.*, 1995).

NNN(N'-Nitrosonornicotine), NNK4-(nitrosomethylamino) - 1 - (3-pyridyl) -1- butanone), are the aromatic hydrocarbons and nitrosamines are the major carcinogenic agents from tobacco smoke that bind with the DNA of keratinocyte

stem cells to form DNA adducts. During the DNA replication, critical mutations are caused by these adducts. Oxygenations of these carcinogens are done by P450 enzymes in cytochromes. Glutathione-S-transferases are involved in the conjugation process. (Warnakulasuriya *et al.*, 1999).

Use of tobacco without combustion (smokeless tobacco) has become a common practice globally. It is placed in different forms in the oral cavity in contact with the oral mucosa to obtain the desired effects of nicotine. In Western countries, it is used as oral snuff in moist form, whereas in Asian countries it is used as betel quid chewing. In west Europe and USA, tobacco used in chewing plug, loose leaf and in twisted forms. The use of smokeless tobacco mainly causes pre-cancerous lesions and cancerous lesions (Kumar *et al.*, 2016a).

Betel quid chewing is mostly practiced in South-East Asian countries. Betel quid also known as paan is made from the leaf of piper betel vine, quenched lime, areca nut and dried tobacco. Areca nut and tobacco are potentially carcinogenic, mutagenic and genotoxic, proven by many studies conducted in the past(Kumar *et al.*, 2016a). The research showed that the ingredients of betel quid are carcinogenic and cause the proliferation of cells. Reactive oxygen species (ROS), reactive metabolic intermediates and methylating agents caused the major DNA impairment(Hecht, 2003)

Consumption of alcohol is major cause of cancer in United Kingdom, especially in the younger population (Hindle *et al.*, 2000).

An increased risk for the development of oral cancer has been seen in many studies, where alcohol and tobacco have been consumed together by the patients. In one study, alcohol proved to be an independent risk factor for the development of oral premalignant lesions (Maserejian *et al.*, 2006). The role of alcohol was evaluated to be crucial in the development of oral epithelial dysplasia when consumed with

smoking, where it was observed to alter the morphology of oral mucosa by causing epithelial atrophy, making it easier for the tobacco carcinogens to penetrate the mucosa (IARC, 2002).

A number of studies have shown that human papillomavirus (HPV) is a major risk factor associated with OSCC, especially in the carcinoma of oropharynx (D'Souza *et al.*, 2007). An increase in OSCC has been seen in patients who have an active sex life since at a younger age, involving multiple partners. A greater number of OSCC cases have been seen in females with cervical carcinoma, (which is associated with HPV) and their partners, proving that it is sexually transmissible(Scully and Bagan, 2009).

The studies that were conducted in USA concluded that HPV (subtypes 6 and 16) is a major risk factor for oropharyngeal and cervical cancer particularly in young people who do not consume tobacco or alcohol (Furniss *et al.*, 2009). Research showed that HPV virus could affect the p53 tumour suppressor gene, reducing the normal apoptotic activity (Andrews, Seaman et al. 2009).

Decreased immunity, such as in patients with recent transplants, or chronically immunocompromised or affected with HIV/AIDS are the other factors which contributed in development of OSCC. It may also be caused by the immune suppressive reagents such as azathioprine and cyclosporin which are used after kidney transplantation(Van Leeuwen *et al.*, 2009). Cancer of tongue is also associated with the prolonged use of immunosuppressants during the management of Crohn's disease (Li *et al.*, 2003).

It is also reported that low intake of fresh vegetables and fruits increased the risk of oral cancer. Approximately 50% decrease in oral cancer occurred only by use of fresh vegetables and fruits(Warnakulasuriya, 2009b). Evidence from recent research

shows that high risk or oral cancer is associated with people from lower socio-economic class independent from the healthy style of living(Conway *et al.*, 2008).

The other important factors that contributed to the development of oral cancer was the poor hygiene of oral cavity. Infections like syphilis and candidiasis increase the bacterial flora and may be responsible for OSCC (Guha *et al.*, 2007). Evidence showed that 62% of patients with proper dental care were less likely to develop OSCC (Holmes Jr *et al.*, 2009).

Another important risk factor for the development of OSCC is genetic susceptibility, Copper *et al* studied that from 105 head and neck cancer patients, 31 of their first-degree relatives were diagnosed with lung cancer. There may be a possibility that some patients did not inherit the ability to metabolize carcinogens or repair their damaged DNAs (Copper *et al.*, 1995).

2.1.2 Development of OSCC (pathogenesis)

Cancer progression in the oral epithelium passes through different stages to eventually become OSCC. These include the initial oral epithelial dysplasia (OED) and carcinoma in situ (CIS). Clinically OED appears as red or white coloured lesions and sometimes a mix of both (Al-Hashimi *et al.*, 2007).

The white lesions are known as leukoplakia, the red ones are as erythroplakia and the combination of both is called speckled leukoplakia. These lesions are surface lesions and are graded according to the level of derangement from normal histological architecture. The grading of the lesion is categorized as mild, moderate or severe; mild grade involving histological aberration to the lower third of epithelium, moderate grade involving half the thickness and severe grade involving two-third of the epithelial thickness (Woolgar and Triantafyllou, 2011). Once the dysplasia involves the entire thickness of epithelium, it is then called CIS. CIS is not determined as

carcinoma because it has not spread beyond the epithelium. Once the lesions breaches basement membrane to enter the connective tissue, it is then turned as cancer and can be classified into well-differentiated, moderately-differentiated or poorly-differentiated carcinoma. Well-differentiated squamous cell carcinomas bear a close resemblance to normal squamous mucosa with only the presence of few additional keratin pearls which provide evidence for high degree of differentiation (Woolgar and Triantafyllou, 2011). Moderately-differentiated carcinoma shows abnormal keratinization, nuclear pleomorphism and atypical forms of mitoses, whereas poorly-differentiated squamous cell carcinoma shows abnormal histological architecture which includes immature cell with a large number of typical and atypical mitoses, minimal keratinization and sometimes necrosis.

Oral cancers possess certain hallmarks like every other cancer. These features include the ability of the cancer cells to replicate and invade constantly through escaping apoptosis, being insensitive to antigrowth signals, being self-sufficient in proliferative signals and constant blood supply through sustained angiogenesis. These characteristics of oral cancer are presented in Fig 2.1 (Hanahan and Weinberg, 2000).

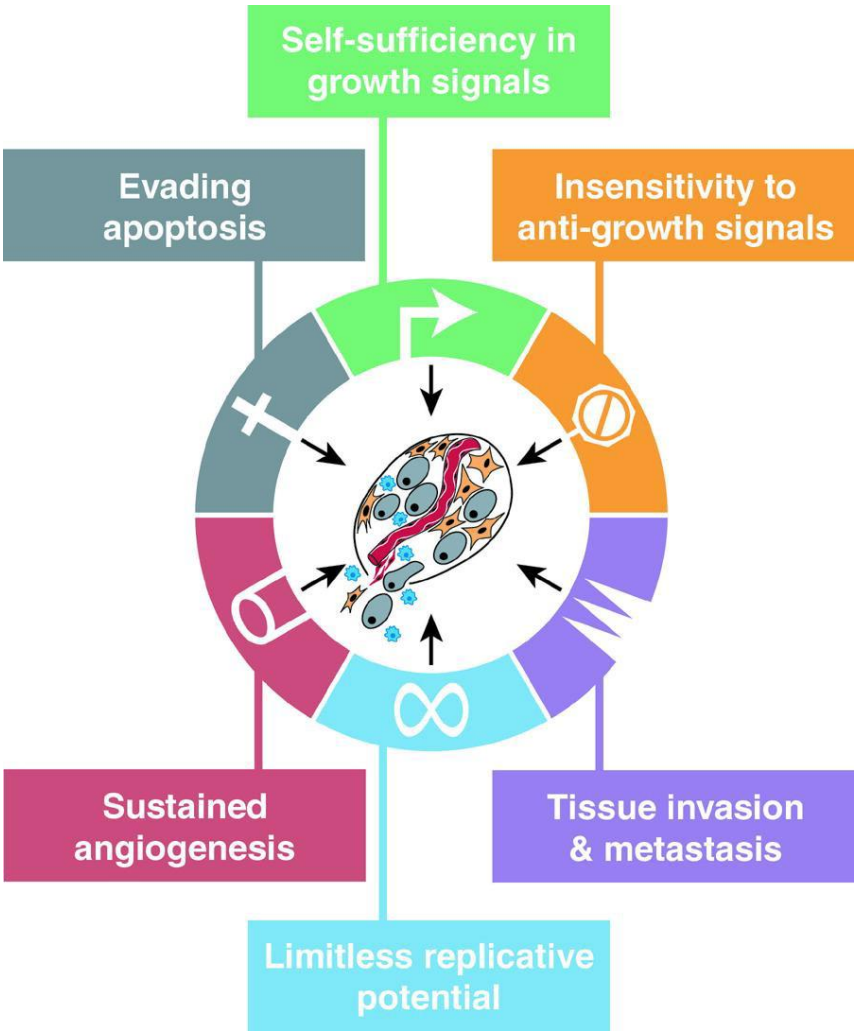


Figure 2.1 Hallmarks of Cancer (Hanahan and Weinberg, 2011)

2.1.3 Clinical Features of Oral Cancer

The major factor for the cancer to go undetected in early stages is because the patients are asymptomatic. Only when the cancer has reached an advanced stage it can be detected by oral potentially malignant disorders (OPMD)(Lingen *et al.*, 2011). OPMD is a pre-cancerous stage and present itself as an ulcer, leukoplakia, erthyroplakia, lump or thickening of mucosa in the throat or mouth, that does not heal, patient presents with the pain, which later undergo histopathological and molecular changes to invade deeper into oral cavity and become advance cancer (Scully and Bagan, 2009). 30-40 % of oral cancer patients present with pain as a chief complaint (Cuffari *et al.*, 2006). The classical features of advanced stages of oral malignancy are

ulceration, nodularity and fixation to underlining tissues (Ribeiro *et al.*, 2009). Other symptoms include difficulty in speaking, chewing, breathing, teeth mobility, trismus, ear pain, neck lump and coughing up blood (Haya-Fernández *et al.*, 2004). Unusual presentation includes paraesthesia of chin, delayed healing after tooth extraction, difficulty in swallowing, weight loss or a lump with abnormal blood supply (Scully and Bagan, 2009).

2.1.4 Prognosis and Treatment

Well-differentiated cancers which have not metastasized, when detected in early stages, they have good prognosis (Scully and Bagan, 2009). Unfortunately, the main reason for poor survival rate of oral cancer patients is because they are diagnosed at advanced stage of disease (Brandizzi *et al.*, 2008). There are a number of factors which affect the prognosis of OSCC that depends on the size, location, histological subtypes of tumour, treatment option and the general wellbeing of the patient (Scully and Bagan, 2009).

The most well-established modes of treatment for early and locally advanced OSCC of oral cavity are surgery and radiation. Surgical approach of the tumour is influenced by a number of factors including location, size, depth of infiltration and proximity to the bone. It may include sacrificing the vital structures (Kalavrezos and Scully, 2016a). Radiotherapy also plays an important role in the treatment of such cases, either alone or combined with surgery or chemotherapy. Surgery combined with radiotherapy in some cases has shown to improve the 5 year survival rates from 40 percent to around 70 percent (Vaughan, 2009). However, radiation comes with its own acute and late toxicity (Kalavrezos and Scully, 2016b). To decrease the related toxicities and to improve the result of radiotherapy, altered fractionated radiotherapy or concomitant chemo-radiotherapy (CT-RT) is used in advanced stage oral cancers

(Mazeron *et al.*, 2009). 50% of the OSCC patients are detected with recurrent or distant metastasis within first two years of their treatment. Lifesaving surgeries can be performed in patients with loco-regional resect able cancer (Weber *et al.*, 2003).

Despite advancement in surgical approach and radiation therapy, the morbidity and mortality rates have continued to increase associated with locally advanced oral cancer (Kim and Li, 2019). Recent advancement in immunotherapy and targeted molecular treatments have opened the treatment options for OSCC patients, new drugs have been developed to target specific molecules. There are several types of molecules in excess amount in the lesion such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and its receptors, these drugs can target these molecules and are highly selective with low toxicities, therefore improving the survival rate of cancer patients. But still these drugs require more research and more patients needs to go under clinical trials to explore their full potential (Liu *et al.*, 2019).

The adverse effects of these standardized treatments (surgery, chemotherapy, radiation therapy) on patients include anaemia, loss of appetite, peripheral neuropathy and poor quality of life. There is a need to develop an effective treatment with potent anti-cancer activity and less adverse effects (Cragg *et al.*, 2009).

A new approach in the management of oral cancer treatment is by antioxidant therapy. Free radicals or oxidative stress in increased levels damages the intracellular structure and DNA of cell leading t many chronic diseases as well as oral cancer. Reduction in oxidative stress can be achieved by antioxidants, which are responsible for the removal of free radicals. Furthermore, it restores the levels of antioxidant enzymes as well as non- enzymes levels, and reduces lipid peroxidation via modulating multiple cell signalling pathways. Diet and medicinal herbs are a major source of antioxidants (Prasad and Srivastava, 2020). Recently the research interest in the natural bioactive

products has increased due to their chemopreventive, chemotherapeutic, immunomodulatory, and radioprotective activity (Mahmoud and Abdelrazek, 2019).

2.1.5 Natural Products

Medicinal plants have been used since many centuries to treat various diseases. Written evidence from the ancient civilization of China, India and North Africa verifies the use of natural products (Phillipson, 2001). Many natural products are still being used all around the world as an alternative medicine. Evidence suggest that these natural products have the properties to trigger autophagy, inhibit angiogenesis, inhibit cell cycle progression and induced apoptosis by modulating signalling pathways which can regulate intracellular reactive oxygen species (ROS), Bcl-2/Bax, p53/p21 pathways. These pathway modulation may be applied for the treatment of cancer diseases (Ding *et al.*, 2016). Natural products can be further classified into three categories, mainly conventional drugs, botanical drugs (medicinal plants) and dietary supplements (Enioutina *et al.*, 2020). A conventional drug has a single recognised bioactive molecule extracted from the natural product and is available as a prescribed or over the counter medicine for the aliment. Whereas botanical drugs contain multiple components extracted from medicinal plants, fungi or algae with their individual pharmacological structure or properties not well characterized (Wu *et al.*, 2020). A dietary supplement may include vitamins, minerals, enzymes, probiotics and other natural herbs. The trend of consuming medicinal drugs and dietary supplements is growing nowadays in both Western and Asian population as many consumers consider these to be harmless and safer alternative to conventional drugs. Unfortunately, the unsupervised usage of medicinal herbs and dietary supplements may cause toxicities mainly due to the interactions with the conventional drugs and over dosage as they are self-prescribed. The safety and regulation of conventional drugs are tightly monitored

by the Food and Drug Administration (FDA) whereas the herbal medicine and dietary supplements are minimally overseen and are commonly available in the supermarkets. Therefore, there is a need to develop a criterion through preclinical and clinical studies for the safety, efficacy and regulation of herbal medicines worldwide (Enioutina *et al.*, 2020).

2.2 Apoptosis

Cancer is an uncontrolled growth of cells due to the imbalance between apoptosis and cell proliferation (Ponder, 2001). Cancer can be viewed as the result of damaged or altered DNA changes, escaping cell death and influencing growth of cells causing malignant transformation (Hanahan and Weinberg, 2000). Programmed cell death or apoptosis is an apparent, inherent cell death program that happens in numerous physical and pathologic conditions. It can be induced by various undesirable stimuli such as radiation, hypoxia, cytotoxic anti-cancerous drugs and ROS (Mousavi and Hersey, 2007).

Apoptosis plays crucial role in the development of cancer as well as in cancer treatment. During carcinogenesis, cells that can escape apoptosis do not die, resulting in uncontrolled cell proliferation. Apoptosis involves many pathways and defect can occur at any given step. A cell can escape apoptosis due to the imbalance in pro-apoptotic and anti-apoptotic proteins, disruption in cell receptor signalling, reduced caspase function and defects or mutations in p53 genes. When p53 gene is mutated, which is responsible for the induction of apoptosis, results in decreased apoptosis and an increase in tumour cell growth and development of cancer (Bauer and Helfand, 2006). However, to find the solution, new drugs that can target the specific aspect of apoptosis has been widely under research. In a study, it was found that when mutant

p53 gene was silenced, a decrease in the growth of human cancer cell was observed which was due to the induction of apoptosis (Vikhanskaya *et al.*, 2007).

2.2.1 Biochemical changes in apoptosis

Biochemical changes that take place during apoptosis include protein breakdown, DNA fragmentation, membrane changes, phagocytotic recognition and activation of caspases pathways (Mollazadeh *et al.*, 2017).

2.2.2 Morphological changes in apoptosis

Morphological changes in apoptotic cell are observed both in nucleus and cytoplasm. These hallmarks include chromatin condensation, nuclear DNA fragmentation, structural alteration of cytoplasmic or organelles, rounding up of the cell, retraction of pseudopods, and membrane blebbing (Kroemer *et al.*, 2009). Cell shrinkage and pyknosis occur during early apoptosis and are the most distinctive features of apoptosis (Mollazadeh *et al.*, 2017).

2.2.3 Mechanisms of apoptosis

Mechanism of apoptosis can be mainly divided into two components; sensors and effectors. Sensors monitor the extracellular and intracellular environment for normal or abnormal stimuli, which will decide whether a cell should live or die. The signals from these sensors regulate the effectors which are responsible for apoptotic death. The effectors are the cell surface receptors which bind with their specific ligands to induce cell death or survival. Example of these receptors/ligand pairs include receptor IGF-1R and the ligand IGF-1/IGF-2 which convey survival signals. Likewise, death signals are conveyed by FAS ligand which bind to their FAS receptor (Hanahan and Weinberg, 2000).

2.2.4 Apoptosis Pathways

There are two major apoptotic pathways that are involved in the activation of the caspase cascade (Hengartner, 2000).

1. Intrinsic Pathways (Mitochondrial Pathway)
2. Extrinsic Pathways (Death Receptor Pathway)

Many anti-cancerous drugs target the signalling pathways resulting in the activation of caspase. Caspases belong to the family of cysteine proteases enzyme that play an essential role as a death effector molecule in the hydrolytic reaction of apoptosis (Mollazadeh *et al.*, 2017).

2.2.4(a) Intrinsic Pathways (Mitochondrial Pathway)

As the name suggests, intrinsic pathway is activated within the cell. Mitochondria are the major organelle that are involved in intrinsic pathway. There are two groups of pro-apoptotic proteins in this pathway, the ones present in the cytoplasm of cell such as Bax and Bad, and the others present in the space between the outer and inner mitochondrial membrane, such as cytochrome c and Smac/DIABLO as seen in the Fig 2.2. When there is an internal or external apoptotic signal, such as, damaged gene, hypoxia, severe oxidative stress, there is a structural change in the outer membrane of mitochondria which is associated with Bax molecule and this results in increased permeability of mitochondrial membrane and the release of cytochrome c into the cytoplasm. Caspase-9 is activated with the release of cytochrome c which in turn triggers the activation of caspase-3, hence, initiation of apoptosis (Hengartner, 2000).

2.2.4(b) Extrinsic Pathways (Death Receptor Pathway)

This pathway is activated when extracellular signals bind to a death receptor in the plasma membrane. For example, a death receptor CD95 lies across the cell membrane and it is triggered when its ligand FasL binds to it. As a result, Fas-associated death domain (FADD) is activated and procaspase-8 molecule is released (which is bound to FADD). The activation of caspase-8 triggers apoptosis (Wong, 2011).

2.2.4(c) The Common Pathway

Caspase-9 activation from the intrinsic pathway and Caspase-8 activation from the extrinsic pathway shares a common final phase of apoptosis by the activation of Caspase-3. Caspase dependent proteins are then cleaved by caspase-3 and producing advanced chromatin condensation causing nuclear apoptosis (Mollazadeh *et al.*, 2017). The summary of these pathways and the important intermediators are shown in the Fig 2.2.

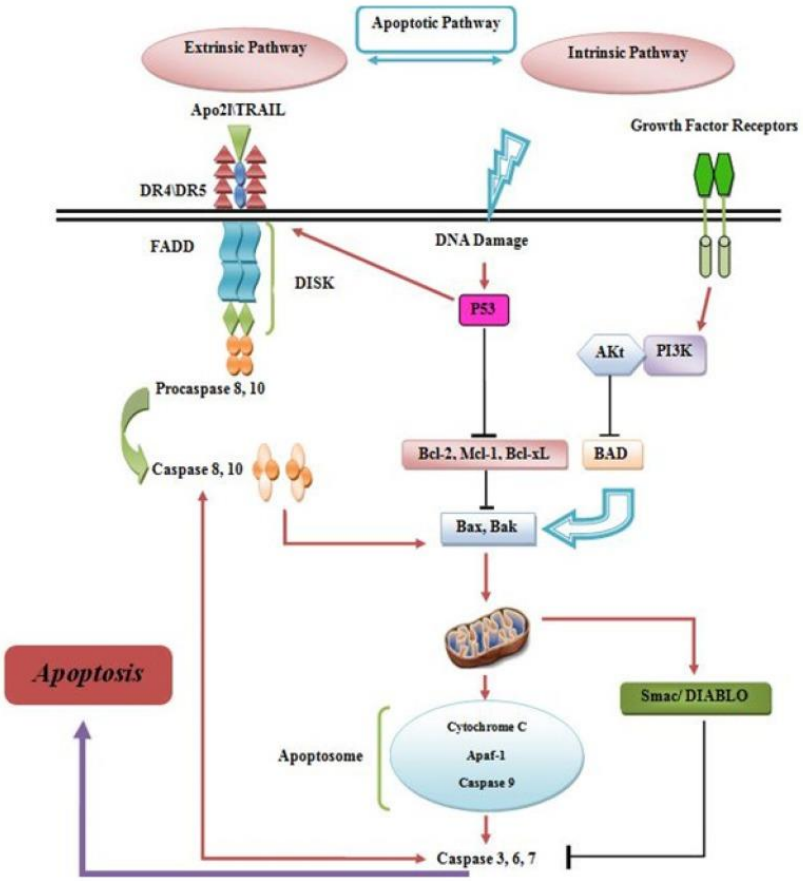


Figure 2.2 Apoptotic Pathway

2.2.5 Apoptosis Inducing Natural Products

Natural product derived compounds could provide an effective treatment which has anti-cancer activities due to their potential ability to induce apoptosis more frequently in cancer compared to normal cells. As a matter of fact, many standard chemotherapeutic drugs including Taxol, epothilones, vinca alkaloids, which are derived from the medicinal plants are still in use to date (Mann, 2002).

2.2.5(a) Black Cumin

Thymoquinone is the main active compound found in Nigella Sativa and have shown a vast therapeutic effects including anti-cancer properties with apoptotic effects and can be used to treat patient with various cancers (Mollazadeh *et al.*, 2017). A study conducted by El-Mahdy et al, thymoquinone has shown to cause reduction in growth and induction of apoptosis by activation of caspase-8, 9 and 3 in myeloblastic

leukaemia HL-60 cells (El-Mahdy *et al.*, 2005). In another similar study, thymoquinone has shown cytotoxic effect in cancer cell lines by induction of apoptosis and cell cycle arrest, whereas, no effect was seen in normal cells (Shoieb *et al.*, 2003).

2.2.5(b) Grapes (Vitis vinifera L.)

Resveratrol is a naturally occurring polyphenolic compound present in significant amount in grapes, berries and peanuts (Biesalski, 2007). Resveratrol has shown promising role in intervening inflammation tumorigenesis and cardioprotective effects (Baur and Sinclair, 2006). Studies have shown that resveratrol has cytotoxic and anti-proliferative effects by inducing cell apoptosis through activation of caspase-3 and other apoptotic signals (Aluyen *et al.*, 2012; Cai *et al.*, 2015).

2.2.5(c) Curcumin

Curcumin is a bioactive polyphenol compound found in turmeric. Curcumin has shown potent anti-cancerous activity by promoting programmed cell death in human malignant brain cancer cells, melanoma cancer cells and T-cell lymphoma cells (Dhandapani *et al.*, 2007; Lu *et al.*, 2010; Zhang *et al.*, 2010). The anti-cancerous and cytotoxic effect of curcumin was found due to the cell cycle disruption and induction of apoptosis (Vietri *et al.*, 2003). Another study has shown the anti-proliferative and anti-angiogenesis effects of curcumin in oral squamous cell carcinoma cell line (Aggarwal *et al.*, 2006).

2.2.5(d) Ginger

Ginger contains many biologically active compounds including certain vallinoids, gingerol, paradol, shogaols and zingerone which are responsible for anti-cancerous properties of ginger (Shukla and Singh, 2007). Ginger has been widely used since very long ago and possesses potent anti-oxidative, anti-inflammatory, anti-

cancerous and anti-mutagenic activities (Surh, 2002). Paradol, one of the phenols presents in ginger has shown to induce cancer cell death by activation of apoptosis and caspase-3 pathway in OSCC cell line (Keum *et al.*, 2002).

2.3 Ajwa date (Phoenix dactylifera)

Date palm (*Phoenix dactylifera*) is a member of Arecaceae plant family. This family has two hundred genera that consists of 3000 species taxonomically (Barreveld, 1993). It is endemic to desert regions of Arab and distributed in Africa, southern parts of Europe, Pakistan, India and South America as well (Baliga *et al.*, 2011).

Date has significant religious, historic al, economical as well as nutritional importance. Across the globe, a huge population consumes this fruit due to its vital performance in healthy metabolic activities as it is a rich source of vitamins, minerals, sugars as well as fibres (Khalid *et al.*, 2016). Dates are also known for their anti-mutagenic, anti-inflammatory, anti-oxidant and immunomodulatory traits. Date also scavenges the free radicals and prevents the occurrence of macromolecular changes in living systems (Al-Farsi *et al.*, 2005).

Ajwa date originates from Holy land of Madinah-Al-Munawwara, Saudi Arabia. It is black coloured, medium sized, sweet and ovoid shaped fruit. It turns green to dark red in early developmental stages while the ultimate mature fruit is black in colour (Al-Khayri *et al.*, 2015).

Studies have found that Ajwa date have anti-oxidant and anti-inflammatory effects due to the presence of flavonoid glycosides (Assirey, 2015). In this regard, activity of Ajwa date was also compared with synthetic anti-inflammatory agents like aspirin, ibuprofen, naproxen and celebrex which resulted in positive evidence (Zhang *et al.*, 2013).

2.3.1 Religious Significance of Ajwa dates

There are certain authentications of Ajwa cultivation and its use as staple food in Saudi-Arabia (A Holy land of Muslims) since 5000 BC. For one and half millennium, around the globe, Muslims use Ajwa date to end their fast in Ramadan period (Khalid *et al.*, 2017).

The health significance and nutritional benefits of this fruit are also documented in Holy Quran. Date is cited twenty times in seventeen Surahs and declared as fruit of heaven in Holy book of Muslims (Qur'an, 55:68). Allah (SWT) in Qur'an recommends Maryam (mother of Prophet Jesus) to eat this fruit. "*The pains of labour drove her to the trunk of a date palm*. She [Maryam] said, "*Oh if only I had died before this time and was something discarded and forgotten*!" A voice called out to her from under her, "*Do not grieve! Your Lord has placed a small stream at your feet. Shake the trunk of the palm towards you and fresh, ripe dates will drop down onto you. Eat and drink and delight your eyes*" (Al-Qur'an, 19:23–26, Surah Maryam).

The Holy Prophet (P.B.U.H) advised Muslims to produce, maintain orchards and eat dates due to its great dietary values. Once he said that "*he who eats seven Ajwa dates every morning will not be affected by poison or magic on the day he eats them*" (Al-Bukhari, 2008). Prophet Muhammad (Peace be upon him) said in another hadith: *"When you break the fast, you should do it with a date fruit, for there is a blessing in it, and if you do not find a date fruit, break it with water, for it is pure"* (Al-Qarawi *et al.*, 2003).

2.3.2 Nutritional significance of Ajwa date fruit parts

Date fruit is one of the most consumed food around the globe and holds great nutritional value as they are rich in many healthy metabolites including dietary fibres, minerals and vitamins (Baliga *et al.*, 2011; Khalid *et al.*, 2016). A lot of research on

its health benefits and pharmacological actions have been done in recent years (Baliga *et al.*, 2011; Khalid *et al.*, 2017; Yasin *et al.*, 2015). Following section presents a detailed review on the chemical composition of Ajwa date flesh and pit focusing on its polyphenols and biological activities.

2.3.3 Proximate Composition of Ajwa date flesh and pits

Soft fleshy, dark brown, edible part of Ajwa contains highest moisture percentage of 22.8%, when compared to the pit of Ajwa which is 14.3%. On the other hand, Ajwa pits shows less ash contents of 2.8% while flesh has 3.2% (Khalid *et al.*, 2016).

2.3.3(a) Carbohydrates

Ajwa dates are well known for their high energy sugar contents. The carbohydrate contents of Ajwa date flesh are listed in Table.2.1 The Ajwa flesh consists of glucose (54.5%), fructose (50.03%), maltose (22.5%) and galactose (12.2%) while the Ajwa pit contains less amount of carbohydrates compared to the flesh which is glucose (20.15%), fructose (16.1%), maltose (6.1%) and galactose (3.4%) (Khalid *et al.*, 2016). Experiments with various methanolic and aqueous extracts described that there are many monosaccharaides present in fleshy part of Ajwa date. In a recent study, proton and carbon nuclear magnetic resonance (NMR) image disclosed that two monosaccharaides mixture of b-D-glucopyranose and a-D-glucopyranose, and b-D-fructopyranose with b-D-fructofuranose were found (Zhang *et al.*, 2017).

Table 2.1 Carbohydrate found in Ajwa date flesh and pit

Ajwa date fruit	**Sugars**	**Khalid *et al. 2016* (%)**
Flesh	Glucose	51.5
	Fructose	52.0
	Maltose	22.5
	Galactose	12.2
	Sucrose	-
Pit	Glucose	20.1
	Fructose	16.1
	Maltose	6.1
	Galactose	3.4
	Sucrose	2.8

2.3.3(b) Minerals and Vitamins

Ajwa date flesh and pit contain high amount of essential minerals which are necessary for the skeletal growth, and maintenance of cellular functions in human body in contrast with other date varieties. A summary of minerals in ADF and ADP is given in Table 2.2 (Assirey, 2015; Khalid *et al.*, 2016). Date fruit is ideal for hypertension patients as it is high in potassium and low in sodium. Magnesium and calcium are important for healthy bone development and selenium helps in protecting against oxidative stress and infections (Mirza *et al.*, 2019). Ajwa dates are also high in iron contents as it is vital for red blood cell production and prevention of anaemia (Hasan *et al.*, 2010). Ajwa pits contains zinc (1.91mg/g), calcium (2mg/g), magnesium (0.8mg/g) and potassium (4.6mg/g). Ajwa flesh contains comparatively higher concentration of potassium (6.4mg/g), magnesium (1.5mg/g) and calcium (2mg/g) (Khalid *et al.*, 2016).

