Immune -Modulatory Effects of Sidr Honey: Implications for Anti-Proliferative Effects on Cancer Cells

by

Danah Almnayan

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Thesis Examiners/Examinateurs de thèse:

Dr. Robert Lafrenie
(Supervisor/Directeur(trice) de thèse)

Dr. Aseem Kumar
(Committee member/Membre du comité)

Dr. Kabwe Nkongolo
(Committee member/Membre du comité)

Dr. Edmond Lui
(External Examiner/Examinateur externe)

Dr. Jian Chen
(Internal Examiner/Examinateur interne)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. Lace Marie Brogden
Madame Lace Marie Brogden
Acting Dean, Faculty of Graduate Studies
Doyenne intérimaire, Faculté des études supérieures

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Abstract

Honey has become popular as a potential treatment for several ailments, including many cancers. Being a natural product, honey is often considered to be a safe and inexpensive adjunct or sometimes even an alternative to the currently available cancer treatments (including chemotherapy and radiotherapy) that have adverse side effects. Honeys from different parts of the world have shown anti-proliferative, immune-modulatory, and anti-inflammatory actions. Yemeni Sidr Honey (YSH) is a world-renowned honey whose anti-inflammatory activity suggests the possibility of underlying anti-cancer and/or immune-modulatory actions. Our studies have shown that treatment with 1% YSH is able to inhibit proliferation, and induce apoptosis in breast cancer cell lines (MDA-MB-231 and MCF-7) and cervical cancer cell lines (Hela). We also showed that THP-1 monocyte-like cells differentiated by treatment with phorbol ester and then treated with YSH affected their polarization into M1 or M2 macrophages: treatment with YSH for 24 h, enhanced the expression of the M1 phenotype while treatment of the macrophages with LPS and YSH for 48 h increased the level of M2 markers of differentiation. Further, co-culture of the M1 differentiated macrophages with breast cancer cells showed that treatment of the macrophages with YSH decreased tumour cell growth and increased apoptosis. These results suggest treatment with YSH is able to impact cancer via two separate mechanisms: direct impacts on cancer cell survival and activation of anti-tumour immune system (monocyte) activation.

Keywords

Honey, Yemeni Sidr Honey, THP-1, anti-proliferative, apoptotic, pro-inflammatory, anti-inflammatory

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List of abbreviations

2-DG	2-deoxyglucosone
APS	ammonium persulfate
β2M	beta-2 microglobulin
BSA	bovine serum albumin
CCL2	chemokine (C-C motif) ligand 2
CCL5	chemokine (C-C motif) ligand 5
CCD	charge-coupled device
CD14	cluster of differentiation antigen 14
cDNA	complimentary DNA
COX	cyclo-oxygenase
CR	cystein-rich domain (of MR)
CRD	carbohydrate recognition domains
Da	daltons
DCFH	dichlorofluorescein
DCFH-DA	2'3'dichlorofluorescein diacetate
DDT	dithiothreitol
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
RJ	royal jelly

GM-CSF	granulocyte-macrophage colony-stimulating factor
HCl	hydrochloric acid
HEPES	buffering agent (4-(2-hydroethyl)-1-piperazineethanesulfonic acid)
IL-1	interleukin 1
IL-10	interleukin 10
IL-1ra	interleukin 1 receptor antagonist
IL-6	interleukin 6
IκB	inhibitor of kapa B
IκK	IκB kinase
LPS	lipopolysaccharide
M1	type 1 macrophage
M2	type 2 macrophage
MALDI	matrix assisted laser desorption ionisation
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MDC	myeloid dendritic cell
MGO	methylglyoxal
MR	mannose receptor
MRJP	major royal jelly protein
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
MW	molecular weight
MyPo	myeloperoxidase
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDS	nonsteroidal anti-inflammatory drugs
OD	optical density
PI3K	phosphatidylinositol 3-kinases
PAGE	polyacrylamide gel electrophoresis

PBMC	peripheral blood monocytes
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PMA	phorbol-12-myristate-13-acetate
PPR	pattern recognition receptor
Q-RT-PCR	quantitative reverse transcription polymerase chain reaction
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SOD	superoxide-dismutase
SR	scavenger receptor
TAE	tris-acetate EDTA
Taq	enzyme isolated from *Thermus aquaticus*
TCA	trichloroacetic Acid
TEMED	N,N,N',N'-Tetramethyl-etylenediamine
THP-1	human acute monocytic cell line
TLR4	toll-like receptor 4
Tm	melting temperature
TNF-α	tumor necrosis factor alpha
UV	ultraviolet
V/V	volume/volume
Vit-D3	1,25-dihydroxyvitamin D-3
W/V	weight/volume
YSH	Yemen Sidr Honey

Preamble

This thesis has been divided into separate chapters, each containing an introduction, methods, results, and discussion section. The references are found at the end of each chapter. A brief introduction **(Chapter 1)** and final discussion **(Chapter 6)** are presented, but the main topics are introduced and discussed in each chapter.

Chapter 2 studies the anti-proliferative effect and the mechanism of action of Yemen Sidr honey on different cancer cell lines.

Chapter 3 identifies the immune-modulatory activity of Yemen Sidr honey, by looking at macrophage polarization, and measuring (pro- and anti-inflammatory) cytokine expression, after THP-1 cell stimulation by PMA, LPS, honey, or a combination of these.

Chapter 4 looks at cancer cell co-culture with macrophages that had been stimulated by treatment with Yemen Sidr honey in an *in vitro* assay assessing cell morphology and confluency.

Chapter 5 is focused on the compositional analysis of Yemen Sidr honey using HPLC.

The majority of this work was carried out using an *in vitro* cell-based culture system.

Chapter 1 . General Introduction.

1 Background.

Cancer is a disease that has been problematic to researchers and clinical professionals for a very long period of time. However, some of the latest developments have indicated that the use of some natural products, such as honey, may be useful as part of the treatment for cancer either to inhibit cancer growth, moderate cancer symptoms, or minimize the side-effects of cancer treatment. Cancer is annually diagnosed in over 11 million people leading to 7.6 million deaths across the world each year (1). The early stages of cancer are characterized by the presence of only one transformed cell before swift proliferation, invasion of neighboring tissues, and eventually metastasis occurs (2). The dynamic process of carcinogenesis is catalyzed by a range of carcinogens, inflammatory agents, and tumour promoters. The entire process is highly controlled via a variety of cellular activities including cyclooxygenase activity, cell-adhesion molecules, angiogenic factors, protein kinases, transcription factors, anti-apoptotic proteins, pro-apoptotic proteins, and other molecular targets.

Ahmed and Othman (3) insist that the only standard treatments for cancer constitute chemotherapy, surgery, and radiotherapy. However, all of these modalities are largely thought to have serious side effects, which prompts the search for new and more specific treatments to focus only on specific molecules. Some of the natural products commonly used in traditional medicine, such as honey, have surfaced as having anti-cancer effects (2). Essentially, honey contains amino acids, various sugars, phenolic acids, flavonoids, proteins, as well as other miscellaneous compounds (4). The phenolic components, for instance, are believed to have an anti-leukemic activity that attack a range of leukemic cell lines (5). The activity can also act on

tissues such as the oral, breast, endometrial, colorectal, and renal tissues (6). Further, honey can potentiate the anti-tumour activity of known chemotherapeutic drugs like cyclophosphamide and 5-fluorouracil (2). According to the observations made by Vit et al. (7), flavonoids identified as constituents of honey are noted to have anti-cancer activity. In this context, cancer chemoprevention is regarded as one critical issue which can be aligned to the dietary components of honey polyphenols.

Changes in cell survival in response to exposure to modulating agents, such as components of natural products, can be mediated by changes in cell signaling and epigenetic control. This implies that flavonoid constituents might affect multiple pathways of action in mediating the chemopreventive action aligned with them (7). Dietary polyphenols have a large number of properties which are not limited to the effects of epigallocatechin-3-gallate, xanthohumol, catechin, and chrysin, although the activity of these polyphenols have been shown to affect a wide range of the molecular mechanisms linked to activity against colorectal cancer cell lines (5). The studies conducted by Ahmed and Othman (2) have indicated potential value in using Tualang Honey in the treatment of breast cancer. Tualang Honey is produced by the commonly known *Apis dorsata* bee species known for building hives on the Tualang trees, also known as *Kompassia excelsa*, which is found in the Malaysian tropical rainforest (8). Tualong Honey is seen as a potential cancer-preventive agent that also has potent anti-oxidant, anti-diabetic, and anti-inflammatory activities (9). The purpose of this thesis is to investigate the immuno-modulatory effects of Yemeni Sidr Honey (YSH) with a focus on the implications for its anti-proliferative effects on cancer cells. The research investigations in the thesis are also based upon the effects that treatment with honey can have on directing monocytes to show activity during the inflammatory stages of tissue repair. In addition, this study is based on the

fact that there is poor level of understanding on the factors that might affect the healing power of honey (10). This investigation will help in understanding the immuno-stimulatory effect of YSH and its actions on the immune system.

1.1 Honey Overview

Honey remains one of the most valued natural products since ancient times. Having been used traditionally as a nutritional product, honey is also currently being used as an alternative clinical treatment option for various diseases including cancer and wound infections (11). Honey has also been used in some medical clinics as a therapeutic anti-oxidant for various ailments. Published data already suggests it has potential as an anti-bacterial, anti-viral, anti-diabetic, anti-fungal, and anti-inflammatory treatment (11). Eteraf-Oskouei and Najafi (12) demonstrated the use of honey in the treatment of neoplasia and cardiovascular diseases. According to Ediriweera and Premarathna. (13), several other diseases including tuberculosis, throat infections, constipation, eye disease, eczema, ulcers, bronchial asthma, hiccups, dizziness, and thirst, can be "cured" by taking honey.

Honey is a naturally sweet substance produced by bees either from plant nectar or from other plant secretions or from the secretion of other related insects. The color of honey varies from white to amber depending on the floral sources and the mineral content collected by the bees (14),(15). This color reflects the components present, for instance, the amber color is dictated by the presence of pollen, polyphenols, and minerals while the dark color is a result of high concentrations of flavonoids. The chemical structure of honey is complex and this allows treatment with honey to inhibit the growth of more than 60 bacterial species alongside a large number of viruses and fungi. Furthermore, its viscosity and physical nature can provide a

protective barrier that promotes the healing of wound infections (16),(17). The anti-microbial activity of honey can be attributed to the production of hydrogen peroxide (18), nonetheless, non-peroxide-producing honey exhibits anti-bacterial activity whose actions are believed to be manifest through the low pH level of honey and the high sugar content/osmolarity that can hinder microbial growth (16),(18). According to Estevincho et al. (19), the high composition of phenolic compounds in honey is positively correlated with the anti-bacterial activity of the honey. The presence of a wide variety of enzymes, phenolics, peptides, organic acids, and Maillard reaction products (sugar-amino acid conjugates) forms the rationale for honey as an anti-oxidant.

Honey has been shown to exhibit anti-cancer effects against a variety of cancer cell lines collected from breast, renal, prostate, oral, cervical, endometrial, and colorectal tissues (3). This activity is attributed to the high content of phenols and tryptophan in honey (2),(9). In addition, the ability of honey to elicit an immune response from inflammatory cells of the immune system may be used as an alternative approach in the fight against cancer (20). This paper examines anti-cancer immuno-stimulatory effects associated with YSH.

1.2 Composition of Honey

According to the classification of the composition of honey, 79.6% of honey is composed of sugars with 31.3% being dextrose sugars and 38.2 % being levulose sugars. Miguel et al. (21) highlighted that 10% of the constituent weight of honey was composed of disaccharides such as maltose, sucrose, isomaltose, nigerose, turanose, trehalose, and kojibiose, and trisaccharide such as melezitose and maltotriose. The minor constituents of honey, however, encompass 1.27% proteins, 0.17% minerals, 0.1 % amino acids, 0.04% nitrogen, and 0.57% acids (14). Kek et al.

(22) characterized the composition of honey and determined that it contained at least 181 different substances whose composition depends on the seasonal changes. Similarly, 17 % of honey is generally composed of water in which the other substances are suspended. Since honey is predominantly made up of a mixture of sugars which are considered to be pro-inflammatory and carcinogenic (23) it is therefore understandable that some of the beneficial effects of honey associated with cancer raises skepticism. However, many of the beneficial effects of honey are associated with other constituent materials.

Other molecules that are present in honey, including phytochemicals like lycopene, curcumin, epigallocatechin-3-gallate, genistein, and resveratrol have been used for prostate cancer treatment. Phytoestrogens, which are classified as a type of flavonoid, and isoflavones are also present in honey and have been implicated in anti-cancer activity (4). Natural honey contains phenolic compounds, such as caffeine, ferulic acids, ellagic acid, and p-coumaric acid, and flavonoids, such as pinocembrin, galangin, apigenin, hesperetin, kaempferol, chrysin, and quercetin. These simple polyphenols including caffeic acid, galangin, quercetin, chrysin (CR), apigenin (AP), pinobanksin (PB), and Kaempferol (KP), have all been used as pharmacological agents in the prevention of cancer (5),(8).

The presence of flavonoids in all types of honey, including YSH, is associated with anti-cancer properties with mechanisms including the induction of apoptosis, inhibition of cell proliferation, as well as lipoprotein oxidation (24). Honey also contains catalase, reduced glutathione, ascorbic acid, tocopherols, peptides, superoxide dismutase, and Millard reaction products (12). These substances are classified as anti-oxidants and can act as free radical scavengers. Considering that there are relatively small differences between various types of honey (19), it is more than probable that these antioxidant activities are also present in YSH.

Honey has been shown to act as a chemoattractant for neutrophils which are able to produce reactive oxygen species that can kill pathogens (16). The amino acid composition of honey is largely composed of radical scavenging capacity which may aid in the destruction of tumour cells.

1.3 Classification of honey

Classification of honeys has been regarded as a rigorous exercise that requires a chain of testing for the presence of activities and chemicals before establishing it as a specific class or quality of honey. Studies conducted by Kek et al. (22), showed that specific physicochemical properties are important in classifying honey. Some of the properties include color, which may have an impact on the price and the moisture content which may support the stability analysis. At some point, the viscosity may be important in the course of designing the processing equipment. All these properties are important in determining the quality of honey. In recent years, the botanical as well as the geographical origins of the honey have become significant considerations in classifying the honey (24). Classification by origin is dependent on the plant nectar type from which the honey is obtained. In addition, honey can be classified as dark or light depending on the kind and quality of nectar from which the honey was obtained. The glucose content is also an important factor in assessing the quality of a particular type of honey. Some honey is generally thick while another type may be less viscous depending on its composition which is an additional key principle considered in classification. Lastly, the method of production of honey predetermines the various types of honey, which range from extraction from the honeycomb using an extractor and extraction by centrifugation (24).

1.4 Varieties of Honey

Varieties of honey differ depending on origin, flavour, and color. Nonetheless, the various ingredients contained in honey are largely preserved although their proportions may be different. Almost all varieties of honey contain 80% sugar by weight including varying amounts of fructose and glucose. Honeys also contain natural substances from different kinds of nectar that dictate the flavour and color of the honey (24). Honeys with different viscosities are also available and they depict a variation in the manner in which the honey is mixed, heated, and filtered. In addition, honey containing large glucose amounts is more crystallizable and hence becomes opaque and more viscous (4). However, this kind of honey can be warmed to reacquire a liquid state. The three basic honey categories based on origin include single-origin or monofloral honey, multi-floral honey, and local honey (15). For single or monofloral honey, the nectar is collected by the bees from a specific plant part such as the clover. Multi-floral honey is one in which the nectar is collected from different plants, whereas local honey is collected from a specific territory or region such as the mountain area or coastal region (26).

1.5 Biological Activity of Honey

1.5.1 Anti-Oxidant Activity of Honey

The anti-oxidant activity of honey is primarily defined by its ability to prevent cell damage by providing compounds with anti-oxidation properties (11). Honey possesses anti-oxidant properties which are able to modulate the production of free radicals thereby protecting cellular components from peroxidation (6),(19). Several phenolic compounds in honey can reduce the concentration of free radicals thus providing an anti-oxidant capacity (8). Results

from electronic paramagnetic resonance studies have proved that honey can execute its quenching activity on free radical species and superoxide anion radicals due to the presence of methyl syringeate. Similarly, honey also manifests its role in protecting against cellular oxidative damage through the reduction in DNA damage (14), and enhanced glutathione peroxidase activity (27). Generally, the anti-oxidant effects are achieved by the modulation of the activity of anti-oxidant enzymes such as catalase and by the presence of phenolic compounds in honey (27). The brightness of honey has been shown to vary with its anti-oxidant properties where dark honey has more anti-oxidant activity than lighter honey As such, phenolic compounds are thought to be the principal components responsible for anti-oxidant activity in honey. Phenolic compounds are also involved in radical absorbance activity values of honey (11).

1.5.2 Anti-microbial Activity

The anti-microbial activities of honey have been related to the actions of its enzymatic glucose oxidation reactions and physical properties (16). Thus, the inhibition of the growth of yeast and bacteria by honey can result from glucose oxidation, water acidity, and hydrogen peroxide production by the honey (19). Nonetheless, several other factors have been associated with the anti-microbial activities of honey including high or low osmotic pressure, low protein content, low pH acidic environment, high carbon to nitrogen content, low redox reactions due to high reducing sugar content, presence of phytochemical and chemical changes, and high viscosity that reduces the content of dissolved oxygen required by bacteria (11).

Many investigations have demonstrated the anti-bacterial ability of honey by measuring the minimum inhibitory concentration that inhibits the growth of bacteria (16). In this regard, honey inhibits bacterial growth, which can be visualized as areas with reduced growth (eg. larger

inhibition zones) in plates containing cultured bacteria. The Manuka type of honey, from New Zealand and Australia, has the highest non-peroxide activity which has been shown to inhibit the growth of both *Staphylococcus aureus* and *Escherichia coli* (16),(28). Olaitan et al. (29) stated that more than 60 bacterial species are currently known to be inhibited by incubation with honey. Some of these species include aerobes, gram positives, anaerobes, and gram negatives. In a recent study on the effect of honey against methicillin-resistant *Staphylococcus aureus*, honey was shown to be effective on this organism with a minimum inhibition concentration found to be 1.8% to 10.8% (w/v) (30).

Honey has also been shown to treat urinary tract infections caused by *Streptococcus faecalis*, *Escherichia coli*, and *Proteus spp* (31). In this regard, honey has both bacteriostatic and bactericidal activity depending on concentrations. At a concentration of between 8 - 15%, honey exhibits bacteriocidal effects whereas, at a concentration of 5 - 10%, it exhibits bacteriostatic effects (13). One proposed mechanism for the action of honey against bacteria, suggests that the high glucose concentration draws moisture from bacteria hence dehydrating them and causing the inhibition of bacterial growth (16).

The pH range of honey varies from 3.2 and 4.5, which means that any bacterium that does not survive within this pH range dies due to a low pH (12). The growth of certain bacteria depends on the pH; for example, *E. coli, Salmonella. spp, P. aeruginosa*, and *S. pyrogens* require a pH of at least 4.3, 4.0, 4.4, and 4.5, respectively, to be able to grow. As such, the acidity contributed by honey provides some anti-bacterial activity (29).

Eteraf-Oskouei and Najafi (12) also demonstrated that the presence of lysozymes, beeswax, volatiles, propolis, organic acids, and pollen contributes to the anti-bacterial activity of honey. There is a relationship between bacterial inhibition by honey and the sugar composition

of bacteria (4). Hydrogen peroxide, which according to Mandal and Mandal, is the predominant antimicrobial agent in bacteria, is synthesized by bees as well as by catalase that originates from the flower pollen. The concentration of hydrogen oxides in honey is dependent on the glucose oxidase levels in honey. The production of hydrogen peroxide in honey is due to the dilution with water that causes the activation of the glucose oxidase enzymes. These enzymes drive the oxidation of glucose to gluconic acid and hydrogen peroxide. Non-peroxide honey lacks hydrogen peroxide and its anti-bacterial activities involve the enzyme catalase. Most often, the presence of methylglyoxal and methyl syringate provides much of the non-peroxide activity in honey (27).

A recent study conducted by Wang et al. (32) demonstrated additional mechanisms through which honey inhibits bacterial infections. First, the quorum sensing of bacteria is inhibited by honey and results in the retardation of *las, rhl,* and *MvfR* regulon expression and the production of the associated virulent factors . The quorum sensing genes (AI2 importer), curli genes, virulence genes (LEE genes), and Indole synthesis genes are also suppressed by honey. Secondly, honey has been shown to be active against some of the antibiotic resistance properties acquired by bacteria and can still actively kill the bacterial cells (32). Biofilms have been known to play a vital role in the emergence of antibiotic resistance. Biofilms are a complex mixture of secreted polymers and bacteria that forms a physical and chemical barrier against antibiotic penetration thereby creating persistent bacterial infections. Honey is capable of penetrating the biofilms to eradicate bacterial colonies (16). In this regard, honey eliminates bacterial film colonization and preserves antibiotic stocks. Among the components of honey involved in the expression of biofilm disruption, fructose and glucose have been considered to be the primary components for the suppression of bacterial resistance (12),(16). Similarly, honey inhibits the

attachment of bacterial strains to fibronectin at the infection sites. The inhibition is accompanied by the reduction of fibronectin surface-binding protein expression such as the *Sof* and *Sfb1* proteins.

Olaitan et al. (29) ascertained a role for honey in the stimulation of blood lymphocytes, T lymphocytes, and phagocytes. Moreover, honey stimulates the activation of several cytokines including the monocyte-derived, interleukin 1(IL-1β) and IL-6, and tumour necrosis factor (TNF-α), which in turn results in the activation of the inflammatory response of the immune system against bacterial infections

1.5.3 Honey and cancer

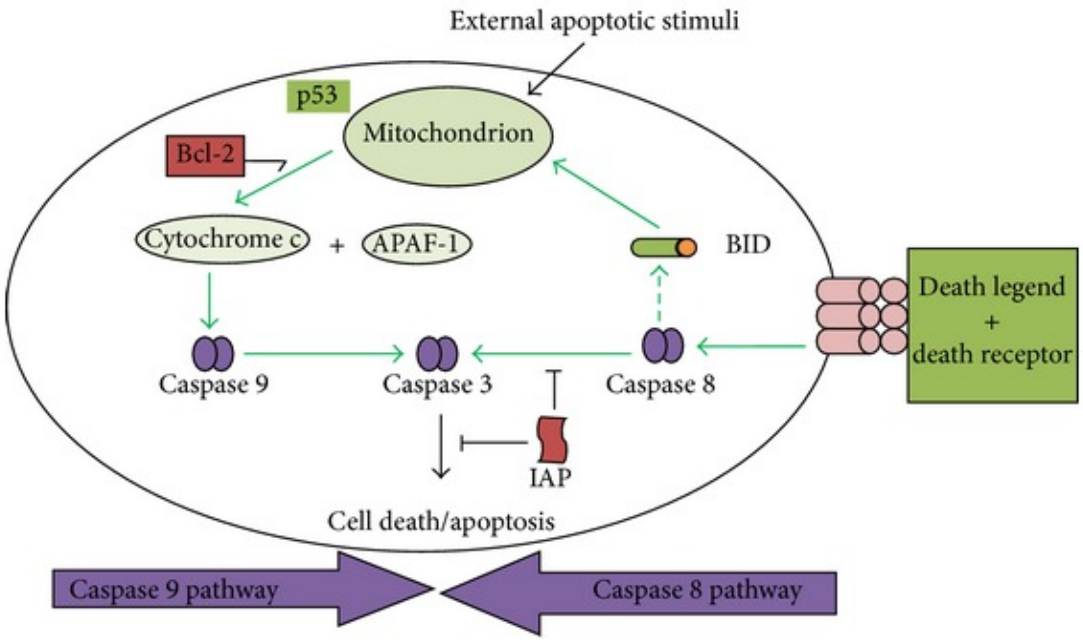
As mentioned, the relationship between honey and cancer is more related to therapeutic support rather than just tumouricidal activity. Honey has largely been regarded as a medicinal used in treating wounds and other infections which may be complications in cancer (2). These observations have prompted many researchers to start thinking in line with how the anti-oxidant and anti-microbial activities can contribute to cancer treatment in the light of useful components of honey. The association between honey and cancer can first be discussed in terms of an induction of apoptotic activity (9). In this sense, it can be asserted that two profound properties of the cancer cells include inadequate apoptotic turnover and uncontrolled cellular proliferation (2). Many drugs known for cancer treatment are said to be apoptosis inducers.

1.5.4 Apoptotic Activity of Honey

Recently, honey has been shown to exhibit anti-cancer properties in animal models and cell cultures. Some of the mechanisms through which anti-cancer effects are achieved include cell cycle arrest, mitochondrial membrane potential disruption, and apoptosis induction (2) Some

of the phytochemicals contained in honey such as the flavonoid and phenolic compounds have been shown to contribute anti-oxidant effects to cancer. Anti-oxidants, as stated earlier, prevent oxidative stress and free radical development, which play important roles in cancer development (20). The main phytochemicals in honey, the phenolic and polyphenols, have also been reported to promote anti-proliferative activity in cancer cells (5),(21).

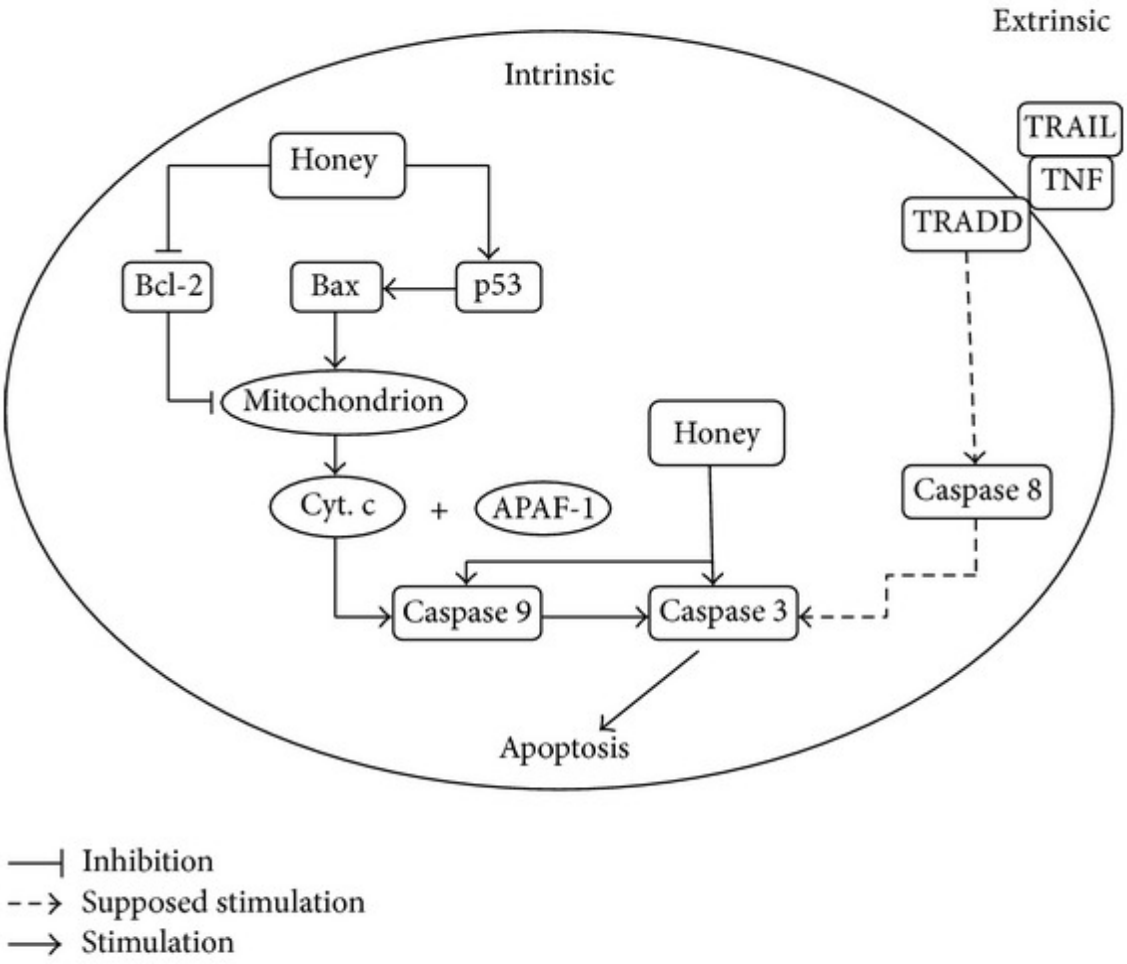
Most cancer therapeutic agents act by inducing cellular apoptosis and/or inhibiting cell proliferation. Apoptosis, or programmed cell death, can be categorized into three main phases: the induction phase, the effector phase, and the degradation phase. During the induction phase, death-inducing signals are produced through the stimulation of a pro-apoptotic signal transduction cascade. The effector phase is involved in cell death via mitochondrial membrane regulation (3).



Schematic Diagram 1.1: The process of apoptosis involving caspase 8 and caspase 9 pathways.

In the degradation phase, the cell shrinks and the nucleus and chromatin condense alongside DNA fragmentation and blebbing of the cell membrane. Similarly, caspase enzymes in the cytosol are activated resulting in cell fragmentation and subsequent phagocytosis of the resulting apoptosomes by macrophages (3). The high tryptophan and phenol content in honey causes an elevation in the levels of *ADP-ribose polymerase (PARP)* cleavage and caspase 3 cleavage. In addition, the induction of apoptosis can be mediated through the regulation and expression of anti-apoptotic and pro-apoptotic proteins in the cancer cells.

Through the generation of reactive oxygen species, honey can cause the activation of p53, which in turn modulates the expression of pro-apoptotic and anti-apoptotic proteins such as Bcl-2 and Bax (9). Studies conducted in Wistar rats highlighted that a combination of honey and *Aloe vera* (33) has significant effects on the induction of the Bax pro-apoptotic proteins and the down-regulation of Bcl-2 expression. Specifically, the Manuka type of honey exerts apoptotic effects through caspase 9 activation that in turn causes the activation of caspase 3, the executor protein. The other mechanisms underlying the anti-cancer effects of honey constitute PARP activation, fragmentation of DNA, and loss of Bcl-2 expression (9).



Schematic Diagram 1.2: The apoptotic effects on honey on cancer cell lines.
The effects of honey are achieved through the modulation and upregulation of pro-apoptotic proteins such as caspase 9, caspase 3, Bax, and P53, and the downregulation of several anti-apoptotic proteins such as Bcl-2 (5).

Fukuda et al. (20) demonstrated the effects of honey constituents on inducing chemotactic activity for neutrophils and generation of reactive oxygen species which proved to pose anti-tumour properties. According to Othman (34), the flavonoids present in honey

stimulate the release of tumour necrosis factor-alpha (TNF-α) and inhibit the proliferation of cells. Similarly, flavonoids and chrysin inhibit lipoprotein oxidation which is one of the major pathways through which cells proliferate to cause cancer. Kadir, et al. (35) stated that Tualang honey is involved in the amelioration of 7,12-dimethyl-benz[a]anthracene (DMBA)-mediated carcinogenesis by causing histological grading and modulation of the severity of the induced tumours. In another study by Yaacob and Ismail (36), honey was shown to increase tamoxifen-induced cytotoxicity against various types of cancers thus demonstrating its anti-cancer effects.

1.5.5 Anti-proliferative Activity

Epithelial cells tend to divide throughout their lifespan. The cell cycle is composed of three different stages including; G_0/G_1, S, and G_2/M. Cell cycle events are monitored and regulated by several different proteins including cyclins and cyclin-dependent kinases. The G_1/S phase is a vital regulator of cell apoptosis, differentiation, and proliferation. Dysregulation and overexpression of cell cycle growth factors, cyclin, and cyclin-dependent kinases are directly associated with tumourigenesis and are a hallmark of cancer (2). Honey can affect the cell cycle arrest process. For instance, the administration of honey together with aloe vera reduced the proliferation of tumour cells in rats (33). Treatment of Wistar rats that had been injected with W256 carcinoma cells with oral administration of aloe vera and honey solutions resulted in smaller tumours and a decrease in Ki-67 staining (a proliferation marker) and increased Bax/Bcl-2 expression ratios in the tumours. This implies that honey therapy for cancer could be used in lowering cell proliferation. Several of the different components present in honey, including phenolics and flavonoids, have been shown to block cell cycle progression in melanoma, glioma, and colon cancer cell lines in the G_0/G_1 phase (37). The inhibitory effect of honey on tumour cell

proliferation also affects other downstream processes affecting various enzymes including tyrosine cyclooxygenase, kinases, and ornithine decarboxylase which are involved in cell proliferation.

1.5.6 Effect of Honey on Tumour Necrosis Factor-α (TNF-α)

Tumour necrosis factor-α (TNF-α) mediates tumour promotion, initiation, and progression. TNF-α pro-inflammatory activity is linked to various diseases due to its activation of NF-kB. Activation of NF-kB results in the expression of inflammatory genes like cyclooxygenase-2 (COX-2), lipoxygenase-2 (LOX-2), inducible nitric oxide synthase, cell-adhesion molecules, and inflammatory cytokines (20). Royal jelly (RJ) proteins contain apalbumin-1 and apalbumin-2 which have been shown to have anti-tumour properties (38). The above proteins have been shown to stimulate macrophages to release various cytokines including interleukin-1(IL-1), IL-6, and TNF-α. Manuka, jelly bush, and pasture honey have also been shown to stimulate monocytes to produce various cytokines including interleukin-1β (IL-1β), IL-6, and TNF-α (17) . The mechanism suggested reveals that the cell surface TNF-receptor binds to TNF-α and other adaptor proteins such as TNFR-associated death domain protein (TRADD) to regulate cellular responses such as inflammation and apoptosis. TNF-α release ensures that cytokines which are essential in regulating inflammation, apoptosis, and cell proliferation are released (17).

1.5.7 Immuno-Modulatory Activity of Honey

Normal wound healing is a process that coincides with several body processes including inflammation, tissue remodeling, replacement of damaged tissues, coagulation, cell proliferation, and inflammation. In this regard, honey has been employed in the treatment of several types of

burns, and diabetic foot, chronic, and split skin wounds (18),(39). During the wound healing inflammatory phase, honey is essential in the elimination of any possible necrotic tissues which is accompanied by the resultant inhibition of bacterial growth and tissue remodeling (18),(40).

Increased chemotactic recruitment of leukocytes such as neutrophils, monocytes, macrophages, and lymphocytes as well as the increased production and release of cytokines including IL-1, TNF-α, and IL-6 is facilitated by honey. These components are important in enhancing the healing process. In a recent study, the increased production of TNF-α and IL-6 at the wound healing site was shown in mouse models of wound healing. In this regard, the release of these cytokines was facilitated by honey (41).

Honey also possesses high osmolarity in which water is dehydrated from the sites of application thereby enhancing wound healing. Osmotic effects, therefore, contribute to the drawing of water from the wound site through the outflow from the lymph thus resulting in wound drying (41). Research has also indicated that honey can improve wound healing through anti-oxidant-mediated responses by specifically activating the 5' adenosine monophosphate-activated protein kinase and several other anti-oxidant enzymes that are involved in the amelioration of oxidative stress (39). These anti-oxidants stimulate the migration and proliferation of dermal fibroblasts that are key during wound healing. Moreover, anti-oxidants modulate mitochondrial function further assisting in wound healing (39).

Esterat-Oskouei and Najafi (12) highlighted that almost all kinds of wounds including diabetic wounds, burns, traumatic wounds, sickle cell ulcers, amputations, cracked nipples, abrasions, septic wounds, surgical wounds, and abscess were responsive to honey. Honey contains a variety of minerals, amino acids, sugars, and vitamins that may have a key role in the support of phagocytosis to engulf bacteria through glucose consumption. Honey can also

stimulate monocytes to release reactive oxygen species that initiates an inflammatory response within the body (40). The result is edema, which restricts the circulation of blood and oxygen in the surrounding tissues. Ultimately, cell growth is restricted to allow for the replacement of dead tissues thus promoting wound repairing (6).

Inflammation refers to the biological responses against detrimental stimuli that are exhibited by the vascular tissues and phagocytes. In other words, it is a defensive mechanism by which organisms and tissues eliminate pathogens that may cause injury to the body (42). The classification of inflammation falls into two broader categories; acute inflammation and chronic inflammation. Acute inflammation is short-acting and presents as an early response towards detrimental pathogenic stimuli by the body. This kind of inflammation may present as pain, loss of function, redness, and itching. Failure to treat acute inflammation can result in tissue damage or may progress to chronic inflammation, which is more prolonged (42). As such, there must be a normal anti-inflammatory action to turn off the inflammatory response to counteract prolonged disease conditions such as cancer, kidney diseases, and liver diseases. For pro-inflammatory responses to take place, several key players are involved including cyclooxygenases (COXs), mitogens, cytokines, microphases, lipoxygenases (LOXs), TNF factors, among other players (6),(43).

The inflammatory pathways involve two main components, the nuclear factor-kappa B pathway and the mitogen-activated protein kinase pathway (40),(42),(43). The activation of these two pathways are the hallmarks for the induction of various proteins, inflammatory mediators, proteins, genes such as lipoxygenase 2, and cytokines including IL-1, IL-10, IL-6, cyclooxygenase 2, TNF-α, and C-reactive proteins (43). Some of these can also act as anti-inflammatory factors that play important roles in angiogenesis-related to disease etiology as well

as inflammation. Documentation of the anti-inflammatory effects of honey has been reported in experiments using various cell cultures and animal models, however, the anti-inflammatory mechanisms underlying the effects of honey are still to be resolved (5),(11).

To understand some of the anti-inflammatory mechanisms exhibited by honey, a study conducted by Hussein et al. (42) demonstrated that treatment with honey decreased NF-kB levels and decreased the levels of pro-inflammatory cytokines and other mediators in blood plasma such as the NO, IL-6, COX-2, PGE2, TNF-α, and INOS. It was indicated that honey causes a decrease in edema and attenuates the translocation of NF-kB to the nucleus as well as decreasing proteolysis of the kappa B inhibitor (42). Suppression of the pro-inflammatory activity associated with certain pro-inflammatory enzymes within the cell is primarily brought about by flavonoids and phenolic acids such as quercetin, chrysin, and galangin. Some of the inhibited enzymes include prostaglandins, cyclooxygenase 2, and nitrogen oxidase synthase.

Flavonoid content in honey has also been linked with down-regulation of the expression of matrix metalloprotease 9, which is a major inflammatory mediator during chronic inflammation. Alongside the down-regulation of matrix metalloprotease 9, honey also inhibits the expression of pro-inflammatory cytokines such as IL-1 and anti-inflammatory cytokines such as IL-10 as well as transforming growth factors and platelet-derived growth factors (44). Other mechanisms by which honey is involved in the inhibition of inflammation are through interfering with the stimulation and the release of monocytes, neutrophils, and microphages which promotes inflammation. Similarly, the inhibition of leucocyte and keratinocyte cytokine release reduces any possible inflammation. Moreover, the stimulation of hydrogen peroxide production by honey enhances the growth of epithelial cells and fibroblasts which promotes the repair of inflammatory damage (45).

Samarghandian et al. (11) stated that the suppression of pro-inflammatory activities executed by nitric acid synthase and cyclooxygenase 2 were primarily mediated by the presence of phenolic compounds and flavonoids in honey. Moreover, the ingestion of honey can result in slow gastrointestinal absorption and lead to the production of short-chain fatty acids due to its ability to stimulate the growth of colonic bacteria which are then able to produce short-chain fatty acids. These fatty acids are involved in immune modulation. Furthermore, the immune responses in the body can be induced by the sugars present in honey. The sugar components in honey have been shown to promote immunopotentiation effects such as the Niger oligosaccharides. The sugar constituents in honey may have potential effects on the modulation of the immune system and immune responses (46).

1.6 Yemini Sidr honey

Findings recorded by Roshan et al. (26) have shown that beekeeping and honey production are regarded as one of the oldest professions in Yemen. The most dominant varieties of honey include the Elb honey and the Sidr honey, which contributes to a total annual production of 5600 tons/year. Sidr honey is largely produced in the Gerdan and Dawan valleys in the Shabwa and Hadramout governorates. Produced with little human interference, Sidr is believed to attain the best quality and appears at the top of the reputation and popularity chart of monofloral honeys across the world. A number of techniques have been developed to determine the authenticity of the honey with the help of pollen analysis (26). This is especially important because the popularity and high value of Sidr honey has made it profitable to sell adulterated or forged products. The activity of YSH has been indicated for the treatment of peptic ulcers. YSH contains a variety of phenolic compounds which have been associated with its health-promoting

characteristics. The polyphenol fraction of YSH also contains another compound that have profound therapeutic properties known for enhancing endothelial function (26).

The pure mountain YSH has been shown to contain high levels of anti-oxidants compared to the other types of honey. Additionally, this type of honey has been described as having anti-inflammatory, pain-relieving, and body cell stabilization activities, as well as being able to provide protection against injury. A pain relief study conducted in rats on the activity of YSH demonstrated high pharmacological effects of YSH compared to aspirin and indomethacin analgesics (47). In this model, pain was generated directly via injection of acetic acid and pre-treatment of the rats with Yemeni Sidr honey reduced the acetic acid –induced writhing response, similar to those of the reference drugs. This type of honey has also been documented to exhibit high anti-bacterial activity against *Staphylococcus. spp*, *E. coli, S. aureus,* and *P. aeruginosa* at a concentration of 10-20% (47). Taha et al. (48) also demonstrated that YSH was effective in wound healing as well as in treating stomach ulcers. Similarly, YSH has been linked to the promotion of menstruation and in the treatment of epilepsy. The antioxidant activity of YSH has provided some of the reason for its prominent use within Saudi Arabia and other areas of the Middle East. In Saudi Arabia, Sidr trees are found in areas close to Yemen, where the Kingdom of Seba used to be "the home of Sidr" which is mentioned in the Holy Quran verse 16 of Sura Saba.