Ajwa dates also contain high amount of pro-vitamin A and vitamin C (Abdul-Hamid *et al.*, 2015; Sawaya *et al.*, 1983), vitamin E (Elmaa *et al.*, 2018). Riboflavin (B2), pyridoxin (B6), niacin (B3), vitamin K, vitamin D and vitamin E were also found in Ajwa date flesh (Al-Radadi, 2019).

Table 2.2 Minerals found in Ajwa date flesh and pit (Khalid *et al.*, 2016)

Ajwa date fruit	**Minerals**	**Khalid *et al. 2016* (mg/100g)**
Flesh	Manganese	0.36 – 0.5
	Magnesium	1.5
	Sodium	7.5 – 8.1
	Potassium	6.45
	Zinc	0.46 – 0.52
	Phosphorous	1.9 – 2.3
	Calcium	2.0
	Iron	0.15 – 0.5
	Cadmium	0.001 – 0.005
	Copper	0.37 – 0.5
Pit	Zinc	1.91
	Potassium	4.6
	Calcium	2.0

2.3.3(c) Proteins

Although a long list of amino acids is known to be present in dates but they are not in high concentrations. Amino acids are highest at initial maturity stage of Ajwa date that might be due to low moisture percentage initially. In a study conducted by Khalid, the Ajwa flesh showed (2.94%) percentage of crude protein which was the highest among two other varieties and the crude protein percentage of Ajwa pits was between 6% to 6.5%. The result of this study suggests higher crude protein in pits compared to the flesh part.(Khalid *et al.*, 2016).

In a study conducted by Assirey and Hamad *et al*, Ajwa flesh showed the higher percentage of essential amino acids than other varieties. A list of essential amino acids that are present in Ajwa dates is presented in Table 2.3. Another study conducted by Ali *et al* showed the presence of proteogenic and non-proteogenic amino acids in Ajwa dates using ion exchange chromatography (Ali *et al.*, 2014). Non-proteogenic amino acids bind with anti-bodies and help in the production of T lymphocytes which help the liver in detoxification of harmful substances and excretion

of creatinine from the body. The non-proteogenic amino acids which were identified in Ajwa date flesh are L-allo-isoleucine, (2S,5R)-5-hydroxypipecolic acid, L-pipecolic acid, 2-aminoethanol, g-amino-n-butyric acid, 1-aminocyclopropane-1-carboxylic acid and (2S,4R)-4-hydroxyproline. Traces of L-ornithine, B-alanine, 5-hydroxylysine, (S)-b-aminoisobutyric acid were also identified in Ajwa date flesh (Ali *et al.*, 2014). However, there was no literature to date present the composition of amino acids on Ajwa date pits.

Table 2.3 Amino Acids presents in Ajwa date flesh (Ali *et al.*, 2014)

Amino Acids	**Ali *et al. 2014* (mg/g) dry matter**
Alanine	0.75 – 1.16
Arginine	0.45 – 1.23
Asparagine	1.29 – 2.80
Cysteine	0.89 – 1.38
Glutamate	1.76 – 3.79
Glycine	1.04 – 1.98
Histidine	0.36 – 0.54
Isoleucine	0.55 – 0.80
Leucine	0.89 – 1.32
Lysine	0.075 – 1.14
Methionine	0.03 – 0.023
Phenylalanine	0.62 – 0.87
Proline	1.04 – 1.98
Serine	0.48 – 0.74
Threonine	0.59 – 0.18
Tyrosine	0.22 – 0.51
Valine	0.66 – 0.95

2.3.3(d) Fiber

Dietary fibres perform an ultimate role in metabolic activities of human body. Absence of fibres in human diet leads towards constipation, cancer and increased cholesterol (Brown *et al.*, 1999), therefore it is important to have dietary fibres in healthy diet. Table 2.4 shows the amount of fibres present in Ajwa date flesh and pits

(Khalid *et al.*, 2016) Ajwa date has abundant amount of dietary fibres in its flesh as well as pits (Al-Shahib and Marshall, 2003).

Table 2.4 Amount of fibres present in Ajwa date flesh and pits. Numbers given in percentage

	Crude fibre %	**Soluble dietary fibre**	**Insoluble dietary fibre**	**Total dietary fibre**
Ajwa flesh	9.01	4.5	8.19	12.73
Ajwa pits	51	19.5	34.6	53.9

In another study by Ahmed, it was described that roasted dates have more dietary fibers as compared to fresh dates. Roasted dates have high fiber content due to low water content (Ahmed *et al.*, 2016).

2.3.3(e) Lipids

The total amount of crude fat in Ajwa flesh was 0.47% and in Ajwa pits was 7.8% which was statistically higher than the flesh part (Khalid *et al.*, 2016). The lipids in Ajwa dates are known as oleic-linoleic lipids and yield about 8.9%. Mostly it contains triacylglycerol species which include dilinoleoyl-1-oleoylsn-glycerol and 1,2-dioleoyl-3-linoleoyl-sn-glycerol (Galeb *et al.*, 2012). Ajwa date pits constitute contains about 21.2% of total saturated fatty acid and 75.26% of un-saturated fatty acids. The saturated fatty acids of Ajwa pits include palmitic acid, myristic acid, lauric acid and steric acid. The unsaturated fatty acids include oleic acid, linoleic acid and linolenic acid (Table 2.5). Ajwa pit oil composition is of better quality when compared with another date variety like Barni. Therefore, it can be used as a vehicle for drugs delivery (Galeb *et al.*, 2012).

Table 2.5 Percentage of fatty acids present in Ajwa date pit (Galeb *et al.*, 2012)

Ajwa date fruit	**Fatty acids**	**Galeb *et al.*, 2012 (%)**
Pit	Palmitic	10.3
	Myristic	5.6
	Lauric	3.2
	Steric	2.1
	Oleic	66.1
	Linoleic	8.3
	Linolenic	0.86

2.3.4 Phytochemicals in Ajwa date fruit

2.3.4(a) Phenolic Acids

Phenolic acids chemically-structured as hydroxylated benzene ring in both direct and indirect ways, bonded with carboxyl groups. This class of major secondary metabolites ranges from 245 to 455 mg/100g in Ajwa dates, based on different solvent and extraction method used (Saleh *et al.*, 2011). In a study conducted by Eid *et al*, it was found that Ajwa contains the highest number of polyphenols when compared to other varieties (Eid *et al.*, 2013). The phenolic acid includes hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid, caffeic acid, sinapic acid, iso-ferulic synergic acid, chlorogenic acid, ferulic acid, p-coumaric acid, cinnamic acid, isovanillic acid and hydroxycinnamic acid (Eid *et al.*, 2013). The structure and the quantity of phenolic acid present in Ajwa date is shown in Fig. 2.3. Out of these p-coumaric acid, gallic acid and ferulic acid derivatives were the most abundant phenolic compounds found in Ajwa date (Hamad *et al.*, 2015).

Ajwa date pits contain gallic acid, caffeic acid, chlorogenic acid, synergic acid, p-coumaric acid, m-coumaric acid and ferulic acid in abundant quantity (Ahmed *et al.*, 2016).

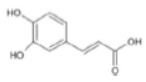
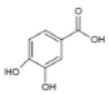
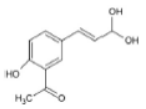
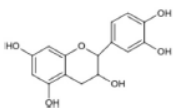
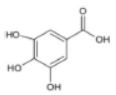
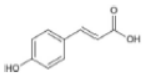
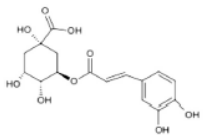
Phenolic acid	Structure	Quantity (mg/100 g DW)	Reference
Caffeic acid		0.026–0.050	Hamad et al. (2015) Saleh et al. (2011) Ragab et al. (2013) Ahmed et al. (2016)
Ferullic acid		2.52–3.20	Hamad et al. (2015) Ahmed et al. (2016)
Protocatechuic acid		1.27–2.20	Hamad et al. (2015) Saleh et al. (2011)
Catechin		0.50–0.80	Hamad et al. (2015) Saleh et al. (2011) Ragab et al. (2013)
Gallic acid		13.90–14.10	Hamad et al. (2015) Ahmed et al. (2016)
p-coumanic acid		3.08–3.50	Hamad et al. (2015) Ahmed et al. (2016)
Chlorogenic acid		0.18–0.20	Hamad et al. (2015) Ahmed et al. (2016)
Resordinol acid		0.03–0.05	Hamad et al. (2015)
Total phenols		22.10–455.80	Hamad et al. (2015) Saleh et al. (2011)

Figure 2.3 Phenolic Acids found in Ajwa date (DW= Dry Weight) (Khalid *et al.*, 2017)

2.3.4(b) Phytosterols

Generally, phytosterols are known as sterols of plant which are closely similar to cholesterol. These phytosterols have certain health benefits. In Ajwa date, β-sitosteryl-3-β-glucopyranoside-6′-*O*-palmitate, β-sitosteryl-3-*O*-β-glucoside and β-Sitosterol are reported by (Zhang *et al.*, 2013) presented in Fig. 2.4.

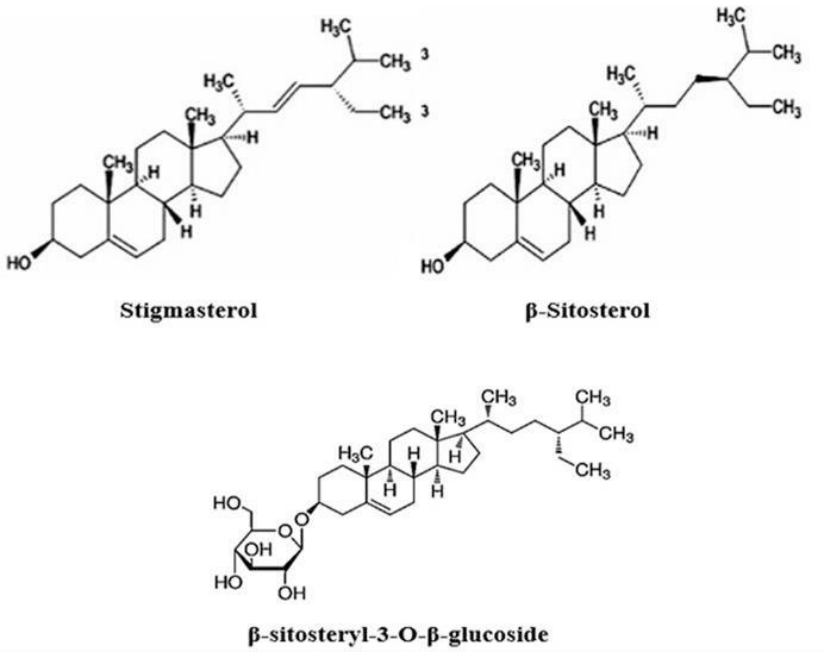


Figure 2.4 Phytosterols present in Ajwa date, reported by (Zhang *et al.*, 2013)

2.3.4(c) Carotenoids

Carotenoids are fats soluble pigments which gives colour to the plant. A study reported that carotenoids are a major part in the lipid fraction of phytochemicals found in dates and are reported to have useful health benefits in preventing chronic diseases (Baliga *et al.*, 2011). The beneficial effects of carotenoids like beta-carotene and lutein are due to their anti-oxidant property in preventing chronic diseases (Johnson, 2002).

2.3.4(d) Flavonoids

Flavonoids (sub-classes are flavones, anthocyanins, iso-flavones and flavanols) perform various health advantages such as radical scavenging, antioxidant activity, anticancer activities and reduction of chronic diseases. Quercetin, iso-quercetin, luteolin, apigenin and rutin were also present in Ajwa date (Hamad *et al.*, 2015; Ragab *et al.*, 2013). These bioactive compounds of Ajwa date also contribute towards anti-inflammatory properties (Eid *et al.*, 2013).

These flavonoids play a major part in the prevention of proliferation of damaged cells and reduction of cardiovascular diseases. (Eid *et al.*, 2014). Hamad et

al. (2015) reported total flavonoid contents of Ajwa date fruit around 2.79mg/100g (Hamad *et al.*, 2015). Ajwa date pit reported to have 1.35mg/100g of quercetin present (Ahmed et al., 2016). However, the amount of these compounds varied between Ajwa flesh and pit (Ahmed *et al.*, 2016; Hamad *et al.*, 2015). It is interesting that dates are the solitary food which contain sulphated-flavanol glycosides. This form of flavanols were not detected before in any fruit as well as vegetables (Chaira *et al.*, 2009; Hong *et al.*, 2006).

Eid *et al.* also reported the presence of significant amount of anthocyanidins with petunidin in Ajwa variety (Eid *et al.*, 2013). Chrysoeriol-7-O-(2,6-dirhamnosyl)-glucoside was also identified in Ajwa date fruit (Zhang *et al.*, 2013). Significant amount of quercetin, myricetin, naringenin, apigenin, luteolin, kaempferol and p-coumaric were found in Ajwa date fruit (Eid *et al.*, 2013; Zhang *et al.*, 2013). Other few important organic acids like succinic acid, oxalic acid, malic acid, citric acid, isobutyric acid and formic acid were also found in Ajwa date fruit which help in enhancing its functionality (Hamad *et al.*, 2015). Anthocyanins have reported to have strong anti-oxidant activity which helps in the prevention and treatment of cancer (Nichenametla *et al.*, 2006).Other than inhibiting the growth of cancer cells they have also shown neuronal and behavioural anti-aging effects (Törrönen and Määttä, 2000). Other health benefits include relief from allergies, healthy cardiovascular effects (Wallace, 2011), and better eye function (Ghosh and Konishi, 2007).

2.3.5 Biological and Pharmacological activities of Ajwa date

2.3.5(a) Anti-Oxidant Action of *Phoenix dactylifera*

The excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), either by increased production or decreased body's ability to remove it is a physiological state known as oxidative stress (Forcados *et al.*, 2017).

Free radicals cause oxidative damage in the proteins, lipids and nucleic acid of the body and cause cancer, mutation, aging, arthrosclerosis, neuro-degenerative diseases and stress induced depression which leads to a poor quality of life. Polyphenols present in *Phoenix dactylifera* act as free radical scavengers and prevent the cellular damage caused by oxidative stress and inflammation in the body (Bladé *et al.*, 2016; Pujari *et al.*, 2011). A dietary intake of Ajwa date provides a treatment for several chronic diseases and cancer, due to its strong anti-oxidant, antimutagenic and hepato-protective properties (Khalid *et al.*, 2017). In a study conducted on albino rats, with lead acetate toxicity induced, Ajwa date inhibited the depletion of antioxidants. Polyphenols, flavonoids and flavones attributed to the Ajwa dates antioxidants and tissue protective properties (Ragab *et al.*, 2013). Several other studies have confirmed that the tissue protective property of Ajwa date is due to its free radicals scavenging activity (Baliga *et al.*, 2011; Hamad *et al.*, 2015; Hasan *et al.*, 2010). Another study conducted with Ajwa date seed extract, reported to have a potential role in balancing the oxidative stress state in diabetic rats (Hasan and Mohieldein, 2016).

Previously it has been established that cancer is caused by carcinogenic substance as a result of oxidative damage to the cells by cell cycle dysregulation. The production of ROS in the body can lead to oncogenic cells via DNA mutation. An *in vitro* study was conducted on Salmonella tester strains with benzo(a)pyrene induced mutagenicity to gauge its cell damage protective property (Vayalil, 2002). Benzo(a)pyrene is a known by-product of cigarette combustion and a carcinogenic substance to cause lung cancer (Denissenko *et al.*, 1996). In another study, oxidative stress induced by hydrogen peroxide in human keratinocytes and melanocytes was tested by date seed oil extract. The date seed oil was found to significantly increase the cell viability which suggests its possible application in the prevention of skin

cancer caused by oxidative damage of UV radiation (Dammak *et al.*, 2009; Ines *et al.*, 2010).

2.3.5(b) Hepatoprotective and antifibrotic property of *Phoenix dactylifera*

Production of ROS by environmental chemicals and chemotherapeutic agents such as methotrexate, 6-mercaptopurine, azathioprine, l-asparaginase, gemtuzumab and many others (Kufe *et al.*, 2010) can lead to liver cirrhosis and cancer (Tanikawa and Torimura, 2006). A study was conducted to test the hepatoprotective effect of Ajwa date extract on rats with hepatotoxicity induced by ochratoxin A. It was concluded that Ajwa date decreased the hepatotoxicity by boosting antioxidant enzymes which provides protection to the liver against oxidative damage. An important factor in this study was the use of exact dose (seven dates per day) recommended by Prophet Muhammad (Peace be upon him) (Bakr Abdu, 2011).

A few more studies evaluated the hepatoprotective effect of date palm fruit on rats and rabbits' models induced with carbon tetrachloride and dimethoate toxicity. Similar results were reported that flavonoids present in *Phoenix dactylifera* were responsible for the liver protective effects (El-Gazzar and El-Far, 2009; Saafi *et al.*, 2011; Sheikh *et al.*, 2014).

2.3.5(c) Chemotherapeutic property of *Phoenix dactylifera*

Phoenix dactylifera has also been used as therapeutic agent. The effect of 1-6 and 1-3 branched beta-glucans isolated from date fruit extract were tested on mice induced with Sarcoma-180 tumour. A significant decrease of tumour growth was found with 0.2 and 5mg/kg doses (Ishurd and Kennedy, 2005). Similar anti-cancerous effects were obtained with beta-glucans from different plant sources from other studies (Chan *et al.*, 2009).

2.3.5(d) Antibacterial, antifungal and antiviral activities

Several studies have reported antimicrobial effect of date palm extract against gram (-) bacteria like Escherichia coli (Kchaou et al., 2016), Pseudomonas aeruginosa, Salmonella abony and gram (+) bacteria like Bacillus subtilis, Bacillus cereus, and Staphylococcus aureus. Phenolic compounds are attributed for these antimicrobial activities (Bammou et al., 2016).

Methanolic extract of date fruit flesh and pit have also reported promising antifungal activity against *Fusarium Oxysporum, Fusarium sp., Fusarium solani, Aspergillus Flavus, Alternaria Alternata, Alternaria sp.* and *Trichoderma sp.* (Bokhari and Perveen, 2012).

Apart from antibacterial and antifungal activity, date palm pit extract has reported to have positive antiviral activity (Jassim and Naji, 2010).

2.3.5(e) Gastroprotective effects of *Phoenix dactylifera*

Date fruits help in secretion of digestive enzymes and protect the gastrointestinal tract (GIT) from constipation. In a study, positive effects of date flesh and seed extract were seen against a laxative (yohimbine) and a drug that decreases the GIT movement (clonidine) (Al-Qarawi *et al.*, 2003).

2.3.5(f) Anti-inflammatory activity

Previously, date fruit was used to treat inflammation-associated diseases (Yasin *et al.*, 2015). It was recently reported that date fruit acted as a pain reliever in a similar manner as ibuprofen and paracetamol (Umar Ibrahim *et al.*, 2015). There is no clear explanation for this mechanism but it has been reported that active substances from date fruit inhibit the production of inflammatory cytokines such as IL-6, IL-8, IL-10, TNF-α, and IGF-1 in prostaglandin synthesis (Al-Yahya *et al.*, 2016). Increased

expression of TGF-β and presence of Vitamin C and E showed analgesic effects (Elberry *et al.*, 2011)

2.3.5(g) Antidiabetic activity

Oxidative stress has been recognized as one of the major factors for the development of diabetes (Evans *et al.*, 2002). Higher level of ROS production and more oxidative cells have been seen in diabetic patients (Martín-Gallán *et al.*, 2003). Study conducted by Hasan and Mohieldein showed that Ajwa pit extract significantly reduced the blood glucose level of diabetic rats by controlling the oxidative stress. However, one of the limitations of their study was the small sample size (Hasan and Mohieldein, 2016).

Strong antioxidant activity of Ajwa date by scavenging the free radicals can have strong antidiabetic effects (Zhang *et al.*, 2017).

2.3.6 *In Vitro* Studies of Ajwa date

A study carried out by Khan *et al.*, mechanism was investigated by which methanolic extract of Ajwa date inhibited the growth of human breast adenocarcinoma (MCF7) cell line. MTT assay showed significant inhibition of cell proliferation in a dose dependent manner. Flow cytometric analysis showed cell cycle arrest at 'S' phase, upregulation of proapoptotic molecules, p53, Bax protein expression, caspase-3 activation and down regulation of mitochondrial membrane potential, explaining the mechanism of anti-cancer effect of Ajwa dates (Khan *et al.*, 2016). Another study reported the anticancer effects of Ajwa date extract in combination with 5-flurouracil in human breast adenocarcinoma cell line *in vitro* (Khan *et al.*, 2018).

Another study was conducted to evaluate the anticancer effects of Ajwa date extract on human prostate cancer cell line (PC3). MTT assay showed the strong anti-proliferative effect of Ajwa date extract on PC3 cells by loss of mitochondrial

membrane potential and increased oxidative stress during apoptosis. Flow cytometry results determined the induction of apoptosis and cell cycle arrest in S phase in cells treated with Ajwa date extract (Mirza *et al.*, 2018).

A study conducted by Eid *et al.* 2014, suggested that the intake of Ajwa dates by humans help in maintaining the bowel health and decreasing chances of colorectal cancer growth (Eid *et al.*, 2014). Few other *in vitro* studies showed similar results of cytotoxic properties of Ajwa date extract with cervical (HeLa) cell line (Kchaou *et al.*, 2016), hepatocellular carcinoma (HepG2) cell line (Siddiqui *et al.*, 2019) and lung cancer (NCI-H460) cell line (Zhang *et al.*, 2017).

2.4 Antioxidant assay

To investigate the antioxidants such as polyphenols/flavonoids, present in natural products, several chemical methods can be used. Chemical tests are based on their property to transfer electron in the presence of an antioxidant during the test.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a simple yet highly sensitive method for the detection of antioxidant from natural products. DPPH• is a stable organic nitrogen radical available commercially (MacDonald-Wicks *et al.*, 2006). In this test, DPPH• accepts a hydrogen from a hydrogen donor which is an antioxidant present in the natural product. DPPH•, upon absorption of hydrogen from an antioxidant turns from purple to yellow. Figure 2.5 represents the equation reaction of DPPH reagent in the presence of antioxidant. The disappearance of DPPH• is directly proportional to the antioxidant effect of the test samples, which can be then measured using a UV spectrometer at a maximum absorption of 517 nm (Chaillou and Nazareno, 2006).

Positive controls are used for the standardization of the results. Ascorbic acid (Vitamin-C) had been used in this study to compare the antioxidant activity of a sample (Lu and Foo, 2000).

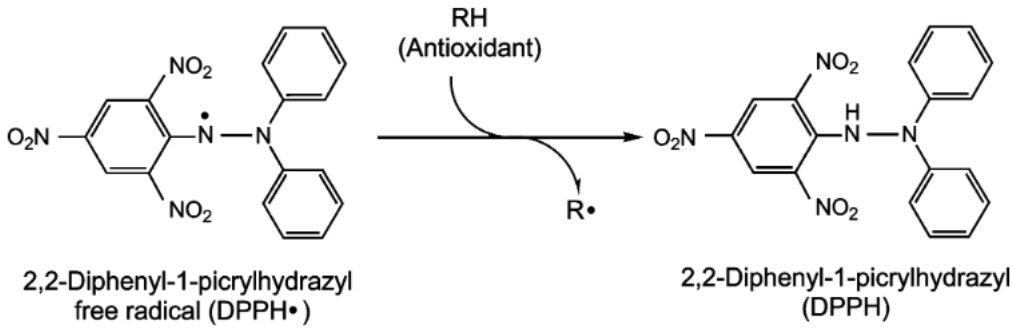


Figure 2.5 Mechanism by which DPPH• accepts hydrogen from an antioxidant

2.5 Bioassays

Bioassays are chosen based on the type of the study performed, use of tissues/cells (*in vitro*) or animals (*in vivo*). The assays are chosen to determine the desired biological activity of a substance. A classical property of bioassay would be its cost effectiveness, selectivity, sensitivity, easy to run and maintain.

2.5.1 Cell proliferation assay

Cell proliferation is defined by which the cells grow and divide to replace the lost cells. Proliferation in normal cells is a positive indication of healthy cells, however, it is highly regulated in normal cells as they remain in a non-proliferative state unless stimulated to divide and replace the lost cells (Cooper, 2000). Various physical and chemical agents may cause cytotoxicity in cells via various mechanisms such as cell membrane disintegration, hindrance in protein synthesis, binding of receptors irreversibly, polydeoxynucleotide elongation inhibition, and some enzyme reactions (Ishiyama *et al.*, 1996). To measure the cell death caused by such

mechanisms, cell viability and cytotoxicity assays are used for drug screening in order to detect whether they have proliferative effects or cytotoxic effects on cells (Yadav *et al.*, 2001). Therefore, this assay is important to determine the cytotoxic effect of *Pheonix dactylifera* extract on human squamous cell carcinoma cell line (HSC-2) in order to test its antiproliferative effects.

Various types of cell proliferation assays are available based on different parameters used to determine the proliferation rate. One such assay uses the reduction potential of cells either by tetrazolium salts or resazurin dyes to measure the cell proliferation activity (McGaw *et al.*, 2014). During cell growth, the production of metabolic intermediate such as nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide (NAD) increases. These intermediates reduces tetrazolium salts to formazan crystals resulting in colorimetric change which can be measured via spectrophotometer (Präbst *et al.*, 2017). In the same way, resazurin dye reduces to resorufin.

Commonly-used tetrazolium salts are 3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt (XTT) (McGaw *et al.*, 2014). Tetrazolium reduction assays are applicable for adherent cell lines, easy to perform and cost effective, giving good performance. Hence, MTT assay was selected to be used in this study. MTT assay was first described by Mosmann (Mosmann, 1983). In this assay, MTT is reduced from yellow to bluish-purple formazan product by metabolically active cells via mitochondrial and cytosolic enzymes (Figure 2.6). Formazan crystals cannot cross the cell membrane, therefore are

accumulated within the cell. These formazan crystals are then solubilized with organic solvents such as dimethyl sulfoxide (DMSO) to enable quantification using a spectrophotometer at a specific wavelength (McGaw *et al.*, 2014).

The amount of formazan crystals formed were directly proportional to the number of living cells in culture as only viable cells can reduce MTT to formazan crystals (Mosmann, 1983).

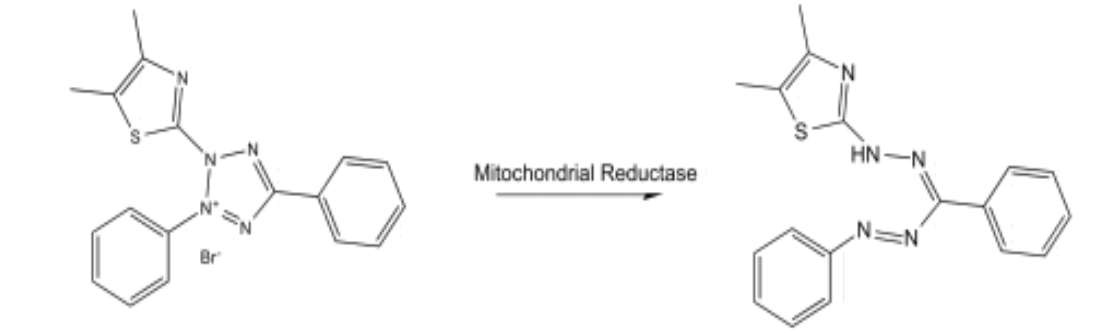


Figure 2.6 Reduction of MTT molecule to purple formazan crystals

2.5.2 Apoptosis detection by flow cytometry assay

Flow cytometry is a laboratory-based method to analyse any changes in cellular structure or function with the use of an appropriate fluorescent probe. Fluorescent probe can be a fluorescent stain/reagent or a monoclonal antibody attached to a fluorochrome. Before analysis, fluorescent probes react with the cells structure of interest; therefore, the amount of fluorescent light emitted by these particles when they pass through flow cytometry, is proportional to the amount of fluorescent probe attached to that specific cellular structure. Flow cytometry has a basic principle when a stained cell passes through a single wavelength light beam, it will scatter light and emit fluorescence rays. Light scattering and fluorescence rays emitted from each individual cell are measured and the data can be used to determine a variety of biochemical and functional aspects of cells. Flow cytometer consists of three parts;

fluid system, excitation source and electron detectors which detects the signals and digitalizes it to be analysed by a certain computer software (Picot *et al.*, 2012)

Annexin -V and propidium Iodide (PI) staining kits are used to determine the stages of apoptosis in cells. One of the hallmark features of apoptotic cell is the exposure of phospholipids which are initially present between the inner and outer lipid bilayers of cell membrane. During apoptosis, this distribution is disrupted and phospholipids are exposed on the outside surface of plasma membrane (van Engeland *et al.*, 1998). Annexin-V conjugated-fluorochrome has high affinity for the phospholipids and binds to an apoptotic cell. Therefore, it can be used as a marker for apoptotic cells during flow cytometry analysis (van Engeland *et al.*, 1998). Cells with a loss of plasma membrane can be detected by cationic dyes such as PI. Therefore, a combination of Annexin V-FITC and PI is used for the detection of apoptosis in flow cytometry. Non-apoptotic cells are presented as (Annexin V-FITC negative/PI negative), early apoptotic cells are presented as (Annexin V-FITC positive/PI negative), late apoptotic cells and necrotic cells are presented as PI positive (Pozarowski *et al.*, 2003).

To the best of our knowledge, there has been no published data regarding the cytotoxic activity of Ajwa dates (flesh and pit) on Human Squamous cell Carcinoma-2 cell line (HSC-2) present.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

This is an *in vitro* experimental study conducted to evaluate the antioxidant activity, cytotoxic effect and pro-apoptotic activity of Ajwa date extracts prepared from the flesh and seeds. The preparation of Ajwa date extracts was prepared at the science laboratory, School of Health Sciences and Craniofacial Science Laboratory, School of Dental Sciences. Cell culture was carried out in the clean room of the Craniofacial Science Laboratory and antioxidant assay was performed at the Microbiology lab, School of Dental Sciences. MTT assay was conducted at the Central Research Laboratory and flow cytometry analysis was carried out at the Immunology Lab, School of Medical Sciences, Universiti Sains Malaysia (USM), Health Campus, Kelantan.

3.2 Overview of Study

The overview of study is given in **Figure 3.1** below

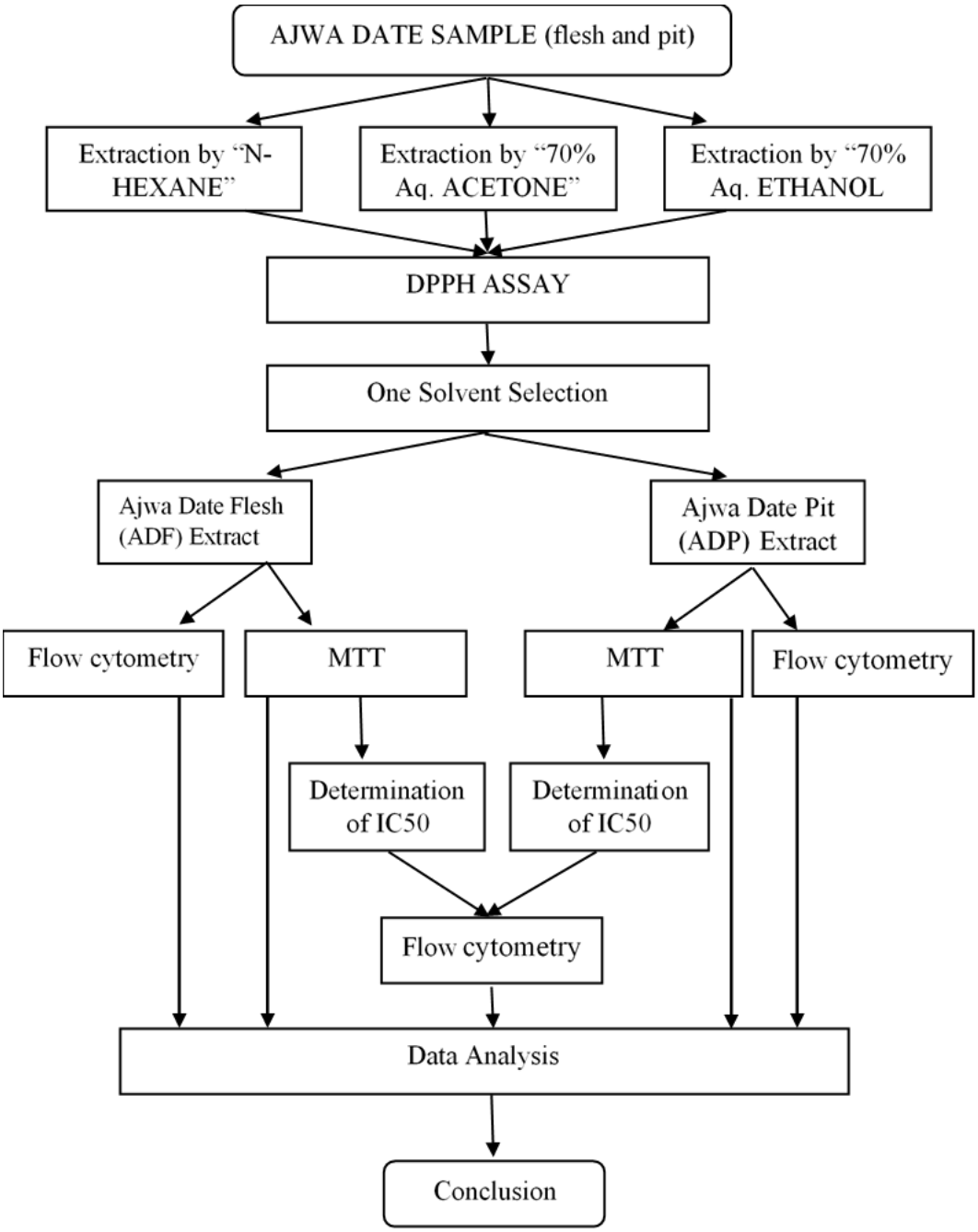


Figure 3.1 The overview of study

3.3 Research Materials

3.3.1 Ajwa dates

Ajwa dates are only cultivated in the outskirts of city of Madinah Al Munawwarah. Four kg of Ajwa dates were purchased from the "Tamar Market" of Al-Madinah Al-Munawwarah, Kingdom of Saudi Arabia (Appendix A). Upon arrival at

the USM laboratory, the Ajwa dates were sealed in a plastic bag and stored in a refrigerator at 4 °C in dark until used. Ajwa dates were black coloured with white basal lines on the exocarp as shown in Figure 3.2. They were also medium to small sized, sweet and ovoid in shape.

Figure 3.2 Ajwa date. Basal white lines on the black exocarp, small in size and ovoid in shape are its classical features

3.3.2 Cells

For this project HSC-2 RCB-1945 cell line was used. HSC-2 is human Oral Squamous Carcinoma Cell line of which was kindly provided by Dr Wan Nazatul Shima Shahidan (Appendix B). Figure 3.3 shows the image of cells taken at 80% confluence.

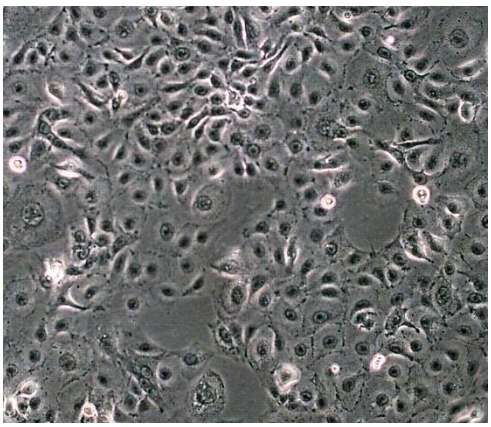


Figure 3.3 HSC-2 cells at 80% confluence. Morphologically cells are epithelial like. Polygonal in shape, cell to cell adhesion, grow as thin monolayer in discrete patches, dark coloured central nucleus and many cells can be seen going under mitotic activity.

3.4 Experimental Materials

All the chemicals and reagents that were used in this study were of analytical grade and are listed in Table 3.1 with their respective suppliers.

3.4.1 Instruments/equipment

All the apparatus and equipment that are used during the study are listed below in the Table 3.2.

3.4.2 Reagents and kits used in antioxidants, cytotoxicity and apoptosis assay

The reagents for antioxidants activity, cytotoxicity assay and apoptosis kit used in these experiments are listed in the Table 3.3 below.

3.4.3 Consumable Materials

The consumables and glassware used in this project are listed below in Table 3.4.

3.4.4 Computer Software

All the software and computer programmes used in this project are listed in the table 3.5 below

Table 3.1 List of chemicals and reagents used in this study

Items	Brand/Manufacturer
Acetone	EMSURE® Merck - Germany
Dimethyl Sulfoxide (DMSO)	Nacalai Tesque - Japan
Ethanol 70% C0311	HmbG® Chemicals - Germany
Fetal Bovine Serum (FBS)	Gibco® Invitrogen – USA
Methanol Absolute (MeOH)	EMSURE® Merck - Germany
Minimum Essential Medium (MEM)	Gibco® Invitrogen – USA
n-Hexane	EMSURE® Merck - Germany
Oral Squamous Cell Carcinoma Cell line (HSC-2) RCB-1945	Riken Cell Bank, Tsukuba - Japan
Penicillin-Streptomycin/Pen Strep	Gibco® Invitrogen – USA

Phosphate Buffer Saline (PBS) pH 7.4	Gibco® Invitrogen – USA
Trypan Blue	Sigma-Aldrich – USA
Trypsin-EDTA (0.25% trypsin, 1mM EDTA with phenol red)	Gibco® Invitrogen – USA

Table 3.2 Apparatus and equipment used in the study

Equipment	Manufacturer
-85 °C deep freezer	ilShinBioBase, Netherlands
-30°C freezer	SANYO, Japan
-20°C freezer	Shin lab, Japan
4°C refrigerator	Panasonic, Japan
37 °C Incubator	SANYO, Japan
Axioplan 2 imaging Microscope	Zeiss, Germany
Autoclave	Hirayama, Japan
Biosafety cabinet class II	Heraeus, Germany
BD Accuri™C6 flow cytometer	BD biosciences, USA
Carl Zeiss Inverted Microscope	Zeiss, Germany
Concentrator plus	Eppendorf, Germany
CO_2 Incubator	NuAire, USA
Electrical powder grinder	Golden Bull, Malaysia
ELISA reader	Tecan, Switzerland
Flask shaker machine	Thomas Scientific, USA
Haemocytometer	Neubauer, Germany
Micropipettes Eppendorf 10, 200, 1000µl	Eppendorf, Germany
Neubauer chamber	Neolab®, Heidelberg
Nitrogen Tank	Cryo Systems, Germany
Semi-Micro Analytical Balance	A&N, Japan
Test tube heater	Stuart, UK
Ultraspec/2100 PRO UV spectrophotometer	Amersham Biosciences, USA
Universal 32R centrifuge	Hettich Zentrifugen, Germany
Vortex	Vortex 2, Genie USA
Water bath TW8	Julabo GmbH, Germany

Table 3.3 Reagents and kits used in this study

Items	Manufacturers
1,1-Diphenyl-2 Picrylhydrazyl Free Radical (DPPH)	Tokyo Chemical Industry, Japan
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT powder)	Calbiochem, Germany
Annexin V-FITC Apoptosis Dtec Kit 1	BD Pharmingen™, BD-Biosciences USA

Table 3.4 Consumables and glassware used in this study

Item	Manufacturer
5ml round bottom polystyrene tubes	Falcon®-BD, USA
6-well culture plate	NEST, China
96-well culture plate	TPP, Switzerland
Cell culture flask (T25) blue filter cap	Nunc®, Denmark
Cell culture flask (T75) yellow filter cap	TPP, Switzerland
Cryovial (2ml)	TPP, Switzerland
Centrifuge tube 15ml, 50ml	Falcon®-BD, USA
Filters	TPP, Switzerland
Laboratory glassware	Favorit®
Microcuvettes	Merck, Germany
Parafilm® M sealing film	Pechiney plastic packaging, USA
Pasteur transfer pipettes (3ml)	Biologix, Changzhou
Pipette tips (100-1000µl)	Labcon, USA
Pipette tips (0.5-10, 1-200µl)	Axygen Scientific, China
Serological Pipettes (5ml, 10ml)	Falcon®-BD, USA
Serological Pipette (25ml)	TPP, Switzerland
Syringe (5, 10, 50ml)	Terumo Corporation, Philippines

Table 3.5 Software and computer programs used in this study

Software	Company
Axio Vision 4.6	Zeiss, Germany
BD Accuri™ C6 software	BD biosciences, USA
GraphPad prism version 7	GraphPad software Inc. USA
IBM SPSS statistics version 24	SPSS Inc, USA
Microsoft Office 2016	Microsoft Corporation, USA

3.5 Methodology

3.5.1 Preparation of Ajwa date

Fresh, medium sized, soft and fleshy ripe Ajwa dates with basal white lines on the black exocarp without any visible physical damage were selected. The selected Ajwa dates were washed first with tap water and then with distilled water three times under sterile environment to remove all the dust and soil particles, then dried with sterile cotton cloth and then air dried under shade for one night. Ajwa dates were then manually pitted and the seeds were washed again to remove any remaining date flesh

and then air dried for a night. Ajwa date flesh was chopped to small pieces approximately 1cm each using a surgical blade no.10. Ajwa pits and the cut date flesh were then further dried completely using freeze drying method for a week separately in order to stabilize the samples and preventing from microbial spoilage and hydrolytic rancidity (Chan *et al.*, 2013). Freeze drying was also performed to preserve the bioactive components of the Ajwa date as the method has given higher yields of antioxidants, phenols and flavonoids in the past. Date pits were milled using laboratory milling grinder with 30~300micron finesse. The flesh part was crushed coarsely using pestle and mortar. The powdered pits and flesh were weighed and made into aliquots of 50g and sealed into airtight plastic bags stored at -40°C in dark until required for extraction.

3.5.2 Ajwa flesh and seed extract

In this study, different solvents such as n-Hexane, acetone/H_2O (70:30, v/v), and ethanol (70:30, v/v) were used successively in order to isolate a wide range of antioxidant compounds (phenols and flavonoids) present in Ajwa date's flesh and pits. These three solvents were selected based on their increasing polarities varying from non-polar, intermediate polarity to polar solvent. In this study the order in which extraction was performed as: n-Hexane, acetone 70% and ethanol 70%. Ajwa date's flesh and pits extractions were performed separately following the protocol described previously by (Khan *et al.*, 2016; Ramasamy *et al.*, 2011) with slight modifications.

A portion of freeze-dried contents (15g) were then extracted in n-Hexane (150ml) with a ratio of 1:10 (weight to volume) for 48 hours at room temperature in a flat bottom flask on a shaking incubator. Following extraction, the resultant extract was filtered using Whatman No.1 filter paper. The remaining residue was further extracted in acetone/H_2O (70:30) for 48 hours at room temperature with continuous

agitation. The solvent containing extract was filtered and the excess solvent was evaporated under vacuum using a concentrator to give a dark brownish extract. Remaining insoluble residue was subjected to ethanol/H_2O (70:30) extraction for 48h at room temperature. The mixture was filtered, and excess solvent evaporated under vacuum to give dark brown concentrate. All the crude extract was frozen and the H_2O removed by freeze-drying to yield a brown solid. All the extracts from Ajwa date's flesh and pits were weighed, sealed, labelled and stored at -20°C in 50ml tubes for analytical purposes.

The best extraction was determined based on the quantities extracted and the antioxidant activity present in each extractants. A flow chart of extraction procedure is shown below in Figure 3.4.

3.5.3 Cell culture procedure

To avoid any contamination, all the cell culturing procedures were carried out following strict aseptic protocols. All the equipment used during cell handling processes were purchased sterile or autoclaved before use. At the start of every experiment the safety cabinet was sprayed with 70% ethanol and sterilized by UV radiation. All the apparatus was also sprayed with 70% ethanol before entering the biosafety cabinet.

3.5.3(a) HSC-2 cell line culture

HSC-2 cells were cultured in minimum essential medium (MEM) containing 2mM L-glutamine, 10% fetal bovine serum (FBS) and 1 % penicillin/streptomycin. The cells were incubated in 5% CO_2 at 37 °C incubation in a humidified CO_2 incubator (Appendix B). Cells were maintained as monolayer and passage was performed every 3rd or 4th day.

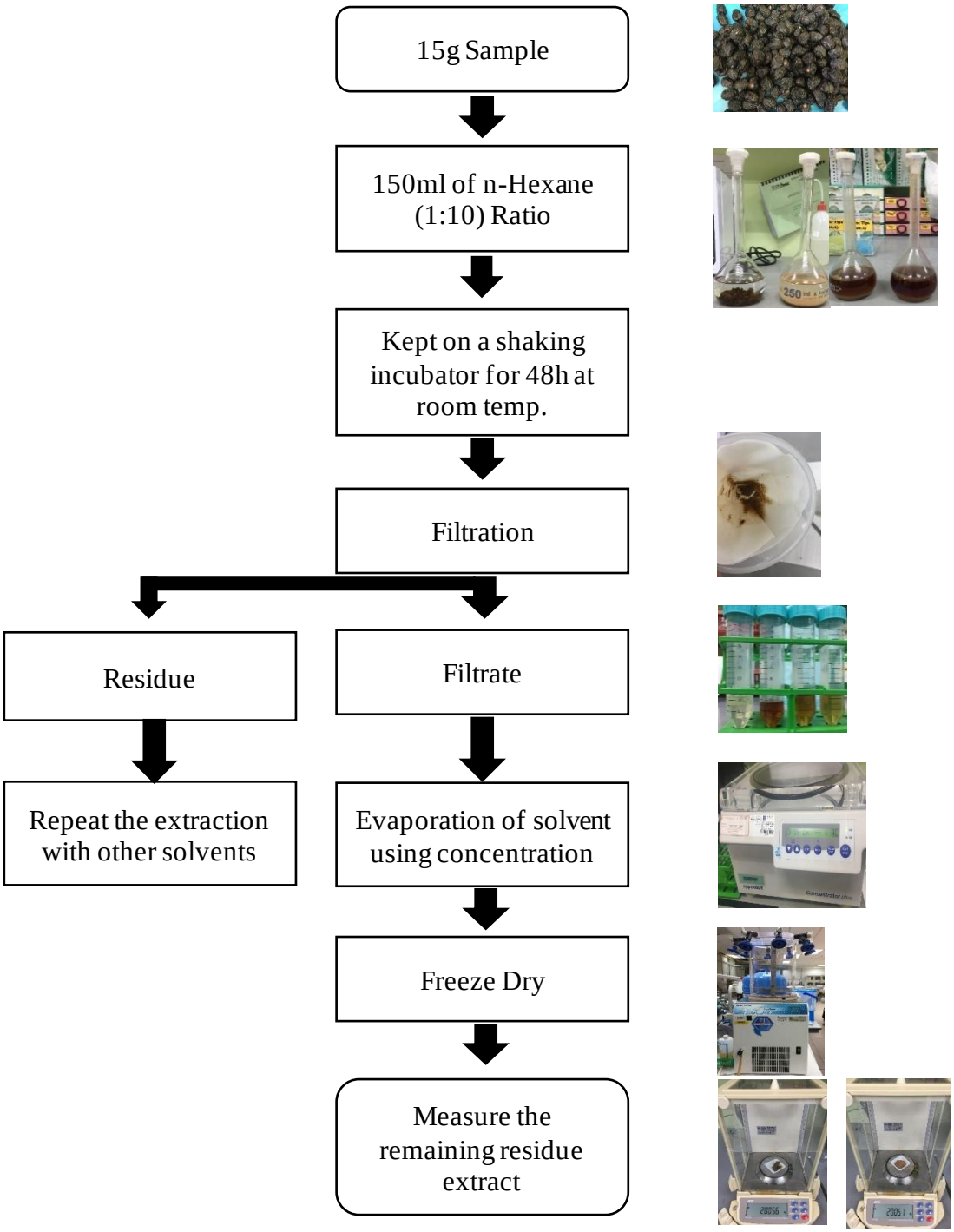


Figure 3.4 Schematic of extraction procedure

3.5.3(b) Thawing frozen cells

HSC-2 cell cryovials were taken out from the liquid nitrogen vapor phase tank for thawing. Before taking out the vials, biosafety cabinet was prepared and about 8ml prewarmed complete growth medium was transferred into a 15ml centrifuge tube and 5ml of complete growth media was transferred into T25 culture flask.

To prepare fresh cell culture from the frozen stock, the vial was taken out of the nitrogen tank, and quickly thawed by half submerged into 37°C water bath. After two minutes of submerging the vial, ice crystals melted and the vial was sprayed with 70 % ethanol and immediately transferred to the biosafety cabinet. The contents of the vial were transferred into a 15ml centrifuge tube drop by drop which already contained prewarmed media and the cell suspension was centrifuged for five minutes at 157 ×g to separate the cryoprotectant agent. After discarding the supernatant, the cell pellet was resuspended into 1 ml of freshly prewarmed complete growth media. In a T25 flask which contained 5 ml of prewarmed media at 37°C, the cell suspension was pipetted gently up and down for equal distribution of cells in the flask. The T25 flask with cells was placed in an incubator at 95% air and 5% CO_2 in a humidified atmosphere at 37°C. The cells were observed after 24 hours.

3.5.3(c) Trypsinization and passaging

Cells can be sub-cultured once they reach 80% confluency. During the cells sub-culture, the used media was discarded and the cells were washed with 2ml prewarmed FBS twice to remove the serum traces. Then, 1ml of 0.25% trypsin + 0.02% EDTA was added into T25 flask and kept in the 37°C incubator. After 7 minutes, the flask was observed under inverted microscope for rounded cells and gently tapped on the side to aid the detachment of remaining cells form the flask surface. To deactivate the trypsin, 5 ml of complete growth media was added to the

flask immediately and pipetted up and down several times and transferred to 15ml tube and centrifuged at 157 ×g for 5 minutes. The supernatant medium was discarded and cell pellet was resuspended in 1ml of medium, which was then divided into 3:1 ratio to be sub-cultured further. The T25 flasks were labelled carefully with date, cell line name and passage number and kept in the incubator at 95% air and 5% CO_2 in a humidified atmosphere. HSC-2 cell can be passaged every 2nd or 3rd day.

3.5.3(d) Cell counting

Haemocytometer was used in this project to count the viable cell before carrying out all the experiments. 10µl of cell suspension from the 1ml of freshly harvested cell suspension was mixed with 10µl of trypan blue dye. After thorough mixing, 10µl of this solution was pipetted to the edge of Neubauer chamber, which was covered with the cover slip. The solution was transferred by capillary action. The haemocytometer was observed under the microscope at 100x magnification and the viable cells were counted in the 1st, 2nd, 3rd and 4th quadrant. Cells which were stained by trypan blue were dead cells, therefore excluded from the counting. To calculate the number of viable cells in 1ml of cell suspension, following formula is used:

Number of cells= Av x 2n x 10^4

Where,

Av = Average number of cells from 4 quadrants

2n = Dilution factor

10^4 = Volume of one grid

3.5.3(e) Cryopreservation and cell stock

After the propagation of cell line, it was important to keep a cryopreserved cell stock as a reservoir for any unexpected sudden cell death or cell culture contamination. Cells were cryopreserved, once the flask reached 80% confluency and the cell count was more than one million cells/ml, 90% of this cell suspension was transferred to a vial and 10% of DMSO was added to it drop by drop and gently mixed. The vial was labelled and placed in -30°C freezer for 30 minutes and then in -80°C freezer overnight and then transferred to nitrogen tank on the next day. 70% ethanol spray was used for sterilization purpose in every step. Throughout the progress of this project, the cells were substituted from the cryopreserved stock to maintain the cells characteristics.

3.5.4 Preparation of extract stock solution

The extractable solids were measured from each solvent at the end of each extraction. The flesh and pits of Ajwa date were extracted separately using three different solvents. After the evaporation of the solvents in the concentrator and completely freeze drying the remaining extracts, the weight of each extract was measured using a weighting scale.

Fresh stock solutions of Ajwa date flesh (50mg/ml) and pits (5mg/ml) extracts were prepared (w/v) by dissolving the dried extract powder with complete growth medium. The first stock solution of extract was sterilized by filtration with 0.22 µm syringe filters and the next dilutions were prepared from this sterile extract solution.

3.5.4(a) Preparation of extract from the flesh (ADF)

Based on the previous studies the concentration range of Ajwa date flesh used were 0.2 to 0.6 mg/ml (Mirza *et al.*, 2018), 5 to 25mg/ml (Khan *et al.*, 2016), and 10 to 30mg/ml (Siddiqui *et al.*, 2019). Thus, it was concluded that a wider range of concentrations was needed to study the anticancerous effects of Ajwa date flesh for a

beneficial outcome. Therefore, the concentration of Ajwa date flesh extract ranged between 0.8 to 50mg/ml (0.8 mg/ml, 1.5 mg/ml, 3.1 mg/ml, 6.3 mg/ml, 12.5 mg/ml, 25 mg/ml and 50mg/ml) were used in this study based on the previous studies. Serial dilutions were performed using complete growth medium (v/v) (Appendix D).

3.5.4(b) Preparation of extract from the pit (ADP)

According to the different concentration ranges of various date seeds used in previous studies from 0 to 0.25mg/ml (Al-Zubaidy *et al.*, 2016) for MCF-7 breast cancer cell line and 0.031 to 4 mg/ml (Thouri *et al.*, 2019) for human cervical and liver cell line. The concentration range for Ajwa date pits to be used in the present study were selected from 0.08 mg/ml to 5mg/ml (0.08 mg/ml, 0.31mg/ml, 0.63 mg/ml, 1.15 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5mg/ml) based on the previous studies. Serial dilutions were performed using complete growth medium (v/v) (Appendix E).

3.6 DPPH assay

To evaluate the antioxidant capacity of Ajwa date's flesh and pit extracts extracted with three different solvents, diphenyl picrylhydrazyl (DPPH) free radical scavenging activity assay was carried out to obtain the highest biologically active extract. The DPPH assay was carried out following the previously described protocol (Brand-Williams *et al.*, 1995). The working solution of DPPH in methanol was prepared daily for the measurement of antioxidants in the extracts using UV spectrophotometer. To prepare the 0.1mM of DPPH (molecular weight 394.32g/mol) solution, 3.94mg of DPPH was dissolved in 100mL of methanol in a flask which was covered by aluminium foil. Three ml of this solution was then mixed with 100µL of various concentrations of sample extract solution in a disposable microcuvettes. The samples were kept in a dark place for 30 min at room temperature before being

measured for absorption at 517 nm using the spectrophotometer. A blank sample containing 3ml of DPPH solution was measured daily to obtain an absorbance of 0.0±0.02 units at 517nm. Ascorbic acid was used to prepare a standard curve for calibration using six concentrations ranging from 1.5 to 50 mg/ml. The total antioxidants are expressed as mg/ml of ascorbic acid. The experiment was run in triplicates and average was taken to calculate the radical scavenging activity using the following formula

$$\% \text{ inhibition} = [(A_b - A_s)/A_b] \times 100$$

Where,

A_b =Absorbance of control

A_s = Absorbance of Sample.

3.7 Cytotoxicity study

3.7.1 Assessment of morphology

Cells were cultured in 6 wells (10mm) cell culture petri dishes at a seeding density of 2×10^5 cells/well. After 24 hours attachment the cells were treated with different concentrations of Ajwa date's flesh and pit extracts separately and in combination, whereas untreated cells served as control. The morphological changes were observed under an inverted microscope and images were taken for the comparison with the untreated cells images at 48 hours treatment.

3.7.2 MTT assay

In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxicity effect of Ajwa date's

flesh and pit extracts separately on the human oral squamous cell line growth. Cytotoxicity assay was carried out after 24h, 48h and 72h incubation durations. MTT assay was performed to determine the 50% inhibitory concentration (IC_{50}) of the ADF and ADP extract.

The HSC-2 cells were harvested and seeded in a 96-well plate at a cell density of 5 x 10^3 cells/well and incubated at 37°C in humidified atmosphere with 5% CO_2 and 95% air. Cells attachment was confirmed under microscope after 24 hours; the cells were treated with different concentrations of ADF and ADP extracts separately (see section 3.5.3a, b). Control wells were treated with the same amount of complete growth media only. For every treatment and untreated control group, complete growth media without cells were added as a blank to reduce the background absorbance values. For every concentration of ADF and ADP extracts, 6 wells were used, three with treatment and three as blanks. All the experiments were carried out three times independently and at least 3 replicates were performed in each experiment.

The cells were grown with sample extracts up to three different time points (24h, 48h and 72h) after which MTT assay was performed by removing the medium gently and adding 10µl of MTT solution with a final concentration of 5mg/ml per well, and incubated at 37°C for about 4 hours until the purple crystals were formed. After that the MTT solution was discarded from every well and 100µl of DMSO was added to dissolve the crystals. The 96-well plate was mounted on a microplate shaker and shaken for 15 minutes until the crystals completely dissolved. The absorbance value for each well was determined at an optical density at 570nm wavelength using ELISA Microplate reader (Khan *et al.*, 2018).

The cell viability (CV) percentage after treatment with ADF and ADP extract was calculated with the formula below:

$$CV(\%)= \frac{absorbance\ of\ treatment\ cells - absorbance\ of\ blanks}{absorbance\ of\ control\ cell - absorbance\ of\ blanks} \times 100$$

3.7.3 Determination of IC_{50}

The IC_{50} value represents the concentration of extract needed to reduce 50% viable cells and was calculated from non-linear regression analysis with GraphPad-Prism™ software.

3.8 FITC Annexin-V apoptosis assay

Only two concentrations from the MTT assays result at 24 hours were selected from ADF and ADP extract, to be further used in FITC Annexin-V apoptosis assay. The first concentration was the IC_{50} value and the second concentration was the next higher value then the IC_{50} value for both the extracts. Further higher concentrations were not used in this assay as they would have only indicated an increased percentage of dead cells and less or no cells in early or late apoptotic phase, which gives no information to whether the cell death was caused due to apoptosis. This being a limitation of this assay.

For negative control, untreated cells were used. The experiment was performed in triplicate at 24h, 48, and 72h. Apoptotic cells were quantified with Annexin V-FITC Apoptosis kit following the manufacturer's instructions.

Briefly, HSC-2 cells at 2 x 10^5 cells/ml density were incubated for 24h to allow adherence of cells to the 6-well culture plate. After 24 hours, the cells were incubated with ADF and ADP extracts for 24 hours, 48 hours and 72 hours. After the given time point, the cells were harvested by washing with PBS and trypsinizing with 400-450µl of trypsin. After that the cells were centrifuged with 1ml of cold PBS twice, and once with 100µl of 1X binding buffer solution for 5 minutes each time at 157 ×g. The cells

were stained with 5µl Annexin V-FITC and 5µl Propidium Iodide (PI) for 15 min at 25°C in the dark. The cells were resuspended in 400µl of 1X binding buffer solution and immediately analysed by BD Accuri ™ C6 flow cytometry in 5ml round bottom polystyrene FACS tubes. Wavelengths of 533nm and 585nm were used for Annexin V-FITC and PI, respectively. 10,000 events per sample were recorded on forward scatter versus side scatter plot using BD Accuri™ C6 software. Control samples were prepared separately for getting purpose of HSC-2 cells according to their granularity and size. Percentage of PI only stained cells represent necrosis, PI and Annexin -V represent late apoptosis, cells stained with Annexin -V only represent early apoptosis and unstained cells were evaluated as viable healthy cells.

3.8.1 Combination treatment

A combination test was performed with both ADF and ADP extract together to analyse if there was any synergistic effect in apoptosis of the cells.

IC_{50} concentration from the MTT assay result of 24 hours for ADF and ADP were combined together and used as a single reagent, and the results were recorded.

Likewise, next higher concentrations were combined for both the extract and used as a single reagent to observe if it had significantly higher apoptotic effect then if used separately. Apoptosis effects of the combination treatments were carried out similarly as described in section 3.8

3.9 Statistical Analysis

The statistical analysis was carried out using SPSS 24 version and Microsoft excel. The mean percentage weight of the extracted yield of Ajwa date (flesh and pit) from three solvents was calculated and compared in a bar graph. For DPPH assay, a graph of antioxidant activity was plotted against concentration and for analysis a non-

parametric test was applied, since the assumption of normality was violated. The half maximal effective concentration (EC_{50}) for 70% aq. ethanol and acetone was calculated using non-linear regression calculation. One-way ANOVA and Tukey's post hoc was used to analyse the significant difference between the two solvents antioxidant activity for both flesh and pits.

For MTT assay One-way ANOVA was conducted followed by Dunnett's Multiple Comparison test to evaluate the variance of all samples at 24, 48 and 72 hours and the IC_{50} from the MTT results were calculated using non-linear regression analysis function with Prism GraphPad software. For apoptosis assay, comparison bar graphs were plotted and One-way ANOVA was performed followed by Dunnett's Multiple Comparison test at 24, 48 and 72 hours.

All experimental data was represented as the mean ± standard deviation (SD) of three independent experiments which were performed in triplicate. A P-value of less than 0.05 ($p<0.05$) was considered statistically significant.