1.7 Activity of Yemini Sidr honey

The capacity or power of honey to kill microorganisms depends on the phytochemical nature of the honey, pH values, and osmolarity. Ghramh et al. (50) performed a large study on the anti-bacterial and immuno-modulatory activity of YSH. YSH is believed to have the ability

to inhibit the growth or survival of at least 62 different species of bacteria including gram negatives, gram positives, anaerobes, and aerobes. YSH is often used for medicinal reasons in the course of treating ulcers, lung infections, liver diseases, burns, digestion problems, and even the consequences of malnutrition, among others. At the same time, YSH is said to have strong anti-bacterial and anti-oxidant activities (26). Such properties have made this type of honey useful as an adjunct to chemotherapy as far as treatment of cancer is concerned. Ghramh et al. (50) also noted that YSH can be used to synthesize honey-silver nanoparticles. Based on their observations, Ghramh et al. noted that YSH incorporated into the silver nanoparticles, can stimulate the development of splenic cells in rats. In addition, treatment with YSH has the capacity of inhibiting the proliferation of the HepG2 cancer cell line. This means that this type of honey can be utilized as an anti-microbial agent as well as a potential anti-cancer agent.

1.8 Macrophages

Macrophages are known to be derived from the bone marrow where hematopoietic stem cells differentiate from progenitors to differentiated leukocytes (51). Macrophages are also regarded as significant components of the innate immune system derived from myeloid progenitor cells. The latter develops into what is referred to as the promonocytes and later differentiates into monocytes. The monocytes eventually migrate via the circulatory system into tissues where they differentiate into macrophages. Monocyte/macrophages can be understood as large phagocytic cells that are ultimately found in static form in tissues. Under normal circumstances, macrophages are found as part of the structure of most of the connective tissues and organs like liver, spleen, skin, central nervous system, and lymph nodes or sinus histiocytes (51). Notable examples of the macrophages in tissues include the Kupffer cells that are found as

part of the structure of the liver as well as the alveolar macrophages found in the lungs. They can also appear as mobile white cells at the sites of infection. They are also known to be a set of immune cells that can change in their functional state based on environmental stimuli. However, macrophages play a focal role in terms of tissue development, wound healing, response to infection, as well as other physiological conditions. Macrophages have gained attention from researchers since they appear to be essential in controlling a wide variety of physiological processes and since there appears to be different types of macrophages that are able to mediate different processes (52). Notable discoveries, that were originally described in the 1970s and 1980s, showed the capacity of macrophages to promote as well as inhibit cell growth in tumours. Macrophages are categorized by various markers including transmembrane glycoproteins, enzymes, scavenger receptors, growth factors, cytokines, hormones, and cytokine receptors (51). Macrophages are frequently classified as M1, or pro-inflammatory type macrophages, or M2, or anti-inflammatory type macrophages. The binary classification of macrophages (M1/M2) does not account for the variety of activities exhibited by the macrophages. Instead, macrophage polarization is a spectrum, rather than a strict binary phenomenon with many cells expressing both M1 and M2 characteristics to different extents. The changes in gene expression reported in tissue resident macrophages and bone marrow-derived macrophages (for example, in microglia and liver macrophages) do not alter the potential biological functions of the macrophages to any significant degree (52). Tumour-assoicated macrophages typically express an M2 (pro-tumourigenic) phenotype, and are considered markers for cancer diagnosis and prognosis, as well as therapeutic target in cancers.

1.8.1 Macrophages in cancer

It is quite interesting to learn how macrophages can be related to cancer. Lin et al. (53) suggested the idea that the macrophage in cancer is associated with studies revolving around the differentiation into tumour-associated macrophages (TAM) and their ability to promote tumour metastasis. Tumour metastasis is a key contributor to the mortality of cancer patients. As the tumours spread and grow in tissues throughout the body they cause significant disruption of organ systems and depletion of energy stores (53). The process of metastasis is influenced by intrinsic alterations in the ability of the tumour cells to migrate and grow, increased cross-talk between the tumour cells, and changes in the microenvironment all promote tumour spread. For example, TAM are regarded as key cells in the creation of an immunosuppressive tumour microenvironment, via the production of growth factors, cytokines, and chemokines, that are thought to promote metastasis. In the course of understanding macrophages in cancer, Lin et al. (53), highlighted the fact that macrophages are versatile immunocytes that have a spectrum of functions including defense against the pathogens, enhancement of wound healing, and modulation of tissue homeostasis.

Macrophages are known to infiltrate the tumour tissues to differentiate to form TAMs. The macrophages, which become the TAMs, are commonly recruited to the tumours through chemotaxis to growth factors as well as to chemokines that are known to be secreted by stroma cells and by the cancer cells. Some clinical studies have indicated a correlation between the presence of TAMs in tumour tissue and poor prognosis for endometrial, bladder, cervical, breast, esophageal, and prostate cancers (45).

1.8.2 Role of Macrophages in Inflammatory Activity

Cytokines are proteins commonly produced by macrophages and lymphocytes which activate signals in other leukocytes (and some other cells) to alter the function of the cell and promote various activities, including inflammatory processes, anti-inflammatory processes, cell growth and differentiation, tissue repair, coagulation, etc. In some instances, cytokines can be produced by endothelial and epithelial cells. Cytokines are essential in the functioning of macrophages through a number of different approaches (51). First, the presence of specific cytokines is required to mediate an effective immune response, influence the macrophages' environment, and link the adaptive and innate immune responses. In addition, specific cytokines can control the differentiation of macrophages into different functional subsets; the M1 macrophage subset which are primarily involved in mediating pro-inflammatory responses can be induced by treatment with interferon-gamma and other cytokines derived from Th1 helper cells; and, the M2 macrophage subset which is more involved in mediating tissue repair can be induced by treatment with IL-4 and IL-5 produced by Th2 helper cells (52). Based on their subset classification as well as their microenvironments, macrophages can be alternatively or classically activated to release different cytokines with these processes being regulated through subset-dependent differences in intra-organellar exchange as well as cytoskeletal remodeling and vesicular trafficking.

The M1 and M2 subtypes of the macrophages play important roles in regulating the immune response and homeostatic functions as well as in cancer therapy (45). In addition, macrophages are phagocytic and they form a major part of the immune system. The polarization mechanism of inflammatory macrophages (M1) is associated with the production of proinflammatory cytokines and reactive oxygen species that are responsible for body defense and

the death of tumour cells (54). M2 macrophages, on the other hand, produce cytokines involved in inhibiting some aspects of inflammation and promoting processes related to tissue repair (54). More recent ideas about macrophage polarization into the M1 and M2 subtypes indicate that individual macrophages may have properties corresponding to both the M1 and M2 phenotypes and that rather than being an all-or-nothing phenomenon, most cells exist somewhere on a spectrum between M1 and M2 (53). TAMs, which resemble the M2 macrophages, have been highly associated with cancer modulation, especially through the role they play in apoptosis and cell proliferation. This class of macrophages is involved in tumourigenic processes such as angiogenesis, invasion, transformation, cell proliferation, and metastasis. Conventionally, macrophages have been considered to be anti-tumourigenic by playing roles in the suppression of various tumours (55).

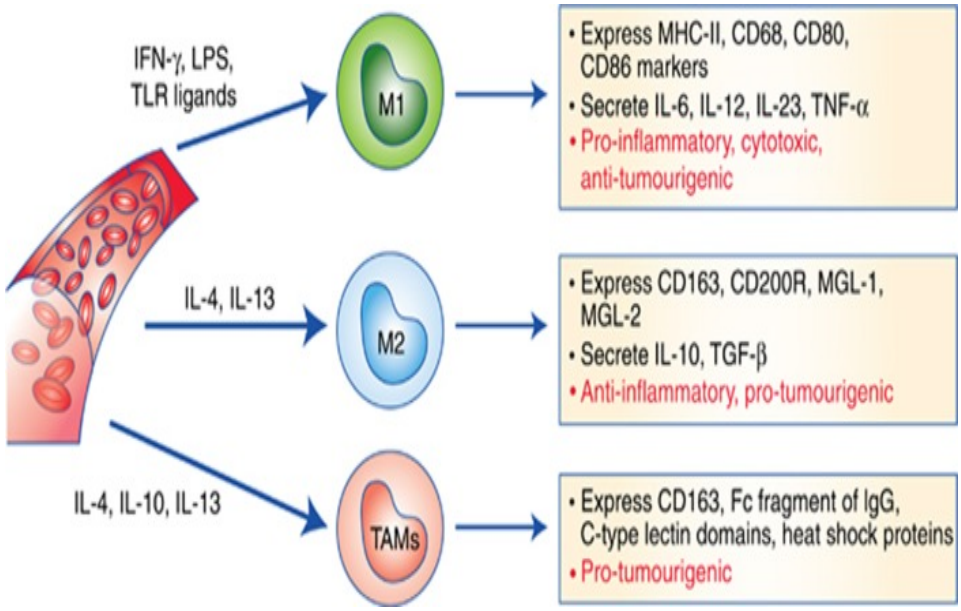
According to Ong et al. (55), macrophages produce pro-inflammatory effects that result in the inhibition of tumour cell growth through the secretion of chemokines that attracts the T cells which are able to prime the type 1 anti-inflammatory responses which have anti-tumour effectors. TAMs also demonstrate anti-tumour functions when stimulated by the anti-CD 40 and TRL ligands, and by interferon-alpha. This can promote macrophage-dependent phagocytosis of tumour cells provided tumour cell phagocytosis is not affected by the expression of CD47 since CD47 is generally considered a marker of "self" that inhibits immune system destruction.

Through the production of reactive oxygen intermediates and reactive oxygen species, TAMs can suppress tumour growth. Moreover, mutations in the genes encoding for colony-stimulating factor and macrophage-specific growth factors expressed in cancer cell lines have been shown to inhibit metastasis and decrease the progression of several tumours (56). Colony-stimulating factor 1 can also cause increased infiltration of TAMs and recruitment of CD8+

cytotoxic T cells, which in turn reduces tumour growth in different body organs (56).

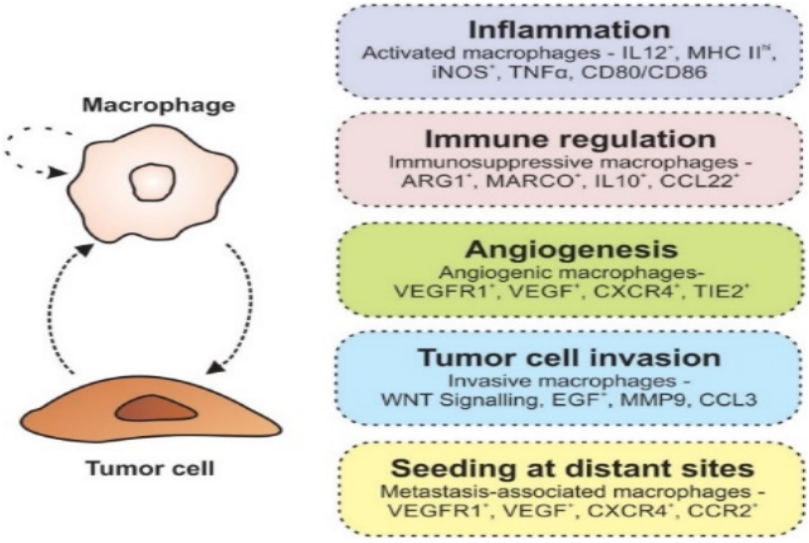
Chemotherapy resulting in the induction of cell death in the cancer cells can be inhibited by the expression of cathepsin by macrophages. Cell death protein 1 (CD1), which is a negative regulator of the phagocytic activity of macrophages against tumour cells, can be produced by TAMs thereby reducing the growth of tumour cells (57).

The secretion of IL-10 from macrophages is stimulated by IL-3 and IL-4 treatment of the M2 macrophages, which in turn acts as a leukocyte chemokine which along with transforming growth factors (TGFs) causes changes in tumour progression and remodeling (54). Similarly, the function of TAMs is controlled by interacting functions of multiple proteins and the activity of the M2-like macrophages within the tumourous environment involves the expression of certain M2-specific markers including CD163, IgG immunoglobulin F_C receptors, heat shock proteins, and lectins including types of the C type domains (54).



Schematic Diagram 1.3: The differentiation of macrophages and their role in tumourigenesis.

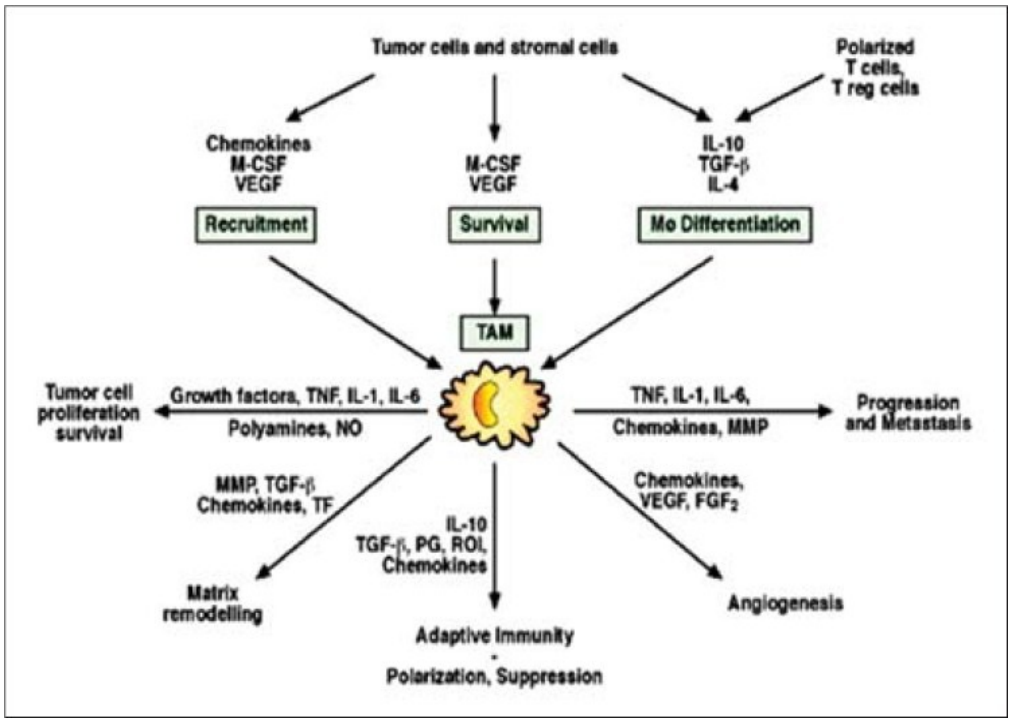
The M1 macrophages are primarily activated by TRL, IFN-α, and LPS which promotes the secretion of the pro-inflammatory cytokines that play important roles in inhibition of tumourigenesis. Activated M2 macrophages alternatively secrete anti-inflammatory cytokines such as TGF-β and IL-10 upon stimulation by 1L-4 and IL-13. The resultant effects are competing tumourigenic roles (45). Poh and Ernst (58) demonstrated the role of macrophages in the promotion and rejection of tumours as well as in metastasis. Macrophages in this regard may contribute to more than 50% of the tumour mass. Some of the circulating cells that include the monocytes and the myeloid-derived suppressor cells can differentiate into tumour-associated cells. For the suppression of tumour growth, monocyte-derived TAMs are involved (45). TAMs have also been shown to cause the death of various tumour cells through the initiation of macrophage migration inhibitor factor production. This component inhibits macrophage recruitment and stimulates important functions involved in tumour inhibition such as phagocytosis, TNF-α secretion, ILK1β release, and enhanced cellular cytotoxicity (45).



Schematic Diagram 1.4: Role of macrophages in tumourigenesis.

The interactions that occur between tumour cells and macrophages causes pro-tumourigenic properties (54).

Vinogradoc et al. (59) highlighted the involvement of myeloid-derived suppressor cells, regulatory T cells, and TAMs in the growth, invasion, and metastasis of tumours. Poor prognosis in various cancer cells has been linked to high levels of TAM mass. TAMs also have an important role in the increased expression of IL-10 and TGF-β thereby contributing to local immunosuppression in the late stages of cancer (60). According to Qian and Pallord (61), macrophages are involved in the promotion of features that are involved in intravasation and motility of tumour cells during the process of tumour progression and promote persistent growth and extravasation of tumour cells. TAMs can be highly correlated to the expression of IL-8 and the resultant anti-tumoural micro-vessel density in lung cancers.



Schematic Diagram 1.5: An illustration of the role of macrophages in cancer regulation (62).

Co-culture of macrophages with breast cancer cell lines causes the up-regulation of MMP-3, 2, 7, and 9 as well as the up-regulation of TNF-α in the M2 macrophages which enhances the invasive activity of the cancer cell lines (59). In the breast cancer cell lines, co-incubation with macrophages activates the c-Jun-NH2 kinase and the NF-kB signaling pathways, which are TNF-α dependent. In addition, the secretion of growth factors by TAMs can promote cancer cell proliferation (59).

1.9 Research Aims

Recently, both patients and cancer researchers are turning towards alternative medicines, such as the use of natural remedies, for cancer cure and management (2). One of the main problems with cancer is that cancer cells use the factors produced by the host's own immune system to grow and spread, and ultimately become deadly. One of these immune cells found in tumour microenvironments is the macrophage. Ideally macrophages work by phagocytosing and destroying whatever they do not recognize as healthy tissue, including pathogens or diseased cells (63). They can be activated into M1 macrophages, which are in a pro-inflammatory state that helps to kill pathogens or damaged cells, such as cancer cells. Alternately, they can be activated into M2 macrophages, which can repair and remodel damaged tissues (61) . The M1 macrophages can kill cancer cells by themselves in a direct manner or indirectly through recruitment of other immune cells, such as cytotoxic T-lymphocytes. However, the cancer cells can communicate with the macrophages and change their function to support the growth of the tumour (64). Recent treatment strategies have focused on harnessing the ability of M1 macrophages to fight cancer cells. However, most of these strategies have severe side effects (58). The aim of this thesis is to use YSH to safely activate macrophages to kill cancer cells

without secondary side effects. The hypothesis of fighting cancer, by utilizing macrophage activity modulated by the use of natural products such as plants and herbs has been suggested. Honey has some promise as a natural product because it is reputed to aid in wound healing and have immuno-modulation activity which seems relevant to anti-cancer activity (36). More recently, several reports have demonstrated that honey, being rich in polyphenols and flavonoids, has anti-proliferative effects against cancer cells. However, the mechanisms for the anti-cancer effects are still to be fully elucidated. Honey from different floral sources may have different heath benefits. That is why this study is investigating the activity of Yemeni Sidr Honey (YSH) which is considered to be one of the rarest and most expensive honeys in the world, in part because of the unique nutritional and medicinal properties of the Sidr tree itself. YSH has potent anti-inflammatory activity, which suggests the possibility of underlying anti-cancer and/or immune-modulatory actions. However, the empirical evidence is lacking regarding the effect and the mechanism of YSH on cancer cells

The study is focused on the following specific objectives to achieve the aim of assessing the potential for YSH as an anti-cancer treatment:

1) To investigate the anti-proliferative, apoptotic, pro-inflammatory, and anti-inflammatory effects of YSH on various human cancer cell lines to report its anti-cancer potential.

For the *in vitro* viability assay, tumour cells grown in culture will be treated with serial dilutions of honey prepared in sterile culture medium and cell viability will be determined by doing MTT and SRB assays to determine viable cell number after 24, 48, and 72 h of incubation at 37^{o} C. Cells treated with camptothecin will serve as positive control. The percent cell viability

of honey-treated groups will be compared with untreated cells and cells treated with equivalent amounts of sugar.

Moreover, morphological determinations of apoptosis will be done through light and fluorescent microscopy after staining cells with acridine orange and ethidium bromide. Flow cytometric analysis to look at the DNA content of propidium iodide-labeled cells will be done to assess apoptotic action. Western blot analysis will also be used to validate changes in the expression of apoptotic regulators.

2) To identify the immune-modulatory activity of Yemeni Sidr honey, by looking at macrophage polarization and measuring (pro- and anti-inflammatory) cytokine expression, after THP-1 cell differentiation by PMA, LPS, honey, or a combination of these.

Macrophages will be obtained by culturing THP-1 cells in the presence of phorbol 12-myristate 13-acetate (PMA) for 3 days. These THP-1-derived macrophages will be incubated with different concentrations of YSH for 12 and 24 h. The positive controls for M1 and M2 polarization will be THP-1-derived macrophages treated with LPS, and with IL-4, respectively. THP-1-derived macrophages differentiated into M1 and M2 subtypes will also be incubated in the presence of YSH to determine whether YSH can interfere with M1 and/or M2 polarization. M1/M2 polarization will be measured using established M1/M2 cell surface markers (CD80, CD68, CD163) by flow cytometric analysis and M1/M2 cytokine secretion levels (TNF-α, IL-1, and IL-6 versus IL-10 and TGF-β) by ELISA.

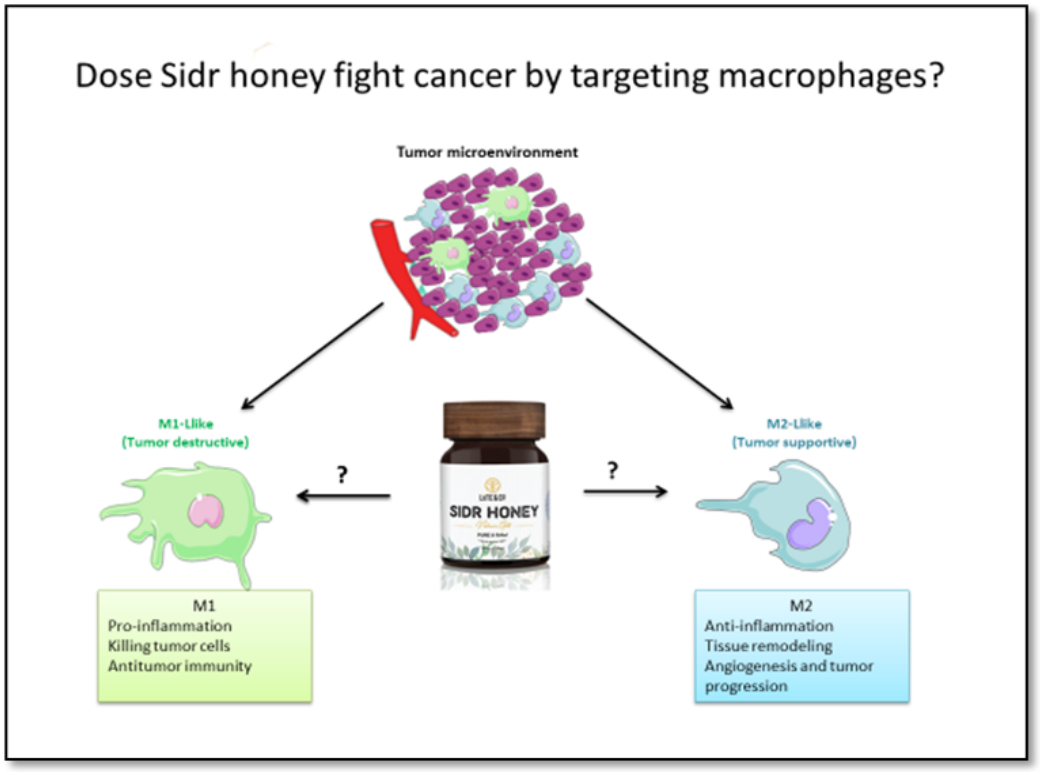
3) To observe if the rate of YSH-induced apoptosis in human cancer cell lines co-cultured with TAM depends on M1/M2 status.

Human breast cancer cell lines will be co-cultured with THP-1- derived macrophages and exposed to YSH to see if the apoptotic effects (as per study 1) depend on M1/M2 polarization.

Also, following co-culture for 48 h, cells will be harvested for subsequent experiments such as migration, and sphere formation assays to determine the effect of co-culture on tumour cell behaviour and function.

4) Chemical analysis of Sugar, phenolic acid, and flavonoid using HPLC

The YSH samples used in these studies will be analyzed for the proportion of various sugars as well as for a variety of phenolic or flavanoid compounds using HPLC. The characterization of YSH composition will be compared to published standards to confirm these samples are consistent with YSH and to show that our YSH samples have similar composition.



Schematic Diagram 1.6: An Overview of the project (65),(66).

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Chapter 2 . Investigate the anti-proliferative, apoptotic effects of Yemen Sidr Honey on various human cancer cell lines

Abstract

Honey has become popular as a potential treatment for several ailments, including many cancers. Honeys from different parts of the world have been shown to have different anti-proliferative, immune-modulatory, and anti-inflammatory actions. For example, Yemeni Sidr Honey (YSH) is world-renowned for its anti-inflammatory activity and has been suggested to have anti-cancer and/or immune-modulatory actions. However, empirical evidence is lacking. We have been investigating YSH's apoptotic, anti-proliferative activities in *in vitro* models of cancer growth. Our studies have shown that YSH is able to inhibit proliferation, and induce apoptosis in several human cancer cells lines. Treatment of breast cancer cell lines (MDA-MB-231 and MCF-7) and cervical cancer cell lines (Hela) with 1% (w/v) YSH in media almost completely inhibits cell proliferation, and promotes cell apoptosis at the 72 h time point.

2 Background

Cancer is one of the most devastating illnesses burdening modern medicine and has become a target of extensive research and practical attention. Among the most challenging aspects in the management of cancer is the tendency of chemotherapeutic agents to induce undesired side effects and decline in efficiency as cancer cells develop physiologic resistance based on a variety of molecular mechanisms **Error! Reference source not found.**,**Error! Reference source not found.**. The frustrating ineffectiveness of mainstream treatments is compounded by the expense accompanying cancer treatment. Driven by these challenges, a combination of researchers, health practitioners, and other professionals have investigated several other substances for potential application to cancer management, a venture that has revealed the capacity for several alternative medicinal practices to be useful in anti-cancer therapy: among these the use of honey appears to have a role in treating patients with cancer (3),(2),(4). Honey has long been appreciated for its utility in a wide range of situations, including wound-healing, antimicrobial, and anti-inflammatory effects. One of the more recent findings is the inhibitory effects of honey on cancer cell growth, an effect that has been speculated to arise from effects on various molecular pathways, including apoptotic pathway modulation, molecular regulation, anti-oxidation, and anti-inflammatory activity by honey components such as propolis (5),(2),(4). However, modern investigations into the role of honey in these conditions, particularly in the form of *in vitro* studies, have yielded several revelations regarding both its nature and on the basis of its properties (6). One such revelation is that the chemical composition of honey (7),(8), as determined by such methods as HPLC, varies widely, mostly according to the geographical location where the sample is obtained and the species of trees /plants which are available to the bees in that locality (9),(10). This realization underlies the interest in Yemen Sidr

honey as an example of a natural product with distinct anti-cancer effects: the determination of the mechanisms by which Yemen Sidr honey inhibits cancer cell growth is the subject of this study.

In Yemen, beekeeping is an ancient profession, dating as far back as the 10th century BCE. Today, the country produces as much as 5600 tons/year of natural honey which, unlike that produced in other countries, is of several varieties. The export of this product, mainly to the Gulf States, generates annual revenues of approximately U.S. $40 million (9). However, a significant proportion of Yemeni honey is the famous Sidr honey, produced by bees from the nectar of *the Ziziphus spina-christi* tree (9),(11). The natural forms of this product obtained from the Dawan and Gerdan areas of Hadramout and Shabwa are of high value since the hives are inhabited by bees with no human interference, culminating in one of the world's best quality monofloral honeys. Consequently, the product has traditionally been highly priced, a situation that has also contributed to by its limited availability (12),(13). In other locations, Sidr honey is harvested from bees that derive the nectars of the jujube tree (also known as Christ's Thorn Jujube), and utilized for a variety of medicinal purposes (14). Like several other honey varieties, Sidr honey has historically grown to occupy an important position in alternative medicine. The product has been hailed as an effective therapy for such ailments as constipation, gastric and duodenal ulcers, as well as for wound healing (15) . Moreover, Sidr honey is used for maintenance of optimal function of the nervous and gastrointestinal systems. Its anti-inflammatory and anti-oxidant effects have been deemed beneficial in preventing hepatic injury (12),(16) In addition, it has been used as an anti-microbial agent in traditional medicine practice. Sidr honey is also considered to be useful in resolving insomnia, allergy, arthritis, as well as anemia. Due to the high demand and high value of Sidr honey, it has commonly been adulterated with other types of

honey or with other products in order to increase its volume for sellers. Consequently, scientific methods to determine the purity of samples of the product have been described by several studies (9),(17). Acknowledgement of this concern is important not only to ensure the accuracy of investigations using samples of Sidr honey but also for potential development of therapeutic derivatives from such samples. Beyond the longstanding rivalry of this variety of honey with other premium types such as the New Zealand's Manuka honey, most of its uniqueness, at least as far as the current study is concerned, lies in the fact that it is monofloral (6). A well-recognized obstacle in any attempts to explain the anti-cancer mechanisms of various honey varieties has been the presence of a vast number of substances that constitute honey and the possibility that each component has a separate activity contributing to the observed effects. As a monofloral moiety, Yemeni Sidr honey offers more promise in terms of isolating active anti-cancer constituents, a step that is of obvious significance in the ultimate goal of developing a practical chemotherapeutic.

2.1 Materials and methods

The following section describes the general methods and routine protocols employed in performing the experiments, to test the effectiveness of the YSH on inhibiting the proliferation of cancer cells.

2.1.1 Cell Lines and tissue culture

MCF-7 (human breast adenocarcinoma, estrogen receptor positive), MDA-MB-231 (human triple-negative breast adenocarcinoma, p53-/-, mutant k-ras), and HeLa (human cervical adenocarcinoma) cells were all obtained from the American Type Culture collection, ATCC, Manassas, VA). The HBL-100 (non-malignant breast epithelial cells) were obtained from Dr K.M. Yamada, National Institutes of Health). These cell lines were maintained in Dulbecco's Modified Essential Medium (DMEM, Hyclone Logan UT) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Burlington, ON). The cells were cultured at 37^{o} C in 5% CO_2. The THP-1 (peripheral blood acute monocytic leukemia) cells were obtained from ATCC and cultured in RPMI 1640 media supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin.

2.1.2 Camptothecin

Camptothecin was dissolved in DMSO to obtain a stock solution of 10 mM for use as a positive control in the cytotoxicity analysis experiments. The stock solution was kept at -20° C.

2.1.3 Honey samples

Three Yemin sidr honey samples were supplied by different commercial vendors. Two of these samples were obtained from commericial markets in the Kingdom of Saudi Arabia while

the third sample was purchased from a company in the United Kingdom that sells characterized and verified product (Lote & Co., UK). The honey samples were stored at ambient temperature until required for further use. The age of the honey samples ranged from 1 to 5.5 years.

2.1.4 Preparation of honey media

Honey samples were weighed using a top-pan balance. Honey samples were diluted in serum-free culture medium (10 ml) to give a final concentration of 10% (w/v). The samples were then incubated at 37° C for 15 min to dissolve the honey into the media. Once the honey had dissolved, further dilutions were carried out in complete culture medium as described in the specific experimental procedures.

2.1.5 Treatment

The experimental design involved dividing the cell samples into test and control groups. The YSH samples were freshly prepared for every experiment. The cells in the test group were treated with different concentration of YSH (ranging in final concentration from 0.1 - 10% (w/v) dissolved in complete culture (DMEM) media. The cells in the positive-treatment control group were treated with 10 μM camptothecin. In experiments where varying concentrations of YSH were used, cells in the negative control group were treated with a concentration of sugar (w/v) equivalent to the concentration of YSH used in that experiment. Another control group included in the experiment is a non-treated group which were grown in complete medium with no added YSH or glucose. Similar to the YSH-treated samples, the non-treated and control samples were collected at the same time points during every experiment. Moreover, the positive control groups varied with each experiment.

2.1.6 Sulphorodhamine b (SRB) assay

SRB assays are mainly performed to determine the relative cell density based on the cellular protein content. The protocol by Vichai and Kritikara (18) was adopted for SRB assays to measure the proliferation of cells treated with YSH or controls. For each experiment, 100 µl/well of each cell line was plated onto replicate 96-well plates: MCF-7, THP-1, and HLB-100 cells were suspended in media at 4000 cells/ml; and, HeLa, and MDA-MB-231 cells were suspended in media at 3000 cells/ml. The plates were then incubated for 24 h in an incubator containing 5% CO_2 at 37° C and then the growth medium in each plate was replaced with a media containing different concentrations of YSH, sugar, or camptothecin, as indicated, and incubated for an additional 48 - 72 h. At the end of the experiment, the cells in each plate were fixed by incubation with 50% ice-cold tricholoracetic acid (TCA) and incubated at 4º C for 1 h, followed by repeated washing in running water and then the plates were air dried. The fixed cells were then stained by incubation in a 0.1% (w/v) SRB solution for 30 min at room temperature, and washed and then re-suspended in 200 µl of 10 mM Tris buffer, pH 10.5. The absorbance of each well was recorded at a wavelength of 530 nm using a plate reader (Molecular devices, Spectra max 340 PC) (18). The IC_{50} values for YSH were calculated using a sigmoidal dose-response curve (variable slope) using Graph Pad Prism V 4.02 software (Graph Pad Software, Inc.). The following formula was used to normalize the data:

$$(\text{Mean OD sample} - Tn) / Tp - Tn$$

(Where, Tp is mean O.D. of positive control and Tn is mean O.D. of negative control)

2.1.7 MTT assay (Methyl Tetrazolium Blue)

The methyl thiazol tetrazolium (MTT) assay was performed to measure cell viability and proliferation rate in cells treated with the YSH. Cancer cells were plated onto replicate 96-well plates at 2000 cells/well, with one plate per day of experiment (19), and incubated overnight in an incubator with 5% CO_2 at 37° C. The cells were treated in complete culture media containing various concentrations of YSH or glucose as control. Each column of the 96 well plate (n = 8) was treated with the same conditions. The cells were incubated in the continued presence of the YSH over the course of 4 - 5 days without a media change. On each day of the experiment, one of the replica plates was treated by adding 10 µl of MTT solution in water into each well, at a final concentration of 0.25 µg/ml, and incubated for 4 h. Following the incubation, the media was removed and 100 µl/well of dimethyl sulfoxide (DMSO) was added to solubilize the converted formazan crystals. The absorbance of each well was read at 540 nm using a (Spectramax 340PC 389) plate reader. To analyze the data, relative cell viability was determined by averaging the reading for each sample and then subtracting the culture medium background from each sample. The amount of absorbance is proportional to cell number. Statistical analysis for the absorbance corresponding to each experimental condition for each day of the experiments was performed using an ANOVA using Graph Pad Prism Software (19).

2.1.8 Cell Proliferation Using Incucyte® Proliferation Assays for Live-Cell Analysis

Cell proliferation assays are generally employed to evaluate the response of a cell population to external factors. The IncuCyte® Proliferation Assays for Live-Cell Analysis (Essen BioScience), was used in a series of cell proliferation experiments Label-free, direct cell

counting using the IncuCyteS3 cell analysis module allows cell proliferation to be quantified by counting the number of phase objects over time. By masking individual cells this enables a label-free count until the point that the cultures are densely packed and cell edges can not be accurately determined. In addition, subsequent classification of cells into subpopulations can be performed based on properties including size and shape. For experiments, cells were collected, cell concentration determined, and then plated onto the wells of a 96 well plate and incubated at 37º C for 24 h. After incubation, these cells were treated with 1% (w/v) YSH, which is the approximate IC_{50} concentration. The Incucyte was set to count the cells from 8 different areas for each sample well, for each day for 7 days, and the change in cell number was calculated and plotted for each condition.

2.1.9 Detection of Apoptosis by Acridine Orange/Ethidium Bromide

To examine changes in cellular morphology for MDA-MB-231 and MCF-7 cells treated with YSH, the cells were stained with acridine orange and ethidium bromide and visualized on a fluorescence microscope (20). The MDA-MB-231 and MCF-7 cells were grown on 6-well plates overnight at 37º C to allow for adherence of cells. Cells were then treated with different concentrations of YSH in culture media for 24 and 48 h. For this experiment, cells were treated with 6 μg/ml of camptothecin for 24 - 48 h as a positive control group for apoptosis. Following treatment, each sample was then stained with 100 μg/ml acridine orange (Sigma-Aldrich) in culture media for 5 min and then stained with 100 μg/ml of ethidium bromide (Sigma-Aldrich) for 5 min. Finally, the cells were washed with PBS, pH 7.4, and then each sample was visualized one at a time, as they were live and not fixed. Pictures were taken using an OLYMPYS fluorescence microscope (21).

2.1.10 Analysis of Cell Cycle Distribution

The cells were stained with the DNA stain propidium iodide and analyzed using flow cytometry to detect apoptotic cells with fractional DNA content and to identify the distribution of cells in the three major phases of the cell cycle (G_1 vs S vs G_2/M) (22). The following protocol was adopted in order to analyze the cell cycle distribution. MCF-7, MDA-MB-231, Hela, B16-BL6, and HBL-100 cells were exposed to YSH, glucose, or camptothecin for different time intervals, ranging from 24 h to 72 h and then harvested using trypsin. The collected cells were washed twice in PBS, pH 7.4, and fixed overnight by incubation in 75% ice-cold ethanol and stored at -20º C until analysis. On the day of analysis, the cells were collected by centrifugation and washed twice with PBS, pH 7.4. The cells were suspended in 0.5 ml PBS, pH 7.4, and 0.5 ml propidium iodide (PI) staining solution (PBS, pH 7.4, 0.3% Nonidet P-40, 100 µg/ml RNase A, and 100 µg/ml propidium iodide) was added and incubated for 1 h. The re-suspended samples were then analyzed by flow cytometry using an FC600 Flow Cytometer (Beckman Coulter) (22). Flow profiles of ten thousand events were collected and gated to accurately measure PI intensity. propidium iodide fluoresces at 623 nm when excited and single parameter displays were obtained using the flow cytometric acquisition software, and the FL3-gated fluorescence signals were recorded and the proportion of cells in the sub-G_1, Go/G_1, S, and G_2/M phases were determined.

2.1.11 Protein Extraction and Quantification

Proteins were extracted and quantified, from cultured cells treated with YSH, glucose, and control following the protocol by Santi and Lee (23). Cells were plated on 10 cm plates (Sarstedt) and incubated at 37º C for 24 h before exposing them to different concentration of

YSH. Post-treatment, exposed cells were collected at different time points (0, 24, 48, and 72 h). Whole cell extracts were prepared by collection in RIPA buffer (PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with a protease cocktail tablet (Roche Diagnostics), 2 μM sodium orthovanadate, and 10 μM sodium fluoride. After incubating the cells in RIPA buffer on ice for 10 min, the lysates were sheared by passage through a 20 Ga needle, and then the cell lysates were cleared by centrifugation (Beckman Coulter, microfuge 22R) at 4º C for 15 min at 14,000 x g (23). Finally, protein quantification was carried out using a BCA Assay kit (Pierce Chemical, Fisher) and the absorbance of the samples and known concentrations of a BSA standard were read at 562 nm using a Spectramax/340 plate reader. The concentration of unknown proteins was analyzed against the BSA standard curve using the GraphPad Prism version 4.03.

2.1.12 SDS-PAGE

Protein samples, corresponding to 25 μg or 50 μg of cellular lysate protein, were subjected to electrophoresis on polyacrylamide gels containing sodium dodecylsulfate (SDS-PAGE), as described by Laemmli (24). Samples were prepared by adding 5X loading buffer (0.1 M Tris-HCl, pH 6.8, 10% [w/v] SDS, 40% [v/v] glycerol, 0.1% bromophenol blue), and heated for 5 min to denature the proteins (24). The samples were loaded per well onto a 6 – 15% polyacrylamide gel, and the proteins were separated by electrophoresis in 1X SDS Running buffer (25 mM Trizma base, 192 mM glycine, 0.1% SDS) at 90 V for approximately 90 min. The proteins in the gel were then transferred onto a PVDF membrane (GE Health Care) which had been immersed in Transfer buffer (48 mM Tris, 39 mM glycine, 20% [v/v] methanol, 0.037%

[w/v] SDS), using a semidry transfer apparatus at 12 volts for 1 h. Transfer was verified by staining the blot with 0.1% Ponceau S in 1% acetic acid and destained in water.

2.1.13 Western Blotting

Western blot analysis was used to identify specific proteins from the complex mixture of proteins extracted from cells. The membrane was "blocked" by incubating it in Blocking buffer, 5% Carnation non-fat skim milk in TBST (50 mM Tris-HCl, pH 7.4, 150 M NaCl, 0.1 % [v/v] Tween 20), for 1 h. The membrane was washed with TBST before incubating it with primary antibodies against Bcl-2, Bax, Caspase 9, and PARP-1 and GAPDH and β-tublin (loading controls) (Santa Cruz Biotech., Santa Cruz, CA) overnight (diluted in 5% Carnation non-fat skim milk, in TBST) at 4º C. After that, the blot was washed three times in TBST, and then incubated with a secondary antibody-horse radish peroxidase conjugate (Santa Cruz Biotech) (diluted in 5% non-fat skim milk in TBST) for 1 h at room temperature. This was followed by two more washes with TBST, and one wash with TBS. The horseradish peroxidase-labelled target proteins were visualized using an ECL kit (Enhanced Chemiluminescence, GE Health care) and exposed to X-Ray film.