CHAPTER 4

RESULTS

4.1 Impact of Extraction Solvents on Extractable Solids

It was observed from the results of this study that the three solvents produced different amounts of extractable solids yield of Ajwa date flesh and pit. The crude solids were extracted from 15g of Ajwa date sample. After the completion of extraction for each solvent, the freeze-dried residues were weighed and the values were noted are given in Table 4.1 and presented as a graph in Figure 4.1. The values are the mean average of three replicate and presented as a percentage.

For the Ajwa date flesh (ADF) sample, 70% aqueous (aq.) acetone had the highest extractable solids (32%) followed by 70% aq. ethanol (10.04%). n-hexane extracted the least amount of solid yield of Ajwa flesh which was (1.87 %) in comparison with 70% aq. acetone and 70% aq. ethanol.

For the Ajwa date pits sample, 70% aq. acetone produced the most (7.71%) extractable solids followed by 70% ethanol (2.7%) and (0.2%) from n-hexane. These results showed that the total extracted solids varied greatly among different solvents. This indicated the possible influence of extracting solvent on total extracted contents. Among all the date extracts, 70% acetone was found to be the most efficient solvent for extracting both flesh and pit samples of Ajwa date when compared with all other solvents used.

Table 4.1 Weight of extracted yield of Ajwa date flesh and pits from three different solvents. Data is presented as a mean percentage from three independent experiments. SD is the standard deviation

Solvent	Ajwa flesh % ± SD	Ajwa pit % ± SD
70% Aq. Acetone	32.00 ± 0.10	7.71 ± 0.05
70% Aq. Ethanol	10.04 ± 0.05	3.18 ± 0.10
n-hexane	1.87 ± 0.03	2.47 ± 0.16

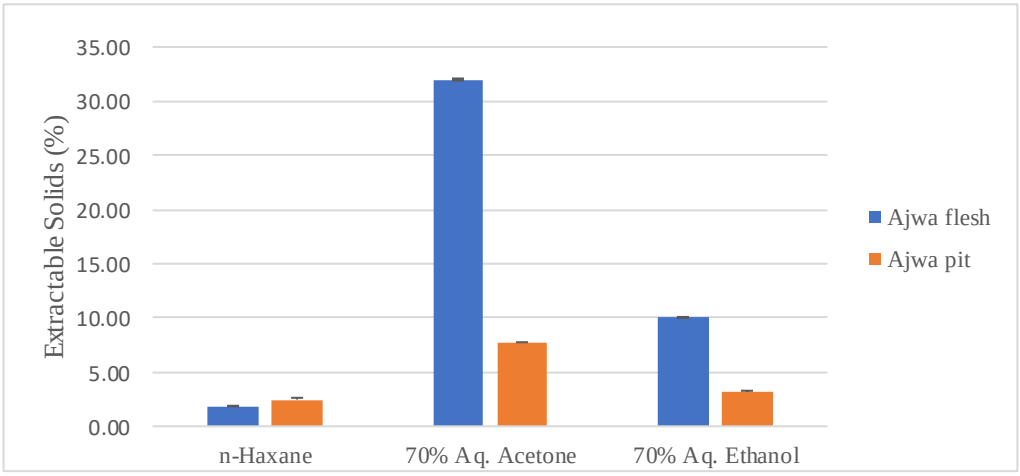


Figure 4.1 Effect of solvents on extractable solids from the Ajwa date flesh and pit. The values are the mean average of three replications for each solvent ± standard deviation

4.2 DPPH assay

The antioxidant activities of the extractable solids were studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. The assay could not be performed with the n-hexane extract due to the insufficient amount of residue present to make a working solution to perform the assay. Therefore, antioxidant activity from the acetone/H2O (70:30, v/v) and ethanol/H2O (70:30, v/v) were studied. Ascorbic acid was used as a standard reference for the comparison of results.

4.2.1 Assessment of the total antioxidant contents of the Ajwa date flesh's extract

The extract from the aq. acetone 70% and aq. ethanol 70% of ADF samples were tested first using concentrations from 1 to 50 mg/ml and the results were plotted against the inhibition percentage of radical scavenging activity. Table 4.2 presents the average of three values with standard deviation.

The graph presented in Figure 4.2 shows the percentage of DPPH free radical scavenging activity against the extract concentration showed that the extract concentrations of both solvents are proportional to the percentage of inhibition, which means the greater the concentration of extracts, the greater the DPPH scavenging activity.

The curve for ascorbic acid also showed the same inhibition pattern, but at a very low concentration of 1.5 mg/ml, it reached the highest inhibition percentage of 89.8%. Data shows that the percentage of inhibition of aq. acetone 70% ADF extract was higher than the aq. ethanol 70% ADF extract (Figure 4.2). To analyse whether there was a significant difference between the extract prepared using these two solvents (aq. acetone 70% and aq. ethanol 70%), a non-parametric test was applied as the assumption of normality was violated.

Table 4.2 Concentration of Ascorbic acid, 70% aq. acetone and 70% aq. ethanol flesh extract inhibition at 517nm

	Inhibition mean ± SD (%)		
Concentration (mg/ml)	**Ascorbic Acid**	**70% Aq. Acetone flesh**	**70% Aq. Ethanol flesh**
0.0	0.00	0.00	0.00
1.5	89.75 ± 1.81	6.28 ± 0.67	6.16 ± 1.56
3	89.87 ± 1.86	12.09 ± 1.22	3.70 ± 0.41
6	89.72 ± 0.55	14.71 ± 1.28	5.08 ± 0.51
12	90.88 ± 0.09	23.01 ± 0.61	6.62 ± 1.04
25	90.96 ± 0.09	36.23 ± 2.46	14.10 ± 3.05
50	90.59 ± 0.06	47.79 ± 2.44	24.72 ± 8.65

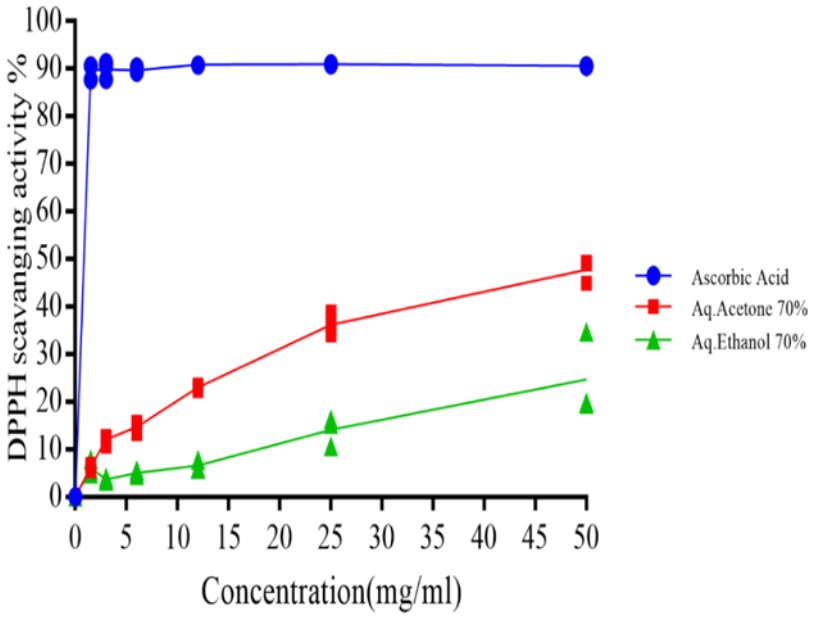


Figure 4.2 DPPH scavenging activity of different solvents of ADF extract at different concentration. The curve for ascorbic acid has been used as a standard. Each value represents the mean of three independent experiments (n= 3)

Table 4.3 and Figure 4.3 shows the result for the inhibition percentage between the two solvents extract of Ajwa flesh. The inhibition percentage was significantly greater for the aq. acetone extract of ADF (median=15.1%), than the aq. ethanol extract of ADF (median= 5.6%), (p=0.011).

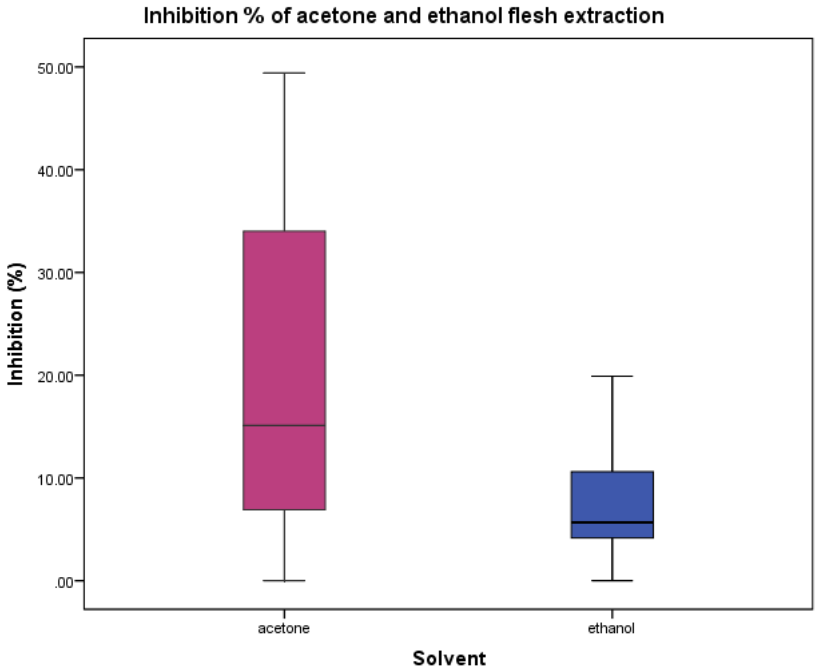


Figure 4.3 Box plot for the percentage of inhibition for aq. acetone and aq. ethanol ADF extraction

Table 4.3 Mann-Whitney U test results for the inhibition percentage of two solvents of ADF extract.

	Test Statistics	***p***
Mann-Whitney U test	119.5	0.011

4.2.1(a) Determination of EC_{50} value for Ajwa date flesh extract with various solvents

Based on the non-linear regression calculation, the half maximal effective concentration (EC50) of 70% aq. acetone extract of Ajwa date flesh was 52.09 mg/ml and EC50 of 70% aq. ethanol extract of Ajwa date flesh was 133.94 mg/ml. Ascorbic acid EC50 = 0.206 mg/ml. The graphical representation is shown in Fig. 4.4. The EC_{50} is the dose concentration of the sample required to reduce 50% of the free radicals of DPPH.

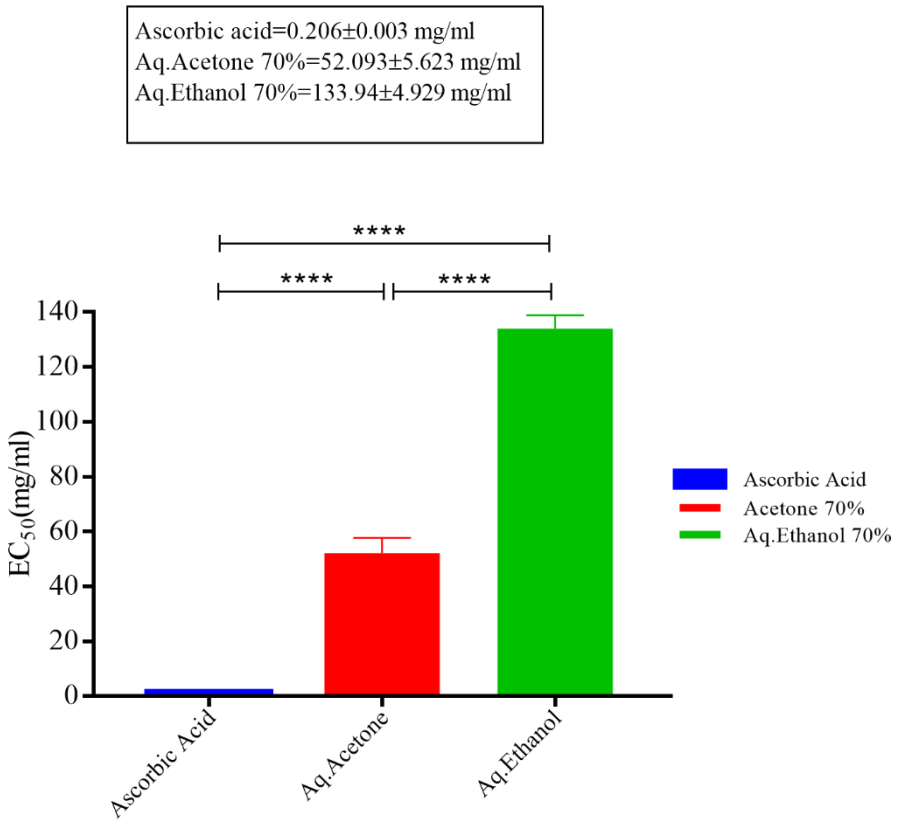


Figure 4.4 Comparison of EC_{50} value of aq. acetone and aq. ethanol extract of ADF with the standard EC50 value of ascorbic acid. **** Significant difference from control at $p < 0.05$

A One -way ANOVA was conducted to compare the EC_{50} value of DPPH free radical scavenging activity of ascorbic acid (positive control) with the EC_{50} of 70% acetone and 70% ethanol extract of Ajwa flesh sample. Significant difference at the $p < 0.05$, there was a significant difference noted between the two solvents and the ascorbic acid [$F(2, 6) = 731.7$, $p < 0.0001$]. Post hoc comparisons using Tukey's HSD test indicated that the mean value for the EC_{50} of ascorbic acid ($M= 0.206$, $SD = 0.003$) was significantly different than the EC_{50} of 70% acetone ($M=52.09$, $SD= 5.62$) with a value ($p<0.0001$) and EC_{50} value of 70% ethanol ($M=133.94$, $SD = 4.92$) extract of Ajwa flesh sample with a value ($p<0.0001$). EC_{50} of 70% acetone extract of Ajwa flesh was also significantly different from the EC_{50} of 70% ethanol extract of Ajwa flesh sample with a value ($p<0.0001$). Taken together, these results suggest that 70%

acetone extract has more antioxidant activity then the 70% ethanol extract of flesh sample. The high EC_{50} value of 70% ethanol extract suggests the minimal amount of antioxidant present in this extract.

4.2.2 Assessment of the total antioxidant contents of the ADP

Total antioxidant activity was assayed spectrophotometrically to evaluate the two extraction solvents for pit sample of Ajwa date, concentrations ranging from 0 to 5 mg/ml were selected for both solvents. Table 4.4 and Figure 4.5 showed that 70% aqueous acetone extract has greater DPPH free radical scavenging activity at a lower concentration than 70% aqueous ethanol extract of ADP, but the highest average yield of the total antioxidants for both the solvents sample were reached at the concentration of 5mg/ml. Figure 5 indicates that both the solvents showed proportional relationship with the percentage of inhibition. It means greater the concentration of extract sample, the more antioxidant in the extract that can reduce free radicals in DPPH. Ascorbic acid showed the same inhibition pattern, the percentage of scavenging activity of ascorbic acid and the 70% acetone extract were similar, followed by the 70% aq. ethanol extract of Ajwa pit.

Table 4.4 Concentration of ascorbic acid, 70% aq. acetone and 70% aq. ethanol of pits extract and their inhibition percentage at 517nm. Values are expressed as mean ± SD (n=3)

	Inhibition mean ± SD (%)		
Concentration (mg/ml)	**Ascorbic Acid**	**70% Aq. Acetone pit**	**70% Aq. Ethanol pit**
0.0	0.00	0.00	0.00
0.2	29.42 ± 1.29	56.53 ± 0.95	11.98 ± 4.61
0.3	87.45 ± 3.02	85.64 ± 1.61	21.84 ± 3.25
0.6	90.56 ± 0.07	88.72 ± 0.20	40.19 ± 2.98
1.0	89.75 ± 1.81	88.78 ± 0.09	54.29 ± 8.85
2.5	89.86 ± 1.86	88.78 ± 0.27	78.23 ± 7.77
5.0	89.72 ± 0.55	84.51 ± 0.11	88.25 ± 0.49

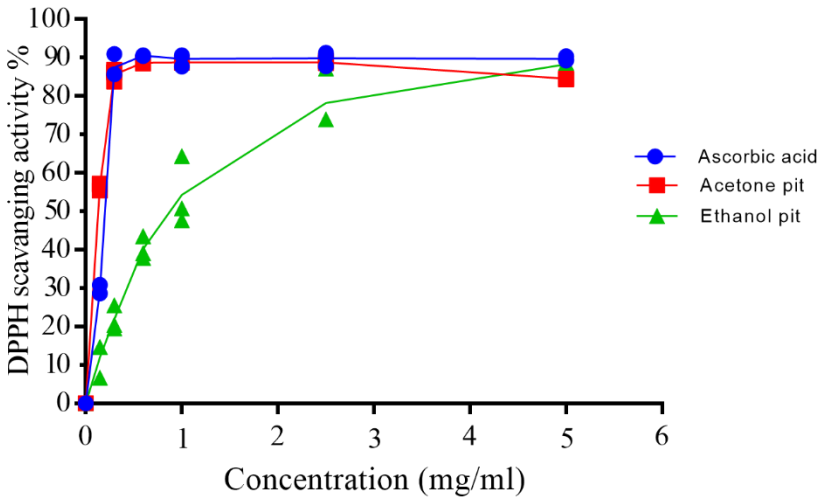


Figure 4.5 DPPH scavenging activity of various fractions of Ajwa date pits at different concentration. The curve for Ascorbic acid is used as a standard. Each value represents the mean of three independent experiments (n= 3)

Table 4.5 and Figure 4.6 showed the result for the inhibition percentage between the 70% acetone and 70% ethanol extracts of Ajwa date pit sample. At the $p<0.05$ level of significance, the results showed that there is significant difference in the inhibition percentage ($p=0.04$) between the 70% acetone (median=86.2 %) and 70% ethanol (median = 39.1 %) solvent extract.

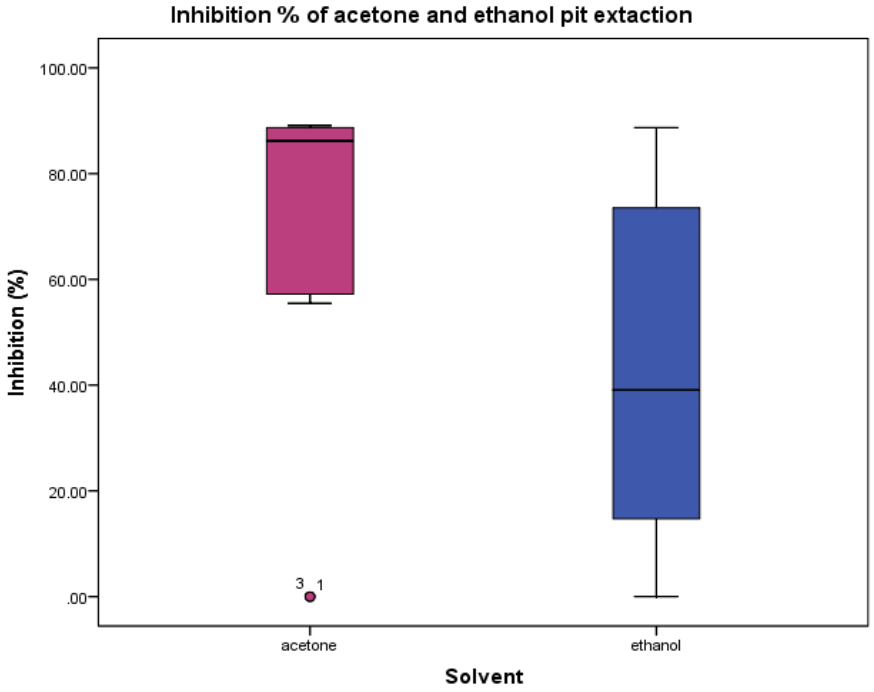


Figure 4.6 Box plot for the percentage of inhibition for aq. acetone and aq. ethanol of ADP extract

Table 4.5 Results for the statistical analysis of inhibition percentage of the two solvents for ADP extract

		Test Statistics	*p*
Mann-Whitney test	U	107.0	0.04

4.2.2(a) Determination of EC_{50} value of Ajwa date pits with various solvents

Based on the non-linear regression calculation, the EC_{50} of 70% aq. acetone extract of Ajwa pit was 0.153 mg/ml and EC_{50} of 70% aq. ethanol extract of Ajwa pit was 0.954 mg/ml. The EC_{50} value of ascorbic acid was 0.206 mg/ml. The EC_{50} is the dose concentration of the sample that is needed to reduce 50% of the free radicals of DPPH. The EC_{50} and the antioxidant results obtained from Tukey's grouping are illustrated in Figure 4.7.

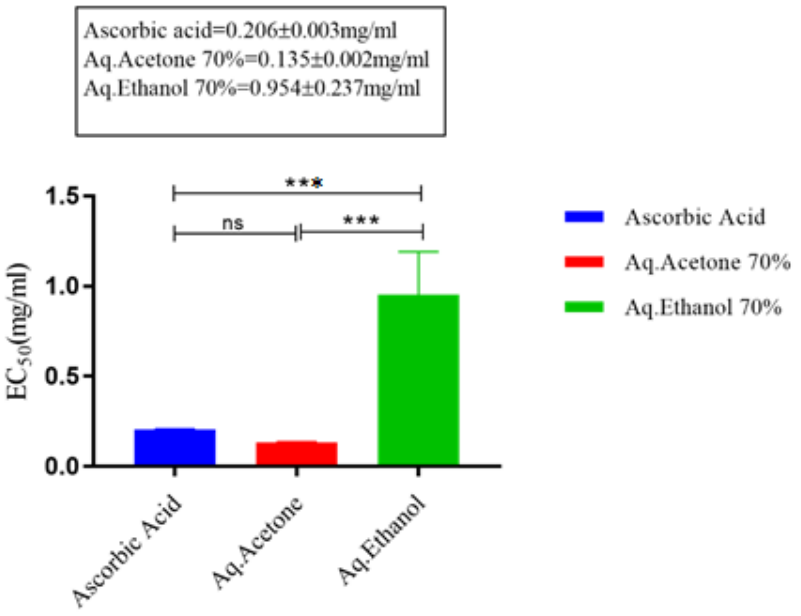


Figure 4.7 Comparison of EC50 value of aq. acetone and aq. ethanol extract of ADP with the standard EC50 value of ascorbic acid. *** Significant difference from control at $p < 0.05$, NS: no significant difference from control

A One-way ANOVA was conducted to compare the EC50 value of DPPH radical scavenging activity of ascorbic acid, 70% acetone and 70% ethanol extract of

Ajwa pit sample. There was a significant difference at the $p < 0.05$ level between the two solvents and the standard reference of ascorbic acid [$F(2, 6) = 33.03$, $p = 0.0006$]. Post hoc comparisons using Tukey's HSD test indicated, that the mean value for the EC50 of ascorbic acid ($M = 0.206$, $SD = 0.003$) was significantly different than the EC50 of 70% ethanol extract ($M = 0.954$, $SD = 0.237$) of Ajwa pit sample.

However, the EC_{50} of ascorbic acid did not significantly differ from the EC_{50} of 70% acetone ($M = 0.135$, $SD = 0.002$). EC_{50} of 70% acetone extract of Ajwa pit was also significantly different from the EC_{50} of 70% ethanol extract of Ajwa pit sample. The comparison of the two solvents with the positive control ascorbic acid suggested that the highest antioxidant activity among the two solvents was 70% acetone extract that contains the highest amounts of polyphenols and flavonoids. However, the high EC50 of 70% ethanolic extract suggest low antioxidant activity. It also concluded that Ajwa date pit has promising antioxidative and anti-free-radical effects.

Table 4.6 The EC50 values of two solvents for the Ajwa date flesh and pits

Solvent	EC_{50} of Ajwa flesh	EC_{50} of Ajwa pit
70% Acetone	52.093 ± 5.623	0.135 ± 0.002
70% Ethanol	133.94 ± 4.929	0.954 ± 0.237

It was concluded from the Table 4.6 that the order of antioxidative effect and polyphenolic content was as follows, 70% acetone pit>70% ethanol pit>70% acetone flesh>70% ethanol flesh as shown in Figure 4.8, indicating that the solvent that yield the largest amounts of polyphenols was 70% acetone. The results from this assay also indicated that the pit part of Ajwa date had more powerful antioxidant effect compared with the flesh part of Ajwa date. Consequently, 70% acetone can be used as an appropriate solvent system to increase the antioxidative effect and concentration of polyphenolic compounds. Therefore 70% acetone was used further to conduct the cellular studies.

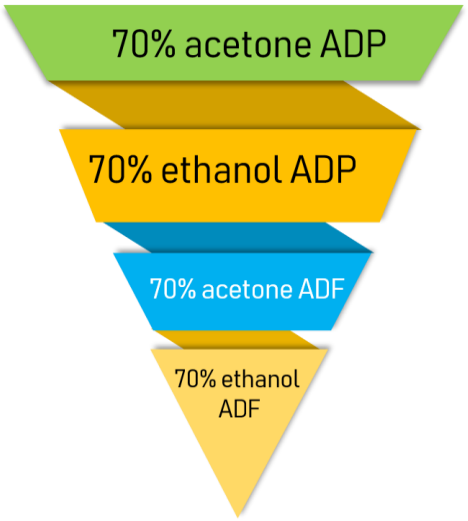


Figure 4.8 Antioxidant activity of the ADF and ADP extract

4.3 Effect on morphology of HSC-2 cells

Images taken from the light microscope of HSC-2 cells of control sample demonstrated characteristic of epithelial nature, and growth proliferation as a monolayer. The cells appeared to be attached together in an ovoid shape with a large central nucleus, dividing cell can also be seen with two or more nucleoli (Figure 4.9-a). In contrast the ADF and ADP extract treated cells showed mild to severe decrease in cell numbers (Figure 4.9, b-g) which was dose dependent leaving behind very few live cells. At a concentration of ADF extract IC_{50}, cell shrinkage and partial cell-to-cell detachment can be seen, cells have also started losing their shape. At a concentration of ADF extract 25 mg/ml, more drastic changes can be seen, cells have become rounded in shape with complete cell-to-cell detachment and with the decrease in number of viable cells. For ADP extract at a concentration of $IC_{50,}$ cytoplasmic vacuolization, nuclear condensation and cluster shrinkage can be seen. At a concentration of 2.5 mg/ml, a lot of cellular fragmentation can be seen with very few viable cells. For the combination treatment with ADF and ADP extract, drastic

morphological changes can be seen. Nuclear condensation, cell membrane blebbing and fragmentation is vastly present with very few viable cells.

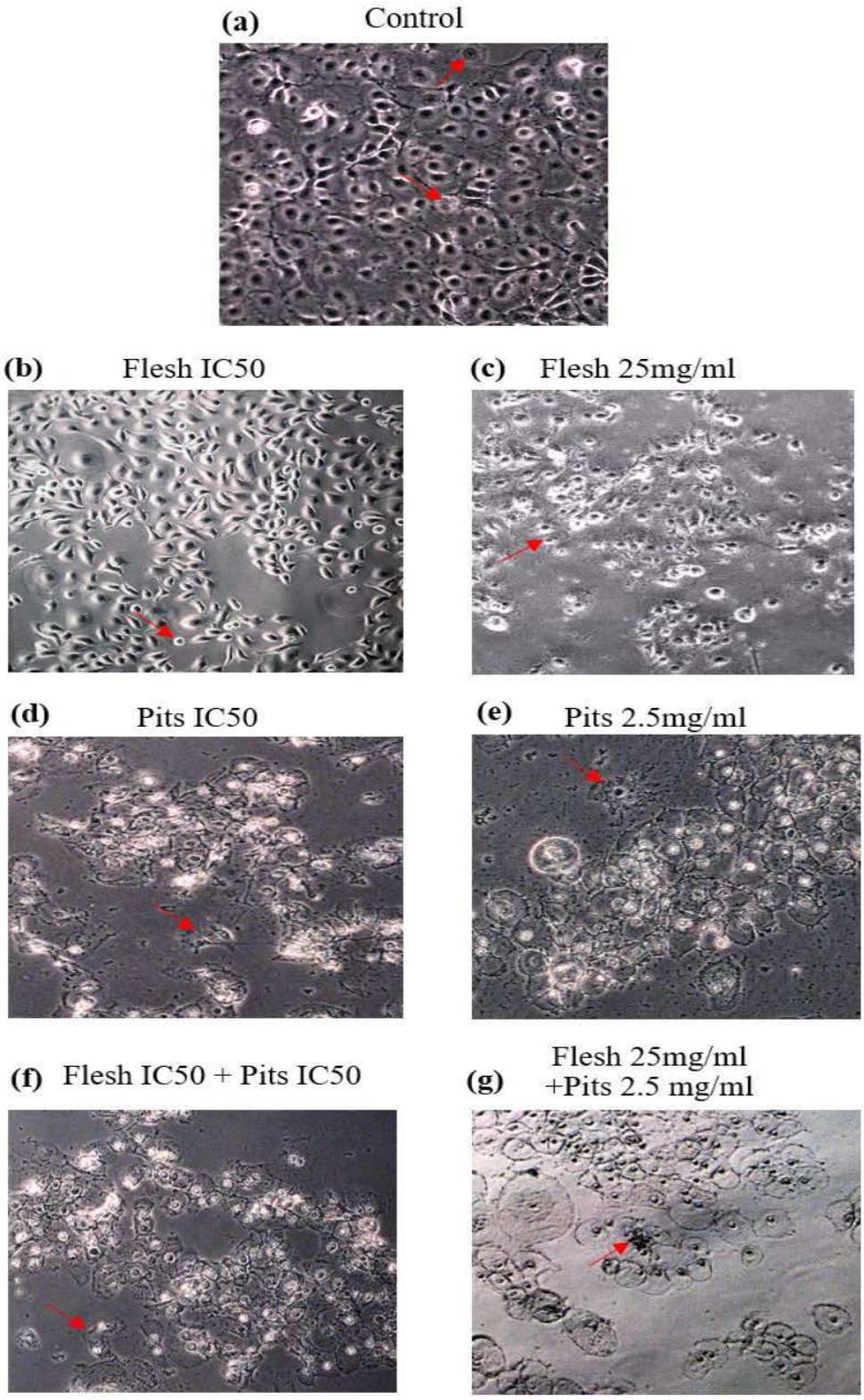


Figure 4.9 Morphological appearance under an inverted light microscope after 48 hours. Mitotic figures can be seen in (a), cell shrinkage and cell detachment in (b)

and (c), membrane disruptions, cytoplasmic condensation and cell death can be seen in (d), (e), (f) and (g) magnification 100X

4.4 MTT assay

4.4.1 Cytotoxic effect of ADF extract on HSC-2 cells

MTT assay was carried out on HSC-2 cells with various concentrations (0.8, 1.5, 3.1, 6.3, 12.5, 25, and 50mg/ml) of ADF extract with 70% aq. acetone after the incubation period of 24h, 48h, and 72h is shown in Figure 4.10. MTT assay demonstrated HSC-2 cells growth inhibition following the treatment with ADF extract in a dose-dependent manner (Figure 4.10a, b, and c). There was a significant decrease in percentage of cell viability starting from 12.5 mg/ml concentration at 24 h, 6.3 mg/ml at 48 hours and 3.1mg/ml at 72 h exhibiting a time-dependent manner. One-way ANOVA was conducted to evaluate variance of samples (Table 4.7). The decrease in the cell viability percentage was statistically significant at p-value less than 0.05.

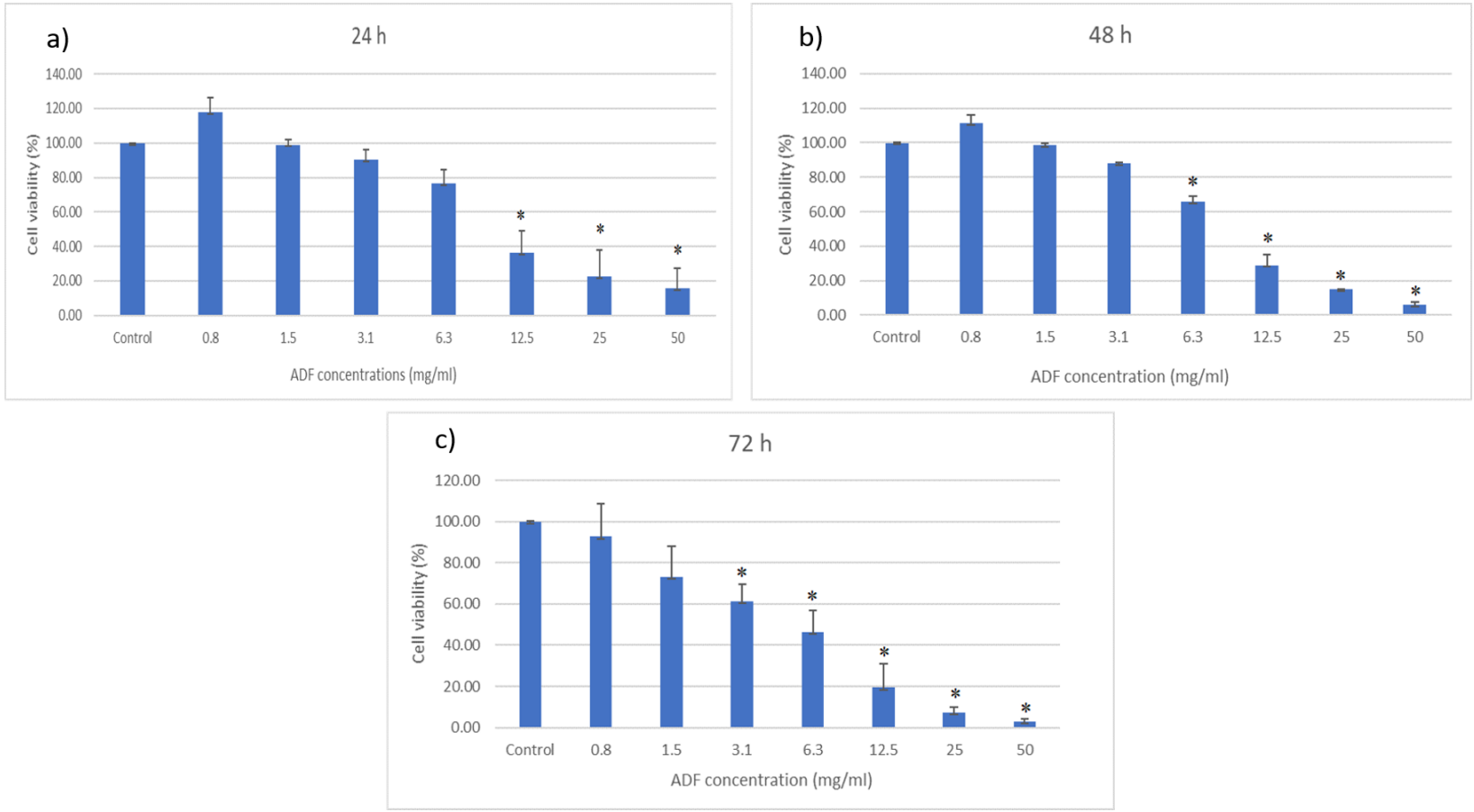


Figure 4.10 Inhibition of proliferation of HSC-2 cell. MTT assay of HSC-2 cells treated with ADF extract at different concentrations (0.8, 1.5, 3.1,6.3, 12.5, 25 and 50 mg/ml) showed decreased cell viability compared to untreated controls in a dose dependent manner at a)24h, b)48h, c)72h. These decreases in percentage of cell viability were statistically significant. The values are expressed as mean SD from triplicate samples of three independent experiments. * indicate $p < 0.05$

Table 4.7 MTT assay statistical analysis for ADF extract for 24h, 48h and 72h. The cell viability percentage is presented as a mean of triplicates of three independent experiments. The p value is significant at the $p < 0.05$ level

Concentrations(mg/ml)	**Cell viability (%) Mean ± SD**	**F(*df*)**	***p***
24h			
Control	100	51.48 (7,16)	0.0001
0.8	117.92 ± 8.4		
1.5	99.02 ± 3.0		
3.1	90.36 ± 5.8		
6.3	76.51 ± 7.9		
12.5	36.55 ± 12.3		
25	22.40 ± 15.7		
50	15.59 ± 11.9		
48h			
Control	100	548.36 (7,16)	0.0001
0.8	111.47 ± 4.79		
1.5	98.51 ± 1.01		
3.1	88.56 ± 0.83		
6.3	65.65 ± 3.57		
12.5	29.07 ± 5.96		
25	14.82 ± 0.46		
50	5.96 ± 1.96		
72h			
Control	100	56.19 (7,16)	0.0001
0.8	92.62 ± 15.90		
1.5	66.50 ± 4.38		
3.1	61.19 ± 8.27		
6.3	46.39 ± 10.29		
12.5	19.44 ± 11.60		
25	7.37 ± 2.70		
50	2.91 ± 1.54		

4.4.1(a) Determination of IC_{50} of ADF extract on HSC-2 cells

HSC-2 cells were treated with ADF extract at 0.8, 1.5, 3.1, 6.3, 12.5, 25 and 50 mg/ml concentration for 24 h and MTT assay was performed. The data acquired was then analyzed using non-linear regression analysis function and the IC_{50} value was determined to be 8.69 mg/ml at 24 h duration, represented in Figure 4.11.

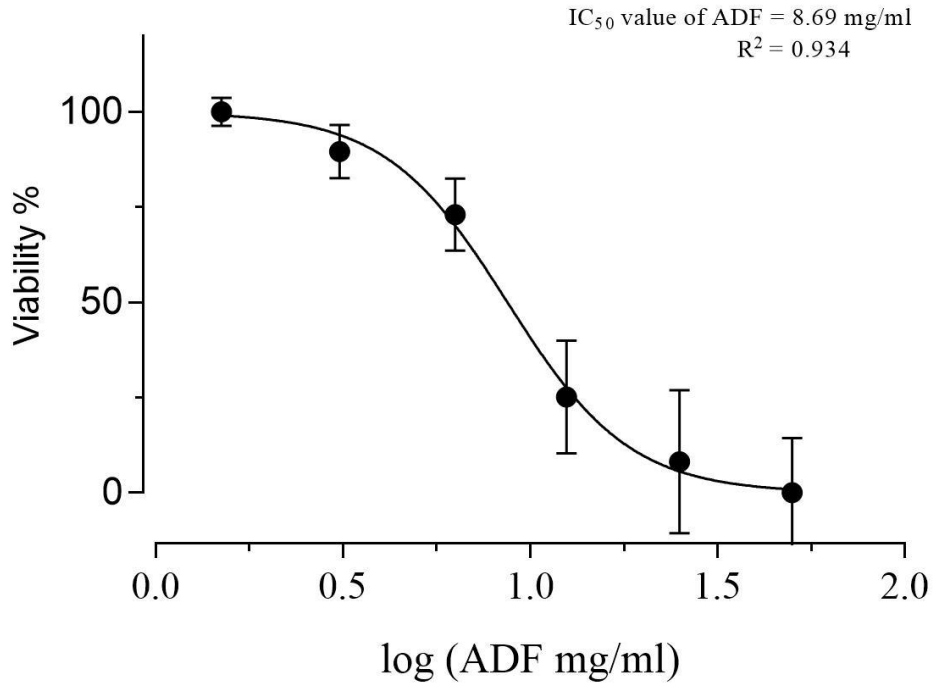


Figure 4.11 Determination of IC_{50} value of Ajwa date flesh extract (ADF) on HSC-2 cells

4.4.2 Cytotoxic effect of ADP extract on HSC-2 cells

MTT assay was carried out on HSC-2 cells with various concentrations (0.08, 0.31, 0.63, 1.15, 1.25, 2.5 and 5 mg/ml) of ADP extract with 70% aq. acetone. After the incubation period of 24h, 48h, and 72h, the result is shown in Figure 4.12. MTT assay demonstrated HSC-2 cells growth inhibition following the treatment with ADP extract in a dose-dependent manner (Figure 4.12 a, b, and c). There was a significant decrease in percentage of cell viability starting from 0.63 mg/ml concentration at 24 h, and at 48 hours and 1.25 mg/ml at 72 h. One-way ANOVA was conducted followed

by Dunnett's Multiple Comparison (Table 4.8). A p-value less than 0.05 was considered as statistically-significant.

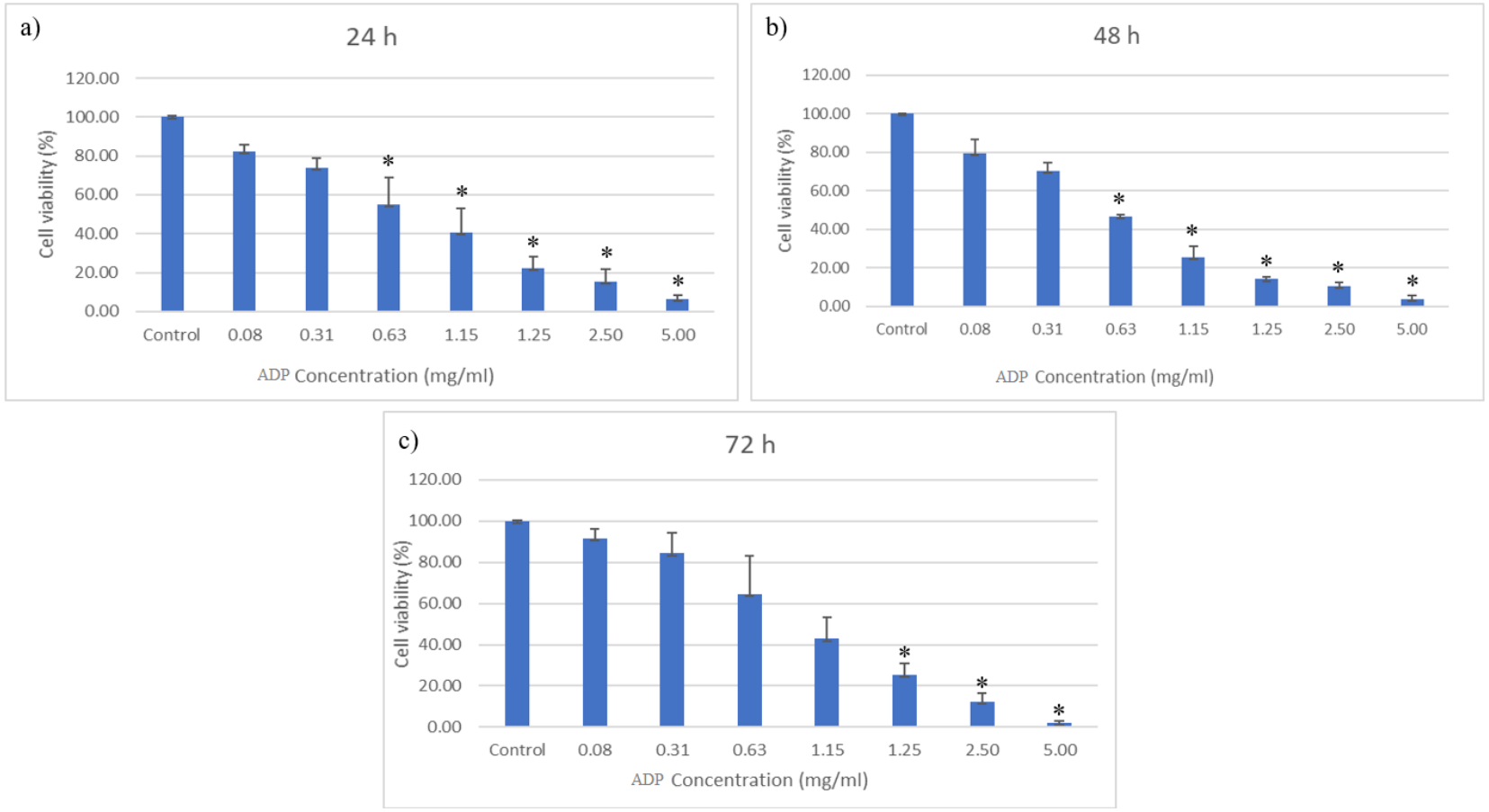


Figure 4.12 Inhibition of proliferation of HSC-2 cell. MTT assay of HSC-2 cells treated with ADP extract at different concentrations (0.08, 0.31, 0.63, 1.15, 1.25, 2.5 and 5 mg/ml) showed decreased cell viability compared to untreated controls in a dose dependent manner at a)24h, b)48h, c)72h. These decreases in percentage of cell viability were statistically-significant. The values are expressed as mean SD from triplicate samples of three independent experiments. * indicate $p < 0.05$.

Table 4.8 MTT assay statistical analysis for ADP extract for 24h, 48h and 72h. The cell viability is presented as mean of three replicates of three independent experiments. The p value is significant at the $p < 0.05$ level

Concentrations(mg/ml)	**Cell viability (%) Mean ± SD**	**F(*df*)**	***p***
24h			
Control	100	58.47 (7,16)	0.0001
0.08	82.17 ± 3.68		
0.31	73.70 ± 4.98		
0.63	54.89 ± 14.22		
1.15	40.70 ± 12.26		
1.25	22.37 ± 5.74		
2.50	15.19 ± 6.75		
5.00	6.57 ± 1.96		
48h			
Control	100	259.69 (7,16)	0.0001
0.08	79.32 ± 7.27		
0.31	70.16 ± 4.65		
0.63	46.50 ± 1.35		
1.15	25.38 ± 5.92		
1.25	14.16 ± 1.39		
2.50	10.56 ± 1.74		
5.00	3.77 ± 1.71		
72h			
Control	100	53.7 (7,16)	0.0001
0.08	91.39 ± 4.68		
0.31	84.32 ± 10.10		
0.63	64.34 ± 18.66		
1.15	42.86 ± 10.42		
1.25	25.32 ± 5.79		
2.50	12.25 ± 4.11		
5.00	2.25 ± 0.54		

4.4.2(a) Determination of IC_{50} of ADP extract on HSC-2 cells

HSC-2 cells were treated with ADP extract at 0.08, 0.31, 0.63, 1.15, 1.25, 2.5 and 5 mg/ml concentration for 24 h and MTT assay was performed. The data acquired was then analysed using non-linear regression analysis function and the IC_{50} value was determined to be 0.97 mg/ml at 24 h duration. The graph is presented in Figure 4.13.

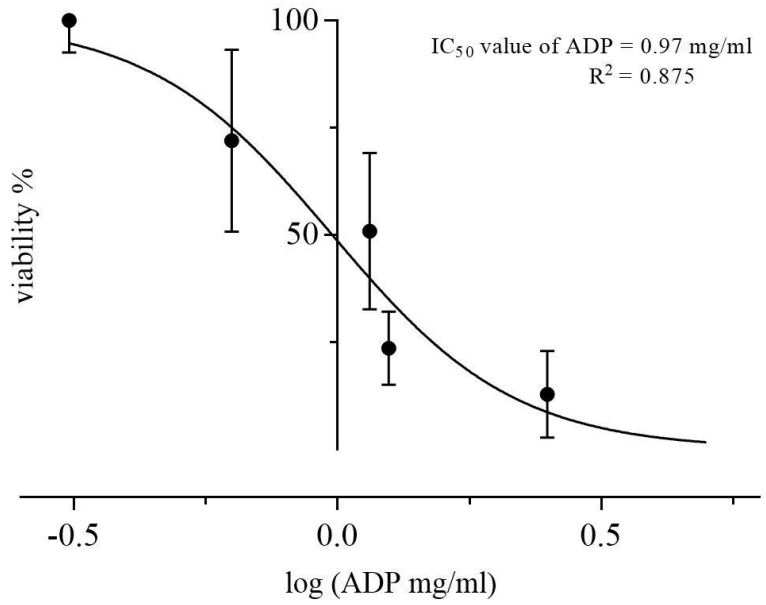


Figure 4.13 The graph was constructed to determine the IC_{50} value of ADP extract on HSC-2 cells using GraphPad Prism software version 7

4.5 Apoptosis assay

The viability and apoptotic activity of HSC-2 cells treated with two different concentrations of ADF extract IC_{50}(8.69 mg/ml) and higher concentration then IC_{50}(25mg/ml), for ADP extract IC_{50}(0.97 mg/ml) and a concentration higher then IC_{50} (2.5mg/ml) and the combination of both ADF and ADP extract as a single treatment (ADF IC_{50} + ADP IC_{50})mg/ml and (ADF 25 + ADP 2.5)mg/ml were analysed by flow cytometry after an incubation period of 24h, 48h and 72h are shown in Figure 4.14, 4.15 and 4.16 respectively and Appendix F. The results indicated that the percentage of cancer cells decreased in the cells treated with Ajwa date extract when compared to

the control cells. The percentage of early apoptotic and late apoptotic cells increased in the treated group when compared to the early and late apoptotic cells of control cells. Among the various extracts ADP 2.5 mg/ml showed the highest percentage of late apoptotic cells and combination of ADF and ADP IC_{50} concentration showed the highest percentage of early apoptotic cells at 24h (Figure 4.14). At 48h, ADP IC_{50} showed the highest percentage of late apoptotic cells and combination ADF 25mg/ml +ADP 2.5mg/ml showed the highest early apoptotic cells (Figure 4.15). One-way ANOVA was performed followed by Dunnett's Multiple Comparison test. A value of $p < 0.05$ was considered statistically significant (Table 4.9, 4.10 and 4.11).

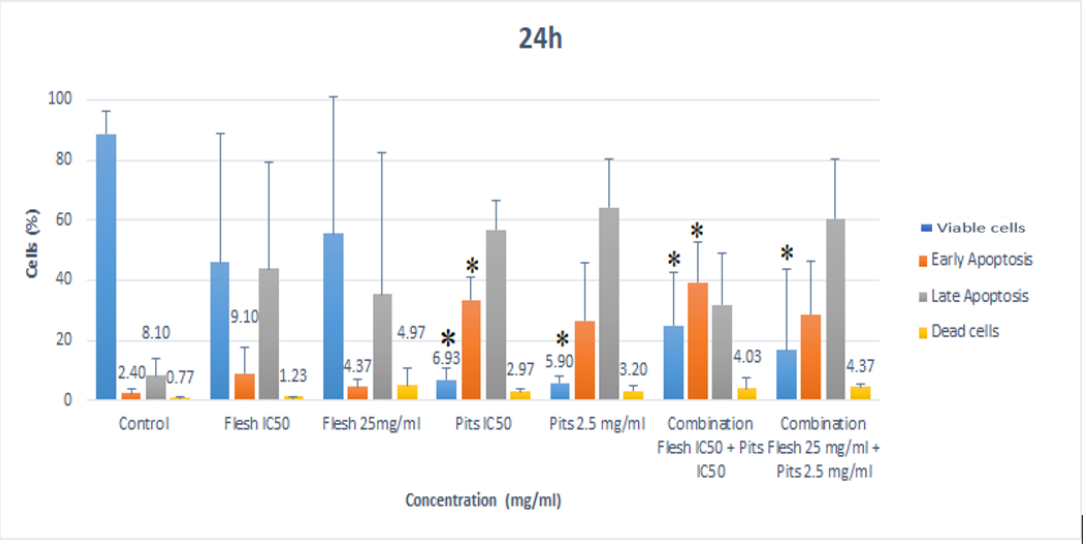


Figure 4.14 The effect of adding various concentration of Ajwa date flesh and pit extracts on the apoptotic activity of HSC-2 cell line at 24h. The percentage of cells are shown in four stages, healthy cells, cells in early apoptotic stage, cells in late apoptotic stage and dead or necrotic cells. The flesh IC_{50} value is 8.69 mg/ml and pits IC_{50} value is 0.97 mg/ml. (* indicates that the treatment is significantly different from the control (untreated) group at $p < 0.05$

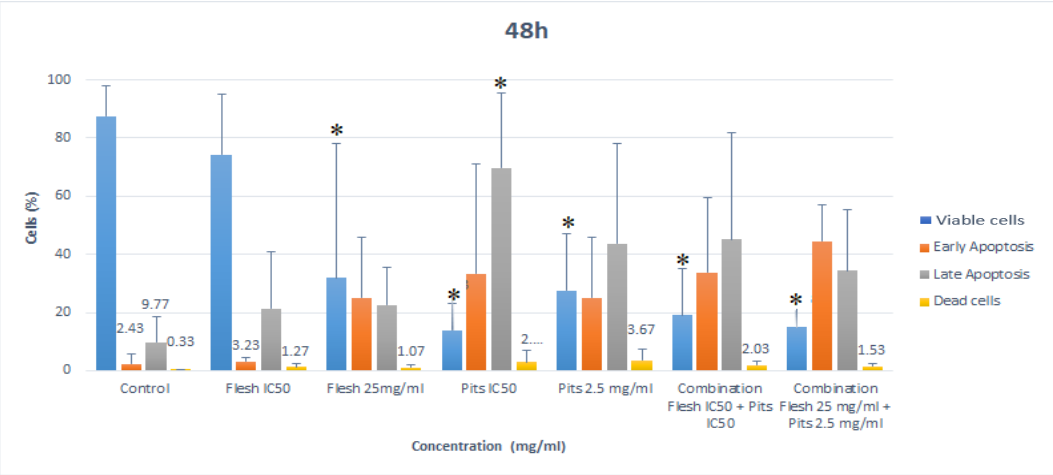


Figure 4.15 The effect of adding various concentration of Ajwa date flesh and pit extracts on the apoptotic activity of HSC-2 cell line at 48h. The percentage of cells are shown in four stages, healthy cells, cells in early apoptotic stage, cells in late apoptotic stage and dead or necrotic cells. The flesh IC_{50} value is 8.69 mg/ml and pits IC_{50} value is 0.97 mg/ml. (* indicates that the treatment is significantly different from the control (untreated) group at $p < 0.05$

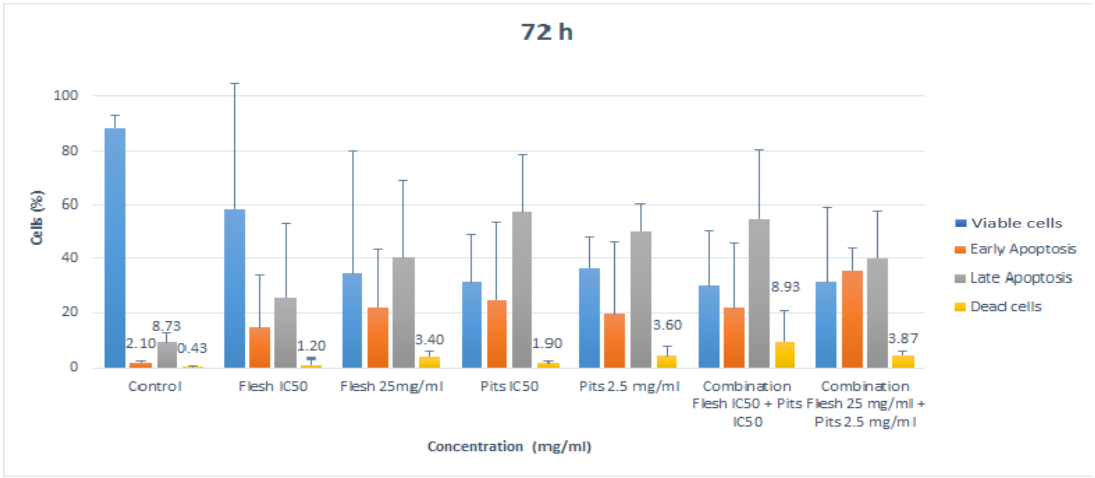


Figure 4.16 The effect of adding various concentration of Ajwa date flesh and pit extracts on the apoptotic activity of HSC-2 cell line at 72h. The percentage of cells are shown in four stages, healthy cells, cells in early apoptotic stage, cells in late apoptotic stage and dead or necrotic cells. The flesh IC_{50} value is 8.69 mg/ml and pits IC_{50} value is 0.97 mg/ml

Table 4.9 Analysis of viability and apoptotic activity of Ajwa date extract of flesh and pits at various concentrations at 24 hours. A p value of less than 0.05 is considered as statistically-significant. Data is expressed as percentage mean of three independent experiment

24 h				
	Concentration mg/ml	**Mean (%) ± SD**	**F *(df)***	***p***
Healthy Cells	**Control**	88.73 ± 4.43	3.85 (6,14)	0.018
	Flesh IC50	46.00 ±2 4.67		
	Flesh 25 mg/ml	55.50 ± 26.12		
	Pits IC50	6.93 ± 2.31		
	Pits 2.5 mg/ml	5.90 ± 1.36		
	Combination Flesh IC50 + Pits IC50	24.90 ± 10.26		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	16.63 ± 15.58		
Early Apoptotic Cells	**Control**	2.40 ± 0.87	4.68 (6,14)	0.008
	Flesh IC50	9.10 ± 4.80		
	Flesh 25 mg/ml	4.37 ± 1.40		
	Pits IC50	33.40 ± 4.48		
	Pits 2.5 mg/ml	26.57 ± 11.10		
	Combination Flesh IC50 + Pits IC50	39.20 ±7 .70		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	28.57 ± 10.23		
Late Apoptotic Cells	**Control**	8.10 ± 3.42	1.82 (6,14)	0.168
	Flesh IC50	43.67 ± 20.42		
	Flesh 25 mg/ml	35.33 ± 27.03		
	Pits IC50	56.67 ± 5.81		
	Pits 2.5 mg/ml	64.30 ± 9.36		
	Combination Flesh IC50 + Pits IC50	31.87 ± 9.72		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	60.40 ± 11.43		
Dead Cells	**Control**	0.77 ± 0.32	0.95 (6,14)	0.491
	Flesh IC50	1.23 ± 0.03		
	Flesh 25 mg/ml	4.97 ± 3.43		
	Pits IC50	2.97 ± 0.59		
	Pits 2.5 mg/ml	3.20 ± 1.10		
	Combination Flesh IC50 + Pits IC50	4.03 ± 2.09		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	4.37 ± 0.69		

Table 4.10 Analysis of viability and apoptotic activity of Ajwa date extract of flesh and pits at various concentrations at 48 hours. A p value of less than 0.05 is considered as statistically-significant. Data is expresses as percentage mean of three independent experiment

48 h				
	Concentrations mg/ml	**Mean (%) ± SD**	**F *(df)***	***p***
Healthy Cells	**Control**	87.50 ± 10.31	5.446 (6,14)	0.004
	Flesh IC50	74.17 ± 20.75		
	Flesh 25 mg/ml	31.87 ± 46.10		
	Pits IC50	13.83 ± 9.33		
	Pits 2.5 mg/ml	27.50 ± 19.75		
	Combination Flesh IC50 + Pits IC50	19.17 ± 16.00		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	15.03 ± 7.55		
Early Apoptotic Cells	**Control**	2.43 ± 3.46	1.626 (6,14)	0.212
	Flesh IC50	3.23 ± 1.30		
	Flesh 25 mg/ml	44.57 ± 36.68		
	Pits IC50	33.30 ± 37.58		
	Pits 2.5 mg/ml	25.07 ± 20.60		
	Combination Flesh IC50 + Pits IC50	33.77 ± 25.78		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	44.40 ± 12.69		
Late Apoptotic Cells	**Control**	9.77 ± 8.70	1.957 (6,14)	0.141
	Flesh IC50	21.30 ± 19.38		
	Flesh 25 mg/ml	22.57±13.06		
	Pits IC50	69.80 ± 25.60		
	Pits 2.5 mg/ml	43.77 ± 34.42		
	Combination Flesh IC50 + Pits IC50	45.10 ± 36.62		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	34.37 ± 20.80		
Dead Cells	**Control**	0.33 ± 0.21	0.738 (6,14)	0.628
	Flesh IC50	1.27 ± 1.16		
	Flesh 25 mg/ml	1.07 ± 1.17		
	Pits IC50	1.98 ± 2.48		
	Pits 2.5 mg/ml	2.44 ± 1.74		
	Combination Flesh IC50 + Pits IC50	2.03 ± 1.19		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	1.53 ± 0.92		

Table 4.11 Analysis of viability and apoptotic activity of Ajwa date extract of flesh and pits at various concentrations at 72 hours. A p value of less than 0.05 is considered as statistically-significant. Data is expresses as percentage mean of three independent experiment

72 h				
	Concentraations mg/ml	**Mean ± SD**	**F *(df)***	***p***
Healthy Cells	**Control**	88.73±3.92	1.721 (6,14)	0.189
	Flesh IC50	58.77±46.07		
	Flesh 25 mg/ml	34.27±45.66		
	Pits IC50	31.50±17.56		
	Pits 2.5 mg/ml	36.37±11.82		
	Combination Flesh IC50 + Pits IC50	30.17±20.40		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	31.63±27.42		
Early Apoptotic Cells	**Control**	2.10±0.44	0.712 (6,14)	0.646
	Flesh IC50	14.27±19.51		
	Flesh 25 mg/ml	21.97±21.33		
	Pits IC50	24.70±29.17		
	Pits 2.5 mg/ml	19.47±26.58		
	Combination Flesh IC50 + Pits IC50	21.67±24.31		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	35.47±8.41		
Late Apoptotic Cells	**Control**	8.73±3.43	2.102 (6,14)	0.118
	Flesh IC50	25.73±27.58		
	Flesh 25 mg/ml	40.40±28.76		
	Pits IC50	57.43±21.53		
	Pits 2.5 mg/ml	50.30±10.45		
	Combination Flesh IC50 + Pits IC50	54.90±25.90		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	40.00±13.23		
Dead Cells	**Control**	0.43±0.25	1.421 (6,14)	0.274
	Flesh IC50	1.20±1.73		
	Flesh 25 mg/ml	3.40±1.90		
	Pits IC50	1.90±0.78		
	Pits 2.5 mg/ml	3.60±3.82		
	Combination Flesh IC50 + Pits IC50	1.17±2.02		
	Combination Flesh 25 mg/ml + Pits 2.5 mg/ml	3.87±1.50		

CHAPTER 5

DISCUSSION

5.1 Impact of Extraction Solvents on Extractable Solids

The results from the present study showed that different solvents had significant effects on the extractable solids yield of Ajwa date (flesh and pits) shown in figure 4.1. 70% aq. acetone had the highest extractable solids for flesh and pits (32% and 7.7%), followed by 70% aq. ethanol (10% and 3.18%) and the least amount with n-hexane (1.9% and 2.5%) respectively. The results demonstrated that total extractable yields from the Ajwa date could be significantly affected by the solvent used for extraction. The solvent used in the extraction procedure has significant effect on the yield and phytochemical composition of the extracted plant. It is necessary the that solvent should be non-toxic and easily evaporable at low heat, since the end product will have traces of residual solvent, so it shouldn't be toxic, or dissociate the bioactive compound or interfere with the bioassays. Phytochemicals present in plants have a wide range of chemical characteristic and different polarities; therefore, it may not be soluble in one particular solvent (Do *et al.*, 2014). Polyphenols are best extracted with a more polar solvent from plant material hence increasing the total yield. Aqueous mixtures of ethanol, methanol, acetone and ethyl acetate are most suitable solvent for maximum extraction yield. Aqueous acetone is a suitable solvent for the extraction of flavanols, whereas ethanol and methanol is used for the extraction of polyphenols, ethanol is preferred even though methanol is more efficient solvent but is toxic to living cells (Dai and Mumper, 2010).The results of the present study are also in agreement with previous studies where 70% acetone was the best solvent to extract

highest amount of phenolic compounds from *Phoenix dactylifera L.* (Kchaou *et al.*, 2013) and *Ocimum basilicum* L. (Złotek *et al.*, 2016).