2.1.14 Wound Healing

Cancer cells (5×10^5 cells/well) were seeded in 6-well plates and grown to form a confluent monolayer for 24 h. A sterile 500 µl pipette tip was held vertically to scratch a cross in each well, Liang et al. (25),(26). The detached cells were removed by washing the well with 500 µl of fresh medium. YSH was added immediately after the wound was created and the plates incubated for 72 h. The scratch closure was monitored and imaged over the period of 24 - 72 h using the IncucyteS3 Live imaging protocol at 4 x magnification and images obtained and time-

lapse videos created for some experiments (Essen BioScience). The recovery of the scratched area can be calculated using commercially available Image J image analysis software (27),(28). The migration of cells toward the wounds was expressed as percentage of wound closure:

$$\% \text{ of wound closure} = [(At = 0\ h - At = \Delta\ h)/At = 0\ h] \times 100\%,$$

where, At = 0 h is the area of wound measured immediately after scratching, and At = Δh is the area of wound measured 18 or 24 h after scratching.

2.1.15 Statistical Analysis

For analysis of each treatment group, data was reported as a mean and standard error of the mean (SEM). IC_{50} values were calculated using a sigmoidal-dose response curve which was generated by GraphPad Prism V.402. Values are means of triplicates of two or three independent experiments.

2.2 Results

2.2.1 YSH Inhibits the Proliferation of Malignant Cell Lines

To determine the anti-proliferative effect of YSH on malignant and non-malignant cell lines, the SRB, MTT, and proliferation assays for Live-Cell Analysis using IncucytS3, were performed. Findings from each assay revealed that YSH exhibits a significant anti-proliferative effect on malignant cells. The MTT assay, and Incucyte results (Fig 2.1 and 2.2) showed significant anti-proliferative activity on several different cancer cells (MCF-7, MDA-MB-231, THP-1, and HeLa cell lines). The SRB assay showed that the IC_{50} concentrations of YSH were (0.99% , 2.1%, 1.95%, and 6.7%) for MCF-7, MDA-MB-231, HeLa, and THP-1 cell lines, respectively (Fig 2.3 and Table 2.1). The IC_{50} represents the concentration of a drug that is required for 50% inhibition of treated cells *in vitro* compared to non-treated controls. IC_{50} values were calculated using the GraphPad Prism sigmoidal dose response curve. Each experiment was performed in triplicate and each value represents the mean ± SD of three independent experiments. Table 2.1 summarizes the IC_{50} concentrations of YSH on the growth of cells lines as determined by the SRB assay.

Moreover, treatment with YSH has dose- and time-dependent effects on the cell growth of breast cancer cells, with significant reductions in the number of cells. The effect of YSH on cell proliferation as measured using MTT assays were consistent with the findings of the SRB assays. All of the malignant cell lines tested showed a significant decrease in cell numbers on each day in response to YSH treatment compared to untreated (or glucose-treated) controls.

2.2.2 YSH Is More Cytotoxic To Malignant Cells than To Non-Malignant Cells

To understand whether YSH selectively targeted cancer cells and did not harm non-cancer cells, the cytotoxic effect of YSH on non-malignant cells was analyzed using a cytotoxic assay. The cytotoxic assays revealed that YSH did not significantly affect non-malignant cells (HBL-100). In addition, HBL-100 cells also did not show any significant changes in their cell proliferation, when the cells were treated with the same dose of YSH as malignant cells. Experimental findings revealed that YSH had lesser toxic effects on non-malignant breast epithelial cells (HBL-100 cells), but had higher toxic effects on malignant breast cancer cells (MCF-7 and MDA-MB-231 cell lines). Fig 2.3 and Table 2.1 shows the IC_{50} concentrations for the malignant MDA-MB-231, MCF-7, and HeLa cells were between 1 – 3% while the IC_{50} for the non-malignant HBL-100 cells was >10%.

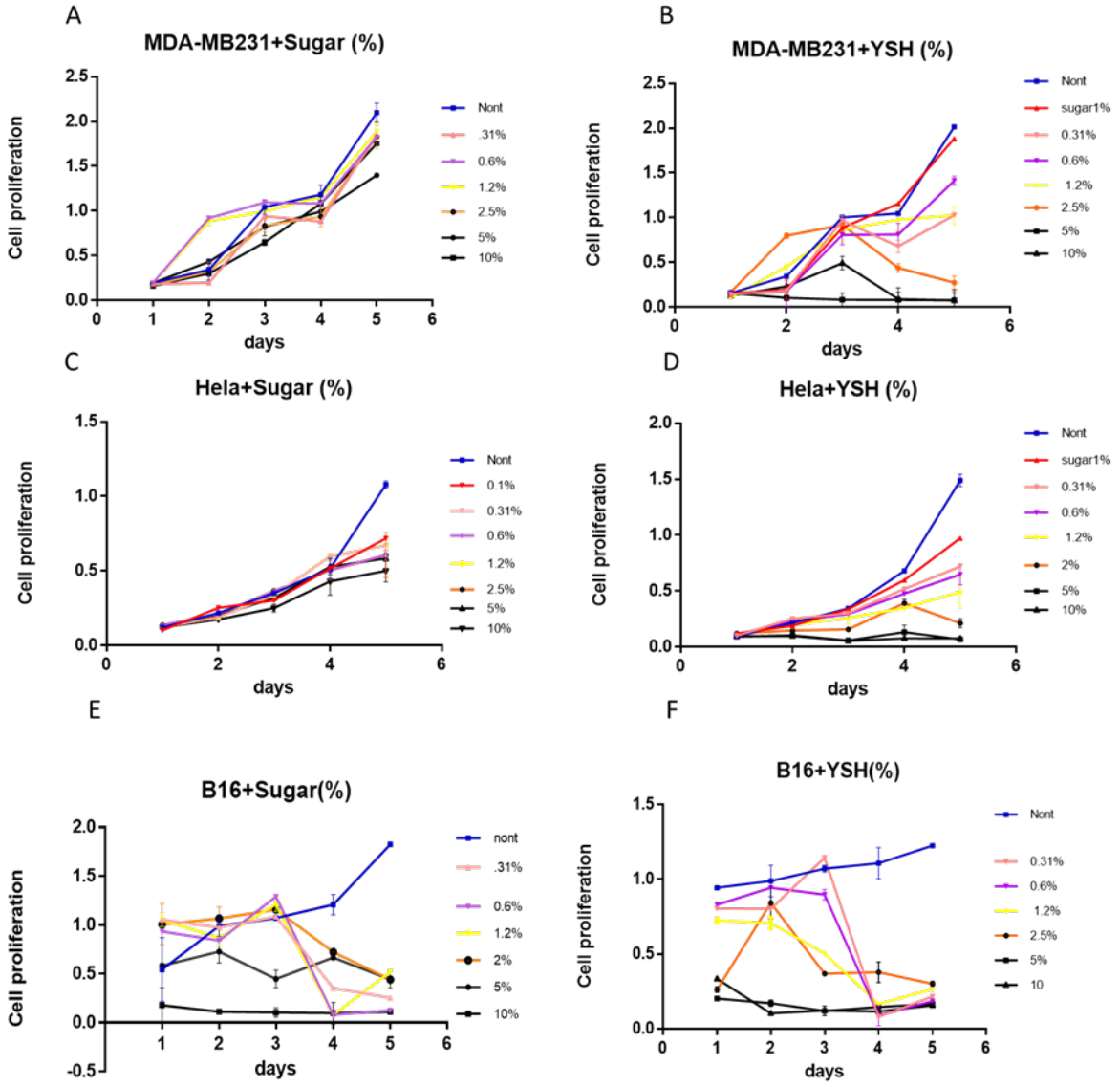
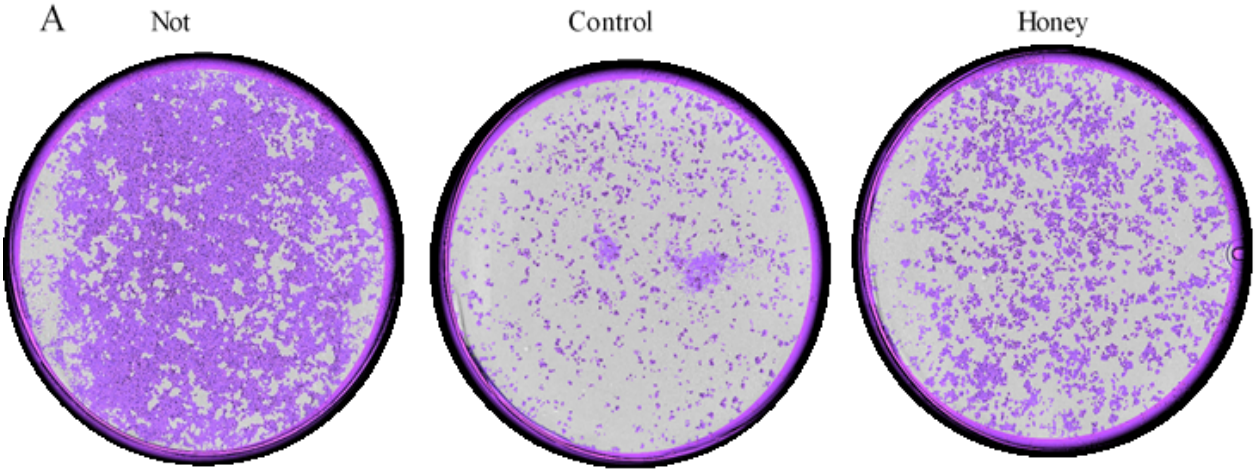


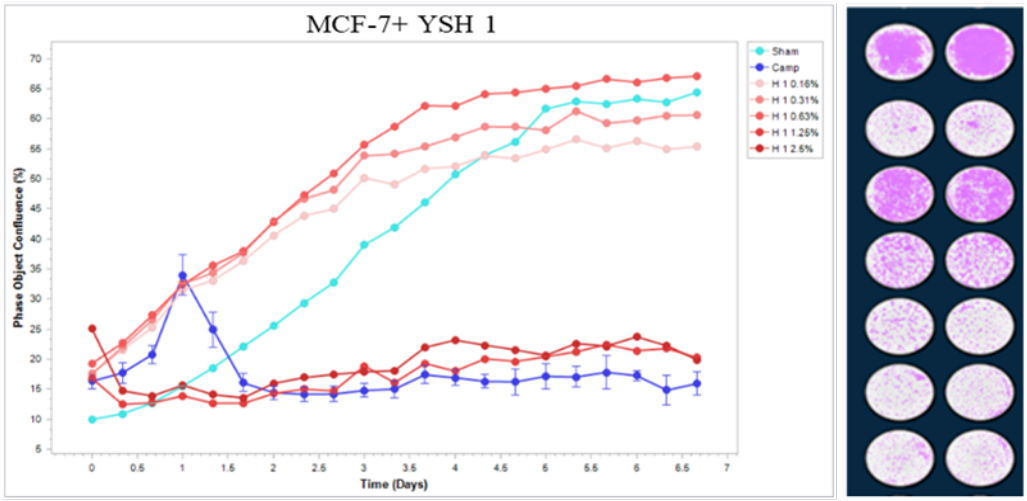
Figure 2.1: Inhibition of cancer cell proliferation following treatment with YSH.

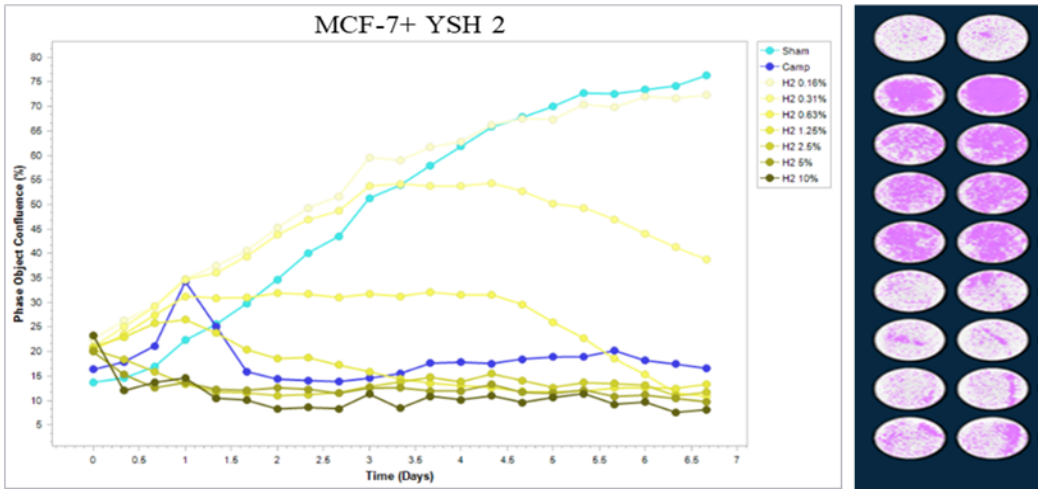
The treated cells were compared to control (untreated cells suspended in media) and cells treated with sugar suspended in DMEM culture media. Relative cell number was determined for replica plates using the MTT assay and absorbance was determined every day at 540 nm. Representative graphs show the mean of triplicate wells using Graph Pad. There were no discernable differences among the two independent experiments.

A


Not
Control
Honey

B


MCF-7+ YSH 1
Phase Object Confluence (%)
Time (Days)
Sham
Camp
H 1 0.16%
H 1 0.31%
H 1 0.63%
H 1 1.25%
H 1 2.5%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Phase Object Confluence (%)
Time (Days)
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Camp
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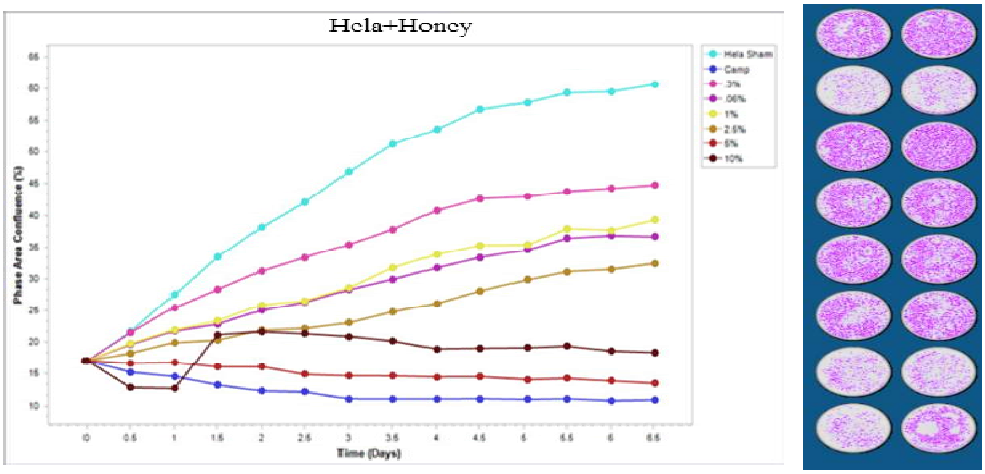
H2 0.31%
H2 0.63%
H2 1.25%
H2 2.5%
H2 5%
H2 10%

C



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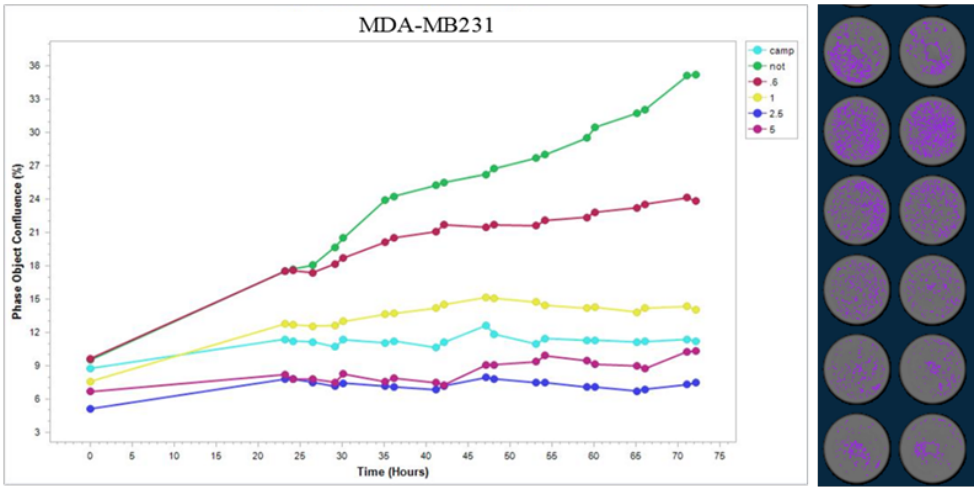


Figure 2.2: Anti-proliferative activity of YSH on different cell lines.

Cells on 96-well plates were grown in medium containing different concentration of YSH. (A) The proliferation of the cancer cells on the plates was monitored in real time using the Cell program by Cell Analysis Software module (mask shown). Cells were masked using the Incucytes protocol and counted for cell number (as measured by cell confluence). (B) two differently sourced YSH were tested on MCF-7 cells with similar results. (B, C, D) Treatment with YSH decreased cell numbers in breast cancer and Hela cells in a dose- and time-dependent manner. The data shows a representative plot of replicated wells.

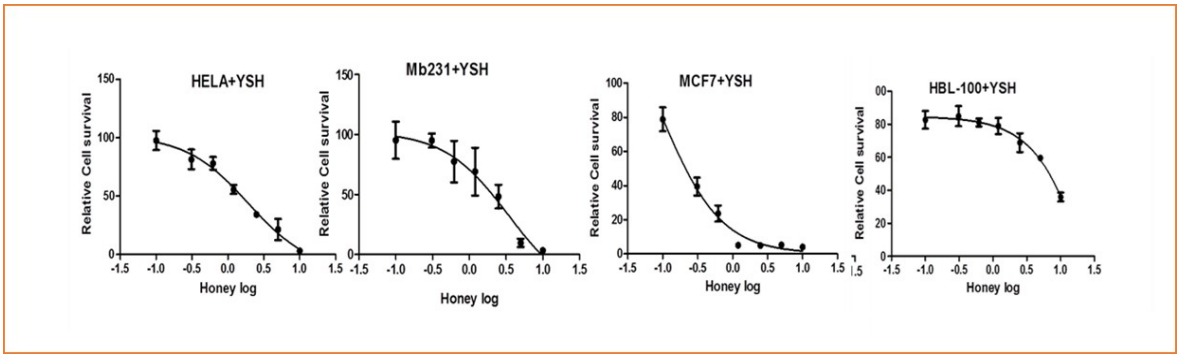


Figure 2.3: The effects of YSH on MDA-MB-231, MCF-7, Hela, and HBL-100 cells as determined by the SRB assay.

The SRB assays were performed to determine the anti-proliferative activity of YSH. The MDA-MB-231, MCF-7, Hela, THP-1, and HBL-100 cell lines were seeded onto 96-well cell culture plates and treated with seven different concentrations of YSH for 48 - 72 h. The relative number of cells, determined using the SRB assay, for each cell line were plotted against the varying concentrations of YSH using a GraphPad Prism sigmoidal dose response curve. Representative graphs show the mean of three independent experiments. Error bars represent 95% confidence intervals.

Table 2.1: The IC50 of YSH on cancer cells as obtained using the SRB assay.

Compound	IC_{50} (%) MCF7	IC_{50} (%) MDA-MB231	IC_{50} (%) HeLa	IC_{50} (%) HBL-100
YSH	**0.99 ± 1.4**	**2.3 ± 1.2**	**1.95 ± 0.78**	**~15**

2.2.3 Induction of Apoptosis by YSH

After confirming that treatment with YSH has the ability to differentiate between malignant and non-malignant cell lines, it was necessary to determine the mechanisms by which YSH affects cell cycle progression and kills malignant cancer cell lines (MDA-MB-231, MCF-7, and Hela). The induction of apoptosis and cell cycle arrest are both anti-proliferative responses, which likely contribute to the anti-cancer activity (29), of YSH. The induction of apoptosis in malignant cancer cell lines was studied using three different experiments namely: morphological evaluation of cells using acridine orange/ethidium bromide staining, flow cytometry of propidium iodide-stained cells, and Western blot analysis for apoptosis-related proteins.

Cells were stained with acridine orange and ethidium bromide to determine if the cells were undergoing apoptosis after being treated with YSH. In addition, the acridine orange-staining experiment provides a means to examine the cell morphology, cell size, and refractive properties of breast cancer cells treated with YSH. The rationale behind the acridine orange/ethidium bromide staining experiment is that both acridine orange and ethidium bromide bind to and stain nucleic acids and that the plasma cell membrane is normally permeable to acridine orange but not to ethidium bromide. Therefore, the nucleus (and other nucleic acids) stain fluorescent green. However, when the cell membranes lose their structural integrity, they become permeable to ethidium bromide and the DNA also stains red (20),(30). Results from the double staining experiment revealed that malignant cancer cells treated with 1% YSH for 48 h underwent chromatin condensation and extensive membrane blebbing, which are hallmarks of apoptosis, while untreated controls did not demonstrate any visible damage to the nuclear membrane. Although there were no significant morphological changes after 24 h of incubation with 1% of YSH (data not shown), there were significant changes after 48 h of incubation. The

data in Fig 2.4 clearly shows that MDA-MB-231, MCF-7, and Hela cells treated with YSH cells have lost their structural integrity after 48 h with bright yellow patches within cells. This clearly demonstrates that these cells are in the late stages of apoptosis because the cell membranes of the breast cancer cell have lost their integrity allowing ethidium bromide to enter the cell causing the red stain.

To confirm that YSH treatment causes malignant cells to undergo apoptosis and nuclear fragmentation, flow cytometry to show the proportion of cells containing sub-G_1 phase DNA content was determined. In addition to the study of DNA content, flow cytometry also helps to study the cell size, number of cells and their granularity by examining each cells' ability to scatter the incident light (31),(22). A total of 10,000 events were analyzed by flow cytometry following propidium iodide (PI) staining. Results are expressed as the percentage of total cells in each phase of the cell cycle. The Sub-G_1 peak displays cells with lower DNA content than an intact cell, which indicates a cell undergoing nuclear fragmentation typical of apoptosis. The G_1 peak corresponds to the 2N content of DNA, the G2/M peak corresponds to 4N DNA content, and the S phase represents cells with intermediate DNA content (replicating DNA).

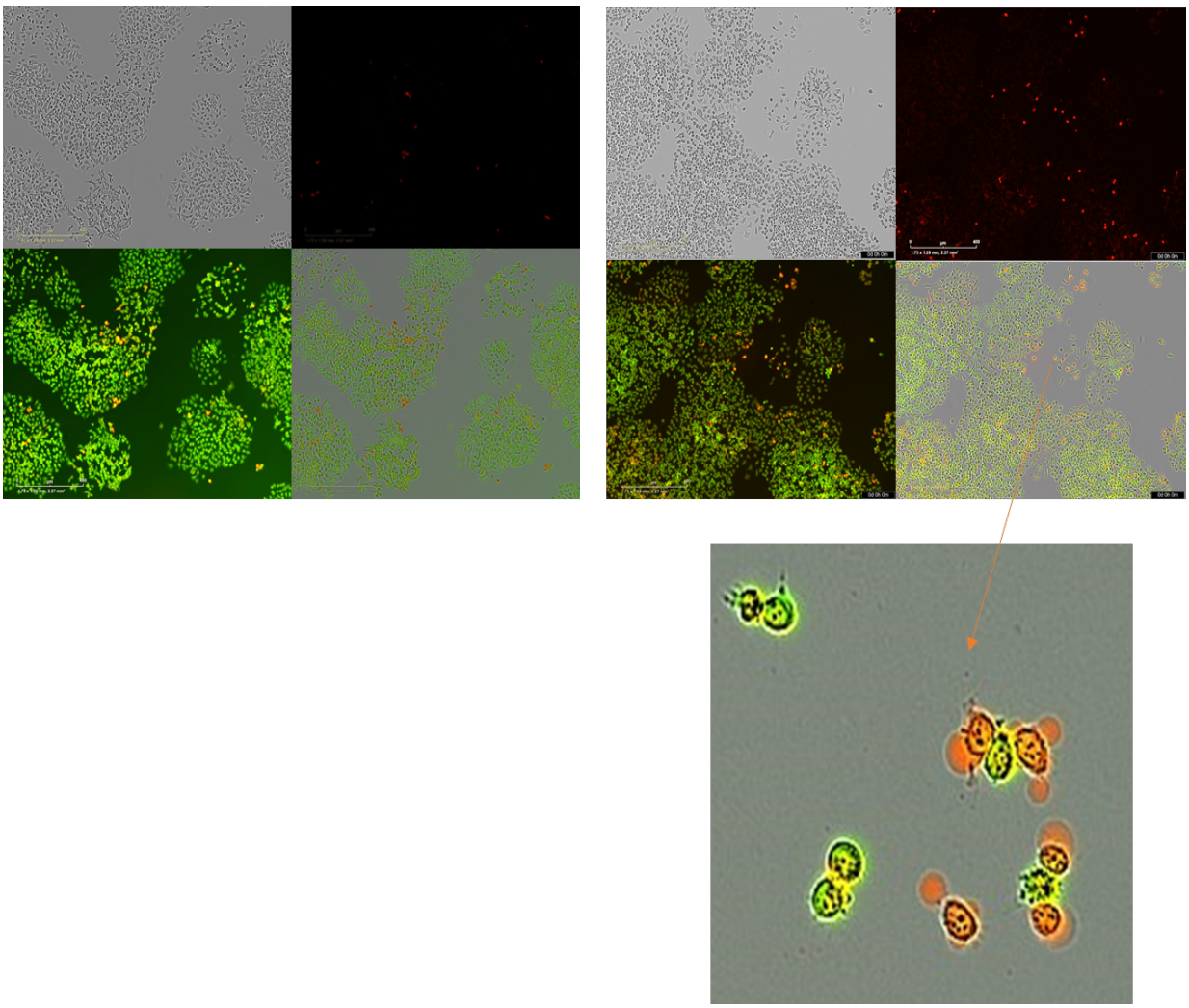
The histograms of breast cancer cells with low forward scatter and side-scatter histogram indicating that they are undergoing apoptosis (data not shown). Flow cytometry results revealed that treatment with YSH induces apoptosis in MDA-MB-231 and MCF-7 cells as shown by an increase in the proportion of cells with sub-G_1 DNA content, which is indicative of apoptosis (Fig 2.5). Flow cytometry results also showed that the percentage of cells with sub-G_1 phase DNA content increased in a dose-dependent manner suggesting increased number of cells undergoing apoptosis.

2.2.4 YSH had a weak effect on cell cycle progression in HBL-100 non-malignant breast cells.

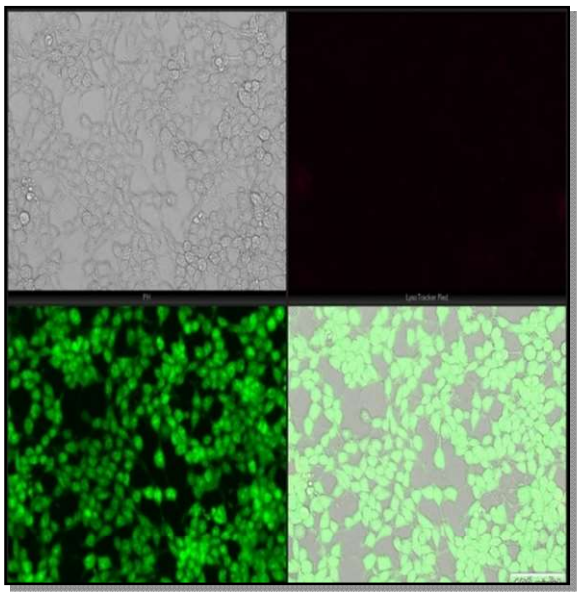
To determine if there was a noticeable change in the cell cycle progression of HBL-100 cells in response to treatment with YSH, cells were exposed to different concentrations of YSH for 24 h and then analyzed for DNA content using flow cytometry. There was no significant induction of cell cycle arrest or apoptosis in response to treatment with 1% YSH at the 24 h time point (Fig 2.5).

From this data it can be inferred that YSH is able to induce killing in a cancer cell-specific manner. After determining that treatment with YSH can differentiate between malignant and non-malignant cells, there is a need to investigate the mechanisms through which YSH kills cancer cells which can be a result of either altered cell cycle progression or cell death.

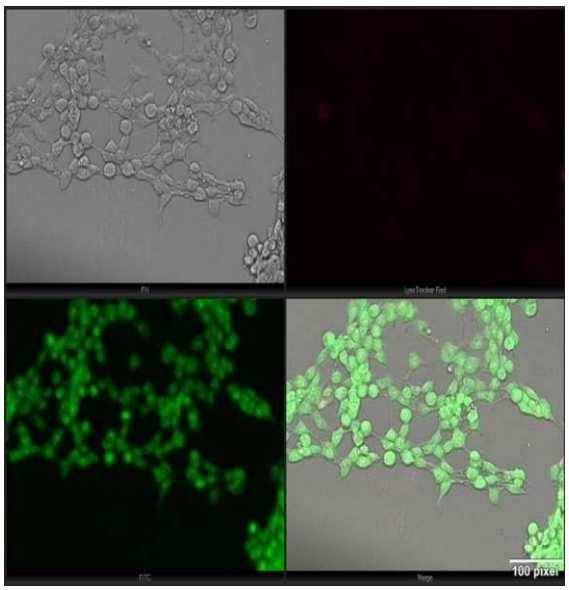
A MCF7-Not 1%YSH



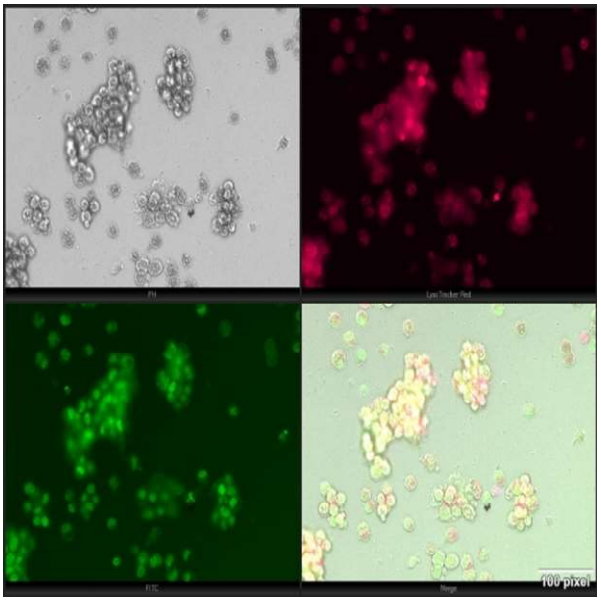
HBL-100-NonT



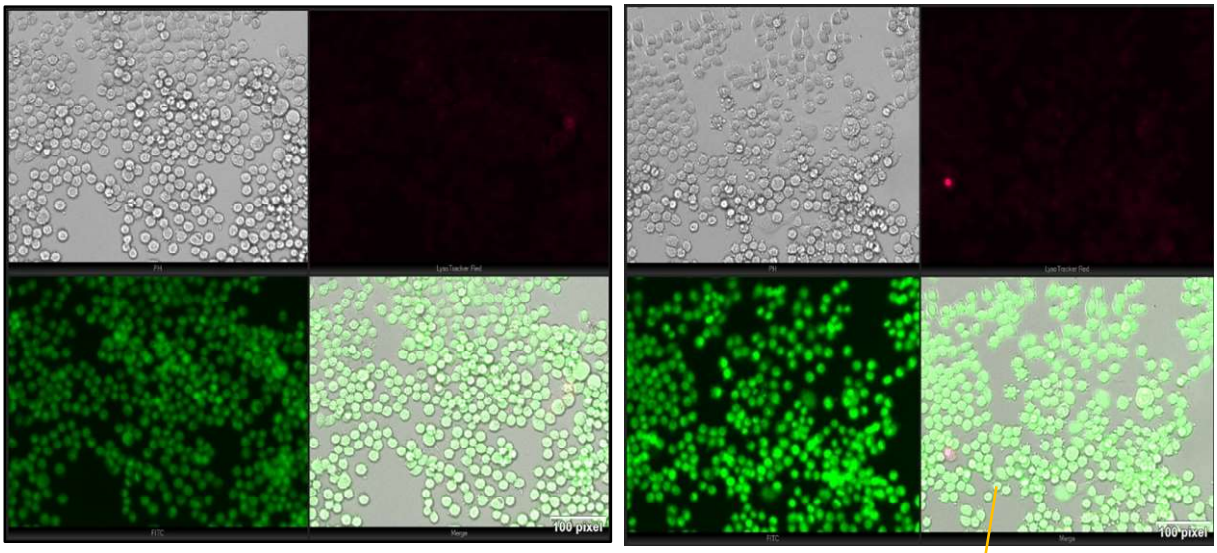
1%YSH



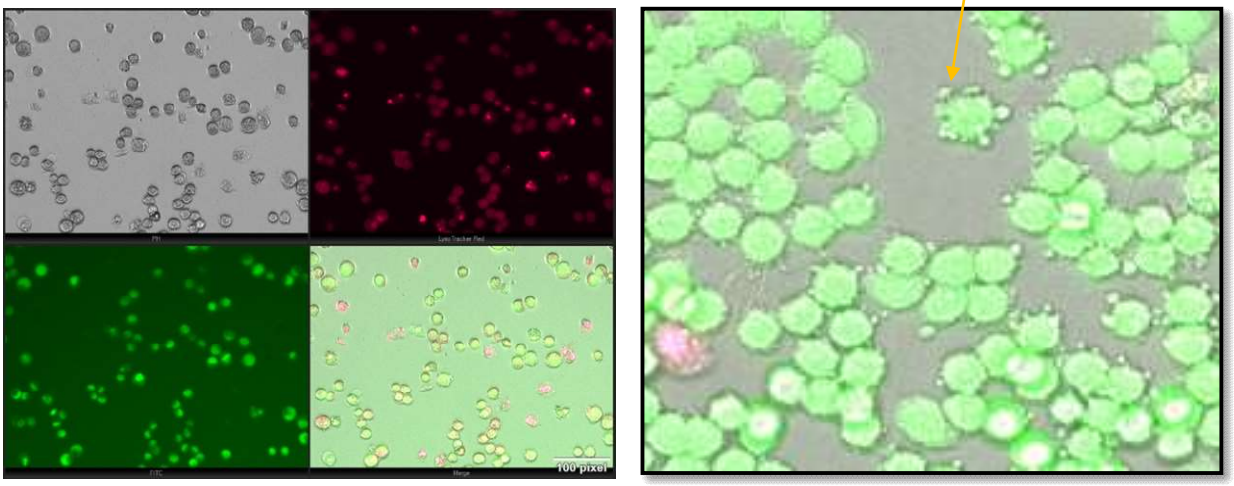
Camptothecin



B Hela-NonT 1%YSH



Camptothecin



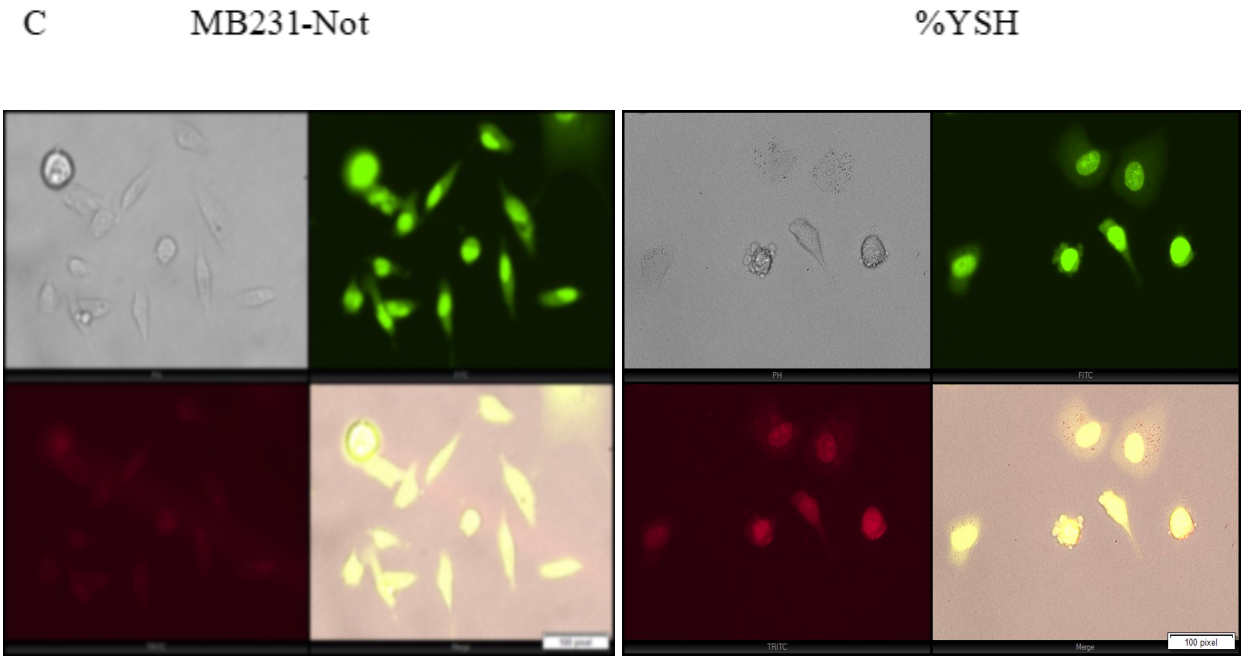


Figure 2.4: YSH induced apoptotic cell death was observed by acridine orange/ ethidium bromide staining.

Following treatments with specific doses of YSH, cells were stained with acridine orange (green) /ethidium bromide (red). Cells were analyzed after 48 hours of treatment. Morphologic changes were observed in (A) MCF-7 breast cancer (B) Hela, and (C) MDA-MB-231 cells, such as nuclear condensation and membrane blebbing. Results were obtained using fluorescent microscopy. There were no significant differences between two independent experiments.

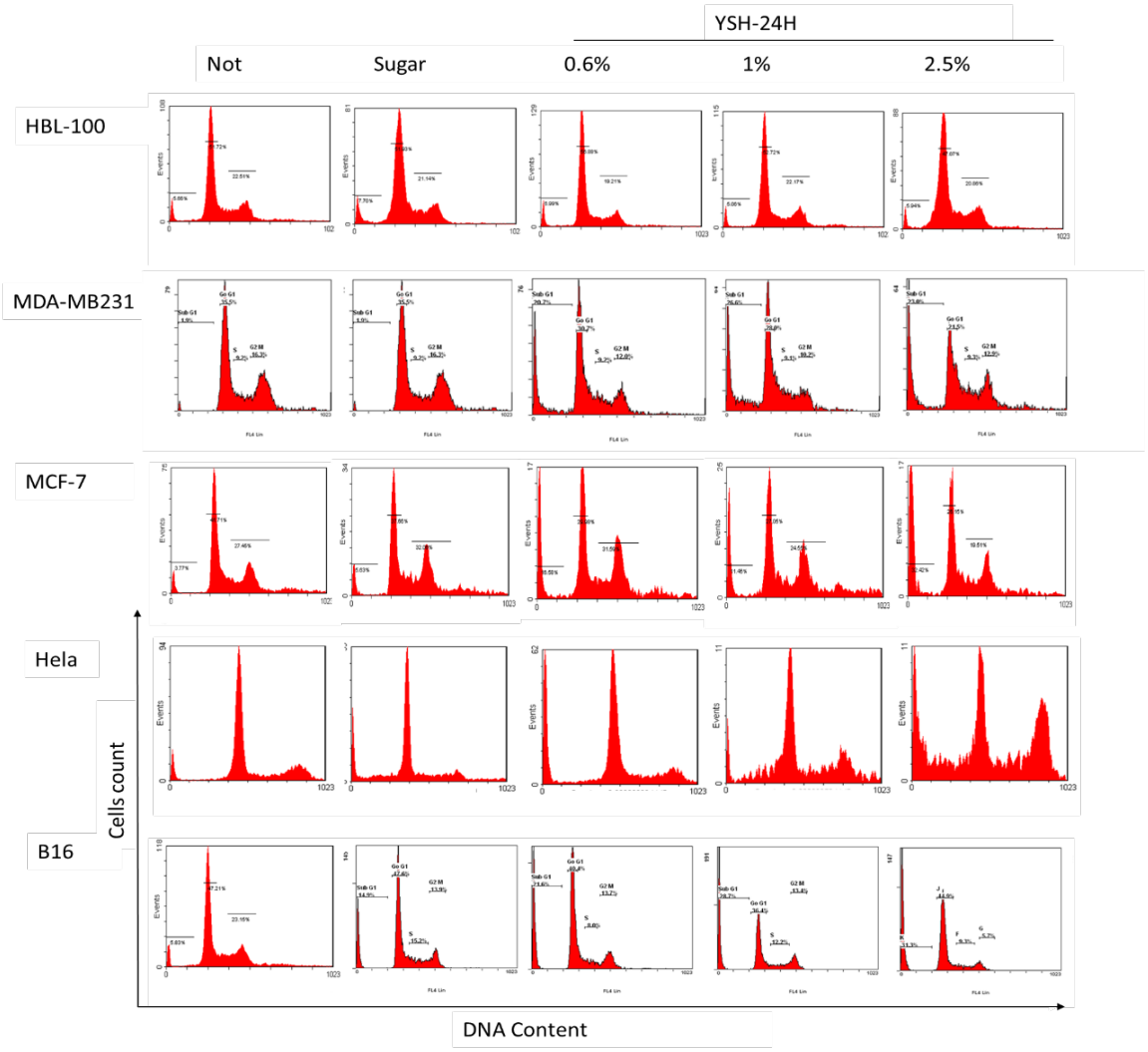


Figure 2.5: Cell cycle analysis of MDA-MB-231, MCF-7, HeLa, B16-BL6, and HBL-100 cells treated with YSH.

Flow cytometry profiles of MDA-MB-231, MCF-7, Hela, and HB-100 cells treated with 0.6, 1.0, and 2.5% YSH for 24 treated cells were compared to Not-treated cells and the 1% sugar control. Cells exposed to higher concentrations of YSH resulted in an increase in the proportion of the sub-G1 cell population indicating that YSH induces apoptosis. The percentage of cells in each phase of the cell cycle was estimated by gating for the fluorescent intensity corresponding to the amount of DNA in each event with respect to non-treated cells. The X-axis represents the

DNA content (PI staining) and the Y-axis corresponds to the number of cells. The three independent experiments did not show any significant differences.

2.2.5 YSH induces apoptosis through alteration in molecular pathways

To further understand the underlying molecular mechanisms through which YSH induces apoptosis, western blot analysis for important molecular markers responsible for apoptosis was performed. Analysis of protein levels in cells undergoing apoptosis revealed that the Bax/Bcl-2 ratio plays a major role in apoptosis (32),(33),(34),(35). Findings revealed that the expression levels of the anti-apoptotic protein Bcl-2 were significantly decreased. However, the Bax pro-apoptotic protein increased in a time-dependent manner. The PARP-1 plot shows the full-length PARP (116 kDa) and the larger fragment (85kDa) of apoptotic cleaved products. Treating cells with YSH also showed an increase in the amount of the caspase 9 cleavage product. These results are consistent with the idea that treatment with YSH induces apoptosis through the mitochondria-dependent signaling pathway. Moreover, apoptosis was evident in MDA-MB-231 and MCF-7 cells treated with YSH compared with control samples.

2.2.6 YSH impairs cancer cell migration *in vitro*

The effect of treatment with YSH on cancer cell migration was examined using a wound-healing assay. MDA-MB-231, MCF-7, and B16-BL6 cells were incubated for 4 days in the presence or absence of YSH (1% final concentration). The concentration was chosen because it is known to cause death to half of these cells over this time period (Fig 1A). The results of the wound-healing assay show that exposure to a 1% solution of YSH led to a significant inhibition in cell migration (up to 26% within 24 h; Fig 6A, B).

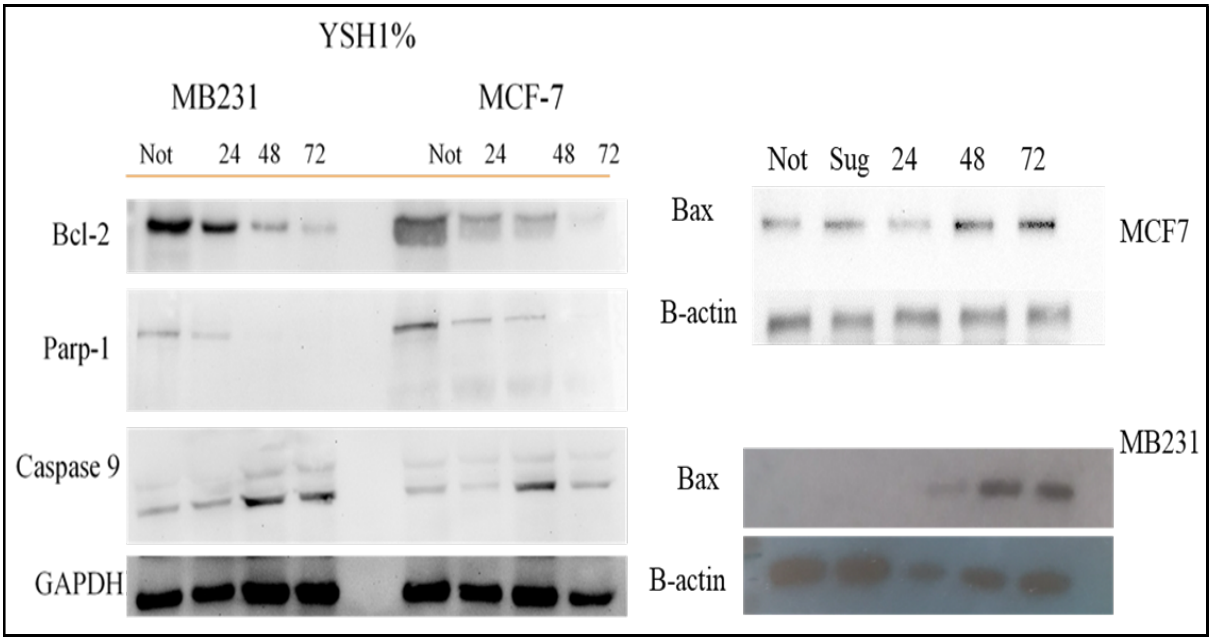
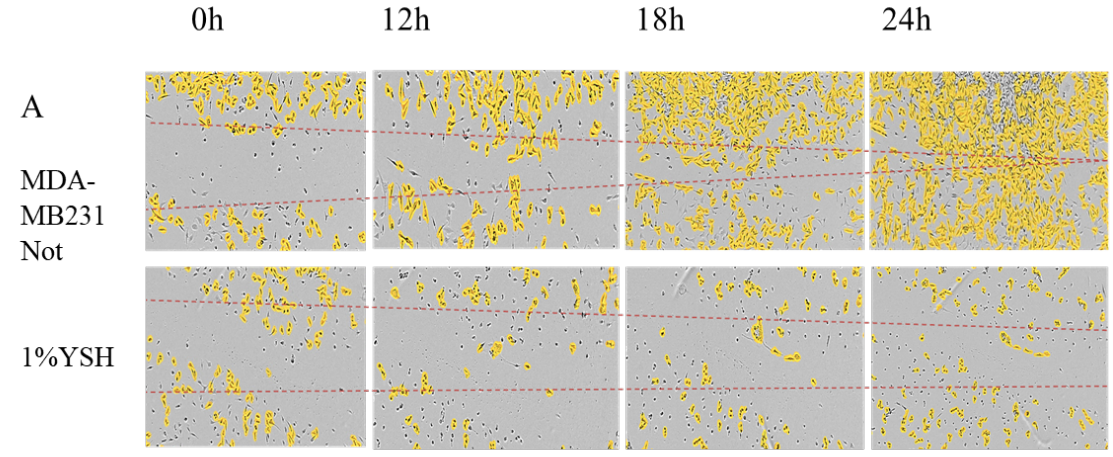
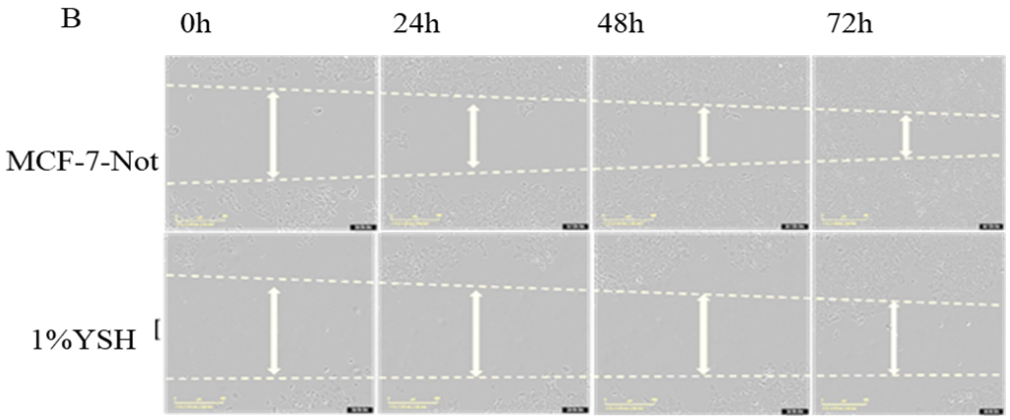
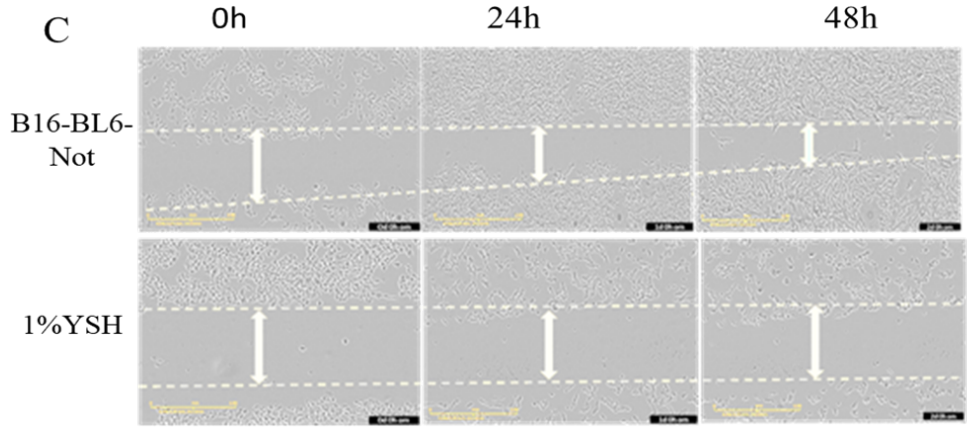


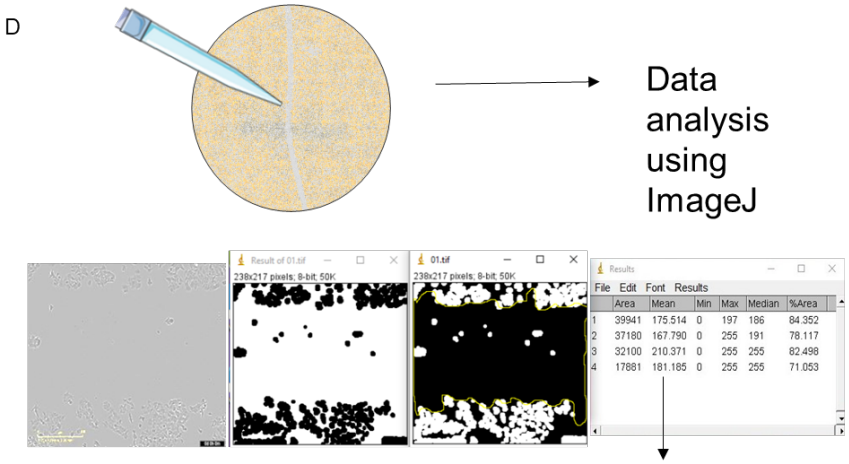
Figure 2.6: YSH induced apoptosis by down regulating anti-apoptotic proteins and up regulating pro-apoptotic proteins in MDA-MB-231, and MCF-7.

Whole cell lysates were prepared after treating MDA-MB-231 or MCF-7 cells with 1% of YSH for 24, 48, and 72 h. Proteins levels and cleavage status were analyzed by western blot analysis using antibodies against Bcl-2, Bax, Caspase 9, and PARP-1. GAPDH and β-tubulin were used as a loading control.


0h
12h
18h
24h
A
MDA-MB231 Not
1%YSH


B
0h
24h
48h
72h
MCF-7-Not
1%YSH


C
0h
24h
48h
B16-BL6-Not
1%YSH



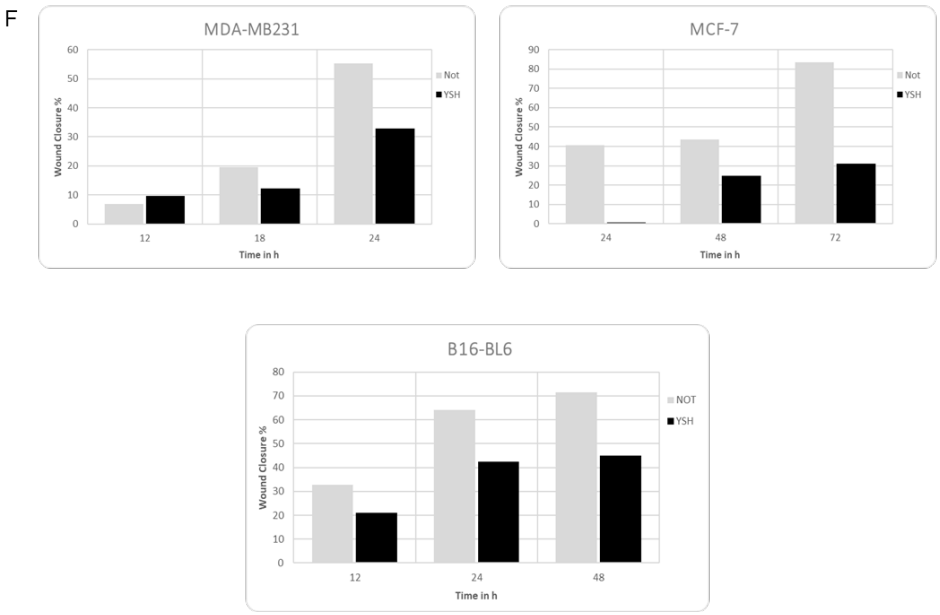


Figure 2.7: YSH inhibits cancer cell migration in wound-healing assay.

Wounds were introduced in (A) MDA-MB-231, (B) MCF-7, and (C) B16-BL6 cell confluent monolayers cultured in the absence (control) or presence of 1% YSH. The distance that the MCF-7 cells traveled from the edge of the scraped area over a period of 6 h at 37° C was measured using ImageJ software to determine the percentage of wound healing, as shown in panel (D,F).

2.3 Discussion

The results described in this chapter show that YSH inhibits the proliferation, viability, and migration capacity of several different cancer cell populations. This outcome, it appears, is reached through a variety of mechanisms, the most significant of which is the induction of apoptosis due to a combination of cell cycle arrest, nuclear condensation and fragmentation, down-regulation of such anti-apoptotic molecules as Bcl-2 and uncleaved PARP, and the up-regulation of promoters of cell-death, such as the Bax class of proteins and upregulation of cleaved caspase 9. YSH was found to have the power to reduce the proliferating impact on malignant cells. This was confirmed through performing SRB and MTT assays. The two tests collectively showed that treatment with YSH had a unique dose-dependent impact on cell growth of cancer cells by significantly minimizing the number of the cells. Every day of treatment the malignant cell lines showed a reduction in cell number compared to non-treated controls samples. A flow cytometry experiment was performed to find out whether YSH inhibited the cell cycle progression of the malignant and non-malignant cell lines. The results showed that treatment of non-malignant HBL-100 cells with 1% YSH did not show any characteristics of cell cycle arrest or apoptosis at varying times. However, treatment of malignant MDA-MB-231, MCF-7, Hela cells with 1% YSH was shown to induce apoptosis, as measured by the increase in the sub-G_1 fraction of the cells, in a dose-dependent manner. This result shows that YSH has the outstanding characteristic of inducing apoptosis in a cancer-cell specific manner. The other question which was addressed by these experiments is whether YSH causes apoptosis through alteration of molecular pathways. This was done through the analysis of key molecular markers of apoptosis which indicates that apoptosis depends on the mitochondrial, intrinsic pathway.

Treatment of MDA-MB-231 and MCF-7 cells with 1% YSH also resulted in an increase in cleaved caspase 9 and a decrease in uncleaved PARP supporting the conclusion that YSH caused apoptosis of the cancer cells.

The research also showed that the YSH inhibited the ability of cancer cells to migrate through the wound-healing assay. Treatment of MDA-MB-231, MCF-7, and B16-BL6 cells with a 1% solution of YSH greatly reduced the cell movement compared to the untreated control cells. This could explain how YSH might help in reducing cancerous cells from spreading to other parts of the body and therefore could be useful in inhibiting metastasis. This unique ability could make YSH a particularly useful way of treating cancer cells.

Similar findings have been obtained by other researchers using comparable *in vitro* studies with different types of honey. For instance, a 2013 study documented the morphologic features of breast cancer cell lines after a 24 h exposure to honey from Indian stingless bees (36), and determined that there was a cell cycle arrest, based on p53-activation. This arrest was the underlying mechanism for the ability of Brazilian honey extracts to induce apoptosis in cancerous cell lines. Similarly, reduced expression of Bcl-2, activation of caspase 3, and Bax up-regulation accounted for the anti-proliferative influence of honey extracts on leukemic cell lines (37),(38).

Another important finding in the current study is that the anti-proliferative effect of YSH is specific to the malignant cells tested, a realization that boosts optimism for its potential as a chemotherapeutic agent. While studies examining the specificity of honey products towards cancer cells are scarce in the literature, Umthong et al. (39), in their investigation of the anti-proliferative activity of Thailand honey extracts, found that the effect is only observed in

neoplastic and not normal cells. Calhelha et al. (5), in a similar *in vitro* study, concluded that Portuguese honey extracts did not exhibit a significant preference for cancerous cells in their ability to inhibit cell proliferation. On the other hand, experiments done by Almeer et al. (11) showed a similar result when Sidr honey from Saudi Arabia was tested on the MDA-MB-231 cell line, as the MTT assay after 48 h showed a reduction in cell viability of 48%. However, Ghramh et al. (16), found a conflicting result with our finding, as in their studies, Sidr honey-coated nanoparticles had anti-cancer activity against HepG2, but not Hela cells. These differences could arise from one or more of several variables, including the cell lines used, the type of honey and geographical locations, or even variations in the experimental methodologies.

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Chapter 3 . The effect of Yemen Sidr honey on Macrophage polarization, and cytokine expression in THP-1 cells

Abstract

Honey might initiate or accelerate the healing of chronic wounds; therefore, it has been claimed to have anti-inflammatory properties. The aim of this study was to investigate the effects of Yemen Sidr honey (YSH) on the activation state of monocyte/macrophages, using the human monocytic cell line, THP-1, as a model. Therefore, THP-1 cells were differentiated by treatment with phorbol ester and then treated with YSH or sugar as a negative control and the monocyte activation state and macrophage polarization to the M1 or M2 states was measured. For these experiments, the level of secreted tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, and transforming growth factor-β (TGF-β) were measured by ELISA and cell surface macrophage markers were examined using flow cytometry. Treatment of THP-1-derived macrophages with YSH for 24 h, enhanced the expression of the CD68 and CD80 cell surface markers and the inflammatory cytokines TNF-α, IL-1β, and IL-6 that are associated with the pro-inflammatory M1 macrophage subtype. However, when the THP-1-derived macrophages were treated with 1% YSH in the presence (or absence) of LPS for an extended period of time of 48 h, the level of the M2 phenotype markers, including CD68 and CD163 cell surface markers and IL-10 and TGF-β cytokines was increased. THP-1-derived macrophages or macrophages treated with LPS all showed an increase in M1 phenotype markers compared to untreated THP-1 cells. The observation that prolonged treatment with YSH increases the expression of M2 macrophage markers may at least partially explain the ability of honey to promote wound healing.

3 Background

The impact on inflammatory and immunological pathways is one of the most well-documented effects of treatment with honey from various sources (1),(2) . The flavonoid components in honey have been found to exert broad anti-inflammatory effects through such mechanisms as modulation of the expression of myeloperoxidase, hyaluronidase, and tyrosine-protein kinase (1). Moreover, treatment with honey can influence the release of leukotrienes, prostaglandins, and cytokines from leukocytes and other immunologic cells (3),(4). These activities have been presumed to form the basis of the utility of honey in treatment of inflammatory conditions such as arthritis and certain forms of pleurisy (5). However, the impact of honey on the release of cytokines, specifically from macrophages, along with how this activity changes according to the inflammatory profile of the macrophages, is a relatively less studied aspect of immune modulation.

Cytokines are a group of low-molecular weight proteins released primarily by several classes of white blood cells to mediate their activities and serve as a means of communication between the cells (6). Originally discovered in the 1960s there are now over 100 types of cytokine molecules currently known. These proteins play roles ranging from regulation of inflammatory processes to modulation of metabolism, chemotaxis, cell growth, and tissue repair (7),(8). The term cytokine is applied generically to a diverse array of molecules, which include lymphokines (referring to the subset of cytokines secreted by lymphocytes), monokines (released by monocytes), chemokines (denoting cytokines that express chemotactic effects), as well as interleukins (referring to cytokines that are formed by a leukocyte and exert their effects on other leukocytes) (6). To exert its effects, a cytokine binds to a unique receptor located on the target

cell's surface, generating a series of signals that orchestrate subcellular effects, including the promotion or inhibition of gene expression and the activation of transcription factors. The outcome of these processes may be the release of more cytokines, an increase in the number of receptors for other active mediators on the cell's surface, or an eventual down-regulation of the cytokine's own influence (7).

Cytokines are made by leukocyte cell populations in response to the introduction of different stimuli. They are mostly commonly released by macrophages and lymphocytes, but adipocytes, connective tissues, endothelial cells, and other polymorphonuclear leukocytes can also secrete them (6). Some cytokines, such as interferon-gamma, are essential for the activation of macrophages, which then secrete other cytokines, such as tumour necrosis factor–α (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, and IL-12 which can then affect innate and adaptive immune responses (9). Several subsets of macrophages are known, their classification being based on their source and the microenvironment (ie. tissue) where they are located. Depending on their activation status, macrophages are typically categorized as either classically or alternatively activated. Conventionally, these categories have been designated M1 (pro-inflammatory macrophages) and M2 (anti-inflammatory macrophages) (which will be described further in Chapter 4 of this report). Consequently, the different classes of macrophages show significant differences in the type of cytokines that they secrete, a feature that can be used to distinguish the actions of each type of macrophage (10). Previous studies examining the impact of honey on cytokine production by macrophages revealed certain findings with respect to differences in cytokine secretion. Tonk et al. (9) reported that treatment with honey promoted an increase in the release of pro-inflammatory cytokines by a culture of the monocytic cell line, MM6. A 2 h exposure of the MM6 cell culture to honey extracts resulted in a significant rise in the expression

of TNF-α and IL-1β (9). The latter is the so-called pyrogenic cytokine that has the capacity to increase body temperature through a stimulation of prostaglandin production. The rise in body temperature, mediated by IL-1β, is thought to correlate with important immunologic events such as the release of acute-phase proteins (8). TNF-α stimulates the production of IL-1β, thus functioning as an autocrine loop and escalating further cytokine release. Both classes of cytokines play a central role in mediating inflammatory responses in response to a wide range of conditions (2),(7). The exposure of MM6 cells to honey also promoted the release of IL-6, which can have either inflammatory or an anti-inflammatory activity, which in this case contributes to the down-regulation of TNF-α secretion and augmentation of acute phase proteins (6). A large number of cytokines are pro-inflammatory, including TNF-α, IL-1β, and IL-6, while others, such as TGF-β and IL-10, serve anti-inflammatory roles. Therefore, the type and number of cytokines released by a population of macrophages gives a reliable indication of whether the white blood cells are pro-inflammatory (M1) or anti-inflammatory (M2) (10).

3.1 Materials and methods

3.1.1 Culturing of THP-1 cells

THP-1 cells were cultured in suspension and passaged as required to maintain cell densities between 5 x 10^5 and 2 x 10^6 cells/ml. Cells were grown in RPMI 1640 complete medium with 10% fetal calf serum, in an incubator at 37° C with humidity control and CO_2 maintained at 5% in air. Cultures were monitored microscopically, refreshed with new growth media every 2-3 days (11), and passaged prior to reaching the end of log phase growth by centrifuging at 130 x *g* for 5 min and resuspending to 1 x 10^6 cells/ml. Cells were counted using a haemocytometer chamber under a phase contrast microscope.

3.1.2 Preparation of cell treatments

3.1.2.1 Lipopolysaccharide (LPS)

LPS from Escherichia coli 0127: B8 was tested in cell culture. The LPS powder was diluted in distilled water to give 1 mg/ml. The solution was aliquoted into 50 µl volumes and kept at –20° C.

3.1.2.2 Phorbol 12-myristate 13-acetate (PMA)

A stock solution at 1 mg/ml (162 µM) PMA was made up in DMSO. It was stored as 20 µl and 50 µl aliquots in small microfuge tubes and kept at – 20° C.

3.1.2.3 Honey and Heat treatment of honey samples

A stock solution of honey (10% (w/v)) was prepared in complete RPMI media, and incubated at 37º C to dissolve the honey in solution. The honey stock solution was prepared fresh for each experiment and dilutions of this stock solution were the used in experiments. For some experiments, the honey solution was then heat-treated by incubation in a water bath at 80º C for 30 min. After the heat-treatment, further dilutions were performed in complete RPMI medium.

3.1.3 Effect of honey or PMA on THP-1 cell viability and proliferation

SRB assays were performed to measure the proliferation of THP-1 cells treated with YSH or controls. For each experiment, 100 µl/well of THP-1 cells were suspended in complete media and plated onto replicate 96-well plates at 4000 cells/well. The plates were then incubated for 24 h in an incubator containing 5% CO_2 at 37° C and then the growth medium in each plate was replaced with a media containing different concentrations of YSH, sugar, or media as indicated, and incubated for an additional 48 - 72 h. At the end of the experiment, the cells in each plate were fixed by incubation with 50% ice-cold tricholoracetic acid (TCA) and incubated at 4º C for 1 h, followed by repeated washing in running water and then the plates were air dried. The fixed cells were then stained by incubation in a 0.1% (w/v) SRB solution for 30 min at room temperature, and washed and then re-suspended in 200 µl of 10 mM Tris buffer, pH 10.5. The absorbance of each well was recorded at a wavelength of 530 nm using a plate reader (Molecular devices, Spectra max 340 PC) (Vichai and Kritikara, 2006) and the IC_{50} values for YSH were calculated using a sigmoidal dose-response curve (variable slope) using Graph Pad Prism V 4.02 software (Graph Pad Software, Inc.).

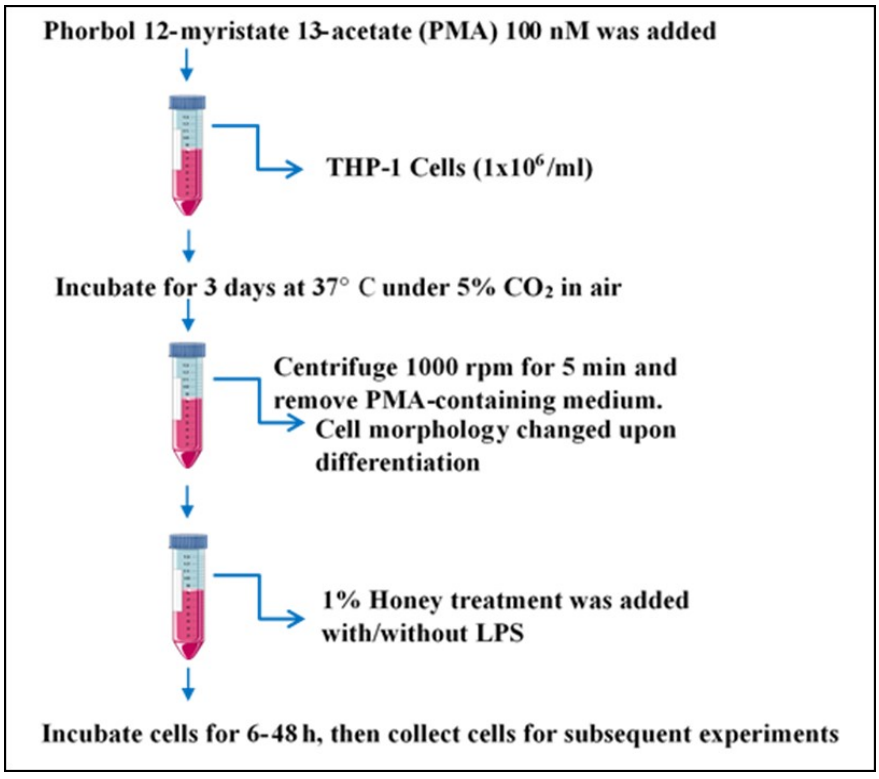
The IncuCyte® Proliferation Assays for Live-Cell Analysis (Essen BioScience) was used to measure cell proliferation of THP-1 cells in response to treatment with phorbol ester by counting the number of phase objects over time. By masking individual cells this enabled a label-free count until the point that the cultures were densely packed and cell edges could not be accurately determined. In addition, subsequent classification of cells into subpopulations can be performed based on properties including size and shape. For experiments, cells were collected, cell concentration determined, and then plated onto the wells of a 6 well plate and incubated at 37º C for 24 h. After incubation, these cells were treated with PMA and the Incucyte was set to count the cells from 8 different areas for each well, for each day, for 7 days, and the change in cell number was calculated and plotted for each condition.

3.1.4 Analysis of DNA content by flow cytometry

The cells were stained with the DNA stain propidium iodide and analyzed using flow cytometry to detect apoptotic cells with fractional DNA content and to identify the distribution of cells in the cell cycle (G_1 vs S vs G_2/M). THP-1 cells were exposed to YSH, glucose, or media for different time intervals, ranging from 24 h to 72 h. The cells were collected and washed twice in PBS, pH 7.4, and fixed overnight by incubation in 75% ice-cold ethanol and stored at -20º C until analysis. For analysis, the cells were collected by centrifugation, washed twice with PBS, pH 7.4, and suspended in 0.5 ml PBS, pH 7.4, and 0.5 ml propidium iodide (PI) staining solution (PBS, pH 7.4, 0.3% Nonidet P-40, 100 µg/ml RNase A, and 100 µg/ml propidium iodide) for 1 h. The re-suspended samples were then analyzed by flow cytometry using an FC600 Flow Cytometer (Beckman Coulter). Flow profiles were recorded and the proportion of cells in the sub-G_1, G_o/G_1, S, and G_2/M phases were determined.

3.1.5 THP-1 cell differentiation

THP-1 cells were harvested by centrifugation and resuspended in complete RPMI medium at 1 x 10^6 cells/ml, after counting using a haemocytometer chamber under a phase contrast microscope. The cells were then plated (1 ml /tube) into a 15 ml tube (Fisher) and 100 nM PMA (Sigma-Aldrich) was added (Schematic diagram 3.1). The cells were incubated for 3 - 5 days in a 37° C incubator with humidity control and 5% CO_2 to allow them to differentiate (12),(13). After incubation, the cells were collected by centrifugation at 130 x *g* for 5 min, washed with fresh RPMI medium, re-centrifuged at 130 x *g* for 5 min to remove the PMA, and fresh RPMI medium (1 ml) added for further analysis. To assess the differentiation of the THP-1 monocytes in this macrophage model, screening was performed on suspension THP-1 monocytes using flow cytometry, and on adherent differentiated THP-1 macrophages using IncucyteS3 imaging.



Schematic Diagrams 3.1: Summary of *in vitro* differentiation protocol.

THP-1 cells were differentiated with phorbol 12-myristate 13-acetate, washed and honey was added in the presence or absence of lipopolysaccharide. Then cells were collected for flow cytometry and the culture supernatant collected for ELISA (14).

3.1.6 Treatment of differentiated THP-1 samples and controls

The THP-1 cells were treated with several culture conditions to generate THP-1-derived macrophages and control cells. **Cells alone:** Cells were incubated in complete RPMI medium (1 ml) and maintained as non-differentiated cells. **Honey alone**: The undifferentiated THP-1 cells were incubated in 1% (w/v) YSH honey (10% YSH stock added at 100 µl/ml) for 3 - 5 days. **PMA**: THP-1 cells were differentiated by incubation with 100 nM PMA for 3 - 5 days. **PMA + LPS:** Undifferentiated THP-1 cells were incubated with 100 nM PMA for 3 days and then treated with 100 ng/ml LPS in complete RPMI culture medium for 24 h. **PMA + Honey:** The THP-1 cells were incubated in 100 nM PMA for 3 days and then 1% YSH for 24 h. **PMA + LPS + Honey:** The THP-1 cells were incubated in 100 nM PMA for 3 days in complete RPMI culture medium and then 100 µg/ml LPS and 1% YSH were added for 24 h. **PMA + IL-4:** THP-1 cells in complete RPMI culture medium were incubated in 100 nM PMA for 3 days and 100 ng/ml IL-4 was added to the differentiated cells and incubated for 18 h before collecting the supernatant. **PMA + IL-4 + Honey:** THP-1 cells were differentiated by incubation with 100 nM PMA for 3 days in complete RPMI culture medium and 100 ng/ml IL-4 was added for 18 h and then honey was added for 24 - 48 h prior to collection.

3.1.7 Conditioned medium preparation

THP-1 cells treated with the different activators, to differentiate them to M1 or M2 polarized macrophages, as described above, were incubated in serum-free medium for 24 h and then centrifuged at 10,000 rpm for 5 min, after which supernatants were collected as conditioned medium and stored at −80° C until analysis of cytokine levels by ELISA (R&D Systems).

3.1.8 Characterization of monocyte differentiation by flow cytometry.

Monocyte differentiation was characterized by measuring the expression of cell surface differentiation markers on THP-1 cells differentiated by treatment with PMA and then treated with LPS and/or honey as described above. The THP-1 cells were aliquoted into 1 ml suspensions at 10^6 cells/ml in 15 ml conical tubes and treated with 100 nM PMA for 3-5 days. The cells were collected by centrifugation and resuspended in 1 ml of RPMI complete media and incubated at 37° C overnight. The cells were then treated with suspending media, 100 ng/ml LPS, and/or 1% YSH, as indicated in Fig 3.2, for 24 - 48 h. For analysis of the cells by flow cytometry, the cells were collected by centrifugation and resuspended in 1 ml BSA blocking reagent (Pierce Chemical Co) or 1% BSA in pH 7.4 and incubated for 10 min. The cells were then incubated for 30 min with specific antibodies against cells surface CD markers at a titre of 1:1000 in block buffer; anti-CD163-phycoerthrine (PE) (clone GH/61), anti-CD68-PER CP (clone KP1), or anti-CD80-fluorescein isothiocyanate (FITC) (clone F7) labelled anti-human monoclonal antibodies. Cells were also incubated with isotype matched, non-specific mouse IgG antibodies labelled with PE, PER-PC, or FITC at a titre of 1:1000 in block buffer to create the negative control histograms for each CD staining antibody. After staining, the cells were washed with PBS, pH 7.4, and resuspended in 500 µl of 1% paraformaldehyde. The cells were then

analyzed by flow cytometery on an FC600 flow cytometer (Beckman). THP-1 cells were gated for analysis by a combination of forward scatter (FSC) and side scatter (SSC). Gated cells were analyzed for CD68 positivity, and $CD68^{+}$ cells were then examined for CD80 (M1) and CD163 (M2) expression using samples stained with the isotype control antibodies (Santa Cruz Biotech). The percentage of cells positive for the (CD68-CD80-CD163) marker was determined by setting a cursor on the negative match isotope or monocyte control to include 98–99% of the population. The histograms for the cells stained for CD68, CD80, and CD163 surface levels were compared using Beckman flow cytometry software. Microsoft® Excel® 2007 (Excel 2007) was used to organize output data of the percent positive values of each surface marker.

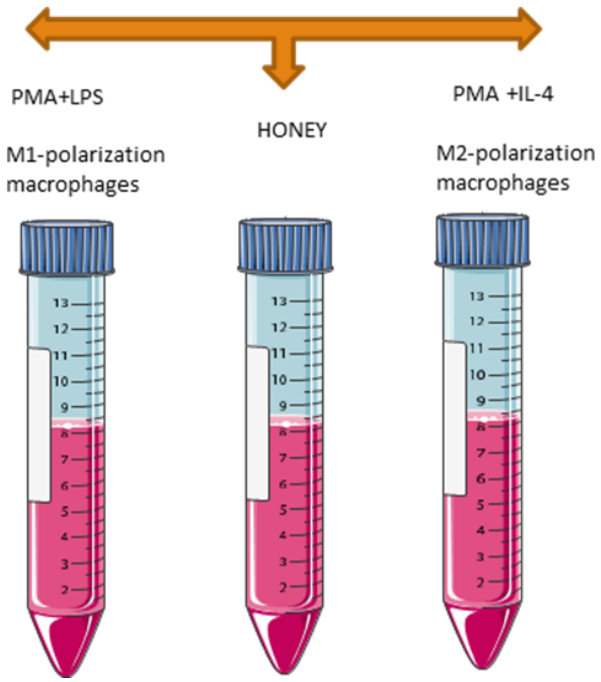
Controls:

THP-1+Meduim Not	**THP1+Meduim** CD45	**THP-1+Meduim** CD68	**THP-1+Meduim** CD80	**THP-1+Meduim** CD163

PMA CD68	**PMA** CD80	**PMA** CD163
PMA+LPS CD68	**PMA+LPS** CD80	**PMA+LPS** CD163
PMA+IL-4 CD80	**PMA+IL-4** CD68	**PMA+IL-4** CD163

Treated samples

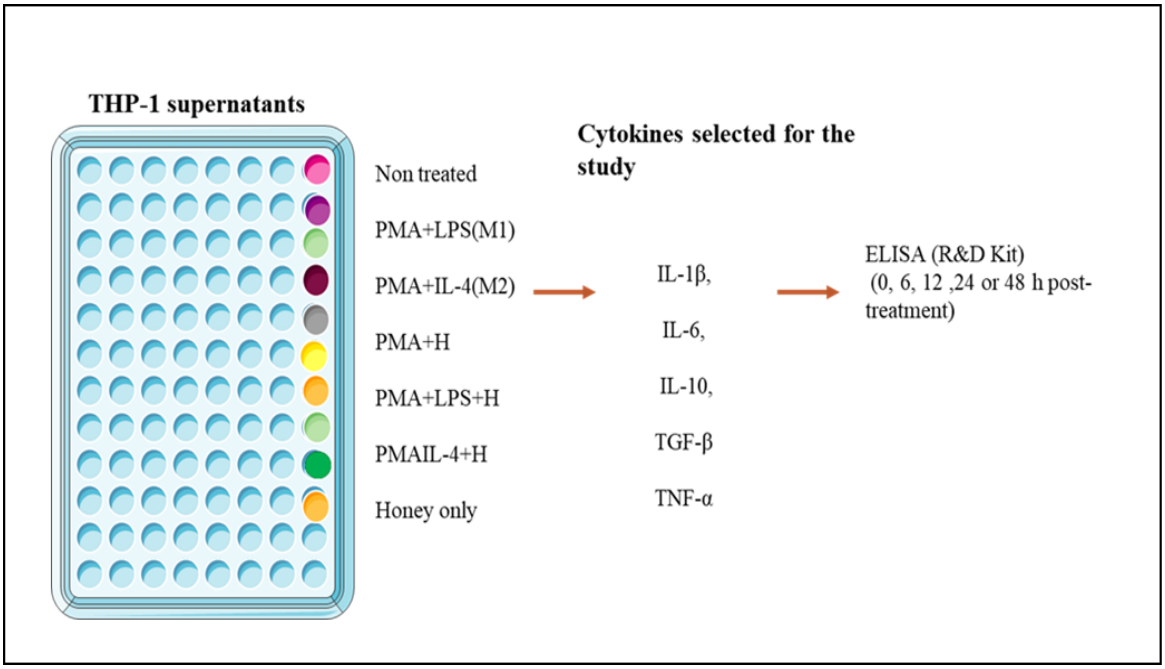
H CD68	**H** CD80	**H** CD163
PMA+H CD68	**PMA+H** CD80	**PMA+H** CD163
PMA+LPS+H CD68	**PMA+LPS+H** CD80	**PMA+LPS+H** CD163
PMA+IL-4+H CD68	**PMA+IL-4+H** CD80	**PMA+IL-4+H** CD163



Schematic Diagram 3.2: Summary of control and honey samples treatments to determine the effects of honey on M1 and M2 polarization surface marker expression levels. The polarization will be determined by comparison to classic established M1/M2 markers (CD68-CD80-CD163) using Flow cytometry (14).

3.1.9 Cytokine production in differentiated THP-1 cells as measured by ELISA.

The honey was analyzed for its ability to stimulate cytokine production from non differentiated and differentiated THP-1 cells, with and without added LPS as described above. The THP-1 cells were differentiated by incubation in 100 nM PMA for 3 days and then treated with 1% YSH (w/v) in complete RPMI medium and/or 100 ug/ml LPS and/or IL-4 for 24 h as indicated in Fig 3.3. In some experiments, the honey had been previously heat treated to determine the potential impact of denaturation on modulation of cytokine production. Cell supernatants were collected for appropriate time points (6, 12, 24, and 48 h) and the level of cytokines produced from the cells were measured using ELISA.



Schematic Diagram 3.3: ELISA summary of THP-1-Honey treated with and without lipopolysaccharide.

THP-1 cells were treated as indicated for 6 - 48 h and supernatants collected to measure inflammatory (IL-1β, IL-6, and TNF-α) and anti-inflammatory (IL-10 and TGF-β) cytokine expression. The honey samples were treated as described (14).

The levels of TNF-α, IL-1β, and IL-6, IL-10, and TGF-β in the THP-1 cell culture supernatants were quantified using commercially available ELISA kits (R&D Systems) according to the manufacturer's instruction. The assay was replicated 3 - 4 times. To prepare the ELISA plates, capture antibody (20 µl) was diluted in 10 ml of coating buffer and 100 µl of the capture antibody solution was added/well to the ELISA plates. The plates were sealed and incubated for 18 h in a darkened 4° C humidity chamber. After incubation, the plates were washed 5 times with PBS, pH 7.4 and 0.05% Tween-20 and then the wells were blocked to

minimize non-specific binding by the addition of PBS, pH 7.4, 0.05% Tween-20, and 1% BSA at 100 µl/well and incubated for 1 h in a darkened humid chamber at room temperature. The plates were washed 5 times with PBS, pH 7.4, and 0.05% Tween-20, air-dried, and stored at 4° C until use. The standards for each cytokine were prepared by dilution in PBS, pH 7.4, containing 1% (w/v) BSA and 0.05% Tween-20 and diluted to provide the standard curve as indicated by the manufacturer. Samples of conditioned media from undifferentiated and differentiated THP-1 cells treated with YSH and/or LPS or samples of the standard were added to the wells (100 µl/well) in duplicate and incubated for 2 h in a darkened humidity chamber. The plates were washed 5 times with PBS, pH 7.4, 0.05% Tween-20 solution and then incubated with 100 µl/well of the secondary antibody-streptavidin conjugate (20 µl of the detection antibody were diluted in 10 ml PBS, pH 7.4 and 0.05% Tween-20) for 1 h at room temperature. The plates were washed 3 times with PBS, pH 7.4 and 0.05% Tween-20 and incubated with 100 µl/well of horseradish peroxidase-streptavidin conjugate (10 µl of the HRP-streptavidin was diluted in 10 ml PBS, pH 7.4 and 0.05% Tween-20) for 1 h at room temperature. The plates were washed again 3 times with PBS, pH 7.4, and 0.05% Tween-20 and stained by incubation with 100 µl/well TMB substrate (prepared by mixing Reagent A and B in equal volumes) for 30 min in the dark until blue colour formation. Then 50 µl of stop solution (2 M H_2SO_4) was added to each well. The absorbance of the samples was measured immediately using a Multiscan MCC/340 plate reader at 450 nm. Microsoft Excel was used to calculate the concentration of cytokines in the supernatant samples using the standard curve from the known concentration.