For the extraction of important bioactive compounds from the plants, the extraction of antioxidant compounds from the date fruit are influenced by their solubility in the extracting solvent and the solvents polarity as the solvents diffuse into the solid plant and solubilize the compounds with similar polarities. (Kchaou *et al.*, 2013). Therefore, it is very crucial to select a single best solvent for the extraction of all polyphenolic compounds from the sample. From the literature it was observed that mixtures of acetone and water were most effective for total phenolic extraction from a plant (González-Montelongo *et al.*, 2010), which was also in agreement with (Al-Farsi *et al.*, 2005) who used it for three different varieties of date fruit. On the contrary, in the work conducted by Bazylina et al. in 2002, ethanolic extract of plant material has the strongest antioxidant potential than other solvents (Bazykina *et al.*, 2002). Whereas phenolic compounds from plants and fruits plays an important role making up the biological properties including the antioxidant activity of plant (Pandey and Rizvi, 2009) and a positive correlation can be observed between the amount of phenolic content present and the total antioxidant activity observed (Kchaou *et al.*, 2013).

5.2 DPPH assay

In the present study, the antioxidant activity of ADF and ADP extracts with acetone and ethanol solvents were determined. The results demonstrated that when acetone was used as a solvent, higher antioxidant activity was obtained in both flesh and pits compared to the values obtained with ethanol at the same solvent concentrations (70%). These results are in line with a previous study conducted by

Nematallah *et al.* in 2018 where 50% aq. acetone yielded the highest antioxidant activity in Ajwa date, followed by ethanolic extract. The variations in the values can be due to the difference in extraction procedure (Nematallah *et al.*, 2018).In contrast to the present results, Gibson *et al.* reported that 75% aq. ethanol extract of ADF had the highest antioxidant activity, the difference in results may be due to the use of fresh dates or different extraction conditions (Gibson *et al.*, 2015). He also reported that Ajwa date flesh had the highest antioxidant activity, phenolic and flavonoid percentage among 4 other varieties.

Another important finding observed from the DPPH assay was that the pit extract exhibited higher antioxidant activity and reducing power with both the solvents when compared with the flesh part. These results are in support with a study reported by Maqsood *et al.* where the concentration of acetone and ethanol between 60 to 80% yielded the highest DPPH free radical scavenging activity from a date pit (Maqsood *et al.*, 2015). But unlike the present study he used Trolox as a standard. However, acetone extract of ADP had significantly higher antioxidant activity when compared with the ethanolic extract of ADP. Therefore, it can be concluded that pit appeared to be richer source for phenols and flavonoids as the extracts from the pits were high in DPPH assay activity compared to the flesh extract. In similarity to our study, Ajwa pit demonstrated higher antioxidant activity than its flesh part when Khalid *et al.* compared the free radical scavenging activity of Ajwa date flesh and pit (Khalid *et al.*, 2016). Based on a previous study (Habib *et al.*, 2014), date pits are the highest source of total polyphenols among tea, flaxseed, nut seeds, grapes and even date flesh (Soong and Barlow, 2004).

5.3 Determination of cytotoxic effects of Ajwa date (flesh and pit) on OSCC Cell line

The present study investigated the cytotoxic and pro apoptotic effect induced by ADF and ADP extracts on HSC-2 cells. It also demonstrated that ADF and ADP mediated a dose and time-dependent anti-proliferative activity against HSC-2 cells. A previous study has reported that the date flesh also contains polyphenols like quercetin and kaempferol (Abdul-Hamid *et al.*, 2018), which possess anticancer activity against oral squamous cell carcinoma (Lin *et al.*, 2013; Zhang *et al.*, 2019). Therefore, it can be predicated that anticancer effect of Ajwa date extract against HSC-2 cells might be due to the integrated or collective effect of the potential bioactive components of Ajwa dates. The anti-cancer activity of these bioactive compounds can be mediated by several molecular mechanisms including free radical scavenging activity, deactivation of carcinogenic metabolites, antiproliferation, induction of apoptosis and cell cycle arrest (Ren *et al.*, 2003). Previously, an extract from the roots of a Chinese herbal plant, *Scutellaria baicalensis* have been evaluated for its anticancer activity against HSC-2 cell line. The root extract demonstrated significant inhibition of cell growth and increased apoptotic activity on human squamous carcinoma cell line. (Sato *et al.*, 2013).

5.3.1 Effect on morphology of HSC-2 cells

The morphological data of the present study revealed severe Ajwa date extract mediated variations. As shown in Figure 4.9 that cells treated with ADF and ADP extracts showed cell shrinkage, non-adherence, partial detachment and rounded morphology, with a decrease in cell number was also seen. As the concentration of ADF and ADP extracts increased, more drastic morphological changes with typical apoptotic features appeared, exhibiting a dose-dependent severity. Cells treated with

combination treatment of ADF and ADP extracts showed cluster shrinkage, nuclear condensation, extensive cytoplasmic vacuolization, membrane blebbing, and detachment from the surface leaving very few attached cells. Cellular fragmentations can also be seen the figure 4.9-g. In contrast the control group exhibited normal features such as regularity in shape, cell-to-cell adhesion, homogeneous and monolayer with prolific growth and mitotic bodies. Interestingly, the present study demonstrated initial characteristic features of apoptotic cell death (Taatjes *et al.*, 2008). A previous study which explored the anticancer effect of Ajwa date on human breast adenocarcinoma (MCF7) cells reported similar apoptotic features and MCF7 cell death. Apoptosis or initiation of apoptotic pathways have been induced by bioactive components and secondary metabolites of natural products (Thomasset *et al.*, 2007).

5.3.2 MTT assay

The data from MTT assay showed that the Ajwa date flesh extract induced a significant decrease in the percentage of cell viability of HSC-2 cells. Interestingly, at a concentration of 0.8 mg/ml of ADF extract there was an increase in cell viability percentage of 117.9 and 111.4 % at 24 and 48 h of incubation period, respectively when compared to the untreated control group. This could be explained by a phenomenon known as hormesis, where some cells might try to adjust to the toxic environment at very low dose resulting in a higher MTT signal compared to the control (Stebbing, 1982). The results from the present study shows that at 24 h treatment period, ADF extract reduced cells viability to 99, 90.3, 76.5, 36.5, 22.4, and 15.6% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml, respectively of the extract concentration. This indicated as the concentration of the ADF extract increases from 1.5 to 50 mg/ml, the percentage of viable cells decreased from 99% to 15.6%. Negative corelation can be seen between the concentration of ADF extract and viable cells of OSCC.

At 48 h treatment period, ADF extract exerted a more pronounced effect, drastically reducing the viability of treated cells to 98.5, 88.6, 65.6, 29, 14.8 and 5.9% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml of extract respectively. Moreover, a further decrease in cells viability percentage was seen at 72 h with a percentage of 66.5, 61.1 46.3, 19.4, 7.3 and 2.9 % at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml of ADF extract respectively indicating as the concentration increases the cell viability decreases, stipulating that ADF extract is more toxic at higher concentrations. From the above results at 50 mg/ml concentration the cell viability decreased to 15.6% at 24h, 5.9% at 48h and 2.9% at 72h indicating that as the time increased the cell viability decreased at the same concentration. Therefore, the cell viability data proposed that treatment with ADF extract significantly reduced HSC-2 cells growth in both dose- and time-dependent manner, signifying its ability to impair proliferation potential. These results are in line with several previously reported studies. Kchaou *et al.* have, for instance reported on the inhibitory potential of *Phoenix dactylifera L.* from Tunisian cultivars. The variation in the inhibition percentage of cytotoxicity could be due to the difference in the variety as well as origin of the dates (Kchaou *et al.*, 2016).

Ajwa date pit extract showed similar but enhanced results compared to flesh part with significant cytotoxic effect on the viability on HSC-2 cells. At 24 h treatment incubation time, ADP extract reduced cell viability to 82.2, 73.7, 54.9, 40.7, 22.3, 15.1 and 6.6% at 0.08, 0.3, 10.6, 31.1, 51.2, 2.5 and 5.00 mg/ml concentration of ADP extract respectively. At 48 h treatment period, ADP extract reduced cells viability 79.3, 70.1, 46.5, 25.3, 14.2, 10.6 and 3.7% at 0.08, 0.3, 10.6, 31.1, 51.2, 2.5 and 5.00 mg/ml concentration of ADP extract respectively. Thus, the data demonstrated cells growth inhibition in a dose- and time-dependent manner. The increased cytotoxicity can be mainly attributed to the presence of high amount of phenolics, flavonoids and vitamin

C in the seeds compared to the flesh part (Habib *et al.*, 2014). The date seeds contain high amount of total polyphenols, close to 3942mg/100 g, whereas date flesh contains 239.5 mg/100g wet weight. (Al-Farsi and Lee, 2008).

5.3.2(a) Determination of IC_{50} value of ADF and ADP extracts on HSC-2 Cells

IC_{50} value is the determination of the extract/drug needed to inhibit 50% of the cells. It is estimated to comprehend the basic pharmacological and biological characteristics, the lower the IC_{50} value, the more potent the drug is. In the present study, IC_{50} value of ADF and ADP extracts were determined using MTT assay(He *et al.*, 2016). Previously, ADF extracts have been evaluated for their anticancer activity against several cell lines. In a study, the IC_{50} value of ADF extract against human hepatocellular carcinoma (HepG2) cells were 20.03 mg/ml after 24 hours exposure (Siddiqui *et al.*, 2019). In comparison to the present study the IC_{50} value of 8.96 mg/ml after 24 hours treatment period indicates that ADF extract was more effective against HSC-2 cells. In another study, the IC_{50} value of ADF extract against the prostate cancer cells was calculated to be 0.388 mg/ml for 24 hours (Mirza *et al.*, 2018). The variation in the IC_{50} value could be due to many reasons. One factor could be the use of different cell lines, as in a study conducted by (Elias *et al.*, 2016) the extract of *Erythroxylum daphnites* (EDH) leaves had an IC_{50} value of 448.9 µg/ml on OSCC cells and IC_{50} value of 737.1µg/ml on keratinocyte cells indicating that EDH was more selective for OSCC cells. Other factors could be the difference in proliferation rate of the cells or the cell density during the assay period (Niepel *et al.*, 2017).

On the contrary, the 50% inhibition of HSC-2 cells by pit extract with an extremely low IC_{50} in the present study confirmed the anticancer property of ADP extract. In a similar study conducted by Amira and colleagues in 2018 showed that date pit extract of two different varieties induced significant growth inhibition and

apoptosis in a human cervical cell line (HeLa) and human liver cell line (HepG2) with an IC_{50} value of 0.028 mg/ml and 0.034 mg/ml, respectively. They also reported that the seed extract had no cytotoxic effects on normal fibroblast cell line (Thouri *et al.*, 2019). Interestingly polyphenols were distinguished by their low cytotoxic effects towards normal cell line and increased cytotoxicity towards cancer cell line (Park *et al.*, 2005).

Another interesting study was conducted by Dalia M. and colleagues in 2014 where a beverage was made out of roasted dates pits and was assessed for its nutritional composition and cytotoxicity against human colon cancer cell line (HCT116) and HepG2 cells. They too reported the dose-dependent inhibition of cell proliferation in both cell lines. Unlike the present study, they determined the cytotoxic level by Sulforhodamine B assay. Their result stated that the cell viability percentage decreased to 53.6 and 79.9% in HCT116 and HepG2 cell lines respectively at a concentration of 100µg/ml (El Sheikh *et al.*, 2014). These results indicated that date pit extract was more sensitive against colon cell line compared to liver cell line. In similarity with the present study, these outcomes commented on the capability of date pits to inhibit the growth of cancer cells effectively because of the presence of high phenolic compounds (Yang *et al.*, 2008).

The mode of action of Ajwa date (flesh and pit) extract on HSC-2 cells is not fully understood. Nonetheless, a likely route of action for the extract could be via modulating oxidative stress and scavenging free radicals within the cells. Various stimuli including reactive oxygen species are known to activate HSC-2 cells (Razzaghi-Asl *et al.*, 2013).

5.4 Apoptosis assay

In order to discover whether the cytotoxic effect noted was due to the apoptosis induction, a flow cytometry analysis of Annexin-V/PI double stain was performed. The result indicated that the percentage of viable cells reduced with a simultaneous increase in the percentage of cells undergoing early apoptosis and late apoptosis. With a lower concentration of both ADF and ADP extracts HSC-2 cells were observed more in early apoptosis stage while late apoptosis stage was identified at a higher concentration of extracts. In figure 4.14, the data also showed that increased percentage of early apoptotic cells were present at 24 h duration of treatment, and as the duration of treatment increased, cells appeared to be more in late apoptotic stages indicating a time-dependent manner. This quantitative data suggests that the death of HSC-2 cells occurred due to the induction of apoptosis. The apoptotic activity exhibited by the ADF and ADP extracts may be attributed to the presence of phenols and flavonoids. According to Saleh *et al.* and Habib *et al.*, Ajwa date flesh and pit consist of high flavonoid and phenolic content like rutin, catechin, caffeic acid, apigenin, and quercetin (Habib *et al.*, 2014; Saleh *et al.*, 2011). Previously literature has shown that these bioactive components play a major role in the prevention of various cancers as well as inhibition of metastasis (Gee *et al.*, 2002).

A previous study has reported the induction of apoptosis in breast cancer MCF-7 cells by the methanolic extract of Ajwa date flesh (Khan *et al.*, 2016). In which it was reported that the percentage of total apoptotic cells were 68.1% at 25mg/ml at 48 h of treatment duration, which correlates with the findings of present study where the total apoptotic cells at 48 h were 67.1% at 25 mg/ml of ADF extract. With the loss of cell membrane asymmetry, the phosphatidylserine (PS) flips towards the outside,

which is considered to be a hallmark of cell in later stages of apoptosis (Saraste and Pulkki, 2000).

Siddiqui *et al.* indicated similar results to the present study when he investigated the role of Ajwa date flesh extract in apoptosis in HepG2 cells. After treating the HepG2 cells with 15mg/ml and 25mg/ml of ADP extract for 48 h, he reported that the percentage of early apoptotic cells was increased from 8.8% to 32.8 % and the percentage of late apoptotic cells increased from 10.5% to 46.7% pointing at the dose dependent increase in early and late apoptotic cells. His results also demonstrated that the number of late apoptotic cells were higher than the early apoptotic cells, which shows similarity to our study at 24 h and 72 h of treatment time, whereas at 48 h in our study the percentage of early apoptotic cells are more than the late apoptotic cells. Furthermore he also reported the overproduction of intracellular reactive oxygen species (ROS) generation in ADF extract treated cells, this evaluation supports the apoptotic mechanism of cell death (Siddiqui *et al.*, 2019). Both intrinsic and extrinsic pathways of cell survival and apoptosis can be regulated by ROS (Davalli *et al.*, 2016). Increased generation of ROS can break the plasma membrane and the cytoskeleton finally leading to chromosomal damage (Guo *et al.*, 2013). The conclusion by this study confirmed that the cell death induced by ADF extract was due to apoptosis.

In addition, annexin V-FITC co-staining with PI assay demonstrated increase in positive cells following treatment with ADP extract. These results support the drastic morphological changes with ADP extract treatment when compared to ADF extract treatment, further pointing towards the increased apoptosis of HSC-2 cells by ADP extract. In contrast to the ADF extract, 6.93 % of viable cells were present with 0.97 mg/ml and 5.9 % with the 2.5mg/ml whereas 0.97mg/ml ADP extract increased

the death by inducing 33.4 % early apoptotic cells and 56.6 % late apoptotic cells at 24 h treatment. These findings indicate a significantly increased induction of apoptosis in ADP extract treated cells. The result from the apoptosis assay supports the results of MTT assay, in which there was significant cell death after the treatment of Ajwa date extract, apoptosis assay elaborates that the cell went through early and late stages of apoptosis before dying, thus confirming that Ajwa date induced apoptotic cell death in oral cancer cells.

In a similar study, the potential of date seed extract was assessed for the induction of apoptosis in HepG2 and HeLa cells. Unlike the present study, western blot analysis was used to investigate the induction of apoptosis by intrinsic pathway. The results demonstrated a decreased level of procaspase-9 and an increase level of cleaved caspase-9, it also showed the decreased level of procaspase-3 and its downstream target poly (ADP-ribose) polymerase (PARP) which confirmed the capacity of date seed to induce apoptosis (Thouri *et al.*, 2019).

In an *in-vivo* scenario, a compelling study conducted by Diab *et al.* in 2012 investigated the possible antigenotoxicity of date seed extract in mice induced with N-Nitroso-N-methylurea (NMU). NMU is a potent carcinogenic and mutagenic agent which was used to damage DNA in mice. The results demonstrated that date pit extract (DPE) significantly reduced the chromosomal aberration, micronuclei and inhibited the hepatic DNA fragmentation, thus restoring the DNA damage induced by NMU (Diab and Aboul-Ela, 2012). All these findings justified the excellent anti-cancerous nature of date seeds, and in our case ADP extract signified its pro-apoptotic nature against OSCC cells.

The present study also investigated the combined effect of ADF and ADP extracts for the induction of apoptosis in HSC-2 cells. At 24 h the percentage of early

apoptotic cells with the combination treatment was 39.2 % whereas when treated separately it was 9.10% and 33.4% for ADF and ADP extract respectively. Although it was not significantly higher than the single treatment but this may suggest that Ajwa date can induce higher level of apoptotic effect when used as whole. Previously many studies have evaluated that the bioactive phenolic compounds of fruit seed more than the fruit flesh. Similar to the present study, grape seed extract had induced apoptotic cell death in OSCC (Aghbali *et al.*, 2013). Not only the seed, but the peel (skin) and the edible part of grape have demonstrated apoptotic activity (Grace Nirmala *et al.*, 2018; Singha and Das, 2015). Many other fruits have demonstrated higher polyphenol content in their seed than the edible flesh (Soong and Barlow, 2004). Therefore, it can be suggested that the fruit as a whole can be more beneficial in providing protection against carcinogenic effects and the seed part can be utilized in many different forms, like recently date pit powder was used to make non-caffeinated coffee with coffee flavour (Baliga *et al.*, 2011).

CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATIONS

Based on the results of the study, the following summary of the key findings was drawn:

- Aqueous acetone (70%) was the most efficient solvent to obtain extract with highest antioxidant activity, while ethanol was inefficient solvent for the yield of antioxidant activity as measured with DPPH assay.
- Total antioxidant activity of Ajwa date pit extract was higher than the flesh extract, therefore it can be a potentially strong candidate as a functional food in human diet.
- Ajwa date (flesh and pit) demonstrated significant cytotoxic and antiproliferative activity when tested *in vitro* utilizing HSC-2 cell line through MTT cell viability assay. The cytotoxic effect was observed in a dose- and time-dependent manner
- The IC_{50} value obtained by MTT assay demonstrated that Ajwa pit had a stronger anti-proliferative effect than the flesh extract.
- Ajwa date (flesh and pit) extract was found to causes cell death via apoptosis in OSCC cells by flow cytometry assay. The percentage of early and late apoptotic cells also increased with increase in concentration and duration of treatment suggesting that apoptotic effect was also dose- and time dependent.
- Ajwa date pit extract showed stronger pro-apoptotic effects than the flesh extract.
- The combination treatment with date flesh and pit extracts together demonstrated increased percentage of early and late apoptotic cells, suggesting Ajwa date as a whole can induce apoptosis more effectively than as separately.

6.1 Limitations of the study

This is an *in vitro* study describing the potential role of Ajwa date flesh and pit on OSCC cell line. Since most of the work was laboratory-based including handling of cells and extraction procedure, as a dentist with no previous laboratory-based research experience, it took a lot of time and practice to learn the aseptic techniques of handling cells and performing the related assays. There are some limitations to the extraction method used in this study. The antioxidant assay was performed only on two solvents i.e., acetone and ethanol and could not be performed on the hexane extract as there was not enough extract residue left after the evaporation of solvent that a working solution with various concentration could be made to run for the assay. Secondly, although the antioxidant activity was measured, but the chemical profile of the extract is lacking and therefore the types of bioactive compounds in each extract could not be identified which could have given a deeper insight in the cytotoxic role of Ajwa date extract.

6.2 Future Recommendations

As research is still on going on the therapeutic and medicinal properties of Ajwa date, there are still many areas unexplored.

- Further studies can be conducted to purify and identify individual components of the Ajwa date flesh and seed that are responsible for the anti-cancer properties, through which novel chemotherapeutic drug can be made with less/no conventional side effects.
- Since the n-Hexane extract was insufficient for performing DPPH assay, different concentrations, and larger samples could be used with different durations and timings to overcome this limitation.

- A wider range of solvents with different concentrations, or different extraction protocols can be used to extract the bioactive components from Ajwa dates.
- Further *in vivo* studies can be conducted to better understand the molecular mechanism involved in apoptosis and the optimal dosage needed to produce significant reduction in various kind of cancers.
- This study can be extended on to the leaves, stems and roots of the plant to investigate if it has same anticancerous effects or not.
- Since it was proven that Ajwa date induced apoptosis in cancer cells, clinical trials can be conducted in cancer patients where Ajwa date (flesh and pit) can be added as a daily nutritional supplement in combination with the conventional chemotherapy treatment to evaluate the synergistic effects.
- *In vivo* and later clinical trials can be carried out with an ointment made from Ajwa date (whole) to use in pre-cancerous and cancerous lesions in oral cavity to evaluate the reduction in the lesion.
- Ajwa date fruit (whole) can be formulated in a capsule form for better bioavailability to be taken as supplements in our daily routine for chemo-preventive purpose against oral cancer and other malignancies.
- Since Ajwa pit can be a potential source of antioxidants, it can replace the synthetic antioxidant compounds in commercial food industries.

REFERENCES

Abdul-Hamid, N. A., Mediani, A., Maulidiani, M., Shadid, K., Ismail, I. S., Abas, F. & Lajis, N. H. (2018). Metabolite characterization of different palm date varieties and the correlation with their NO inhibitory activity, texture and sweetness. *Journal of food science and technology*, **55(4)**, 1541-1551.

Abdul-Hamid, N. A., Abas, F., Ismail, I. S., Shaari, K. & Lajis, N. H. (2015). Influence of different drying treatments and extraction solvents on the metabolite profile and nitric oxide inhibitory activity of Ajwa dates. *Journal of food science*, **80(11)**, H2603-H2611.

Adeosun, A. M., Oni, S. O., Ighodaro, O. M., Durosinlorun, O. H. & Oyedele, O. M. (2016). Phytochemical, minerals and free radical scavenging profiles of Phoenix dactilyfera L. seed extract. *Journal of Taibah University Medical Sciences*, **11(1)**, 1-6.

Aggarwal, B. B., Bhatt, I. D., Ichikawa, H., Ahn, K. S., Sethi, G., Sandur, S. K., Natarajan, C., Seeram, N. & Shishodia, S. (2006). 10 Curcumin—Biological and Medicinal Properties.

Aghbali, A., Hosseini, S. V., Delazar, A., Gharavi, N. K., Shahneh, F. Z., Orangi, M., Bandehagh, A. & Baradaran, B. (2013). Induction of apoptosis by grape seed extract (Vitis vinifera) in oral squamous cell carcinoma. *Bosnian journal of basic medical sciences*, **13(3)**, 186-191. doi: 10.17305/bjbms.2013.2360

Ahmed, A., Arshad, M. U., Saeed, F., Ahmed, R. S. & Chatha, S. A. S. (2016). Nutritional probing and HPLC profiling of roasted date pit powder. *Pakistan Journal of Nutrition*, **15(3)**, 229.

Al-Bukhari, S. (2008). Sahihalbukhari, Hadith Nos. 5396 and 5403. *http: www. sahihal bukhari. com/sps/sbk/last accessed on*, **19(09)**, 2008.

Al-Farsi, M., Alasalvar, C., Morris, A., Baron, M. & Shahidi, F. (2005). Comparison of antioxidant activity, anthocyanins, carotenoids, and phenolics of three native fresh and sun-dried date (Phoenix dactylifera L.) varieties grown in Oman. *J Agric Food Chem*, **53(19)**, 7592-7599. doi: 10.1021/jf050579q

Al-Farsi, M. A. & Lee, C. Y. (2008). Nutritional and functional properties of dates: a review. *Critical reviews in food science and nutrition*, **48(10)**, 877-887.

Al-Hashimi, I., Schifter, M., Lockhart, P. B., Wray, D., Brennan, M., Migliorati, C. A., Axéll, T., Bruce, A. J., Carpenter, W. & Eisenberg, E. (2007). Oral lichen planus and oral lichenoid lesions: diagnostic and therapeutic considerations. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, **103**, S25. e21-S25. e12.

Al-Khayri, J. M., Jain, S. M. & Johnson, D. V. (2015). *Date palm genetic resources and utilization*: Springer.

Al-Qarawi, A., Ali, B., Al-Mougy, S. & Mousa, H. (2003). Gastrointestinal transit in mice treated with various extracts of date (Phoenix dactylifera L.). *Food and Chemical Toxicology*, **41(1)**, 37-39.

Al-Radadi, N. S. (2019). Green synthesis of platinum nanoparticles using Saudi's Dates extract and their usage on the cancer cell treatment. *Arabian journal of chemistry*, **12(3)**, 330-349.

Al-Shahib, W. & Marshall, R. J. (2003). The fruit of the date palm: its possible use as the best food for the future? *International journal of food sciences and nutrition*, **54(4)**, 247-259.

Al-Yahya, M., Raish, M., AlSaid, M. S., Ahmad, A., Mothana, R. A., Al-Sohaibani, M., Al-Dosari, M. S., Parvez, M. K. & Rafatullah, S. (2016). 'Ajwa'dates (Phoenix dactylifera L.) extract ameliorates isoproterenol-induced cardiomyopathy through downregulation of oxidative, inflammatory and apoptotic molecules in rodent model. *Phytomedicine*, **23(11)**, 1240-1248.

Al-Zubaidy, N., Al-Zubaidy, A. & Sahib, H. (2016). The anti-proliferative activity of phoenix dactylifera seed extract on MCF-7 breast cancer cell line. *Int J Pharm Sci Rev Res*, **41(2)**, 358-362.

Ali, H. S. M., Alhaj, O. A., Al-Khalifa, A. S. & Brückner, H. (2014). Determination and stereochemistry of proteinogenic and non-proteinogenic amino acids in Saudi Arabian date fruits. *Amino acids*, **46(9)**, 2241-2257.

Aluyen, J. K., Ton, Q. N., Tran, T., Yang, A. E., Gottlieb, H. B. & Bellanger, R. A. (2012). Resveratrol: potential as anticancer agent. *Journal of dietary supplements*, **9(1)**, 45-56.

Assirey, E. A. R. (2015). Nutritional composition of fruit of 10 date palm (Phoenix dactylifera L.) cultivars grown in Saudi Arabia. *Journal of Taibah University for science*, **9(1)**, 75-79.

Bakr Abdu, S. (2011). THE PROTECTIVE ROLE OF AJWA DATE AGAINST THE HEPATOTOXICITY INDUCED By OCHRATOXIN A. *Egyptian Journal of Natural Toxins*, **8**.

Baliga, M. S., Baliga, B. R. V., Kandathil, S. M., Bhat, H. P. & Vayalil, P. K. (2011). A review of the chemistry and pharmacology of the date fruits (Phoenix dactylifera L.). *Food research international*, **44(7)**, 1812-1822.

Bammou, M., Sellam, K., Benlyas, M., Alem, C. & Filali-Zegzouti, Y. (2016). Evaluation of antioxidant, antihemolytic and antibacterial potential of six Moroccan date fruit (Phoenix dactylifera L.) varieties. *Journal of King Saud University-Science*, **28(2)**, 136-142.

Barreveld, W. H. (1993). Date Plam Products.Bulletin No 101. *Food and Agriculture Organization of the United Nations Rome,Italy*.

Bauer, J. H. & Helfand, S. L. (2006). New tricks of an old molecule: lifespan regulation by p53. *Aging cell*, **5(5)**, 437-440.

Baur, J. A. & Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nature reviews Drug discovery*, **5(6)**, 493-506.

Bazykina, N., Nikolaevskii, A., Filippenko, T. & Kaloerova, V. (2002). Optimization of conditions for the extraction of natural antioxidants from raw plant materials. *Pharmaceutical Chemistry Journal*, **36(2)**, 46-49.

Biesalski, H. K. (2007). Polyphenols and inflammation: basic interactions. *Current Opinion in Clinical Nutrition & Metabolic Care*, **10(6)**, 724-728.

Bladé, C., Aragonès, G., Arola-Arnal, A., Muguerza, B., Bravo, F. I., Salvadó, M. J., Arola, L. & Suárez, M. (2016). Proanthocyanidins in health and disease. *Biofactors*, **42(1)**, 5-12.