3.1.10 Statistical analysis

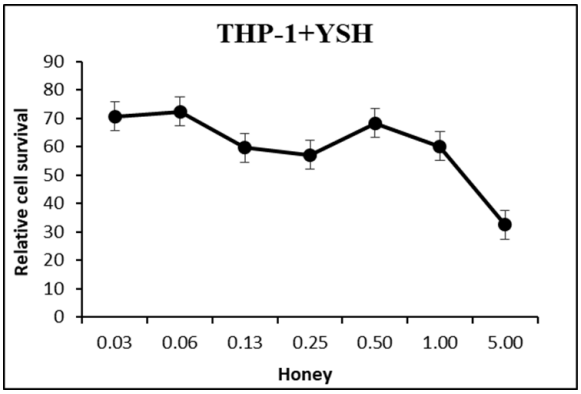
Data was analyzed and graphed using Graph Pad (Prism) software and Microsoft Excel. Results were expressed as means ± SD, and the overall differences between group means were analyzed using one-way ANOVA. Multiple comparisons were carried out using t-tests.

3.2 Results

3.2.1 Cytotoxicity Effect of YSH on THP-1 (IC50) SRB

To determine the effect of Yemen Sidr honey on THP-1 cells, the cells were treated with different concentration of YSH (0.3, 0.6, 1.25, 2.5, 5, and 10%), and relative cell number examined using an SRB assay (Tetrazolium hydroxide-Neutral Red-Sulforhodamine B). The viability of THP-1 cells was reduced in a dose-dependent manner (Fig 3.1A). (When YSH activity was compared to the effect against cancer cells (Figure 2.1A, D), THP-1 cell were less affected). The IC_{50} for YSH-induced THP-1 cell proliferation was determined to be 6.5% at 48 h. Therefore, THP-1 cells treated with 1% YSH maintained a high cell viability (Fig 3.1B) and therefore cells were treated with 1% YSH for the rest of the study to induce behavioural changes in the differentiation of THP-1 cells.

A



B

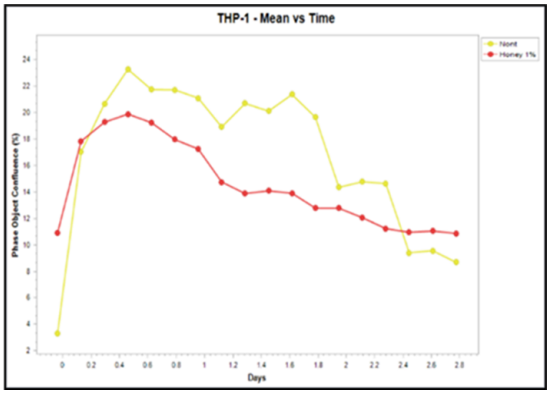


Figure 3.1: The concentration-dependent effect of Yemen Sidr honey treatment on THP-1 cells. Cells were treated with freshly prepared YSH at different concentrations to determine the half maximal inhibitory concentration (IC50). (A) An IC_{50} of 6.5% for cell viability was determined using an SRB assay. The results were presented as the mean $\pm$ SEM of three independent experiments. (B) The cell proliferation assay was performed using the Incucyte live cell imaging count apparatus and software in cells treated with 1% YSH (red) or media control (yellow).

3.2.2 Effect of treatment with YSH on cell cycle progression on THP-1 cells.

To determine if there was a change in the cell cycle progression of THP-1 cells in response to treatment with YSH, cells were exposed to 1% or 2.5% YSH or 1% sugar for 24, 48, and 72 h and then analyzed for DNA content using flow cytometry. There was no significant induction of cell cycle arrest or apoptosis in response to treatment with YSH at different time points. Treated cells were compared to controls (non-treated cells and 1% sugar). A total of 10,000 events were analyzed by flow cytometry following propidium iodide staining. Results are expressed as the percentage of total cells in each phase of the cell cycle and show no differences in the proportion of cells in the Sub-G_1 peak or in G_1, S or G_2M.

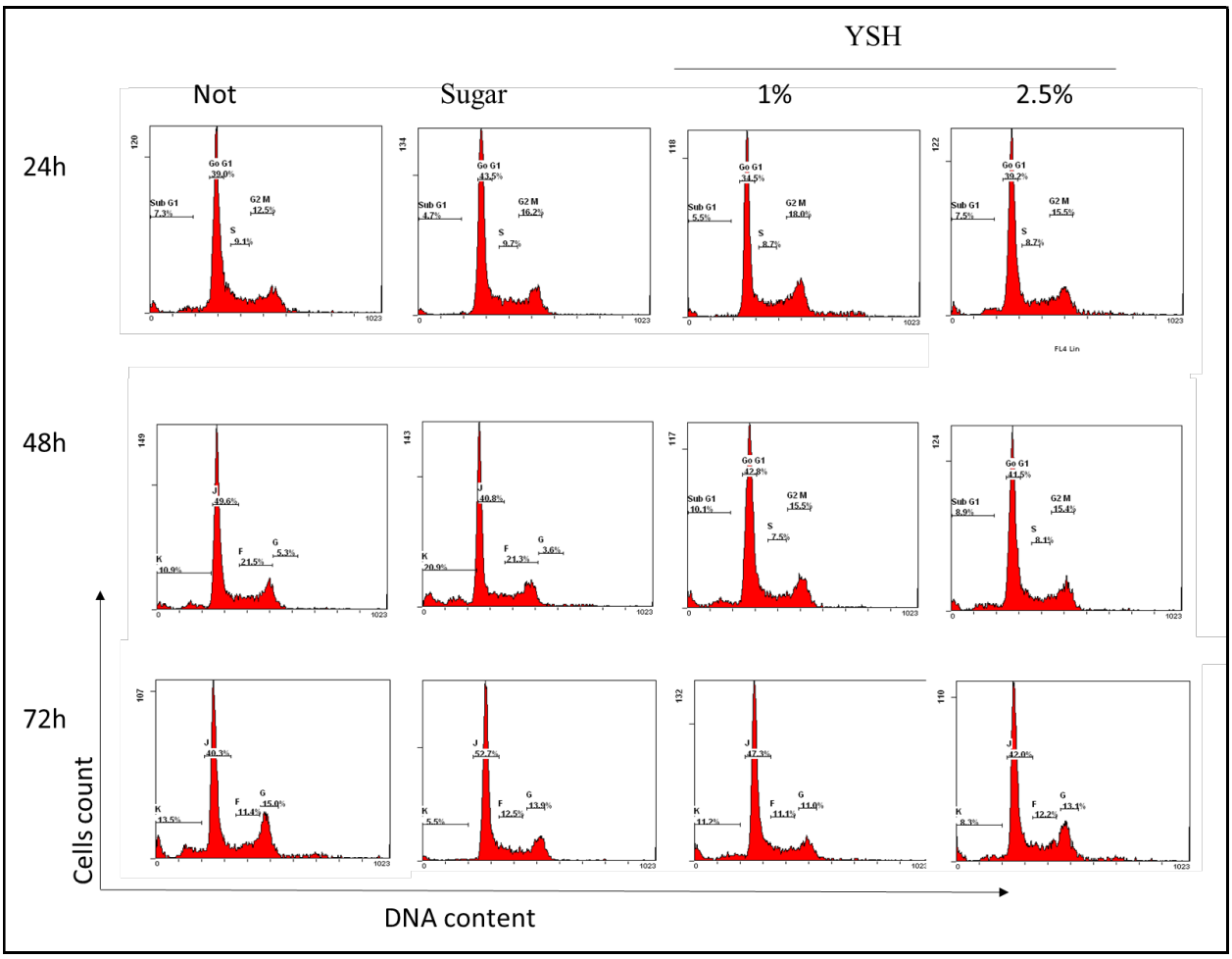


Figure 3.2: Cell cycle analysis of THP-1 cells treated with YSH by flow cytometry.

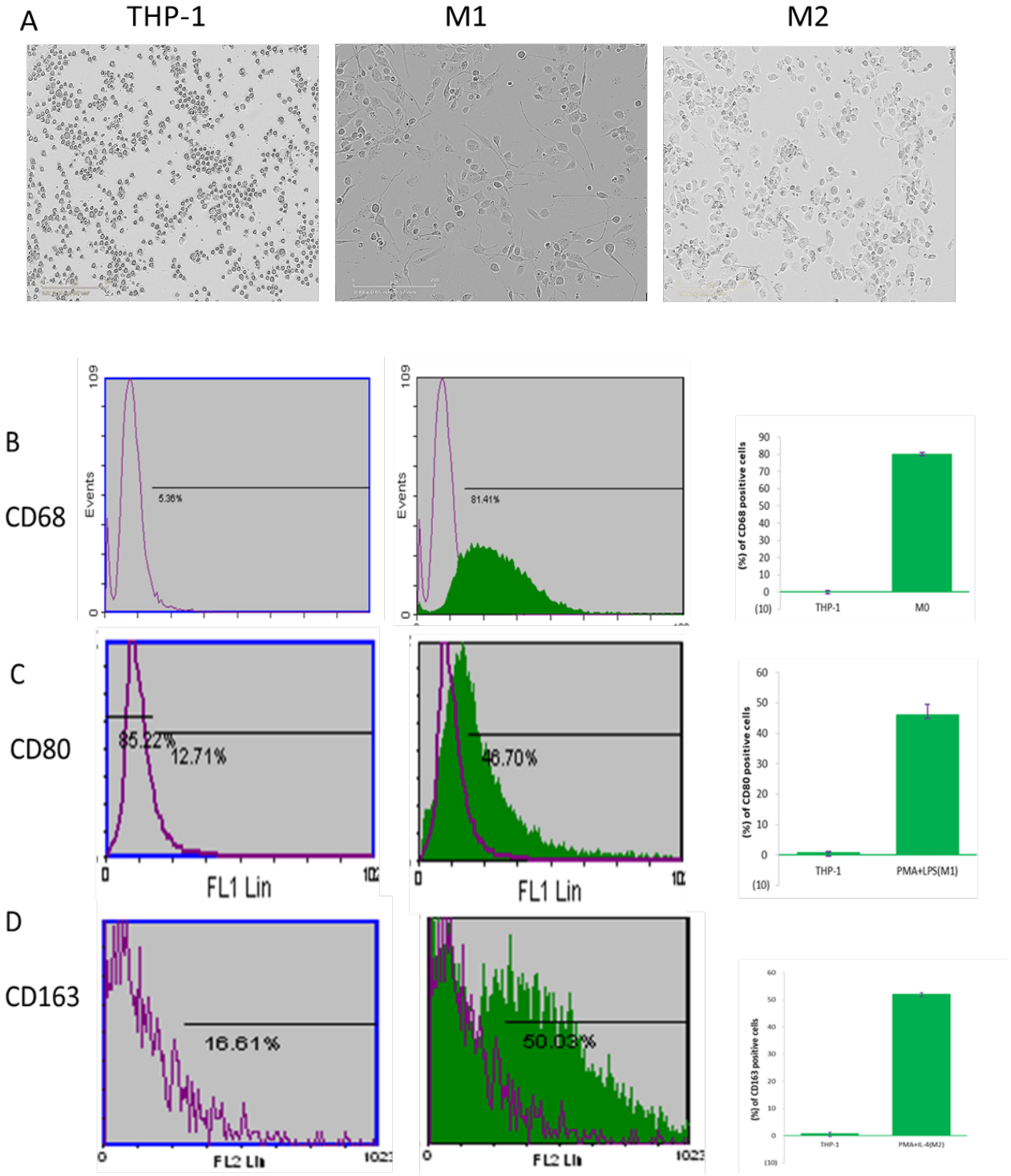
Flow cytometry profiles of THP-1 cells treated with 1 or 2.5 % YSH or 1% sugar for (24 - 72 h. The percentage of cells in each phase of the cell cycle was estimated by gating for the fluorescent intensity corresponding to the amount of DNA in each event with respect to non-treated cells. The X-axis represents the DNA content (PI staining) and the Y-axis corresponds to the number of cells.

3.2.3 Macrophage morphology, and polarization after activation of THP-1 cells

To generate a model of macrophage polarization, we used PMA-differentiated human THP-1 monocytes. THP-1 cells were treated with phorbol ester (PMA) for 3 - 5 days as indicated and the transformation from monocyte to macrophage was expected to have been completed as indicated by the changes in cell morphology that were observed. Representative images of the cell morphology before and after treatment are shown (Fig. 3.3A). To assess the morphological change in cell shape from the typical spherical monocyte phenotype to the amoeboid, adherent macrophage phenotype, cells were examined daily using the Incucyte live imaging system (Phase), until their shape was no longer considered spherical. The cells shown in Fig. 3.3A, were all at this macrophage stage. Control experiments were performed to confirm that the THP-1 cells treated with PMA showed the monocyte to-macrophage differentiation and the cells were characterized for increased expression of the CD68 cell surface macrophage marker using flow cytometry analysis (Fig. 3.3B). Untreated THP-1 cells do not express significant levels of CD68, but after 3 days of treatment with PMA, the cells were positive for CD68 staining. The PMA-differentiated human THP-1 monocytes (designated M0 macrophages), were treated with LPS to generate M1 (inflammatory) polarized macrophages or treated with IL-4 to generate M2 polarized macrophages, which represent the two opposite polarized states. The M1 macrophages showed cellular elongation in cell morphology, while the M2 macrophages showed a more rounded morphology (Fig. 3.3A). The differentiated THP-1 cells were also tested for expression of the surface markers CD80 (M1 phenotype) and CD163 (M2 phenotype) using flow cytometry: increased expression of CD80 was seen on the M1 macrophages and increased expression of

CD163 was seen on the M2 macrophages (Fig. 3.3 C, D). Taken together, these finding indicated the successful polarization of THP-1 monocyte-like cells into M1 and M2-polarized macrophages.

Macrophages are not proliferative cells unlike the monocyte precursor. The THP-1 cells show reduction in proliferation after receiving the activating treatment (PMA), which is also an indicator of the change in phenotype as these cells differentiate. Cells that received treatment with PMA were compared with the non-treated control cells (monocytes) using Incucyte live imaging counting. The data in (Fig. 3.3F) shows that PMA-activation generated a significantly less proliferative cell population than untreated cells.


A
THP-1
M1
M2
B
CD68
Events
5.36%
81.41%
(%) of CD68 positive cells
THP-1
M0
C
CD80
85.22%
12.71%
46.70%
FL1 Lin
(%) of CD80 positive cells
THP-1
PMA+LPS(M1)
D
CD163
16.61%
50.03%
FL2 Lin
(%) of CD163 positive cells
THP-1
PMA+IL-4(M2)

E

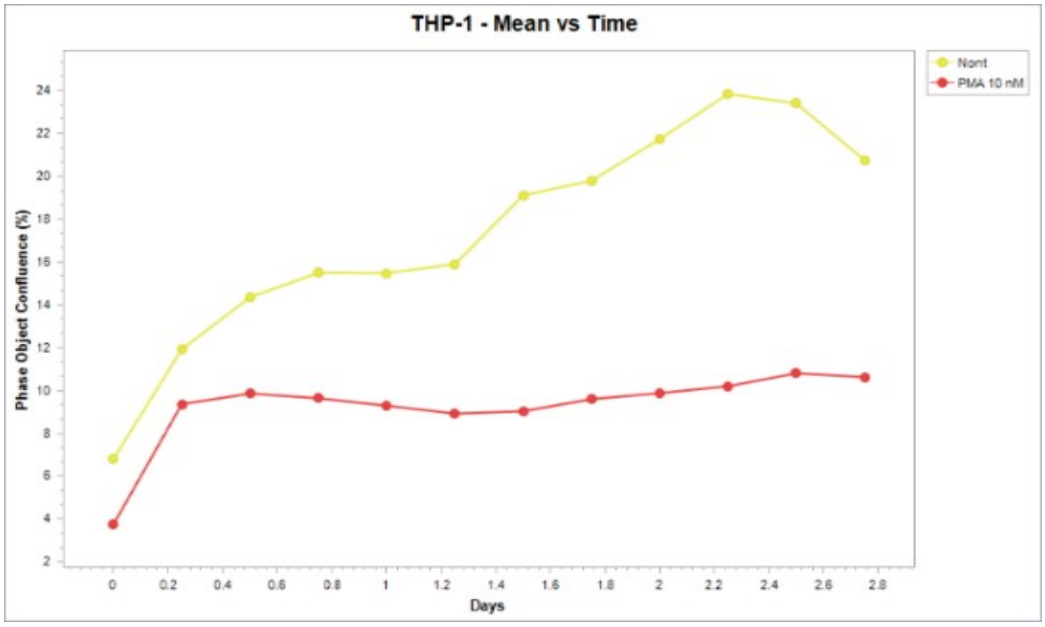


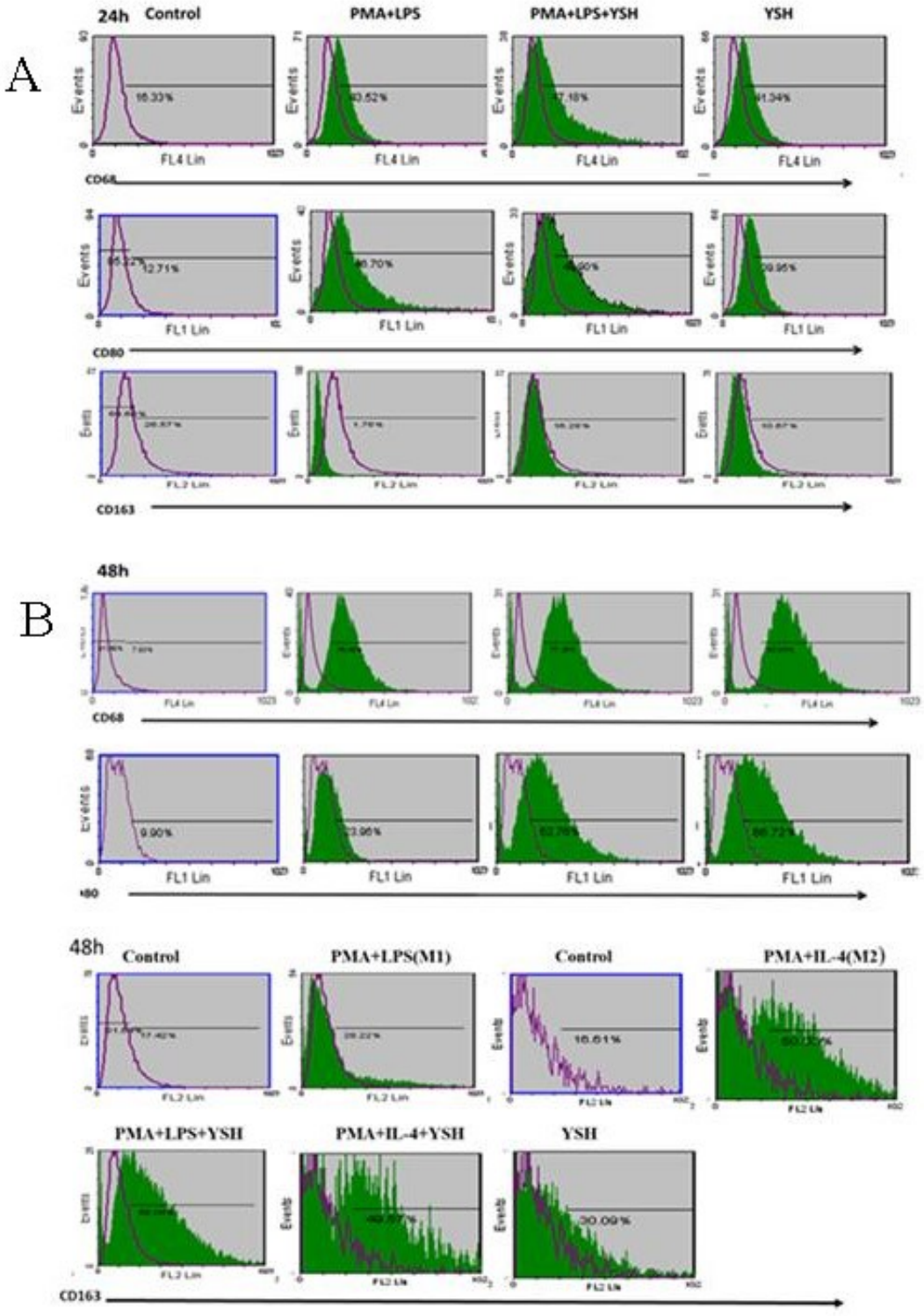
Figure 3.3: Detection of macrophage activation, M1/M2 polarization, and proliferation.

(A). Morphology of THP-1 monocytes, (non-differentiated THP-1 cells), THP-1 cells treated with PMA and LPS (M1), and THP-1 cells treated with PMA and IL-4 (M2) were examined using the Incucyte imaging system (100×). (B) the expression of the CD68 macrophage cell surface marker (green) on untreated cells or THP-1 cells treated with PMA was measured by flow cytometry. (C, D) Flow cytometry of THP-1-derived macrophages treated with LPS or IL-4 for cell surface expression of CD80, the marker for M1 polarized macrophages and CD163, the marker for M2 polarized macrophages. Data are presented the mean % of cells, ±SEM. All results show the relative change compared to untreated THP-1 cells ($p < 0.05$). (E) Incucyte proliferation assay showing macrophage counts in THP-1 cells treated in the absence (yellow) or presence of PMA (red) for 3 days.

3.2.4 Effects of honey on M1 and M2 polarization surface marker expression

Having shown that THP-1 cells can be used as a model of macrophage polarization, we examined the role of honey on this differentiation. PMA-differentiated human THP-1 monocytes were treated with LPS (M1), IL-4 (M2), or honey with and without LPS and the cells examined for expression of the M1 and M2 cell surface markers. In addition, undifferentiated THP-1 were treated with honey and the expression of the cell surface antigens examined. It was hypothesized that the honey treatment may be acting as an immune activator by causing the differentiation of monocytes into macrophages. When treated with 1% YSH, THP-1 cells maintained high viability, yet displayed characteristics which would indicate successful differentiation to a macrophage phenotype as indicated by the increase in CD68 expression (Fig. 3.4A).

Treatment of THP-1-derived macrophages with YSH for 24 h, enhanced the expression of the CD80 cell surface marker that is associated with the pro-inflammatory M1 macrophage subtype in all conditions of honey treatment compared to controls. However, treatment with YSH for 24 h only weakly enhanced the expression of the CD163 a marker associated with the M2 repair subtype suggesting YSH may have an anti-cancer potential. However, when the THP-1-derived macrophages were treated for an extended period of time of 48 h, the level of the M2 marker was increased on those cells that were also treated with LPS (Fig.3.4A), which may at least partially explain the ability of honey to promote wound healing**.**


A
24h
Control
PMA+LPS
PMA+LPS+YSH
YSH
Events
FL4 Lin
CD68
FL1 Lin
CD80
FL2 Lin
CD163
B
48h
CD68
CD80
48h
Control
PMA+LPS(M1)
Control
PMA+IL-4(M2)
PMA+LPS+YSH
PMA+IL-4+YSH
YSH
CD163

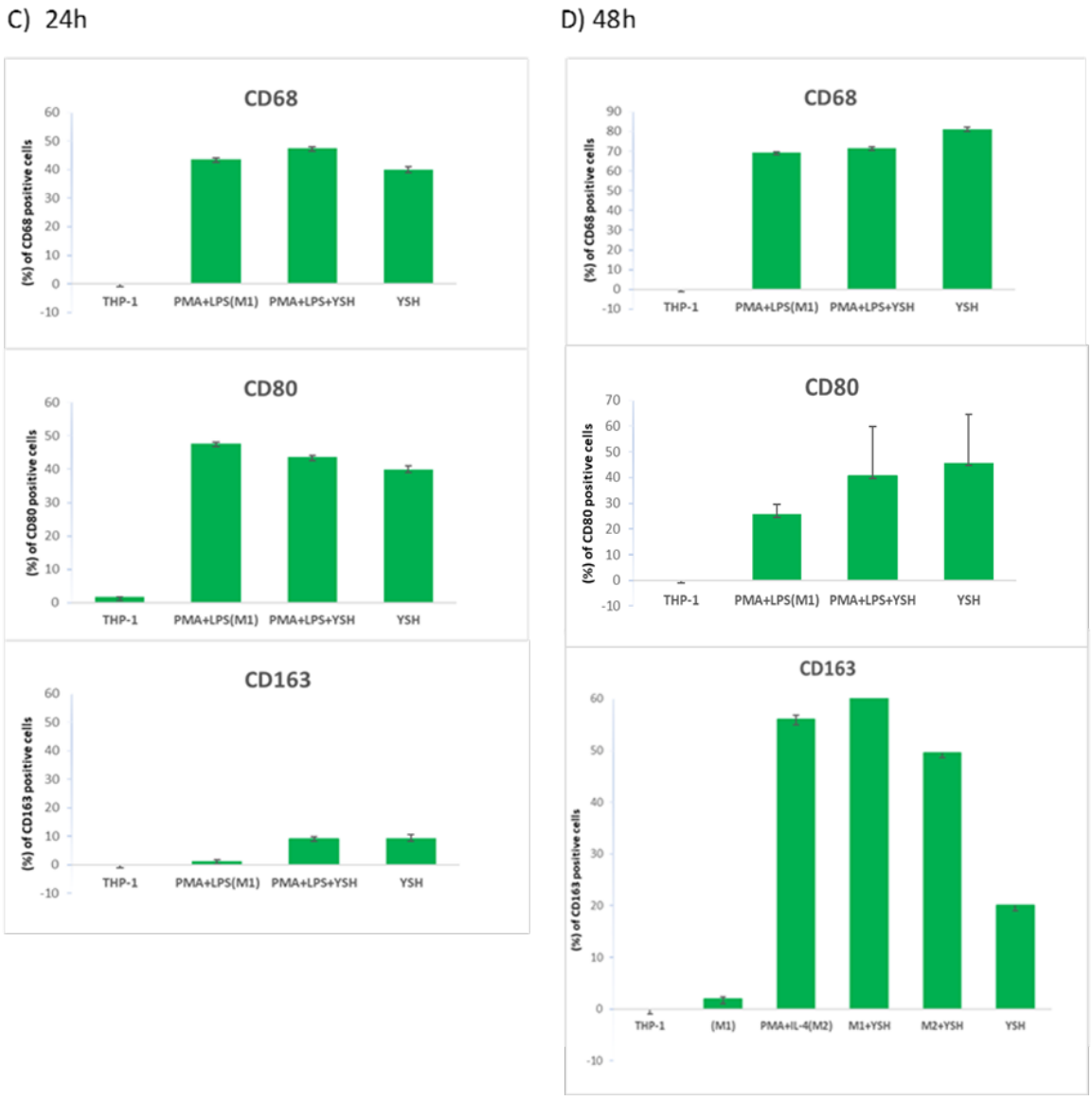


Figure 3.4: Effects of honey on M1 and M2 polarization surface marker expression.

(A, B) Cells were exposed to 1% YSH under different condition for 24 h, and 48 h. The cell surface expression of CD68, CD80, and CD163 were compared by flow cytometry. Macrophage cell populations were electronically gated according to their forward/side scatter properties. Gated cells were analyzed for CD68 positivity, and then the $CD68^{+}$ cells were then examined for CD80 (M1) or CD163 (M2) expression. The green histograms represent the

fluorescent profile of THP-1 cells stained for the indicated cell surface marker, whereas the purple histograms represent the fluorescent profile of cells stained with the isotope-matched control antibody, or untreated THP-1 cells that stained with the cell surface marker antibody. Quantification of the flow cytometry assay was analyzed using EPIC Flow software, and statistical analysis for the relative number of positive cells is presented in (C, D). Data are presented as the mean ± SD from three independent experiments. All results show the relative change compared to untreated THP-1 cells ($p < 0.05$). YSH enhanced the expression of the cell surface markers associated with the pro-inflammatory M1 macrophage subtype, but had a weaker effect on enhancing markers associated with the M2 repair subtype suggesting YSH may have an anti-cancer potential.

3.2.5 The effect of YSH on secretion of cytokines by THP-1-derived macrophages.

Treatment of THP-1 cells with YSH (in the presence or absence of LPS and PMA treatment) at a concentration of 1% (w/v) induced or stimulated the release of TNF-α, IL-1β, and IL-6 when compared to the untreated THP-1 cells or the THP-1 cells treated with PMA (THP-1-derived macrophages) (Fig 3.5). The levels of TNF-α, IL-6 , and IL-1β released into the media peaked at 24 h following treatment with honey. The control THP-1 cells treated with sugar showed only baseline levels of TNF-α, IL-1β, and IL-6 production (5 pg/ml), which were the same as those from untreated cells.

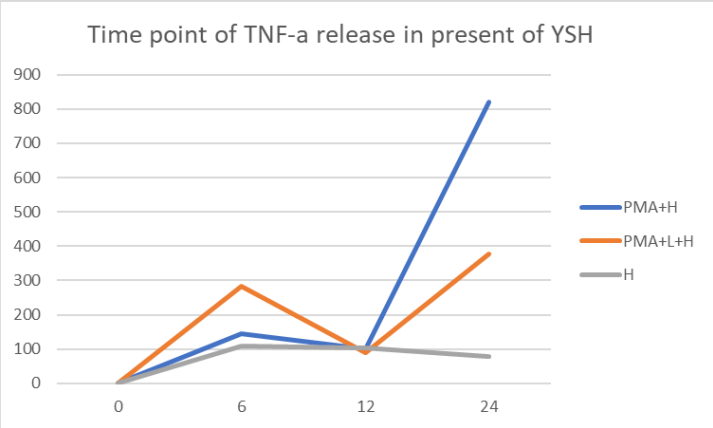


Figure 3.5: Time course effect of 1 (w/v) YSH on TNF-α released from THP-1 cell. THP-1 cells were treated with YSH (H, grey) or THP-1 cells treated with PMA and honey (PMA+H, blue) or PMA, LPS, and honey (PMA+L+H, orange) and incubated for 6, 12, and 24 h. Conditioned media was obtained, after removing the cells by centrifugation, and subjected to ELISA to measure secreted TNF-α release.

THP-1-derived macrophages activated by treatment with pro-inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) also increased the secretion of a variety of cytokines. To analyse the immuno-modulatory effects of honey further, the production of the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, and anti-inflammatory cytokines, IL-10 and TGF-β, were measured in the cell culture supernatants of undifferentiated and PMA-differentiated THP-1 cells treated with 1% YSH for 6 - 24 hours in the presence and absence of LPS (100 ng/ml). The YSH incubation times were chosen based on those shown to stimulate maximal cytokine production in THP-1 cells. Treatment of these cells with 1% (w/v) YSH induced a significant increase in the release of TNF-α, IL-1β, and IL-6 (Fig 3.6, 3.7, and 3.8), compared to untreated THP-1 cells or cells treated with a sugar solution which did not, (sugar not shown) (Fig 3.6). The amount of TNF-α spontaneously released by the untreated and the PMA-differentiated THP-1 cell line was

barely detectable. (Note that the PMA-treated cells had been media changed after 3 days of treatment and before the other activators were added.) When YSH was added to the PMA-differentiated macrophages, there was a significant increase in the amount of TNF-α secreted. The levels of TNF-α were further increased when the cells were also treated with 100 ng/ml LPS. Results show that LPS and YSH each significantly ($p<0.001$) increased the expression of TNF-α compared to non-treated control monocytes. Treatment of THP-1 cells with only YSH induced the release of TNF-α, compared to controls, which was further increased by approximately 25% when the THP-1 cells had been previously differentiated by treatment with PMA . Yet the data suggest that in the absence of LPS, all of the tested YSH honey samples have pro-inflammatory activity.

The data in (Fig 3.7) shows that YSH treatment of PMA-activated macrophages had the most significant effect ($p<0.001$) on increasing the expression of IL-1β compared to controls and other YSH-treated conditions. In addition, YSH treatment of both THP-1 and PMA-differentiated THP-1 cells induced secretion of higher levels of IL-1β than untreated THP-1 cells. The expression of IL-1β in LPS-activated macrophages had an additive increase following the addition of YSH at the 24h time point. Cells treated with only YSH induced the production IL-6, while treatment with only LPS further increased IL-6 production by 2 fold after 24 h (Fig 3.8). On the other hand, YSH treatment inhibited the LPS-stimulated production of IL-6, corroborating the idea that some honeys might exhibit an anti-inflammatory effects.

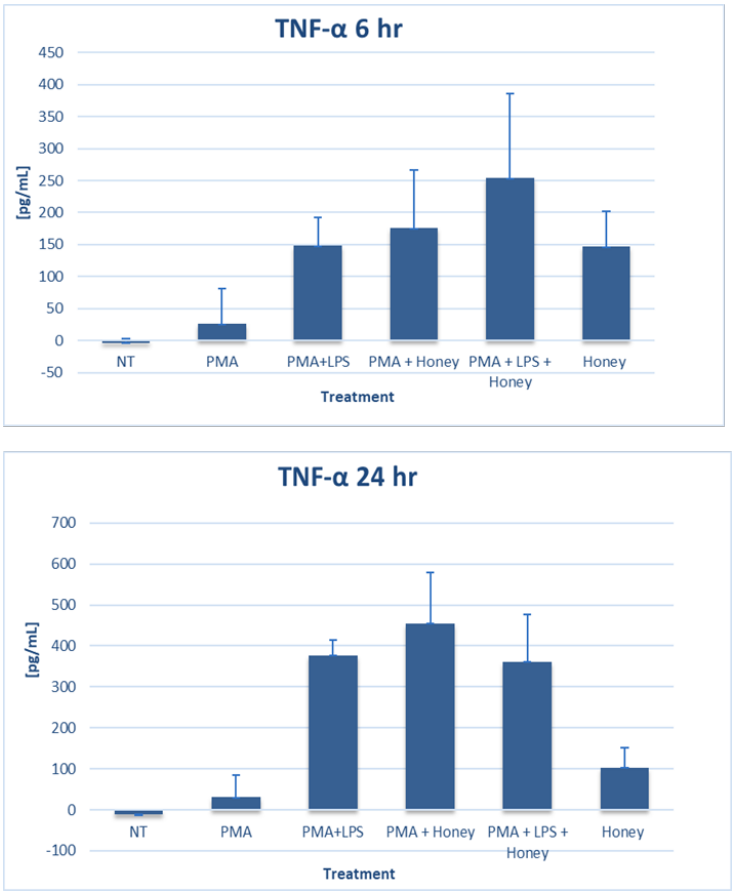


Figure 3.6: YSH induced the release of TNF-α from undifferentiated and PMA-differentiated THP-1 cells.

THP-1 cells were left unstimulated (NT) or were stimulated for 3 days with PMA as controls. PMA-differentiated THP-1treated samples were stimulated for 6 - 24 h with 1% (w/v) YSH, 100 ng/mL LPS, or YSH in combination with LPS. Undifferentiated THP-1 samples were treated with YSH only, as indicated. TNF-α release was measured by ELISA and presented as mean + SD of three replicates. Significant differences between the samples and controls ($p \leq 0.05$).

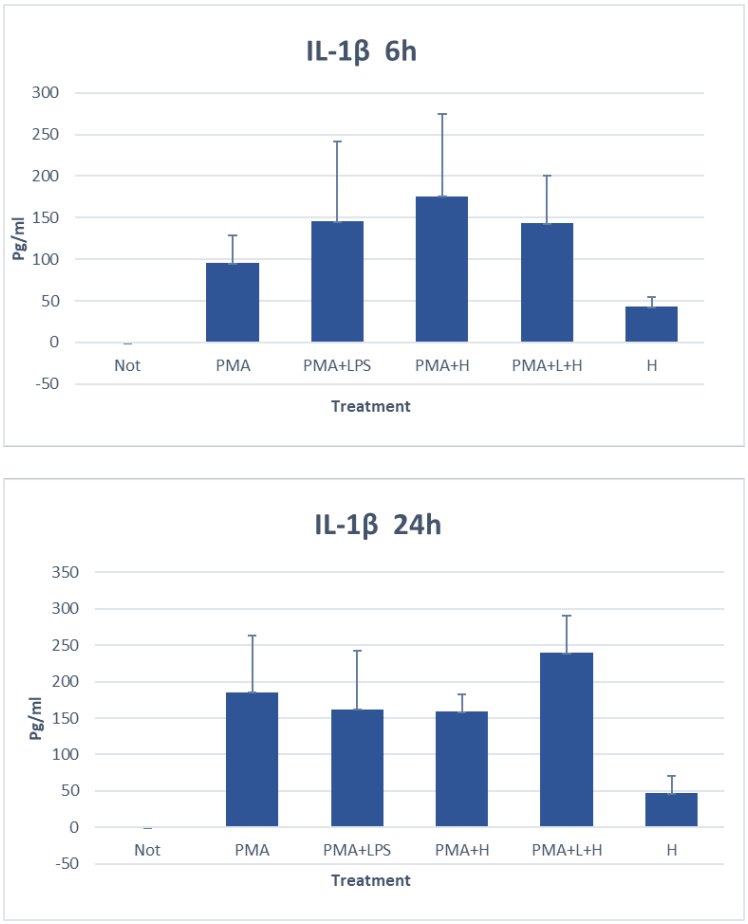


Figure 3.7: Effect of YSH on IL-1β production in THP-1 cells.

The effect of 1% YSH (H) and 100 ng/ml LPS (L), separately and in combination, on IL-1β expression after 6 - 24 h treatment of THP-1 cells (Not) or THP-1-derived macrophages (PMA). Results were calculated for individual experiments from the same cell population. Error bars show mean ± SD of three individual experiments.

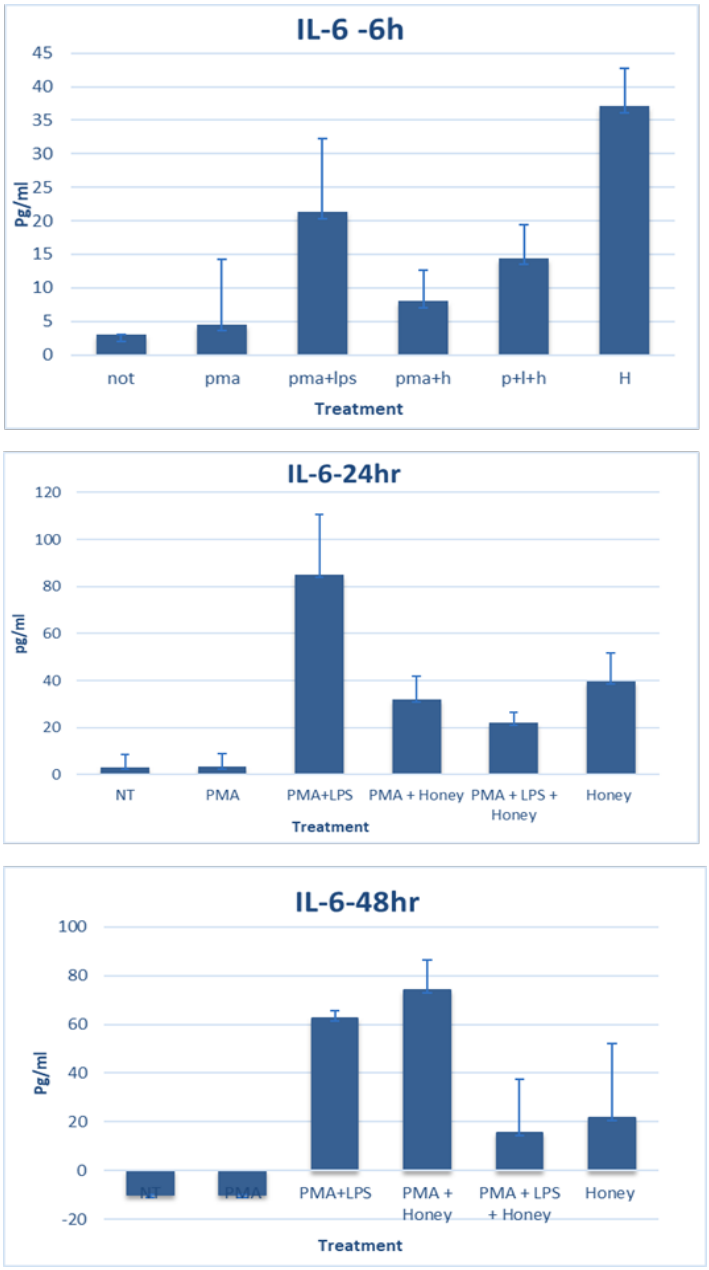


Figure 3.8: Effect of honey on IL-6 production in THP-1 cells.

The effect of 1% YSH (honey) and 100 ng/ml LPS, separately and in combination, on IL-6 expression after 6 - 48 h treatment of THP-1 cells (NT) or THP-1-derived macrophages (PMA). Results were calculated for individual experiments from the same cell population. Error bars show mean ± SD of three replicated experiments for 6 - 24 h, and two replicates for 48 h.

To further analyse the immuno-modulatory effects of YSH, the production of the anti-inflammatory cytokines, TGF-B and IL-10, in undifferentiated and PMA-differentiated THP-1 cells was measured. The level of secreted IL-10 was increased in YSH-treated THP-1 cells compared to untreated THP-1 cells ($p<0.001$) after 6 h (Fig 3.9). YSH or LPS treatment of activated THP-1 cells had approximately the same effect on IL-10 expression levels, but when the cells were treated with both YSH and LPS there was a significant ($p<0.001$) additive effect on IL-10 expression. These results may suggest that honey may have an immunosuppressive effect in the presence of local or systemic pathologic inflammation. On the other hand, without inflammation, honey may enhance local or systemic immune responses as in the presence of LPS treatment, YSH enhanced IL-10.

The results in (Fig 3.10) show that YSH treatment for 48 h does not have a significant effect on TGF-β expression compared to untreated controls, however, the results shows that treatment with both LPS and YSH for 48 h each significantly ($p<0.001$) increased the expression of TGF-β compared to the expression in PMA-treated control monocytes. In LPS-activated macrophages, treatment with YSH for 24 h also increased the expression of TGF-β compared with that caused by YSH only, and this expression level was still significantly ($p<0.001$) higher than in PMA-activated THP-1 cells. Taken together, expression of TGF-β was observed to be significantly increased when THP-1-derived macrophages were treated with either honey or LPS for 48 h. However, while treatment with YSH induced less TGF-β expression than the positive control, treatment with PMA and IL-4, caused its expression to be significantly higher than in the untreated control.

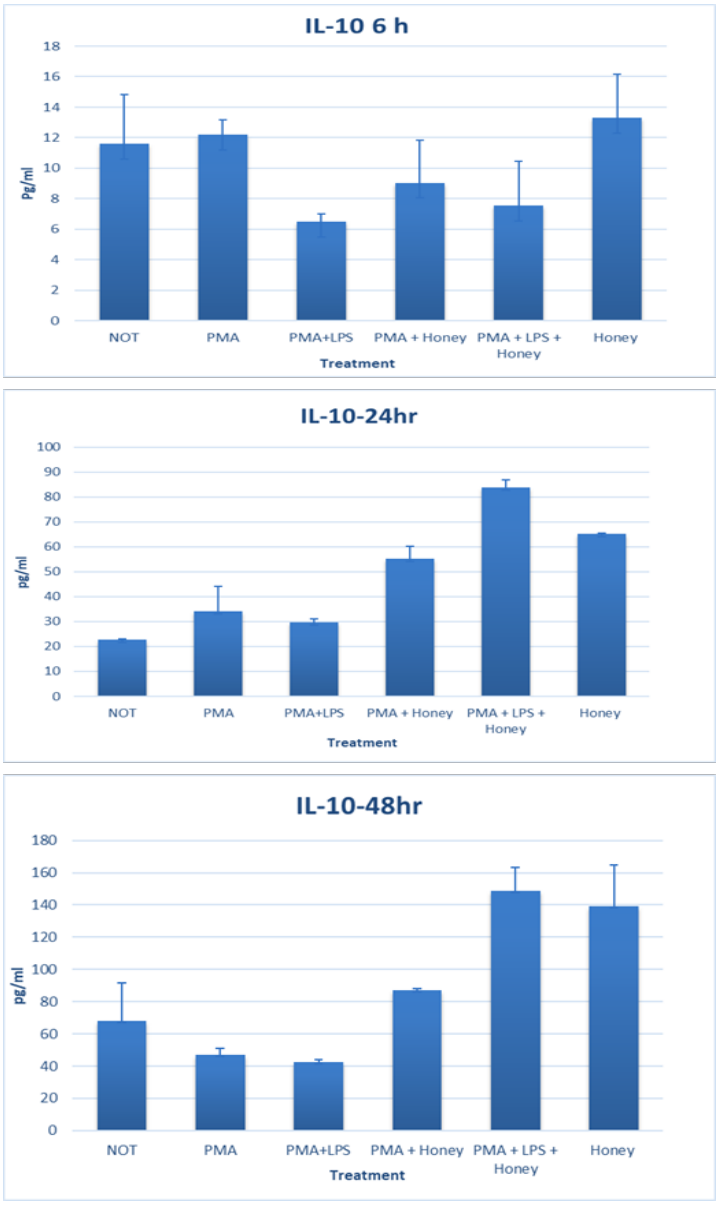


Figure 3.9: Effect of YSH on IL-10 in THP-1 cells.

The effect of 1% YSH (honey) and 100 ng/ml LPS, separately and in combination, on IL-10 expression by THP-1 cells (Not) or THP-1-derived macrophages (PMA) after 24 – 48 h treatment. Error bars show the mean ± SEM of two individual experiments. $p<0.001$ analysed by t-tests multiple pairwise comparisons with the untreated monocytes and PMA -treated samples.

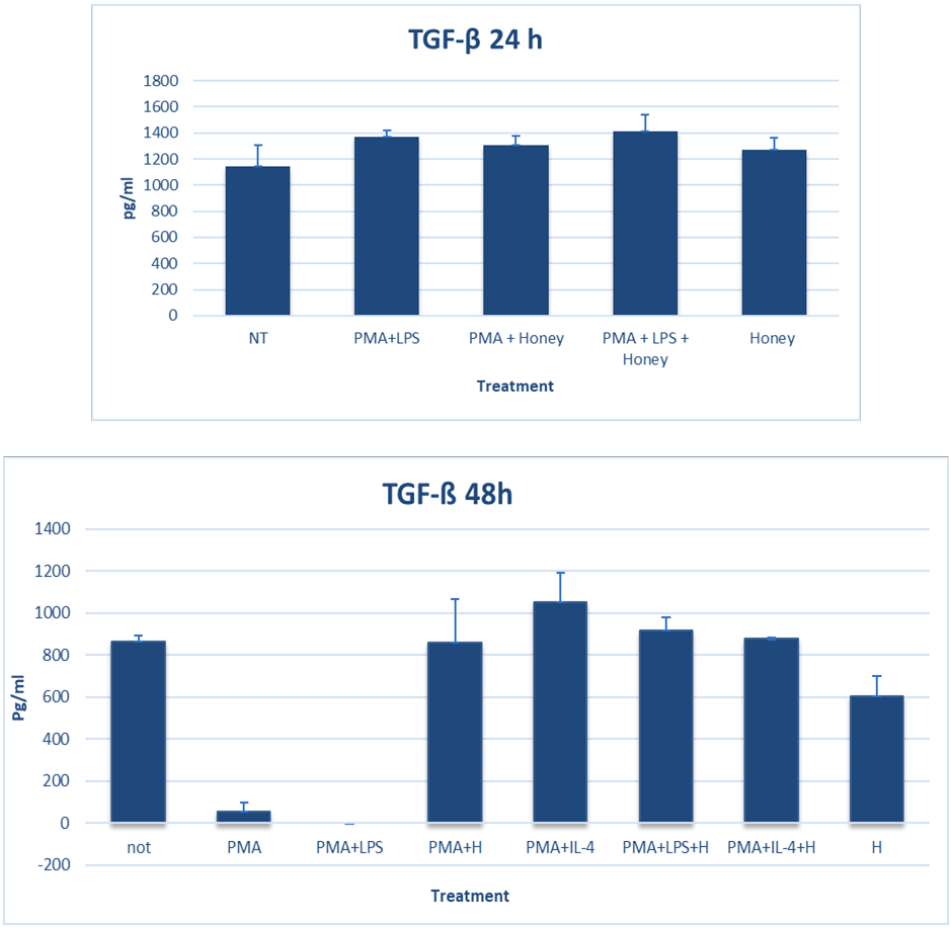


Figure 3.10: Effect of honey on TGF-β in THP-1 cells.

The effect of 1% YSH, and 100 ng/ml LPS, separately and in combination, on treatment of THP-1 cells (not) or THP-1-derived macrophages (PMA) on TGF-B expression after 24 – 48 h treatment. Error bars show the mean ± SD for three individual experiments. $p<0.001$ analysed by t-tests for multiple pairwise comparisons with the untreated monocytes and PMA-treated samples.

3.2.6 Effect of heat treatment on Honey activity.

It is important to determine whether the YSH component(s) that are responsible for the immuno-stimulatory effect is heat stable or labile, as this will further indicate whether the immuno-stimulatory activity of honey is due to endogenous heat labile LPS or other heat labile components, such as proteins. Therefore, the aim of the study was to determine whether the heat-treated YSH were able to elicit a cytokine response from monocytic (THP-1) cells. The YSH samples were each heated at 80°C for 30 min, and used to treat the cells in parallel to the unheated YSH samples.

The ability of heated YSH to induce TNF-α and IL-β release from cells was reduced by 35–50% compared to unheated YSH controls indicating that the immuno-stimulatory activity is mostly heat stable (Fig 3.11). Interestingly, only the secretion of IL-6 was reduced significantly when the YSH was heated. However, the quantified levels of heat labile LPS in the YSH cannot explain the activities observed. In addition, the level of anti-inflammatory cytokines IL-10 and TGF-β weren't affected by the treatment with honey that had been headed, and had the same expression results as the cells treated with unheated honey (Fig 3.12).

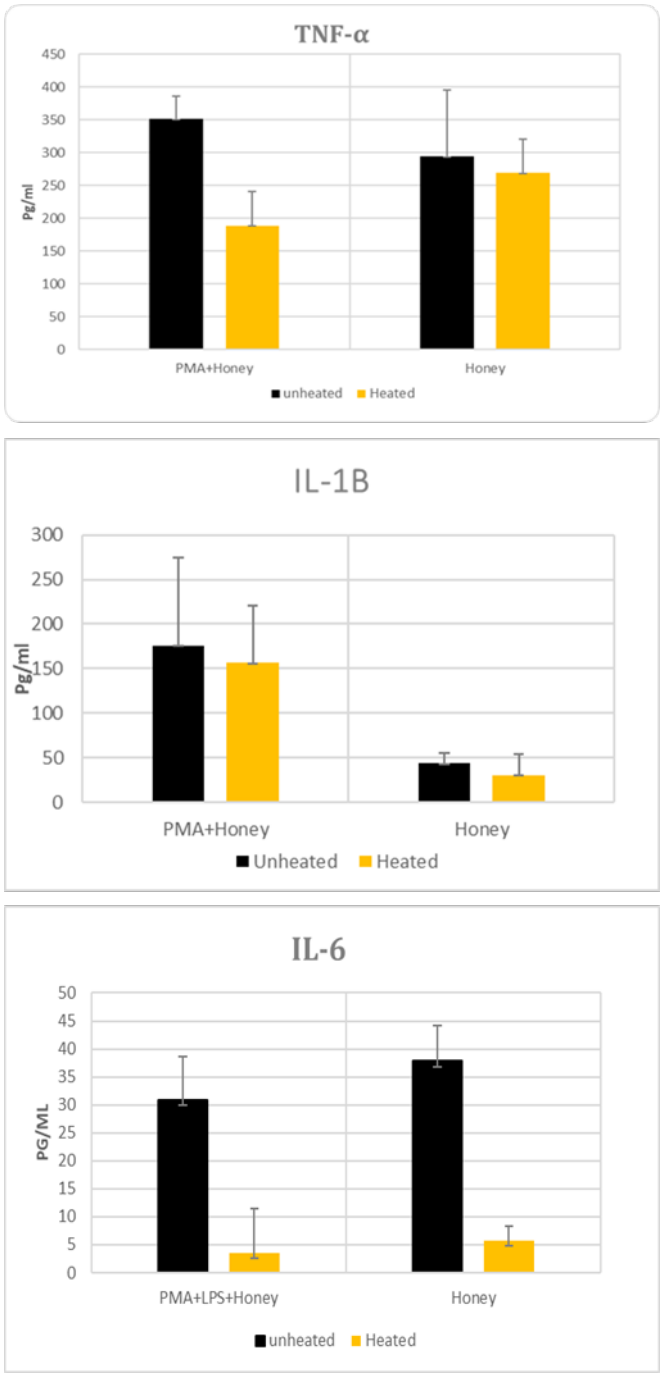
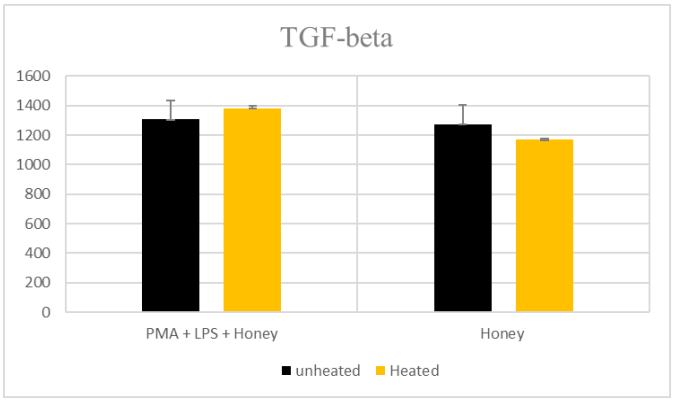


Figure 3.11: The immunostimulatory activities of YSH are partially heat labile.

THP-1 cells or (honey) or PMA-differentiated THP-1 cells (PMA+LPS+Honey) were treated with 1% (w/v) of YSH, honey that had been unheated (black bars) or heat-treated (orange bars)

at 80° C for 30 min. The levels of (A) IL-1β, (B) TNF-α, and (C) IL-6, release was measured by ELISA and plotted as mean + SD of three individual experiments.



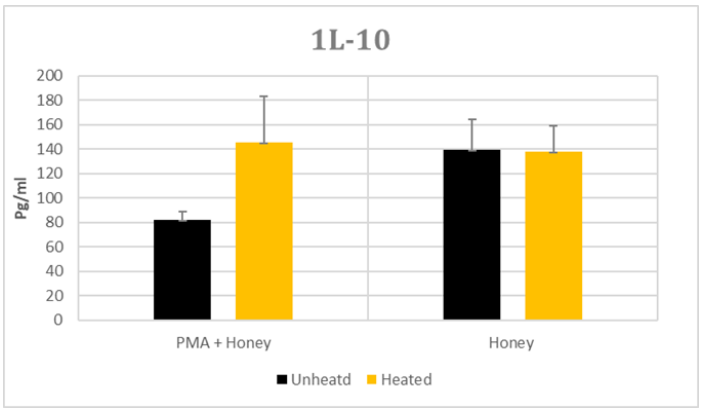


Figure 3.12: The anti-inflammatory activities of YSH are partially heat labile.

THP-1 (honey) and PMA-differentiated THP-1 cells (PMA+LPS+honey) were treated with 1% (w/v) of YSH that had been unheated (black bars) or heat-treated (orange bars) at 80°C for 30 min. (A, B) TGF-β ,IL-10 release was measured by ELISA and plotted as mean + SD of three individual experiments for TGF-β, and two for IL-10.

4.0 Discussion

Collectively, the findings in this chapter point to an enhancement of the M1 profile of macrophages by treatment with Yemeni Sidr honey (YSH) for 24 - 48 h. Particularly, these results include identification of M1 macrophage types distinguished by flow-cytometry-based cell surface markers, and by an increase in the ratio of M1/M2 cytokine profile. In these studies, the THP-1 cell line was used to model different subtypes of macrophages and to determine the effect of treatment with honey on macrophage differentiation. THP-1 cells treated with phorbol ester for 3 days were shown to undergo a change in cell shape and to stop proliferating consistent with differentiation to macrophages. These THP-1-derived macrophages also expressed the macrophage-specific cell surface marker CD68 but expressed low levels of cytokines. THP-1 cells differentiated into the M1 subtype of macrophage by treatment with PMA and lipopolysaccharide (LPS) for 3-5 days were shown to express the macrophage marker CD68 and relatively high levels of the M1 cell surface marker CD80 (47% positive) but low levels of the M2 cell surface marker CD163 (only 7% positve). There is also a relative change in the shape of the cells as compared to the untreated THP-1 cells. Further, the THP-1-derived M1 macrophages were also shown to express relatively high levels of the pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6 but not significant levels of the anti-inflammatory cytokines IL-10 and TGF-β which is consistent with published characterization of M1 polarized macrophages *in vitro* and *in vivo*. THP-1 cells were differentiated into the M2 subtype of macrophage by treatment with PMA and the Th2 helper cell cytokine, IL-4, for 3-5 days. These cells were confirmed to be M2-like because they expressed high levels of the macrophage marker, CD68, relatively high levels of the M2 cell surface marker, CD163 (50% positive), and relatively low levels of the M1 cell type marker CD80 (1% positive). The M2 polarized THP-1 macrophages were also shown to

secrete significant levels of TGF-βI. These results show that THP-1 cells can be differentiated into both M1 and M2 type macrophages which means that they are suitable model system to study macrophage polarization.

Treatment of THP-1-derived macrophages with YSH was shown to impact macrophage polarization and cytokine expression. The THP-1 cells were treated with 1% YSH which was shown not to affect cell growth or increase cell death. The relative cell survival decreases as the concentration of honey increases and shows and IC_{50} of around 6.5% honey. As hypothesized, honey treatment of the monocytes is an activator for differentiation from monocytes into macrophages. Treatment of the cells with 1% YSH, promotes a higher viability of THP-1 cells and the transformation of the monocytes into macrophages. Treatment of the differentiated macrophages with YSH for 24 h was shown to enhance the proportion of cells that express the M1 profile. Flow-cytometry-based cell surface markers analysis showed an increase in the cell surface level of the M1 marker CD80 with no significant increase in the M2 marker CD163 (ie an increase in the M1/M2 ratio).

Treatment of THP-1-derived M2 macrophages with YSH for 24 h also led to a relative increase in the release of pro-inflammatory cytokines such as IL-1β and TNF-α, and a failure to significantly increase the expression of anti-inflammatory cytokines such as IL-10 and TGF-β. The failure of treatment with YSH to stimulate a rise in IL-10 and TGF-β indicates that it does not stimulate THP-1 monocytes to transform into M2 macrophages, which are the principle cells that secrete these cytokines. In the presence of LPS, however, the secretion of both IL-10 and TGF-β increased significantly. This observation draws an interesting inference on the immuno-modulatory effect of honey: it appears that the impact of honey on inflammatory cytokine secretion is dependent on whether the surrounding milieu is already pro-inflammatory or anti-

inflammatory. LPS acts in a similar way to many other naturally occurring pathogen-associated molecular patterns (PAMPs) and binds to and activates their respective cell surface pattern response receptors (PRRs); LPS activates the TLR4 receptor and induces the polarization of monocytes towards the M1 phenotype (15). In this *in vitro* inflammatory situation, honey drives the formation of an anti-inflammatory state, which is marked in this experimental setup by increased expression of such anti-inflammatory cytokines, such as IL-10 and TGF-β.

This observation that treatment of THP-1-derived macrophages with YSH promotes a pro-inflammatory phenotype and secretion of inflammatory cytokines, seems to contradict the commonly held notion that honey has anti-inflammatory properties. This suggests that in the absence of pre-existing inflammation, the substance induces inflammation. A 2003 study reached a similar conclusion on the influence of honey on the expression of cytokines by MM6 macrophages (9). The researchers, after exposing the cells to three types of honeys, found that pro-inflammatory cytokines such as TNF-α, IL-β, and IL-6 were released from the MM6 macrophages at significantly increased levels. However, in THP-1-derived macrophages also treated with the pro-inflammatory mediator, LPS, honey acts as an immuno-modulatory agent and increases the release of anti-inflammatory cytokines. These results show that honey drives the release of either pro-inflammatory or anti-inflammatory cytokines depending on the preexisting environmental condition. The release of these molecules plays a role in the substance's wound-healing properties.

Wound-healing involves removal of damaged tissue components and replacement with restorative tissue, a process that relies on a combination of inflammation and cell proliferation. Following injury, an inflammatory phase ensues in which neutrophils migrate into the affected region to kill any pathogens and remove tissue materials that have been damaged. This

elimination of debris is mostly done by macrophages through phagocytosis and proteolytic activity (10). Once this initial phase lapses, the healing process becomes dominated by macrophages, which produce such mediators as reactive oxygen species and cytokines (9). The release of inflammatory cytokines is necessary for both stimulation and regulation of the delicate processes directing wound repair. TNF-α is one of the principle inflammatory cytokines released in this process, with others including IL-1β. The release of these mediators on a large scale during substantial injury is responsible for the accompanying rise in body temperature, which is induced through stimulation of prostaglandin production (6),(9). Overall, the stimulation of the production of pro-inflammatory cytokine release by macrophages is an important mechanism by which honey and other products promote wound healing. In addition, considering that pro-inflammation is mainly executed by the M1 subtype of macrophages, this observation also points towards the preferential support of M1 macrophage differentiation by treatment with YSH in the samples studied. An important question that arises, however, is whether this property also has a role in the anti-proliferative effect of YSH on cancer cells. The investigation of the role of macrophage subtypes in the tumour microenvironment through co-culture setups in Chapter 4 is an appropriate approach to assessing this subject.

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Chapter 4 . Breast cancer cell lines co-cultured with THP-1-derived Macrophages treated with YSH to induce M1/M2 polarization.

Abstract

Signals from the tumor microenvironment can polarize macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype. The M2 phenotype promotes tumor growth/invasion in several model systems and has been correlated negatively with the success of treatment. Since immune system modulation plays a significant role in carcinogenesis, the proposed study will focus on understanding Yemeni Sidr Honey's (YSH's) effect on monocytes co-cultured with breast cancer cells, in part, to see if YSH's anti-tumor activities are dependent on macrophage polarization. The human monocytic leukemia cell line, THP-1 was used as a model for studying immune-modulation in response to polarization. The effect of THP-1 M1 or THP-1 M2 polarization on the ability of monocytes to affect cancer cell proliferation and apoptosis was investigated. Interestingly, the rate of YSH-induced apoptosis in human breast cancer cell lines was higher than in the co-cultured conditions, and did not depend on the M1/M2 status. However, co-cultures comparing non-treated and YSH-treated macrophages showed that treatment with YSH enhances the ability of the macrophages to inhibit cancer cell growth and survival. These results suggest treatment with YSH is able to impact cancer via two separate mechanisms: direct impacts on cancer cell survival and activation of anti-tumour immune system (monocyte) activation.

4 Background

The ability of neoplastic cells to maintain the unnaturally high rate of proliferation required to form tumours relies on a microenvironment that supports cell proliferation, such as the excessive formation of blood vessels which increases the nutrient supply and allows removal of metabolic waste that is required to support a higher metabolic milieu (1). This tumour microenvironment consists of a variety of cells, including fibroblasts, adipocytes, neuroendocrine cells, inflammatory cells, myofibroblasts, extracellular matrix proteins, and macrophages (1). The macrophages isolated from such tumour milieu have been designated tumour-associated macrophages (TAMs), and they bear resemblance to those occurring in regenerating tissues. Their presence in tumour microenvironments has been described as enhancing the proliferation, invasion, and metastasis of the cancer cell population (2).

Macrophages are normally found to play important structural and physiological roles in nearly all tissue types and typically infiltrate injured or infected organs (3). Their precursors, monocytes, are formed by embryonic forerunners in fetal developmental stages and from hematopoietic stem cells located in the bone marrow during adult life. Migrating from blood into specific tissues, monocytes can transform into various types of macrophages, such as bone osteoclasts, alveolar macrophages, and hepatic Kupffer cells (3). According to their functionality and activation status, macrophages are characterized to be classically activated (M1) or alternatively activated (M2) (2),(3). In many cases, the physiological response to a given stimuli is determined by the relative proportion of the M1 and M2 subsets of macrophages present in the tissue (2),(3). Macrophages have a wide range of functional plasticity and although their assembly around tumour cells was originally thought to mean that they played a role in anti-

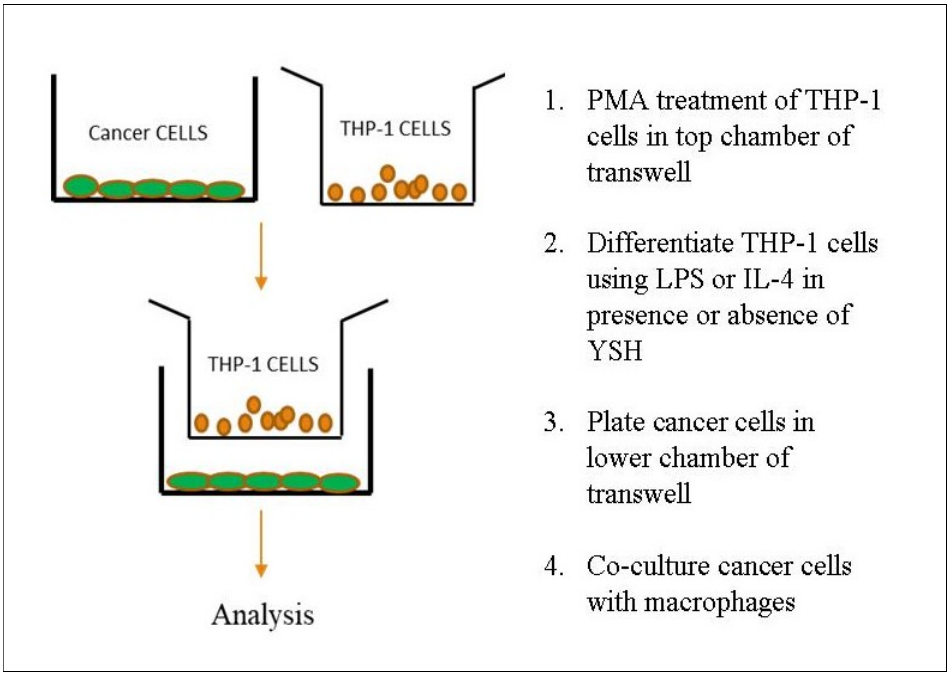
tumour immunity, they have been found to promote cancer initiation, suppress the immune reaction to neoplastic cells, and stimulate angiogenesis in tumours (1),(2). The cytokines released by Th1 and Th2 helper cells also have separate effects on the M1 and M2 classes of macrophages. Along with such stimuli as lipopolysaccharide (LPS), cytokines related to Th1 helper cells, such as interferon-γ, can polarize macrophages into the M1 category, which has pro-inflammatory, tumour suppressive, and microbicidal properties (3), (4),(5). M1 macrophages can also participate in antigen presentation, produce large amounts of interleukins such as IL-6, IL-23, and IL-12, produce large amounts of toxic radicals and nitric oxide, and express matrix metalloproteinases (MMP), including MMP-12. Conversely, the M2 phenotype of macrophages can be induced by treatment with Th2 helper cell-associated cytokines such as IL-13, IL-4, and IL-10. M2 macrophages are most often thought to be involved in wound repair and tissue reorganization after injury (4),(5). The tumour associated microphages are most closely related to a type of the M2 category (3). Recently, studies have revealed that the inflammatory pathway can be influenced by substances that modulate macrophage transformation into the M1 or M2 subtypes. For example, a frequent mechanism used by these substances is to alter the tumour microenvironment by decreasing the proportion of the M2 cell type, which results in the down-regulation of pro-tumour activities and promotion of the suppression of certain properties of neoplastic cells (3),(6), (7). Since important tumour characteristics such as growth and metastasis rely on the presence of an optimal microenvironment and are dependent on the expression of the M2 subtype of the macrophages, they are liable to disruption by substances that influence the polarization of macrophage (7): among the substances that are shown to influence macrophage polarization is honey (as shown in the results from Chapter 3 of this thesis). The induction of the M1 phenotype would not only make use of the role of this class of macrophages

in launching anti-tumour immune responses but also inhibit the production of growth factors and proteases that promote the cancer cells' ability to grow, invade, and metastasize. Therefore, it would be desirable to use honey to induce this effect in clinical cancers by discouraging a switch towards the presence of M2 macrophages in the tumour microenvironment.

4.1 Materials and methods

4.1.1 Preparation of M1/ M2 phenotype THP-1-derived macrophages and co-culture.

The M1/M2-polarised THP-1-derived macrophages were generated as previously described (8),(9). Briefly, the THP-1 cells (1 x 10^6 cells/ml) in RPMI 1640 media supplemented with 10% FBS were seeded into conical tubes and were differentiated by treatment with 10 nM PMA for 3 days at 37˚C. The samples were then collected by centrifugation and washed with complete RPMI to remove all PMA. The cells were then treated by incubation with 20 ng/ml IL-4 for an addition 18 h to polarize them to the M2 phenotype, or by incubation with 100 ng LPS for 24 h to polarize them to the M1 phenotype (10). For the co-culture experiments, the M1/ M2-THP-1-derived macrophages were added to the upper insert of a six-well Trans-Well plate (Corning Inc) and monolayers of MDA-MB-231 or MCF-7 cells (2 x 10^5 cells/well) were cultured on the bottom of the Trans-Well without direct contact for 48 h at 37˚ C. In some experiments, the conditioned media from the treated macrophages was added to the upper well of the Trans-Well plate after removal of the macrophages. The co-cultured breast cancer cells were then washed and harvested for subsequent experiments (11).



Schematic Diagram 4.1: Setting up trans well co-culture system of THP-1 macrophages and cells breast cancer was used in this study.

THP-1 cells and cancer cells were separately cultured for and then co-cultured in the top and bottom, respectively of a trans-well plate in serum free medium for a further 12 h or 24 h. In some experiments, the THP-1 cells were replaced by addition of conditioned media derived from appropriately treated THP-1 cells. The breast cancer cells were assessed for changes in confluency and morphology and then were harvested for experiments to look at migration and Sphere formation activity. Adapted from (10).

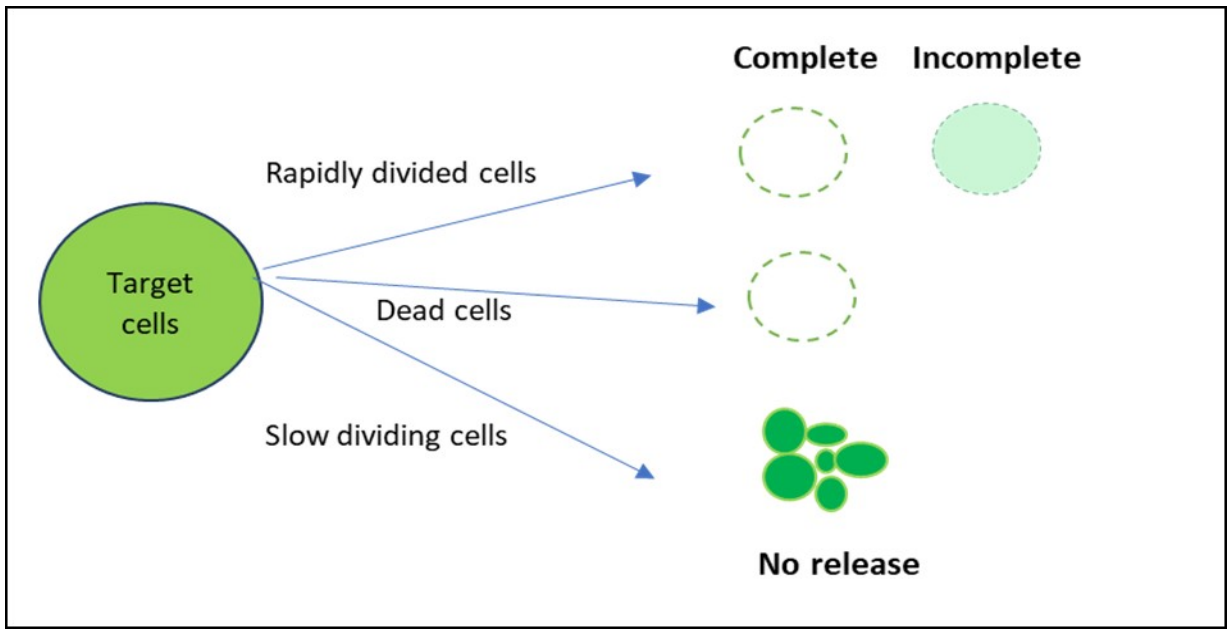
4.1.2 Cell morphology and confluency analysis

In this experiment the shape, appearance and confluence of the breast cancer cell lines following co-culture with the THP-1-derived macrophages was examined. MDA-MB-231 or MCF-7 breast cancer cells were treated with honey only or co-cultured with THP-1-derived

macrophages or treated with macrophage-conditions media and then harvested and replated in 6-well plates in culture media and incubated at 37° C for 24 h. Images of the cells were captured 24 - 48 h after the treatment using live imaging by phase contrast microscopy using the IncucyteS3 device and the number of cells and their morphology documented.

4.1.3 Calcein Staining

Calcein labeling allows for detection of viable cells (12),(13). The cells were stained by incubation in 20 uM calcein-AM as a dilution of a fresh 20 mM stock of calcein AM aminomethyl fluorescein, (C0875, Sigma-Aldrich) in dimethyl sulfoxide (DMSO) for at least 30 min. However, in some cell cultures the amount of staining can decrease over time. For example, cells in undergoing cell division show dilution of the stain as it is partitioned in the daughter cells. Alternately, some cell types can actively pump the cytoplasmic stain out of the cell resulting in a decreased level of staining. Cell morphology was also an important fluorescence-altering factor, as intracellular fluorescence was greater in nonadherent or rounded cells as compared to adherent cells.



Schematic Diagram 4.2: Calcein-AM is a good choice for cell tracking and as a general cytoplasmic stain (13).

However, because calcein does not bind to anything in the cell, it may be actively pumped out of the cells, diluted in daughter cells during cell proliferation, or lost from dying cells. Calcein fluorescence is influenced by cell type and morphology.

4.1.4 Sphere formation assay of MCF-7 cells.

The MCF-7 cells with or without co-culture with THP-1-derived macrophages were harvested and then plated on 96-well culture plates, coated with 1% agarose at a density of 10,000 cells/ml in serum-free media (14). Images of the spheres were captured using a light microscope (Olympus), and the spheres were quantified following 10 days of culture.

4.1.5 Matrigel Trans-well assay.

MCF-7 or MDA-MB-231 cells that had been co-cultured with THP-1-derived macrophages were harvested and then suspended at 5 x10^5 cells/ml in serum-free DMEM and plated into the upper insert of a six-well Trans well plate (Corning Inc.) (11). Serum-containing medium was added to the lower chamber as the chemoattractant and the cells were incubated at 37˚ C for 24 h. In the upper insert, the non-migratory cells were gently removed using cotton swabs and then migratory cells were fixed with 4% paraformaldehyde in room temperature for 10 min, followed by Crystal violet staining. Images were captured using a light microscope (Olympus).

4.1.6 Statistical analysis

The graph packages used were Graph Pad (Prism) software, Incucyte, and Microsoft Excel. Results were expressed as means ± SD, and the overall differences between group means were analyzed using one-way ANOVA.

4.2 Results

4.2.1 Co-culture of THP-1-derived macrophages treated with YSH with Calcein-AM-labelled cancer cell monolayers.

The ability of THP-1-derived macrophages to affect the proliferation and survival of breast cancer cells (MDA-MB-231 and MCF-7 cells) was studied in cancer cell monolayers labelled with calcein-AM. Calcein-AM is a green fluorescence stain used for cell tracking and as a general cytoplasmic stain (12). The idea behind using calcein-staining in this experiment was to be able to discriminate between labeled breast cancer cells and unstained THP-1-derived macrophages and to verify that the cancer cells had not lost integrity (13). The stain is passively taken up by the cells and acted upon by cytoplasmic esterases which traps the stain in the cytoplasm allowing a specific cell type to be easily detected in the co-culture system by imaging using fluorescence microscopy. The stability of calcein-AM fluorescence is influenced by cell type, the duration of post-labelling, and cell morphology. In some cells, calcein-AM can be actively pumped out of the cells decreasing the level of fluorescence while dying cells lose their stain. Rapidly dividing cells will become less fluorescent as the stain is divided among daughter cells faster than for slower dividing cells as shown by the MDA-MB231 cells in (Fig 4.1). Therefore, slightly different results can be observed in both breast cancer cell lines.

Untreated MDA-MB-231 cells, labelled with calcein-AM, were co-cultured with THP-1-derived macrophages for 24 h and the number of green fluorescent cells and phase-contrast identified cells were determined using images from the Incucyte apparatus (Fig 4.3A). The

images acquired during the early stages of the experiment (Fig 4.1) showed that the number of unstained THP-1-derived macrophages detected in the images was not significant: all of the cells in the images were calcein-labelled MDA-MB-231 cells. Therefore, the cell proliferation assay was quantitated by measuring the number of cells as identified from phase contrast micrographs using the Incucyte apparatus (Fig 4.3B left). Incubation of MDA-MB-231 cells with untreated THP-1 cells showed the highest proliferation of MDA-MB-231 cells, which was close to the number of MDA-MB-231 cells incubated with THP-1 cells differentiated to macrophages in the presence of PMA and IL-4 (M2-like macrophages). Incubation of MDA-MB-231 cells with THP-1 cells differentiated in the presence of PMA and LPS (M1-like macrophages) showed a decrease in the proliferation of MDA-MB-231 cells (Fig 4.5A, B). However, treatment of THP-1 cells with honey or M1-like macrophages treated with honey prior to incubation with the MDA-MB-231 cell monolayers all decreased the proliferation of the MDA-MB-231 cells: co-culture with M1-honey treated THP-1-derived macrophages decreased MDA-MB-231 cell growth compared to co-culture with M2-honey treated THP-1-derived macrophages or untreated THP-1-derived macrophages (Fig 4.3B left). In addition, the morphology of the MDA-MB-231 cells co-cultured with the M1-honey treated THP-1-derived macrophages, was different in shape from the cancer cells co-incubated with the untreated THP-1 cells and honey-only treated THP-1 cells (Fig 4.1, 4.5). Incubation of MDA-MB-231 cells with conditioned media from THP-1-derived macrophages also had effects on MDA-MB-231 cell proliferation which was somewhat similar to the effects of incubation with the macrophages.

The results measuring the number of calcein-labeled (green) MDA-MB-231 cells showed somewhat different results. Incubation of MDA-MB-231 cells with THP-1 cell differentiated to macrophages in the presence of PMA, IL-4, and YSH (M2-honey treated) or untreated THP-1

cells showed the highest number of calcein-labelled cells, compared to the rest of the conditions (Fig 4.3C, the red and violet bars) . However, comparison to the phase contrast microscopic images of the MDA-MB-231 monolayers indicate that the some of the differences in fluorescent labeling were an artifact of how the stain was maintained in the cells: the MDA-MB-231 cells incubated with the M2-like THP-1 cells (PMA + IL-4) showed a large number of relatively normal looking cells by phase contrast imaging but a low level of fluorescence labeling due to either diffusion among the rapidly growing cells or active pumping out of the dye; while the MDA-MB-231 cells incubated with the M1-honey treated (PMA + LPS + YSH) or YSH-treated only macrophages showed low levels of fluorescence staining which corresponded to cells that were clearly severely damaged in the phase contrast images (Fig 4.1, 4.5).

On the other hand, MCF-7 results were somewhat different in that the results obtained following co-culture with THP-1-derived macrophages were more similar between the phase contrast and calcein-labeled cells (Fig 4.2). MCF-7 cells treated with honey only or co-cultured with untreated THP-1 cells or M2-honey treated (PMA+IL-4+H) showed a lower number of fluorescently labelled cells that the other conditions (Fig 4.4A). The non-treated MCF-7cells had the highest proliferation (Fig 4.4B). Incubation with THP-1 derived macrophages treated with honey showed an early increase in MCF-7 cell proliferation which then slowed over the next day while co-culture with M1-differentiated THP-1 cells treated with honey showed a consistent decrease in cell proliferation. MCF-7 cells incubated with honey only showed very little proliferation which was similar to incubation of MCF-7 cells treated with camptothecin. These results are similar to those shown for the number of calcein-labelled MCF-7 cells 24 h after co-incubation with the THP-1 cells except that incubation with untreated THP-1 cells was lower at 24 h (Fig 4.4C left). The bar graph shows that co-incubation of MCF-7 cells with M2-

differentiated THP-1 cells increased the number of calcein-labelled cells while incubation with M2-differentiated THP-1 cells treated with honey strongly inhibited the number of calcien-labelled MCF-7 cells. Incubation of calcein-labelled MCF-7 cells with conditioned media from the differentiated THP-1 cells was very similar to incubation with the treated cells except that the effect of honey on MCF-7cells resulted in significantly lower levels of inhibition (Fig 4.4C right).

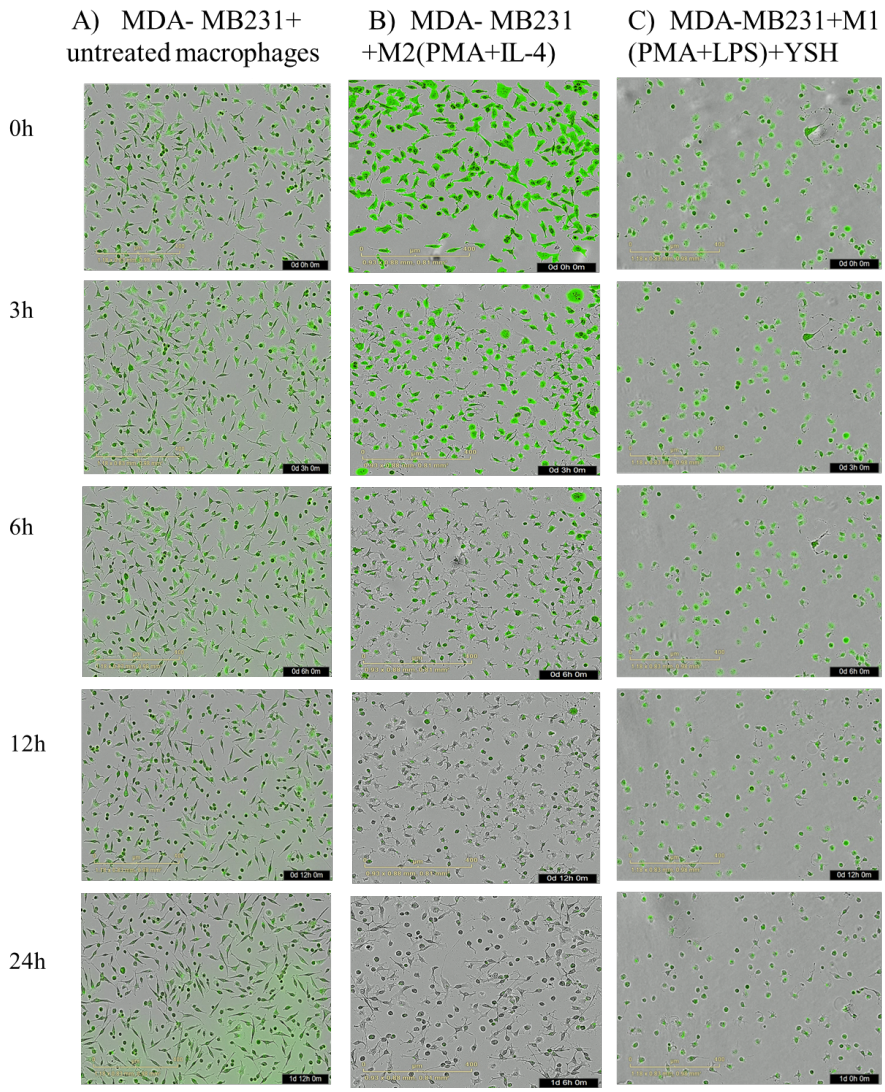


Figure 4.1: Calcein-AM fluorescence intensity is variable with time, and morphology change. (A) Sub confluent of MDA-MB-231 monolayer cell cultures were loaded with calcein-AM and Co-cultured with THP-1-drived macrophages in the presence of LPS, IL-4, and honey. Incucyte microscopy (10×) was used to capture changes in intracellular calcein-AM fluorescence over time (3, 6, 12, and 24 h). Calcein-AM loaded cells were lost at the respective time points and fluorescence intensity measured using an Incucyt live imaging protocol. (A) MDA-MB-231 with non-treated THP-1 cells. (B) MDA-MB-231 cells co-cultured with M2 macrophages (PMA+IL-4). (C) MDA-MB-231 cells co-cultured with M1 macrophages and 1% YSH.