Bokhari, N. A. & Perveen, K. (2012). In vitro inhibition potential of Phoenix dactylifera L. extracts on the growth of pathogenic fungi. *Journal of Medicinal Plants Research*, **6(6)**, 1083-1088.

Brand-Williams, W., Cuvelier, M.-E. & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food science and Technology*, **28(1)**, 25-30.

Brandizzi, D., Gandolfo, M., Velazco, M. L., Cabrini, R. L. & Lanfranchi, H. E. (2008). Clinical features and evolution of oral cancer: A study of 274 cases in Buenos Aires, Argentina. *Age (in years)*, **40(3)**, 9.

Brown, L., Rosner, B., Willett, W. W. & Sacks, F. M. (1999). Cholesterol-lowering effects of dietary fiber: a meta-analysis. *The American Journal of Clinical Nutrition*, **69(1)**, 30-42. doi: 10.1093/ajcn/69.1.30

Cai, Y., Zhao, L., Qin, Y., Zhang, M. & He, Y. (2015). Resveratrol inhibits proliferation and induces apoptosis of nasopharyngeal carcinoma cell line C666-1 through AMPK activation. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, **70(6)**, 399-403.

Chaillou, L. L. & Nazareno, M. A. (2006). New method to determine antioxidant activity of polyphenols. *Journal of Agricultural and Food Chemistry*, **54(22)**, 8397-8402.

Chaira, N., Smaali, M. I., Martinez-Tomé, M., Mrabet, A., Murcia, M. A. & Ferchichi, A. (2009). Simple phenolic composition, flavonoid contents and antioxidant capacities in water-methanol extracts of Tunisian common date cultivars (Phoenix dactylifera L.). *International journal of food sciences and nutrition*, **60(sup7)**, 316-329.

Chan, G. C.-F., Chan, W. K. & Sze, D. M.-Y. (2009). The effects of β-glucan on human immune and cancer cells. *Journal of hematology & oncology*, **2(1)**, 25.

Chan, K. W., Khong, N. M., Iqbal, S. & Ismail, M. (2013). Isolation and antioxidative properties of phenolics-saponins rich fraction from defatted rice bran. *Journal of Cereal Science*, **57(3)**, 480-485.

Chen, C., Zimmermann, M., Tinhofer, I., Kaufmann, A. M. & Albers, A. E. (2013). Epithelial-to-mesenchymal transition and cancer stem (-like) cells in head and neck squamous cell carcinoma. *Cancer letters*, **338(1)**, 47-56.

Conway, D. I., Petticrew, M., Marlborough, H., Berthiller, J., Hashibe, M. & Macpherson, L. M. (2008). Socioeconomic inequalities and oral cancer risk: a systematic review and meta-analysis of case-control studies. *International journal of cancer*, **122(12)**, 2811-2819.

Cooper, G. (2000). *The Cell: A Molecular Approach*. Sunderland: Sinauer Associates.

Copper, M. P., Jovanovic, A., Nauta, J. J., Braakhuis, B. J., de Vries, N., van der Waal, I. & Snow, G. B. (1995). Role of genetic factors in the etiology of squamous

cell carcinoma of the head and neck. *Archives of Otolaryngology–Head & Neck Surgery*, **121(2)**, 157-160.

Cragg, G. M., Grothaus, P. G. & Newman, D. J. (2009). Impact of natural products on developing new anti-cancer agents. *Chemical reviews,* **109(7)**, 3012-3043.

Cuffari, L., de Siqueira, J. T. T., Nemr, K. & Rapaport, A. (2006). Pain complaint as the first symptom of oral cancer: a descriptive study. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology,* **102(1)**, 56-61.

D'Souza, G., Kreimer, A. R., Viscidi, R., Pawlita, M., Fakhry, C., Koch, W. M., Westra, W. H. & Gillison, M. L. (2007). Case–control study of human papillomavirus and oropharyngeal cancer. *New England Journal of Medicine,* **356(19)**, 1944-1956.

Dai, J. & Mumper, R. J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules,* **15(10)**, 7313-7352.

Dammak, I., Boudaya, S., Abdallah, F. B., Hamida, T. & Attia, H. (2009). Date seed oil inhibits hydrogen peroxide-induced oxidative stress in normal human epidermal melanocytes. *Connective tissue research*, **50(5)**, 330-335.

Davalli, P., Mitic, T., Caporali, A., Lauriola, A. & D'Arca, D. (2016). ROS, cell senescence, and novel molecular mechanisms in aging and age-related diseases. *Oxidative medicine and cellular longevity,* **2016**.

Denissenko, M. F., Pao, A., Tang, M.-s. & Pfeifer, G. P. (1996). Preferential formation of benzo [a] pyrene adducts at lung cancer mutational hotspots in P53. *Science,* **274(5286)**, 430-432.

Dhandapani, K. M., Mahesh, V. B. & Brann, D. W. (2007). Curcumin suppresses growth and chemoresistance of human glioblastoma cells via AP-1 and NFκB transcription factors. *Journal of neurochemistry*, **102(2)**, 522-538.

Diab, K. & Aboul-Ela, E. (2012). In vivo comparative studies on antigenotoxicity of date palm (Phoenix dactylifera l.) pits extract against DNA damage induced by N-Nitroso-N-methylurea in mice. *Toxicology international,* **19(3)**, 279.

Ding, Y., Ding, C., Ye, N., Liu, Z., Wold, E. A., Chen, H., Wild, C., Shen, Q. & Zhou, J. (2016). Discovery and development of natural product oridonin-inspired anticancer agents. *European journal of medicinal chemistry*, **122**, 102-117.

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S. & Ju, Y.-H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of food and drug analysis,* **22(3)**, 296-302.

Eid, N., Enani, S., Walton, G., Corona, G., Costabile, A., Gibson, G., Rowland, I. & Spencer, J. P. (2014). The impact of date palm fruits and their component polyphenols, on gut microbial ecology, bacterial metabolites and colon cancer cell proliferation. *J Nutr Sci,* **3**, e46. doi: 10.1017/jns.2014.16

Eid, N. M., Al-Awadi, B., Vauzour, D., Oruna-Concha, M. J. & Spencer, J. P. (2013). Effect of cultivar type and ripening on the polyphenol content of date palm fruit. *Journal of agricultural and food chemistry,* **61(10)**, 2453-2460.

El-Far, A., Shaheen, H., Abdel-Daim, M., Al Jaouni, S. & Mousa, S. (2016). Date palm (Phoenix dactylifera): protection and remedy food. *Curr Trends Nutraceuticals,* **1**, 2.

El-Gazzar, U. & El-Far, A. (2009). The ameliorative effect of Phoenix dactylifera extract on CCl4 hepatotoxicity in New Zealand rabbits. *Journal of Applied Sciences Research***(September)**, 1082-1087.

El-Mahdy, M. A., Zhu, Q., Wang, Q. E., Wani, G. & Wani, A. A. (2005). Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells. *International journal of cancer,* **117(3)**, 409-417.

El Sheikh, D. M., El-Kholany, E. A. & Kamel, S. M. (2014). Nutritional value, cytotoxicity, anti-carcinogenic and beverage evaluation of roasted date pits. *World J. Dairy Food Sci,* **9(2)**, 308-316.

Elberry, A. A., Mufti, S. T., Al-Maghrabi, J. A., Abdel-Sattar, E. A., Ashour, O. M., Ghareib, S. A. & Mosli, H. A. (2011). Anti-inflammatory and antiproliferative activities of date palm pollen (Phoenix dactylifera) on experimentally-induced atypical prostatic hyperplasia in rats. *Journal of Inflammation,* **8(1)**, 40.

Elias, S. T., Macedo, C. C. S., Simeoni, L. A., Silveira, D., Magalhães, P. O., Lofrano-Porto, A., Coletta, R. D., Neves, F. A. R. & Guerra, E. N. S. (2016). Cytotoxic effect of Erythroxylum daphnites extract is associated with G1 cell cycle arrest and apoptosis in oral squamous cell carcinoma. *Cell cycle (Georgetown, Tex.),* **15(7)**, 948-956. doi: 10.1080/15384101.2016.1151583

Elmaa, S. N., Badarushama, K., Roslib, D., Salvamania, S., Hassana, M. S. & Hashima, R. (2018). Solvents extraction effects on bioactive compounds of

Ajwa date (Phoenix dactylifera L.) flesh using mixture design. *CHEMICAL ENGINEERING*, **63**.

Enioutina, E. Y., Job, K. M., Krepkova, L. V., Reed, M. D. & Sherwin, C. M. (2020). How can we improve the safe use of herbal medicine and other natural products? A clinical pharmacologist mission. *Expert Review of Clinical Pharmacology*, 1-10.

Evans, J. L., Goldfine, I. D., Maddux, B. A. & Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocrine reviews*, **23(5)**, 599-622.

Forcados, G. E., James, D. B., Sallau, A. B., Muhammad, A. & Mabeta, P. (2017). Oxidative stress and carcinogenesis: potential of phytochemicals in breast cancer therapy. *Nutrition and cancer*, **69(3)**, 365-374.

Furniss, C., McClean, M., Smith, J., Bryan, J., Applebaum, K., Nelson, H., Posner, M. & Kelsey, K. T. (2009). Human papillomavirus 6 seropositivity is associated with risk of head and neck squamous cell carcinoma, independent of tobacco and alcohol use. *Annals of oncology*, **20(3)**, 534-541.

Galeb, H. A., Salimon, J., Eid, E. E., Nacer, N. E., Saari, N. & Saadi, S. (2012). The impact of single and double hydrogen bonds on crystallization and melting regimes of Ajwa and Barni lipids. *Food research international*, **48(2)**, 657-666.

García-Martín, J. M., Varela-Centelles, P., González, M., Seoane-Romero, J. M., Seoane, J. & García-Pola, M. J. (2019). Epidemiology of oral cancer. In, *Oral Cancer Detection*: Springer, pp 81-93.

Gee, J., Williamson, G. & Johnson, I. (2002). Evidence for consistent patterns between flavonoid structures and cellular activities.

Ghani, W. M. N., Doss, J. G., Jamaluddin, M., Kamaruzaman, D. & Zain, R. B. (2013). Oral cancer awareness and its determinants among a selected Malaysian population. *Asian Pacific Journal of Cancer Prevention*, **14(3)**.

Ghani, W. M. N., Ramanathan, A., Prime, S. S., Yang, Y.-H., Razak, I. A., Abdul Rahman, Z. A., Abraham, M. T., Mustafa, W. M. W., Tay, K. K. & Kallarakkal, T. G. (2019). Survival of oral cancer patients in different ethnicities. *Cancer investigation*, **37(7)**, 275-287.

Ghosh, D. & Konishi, T. (2007). Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. *Asia Pacific journal of clinical nutrition,* **16(2)**.

Gibson, M., Ghanaem, A., Brooks, S.-L. & Ghaly, D. A. (2015). SOLVENT EXTRACTION OF ANTIOXIDANTS, PHENOLS AND FLAVONOIDS FROM SAUDI ARABIA DATES.

González-Montelongo, R., Lobo, M. G. & González, M. (2010). Antioxidant activity in banana peel extracts: Testing extraction conditions and related bioactive compounds. *Food Chemistry,* **119(3)**, 1030-1039.

Grace Nirmala, J., Evangeline Celsia, S., Swaminathan, A., Narendhirakannan, R. T. & Chatterjee, S. (2018). Cytotoxicity and apoptotic cell death induced by Vitis vinifera peel and seed extracts in A431 skin cancer cells. *Cytotechnology,* **70(2)**, 537-554. doi: 10.1007/s10616-017-0125-0

Guha, N., Boffetta, P., Wünsch Filho, V., Eluf Neto, J., Shangina, O., Zaridze, D., Curado, M. P., Koifman, S., Matos, E. & Menezes, A. (2007). Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies. *American journal of epidemiology,* **166(10)**, 1159-1173.

Guo, C., Sun, L., Chen, X. & Zhang, D. (2013). Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural regeneration research,* **8(21)**, 2003.

Gupta, P., Murti, P., Bhonsle, R., Mehta, F. & Pindborg, J. J. (1995). Effect of cessation of tobacco use on the incidence of oral mucosal lesions in a 10–yr follow-up study of 12 212 users. *Oral diseases,* **1(1)**, 54-58.

Habib, H. M., Platat, C., Meudec, E., Cheynier, V. & Ibrahim, W. H. (2014). Polyphenolic compounds in date fruit seed (Phoenix dactylifera): characterisation and quantification by using UPLC-DAD-ESI-MS. *Journal of the Science of Food and Agriculture,* **94(6)**, 1084-1089.

Hamad, I., AbdElgawad, H., Al Jaouni, S., Zinta, G., Asard, H., Hassan, S., Hegab, M., Hagagy, N. & Selim, S. (2015). Metabolic analysis of various date palm fruit (Phoenix dactylifera L.) cultivars from Saudi Arabia to assess their nutritional quality. *Molecules,* **20(8)**, 13620-13641.

Hanahan, D. & Weinberg, R. A. (2000). The hallmarks of cancer. *cell,* **100(1)**, 57-70.

Hanahan, D. & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *cell,* **144(5)**, 646-674.

Hasan, M. & Mohieldein, A. (2016). In vivo evaluation of anti diabetic, hypolipidemic, antioxidative activities of Saudi date seed extract on streptozotocin induced diabetic rats. *Journal of clinical and diagnostic research: JCDR,* **10(3)**, FF06.

Hasan, N. S., Amom, Z. H., Nor, A., Mokhtarrudin, N., Esa, N. M. & Azlan, A. (2010). Nutritional composition and in vitro evaluation of the antioxidant properties of various dates extracts (Phoenix dactylifera L.) from Libya. *Asian J. Clin. Nutr,* **2**, 208-214.

Haya-Fernández, M. C., Bagan, J., Murillo-Cortés, J., Poveda-Roda, R. & Calabuig, C. (2004). The prevalence of oral leukoplakia in 138 patients with oral squamous cell carcinoma. *Oral diseases,* **10(6)**, 346-348.

He, Y., Zhu, Q., Chen, M., Huang, Q., Wang, W., Li, Q., Huang, Y. & Di, W. (2016). The changing 50% inhibitory concentration (IC50) of cisplatin: a pilot study on the artifacts of the MTT assay and the precise measurement of density-dependent chemoresistance in ovarian cancer. *Oncotarget,* **7(43)**, 70803.

Hecht, S. S. (2003). Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nature Reviews Cancer,* **3(10)**, 733-744.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature,* **407(6805)**, 770-776.

Hindle, I., Downer, M., Moles, D. & Speight, P. (2000). Is alcohol responsible for more intra-oral cancer? *Oral oncology,* **36(4)**, 328-333.

Holmes Jr, L., DesVignes-Kendrick, M., Slomka, J., Mahabir, S., Beeravolu, S. & Emani, S. (2009). Is dental care utilization associated with oral cavity cancer in a large sample of community-based United States residents? *Community dentistry and oral epidemiology,* **37(2)**, 134-142.

Hong, Y. J., Tomas-Barberan, F., Kader, A. A. & Mitchell, A. E. (2006). The flavonoid glycosides and procyanidin composition of Deglet Noor dates (Phoenix dactylifera). *Journal of agricultural and food chemistry,* **54(6)**, 2405-2411.

IARC (2002). IARC Monographs Programme Declares Second-Hand Smoke

Carcinogenic To Humans. *IARC Press Release,* **No. 141.**

Inagi, K., Takahashi, H., Okamoto, M. A., Nakayama, M., Makoshi, T. & Nagai, H. (2002). Treatment effects in patients with squamous cell carcinoma of the oral cavity. *Acta Oto-Laryngologica,* **122(4)**, 25-29.

Ines, D., Sonia, B., Fatma, B. A., Souhail, B., Hamadi, A., Hamida, T. & Basma, H. (2010). Date seed oil inhibits Hydrogen peroxide-induced oxidative stress in human epidermal keratinocytes. *International journal of dermatology,* **49(3)**, 262-268.

Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y. & Ueno, K. (1996). A combined assay of cell vability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biological and Pharmaceutical Bulletin,* **19(11)**, 1518-1520.

Ishurd, O. & Kennedy, J. F. (2005). The anti-cancer activity of polysaccharide prepared from Libyan dates (Phoenix dactylifera L.). *Carbohydrate Polymers,* **59(4)**, 531-535.

Jassim, S. A. & Naji, M. A. (2010). In vitro evaluation of the antiviral activity of an extract of date palm (Phoenix dactylifera L.) pits on a Pseudomonas phage. *Evidence-Based Complementary and Alternative Medicine,* **7(1)**, 57-62.

Johnson, E. J. (2002). The role of carotenoids in human health. *Nutr Clin Care,* **5(2)**, 56-65. doi: 10.1046/j.1523-5408.2002.00004.x

Kalavrezos, N. & Scully, C. (2015). Mouth cancer for clinicians part 5: risk factors (other). *Dental update,* **42(8)**, 766-778.

Kalavrezos, N. & Scully, C. (2016a). Mouth Cancer for Clinicians Part 10: Cancer Treatment (Surgery). *Dent Update,* **43(4)**, 375-378, 381-372, 385-377. doi: 10.12968/denu.2016.43.4.375

Kalavrezos, N. & Scully, C. (2016b). Mouth Cancer for Clinicians. Part 11: Cancer Treatment (Radiotherapy). *Dent Update,* **43(5)**, 472-474, 476-477, 479-481. doi: 10.12968/denu.2016.43.5.472

Kchaou, W., Abbès, F., Blecker, C., Attia, H. & Besbes, S. (2013). Effects of extraction solvents on phenolic contents and antioxidant activities of Tunisian date varieties (Phoenix dactylifera L.). *Industrial crops and products,* **45**, 262-269.

Kchaou, W., Abbès, F., Mansour, R. B., Blecker, C., Attia, H. & Besbes, S. (2016). Phenolic profile, antibacterial and cytotoxic properties of second grade date

extract from Tunisian cultivars (Phoenix dactylifera L.). *Food chemistry,* **194**, 1048-1055.

Keum, Y.-S., Kim, J., Lee, K. H., Park, K. K., Surh, Y.-J., Lee, J. M., Lee, S.-S., Yoon, J. H., Joo, S. Y. & Cha, I. H. (2002). Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells. *Cancer letters,* **177(1)**, 41-47.

Khalid, S., Ahmad, A., Masud, T., Asad, M. & Sandhu, M. (2016). Nutritional assessment of ajwa date flesh and pits in comparison to local varieties. *Journal of Plant and Animal Sciences,* **26(4)**, 1072-1080.

Khalid, S., Khalid, N., Khan, R. S., Ahmed, H. & Ahmad, A. (2017). A review on chemistry and pharmacology of Ajwa date fruit and pit. *Trends in food science & technology,* **63**, 60-69.

Khan, F., Ahmed, F., Pushparaj, P. N., Abuzenadah, A., Kumosani, T., Barbour, E., AlQahtani, M. & Gauthaman, K. (2016). Ajwa date (Phoenix dactylifera L.) extract inhibits human breast adenocarcinoma (MCF7) cells in vitro by inducing apoptosis and cell cycle arrest. *PloS one,* **11(7)**, e0158963.

Khan, F., Aldhahri, M., Hussain, M. A., Gauthaman, K., Memic, A., Abuzenadah, A., Kumosani, T., Barbour, E., Alothmany, N. S. & Aldhaheri, R. W. (2018). Encapsulation of 5-flurouracil into PLGA nanofibers and enhanced anticancer effect in combination with Ajwa-dates-extract (Phoenix dactylifera L.). *Journal of biomedical nanotechnology,* **14(3)**, 553-563.

Kim, D. & Li, R. (2019). Contemporary Treatment of Locally Advanced Oral Cancer. *Current treatment options in oncology,* **20(4)**, 32.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P. & Green, D. (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell death & differentiation,* **16(1)**, 3-11.

Kufe, D. W., Hait, W., Holland, J. F., Frei, E. & Pollock, R. E. (2010). *Holland Frei cancer medicine 8* (Vol. 8): PMPH-USA.

Kumar, M., Nanavati, R., Modi, T. & Dobariya, C. (2016a). Oral cancer: Etiology and risk factors: A review. *Journal of Cancer Research and Therapeutics,* **12(2)**, 458-463. doi: 10.4103/0973-1482.186696

Kumar, M., Nanavati, R., Modi, T. G. & Dobariya, C. (2016b). Oral cancer: Etiology and risk factors: A review. *Journal of cancer research and therapeutics,* **12(2)**, 458.

Li, A. C., Warnakulasuriya, S. & Thompson, R. P. (2003). Neoplasia of the tongue in a patient with Crohn's disease treated with azathioprine: case report. *European journal of gastroenterology & hepatology,* **15(2)**, 185-187.

Lin, C.-W., Chen, P.-N., Chen, M.-K., Yang, W.-E., Tang, C.-H., Yang, S.-F. & Hsieh, Y.-S. (2013). Kaempferol reduces matrix metalloproteinase-2 expression by down-regulating ERK1/2 and the activator protein-1 signaling pathways in oral cancer cells. *PLoS One,* **8(11)**.

Lingen, M., Pinto, A., Mendes, R., Franchini, R., Czerninski, R., Tilakaratne, W., Partridge, M., Peterson, D. & Woo, S. B. (2011). Genetics/epigenetics of oral premalignancy: current status and future research. *Oral diseases,* **17**, 7-22.

Liu, J. K. (2014). Anti-cancer vaccines—a one-hit wonder? *The Yale journal of biology and medicine,* **87(4)**, 481.

Liu, L., Chen, J., Cai, X., Yao, Z. & Huang, J. (2019). Progress in targeted therapeutic drugs for oral squamous cell carcinoma. *Surgical Oncology,* **31**, 90-97.

Lu, C., Song, E., Hu, D.-N., Chen, M., Xue, C., Rosen, R. & McCormick, S. A. (2010). Curcumin induces cell death in human uveal melanoma cells through mitochondrial pathway. *Current eye research,* **35(4)**, 352-360.

Lu, Y. & Foo, L. Y. (2000). Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food chemistry,* **68(1)**, 81-85.

MacDonald-Wicks, L. K., Wood, L. G. & Garg, M. L. (2006). Methodology for the determination of biological antioxidant capacity in vitro: a review. *Journal of the Science of Food and Agriculture,* **86(13)**, 2046-2056.

Mahmoud, Y. K. & Abdelrazek, H. M. (2019). Cancer: Thymoquinone antioxidant/pro-oxidant effect as potential anticancer remedy. *Biomedicine & Pharmacotherapy,* **115**, 108783.

Mann, J. (2002). Natural products in cancer chemotherapy: past, present and future. *Nature Reviews Cancer,* **2(2)**, 143-148.

Maqsood, S., Kittiphattanabawon, P., Benjakul, S., Sumpavapol, P. & Abushelaibi, A. (2015). Antioxidant activity of date (Phoenix dactylifera var. Khalas) seed and its preventive effect on lipid oxidation in model systems. *International Food Research Journal,* **22(3)**.

Martín-Gallán, P., Carrascosa, A., Gussinyé, M. & Domínguez, C. (2003). Biomarkers of diabetes-associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. *Free Radical Biology and Medicine,* **34(12)**, 1563-1574.

Maserejian, N. N., Joshipura, K. J., Rosner, B. A., Giovannucci, E. & Zavras, A. I. (2006). Prospective study of alcohol consumption and risk of oral premalignant lesions in men. *Cancer Epidemiology and Prevention Biomarkers,* **15(4)**, 774-781.

Mazeron, R., Tao, Y., Lusinchi, A. & Bourhis, J. (2009). Current concepts in management in head and neck cancer; radiotherapy. *Oral Oncol,* **45(4-5)**, 402-408.

McGaw, L. J., Elgorashi, E. E. & Eloff, J. N. (2014). Cytotoxicity of African medicinal plants against normal animal and human cells. In, *Toxicological survey of african medicinal plants*: Elsevier, pp 181-233.

Mirza, M. B., Elkady, A. I., Al-Attar, A. M., Syed, F. Q., Mohammed, F. A. & Hakeem, K. R. (2018). Induction of apoptosis and cell cycle arrest by ethyl acetate fraction of Phoenix dactylifera L.(Ajwa dates) in prostate cancer cells. *Journal of ethnopharmacology,* **218**, 35-44.

Mirza, M. B., Syed, F. Q., Khan, F., Elkady, A. I., Al-Attar, A. M. & Hakeem, K. R. (2019). Ajwa Dates: A Highly Nutritive Fruit with the Impending Therapeutic Application. In, *Plant and Human Health, Volume 3*: Springer, pp 209-230.

Mollazadeh, H., Afshari, A. R. & Hosseinzadeh, H. (2017). Review on the potential therapeutic roles of nigella sativa in the treatment of patients with cancer: Involvement of apoptosis:-black cumin and cancer. *Journal of pharmacopuncture,* **20(3)**, 158.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods,* **65(1-2)**, 55-63.

Mousavi, S. & Hersey, P. (2007). Role of caspases and reactive oxygen species in rose Bengal-induced toxicity in melanoma cells.

Nagler, R. M. (2009). Saliva as a tool for oral cancer diagnosis and prognosis. *Oral Oncology*, **45(12)**, 1006-1010. doi: 10.1016/j.oraloncology.2009.07.005

Nematallah, K. A., Ayoub, N. A., Abdelsattar, E., Meselhy, M. R., Elmazar, M. M., El-Khatib, A. H., Linscheid, M. W., Hathout, R. M., Godugu, K. & Adel, A. (2018). Polyphenols LC-MS2 profile of Ajwa date fruit (Phoenix dactylifera L.) and their microemulsion: Potential impact on hepatic fibrosis. *Journal of functional foods*, **49**, 401-411.

Nichenametla, S. N., Taruscio, T. G., Barney, D. L. & Exon, J. H. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical reviews in food science and nutrition*, **46(2)**, 161-183.

Niepel, M., Hafner, M., Chung, M. & Sorger, P. K. (2017). Measuring Cancer Drug Sensitivity and Resistance in Cultured Cells. *Current protocols in chemical biology*, **9(2)**, 55-74. doi: 10.1002/cpch.21

Pai, S. I. & Westra, W. H. (2009). Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. *Annual Review of Pathology: Mechanisms of Disease*, **4**, 49-70.

Pandey, K. B. & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, **2(5)**, 270-278.

Park, H.-K., Han, D.-W., Park, Y. H. & Park, J.-C. (2005). Differential biological responses of green tea polyphenol in normal cells vs. cancer cells. *Current Applied Physics*, **5(5)**, 449-452.

Petersen, P. E. (2003). The World Oral Health Report 2003: continuous improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme. *Community Dent Oral Epidemiol*, **31 Suppl 1**, 3-23.

Petti, S. (2009). Lifestyle risk factors for oral cancer. *Oral oncology*, **45(4-5)**, 340-350.

Phillipson, J. D. (2001). Phytochemistry and medicinal plants. *Phytochemistry*, **56(3)**, 237-243.

Picot, J., Guerin, C. L., Le Van Kim, C. & Boulanger, C. M. (2012). Flow cytometry: retrospective, fundamentals and recent instrumentation. *Cytotechnology*, **64(2)**, 109-130. doi: 10.1007/s10616-011-9415-0

Ponder, B. A. (2001). Cancer genetics. *Nature*, **411(6835)**, 336-341.

Popkin, B. M. (2007). Understanding global nutrition dynamics as a step towards controlling cancer incidence. *Nature Reviews Cancer,* **7(1)**, 61-67.

Pozarowski, P., Grabarek, J. & Darzynkiewicz, Z. (2003). Flow cytometry of apoptosis. *Current protocols in cell biology,* **21(1)**, 18.18. 11-18.18. 33.

Präbst, K., Engelhardt, H., Ringgeler, S. & Hübner, H. (2017). Basic colorimetric proliferation assays: MTT, WST, and resazurin. In, *Cell Viability Assays*: Springer, pp 1-17.

Prasad, S. & Srivastava, S. K. (2020). Oxidative Stress and Cancer: Chemopreventive and Therapeutic Role of Triphala. *Antioxidants,* **9(1)**, 72.

Pujari, R. R., Vyawahare, N. S. & Kagathara, V. G. (2011). Evaluation of antioxidant and neuroprotective effect of date palm (Phoenix dactylifera L.) against bilateral common carotid artery occlusion in rats.

Ragab, MA, E., BY, S. & HN, B. (2013). Antioxidant and Tissue-Protective Studies on Ajwa

Extract: Dates from Al-Madinah Al-Monwarah, Saudia Arabia. *Journal of Environmental & Analytical Toxicology,* **3(1)**.

Ramasamy, S., Wahab, N. A., Abidin, N. Z. & Manickam, S. (2011). Cytotoxicity evaluation of five selected Malaysian Phyllanthaceae species on various human cancer cell lines. *Journal of Medicinal Plants Research,* **5(11)**, 2267-2273.

Razak, A. A., Saddki, N., Naing, N. N. & Abdullah, N. (2010). Oral cancer survival among Malay patients in Hospital Universiti Sains Malaysia, Kelantan. *Asian Pac J Cancer Prev,* **11(1)**, 187-191.

Razzaghi-Asl, N., Garrido, J., Khazraei, H., Borges, F. & Firuzi, O. (2013). Antioxidant properties of hydroxycinnamic acids: a review of structure-activity relationships. *Current medicinal chemistry,* **20(36)**, 4436-4450.

Ren, W., Qiao, Z., Wang, H., Zhu, L. & Zhang, L. (2003). Flavonoids: Promising anticancer agents. *Medicinal Research Reviews,* **23(4)**, 519-534. doi: 10.1002/med.10033

Ribeiro, A. C. P., Silva, A. R. S., Simonato, L. E., Salzedas, L. M. P., Sundefeld, M. L. M. M. & Soubhia, A. M. P. (2009). Clinical and histopathological analysis

of oral squamous cell carcinoma in young people: a descriptive study in Brazilians. *British Journal of Oral and Maxillofacial Surgery,* **47(2)**, 95-98.

Rivera, C. (2015). Essentials of oral cancer. *International journal of clinical and experimental pathology,* **8(9)**, 11884.

Saafi, E. B., Louedi, M., Elfeki, A., Zakhama, A., Najjar, M. F., Hammami, M. & Achour, L. (2011). Protective effect of date palm fruit extract (Phoenix dactylifera L.) on dimethoate induced-oxidative stress in rat liver. *Experimental and Toxicologic Pathology,* **63(5)**, 433-441.

Saleh, E. A., Tawfik, M. S. & Abu-Tarboush, H. M. (2011). Phenolic Contents and Antioxidant Activity of Various Date Palm (<i>Phoenix dactylifera </i> L.) Fruits from Saudi Arabia. *Food and Nutrition Sciences,* **02(10)**, 1134-1141. doi: 10.4236/fns.2011.210152

Saraste, A. & Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovascular research,* **45(3)**, 528-537.