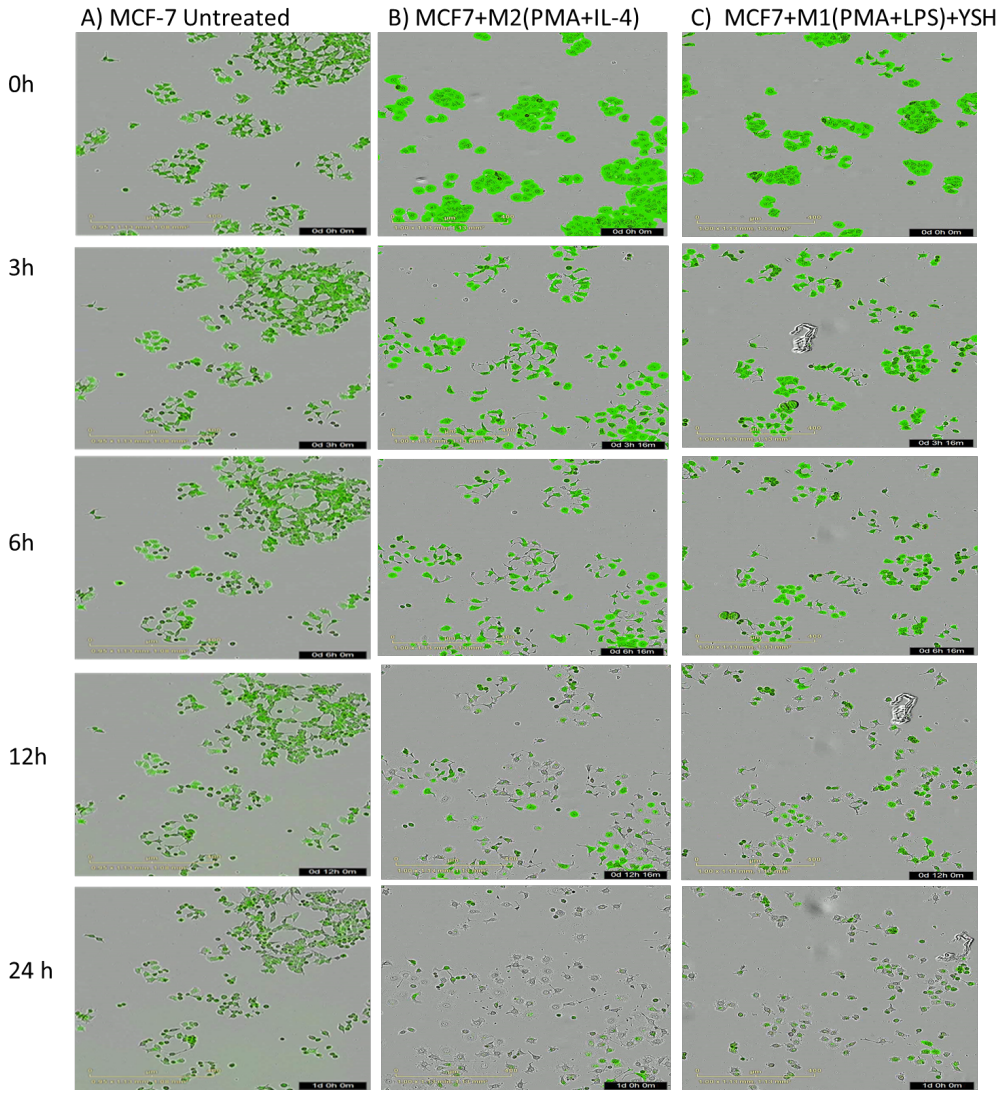


Figure 4.2: Calcein-AM labelled MCF-7 cells fluorescence intensity is variable with time, and morphology change Sub confluent of MCF-7 monolayer cell cultures were loaded with calcein-AM and Co-cultured with THP-1-drived macrophages in the presence of LPS, IL-4, and honey. Incucyte microscopy (10×) was used to capture changes in intracellular calcein-AM fluorescence over time (3, 6, 12, and 24 h). Calcein-AM loaded cells were lost at the respective time points and fluorescence intensity measured using an Incucyte live imaging protocol. (A) MCF-7 non-treated cells. (B) MCF-7 cells co-culture with M2 macrophages (PMA+IL-4). (C) MCF-7 cells co-culture with M1 macrophages in the presence of 1% of YSH.

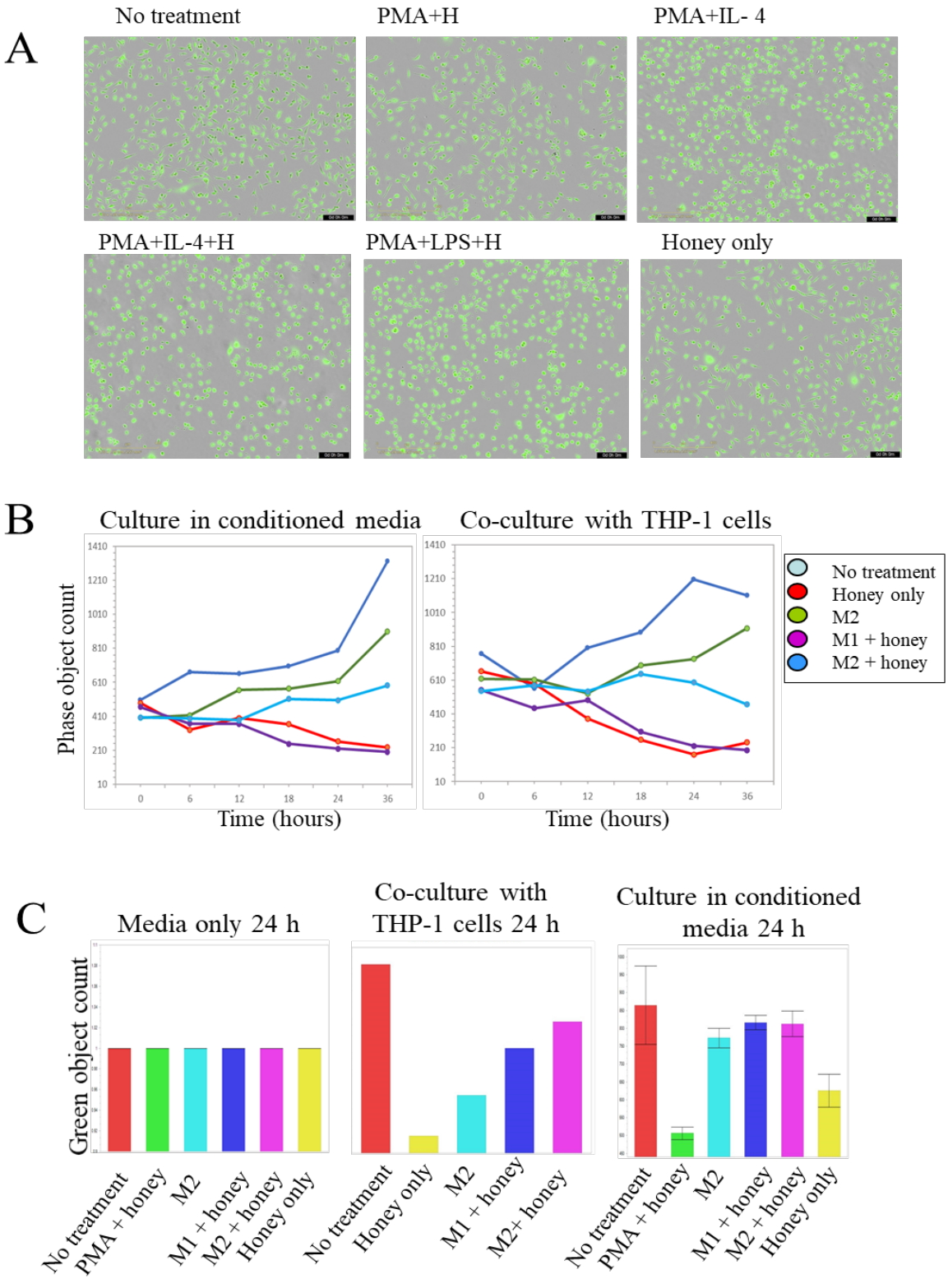
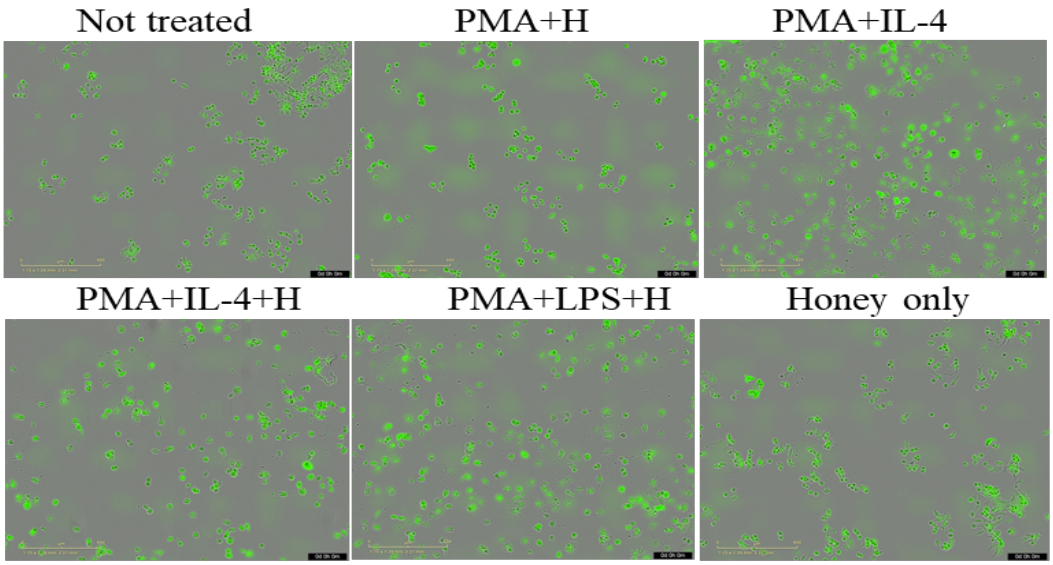
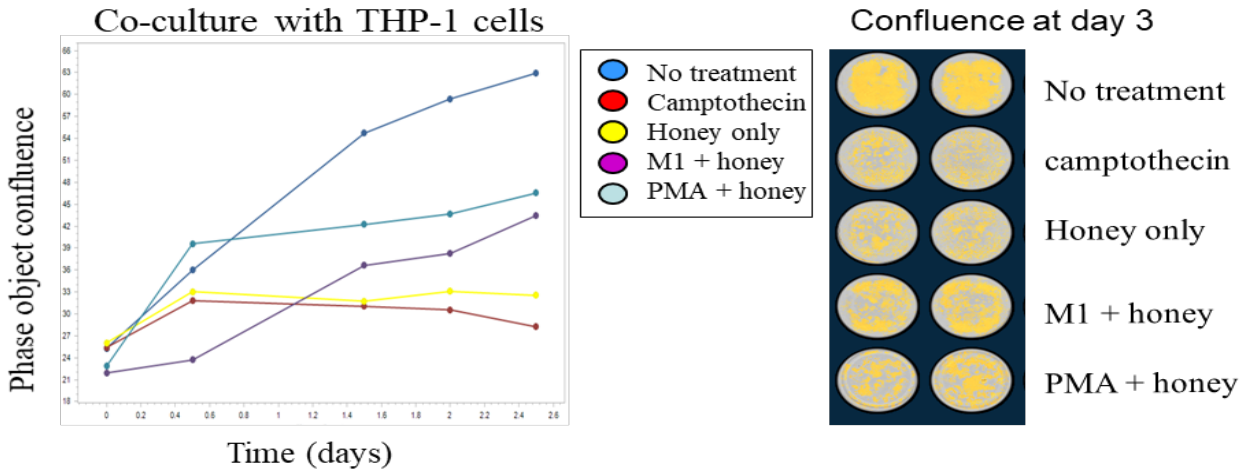

A
No treatment
PMA+H
PMA+IL- 4
PMA+IL-4+H
PMA+LPS+H
Honey only
B
Culture in conditioned media
Co-culture with THP-1 cells
Phase object count
Time (hours)
Time (hours)
No treatment
Honey only
M2
M1 + honey
M2 + honey
C
Media only 24 h
Co-culture with THP-1 cells 24 h
Culture in conditioned media 24 h
Green object count
No treatment
PMA + honey
M2
M1 + honey
M2 + honey
Honey only
No treatment
Honey only
M2
M1 + honey
M2+ honey
No treatment
PMA + honey
M2
M1 + honey
M2 + honey
Honey only

Figure 4.3: Calcein-AM fluorescence intensity in MDA-MB-231 cells.

(A) Sub confluent cell cultures were loaded with calcein-AM and Incucyte microscopy (10×) was used to capture changes in intracellular calcein-AM fluorescence over time (top). Calcein-AM-loaded cells were imaged at the respective time points and fluorescence intensity measured using an Incucyte apparatus. Data are normalized to total fluorescence to day 1 after calcein-AM loading (bottom). (B). Proliferation of breast cancer cells was monitored in real time with confluence image mask using IncuCyte® cell proliferation assays and the confluency of phase contrast-detected cells was reported over 48 h. Graphs were generated by counting cells using IncuCyte® Images, and ImageJ software. (C) Calcein-AM fluorescence intensity, measured 24 h post-labeling and co-culture, was compared among several conditions and normalized to day 0.


Not treated
PMA+H
PMA+IL-4
PMA+IL-4+H
PMA+LPS+H
Honey only


Co-culture with THP-1 cells
Phase object confluence
Time (days)
No treatment
Camptothecin
Honey only
M1 + honey
PMA + honey
Confluence at day 3
No treatment
camptothecin
Honey only
M1 + honey
PMA + honey

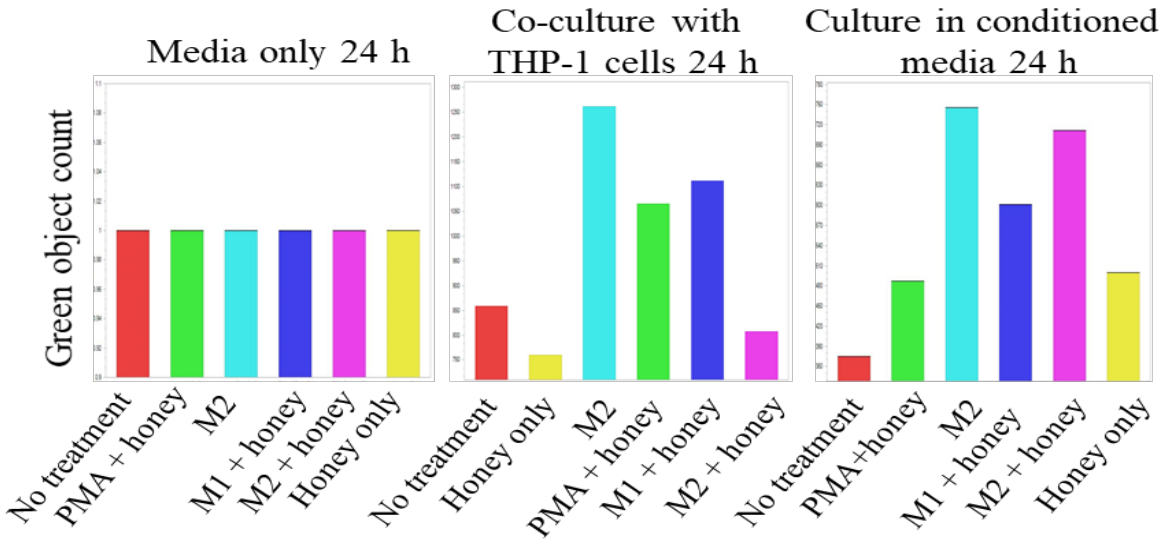

Media only 24 h
Co-culture with THP-1 cells 24 h
Culture in conditioned media 24 h
Green object count
No treatment
PMA + honey
M2
M1 + honey
M2 + honey
Honey only
No treatment
Honey only
M2
PMA + honey
M1 + honey
M2 + honey
No treatment
PMA+honey
M2
M1 + honey
M2 + honey
Honey only

Figure 4.4: Calcein-AM fluorescence intensity in MCF-7 cells.

(A) Sub confluent cell cultures were loaded with calcein-AM and Incucyte microscopy (10×) was used to capture changes in intracellular calcein-AM fluorescence over time (top). Calcein-AM-loaded cells were lost the green florescent at the respective time points and fluorescence intensity measured using an Incucyte apparatus. Data are normalized to total fluorescence at day 1 after calcein-AM loading (bottom). (B) Proliferation of breast cancer cells was monitored in real time with a confluence image mask (Gold) using IncuCyte® cell proliferation assays and the confluency of phase contrast-detected cells was reported over 48 h. (C) Calcein-AM fluorescence intensity was measured 24 h post-labeling and co-culture and was compared among several conditions and normalized to day 0.

4.2.2 Degrees of apoptosis of cancer cells post-THP-1-derived macrophages honey treated co-culture

MDA-MB-231 and MCF-7 breast cancer cells co-incubated with the THP-1-derived macrophages treated with honey had a different morphology from cells co-incubated with untreated THP-1 cells or cancer cells treated with honey only (Fig 4.5A, 4.6A). The cells become flat, rounded and irregular in shape suggesting a morphological response to a stressful condition. In addition, the number of MDA-MB-231 and MCF-7 cells was decreased by co-incubation with THP-1 derived macrophages for 48 h. Co-incubation with M1-like THP-1 cells as well as co-incubation with both M1- and M2- like THP-1 macrophages treated with honey was associated with a significant decrease in cell number (Fig 4.5B, 4.6B).

To assess the effects of incubation with honey only or with THP-1-derived macrophages on the death of breast cancer cells, the cells were stained with acridine orange/ethidium bromide (AO/EB) 24 h after co-incubation as described in methods. Cells were viewed and the number of surviving green-stained cells which did not stain red were counted to quantify the extent of apoptosis using the Incucyte apparatus and software (Fig. 4.7A). When MDA-MB-231 cells were co-cultured with M1-honey treated macrophages (PMA+LPS+H), they were shown to be undergoing apoptosis (red ethidium bromide staining) at a much higher rate than when co-cultured with M2-honey treated macrophages (PMA+IL-4+H) (Fig 4.7B). However, when the cancer cells were treated with only 1% YSH, in the absence of THP-1 cells, the MDA-MB-231cells underwent apoptosis at a significantly higher level than they did for any of the co-culture conditions with THP-1-derived macrophages. These results suggest the mechanisms by which honey directly kills cancer cells are different from the mechanisms by which THP-1-derived macrophages kill the cancer cells. Since M2-type THP-1-derived macrophages are much less effective at killing the cancer cells compared to the M1-type THP-1-derived macrophages, it appears that the induction of cell death is dependent on the M1 inflammatory phenotype.

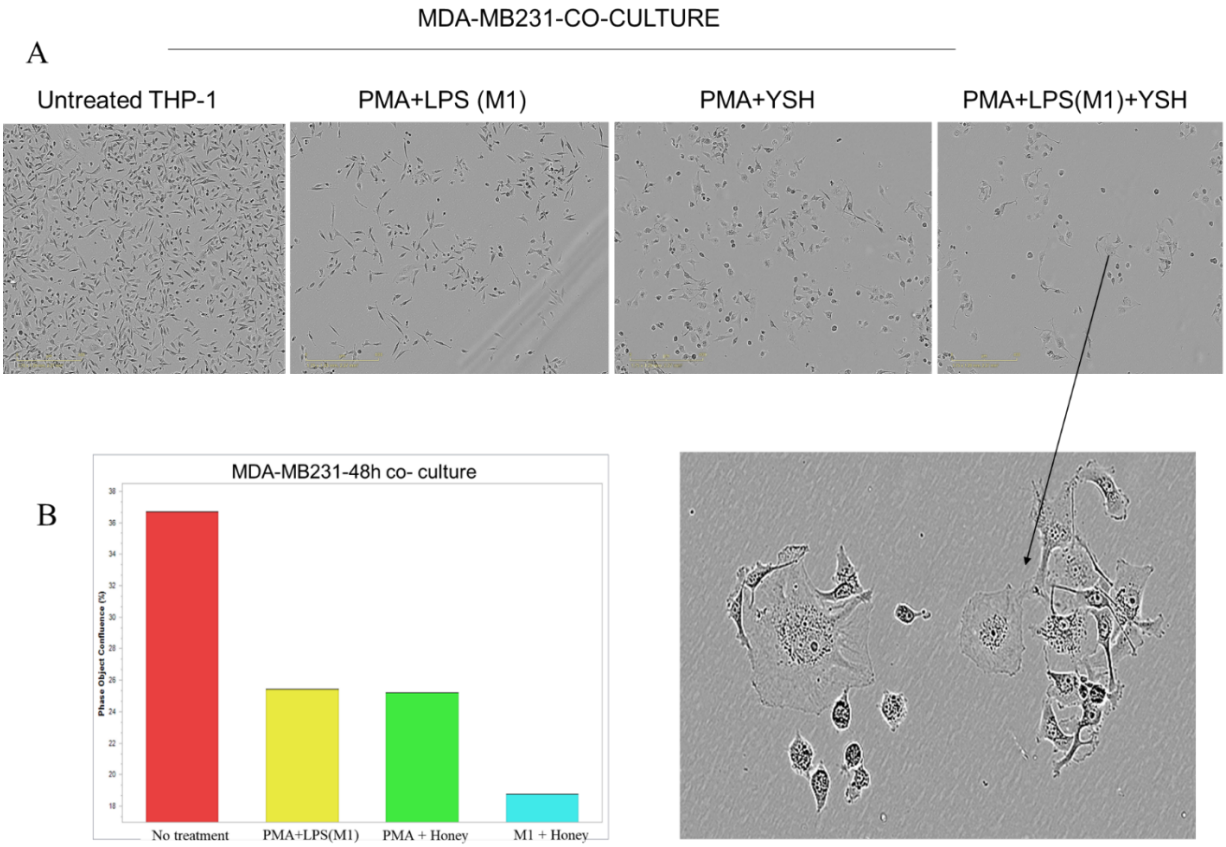


Figure 4.5: MDA-MB-231 cells morphology in co-culture with macrophage-honey treated samples.

(A) Phase contrast-shows cancer cells morphology after co-culture in macrophage-honey treated conditions. (B) Relative cell counts was monitored using IncuCyte® cell proliferation assays over 48 h.

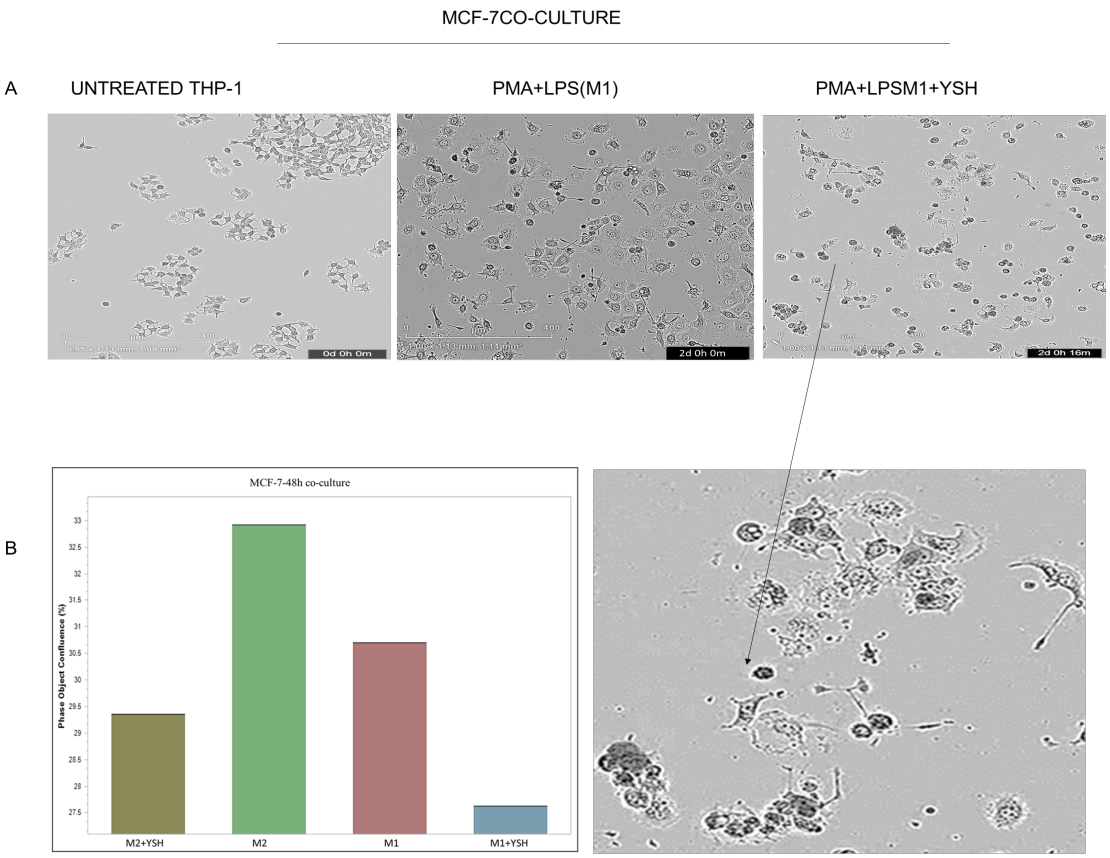


Figure 4.6: MCF-7 cells morphology in co-culture with macrophage-honey treated samples.

(A) Phase contrast-shows cancer cells morphology in co-culture with macrophage-honey treated conditions. (B) Relative cell counts was monitored using IncuCyte® cell proliferation assays over 48 h.

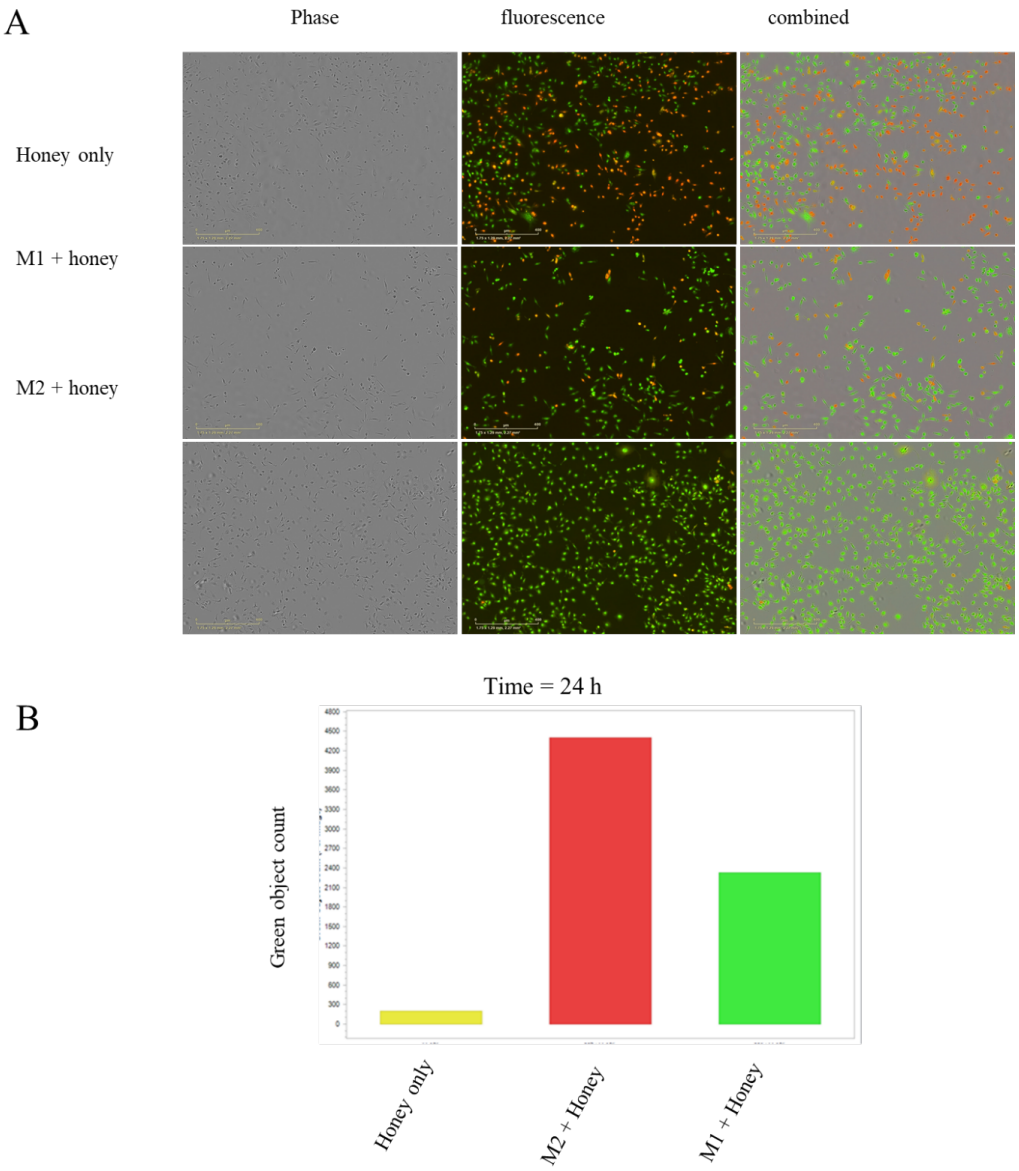


Figure 4.7: YSH alone exhibits significantly different degrees of apoptosis of cancer cells (MB231) than co-culture them with M1/M2

(A) Apoptosis assessment using Acridine and Ethidium bromide fluorescence intensity 24 h post co-culture (B) The relative number of viable to dead MDA-MB-231 cancer cells was monitored in real time images using IncuCyte®.

4.2.3 Co-culture with M1-THP-1-derived macrophages treated with Honey reduces the migration of breast cancer cells.

The tumour microenvironment is important in facilitating tumour migration. A trans-well migration assay, with breast cancer cells in the upper well and THP-1-derived macrophages in the lower well were used to examine breast cancer cell migration (11). The presence of untreated macrophages in the lower well had the strongest effect on inducing breast cancer migration while the presence of M2-THP-1-derived macrophages in the lower chamber slightly inhibited the migration of both the MCF-7 (Fig 4.8A) and MDA-MB-231 (Figure 4.8B) breast cancer cells compared to controls. Treatment of the M2-THP-1-derived macrophages with honey further inhibited their ability to induce breast cancer cell migration (compared to untreated, and M2-like macrophages). The presence of M1-THP-1-derived macrophages had a stronger effect on inhibiting the migration of the breast cancer cell lines compared to the M2-macrophages and the M1-honey-treated macrophages further decreased the migration of both breast cancer cell lines. These results indicated that honey can affect the ability of THP-1-derived macrophages to facilitate the migration abilities of the breast cancer cells (Fig 4.8C).

4.2.4 M1-THP-1-derived macrophages treated with Honey effect mammosphere formation by MCF-7 breast cancer cells.

To demonstrate the influence of THP-1-derived macrophages treated with honey on cancer cells, a co-culture system to assess mammosphere formation was prepared (11). Mammospheres typically form when some cancer cells are cultured on non-adherent substrates

in the presence of growth factors. Following co-culture with THP-1-derived macrophages for 24 h, the MCF-7 breast cancer cells were harvested, and plated on agar-coated culture plates and the cells allowed to grow into tumour-like formations as assessed by the sphere formation assay. MCF-7 cells co-cultured with untreated THP-1-derived macrophages were able to form a large mammospheres on the soft agar: the MCF-7 cells all appeared to aggregate into a single structure. Co-culture with THP-1-derived macrophages treated with honey resulted in the formation of a smaller single mammosphere (Figure 4.9) However, co-culture with M2-THP-1-derived macrophages resulted in the formation of multiple smaller spheres instead of one compared to controls, while M1- and M2-THP-1 derived macrophages almost completely inhibited the formation of mammospheres. When honey was added to both M1- and M2-THP-1-derived macrophages the cells were not able to form any spheres. These results indicated that honey affected the ability of THP-1-derived macrophages to affect the ability of MCF-7 breast cancer cells to differentiate into mammospheres.

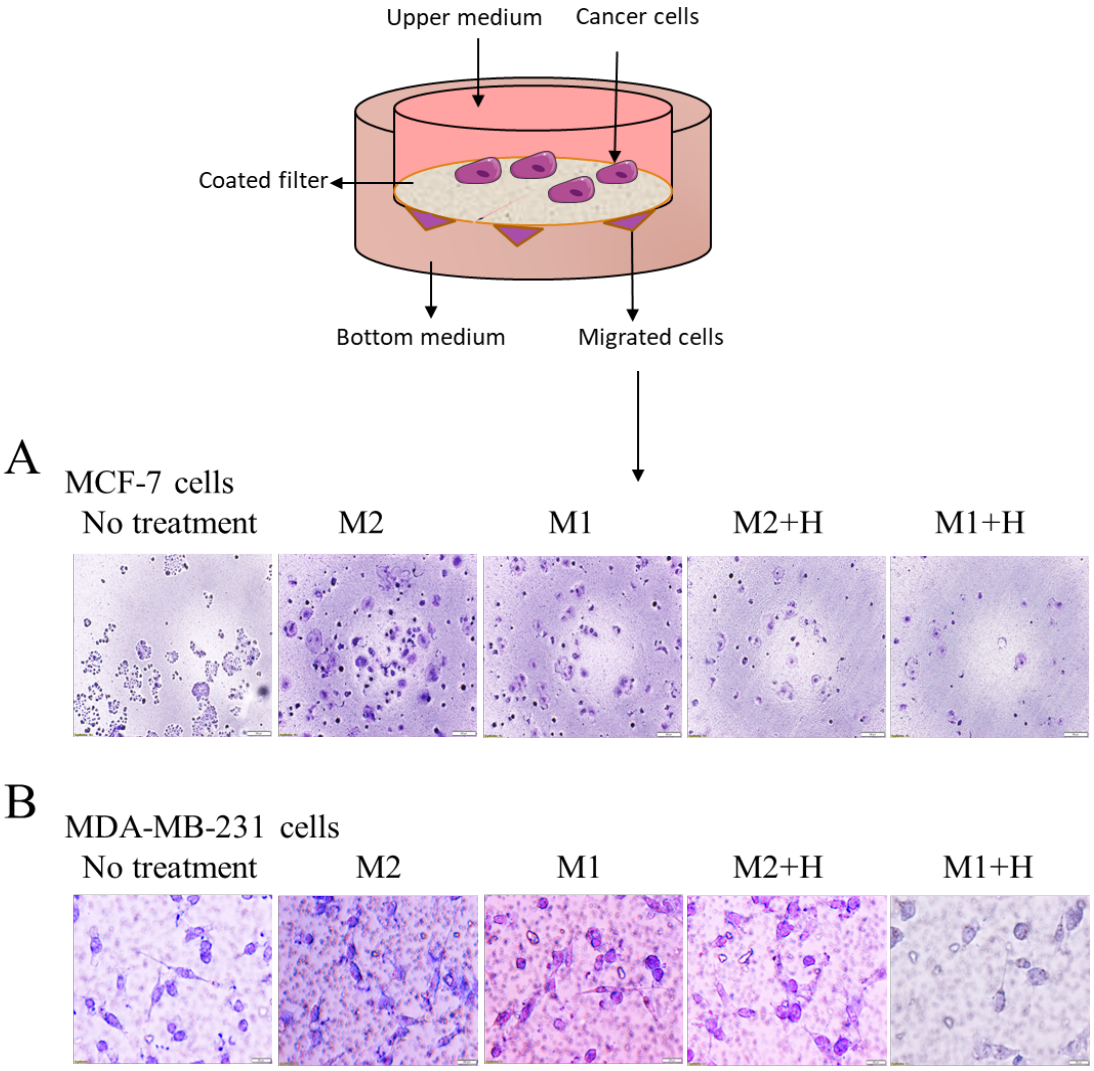


Figure 4.8: Transwell migration assay.

Cancer cells were plated in the upper chamber of the Trans-well insert. Cells were allowed to migrate for 24 h toward the serum-containing medium present in the lower chamber. (A, B) Migration ability of breast cancer cells was decreased following M1-YSH co culture. Scale bar = 100-200 µm. Diagram adapted from (15)(16).

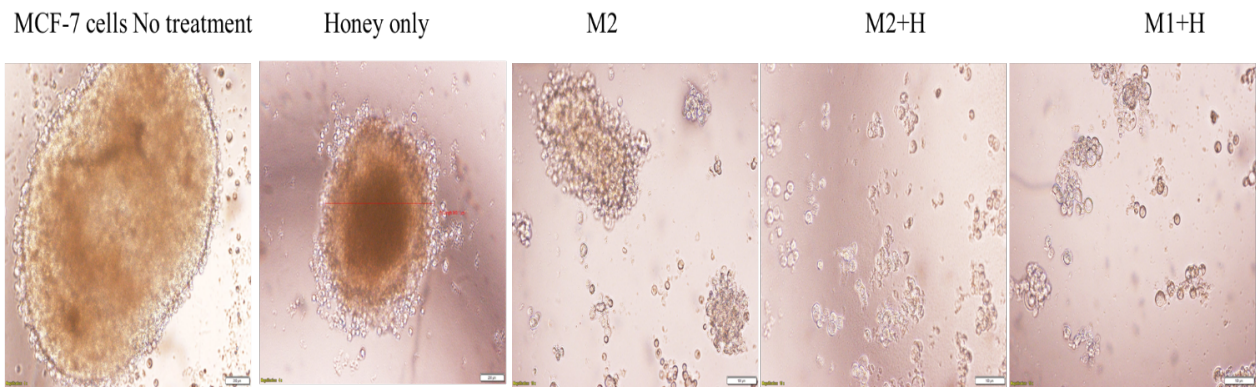


Figure 4.9: MCF-7 cancer cells, co-cultured with M1/M2-YSH exhibited a notable decrease in sphere formation ability.

96 well plate coated with 1% agarose, alternatively to non adhesive plate. MCF-7 cells previously were cultured with Macrophages-honey treated or honey alone. Cells sphere formation for each condition was captured after 10-days and compared to non -treated.

4.3 Discussion

Overall, the results in this chapter indicate that treatment with honey has the potential to disrupt the microenvironment associated with tumour cells by influencing the presence and activity of M2 macrophages in tumours. In these experiments, THP-1 cells were differentiated to macrophages, M1 macrophages, or M2 macrophages and then co-cultured with fluorescently labeled breast cancer monolayers to look at difference in cell survival and growth. In summary, based on flourescent cell counts and cell morphology, breast cancer cells co-cultured with M2 macrophages were less susceptible (continued to divide at a higher rate) to the suppressive effects of honey than cancer cells co-cultured with M1 macrophages, macrophages cultured with honey alone, or macrophages in other control conditions, which showed higher rates of cancer cell death (about 60%). A higher rate of death was observed in cancer cells treated with honey only (ie. in the absence of macrophages) and in those cancer cells that were co-cultured with M1 macrophages in the presence or absence of honey. However, when the cancer cells were co-cultured with M2 macrophages only, they showed a higher cell number. However, the addition of honey to the M2 macrophages resulted in a decrease in cancer cell number as the main impact of exposure to honey. This means that the presence of the M2 macrophages conferred a certain degree of resistance to killing to the cancer cells. Treatment with YSH preferentially disrupts the microenvironment in which an M2 phenotype of macrophages has already been induced as compared with an M1 macrophage phenotype. As a result, it was shown that YSH was able to increase the number of tumour cells that undergo apoptotic cell death, and cause them to become less capable of proliferating, invading nearby tissues, or metastasizing. Similar findings about the

impact of honey on cancer cell profiles have been documented in the literature. For instance, in a 2017 *in vivo* experimental setting, researchers investigated the impact of exposure of a lung carcinoma model to Melittin, an alcohol extract from honey (7). They found that the extract induced significant alterations in the tumour environment by influencing the ratio of M1 to M2 macrophages. Specifically, treatment with Melittin promoted preferential binding to M2 macrophages, with the subsequent reduction in the population of the M2 type of cells, while the population of M1 macrophages remained unchanged. Moreover, the expression of angiogenetic markers such as VEGF and CD206 in the cancer cell population were reduced significantly following exposure to Melittin (7). As a result, the growth, viability, invasiveness, and metastatic potential of the neoplastic cells was diminished. These findings are similar to those recorded in the current study, with the reduction in sphere formation, the decrease in cell migration, and the higher apoptotic rates in the cells with an M2 dominant microenvironment appearing to be more affected by exposure to YSH.

Other experiments have shown that exposure to the natural product, Astragaloside IV, can also affect the polarization of THP-1-induced macrophages. Astragaloside IV is a component extracted from Astragali radix a plant that has been used as a type of Chinese Traditional medicine (17). Although the substance is not derived from honey, it has been found to possess several properties that are strikingly similar to those of honey extracts, including anti-tumour, anti-oxidant, and anti-inflammatory effects (18). In the experiment, researchers exposed THP-1-induced macrophages to Astragaloside IV, and then assessed the effect of this treatment on the subset distribution of M1 and M2 macrophages and on the growth of co-cultured lung cancer cells. The investigators used treatment with IL-4 and PMA to induce the polarization the THP-1 monocytes in the presence and absence of Astragaloside IV, followed by the measurement of the

levels of markers associated with M1 and M2 phenotypes (17). The researchers found that Astragaloside IV prevented the induction of the M2 phenotype by treatment with IL-13 and IL-4, such that the proportion of the M2 cells was significantly lower than the proportion of the M1 subtype. Moreover, the study revealed that markers of angiogenesis, such as AMPK-alpha, were also reduced in co-cultures with lung neoplastic cells treated with Astragaloside IV. The reduction in the proportion of the macrophages with the M2 subtype and the activation of AMPK-alpha resulted in a less supportive microenvironment for the tumour cells, subsequently lowering the capacity of the cancer cells in the co-culture to grow, migrate, and induce the formation of new blood vessels. These findings are comparable to those recorded in the current study. The results sufficiently inform the conclusion that the effect of honey on a neoplastic cell population depends on the preexisting inflammation status, and consequently, honey induces differential rates of death in tumour cells depending on the ratio of M1/M2 macrophage polarization in tumour-associated macrophages. This, therefore, suggests that honey has different mechanisms of killing cancer cells and does not have to depend on TAMs. However, our finding suggest that honey potentially can target tumour-associated macrophages and provides new insight in treating cancer specifically breast cancer.

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Chapter 5 . Characterization of the composition of Sidr Honey by HPLC

Abstract

To gain more knowledge about Yemeni Sidr honey composition, this work has been carried out HPLC analysis of honey from 3 different locations and same floral backgrounds, and analysis of one commercial kinds of honey. The relative amount of specific sugars and phenolic acids was determined in different honey samples and comparison done to standards. The results of this study are consistent with previous studies which looked at the composition of various honey samples, including Sidr honey samples.

Keywords: Sidr, Honey, Sugars, phenolic acids, HPLC.

5 Background.

Honey is a complex mixture of sugars, amino acids, minerals, and biologically active components. The composition of honey is dependent on its botanical and geographical origin as well as on the processing and storage conditions. Further, the price of honey is often related to its botanical and geographical origin and therefore there have been problems with inappropriate labelling or adulteration to increase value (1),(2). Therefore, it has become important to characterize the chemical composition of honey to validate its quality as well as to identify any potentially bioactive components.

Sugars represent the main chemical constituent of honey. Honey sugars are complex mixtures which include approximately 70% monosaccharides, primarily fructose and glucose, and 10-15% disaccharides such as sucrose and maltose (3),(4). The sugar components also depend on the botanical origin of the honey and can show several differences in the types and proportions of sugars depending on the plant sources and the environmental conditions when their nectar is collected (5) . The sugar composition of honey also affects its physical properties: for example, differences in hydroscopic properties, viscosity, and crystallization all depend on the sugar profiles (6),(7). The sugar composition of honey is commonly analyzed using HPLC on amide columns and detected using either absorbance with ultraviolet light or refractive index detector.

Honey is also rich in phenolic acids and flavanoids which are best known as the natural anti-oxidants present in honey and which have been implicated as the active agents in honey's role as a benefit to human health, in particular in wound healing and anti-inflammatory activity (2), (8),(9). Honey phenolics and flavonoids can originate from the nectar, pollen, or propolis from the botanical sources and therefore these components are expected to vary depending on the

plant, the geography, and the time of year the honey is produced(8),(9) ,(10). It is well established that HPLC analysis is the best method for analyzing the phenolic or flavonoid content of honey although the very high sugar content makes direct analysis of these minor components quite difficult. Therefore, methods to remove the sugars from honey prior to HPLC analysis, such as chromatography on Amberlite resins, have been developed (13), (11),(12). Subsequent analysis of the honey extracts with HPLC and detection using UV absorption can provide a good estimate of concentration by comparison to a standard curve of a known sample.

5.1 Methods

5.1.1 Characterization of honey sugars

The relative amount of specific sugars was determined in different honey samples using HPLC and comparison to purified standards (7),(13),(14) . Four honey samples were subjected to analysis: honey 1, a commercial clover honey; honey 2, a SIDR honey purchased in Saudi Arabia sourced from Saudi Arabia; honey 3, a SIDR honey purchased with a certificate of analysis purchased in UK sourced from Yemen; and, honey 4, a SIDR honey purchased in Saudi Arabia sourced from Yemen . The honey samples were diluted to 5% (w/v) in water, dissolved by incubation at 37^{o} C, and filtered through a 0.22 μm syringe filter. The purified standards (fructose, glucose, sucrose, maltose, and trehalose [Fisher Scientific]) were similarly dissolved in water to 1% (w/v) and filtered through a 0.22 μm filter. The samples were subjected to HPLC on a Breeze 2 chromatography system (Waters Limited, Mississauga, ON) including a spectrophotometer, auto-injector, and column heater set to 60^{o} C fitted with an X-Bridge Amide 3.5 μm, 4.6 x 150 mm column and an X-Bridge 3.5 μm, 4.6 x 20 mm guard column (Waters Limited) (7),(14). The samples (5 - 20 μl) were injected onto the column and the mobile phase (a solution of 80% acetonitrile and 20% water) was run in an isocratic manner at 1 ml/min for 20 min (1),(7) and peaks detected as absorbance at 195 nm (15),(16). Two independent preparations of each solution were analyzed using different amounts of sample to ensure consistent separation and quantitation. A standard curve of different concentrations of the purified sugar standards was created and the amount of the corresponding sugar peak in the

honey preparation was determined using the area under the peak. The relative amounts of each sugar were determined as a percentage of total amount of honey analyzed.

5.1.2 Characterization of honey phenolics

A proportion of honey is made up by a complex group of phenolic and flavanoid molecules. Many researchers have implicated this group of compounds in many of the bioactivities mediated by treatment with honey. However, any analysis of this group of compounds requires that they be purified away from the large amount of sugars which would obscure characterization. For purification, 25 g of each type of honey was dissolved in 250 ml of 1% HCl at 37^{o} C for 30 min and combined with 35 g of Amberlite-2AD resin (Sigma-Aldrich Canada) and mixed by stirring for 30 min and then poured and collected into a 3 x 20 cm column (14),(16) . The honey mixture was passed through the column and then the column was washed sequentially with 250 ml of 1 % HCl and 250 ml of water. The phenolics on the column were eluted with 250 ml of methanol and the methanol fraction concentrated to a volume of 4 - 8 ml under reduced pressure and water added to give a final concentration of 80% methanol.

The purified samples were subjected to HPLC on a Breeze 2 chromatography system including a spectrophotometer and auto-injector, fitted with a Sunfire C18 3.5 μm, 4.6 x 100 mm column and a Sunfire C18 3.5 μm, 4.6 x 20 mm guard column (Waters Limited). The samples (2 - 5 μl) were injected onto the column and the mobile phase applied at 1 ml/min: the mobile phase was comprised of a gradient between Buffer A (5% acetic acid in water) and Buffer B (5% acetic acid in acetonitrile) which was set to change from 100% Buffer A to 50% Buffer A: 50% Buffer B after 40 min, to 100% Buffer B from 40 - 50 min, and then to 100% Buffer A from 50 - 60 min)(13),(14). The absorbance of the eluent was measured at 280 nm and 320 nm and at 370

nm and 425 nm in two separate runs. Two independent preparations of each phenolic purification were analyzed at least two separate times for each type of honey to ensure consistent separation and quantitation. A standard curve of different concentrations of multiple phenolic and flavonoid compounds which have been previously identified in honey samples was created. These standards included: galllic acid, chlorogenic acid, caffeic acid, vanillic acid, trans-p-coumaric acid, 4-hydroxy-3-methoxycinnamic acid, transferulic acid, ellagic acid, and quercitin (Fisher Scientific) (13),(14). Each standard was suspended at a known concentration and separately subjected to fractionation on HPLC (to identify the retention time) and then several standards were combined at equal concentrations and serially diluted before being fractionated on HPLC to create a standard curve based on using the area under the peak for each concentration. Individual peaks in the honey chromatographs were identified based on comparison to the retention time of the standard as well as by comparing the change in peak area between chromatographs obtained at different wavelengths. The relative amounts of each component was then determined based on the area of the corresponding peaks in the honey chromatographs in comparison to the standard curve (quantitation was performed based on the chromatographs obtained at 280 nm).

5.2 Results

5.2.1 Characterization of honey sugars

HPLC analysis of the 4 different samples of honey for the presence of sugars in the honeys showed the presence of several different peaks with two prominent peaks at retention times of 4.5 min and 5.5 min (Fig 5.1). Closer examination of the chromatographs (see inset in the figures) identifies at least three additional peaks with retention times of 9.5 min, 10.5 min, and 14 min. Parallel HPLC analysis of sugar standards (Fig 5.2) showed that these peaks corresponded to fructose, glucose, sucrose, maltose, and trehalose. Determination of the relative amounts of each identified sugar in the honey samples showed that the clover polyfloral honey (honey 1) was different in composition from the monofloral Sidr honey samples (honey 2 - 4) (Table 5.1). Honey 1 contained 37.5% fructose which was higher than for the other 3 honey samples which contained 28 - 30.7% fructose. Honey 1 contained a much lower proportions of glucose. sucrose, and maltose compared to the 3 Sidr honey samples which contained similar levels of these sugars. Honey 2 contained a much higher concentration of trehalose than the other honeys.

Table 5.1: Sidr Honey samples collected from different regions

Honey type	Botanical origin	Honey code	Honey source
Clover honey		**1**	Commercial
		2	Saudi Arabia (Mountain Sidr)
Sidr honey	*Ziziphus spina-christi L*	**3**	UK market (Yemen)
		4	Saudi Arabia market (Yemen)

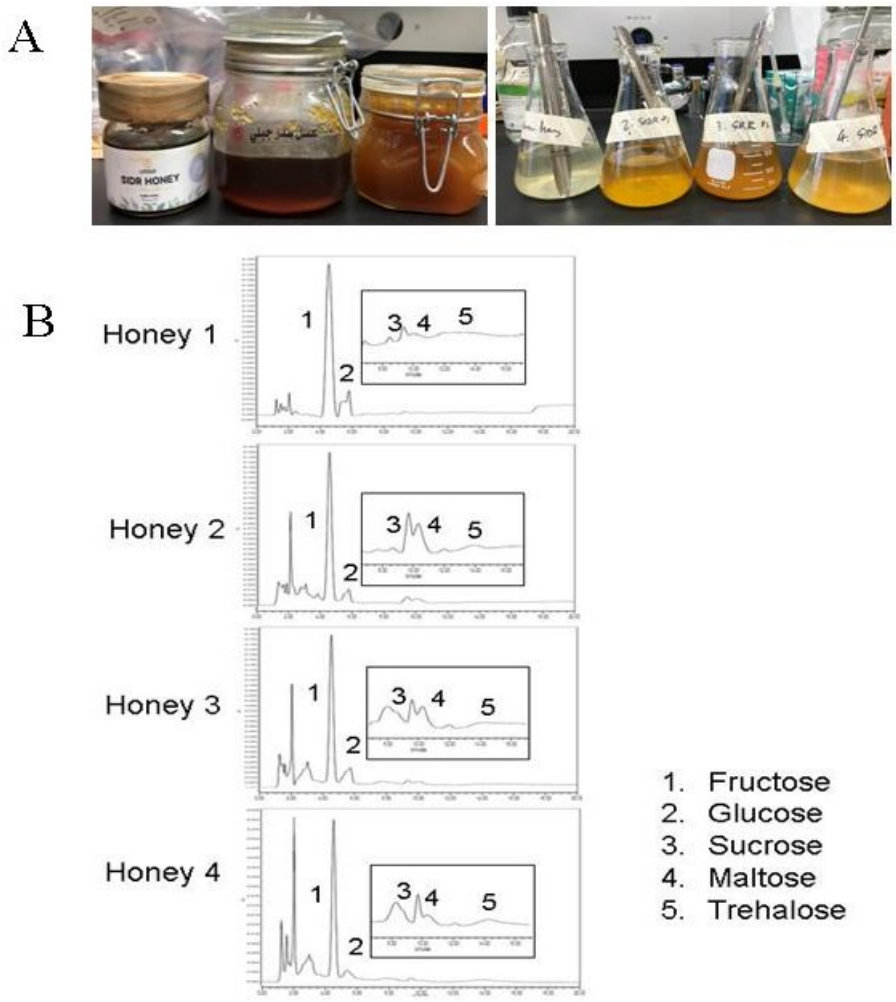

Figure 5.1: HPLC analysis of sugars in honey samples.

(A) Samples of honey and diluted samples prior to analysis show differences in the colour. (B) Samples of honey were analyzed by HPLC on an X-Bridge amide column at an absorbance of 195 nm. Honey 1 corresponds to a sample of a polyfloral clover honey while Honey 2 - 4 corresponds to different samples of Sidr honey obtained from different suppliers. The inset indicates an enlargement of the chromatograph to emphasize smaller peaks. The numbers indicate the position of the peak corresponding to the indicated sugar.

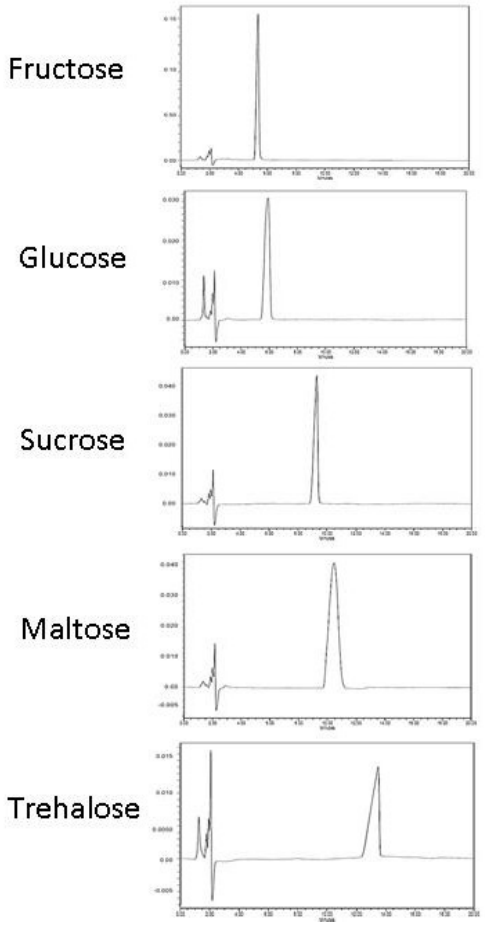

Figure 5.2: HPLC analysis of known sugar standards.

Pure samples of sugar standards were analyzed by HPLC on an X-Bridge amide column at an absorbance of 195 nm in order to identify the retention time for each standard.

Table 5.2: The weight percent for specific sugar components in samples of honey.

	honey 1	honey 2	honey 3	honey 4
fructose	37.5	30.0	28.0	30.7
glucose	7.7	19.1	16.8	21.3
sucrose	1.6	3.8	3.6	3.9
maltose	0.1	4.5	4.8	5.7
trehalose	0.3	3.2	0.7	0.4

5.2.2 Characterization of honey phenolics

HPLC analysis of the phenolic and flavonoid components in the 4 different samples of honey showed the presence of several different peaks which absorbed at the 4 different tested wavelengths (Fig 5.3). Interestingly, the chromatographs looked quite different for all of the different honey samples: all of the samples contained multiple peaks which showed characteristic differences depending on the absorbance wavelength. For example, all four samples of honey showed a major peak at 280 nm, with a retention time of 2.4 min, which was much lower in intensity at 320 nm corresponding to ellagic acid. Honey 1, the commercial clover honey was the only sample that did not show peaks at 8.4 min and 24.7 min at both 280 nm and 320 nm, corresponding to chlorogenic acid and quercitin, respectively. All 4 honeys contained peaks that corresponded to the remaining standards. However, all of the honey samples also contained several peaks that have not been identified.

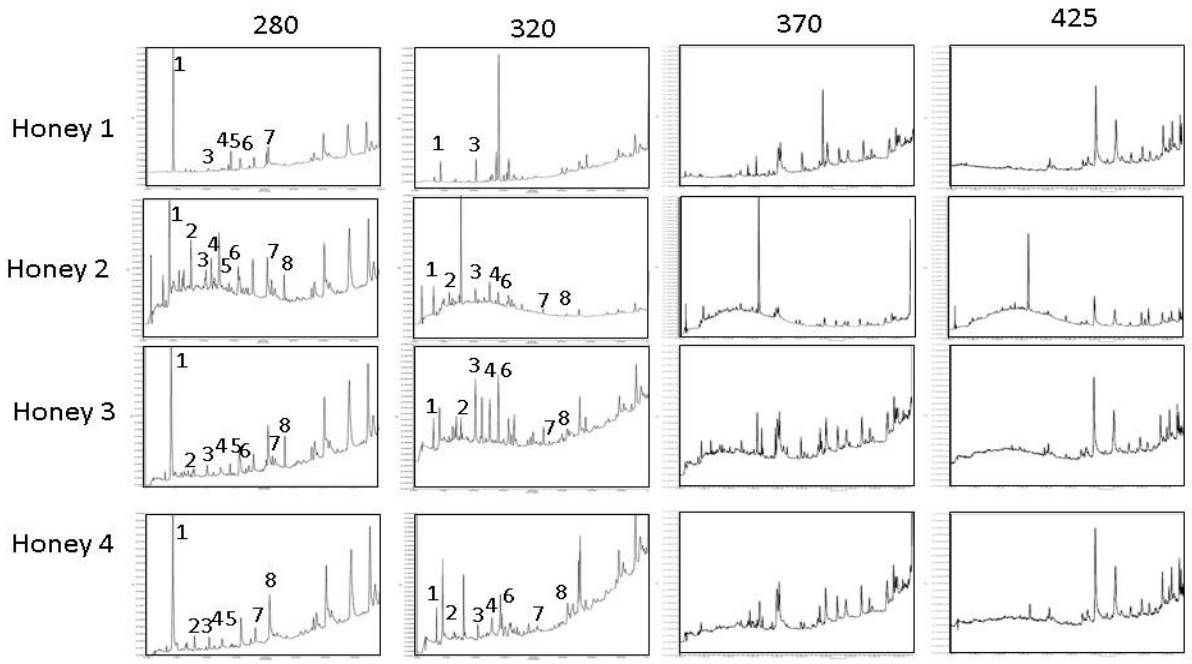

Figure 5.3: HPLC analysis of phenolic and flavanoid compounds in samples of honey. Purified samples of honey were analyzed by HPLC on an Sunfire C18 column at absorbances of 280, 320, 370, and 425 nm. Honey 1 corresponds to a sample of a polyfloral clover honey while Honey 2 - 4 corresponds to different samples of Sidr honey obtained from different suppliers. The numbers indicate the position of the peaks corresponding to the indicated standard component.

Multiple phenolic and flavonoid compounds which have been previously identified in honey samples was including galllic acid, chlorogenic acid, caffeic acid, vanillic acid, trans-p-coumaric acid, 4-hydroxy-3-methoxycinnamic acid, transferulic acid, ellagic acid, and quercitin were separately subjected to fractionation on HPLC and their retention times identified (Fig 5.4). To confirm the retention times, all of the standards were mixed together at 100 μg/ml each and subjected to separation on HPLC and the relative position identified (Fig 5.5). This showed that combination of all of the standards did not affect their retention time on the HPLC chromatographs. Two different combinations of standards, each containing 5 separate components, were created with each component at 100 μg/ml and then these mixes were serially diluted before being fractionated on HPLC (Fig 5.6). The areas under the peak for each standard component in the mixture was obtained for each of the known concentrations and these values were used to create a standard curve for each component (Fig 5.7). Individual peaks in the honey chromatographs were identified based on comparison to the retention time of the standard as well as by comparing the change in peak area between chromatographs obtained at different wavelengths. The relative amounts of each component was then determined based on the area of the corresponding peaks in the honey chromatographs in comparison to the standard curve (quantitation was performed based on the chromatographs obtained at 280 nm) and then calculated based on the dilution of honey required for the original purification of the phenolic fraction (Table 5.2).

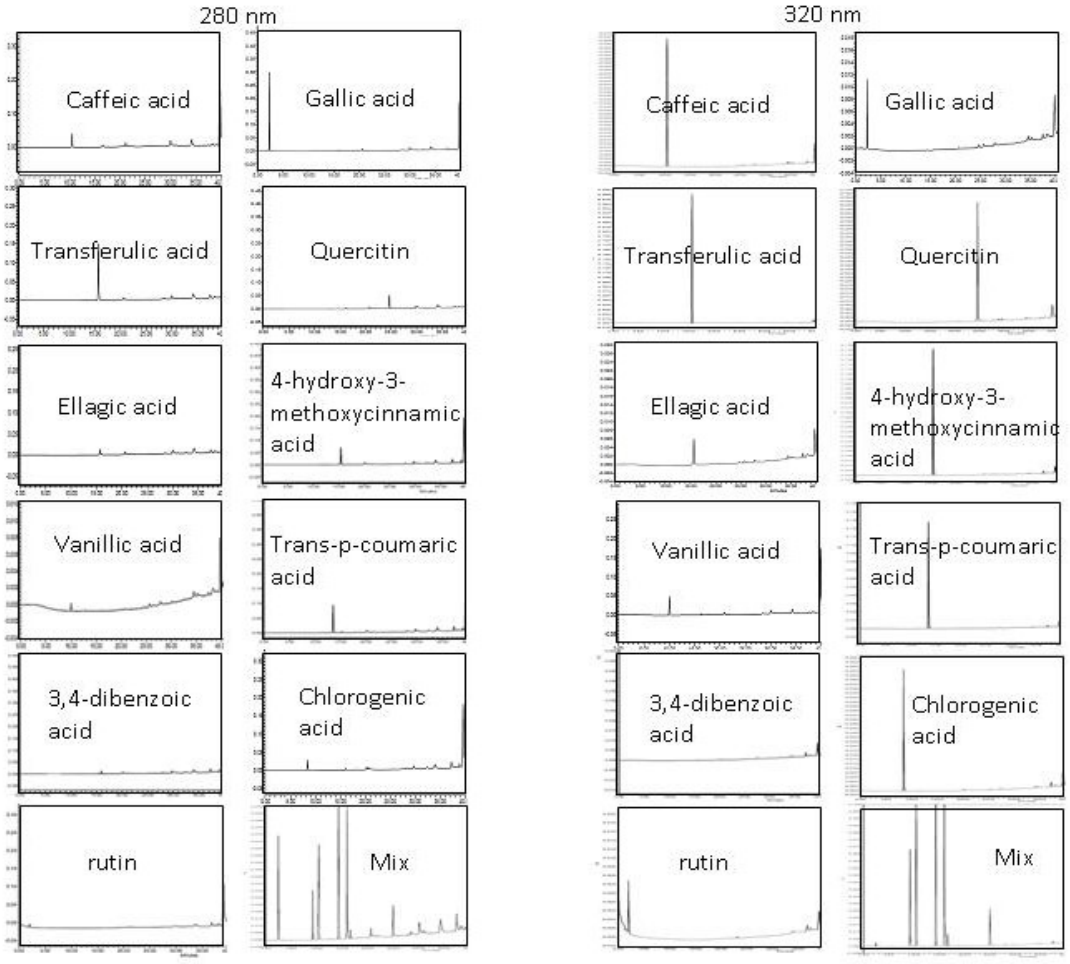

Figure 5.4: HPLC analysis of individual phenolic and flavonoid standards on an Sunfire C18 column at absorbances of 280 and 320 nm.

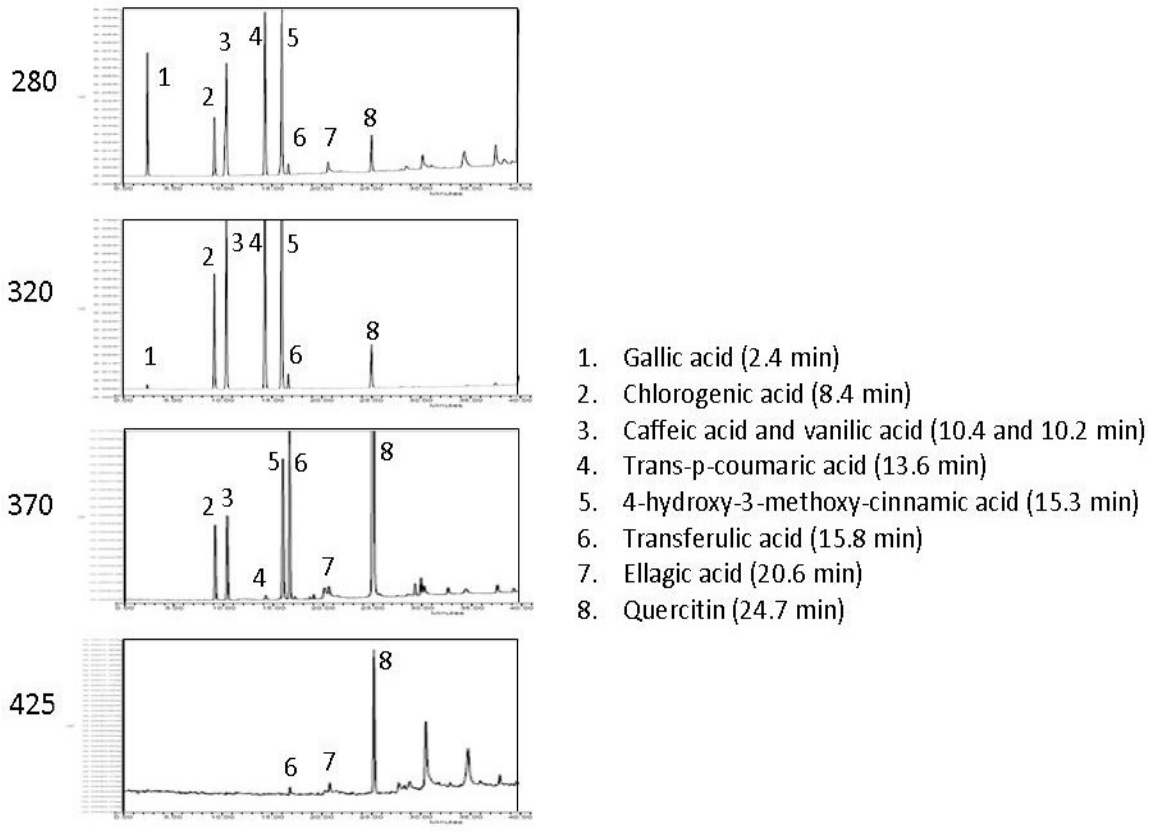

Figure 5.5: HPLC analysis of the combined mixture all of the standard components on an Sunfire C18 column at absorbances of 280, 320, 370, and 425 nm.

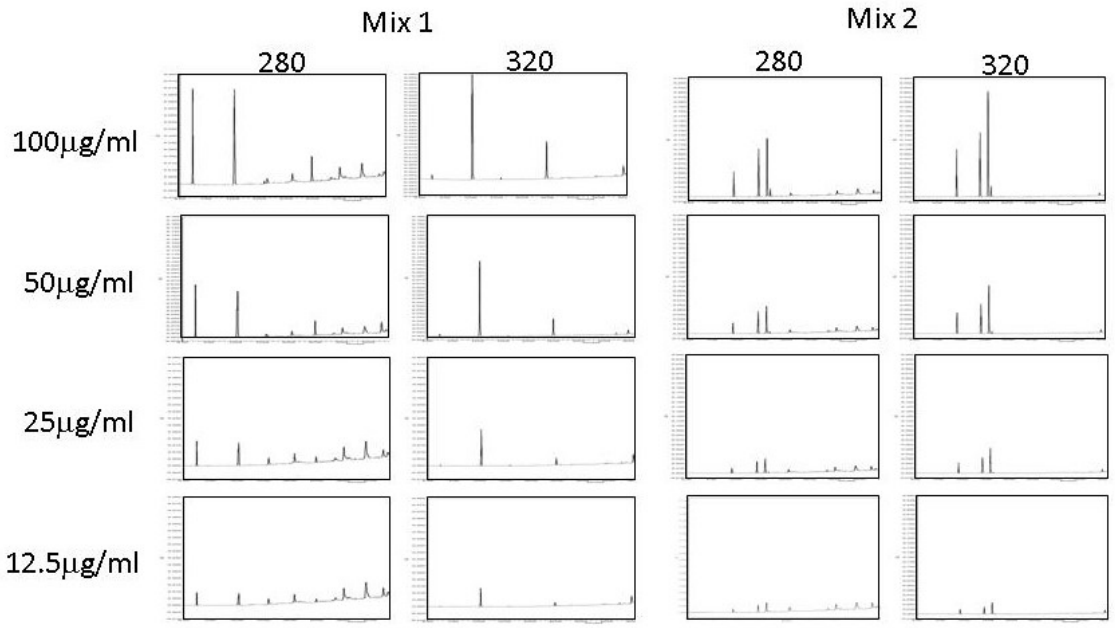

Figure 5.6: Creating a standard curve for the HPLC standards using analysis of serially diluted standard solutions.

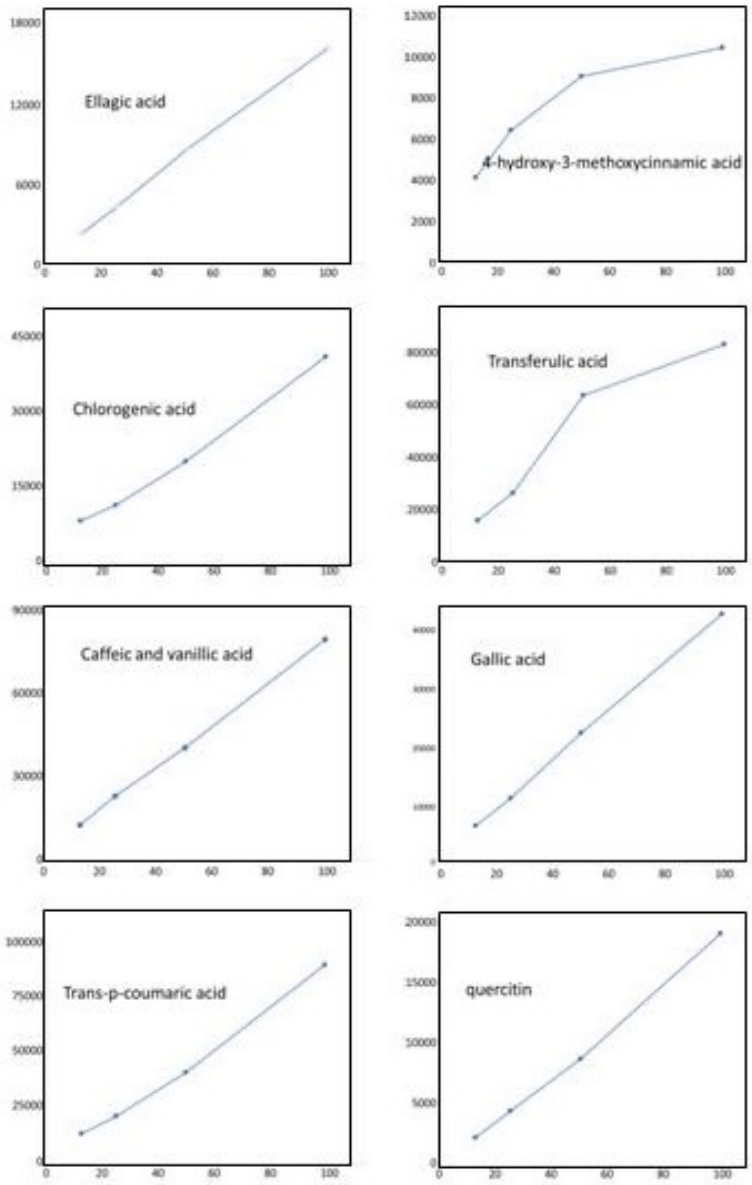

Figure 5.7: Standard curves for concentration versus absorbance readings for HPLC standards.

Table 5.3: Quantitation of various phenolic and flavonoid components in the different honey samples.

	honey 1	honey 2	honey 3	honey 4
gallic acid	14.8	18.24	34.65	42.4
chlorogenic acid	nd	3.84	1.35	1.6
caffeic and vanillic acid	3.6	17.6	9	8
trans-p-coumaric acid	2.8	3.84	5.85	4.8
4-hydroxy-3-methoxycinnamic acid	40	38,4	9.9	7.2
transferulic acid	9.6	16	22.25	17.6
ellagic acid	3	4.16	5.85	
quercitin	1.2	7.68	10.8	3.2

Determination of the relative amounts of each identified standard in the honey samples showed that all of the honey samples contained different levels of the various components. The levels of chlorogenic acid, caffeic and vanillic acid, trans-p-coumaric acid, and transferulic acid in the clover polyfloral honey (honey 1) were all lower than in all 3 of the Sidr honey samples (honey 2 - 4) which had similar levels (Table 5.2). Interestingly, the clover polyfloral honey (honey 1) showed similar levels of gallic acid, 4-hydroxy-3-methoxycinnamic acid, and ellagic acid to one of the monofloral Sidr honey samples (honey 2) but which were different from the levels in the other Sidr honey samples (honey 3 and 4). These results suggest that the level of some of the components may be associated with the identity of a particular type of honey while others are more variable.

5.3 Discussion

The results of this analysis show that Sidr Honey contains sugar and phenolic components consistent with the composition of many other types of honey as reported in the literature. We analyzed 4 separate honey samples; one sample of a commercial clover polyfloral honey and three samples of Sidr honey obtained from different commercial suppliers. In general, these results show that the polyfloral honey sample is somewhat different from the Sidr honey samples. Further, these results show that in spite of some similarities, there are also some differences among the three different Sidr honey samples. Interestingly, all of these honey samples have been shown to have anti-proliferative effects on breast cancer cells, although the Sidr honey samples were shown to be more active than the polyfloral sample (Chapter 2 in this thesis). All of the honey samples show that the most abundant of the sugar components is fructose, however, the polyfloral honey is shown to contain a higher level of fructose (37.5%) compared to the Sidr honey samples which all contained similar amounts between 28 and 30.7%. The polyfloral honey was shown to contain lower levels of glucose, sucrose, and maltose compared to the Sidr honey samples which all contained similar levels of the sugars. Therefore, these results show that the Sidr honey samples all have similar sugar concentration profiles.

The phenolic acid and flavonoid profiles in the purified honey samples were all somewhat complex and, on inspection, appeared to be somewhat different between the different samples. Each of the spectra contained at least 10-20 major peaks and multiple minor peaks. While is seems clear that several of the peaks are shared among all four honey samples, there are several which appear to be expressed at distinctly different levels. This was confirmed by

comparison among different identified components in the honey samples. Ten different standard components, known to be present in previously studied honey samples were used to identify some of the peaks in the current samples. The identity of the peaks in the honey samples were determined by comparison to the HPLC retention times for the standards as well as by comparison of the relative peak heights determined at the different wavelengths. The quantities of the identified components were then calculated by comparing the areas under the peaks to the standards and the amount of the component in the honey determined by multiplying by the dilution that occurred during sample preparation. The relative amounts of each identified standard in the honey samples showed that all of the honey samples contained different levels of the various identified components. This analysis showed that the polyfloral honey was distinct from all three Sidr honey samples. For example, the levels of chlorogenic acid, caffeic and vanillic acid, trans-p-coumaric acid, and transferulic acid were lower in the polyfloral honey compared to the Sidr honey samples which had similar levels. The level of ellagic acid and quercitin were also higher in the Sidr honey samples although these levels were quite variable between these Sidr honey samples. Interestingly, the polyfloral honey showed similar levels of gallic acid and 4-hydroxy-3-methoxycinnamic acid to one of the monofloral Sidr honey samples but which were different from the levels in the other two Sidr honey samples. These results suggest that the level of some of the components may be associated with the identity of a particular type of honey while others are more variable. It is known that the levels of different components in a particular type of honey can vary significantly depending on the time of year the nectar was collected and by the collection and processing techniques used to produce the final product.

The results of this study are consistent with previous studies which looked at the composition of various honey samples, including Sidr honey samples. A more comprehensive study of a larger number of different honey samples with a wider range of identified standards would be useful for verifying that Sidr honey samples have a more unique pattern of chemical composition which can be used to distinguish them from other types of honey.

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Chapter 6

6 Conclusion

Cancer is one of the most overwhelming conditions in 21st century health practice, with the increase in its prevalence being driven by changes in lifestyle and developments in diagnostic capabilities. Recently, there has been a renewed interest in using traditional and alternate medicines to treat patients with cancer due to perceived limitations of current treatment options or as an adjunct to treatments. As the potential for the application of alternative medicine practices increased, the role of honey as a possible chemotherapeutic agent drew more scholarly attention. The use of honey for medicinal purposes is a practice that has been conducted in many communities for a long time, and extracts from honey have been applied to the management of such conditions as wound healing, arthritis, allergy, and gastrointestinal disturbance. However, as the emphasis on scientific evidence in medical practice has increased, the mechanisms underlying many of the therapeutic applications of honey have been investigated using *in vitro*, *in vivo*, and even clinical trial models. Some of these studies have been used to determine the mechanism of action and have validated the effectiveness of honey in treating some of the diseases it has been traditionally used to treat. One such effect is anti-tumour activity, which is one of the recognized properties of many varieties of natural honey. Research studies have revealed several features that are related to the anti-tumour effects of honey, including the composition of the honey from different regions, the active constituents, and the effect of these components on the signaling pathways that mediate its anti-tumour activities.

In the currents studies *in vitro* models were used to investigate the effect of one variety of honey, Yemeni Sidr (YSH), on cancer cell lines. Sidr honey is a premium monofloral honey

derived from the nectar of the *Ziziphus spina-christi* tree. Its derivation from a single tree species sets it apart from other varieties of honey and makes its potential for application in cancer treatment appealing since the multiplicity of active compounds in other types of honey is an important barrier to successful isolation and generation of a chemotherapeutic formulation. The current study was aimed to investigate the anti-proliferative, apoptotic, and immuno-modulatory effects of YSH on various cancer cell lines. Moreover, the impact of YSH on the polarization of macrophages and its effect on THP-1-derived macrophages co-cultured with cancer cells was examined. The limitations of this study, however, lie in the uncertainty regarding its composition. Despite being monofloral, Sidr honey was shown to contain several active components whose isolation was not achieved by this study.

The results of this study indicated that treatment with YSH induces apoptosis in cancer cells, an effect that is evident from morphological changes and alterations detected using such viability assays as MTT and SRB assays. The cell-killing activity of YSH is comparable to that previously shown for other honey varieties, and has been shown to occur through various mechanisms. The dominant pathway of apoptosis induced by YSH involves cell cycle arrest at different phases. Others have shown that the activation of p53 and its decoupling from inhibitory molecules induced by treatment with honey can play a role in the escape of regulation in some cancer cells and can increase the cell's ability to detect defective genetic material and halt division. Other molecular changes that mediate apoptosis under the influence of YSH include the down-regulation of anti-apoptotic Bcl-2, as well as the up-regulation of Bax and the activation of the activated form of caspase-3. The apoptotic effect of YSH was found to be limited to neoplastic cells in our study, a trait that is highly desirable in considerations for chemotherapeutic applications. In addition to directly inducing apoptotic effects in cancer cells,

treatment with YSH affected the polarization of macrophages towards the M1 subtype which increased cancer cell death in cancer cell/macrophage co-cultures. However, in THP-1-derived macrophages activated in the presence of the inflammatory mediator, LPS, prolonged treatment with YSH increased polarization to the M2 subtype. The M2 subtype of macrophages has anti-inflammatory effects and is formed in association with the presence of Th2 helper cell-derived cytokines. The M2 phenotype of macrophages is the dominant form of tumour associated macrophages (TAM), and has been shown to enhance tumour growth, viability, invasion, metastasis, and resistance to immune responses. These results show that the effects of YSH on macrophages depends on the microenvironment of the macrophage: unactivated macrophages exhibit pro-inflammatory (M1) characteristics when treated with YSH while LPS-treated (pro-inflammatory) macrophages exhibit an increase in M2 characteristics when treated with YSH. Because YSH can induce proinflammatory characteristics in unactivated tissues it can disrupt the M2 macrophage-enhanced tumour microenvironment, and diminish the angiogenetic, metastatic, growth, and invasive capacity of certain cancer cell lines. Moreover, YSH was found to enhance the release of inflammatory cytokines by macrophages in environments where the inflammatory response normally does not prevail. However, YSH can induce a greater M2-like phenotype in environments where there is an existing inflammatory response, an effect that is useful in its wound healing effects.

The findings in this study constitute an important addition to the existing literature describing the anti-tumour properties of honey. In particular, the ability of Sidr honey to disrupt tumour microenvironments by influencing the polarization of macrophages represents an important and unique pathway for potential application in cancer treatment. However, despite the hope ignited by these findings, the use of honey in cancer management still has a long way to go.

This is because, for a honey-based therapeutic drug to be developed, the specific active compound in honey has to be isolated, an appropriate formulation and route of administration determined, and clinical trials conducted to determine its efficacy and side effects profile needs to be performed. Considering that most of these stages have yet to be completed, it is unlikely that the anti-tumour activity of honey will find its way to a clinical anti-cancer application soon. This scientific procedure is further lengthened by the fact that few studies have been dedicated to studying the harmful side effects profile of honey.