Sato, D., Kondo, S., Yazawa, K., Mukudai, Y., Li, C., Kamatani, T., Katsuta, H., Yoshihama, Y., Shirota, T. & Shintani, S. (2013). The potential anticancer activity of extracts derived from the roots of Scutellaria baicalensis on human oral squamous cell carcinoma cells. *Molecular and clinical oncology,* **1(1)**, 105-111.

Sawaya, W., Miski, A., Khalil, J., Khatchadourian, H. & Mashadi, A. (1983). Physical and chemical characterisation of the major date varieties grown in Saudi Arabia: I. Morphological measurements, proximate and mineral analyses. *Date Palm Journal*.

Scully, C. & Bagan, J. (2009). Oral squamous cell carcinoma overview. *Oral oncology,* **45(4/5)**, 301-308.

Sheikh, B. Y., Elsaed, W. M., Samman, A. H., Sheikh, B. Y. & Ladin, A.-M. M. B. (2014). AJWA DATES AS A PROTECTIVE AGENT AGAINST LIVER TOXICITY IN RAT

European Scientific Journal, **3**.

Shoieb, A. M., Elgayyar, M., Dudrick, P. S., Bell, J. L. & Tithof, P. K. (2003). In vitro inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. *International journal of oncology,* **22(1)**, 107-113.

Shukla, Y. & Singh, M. (2007). Cancer preventive properties of ginger: a brief review. *Food and chemical toxicology*, **45(5)**, 683-690.

Siddiqui, S., Ahmad, R., Khan, M. A., Upadhyay, S., Husain, I. & Srivastava, A. N. (2019). Cytostatic and anti-tumor potential of Ajwa date pulp against human hepatocellular carcinoma HepG2 cells. *Scientific reports*, **9(1)**, 1-12.

Singha, I. & Das, S. K. (2015). Grapevine fruit extract protects against radiation-induced oxidative stress and apoptosis in human lymphocyte.

Society, A. C. (2020). Cancer Facts & Figures 2020. . *American Cancer Society*.

Soong, Y.-Y. & Barlow, P. J. (2004). Antioxidant activity and phenolic content of selected fruit seeds. *Food chemistry*, **88(3)**, 411-417.

Stebbing, A. (1982). Hormesis—the stimulation of growth by low levels of inhibitors. *Science of the total environment*, **22(3)**, 213-234.

Surh, Y.-J. (2002). Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. *Food and Chemical Toxicology*, **40(8)**, 1091-1097.

Taatjes, D. J., Sobel, B. E. & Budd, R. C. (2008). Morphological and cytochemical determination of cell death by apoptosis. *Histochemistry and cell biology*, **129(1)**, 33-43.

Tanikawa, K. & Torimura, T. (2006). Studies on oxidative stress in liver diseases: important future trends in liver research. *Medical molecular morphology*, **39(1)**, 22-27.

Thomasset, S. C., Berry, D. P., Garcea, G., Marczylo, T., Steward, W. P. & Gescher, A. J. (2007). Dietary polyphenolic phytochemicals—promising cancer chemopreventive agents in humans? A review of their clinical properties. *International Journal of Cancer*, **120(3)**, 451-458.

Thouri, A., La Barbera, L., Canuti, L., Vegliante, R., Jelled, A., Flamini, G., Ciriolo, M. R. & Achour, L. (2019). Antiproliferative and apoptosis-inducing effect of common Tunisian date seed (var. Korkobbi and Arechti) phytochemical-rich methanolic extract. *Environmental Science and Pollution Research*, **26(36)**, 36264-36273.

Törrönen, R. & Määttä, K. (2000). Bioactive substances and health benefits of strawberries. Proceedings from *IV International Strawberry Symposium 567*

Umar Ibrahim, M., Nordin, S. & Abdulkareem, U. (2015). Ibrahim Haruna. S, Atif Amin. B, Thant. Z. Anti-inflammatory and analgesic activities of aqueous extract date palm (phoenix dactylifera l) fruit in rats. *Int J Novel Res Healthcare Nurs,* **2(3)**, 166-172.

van Engeland, M., Nieland, L. J., Ramaekers, F. C., Schutte, B. & Reutelingsperger, C. P. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry,* **31(1)**, 1-9. doi: 10.1002/(sici)1097-0320(19980101)31:1<1::aid-cyto1>3.0.co;2-r

Van Leeuwen, M. T., Grulich, A. E., McDonald, S. P., McCredie, M. R., Amin, J., Stewart, J. H., Webster, A. C., Chapman, J. R. & Vajdic, C. M. (2009). Immunosuppression and other risk factors for lip cancer after kidney transplantation. *Cancer Epidemiology and Prevention Biomarkers,* **18(2)**, 561-569.

Vaughan, D. (2009). The role of functional reconstruction after 30 years of free flap experience. *Oral Oncol,* **45(4–5)**, 421-430.

Vayalil, P. K. (2002). Antioxidant and Antimutagenic Properties of Aqueous Extract of Date Fruit (Phoenix dactylifera L. Arecaceae). *Journal of Agricultural and Food Chemistry,* **50(3)**, 610-617. doi: 10.1021/jf010716t

Vietri, M., Pietrabissa, A., Mosca, F., Spisni, R. & Pacifici, G. (2003). Curcumin is a potent inhibitor of phenol sulfotransferase (SULT1A1) in human liver and extrahepatic tissues. *Xenobiotica,* **33(4)**, 357-363.

Vikhanskaya, F., Lee, M. K., Mazzoletti, M., Broggini, M. & Sabapathy, K. (2007). Cancer-derived p53 mutants suppress p53-target gene expression—potential mechanism for gain of function of mutant p53. *Nucleic acids research,* **35(6)**, 2093-2104.

Wallace, T. C. (2011). Anthocyanins in cardiovascular disease. *Advances in Nutrition,* **2(1)**, 1-7.

Warnakulasuriya, K., Johnson, N., Linklater, K. & Bell, J. (1999). Cancer of mouth, pharynx and nasopharynx in Asian and Chinese immigrants resident in Thames regions. *Oral Oncology,* **35(5)**, 471-475.

Warnakulasuriya, S. (2009a). Causes of oral cancer–an appraisal of controversies. *British dental journal*, **207(10)**, 471-475.

Warnakulasuriya, S. (2009b). Food, nutrition and oral cancer. In, *Food constituents and oral health*: Elsevier, pp 273-295.

Warnakulasuriya, S. (2009c). Global epidemiology of oral and oropharyngeal cancer. *Oral oncology*, **45(4-5)**, 309-316.

Weber, R. S., Berkey, B. A., Forastiere, A., Cooper, J., Maor, M., Goepfert, H., Morrison, W., Glisson, B., Trotti, A. & Ridge, J. A. (2003). Outcome of salvage total laryngectomy following organ preservation therapy: the Radiation Therapy Oncology Group trial 91-11. *Archives of Otolaryngology–Head & Neck Surgery*, **129(1)**, 44-49.

Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research*, **30(1)**, 87.

Woolgar, J. A. & Triantafyllou, A. (2011). Squamous cell carcinoma and precursor lesions: clinical pathology. *Periodontology 2000*, **57(1)**, 51-72.

Wu, C., Lee, S.-L., Taylor, C., Li, J., Chan, Y.-M., Agarwal, R., Temple, R., Throckmorton, D. & Tyner, K. (2020). Scientific and regulatory approach to botanical drug development: A US FDA Perspective. *Journal of Natural Products*, **83(2)**, 552-562.

Yadav, K., Singhal, N., Rishi, V. & Yadav, H. (2001). Cell Proliferation Assays. *eLS*.

Yakob, M., Fuentes, L., Wang, M. B., Abemayor, E. & Wong, D. T. (2014). Salivary biomarkers for detection of oral squamous cell carcinoma: current state and recent advances. *Current oral health reports*, **1(2)**, 133-141.

Yang, S.-F., Yang, W.-E., Kuo, W.-H., Chang, H.-R., Chu, S.-C. & Hsieh, Y.-S. (2008). Antimetastatic potentials of flavones on oral cancer cell via an inhibition of matrix-degrading proteases. *Archives of oral biology*, **53(3)**, 287-294.

Yasin, B. R., El-Fawal, H. A. & Mousa, S. A. (2015). Date (Phoenix dactylifera) Polyphenolics and Other Bioactive Compounds: A Traditional Islamic Remedy's Potential in Prevention of Cell Damage, Cancer Therapeutics and Beyond. *Int J Mol Sci*, **16(12)**, 30075-30090. doi: 10.3390/ijms161226210

Zhang, C.-R., Aldosari, S. A., Vidyasagar, P. S., Nair, K. M. & Nair, M. G. (2013). Antioxidant and anti-inflammatory assays confirm bioactive compounds in Ajwa date fruit. *Journal of agricultural and food chemistry*, **61(24)**, 5834-5840.

Zhang, C.-R., Aldosari, S. A., Vidyasagar, P. S., Shukla, P. & Nair, M. G. (2017). Health-benefits of date fruits produced in Saudi Arabia based on in vitro antioxidant, anti-inflammatory and human tumor cell proliferation inhibitory assays. *Journal of the Saudi Society of Agricultural Sciences*, **16(3)**, 287-293.

Zhang, C., Hao, Y., Sun, Y. & Liu, P. (2019). Quercetin suppresses the tumorigenesis of oral squamous cell carcinoma by regulating microRNA-22/WNT1/β-catenin axis. *Journal of Pharmacological Sciences*, **140(2)**, 128-136. doi: https://doi.org/10.1016/j.jphs.2019.03.005

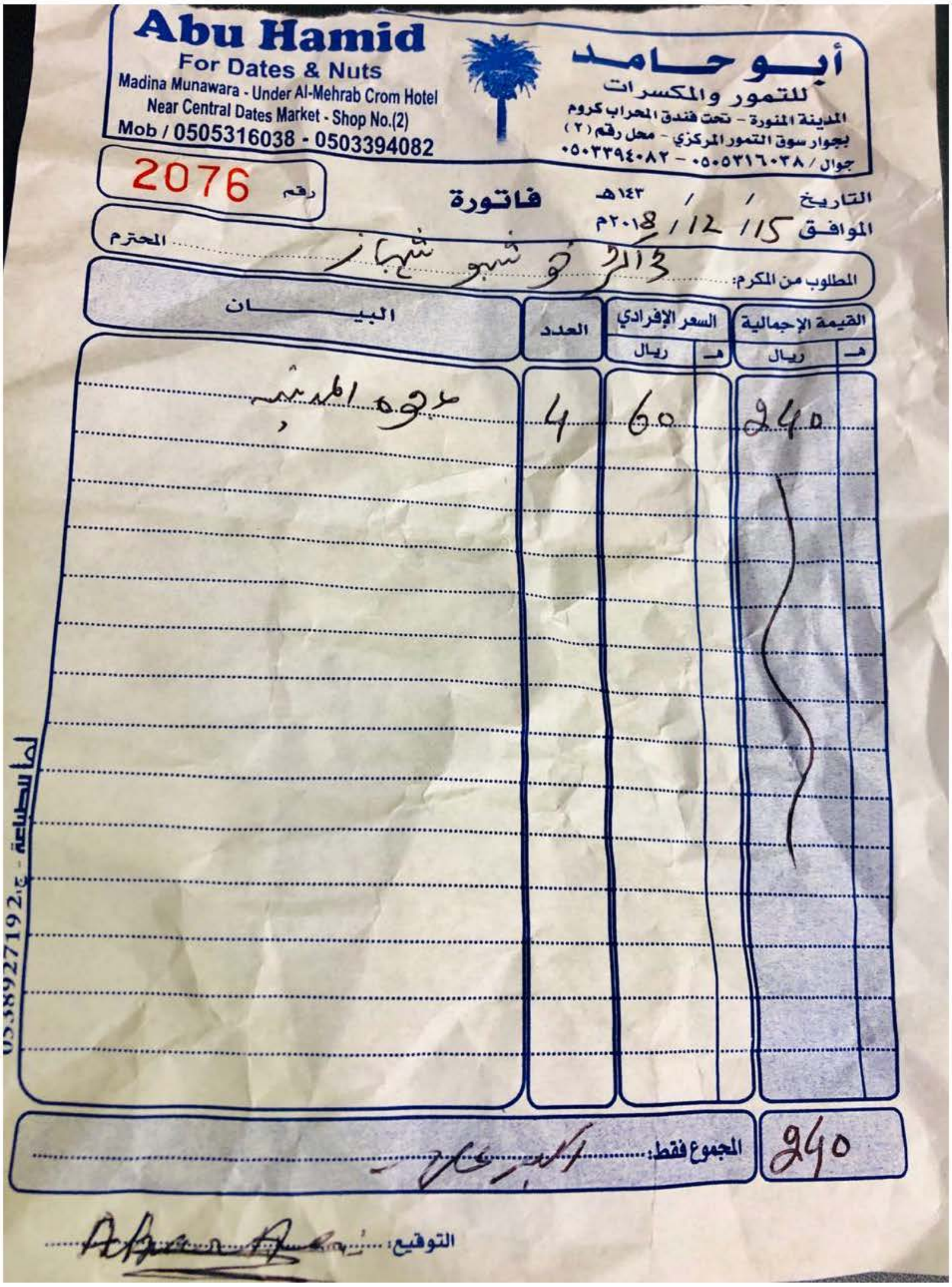
Zhang, C., Li, B., Zhang, X., Hazarika, P., Aggarwal, B. B. & Duvic, M. (2010). Curcumin selectively induces apoptosis in cutaneous T-cell lymphoma cell lines and patients' PBMCS: Potential role for STAT-3 and NF-κB signaling. *Journal of Investigative Dermatology*, **130(8)**, 2110-2119.

Zini, A., Czerninski, R. & Sgan-Cohen, H. D. (2010). Oral cancer over four decades: epidemiology, trends, histology, and survival by anatomical sites. *Journal of oral pathology & medicine*, **39(4)**, 299-305.

Złotek, U., Mikulska, S., Nagajek, M. & Świeca, M. (2016). The effect of different solvents and number of extraction steps on the polyphenol content and antioxidant capacity of basil leaves (Ocimum basilicum L.) extracts. *Saudi journal of biological sciences*, **23(5)**, 628-633.

APPENDICES

APPENDIX A AJWA DATES RECIPT FOR AUTHENTICATION


Abu Hamid
For Dates & Nuts
Madina Munawara - Under Al-Mehrab Crom Hotel
Near Central Dates Market - Shop No.(2)
Mob / 0505316038 - 0503394082

أبـــو حـــامـــد
للتمور والمكسرات
المدينة المنورة – تحت فندق المحراب كروم
بجوار سوق التمور المركزي – محل رقم (٢)
جوال / ٠٥٠٥٣١٦٠٣٨ – ٠٥٠٣٣٩٤٠٨٢

رقم 2076
فاتورة
التاريخ / / ١٤٣ هـ
الموافق 15 / 12 / 2018 م
المطلوب من المكرم: .... المحترم

القيمة الإجمالية (ريال)	هـ	السعر الإفرادي (ريال)	هـ	العدد	البيـــــان
240		60		4	

المجموع فقط: ....
240
التوقيع: ....

APPENDIX B OSCC CELL LINE

20040907

RIKEN BRC CELL BANK

(FormC-0010)

RECEIPT FORM(CELL)

Date: _____

To: Nakamura. Yukio

Head. Cell Bank. Riken BioResource Center

Name: Dr. Khairul Bariah Ahmad Amin Noordin

Telephone Number: +60-97672435 Fax Number:

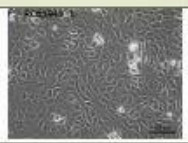
Department Division: School of Dental Science

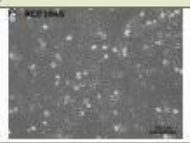
Organization: Universiti Sains Malaysia

Address: School of Dental Science.Health Campus,University Sains Malaysia.16150 Kubang Kerian Kelantan Malaysia

Following cell lines have been received on Month_____Day_____Year_____.

RCB1945 : HSC-2			update : 2018/08/01
Comment		Human cell line derived from oral squamous cell carcinoma occurred in 69-yo, male patient. HLA-A 24/, TKG0487 (Deposited from Tohoku Univ.).	
Comment from the depositor			
Terms and conditions		Basically, there is no restriction regarding academic use.	
Remarks			
Order Form		Regarding MTA between user institutions and RIKEN BRC, there are two kinds of MTA, Category I and II, depending on the sort of user institutions and the purposes of use.Please use an appropriate MTA(to see).In case of Restriction "a" or "f", please contact RIKEN BRC(cellbank.brc@riken.jp) regarding any kind of for-profit use.	
Basic information	Depositor	Obinata, Masuo	
	Originator	Hiraki S	
	Year of deposit	2004	
	Original cell	TKG0487	
	Animal	human < Mammals	
	Genus	Homo	
	Species	sapiens	
	Gender	Male	
	Age at sampling	69 years	
	Tissue	mouth	
	Case history	squamous cell carcinoma of mouth	
	Classification	cancer	
	History	Cell Resource Center for Biomedical Research, Tohoku University (TKG0487)	
	Lifespan	infinite	
	Morphology	epithelial-like	
		deposit info	lot info
Culture information	Medium	Medium List	
	Culture type		Adherent cells
	Medium and additives		MEM + 10% FBS
	Antibiotics		Free
	Passage method		(0.05% trypsin + 0.02% EDTA) or (0.25% trypsin + 0.02% EDTA)
	Passage ratio		1 : 8 split
	SC frequency		Subculture : 2 times/week
	Temperature		37 °C
	CO2 concentration		5 %
	Freeze medium		Medium + 10% DMSO
	Freezing method		Slow freezing
	Mycoplasma		(-)
	STR(human)		OK
	Isozyme		LD, NP
Images		deposit info	lot info





RIKEN Cell Bank

Frozen cells/ampoule	2.2x10^(6)
Viability at thawing	78%
Adhesion efficiency	85%
Antibiotics conc.	Free
Passage number	U+7
Population doublings	
Mycoplasma	-
Bacteria	-
Fungi	-
Protozoa	-
Virus contaminated	
Name of virus	
Isozyme analysis	Lot.1: LD, NP
Chromosome diversion	
Marker chromosome	
Surface antigen	
Contact inhibiton	
Anchorage dependency	Yes
Tumomrigenicity	
Exogenous gene	
Short Tandem Repeat	OK
Special comments	
Memo	
B_Ig subclass	
B_Ig production	

Remainder of page intentionally left blank

APPENDIX C

PREPRARATION OF COMPLETE GROWTH MEDIA FOR HSC-2 CELLS

To prepare 50ml of complete growth medium, add 45.5 ml of basal MEM complete growth medium in a 50 ml conical centrifuge tube, then add 5 ml of 2mM L-glutamine with 10% FBS and 500 µl of penicillin/streptomycin into the conical tube.

Pipette the solution up and down to mix it. This complete medium can be stored at 4 C for up to 3 weeks. Complete growth medium was prepared under biological safety cabinet

APPENDIX D

PREPARATION OF ADF EXTRACT

To prepare 10 ml of ADF extract stock solution with a concentration of 50 mg/ml, dissolve 500 mg of ADF freeze dried powder in 10 ml complete growth medium (w/v). Filtered the stock solution with 0.22 µm syringe to sterilize the solution. Further dilutions were prepared from this sterile stock solution (v/v). To prepare 1 ml of ADF extract, concentration of 0.8 mg/ml, 1.5 mg/ml, 3.1 mg/ml, 6.3 mg/ml, 12.5 mg/ml, 25 mg/ml and 50mg/ml were formulated by using a formula as below.

$M_1 V_1 = M_2 V_2$

Where,
M_1 = Concentration of the initial solution
V_1 = Volume of the initial solution
M_2 = Concentration of the final solution (required)
V_2 = Volume of the final solution

Concentration	CGM (µl)	ADF (µl)
Control	1000	-
0.8	984	16
1.5	970	30
3.1	938	62
6.3	874	126
12.5	750	250
25	500	500
50	-	1000

CGM = Complete Growth Medium

APPENDIX E

PREPARATION OF ADP EXTRACT

To prepare 10 ml of ADP extract stock solution with a concentration of 5 mg/ml, dissolve 50 mg of ADP freeze dried powder in 10 ml complete growth medium (w/v). Filtered the stock solution with 0.22 µm syringe to sterilize the solution. Further dilutions were prepared from this sterile stock solution (v/v). To prepare 1 ml of ADP extract, concentration of 0.08 mg/ml, 0.31mg/ml, 0.63 mg/ml, 1.15 mg/ml, 1.25 mg/ml, 2.5 mg/ml and 5mg/ml were formulated by using a formula as below.

$$M_1 V_1 = M_2 V_2$$

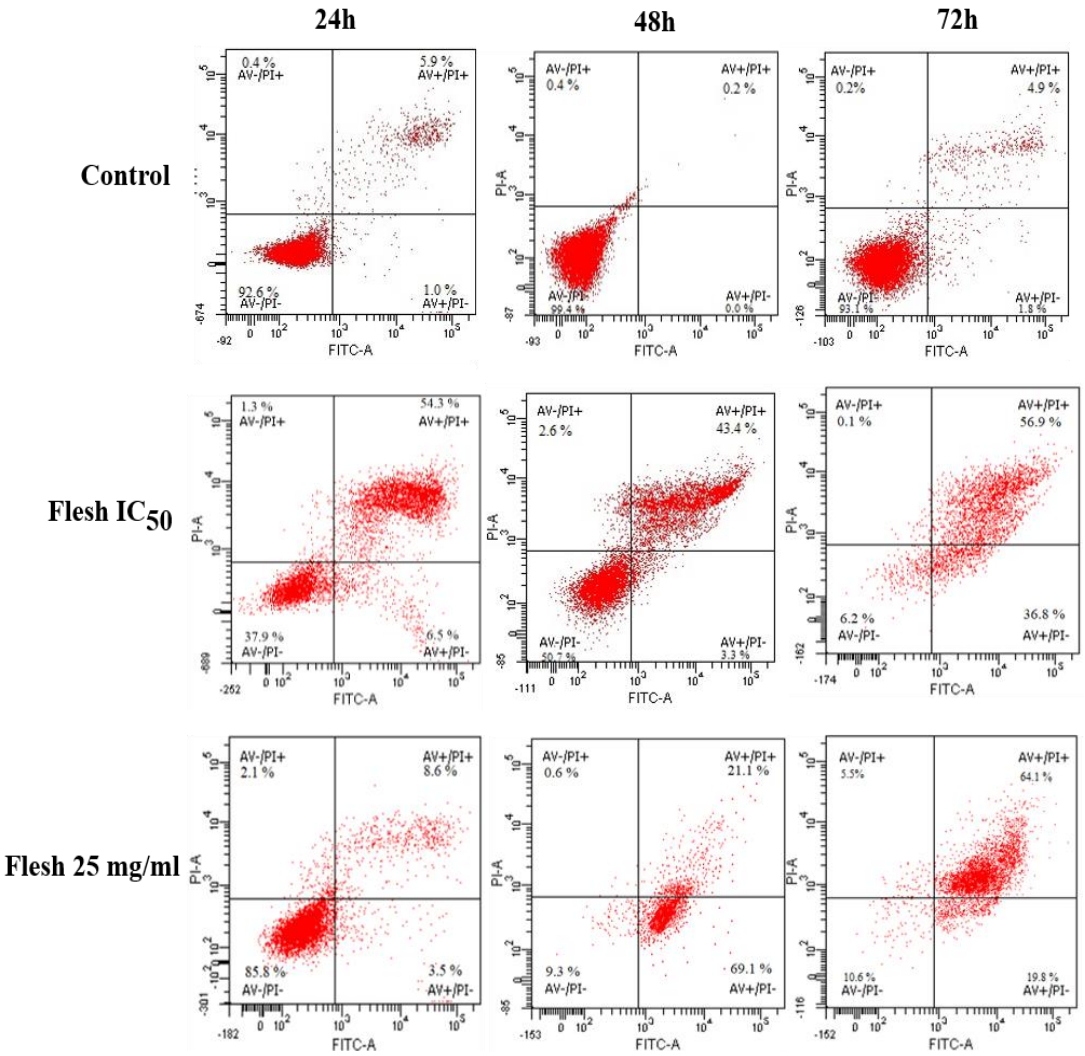
Where,
M_1 = Concentration of the initial solution
V_1 = Volume of the initial solution
M_2 = Concentration of the final solution (required)
V_2 = Volume of the final solution

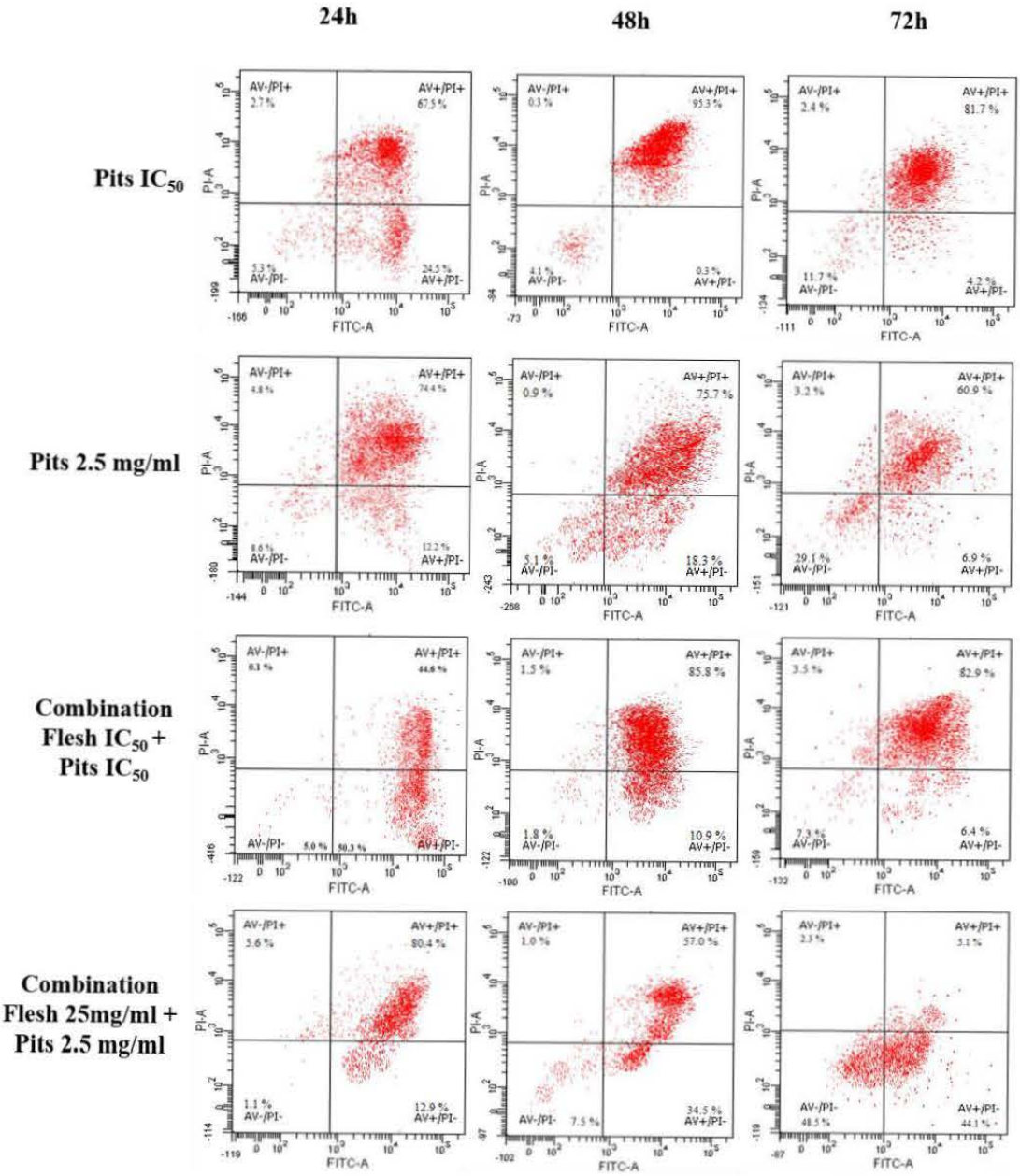
Concentration	CGM (µl)	ADF (µl)
Control	1000	-
0.08	984	16
0.31	938	62
0.63	874	126
1.15	770	230
1.25	750	250
2.5	500	500
5	-	1000

CGM = Complete Growth Medium

APPENDIX F

APOPTOSIS DATA BY FLOWCYTOMETRY




24h
48h
72h
Pits IC50
Pits 2.5 mg/ml
Combination Flesh IC50 + Pits IC50
Combination Flesh 25mg/ml + Pits 2.5 mg/ml
AV-/PI+
AV+/PI+
AV-/PI-
AV+/PI-
PI-A
FITC-A

APPENDIX G

WORKSHOPS AND CONFERENCES ATTENDED

PRISM

UNIVERSITI SAINS MALAYSIA

APEX™

CERTIFICATE OF PARTICIPATION

This is to certify that

Khusboo Shahbaz

has participated
in the

ONE DAY WORKSHOP ON
GRAPHPAD PRISM ANALYSIS
FOR EXPERIMENTAL SCIENTIST

On the
11 April 2019
Held in School of Dental Sciences

Organized by
Craniofacial Science Laboratory,
School of Dental Sciences, Universiti Sains Malaysia

Assoc. Prof. Dr. Mohd Fadhli Bin Khamis
Dean
School of Dental Sciences
Health Campus, Universiti Sains Malaysia

Dr. Wan Nazatul Shima Shahidan
Chairperson
One Day Workshop on GraphPad PRISM
Analysis for Experimental Scientist

CERTIFICATE

This is to certify that

DR. KHUSHBOO SHAHBAZ
participated in the

10TH MDA - USM TALK 2018

held on
19th January 2018

at
School of Dental Sciences,
Universiti Sains Malaysia

MDC CPD: 3 POINTS

Prof. Dr. Adam Husein
Dean
School of Dental Sciences,
Universiti Sains Malaysia

Dr. Ng Woan Tyng
President
Malaysian Dental Association
(MDA)

CERTIFICATE of ATTENDANCE

This is to certify

DR. KHUSHBOO SHAHBAZ

is the **Participant** for

BLEACHING WORKSHOP
(Lectures, Demonstration & Hands-on)

organized by:
SCHOOL OF DENTAL SCIENCES
UNIVERSITI SAINS MALAYSIA

on 26th April 2018

at
UNIVERSITI SAINS MALAYSIA, HEALTH CAMPUS
KUBANG KERIAN, KELANTAN

4 MDE CPD POINTS

PROFESSOR DR.
ADAM BIN HUSEIN
Dean
School of Dental Sciences
Universiti Sains Malaysia

ASSOCIATE PROFESSOR DR.
WAN ZARIPAH BT. WAN BAKAR
Chairperson

APPENDIX H

PLAGIARISM REPORT (TURNITIN)

CYTOTOXIC EFFECT OF PHOENIX DACTYLIFERA L. (AJWA DATE EXTRACT) ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE

ORIGINALITY REPORT

20%	9%	12%	14%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS