An-Najah National University

Faculty of Graduate Studies

The Effects of *Ficus Sycomorus* Extracts on Human Keratinocytes as a Potential Antipsoriasis Therapy

By

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This thesis was Defended successfully on 8/7/2021 and approved by:

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Dedication

Then, after many months, even after many years of work, study and research I dedicate this achievement to my father Adnan and my mother Taghreed, who with love and effort have accompanied me in this process, without hesitating at any moment of seeing my dreams come true, which are also their dreams.

To my beautiful sisters Ruba, Inas, Ghayd and Hala, to my gorgeous brother Jawad and my grandmother.

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الاقرار

أنا الموقعة أدناه، مقدّمة الرسالة التي تحمل العنوان:

The Effects of *Ficus Sycomorus* Extracts on Human Keratinocytes as a Potential Antipsoriasis Therapy

أقر بأن ما اشتملت عليه هذه الأطروحة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الاشارة إليه حيثما ورد. وأن هذه الرسالة كاملة، أو اي جزء منها لم يقدم من قبل لنيل اي درجة او لقب علمي او بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالبة:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

FS	*Ficus sycomorus*
HaCaT	Human epidermal keratinocyte cell line
THP-1	Human monocytic cell line
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute
FCS	Fetal calf serum
PMA	Phorbol 12-myristate 13-acetate
vitamin D3	1α, 25-dihydroxyvitamin D3
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
PBS	Phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
µL	Microliter
µg	Microgram
nm	Nanometre
LPS **GPP** **EP**	Lipopolysaccharide Generalized Pustular Psoriasis Erythrodermic psoriasis
NO **NOS**	Nitric Oxide Nitric Oxide Synthase
KC	Keratinocytes
OS	oxidative stress
ROS	Reactive oxidative species
NF-κB	pathway Nuclear Factor κ-light-chain-enhancer of activated B cells

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Abstract

Introduction: Psoriasis is a chronic hyperproliferative inflammatory skin disease that leads to over proliferation in keratinocytes. Psoriasis which affects 2 to 3% of the population is caused by several factors including, genetics, epigenetics, environments and lifestyles as well as stress, drugs, infections and trauma. Nitric oxide is a principal biomarker for psoriasis. Since eternity, human used plants and natural product as source of food and medicines for treating and preventing the diseases. One of which FS which is used in the tradition for the treatment of psoriasis.

Aim: This in vitro study aimed to evaluate cytotoxic, cytostatic, and anti-inflammatory properties of FS leaves and fruits water-ethanol extracts on the keratinocytes cell line (HaCaT) and THP-1 derived macrophages.

Methods: Cell viability for both monoculture and co-culture was conducted by MTT assay. In addition to that LPS-induced THP-1-derived macrophages and keratinocytes cell line (HaCaT) as monocultures and as co-cultures were used to assess the effects of the FS plant extracts on the production of pro-inflammatory NO by Griess reagent. Moreover, phytochemical analysis of FS fruit and leaf extracts was carried out.

Results: Obtained results are revealed that there is no significant difference between all examined concentrations of the FS plant parts under the study on both investigated monocultures and co-culture based on the conducted statistical analysis ($p< 0.05$) with no variations between FS leaf and fruit extracts. However, significant cytostatic effect of FS fruit extract on HaCaT cells ($p < 0.05$) at all studied concentrations with more pronounced impact at 1000 and 500 µg/ml. This effect was higher on the HaCaT monoculture system than on THP-1 in concentration dependent manner with cell viability of 69.6% at concentration of 31.8 µg/ml reaching to 57% at concentration of 1000 µg/ml. While their co-cultures showed significant decrease in cell viability was at 250,500 and 1000 µg/ml with 64.2%, 63.1% and 64.2%, respectively. This recorded pronounced cytostatic effect is similar between leaf and fruit extract types. Therefore, salient noteworthy cytostatic effect of the FS fruit and leaf extracts is more than the cytotoxic one on both HaCaT cell line monoculture and co-culture cell lines system. Similarly, is observed in the FS fruit extract effect on THP-1 cell line monoculture in concentration dependent manner. On the contrary is recognized by the FS extract effect on THP-1 cell line monoculture as more cytotoxic effect is recognized rather than cytostatic one in concentration dependent manner. In addition, a scavenging power of FS fruit and leaf extracts was documented by the NO production reduction. The marked NO inhibitory activity on macrophages was observed by FS fruits at 250 µg/ml as it caused only 71.46% production in respect to the positive control. So, the fruits extract had resulted in NO inhibitory

activities of 28.54% and 16.57% at 250, 125 μg/ml, respectively. While, the FS leaves extract revealed the NO inhibition of 18.64 and 16.24% at 250, 125 μg/ml, respectively. Furthermore, the qualitative phytochemicals evaluation of both FS fruit and leaf extracts indicated the presence of glycoside, phenols, flavonoids, steroids, saponins and tannins. None the less, minor variations between both extract types were recorded in those carbohydrates, proteins and amino acids as well as terpenoids are found in FS fruit extract only. While, reducing sugars are in leaf extract only. Alkaloids are found in neither fruit nor leaf extracts.

Conclusion: In conclusion, this in vitro study indicated that fruits and leaves extract from FS was not toxic at all tested concentrations in association with higher cytostatic effects in general and of fruits in particular on the HaCaT and THP-1 monoculture system than the co-culture. While, its leave extract effects were on HaCaT monoculture more than on co-culture. In addition, the pronounced inhibition of LPS-induced NO production on THP-1 monoculture. Hence all in all FS fruits and leaves extracts positive recorded data would serve as a source of a novel, effective antiproliferative and NO production inhibitory bioactivity potential agents against hyperproliferative of skin. From this point of view, this study supports to a certain degree the traditional medicinal uses of the plants in diseases therapy and reinforces the concept that ethno botanical approach to screen plants as potential sources of bioactive substances is successful.

Chapter One
Introduction

1.1 General background

Psoriasis is a chronic hyper proliferative inflammatory skin disease that resulted from an excessive activity in the immune system (1,2). This excessive activity leads to over proliferation in keratinocytes which play a critical role in the early stage of psoriasis and in maintaining the chronic course. Destruction of the epidermal barrier in psoriasis makes keratinocytes at more risk by numerous external dangerous materials, causing cellular harm or even cell dying (3). Psoriasis is a non-communicable inflammatory disease of skin that is derived from genetics factors; Scans of the human genome reveal at least nine distinctive loci with susceptibility to psoriasis (PSORS1-9). PSORS-1, a place of the foremost histocompatibility complex on chromosome 6p21.3, is the main genetic determinant of psoriasis, and represents up to 50% of the genetic susceptibility to disease (4).

Nevertheless, the environmental elements concurring in starting up the sickness are ill-described; Among recognised environmental factors that contributes in triggering psoriasis are, pills, infections, physical trauma, smoking, alcohol, and pressure as well as drugs, such as the anti-proliferative agent imiquimod, antidepressants (lithium) and antiviral agents (5). There is a relationship between preceding streptococcal throat infection and psoriasis, mainly with guttate psoriasis, surgical incisions

also give rise to the psoriasis plaques at the site of the trauma, other personal behaviour increase the risk of having psoriasis including smoking and alcohol (5). It should be noted that there is no consensus on whether these factors actually cause psoriasis or exacerbate psoriasis.

According to that the factors that cause pathogenesis of psoriasis can be considered in the three factors: 1) is an interaction between genetic and environmental factors.2) is an interface between innate or adaptive immunity and the resident skin cells.3) consists of epidermal and dermal reforming (1, 4, 5).

Psoriasis is categorized at the cellular level by increased epidermal proliferation, incomplete differentiation, elongation, expansion, "leakage" of the superficial plexus of dermal capillaries and infiltration of inflammatory and immune cells of the epidermis and papillary dermis (1, 2, 3).

Psoriasis vulgaris is a hereditary immune system disorder in the skin that Clinically distinguished by red plaques with silver or white multi layered scales and a thickened acanthotic epidermis in patients who are notably outlined by nearby non lesion skin (1,5). Patients usually report the manifestations of tingling, torment sensation, and draining. This sickness has the highlights of high predominance, chronicity, disfiguration, handicap, and comorbidity (5, 6, 7). Psoriasis can happen at any skin site; however, it appears for the most part of skin includes on the knees, elbows, trunk, back, and scalp. The fingernail and toenail areas are additionally

frequently influenced. The histopathological perception of psoriasis sores uncovers epidermal acanthosis, rete edges, invulnerable cell penetration in the dermis, and expanded angiogenesis. The acanthosis is controlled by keratinocyte expansion that is related with the modified separation technique, as the development of keratinocytes happens from the basal to the cornified layer (1,7). Psoriatic patients are in danger of creating comorbid infections, as well as psoriatic diabetes, joint pain cardiovascular disarranges, Crohn's illness, uneasiness, lymphoma and gloom. psoriasis consistently diminishes the personal satisfaction, and patients are presented to social shame and isolation (5,8).

1.2 Varieties of psoriasis

Psoriasis is a worldwide disease that affects 2% to 3% of the population in the world. Historically, the disorder classification has been based on medical appearance, particularly differentiating in line with localization and morphology. Here, we follow the latest grouping proposed by the International Psoriasis Council, which identifies 4 important varieties of psoriasis: plaque-type, guttate, generalized pustular psoriasis (GPP), and erythroderma, in addition to several sub phenotypes concern with distribution and anatomical localization; (scalp/fingers /flexural, /soles/nail), (localized vs. Good sized), size (big vs. Small) and thickness (thick vs. Skinny) of plaques, onset (early vs. Overdue), and sickness pastime (lively vs. Stable) (6).

Psoriasis is a dynamic disorder; morphological changes follow the evolution of a newly shaped lesion into an advanced plaque that may slowly extend (active lesions, sharing maximum of the histological functions of newly fashioned lesions) or remain static (strong lesions, keeping the morphology of the superior level) (8, 9).

1.2.1 Plaque-type psoriasis

Plaque-type psoriasis, happening in 85%–90% of influenced patients, is the most well-known type of psoriasis and is described by oval or sporadically formed, red, forcefully differentiated, raised plaques secured by shiny scales. Plaques happen for the most part on the extensor surface of elbows and knees on the scalp and in the lower back, it can influence any area of the skin, frequently with a balanced dissemination. Size of the injuries can fluctuate, from pinpoint to bigger individual sores or blended regions prompting two clinical sub phenotypes. A further characterization considers the time of beginning; Type I psoriasis has beginning stage (<40 yr), is regularly connected with recognizable infection history and shows high relationship with the human leukocyte antigen (HLA)- Cw0602 allele, while type II psoriasis creates after the age of 40 (8, 9).

At the beginning of a recently creating plaque, the main changes happen in the highest layer of the dermis (the papillary dermis), Veins then become expanded and convoluted with lymphocytes and neutrophils rising up out of their lumen and going after the epidermis, the aforementioned still looks very ordinary at this stage. Not long after, nonetheless, variant keratinocyte

expansion and relocation start, bringing about epidermal thickening, fragmented terminal separation with beginning loss of the "stratum granulosum," and the presence of foci of parakeratosis, that is, the maintenance of the core by corneocytes. Parakeratosis gets blended, the stratum granulosum is missing, lymphocytes, fundamentally CD8+ T cells, are sprinkled among KCs, and neutrophils amass into the parakeratotic scales, shaping Munro micro abscesses. The expanded veins broaden high into papillae, representing pinpoint draining when a scale is expelled, known as Auspitz sign. The dermis is vigorously penetrated by T cells and dendritic cells. Injuries can immediately resolve, albeit once in a while. Settling injuries after treatment can be encased by an unmistakable edge of whitening prescient of clearing and histologically described by orthokeratosis, that is thickening of the stratum corneum without parakeratosis and rebuilding of the stratum granulosum (5, 8).

1.2.2 Guttate psoriasis

Guttate psoriasis is another type of psoriasis that appears as small point-like lesions. Guttate psoriasis often begins in childhood or youth, and can occur due to a bacterial infection, usually streptococcus (sore throat) (5).

The immune system responses that cause stains on the skin begins. In some cases, guttate psoriasis is genetic, the point psoriasis is hereditary in this case. Other factors can cause guttate psoriasis: Tonsillitis, stress, cuts, burns, or bites on the skin, upper respiratory infections, and some drugs

such as antimalarials and beta-blockers. About 10 percent of people who are suffering psoriasis develop guttate psoriasis (5, 8, 10).

1.2.3 Generalized pustular psoriasis (GPP)

GPP, also identified as von Zumbusch type, is rare but could be life-threatening disease, it is characterized by generalized sterile pustule formation with widespread inflammation and erythema. These pustules often increase and coalesce and form lakes of pus. Acute GPP is often associated with systemic symptoms such as chills, fever anorexia, nausea, severe pain and malaise. Many complications may triggers generalized pustular psoriasis including: Upper respiratory tract infections, stress, nonsteroidal anti-inflammatory drugs, pregnancy, corticosteroid hormones. GPP may also be closely related to some predisposing genetic factors. A genetic mutation in the IL36RN gene leading to an atypical antigen of the IL-36 receptor (IL-36RA) was detected in all studied patients, the mutation was observed to cause an unstable IL-36RA protein with a low affinity for its receptor. IL-36RA mutation also led to an increase in inflammatory cytokines. GPP has been described in the mode of this specific genetic mutation "DITRA" or "IL-36RA deficiency (6, 8, 9).

Pustular psoriasis of pregnancy

Pustular psoriasis of pregnancy is a rare autoimmune dermatological disease observed in the second to third trimesters of pregnancy, the presence of pustular psoriasis of pregnancy has been historically associated

with poor neonatal people together with placental insufficiency, stillbirth, fetal abnormalities, and early neonatal death, with a correlation between severity and period of the disease and terrible neonatal prognosis. Maternal death has additionally been stated. There is a relation between disease in pregnant female and hypocalcaemia or low serum vitamin D levels (8).

1.2.4 Erythrodermic psoriasis (EP)

EP is a rare and severe form of the disease, with an expected prevalence among psoriasis patients ranging from 1% to 2.25%. This type of psoriasis affects most of the body surface, this type of psoriasis is distinguished by fiery redness and diffuse peeling of the skin, it is often accompanied by severe itching and pain, so can be life -threatening, EP is associated with different symptoms including: Severe redness and shedding of skin over a large area of the body so skin looks as if it has been burned, heart rate increases and body temperature goes up and down particularly on very cold or hot days (8, 11).

Patients whose suffering from psoriasis are at risk of developing comorbid diseases, including psoriatic arthritis, diabetes, cardiovascular disorders, Crohn’s disease, lymphoma, anxiety, and depression (5).

About 20% - 30% of psoriasis patients develop chronic musculoskeletal disorder called psoriatic arthritis (PSA), which occurs, in most cases, about a decade after the appearance of psoriasis (9).

The pathophysiology of this chronic autoimmune inflammatory disease is often not clear, but dendritic cells or antigen presenting cells (APC) are seen to sense the stress signals that keratinocytes generate when the antigen comes into contact with them. This also activates T cells, resulting in the release of several cytokines that allow more differentiation between the T cells in response cells such as Th1, Th2, and Th17 (7,11). On the cellular level, psoriasis is characterized by pronounced increased epidermal proliferation, elongation, incomplete differentiation, expansion, "leakage" of the superficial plexus into the skin capillaries and a mixed infiltration of inflammation and the immune cells of the epidermis (12).

The direct pathogenesis of psoriasis is still unknown right now, but sideways with genetic and environmental factors, an immuno-mediated process involving multiple mediators are included, nitric oxide (NO) is one of the signalling mediators responsible and play a crucial role in psoriasis pathogenesis (8).

1.3 Nitric Oxide (NO)

The multifunctional signal NO was considered a powerful candidate for psoriasis via keratinocyte growth and differentiation. This is achieved by increasing the release and actions of peptides and P-linked genes and calcitonin. They are considered important factors in the pathological mechanisms of psoriasis. This is achieved by inducing the production of hyperkeratosis of keratinocytes, chemical reproduction of neutrophils, mast cell degeneration, adhesion molecules and vasodilation (13).

Therefore, NO is a gaseous signalling molecule produced both in and on skin of human. It is created enzymatically by synthetic compounds NOS, which oxidize l-arginine to l-citrulline. NOS has three isoforms: NOS I (neuronal NOS), NOS II (Inducible NOS) and NOS III (Endothelial NOS). In this study the main NOS related isoform is the expression of NOS II as it is inducible via the stimulation by several factors such as inflammatory cytokines. It is strongly related to the psoriasis, as present in many cell types during inflammatory stimulation like macrophages. In addition, In the skin, NO is produced by NOS II in several cells, namely in keratinocytes, Langerhans cells, fibroblasts and other dendritic cells (14).

In spite that NO plays a vital role in cytokine activity, the vascular endothelial growth factor (VEGF) which is the most effective vascular factor in psoriasis depends on the NO itself rather than its production by NOS II regulation (7).

The effects of NO depend on many parameters such as the source of NO, targeted cells in the tissue, form of oxidation and pH of the microenvironment (12, 15, 16).

Through the inflammatory process, iNOS, which is caused by bacterial products and cytokines, plays an important role in the early response. Recent data indicate that the iNOS pathway is involved in the synthesis of acid cytokines during the inflammatory response. One of these cytokines, IL-6 is involved in the pyrogenic response as well as in the catabolic

response, especially glycolysis, caused by severe bacterial infection (7, 17).

NO, itself is measurable at the surface of the psoriatic plaque at up to 100 times the concentration of non-psoriatic skin.

1.4 Treatments of psoriasis

Previously researchers conducted several trials based on the physical and genetic characteristics of psoriasis to discover effective drugs. However, all recorded drugs so far were able only to reduce symptoms and limit the psoriasis spread to other areas which could only restrain its severity. As a consequence, those available treatments only relieve symptoms. The choice of a particular treatment depends entirely on the type, shape and severity of the disease along with the patient's general health and age. Moreover, psoriasis treatments are assigned to each patient on the basis of associated concomitant diseases, negative effects, current quality of life, ability to self-care, medication history, provider status, financial needs and feasibility of follow-up. Treatment usually begins with economic treatments and then escalates to newer/more expensive treatments until an acceptable and effective treatment is reached in good agreement. Treatment methods are as follows:

1.4.1 Topical therapy

Are rubbed directly into the affected skin for local relief. Corticosteroids are the most common medications used to treat mild to moderate psoriasis. They slow down cell turnover by suppressing the immune system, which reduces inflammation and itching. Another medication is Anthralin (Dithranol) which is characterized by an inhibitory effect on hyper proliferating keratinocytes. In addition, Coal Tar- Coal tar which is one of the oldest topical treatments used as monotherapy and in combination with other topical agents causing anti- proliferative and anti- inflammatory effect (9,18).

1.4.2 Photo therapy

It is recommended for patients who do not respond to topical treatments or for patients with psoriasis plaques covering 20% or more of the body's surface. It showed a good success rate as more than 80% of patients were cured. Patients will be subjected to the phototherapy 2 to 3 times a week for 2-3 Months. For example, ultraviolet B (UVB) is used in combination with topical therapies such as coal tar which revealed effective treatment for moderate to severe psoriasis patients. Moreover, ultraviolet A (UVA) is another type of phototherapy which is also used in association with systemic therapies psoralen (PUVA therapy). It is very effective in removing skin lesions in spite of the correlated side effects as redness, itching, dry skin, wrinkled skin, freckles, and skin cancer (8,19).

Ultraviolet light therapy (311-313 nm) is more effective than Broadband UV treatment.

1.4.3 Systemic therapy (Oral or injected medication)

These medications used for only brief periods and could be alternated with other treatments due to potential for severe side effects. this type of medications used if other treatments haven't worked. Used to treat moderate to severe psoriasis.

Like retinoids are pills used to reduce the production of skin cells, the side effects of this treatment might include dry skin and muscle soreness. Methotrexate, it administrated weekly as a single dose, these medications decrease the hyperproliferation of skin and suppresses the inflammation. Biologics, these drugs are expensive such as infliximab (Remicade)and ixekizumab(Taltz), usually administrated by injection, this treatment alter the immune system in way that interruptthe disease cycle and improves symptoms and sign of disease through weeks. Also, this treatment hascarried risk of suppressing the immune system (8, 20).

1.4.4 Medicinal plants

Since eternity, plants and natural products were used as food resources as well as medicines. In spite that the exact date of starting using medicinal plants is unknown it is thought that the ancients Babylon (Iraq),depending on carbon dating, indicated that plants were grown as medicines 60,0000 years ago. This medicinal plant usage was certified date back nearly 5,000

years in India, China and Egypt while, at least 2,500 years in Greece and Central Asia. Therefore, with the passage of time it began to take the name traditional medicine which is “the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness” (24,25).

Today, more than 80% of the world's population depends on traditional medicines, especially plants, that act as a major source of health care (26, 27).

There are no limitations to which part of the medicinal plants that can be used, since it has been documented those different parts were used such as seeds, fruits, roots, flowers, leaves, stems or even the plant as a whole. The recorded observed medicinal plants bioactive ingredients have either direct or indirect therapeutic effects (27). The functional effective phytochemicals could be targeted via different ways and shapes as include whole herbs, syrups, ointments, sauces, essential oils massages, capsules, and tablets containing crushed form or powdered from raw herb or its dried extract. The various phytochemicals extraction varies considerably based on the used extraction solvent, temperature and time. For example, alcoholic extracts (tinctures), vinegar (acetic acid extracts), hot water extract (Ticino), long-term boiled extract, usually the roots or bark (boiled), and cold infusion of plants (macerates) were used (26, 27).

Despite all this, herbal medicine proved the ability to treat many diseases or reduce their symptoms by having an important pharmaceutical value. This is achieved through many effective agents such as bleomycin, dactinomycin, doxorubicin, irinotecan, topotecan, vinblastine, dihydro artemisinin, etoposide, and paclitaxel (anticancer), amodiaquine artemisinin, artemether, and arteether (antimalarial), Mefloquine chloroquine and metformin (26,27).

Hence since there is no absolute cure for psoriasis researchers looked after treatment and natural remedies which could help to manage their symptoms. Many herbs have the potential to reduce inflammation or slow down skin cell growth, which can aid with psoriasis symptoms reduction. One of those plants is *Mahonia aquifolium* (Oregon grape) due to its history in treating inflammatory situations, as psoriasis. Its effectiveness is referred to the presence of anti-inflammatory berberine substance in addition to the anti-proliferative factors (22,23). Furthermore, another medicinal plant used for the treatment of psoriasis is *Indigo naturalis*. It is a traditional Chinese herbal medicine that people use for treating skin conditions. This has been further investigated as 24 people with moderate psoriasis were treated by *Indigo naturalis* for 8 weeks who had then significantly fewer symptoms. In addition, lower levels of interleukin-17 (IL-17) was observed, which was a marker of inflammation decrease (24). Moreover, *Aloe vera* and curcumin were known with their effectiveness in reducing the severity of psoriasis. The previous mentioned herbal medicinal plants examples are only an example, and not exhaustion, as

there are many and many plants are used in the treatment of psoriasis. One of such is *Ficus sycomorus* as its ethnic medical uses include the white latex usefulness to treat warts and inflammation as well as treating the cancer in Nigeria (28,29,30). Moreover, as the synthetic drugs used to treat psoriasis have side-effects, there is a need for novel and efficient anti-psoriatic herbal-based drug candidates with reduced side effects. From this point of view, this research popped out to examine FS bioactivity against psoriasis in Palestine.

1.5 *Ficus sycomorus* (FS)

Ficus sycomorus (FS) is vascular flowering fruit plant belongs to the family Moraceae which has 40 genera and 1400 species, (Fig. 1.5.1). The word Ficus is derived from the Latin language which means fig, which originates from the Persian ‘fica’. The species, syco-morus, derived from the Greek name sykamorea (i.e., sycamore), also known as fig-mulberry. It grows to a length of 20 -46 m, the leaves are heart-like with dark green colour, the colour of the bark is ranges between yellow, orange, and green. The diameter of its fruit ranges between 2.5 and 5 cm. Although it is African plant but this tree is a widespread through the world as it is very common in many countries like Egypt, Sudan, Oman, Nigeria, Cameroon, Congo and Palestine (28,29).

FS has several medicinal activities such as, antimicrobial, antioxidant, hypotensive, neuroprotective, hepatoprotective, anti-diarrheal and anticancer (28, 29, 30). Also, ethnic medical uses of FS (folkloric claim)

include: the white latex is useful to treat burns, ulcers, warts and inflammation, latex also used to avoid infections and promote healing amongst the Africans, boiled bark of FS used in the treatment of scrofula, sore throat, respiratory and chest diseases, the leaves play a role as an antidote for snakebite. The roots are used as a laxative, eating fruits activates the lactation, eating the seed prevents microbial infection (28, 29, 31).

In vitro investigations on psoriasis by isolating the cells from the patients themselves involves many conflicts which are related to the donor variations, relatively short lifespan of culture, and methodology differences. Since cultured human keratinocyte cells are commonly used in the studies of immune and inflammatory responses. Therefore, to standardize laboratory studies on keratinocytes, the use of HaCaT cells was suggested. They are a long-term, human-induced and automatically identifiable keratinocyte cell line leading to consider them as a model for follow-up to the release of inflammatory mediators and repair. So, the HaCaT cells are a beneficial model to study anti-inflammatory effectivity and therapies on skin diseases specially psoriasis (32,33).

1.6 Aim of the study

The aim of this study is to investigate *Ficus sycomorus* fruit and leaves water-ethanol extracts as a potential psoriasis therapy. This isevaluated via the cytotoxic and cytostatic effects assessments to determine non-toxic concentrations and mood of the plant extracts bioactivity in both

monoculture and co-culture systems of cells from the human skin keratinocyte cell line (HaCaT) and cells from the monocyte cell line (THP-1) and their co-cultures. This is associated with anti-inflammatory properties examination of the plant extracts under study through measurements of pro-inflammatory NO production.

Chapter Two
Material and Methods

2.1 Reagents and kits

Cell culture medium Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) and their supplements purchased from (Biological industries, Israel). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT)purchased from (Sigma-Aldrich,USA).No kit (Canyan Chemicals, Ann Arbor, MI, USA).

2.2 Plants material

FS plant specimens (Fig.2.1) were collected from West Bank/ Palestine during periods in September –October, 2019. The samples of leaves and fruits of FS were air dried in the shade for 7-10 days. The dried plants parts were then ground to a powder and stored in cloth bags at 4 ˚C till use.

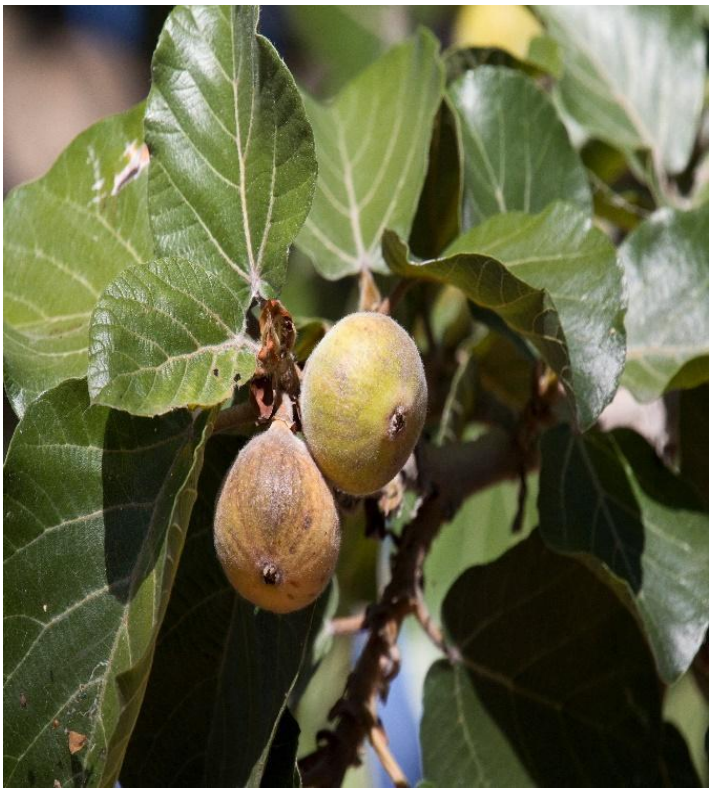

Figure 2.1. Plant parts: *Ficus sycomorus* fruits and leaves.

2.3 Plant extracts

Sixteen grams of air-dried powder of leaves and fruit of FSwere dissolved in 100 ml of 50% water-ethanol solution with continuous stirring for 20 min at 70 ˚C. The extracts were allowed to cool at room temperature then centrifuged at 4000 rpm for 15 min. The obtained supernatants were dried and subsequent different serial dilutions were prepared 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 µg/ml in freshly prepared RPMI media. Then all were sterilized by passing them through a 0.2 mm microfilter.

2.4 Cell Lines

The human monocytic cell line THP-1 which is derived from an acute monocytic leukemia patient were obtained from Arab American University lab. The human keratinocytes cell line HaCaT (ATCC PCS 202-010) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) (Fig.2.2, A and B, respectively). Differentiated THP-1 cells to macrophages were obtained in the lab (Fig.2.2, C).

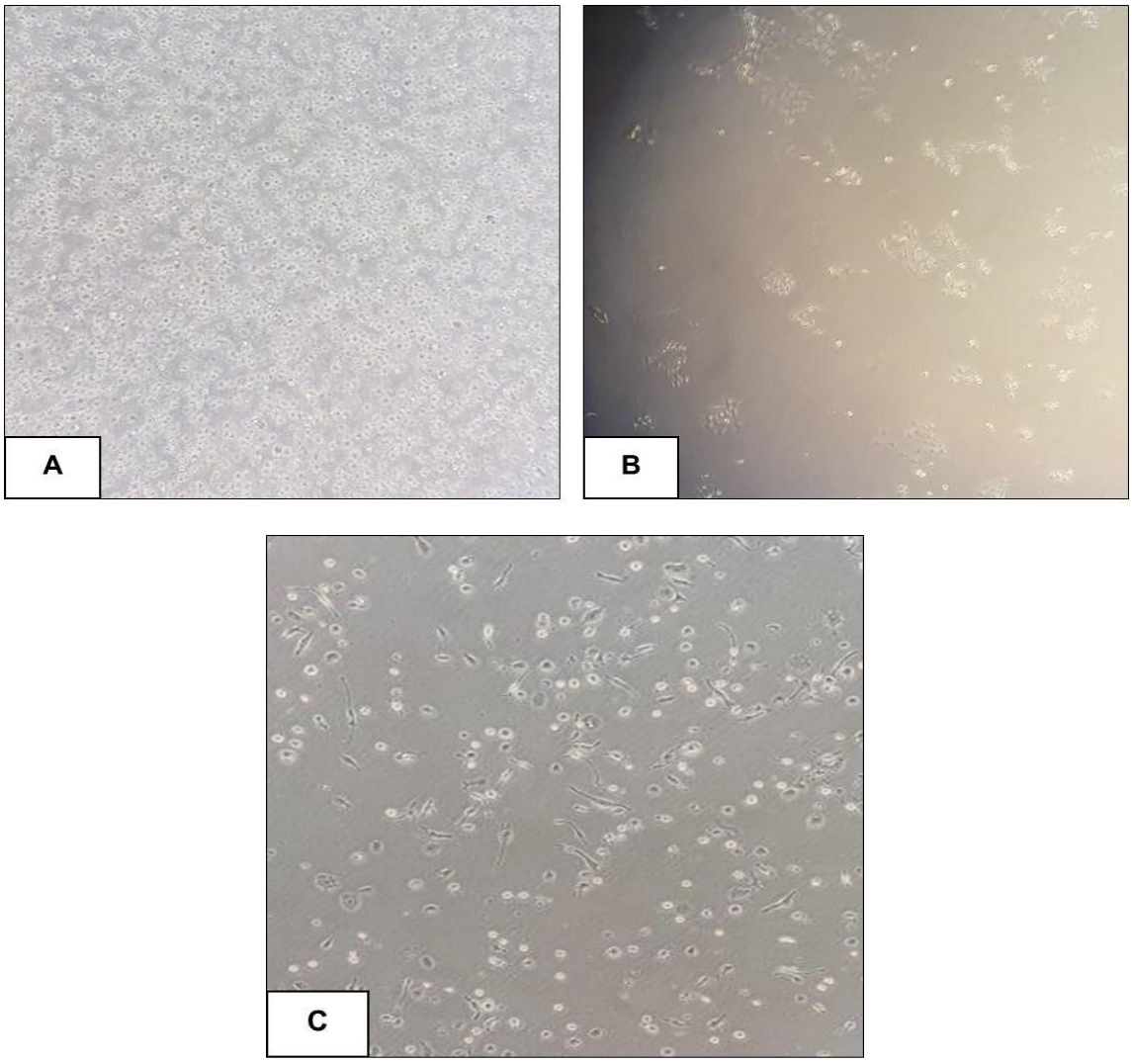

Figure 2.2: Cell lines (A) THP-1 cells, (B) HaCaT cells and (C) Macrophages derived from THP-1 cells.

2.5 Cell cultures

THP-1 cells and HaCaT cells were grown in RPMI-1640 and DMEM-5671 medium, respectively with a high glucose content (4.5 g/L), supplemented with 10% vol/vol inactivated fetal calf serum (FCS), 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin (Sigma-Aldrich). All cell lines were grown in humidified atmosphere of 5% CO_2 at 37°C. The cultured cells were observed routinely under inverted microscope for checking their attachment to the media substratum in the culture flask ensuring their confluence and if any contamination has occurred. Every three days fresh culture medium was replaced until cell confluence was reached to 90%. THP-1 cells were then differentiated to macrophages by the addition of PMA (100 ng/ml) and vitamin D3 (0.1μM).

2.6 Determination of cell viability

2.6.1 MTT Assay

This is a colometric and viability assay which uses reduction (conversion) of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) by NAD(P)H-dependent oxido-reductase enzymes in the viable cells which reduce the MTT reagent (MTT solvent) to a deep purple coloured insoluble crystalline formazan. Formazan crystals were then dissolved using a solubilizing solution (MTT solution) and absorbance was measured at 500-600nanometers using a plate-reader (ELISA reader). The

higher recorded absorbance is the higher the formazan concentration which indicates the higher cell viability as indicated by higher metabolic activity.

2.6.2 MTT procedure

Cells were detached and counted manually using haemocytometer and tryban blue stain. Cells were then inoculated in 96 – well microtiter plate at a density of 20,000 cells/100 µL total volume/well (cytotoxic test). While in (cytostatic test) the density was 5,000 cells/100 µL total volume/well. Cells cultured in RPMI media only were used as normal control. Each treatment was carried out in duplicates. Then cultured plates were incubated in CO_2 incubator of 5% CO_2 at 37 ˚Cfor 24 hrs in cytotoxic test and for 72 hrs in cytostatic test. After the required incubation period (24 or 72 hrs) the media was removed from each well with subsequent washing with PBS. Then the cultured cells under study were re-cultured in 100 µL serum free RPMI media to which 100 µL MTT solution (0.5 mg/ml) was added to each well and incubated for 4 hours at 37 ˚C. After that, media was removed, washed and cells were incubated for 20 min. with 100 µL of acidic isopropanol (0.08N HCL) to dissolve the formazan crystals. The absorbance of MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was calculated as percentage of absorbance of treated cells to absorbance of normal control (untreated cells) (33,34).

2.6.3 Cytotoxicity Assay for Mono-culture and Co-culture

Cells at 70-90% confluence were detached from culture flask by removing the culture medium then adding trypsin-EDTA. A suspension of 100 µl (2.0×10^4 cells/well) of viable cells (THP-1and HaCaT) were seeded in a 96 -well plate and incubated for 24 hrs at 37°C for mono culture. While in co-culture (1.0×10^4 THP1 cells/well) with PMA (100 ng/ml) and vitamin D3 (0.1µM) seeded in in a 96 -well plate incubated for 24 hrs at 37°C. after then the media were removed with subsequent seeding with HaCaT cells (1.0×10^4 HaCaT cells/well) in the 96 -well plate and incubated for 24 hrs at 37°C. After that the media were removed and the attached cells were treated with100µl stock solution of fruit and leaves serially diluted up to the following concentrations 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 µg/ml. All then Then cultured plates were incubated at CO_2 incubator at 5% CO_2 and 37 ˚C for 24 hrs to perform MTT assay.

2.6.4 Cytostatic Assay for Mono-culture and Co-culture

In order to determine the cytostatic effect of the fruit and leaves different extracts concentration under study of FS a smaller number of cell were seeded in each well (0.5×104 cells/well) for mono culture and (0.25×104 cells/well) for co-culture. Then cultured plates were incubated at CO2 incubator at 5% CO2 and 37 ˚C for 24 hrs. Then the medium was removed and cells were treated with 100 µl stock solution of fruit and leaves serially diluted up to the following concentrations 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 µg/ml

Then cultured plates were incubated at CO2 incubator at 5% CO2 and 37 ˚C for 72 hrs to perform MTT assay.

2.7 Determination of NO production

THP-1 cells were grown in freshly prepared RPMI-1640 medium, (0.5×10^6 cells /well) for mono culture which were seeded in 6-well plates. THP-1 cells were differentiated into macrophages with PMA (100ng/ml) and vitamin D3 (0.1 µM) incubated for 24 hrs at 5% CO_2 and 37 ˚C incubator. Then treated with extract concentration 250 and 125 µg/ml of fruit and leaves and with lipopolysaccharide (5 µgLPS/ml) all prepared in a fresh serum-free medium. Then incubated for 72 hrs at 5% CO_2 and 37 ˚C incubator to determination NO. After that, 50 µl of the suspension were placed in 96 -well plate with an equal volume of Griess reagent (100µl A and 100µl B) and incubated at room temperature for 15 minutes at dark. NO production was detected by ELISA reader absorbance at 550 nm.

2.8 phytochemical tests

1. Test for alkaloids: 0.5 g of extract was mixed in 8 ml of 1% HCl, warmed and filtered. 2 ml of the filtrate were treated separately with reagent (Maeyer's), after which it was observed whether the alkaloids were present or absent in the turbidity or precipitate formation.

2. Test for Glycosides:5ml each of various extract were hydrolysed separately with 5 ml each of conc. HCl and boiled for few hours on a water bath and hydrolysates were subjected to the following test: A small amount of

alcoholic extract of samples was dissolved in 1ml water and then aqueous 10% sodium hydroxide was added. Formation of a yellow colour indicated the presence of glycosides.

3. Test for carbohydrates:2ml conc. HCl with little amount of phloroglucinol and equal amount of extract solution then heated over flame. Formation of a red colour indicated the presence carbohydrates.

4. Test for Proteins and Amino acids: 1 ml plant extract with few drops of conc. Nitric acid. Formation of a yellow colour indicated the presenceProteins and Amino acids.

5. Test for Reducing sugars: 0.5ml filtrateb + 0.5mL Benedict's reagent + Boiled for 2 min Formation of a green colour indicated the presence Reducing sugars.

6. Phenols (Ferric chloride test): To 1ml of extract 2ml of distilled water were added followed by few drops of 10% ferric chloride (FeC13). Appearance of blue or green colour indicates presence of phenols.

7. Test for Flavonoids: 3 ml of the filtrate was mixed with 4 ml of 1% aluminium chloride in methanol in a test tube and the colour was observed. Formation of yellow colour indicated the presence of flavanols, flavones and chalcones.

8. Test for steroids: 0.5 g of the various solvent extract plant was mixed with 2 ml of acetic anhydride followed by 2 ml of sulphuric acid. The colour

changedfrom violet to blue or green in some samples indicated the presence of steroids.

9. Test for Saponins: 3 ml of the filtrate was mixed with 5 ml of distilled water and was shaken vigorously to the formation of stable persistent froth. The frothingwas mixed with 3 dropsof olive oil and shaken vigorously for the formation of emulsion thus a characteristic of saponins.

10. Test for Terpenoids (Salkowski test): 5 ml of various solvent extract was mixed in 2 ml of chloroform followed by the careful addition of 3 ml concentrated (H_2SO_4). A layer of the reddish-brown colouration was formed at the interface thus indicating a positive result for the presence of terpenoids.

11. Test for Tannins :0.25 g of various solvent extract was dissolved in 10 ml distilled water and filtered. 1% aqueous Iron chloride ($FeCl_3$**)**solution was added to the filtrate. The appearance of intense green, purple, blue or black colour indicated the presence of tannins in the test samples.

2.9 Statistical Analysis

Error limits mentioned and error bars plotted represent simple standard deviations of the mean. Usually, numerical results are given only to accuracy succentor specify the least significant digit. When comparing the different samples, results were considered to be statistically different when $P<0.05$ (Student’s t-test for unpaired samples).

Chapter Three
Results

The anti-proliferative and anti-inflammatory effects of FSleaves and fruits was examined via the evaluation of their cytotoxic and cytostatic effects using monocultures and co-cultures of cells from the human keratinocytes cell line (HaCaT) and cells from the human monocyte cell line (THP-1). In this co-culture system both cell types have direct cell-to-cell contacts and are maintained in more “in vivo like” culture conditions than in the monoculture system.

3.1 Cytotoxic Effect of FS in Monocultures and Co- cultures of HaCaT and THP-1 Cell Lines

MTT assay was carried out in order to estimate the cytotoxic effect of the FS fruit and leaf water-ethanol extracts with different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 µg/ml) on the immortalized human keratinocytes cell line (HaCaT) and monocyte cell line (THP-1) monocultures as well as their co-cultures. Obtained results are summarized in Table 3.1. The shown data revealed that there is no significant difference between all examined concentrations of the FS plant parts under the study on both investigated monocultures and co-culture. Based on the conducted statistical analysis (> 0.05) (Figures 3.1and 3.2). This data proves the safety of using FS plant fruit and leaf extracts concentrations up to 1000mg/ml.

Table 3.1: Cell viability assay % absorbance of water-ethanol extracts in THP-1 and HaCaT cell lines monocultures and co-cultures under different concentrations of FS fruit and leaf 24 hrs (cytotoxicity after).

Ficus sycomorus Plant Part	Cell line	Studied Concentration (µg/ml)							
		control	15.9	31.8	63.6	125	250	500	1000
Fruit	HaCaT	100	99.3	103.5	103.6	104.3	104.9	104.9	108.5
	THP-1	100	101.9	99.7	103.7	100.2	102.1	104.1	102.1
	Coculture	100	98.7	97.4	95.5	93.2	101.4	104	103.3
Leaf	HaCaT	100	103.2	104.3	103.1	102.6	98.5	99.1	96.1
	THP-1	100	99.5	98.2	96.6	98.2	99.6	99	97.1
	Coculture	100	100	97.3	96.7	97.1	95.8	98.3	98.2

* Cell viability was calculated as percentage of absorbance of treated cells to absorbance of normal control (untreated cells)**.**

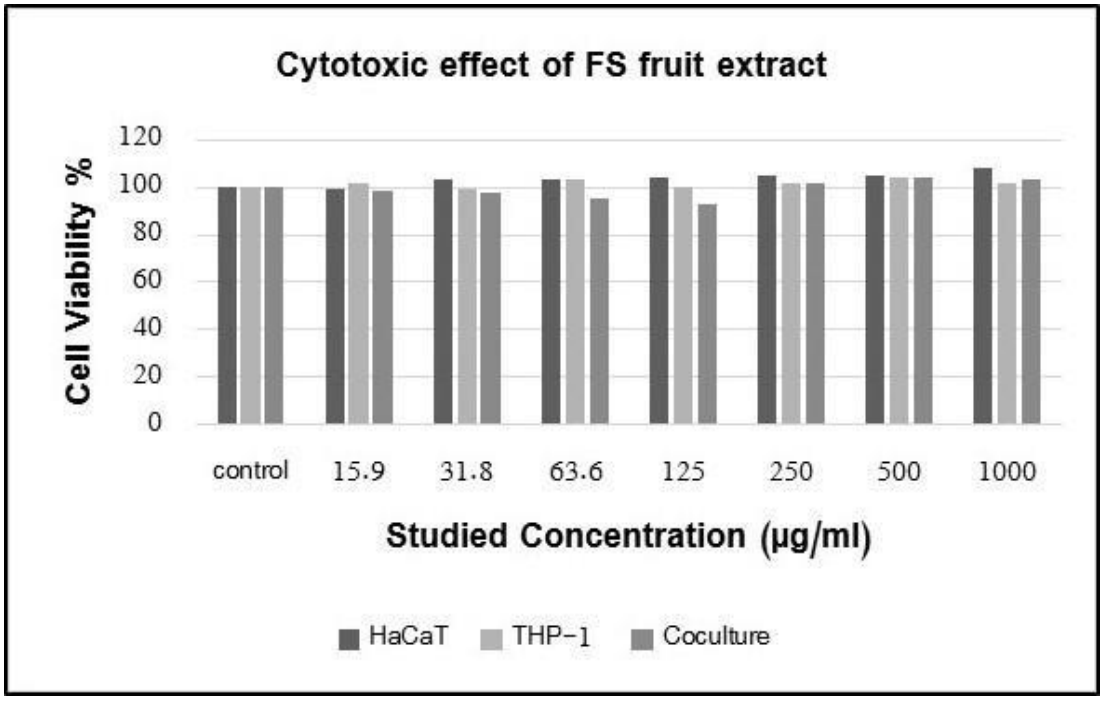

Figure 3.1: MTT in THP-1-derived macrophages, HaCaT cells and their co-cultures after 24h. Treatments were carried out with increased concentrations of extract from FS fruit. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

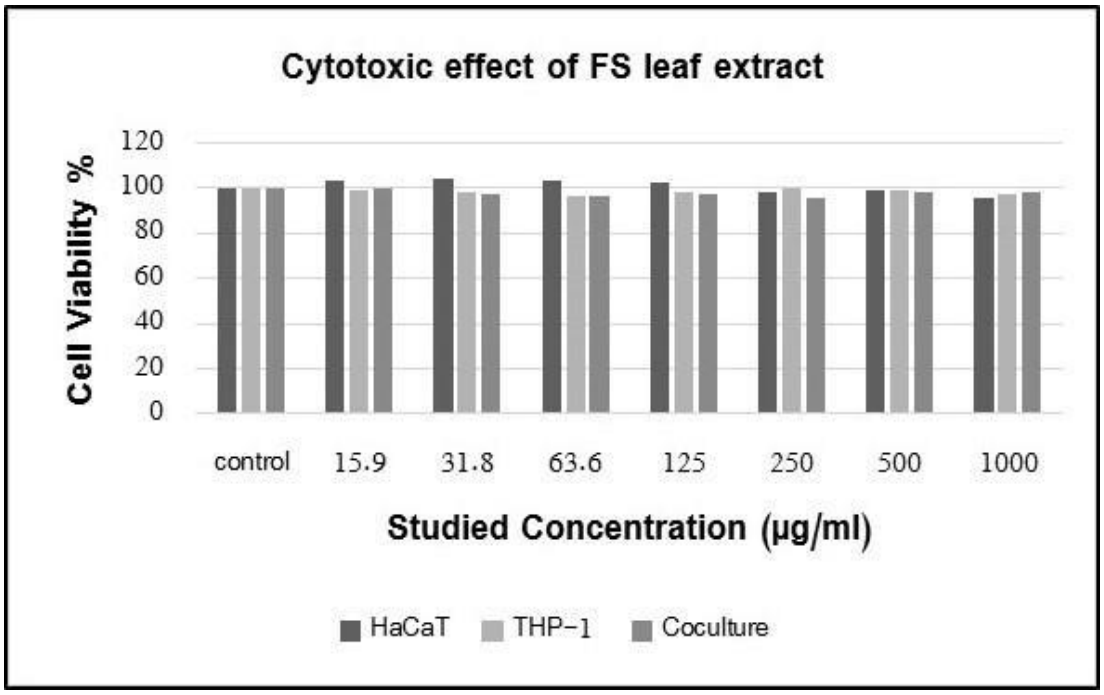

Figure 3.2: MTT in THP-1-derived macrophages, HaCaT cells and their co-cultures after 24h. Treatments were carried out with increased concentrations of extract from FS leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

However, no significant cytotoxic effect variations were observed between the FS fruit and leaf extracts at all concentrations under study on HaCaT cell line monoculture (Fig. 3.3), THP-1 cell line monoculture (Fig. 3.4) and their co-cultures (Fig. 3.5).

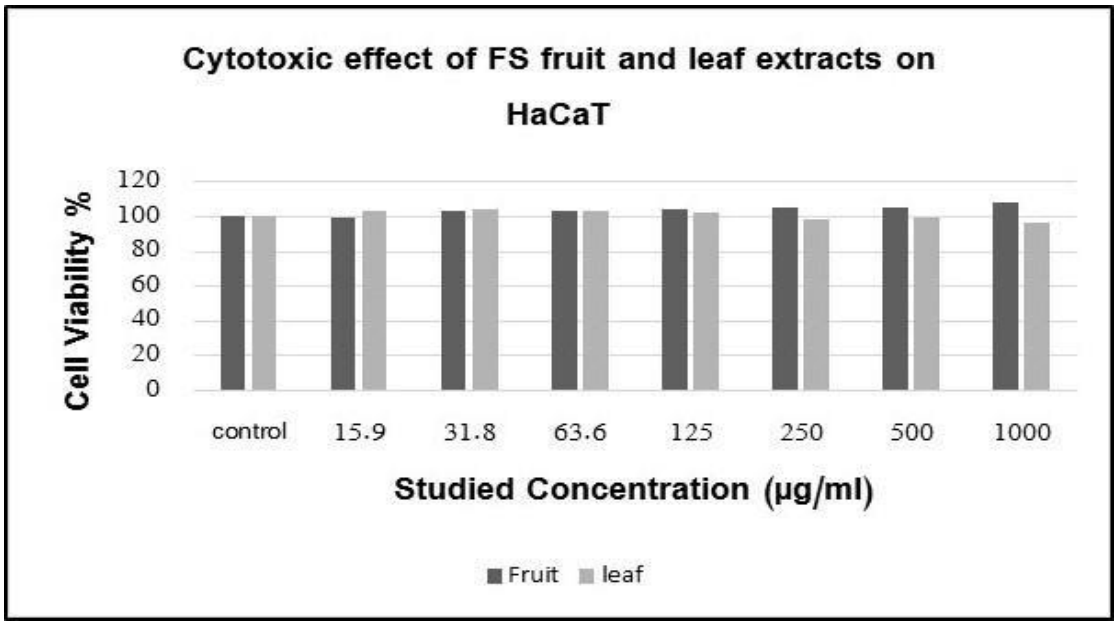

Figure 3.3: MTT inHaCaT cells after 24h. Treatments were carried out with increased concentrations of extract from FS fruit and leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

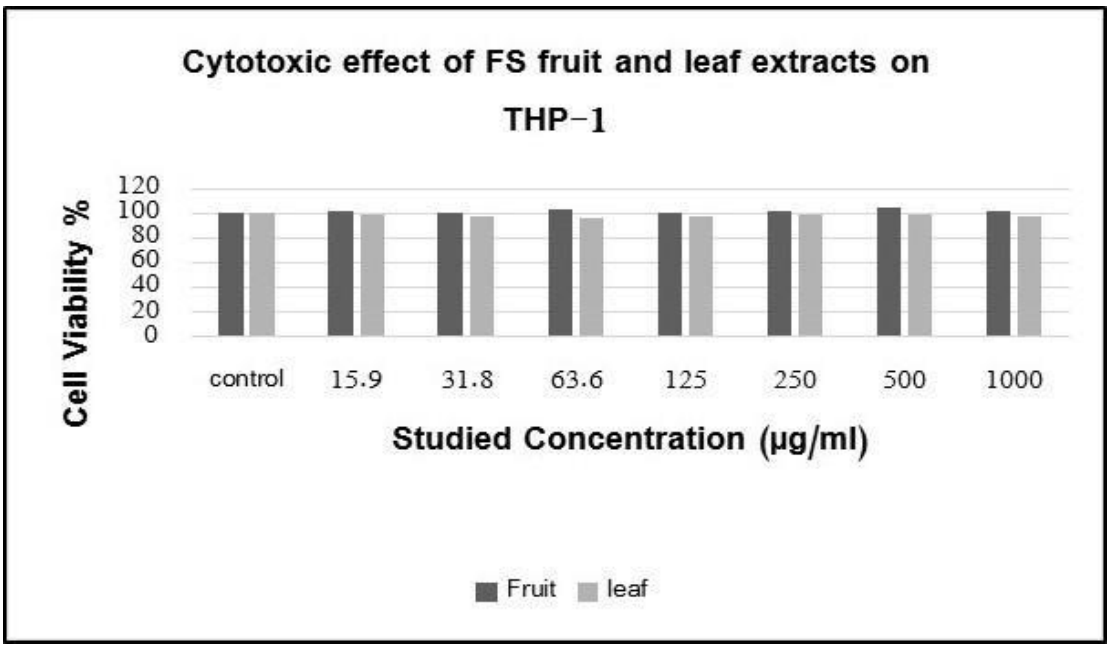

Figure 3.4: MTT in THP-1-derived macrophages after 24h. Treatments were carried out with increased concentrations of extract from FS fruit. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

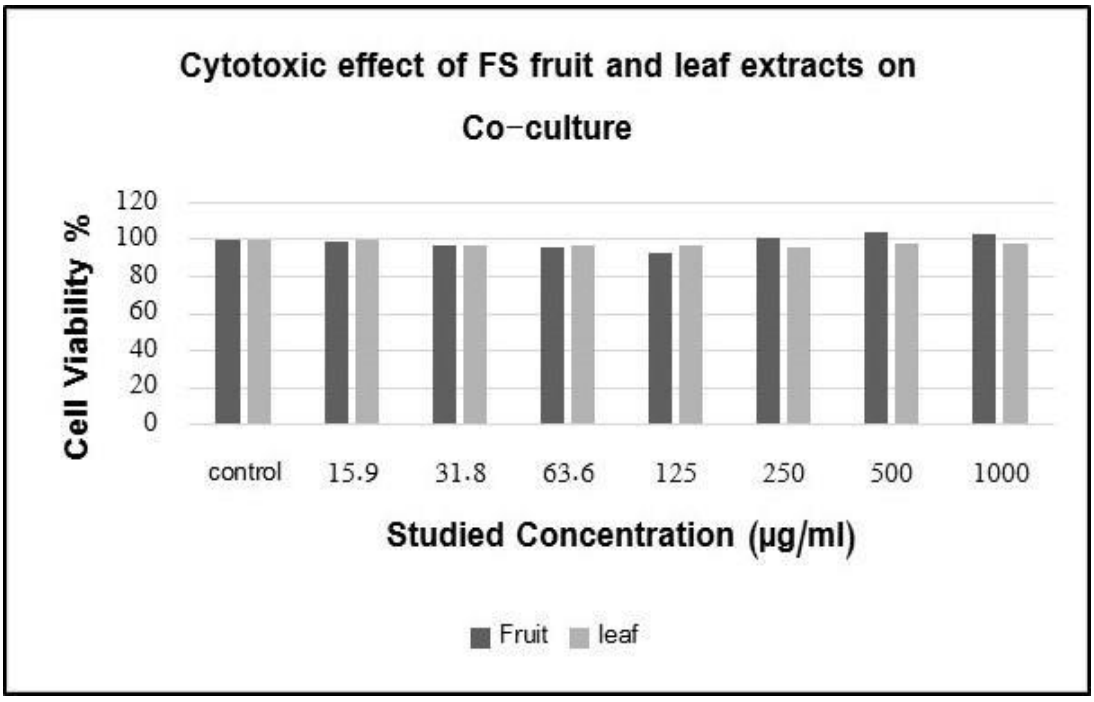

Figure 3.5: MTT in THP-1-derived macrophages with HaCaT cells (co-cultures) after 24h. Treatments were carried out with increased concentrations of extract from FS fruit and leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

3.2 Cytostatic Effect of FSin Monocultures and Co-cultures of HaCaT and THP-1 Cell Lines

Moreover, MTT cytostatic assay was carried out in order to determine if the FSfruit and leaf water-ethanol extracts have cytostatic (anti-proliferative) on the immortalized human keratinocytes cell line (HaCaT) and monocyte cell line (THP-1) monocultures and their co-cultures. Therefore, the examined cell lines were subjected to different serial concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 µg/ml) for 72 hrs. The observed results revealed significant cytostatic effect of FSfruit extract on HaCaT cells ($p < 0.05$) at all studied concentrations with more

pronounced impact at 1000 and 500 µg/ml. Results are represented in Table 3.2.

Table 3.2: Cell viability % of THP-1 and HaCaT cell line monocultures and co-cultures under different studied concentrations of *Ficus sycomorus* fruit and leaf water-ethanol extracts after 72 hrs (cytostatic).

Ficus sycomorus Plant Part	Cell line	Studied Concentration (µg/ml)							
		control	15.9	31.8	63.6	125	250	500	1000
Fruit	HaCaT	100	88.3	69.6	73.7	71.1	71	59.2	57
	THP-1	100	100.3	103.8	101.8	100.3	97.2	95.6	92.8
	Coculture	100	84.9	84.3	83.7	83.1	64.2	63.1	64.2
Leaf	HaCaT	100	93.1	80. 6	74.2	69.7	69.3	66.9	66.6
	THP-1	100	100.6	100.9	100.8	103.8	104.2	104.6	105.9
	Coculture	100	99	89.8	85.4	85	82.9	81.3	74.5

* Cell viability was calculated as percentage of absorbance of treated cells to absorbance of normal control (untreated cells).

The studied plant extracts cytostatic efficacy was higher on the HaCaT monoculture system than on THP-1 in concentration dependent manner with cell viability of 69.6% at concentration of 31.8 µg/ml reaching to 57% at concentration of 1000 µg/ml. While their co-cultures showed significant decrease in cell viability was at 250,500 and 1000 µg/ml with 64.2%, 63.1% and 64.2%, respectively. (Fig. 3.6 and 3.7).

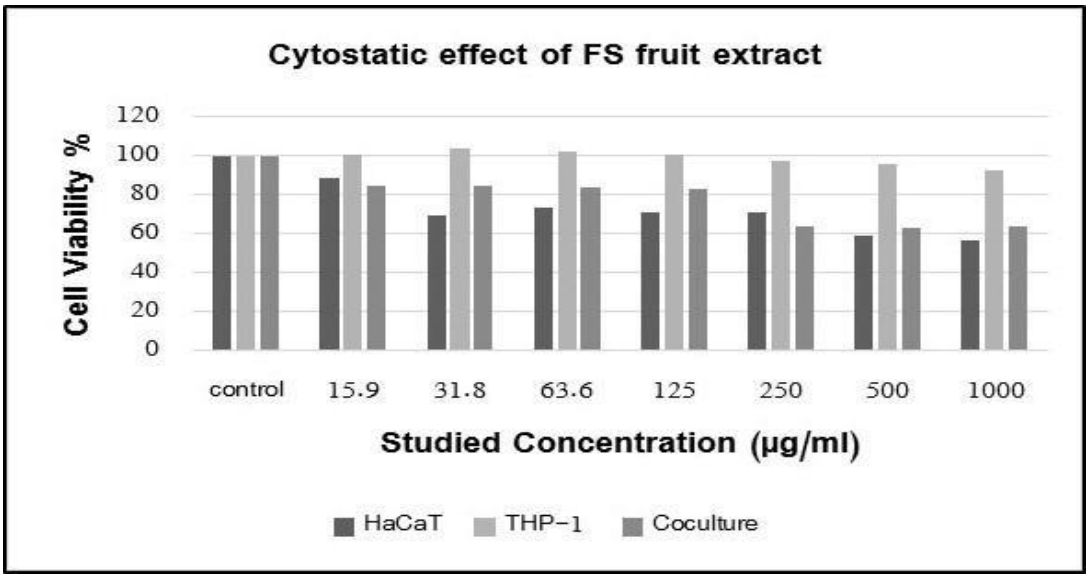

Figure 3.6: MTT in THP-1-derived macrophages, HaCaT cells and their co-cultures after 27h. Treatments were carried out with increased concentrations of extract from FS fruit. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

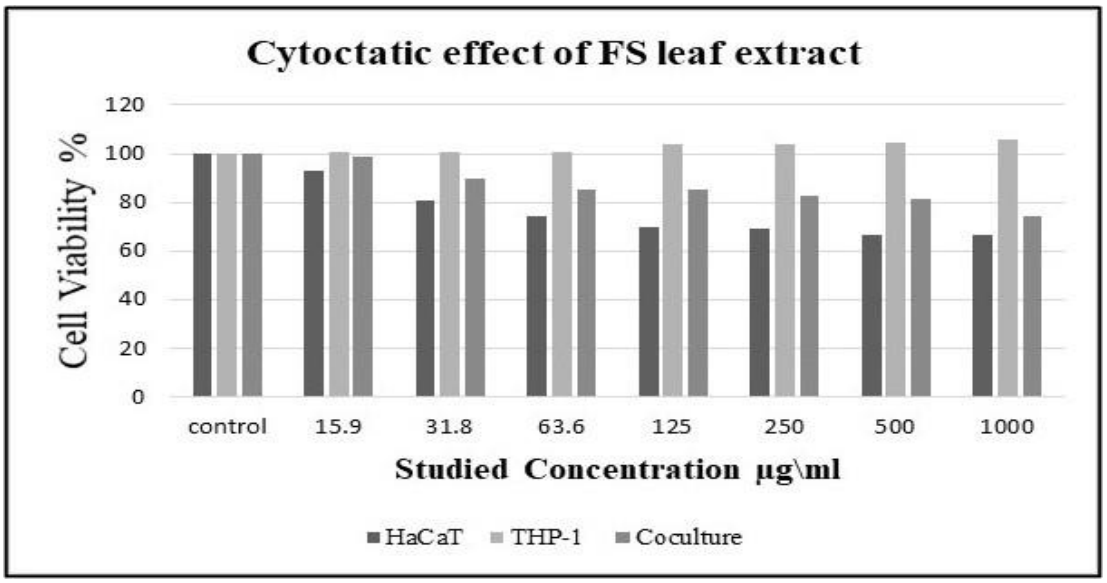

Figure 3.7: MTT in THP-1-derived macrophages, HaCaT cells and their co-cultures after 27h. Treatments were carried out with increased concentrations of extract from FS leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

The concentration dependent manner pronounced cytostatic effect on both HaCaT monoculture cell line and the co-culture cell lines system was more recognised by the fruit extract rather than the leaf one (Fig. 3.8 and 3.9, respectively).

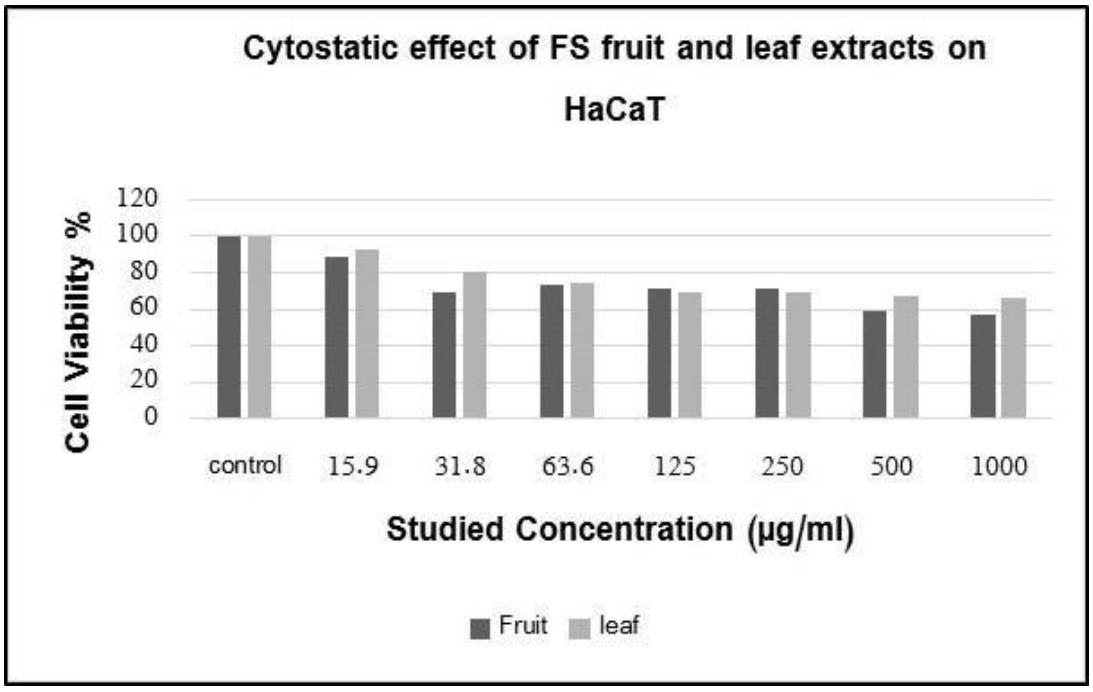

Figure 3.8: MTT in HaCaT cells after 27h. Treatments were carried out with increased concentrations of extract from FS fruit and leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

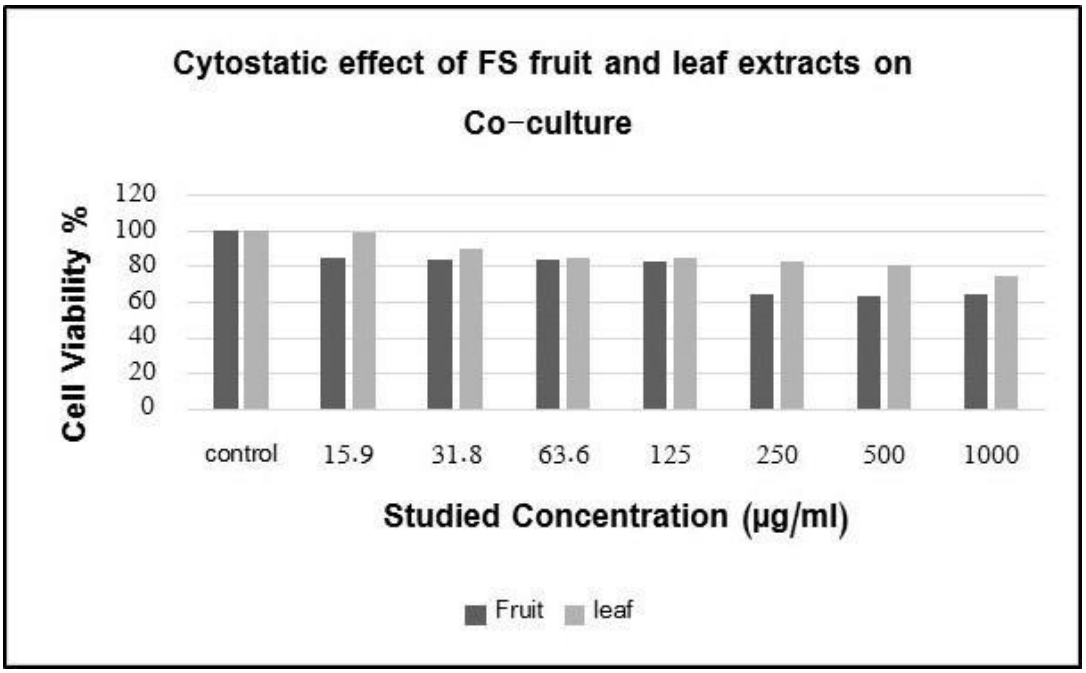

Figure 3.9: MTT in THP-1-derived macrophages with HaCaT cells (co-cultures) after 27h. Treatments were carried out with increased concentrations of extract from FS fruit and leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

In spite that the cytostatic effect of both studied plant extracts different concentrations showed no significant effect on THP-1 cell line monoculture, the fruit extract showed more noticeable effect than the leaf one at higher concentrations (250-1000 µg/ml) (Fig. 3.10).

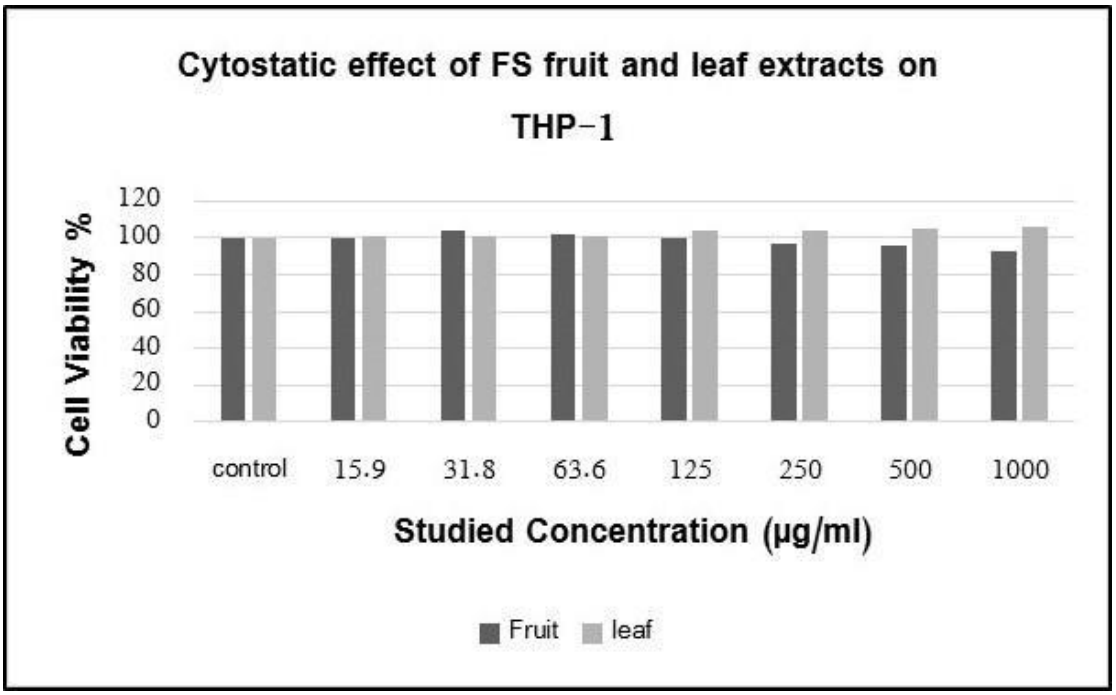

Figure 3.10: MTT in THP-1-derived macrophages after 27h. Treatments were carried out with increased concentrations of extract from FS fruit and leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

3.3 Cytotoxic vs. Cytostatic Effect of FS in Monocultures and Co-cultures of HaCaT and THP-1 Cell Lines

This study results indicated noteworthy cytostatic effect of the FSfruit extracts is more than the cytotoxic one on both HaCaT cell line monoculture and co-culture cell lines system (Fig. 3.11 and 3.12, respectively). This salient bioactivity also observed by the leaf extract on HaCaT cell line monoculture and co-culture cell lines system (Fig. 3.13 and 3.14, respectively).

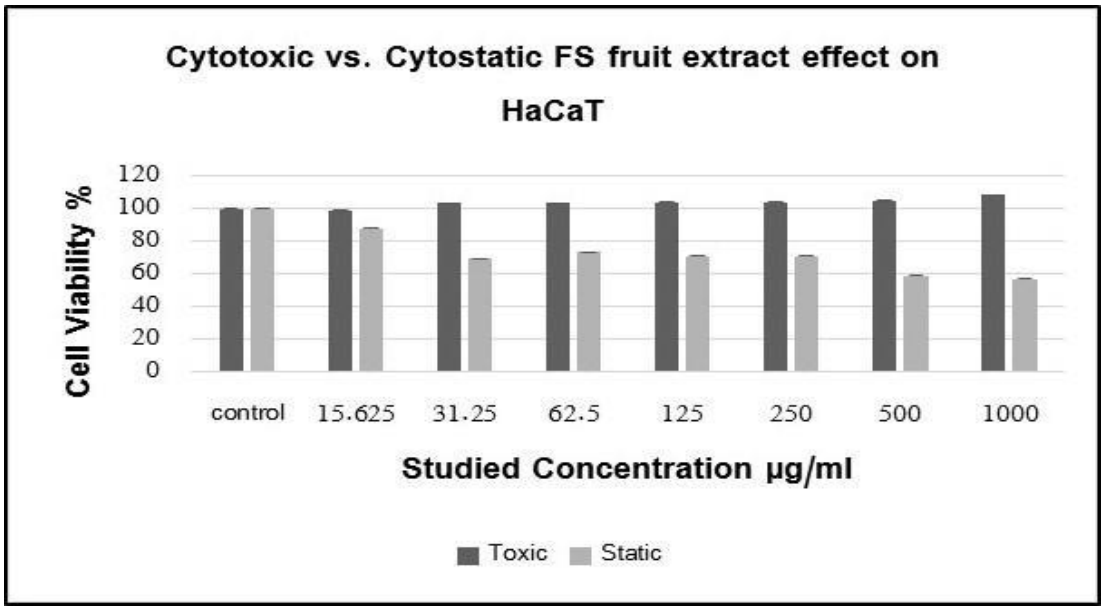

Figure 3.11: MTT in HaCaT cells after 24h(cytotoxic) and 72h (cytostatic). Treatments were carried out with increased concentrations of extract from FS fruit. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

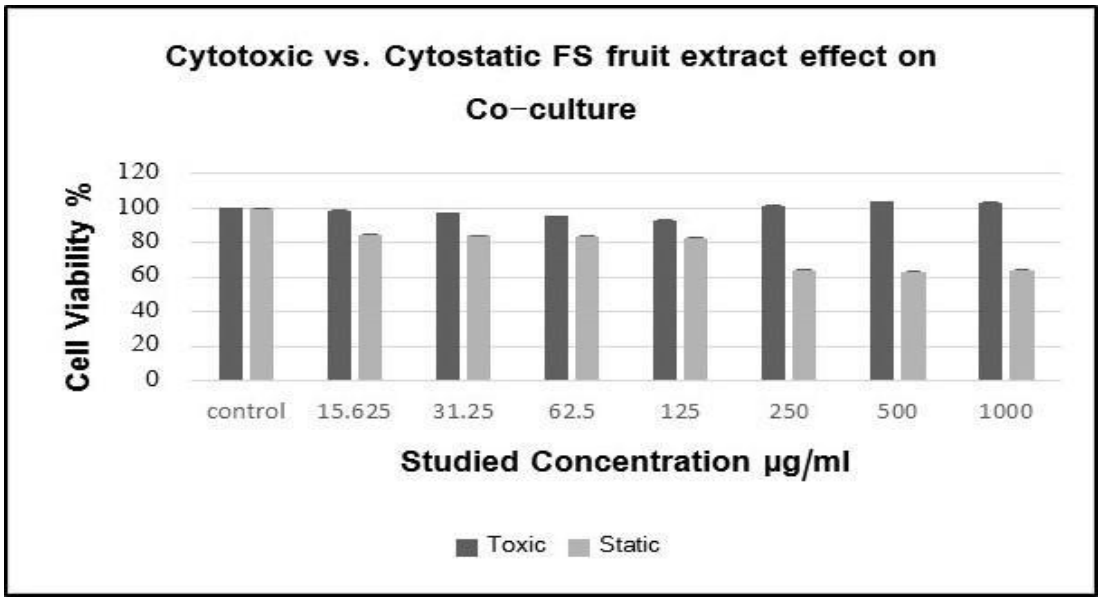

Figure 3.12: MTT in THP-1-derived macrophages with HaCaT cells (co-cultures) after 24h (cytotoxic) and 72h(cytostatic). Treatments were carried out with increased concentrations of extract from FS fruit. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

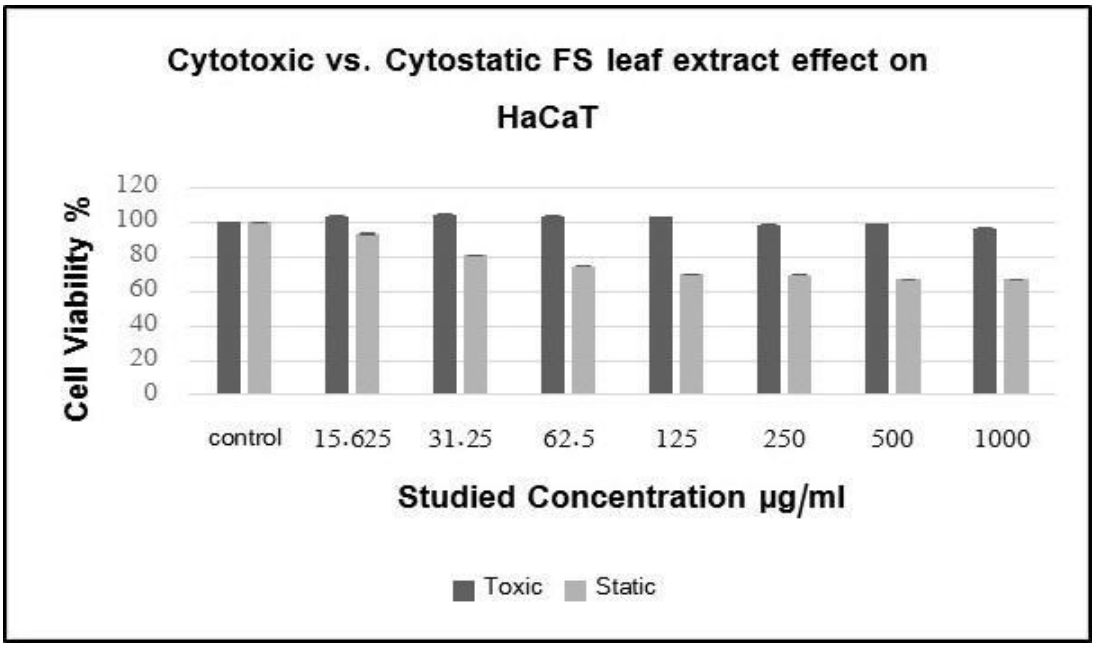

Figure 3.13: HaCaT cells after 24h(cytotoxic) and 72h(cytostatic). Treatments were carried out with increased concentrations of extract from FS leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

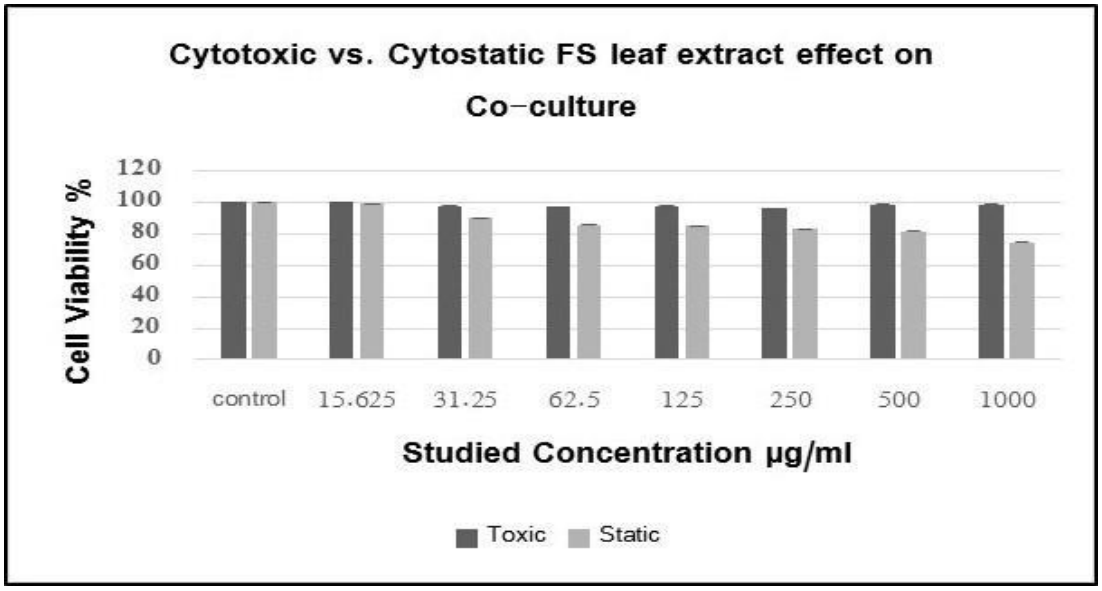

Figure3.14: MTT in THP-1-derived macrophages with HaCaT cells (co-cultures) after 24h(cytotoxic)and 72h(cytostatic). Treatments were carried out with increased concentrations of extract from FS leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

Similarly, is observed in the FS fruit extract effect on THP-1 cell line monoculture in concentration dependent manner (Fig.3.15). On the contrary is recognized by the FS extract effect on THP-1 cell line monoculture as more cytotoxic effect is recognized rather than cytostatic one in concentration dependent manner (Fig.3.16).

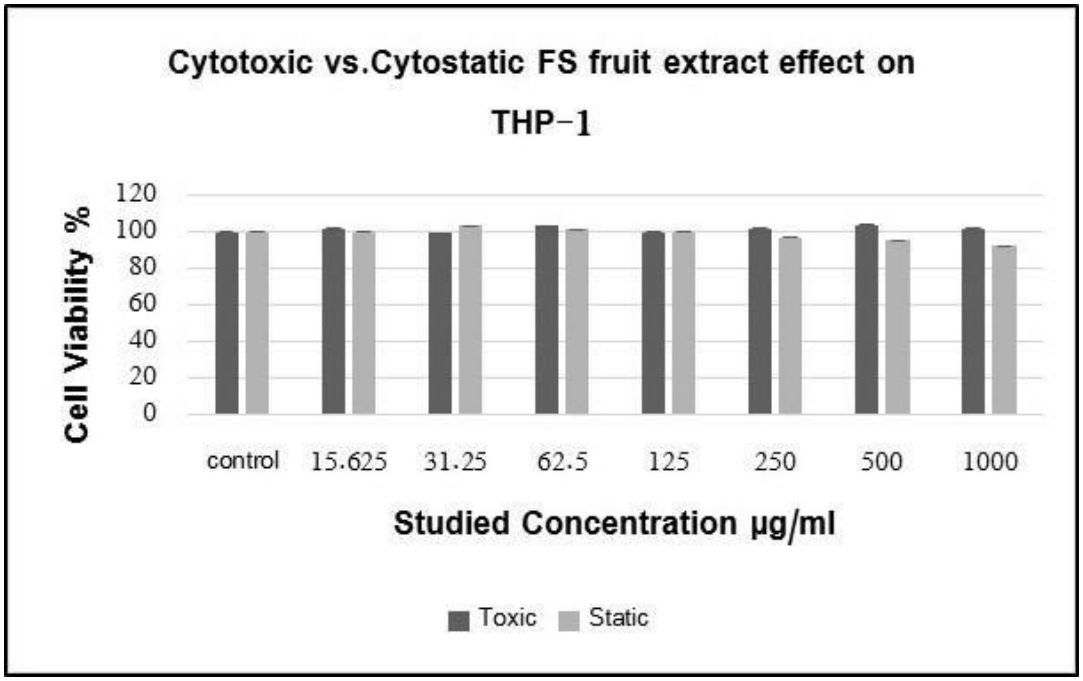

Figure 3.15: MTT in THP-1-derived macrophages after 24h (cytotoxic) and 72h (cytostatic). Treatments were carried out with increased concentrations of extract from FS fruit. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

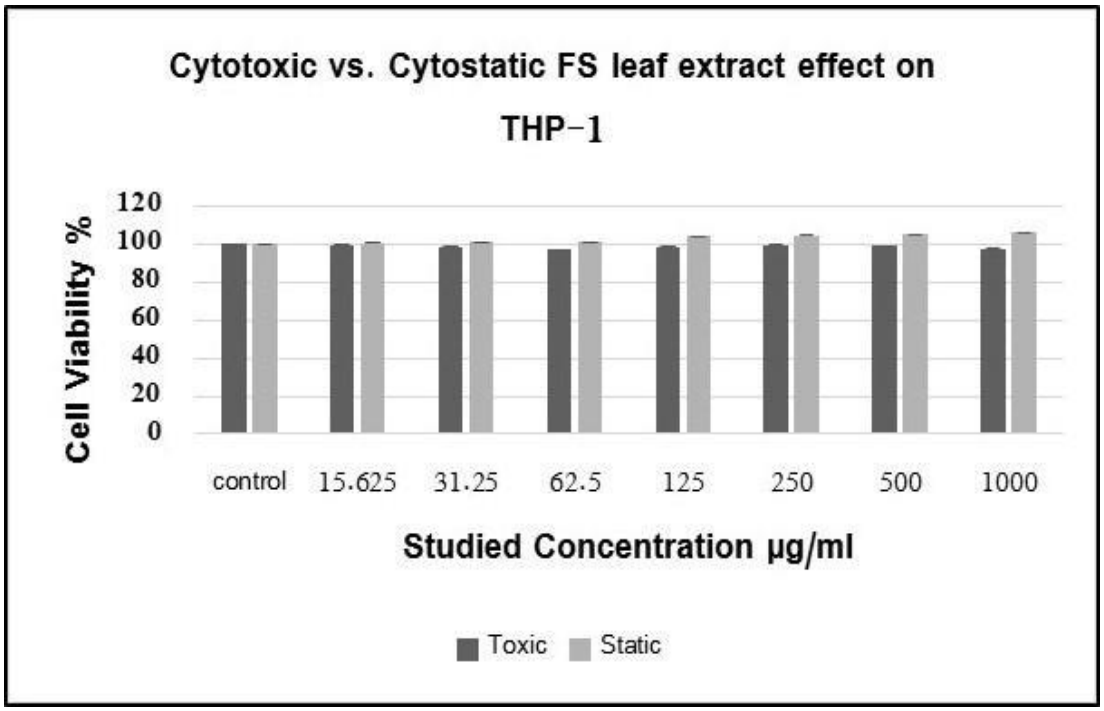

Figure 3.16: MTT in THP-1-derived macrophages after 24h(cytotoxic) and 72h(cytostatic). Treatments were carried out with increased concentrations of extract from FS leaf. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of FS fruit extracts treated cells relative to untreated cells. Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

3.4 Nitric Oxide Determination

Nitric oxide (NO) is a signalling molecule that plays an important role in prolonging inflammation and immune responses. During inflammation, nitrogen oxide is released and maintained at very high levels.NO is generated by endothelial cells, macrophages, neurons and regulation of many physiological processes, including inflammation. Immoderate production and release of NO is associated with several diseases such as diabetes and psoriasis. Which is created in biological tissues by specific nitric oxide synthase (NOSs).

The scavenging ability of FS fruit and leaf extracts for NO production was determined by decrease in the absorbance at 550 nm (Fig. 3.17), which resulted in reduced NO production.

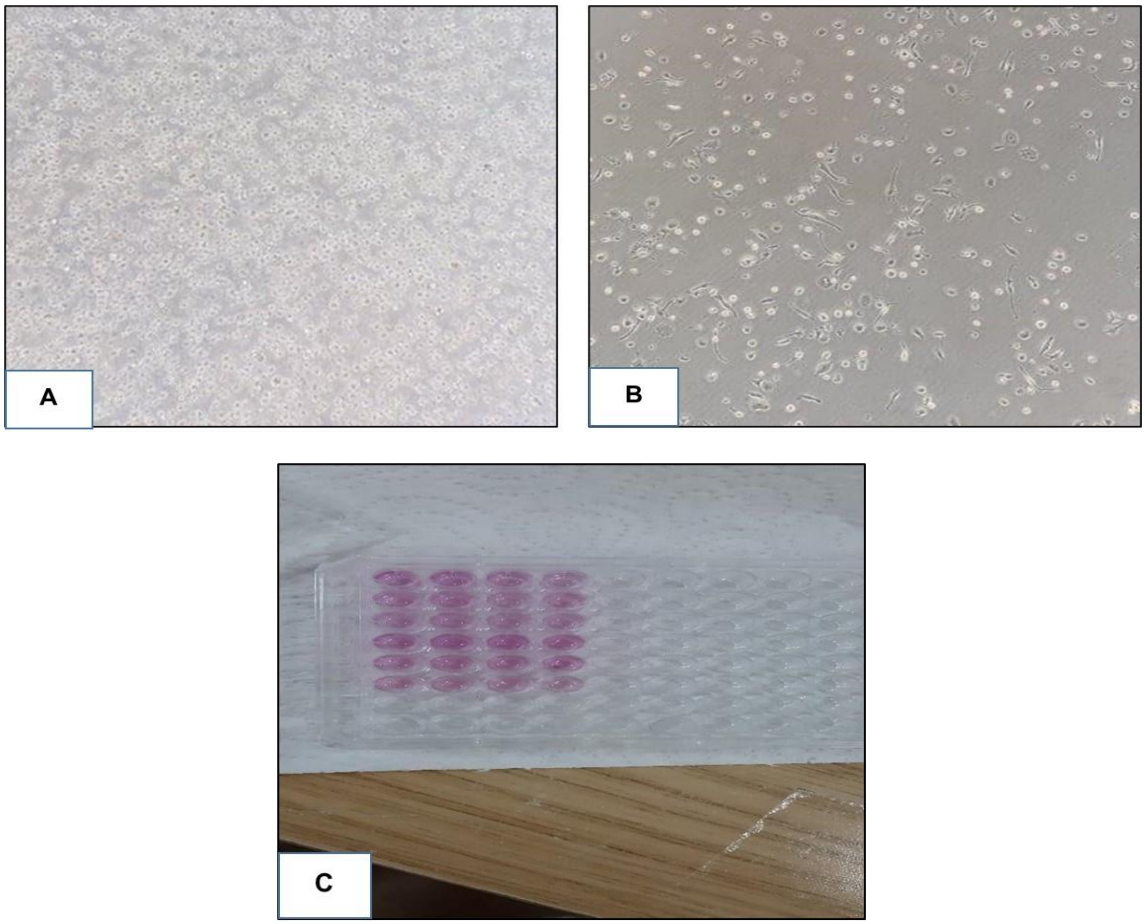

Figure 3.17: (A) THP-1 cells before differentiated to macrophages. (B) THP-1 cells after differentiated to macrophages and without lps. (C) Supernatants of cell after treatment with griess reagent.

In addition, a scavenging power of FS and leaf extracts was documented by the NO production reduction.

The marked NO inhibitory activity on macrophages (differentiated THP-1 cell line) was observed by FS fruits at 250 µg/ml as it caused only 71.46% production in respect to the positive control. So, the fruits extract had resulted in NO inhibitory activities of 28.54% and 16.57% at 250, 125

µg/ml, respectively. While, the FS leaves extract revealed the NO inhibition of 18.64and 16.24% at 250, 125 µg/ml, respectively. Results are represented in Table 3.3. and (Fig. 3.18).

Table 3.3: NO production and NO inhibition in LPS-activated THP-1 derived macrophages by *Ficus sycomorus* fruit and leaves extracts at 125 and 250 µg\ml.

Sample	Treatment	O.D. 550nm	NO Production%	NO Inhibition %
Negative control	-LPS – Ext.	0.57	68.75	31.25
Positive control	+LPS – Ext.	0.82	100	0
Fruit 125 µg/ml	+LPS -125 Ext.	0.69	83.43	16.57
Fruit 250 µg/ml	+LPS -250 Ext.	0.58	71.46	28.54
Leaf 125 µg/ml	+LPS -125 Ext.	0.69	83.76	16.24
Leaf 250 µg/ml	+LPS -250 Ext.	0.67	81.36	18.64

*NO production %= O.D. Experimental/O. D Positive control x 100

*NO Inhibition %=Positive control NO production – Sample NO production *100%

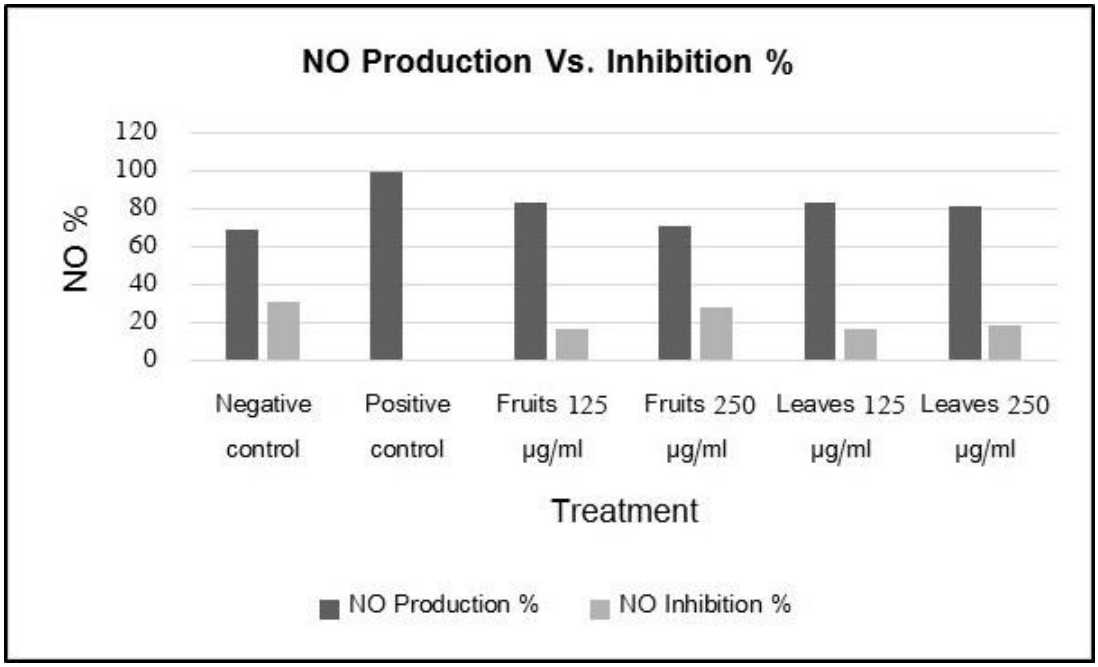

Figure3.18. Effects of *Ficus sycomorus* fruit and leaves extracts NO mediators release by LPS-activated THP-1 cells (monoculture system). Negative control: cells with no additives. Positive control: cells trated with 5 µg/ml LPS only. Treated cells with 125 or 250 µg/ml of fruits and leaves plant extract and5 µg/ml LPS.Values represent means ± SD (*P< 0.05 was considered significant compared to control) of three independent experiments carried out in triplicates.

3.5 Phytochemical evaluation

The performed qualitative phytochemical analysis of FS fruit and leaf extracts under study revealed the presence of several compounds as represented in the following Table 3.4.

Table 3.4: Phytochemical analysis results of FS fruit and leaf extracts under study

Tested Phytochemicals	Fruit	Leaf
Alkaloids	-v	-v
Glycosides	+v	+v
Carbohydrate	+v	-v
Protein & Amino acid	+v	-v
Reducing sugar	-v	+v
Phenol	+v	+v
Flavonoids	+v	+v
Steroids	+v	+v
Saponins	+v	+v
Terpenoids	+v	-v
Tannins	+v	+v

'+v' Indicates Presence, '-v' Indicates Absence

Chapter four
Discussion and Conclusion

Since the concern towards the ethno-medicinal scientific justification, as well as, the over whiling seeking for remedies devoid for unfavourable side effects have prompted the fascination growth for natural products for the treatment of several diseases one of which is psoriasis. The plants natural constituent's phytochemicals are produced by plants due to several metabolic activities (36).

Many recent literatures supported the fact that polyphenols which are one of the phytochemicals which have a positive effect on many chronic diseases as they are known by powerful antioxidants effect that can act as anti-inflammatory and anti-proliferative agents by modulating multiple signalling pathways (37). This property might be useful for treating ailments of multiple causes, such as psoriasis.

Another important phytochemical's composition such as flavonoids, phenolics, tannins, saponins, steroids, glycosides, terpenes, etc of fruits and leaves parts of FS had been documented on several previous studies (28). More than 130 bioactive compounds have been isolated from various parts of *Ficus* species since earliest times. The phytochemical compounds found out are known to have useful importance in medicinal sciences (30). Flavonoids from medicinal plants exert various pharmacological effects in vitro and in vivo, including serving as antioxidants, free radical scavenging,

anti-inflammatory activity, have antitumorigenic and antitumor metastatic activities (38).

The broad therapeutic effects of flavonoids can be largely attributed to their antioxidant properties. Also, have been demonstrated to have anti-inflammatory and improve the immune system (39).

An oxidative stress (OS) condition typical for psoriasis. Oxidative stress leads to the overproduction of reactive oxidative species (ROS) that can damage DNA, proteins, and other cell contents also results in the activation of many signalling pathways including nuclear factor kappa-light-chain-enhancer (NF-κB) andmitogen-activated protein kinase (MAP kinase). This stimulation of Th1 and Th17 cells that's lead to secretion of pro-inflammatory cytokines and then increase keratinocyte proliferation (39,40). All of this can lead to psoriatic inflammation.

ROS is moulded in normal amounts as a portion of the basic metabolism and play a role in numerous physiological mechanisms. The turbulent ratios between the number of oxidants and antioxidants lead to OS (40). In psoriasis, an increased level of total oxidative stress. Thus, oxidative stress should be suspected an important point in psoriasis (39).

Flavonoids perform an important role in the suppression of numerous inflammatory pathways most importantly NF-kappa B pathway (39), suppresses the transcriptional activity of NF-κB in cells so reduces inflammatory cytokines production, including TNFα, IL-6, IL-8, and IL-1β.

This can be controlled by various ways and one way is the direct scavenging of free radicals. Flavonoids are oxidised by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilise the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive (39,40). Moreover, Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response and tissue damage (39,41).

Additionally, many studied demonstrated the effects of flavonoid that found in other plants on psoriasis. such as a study on the *Cassia taura* leaves shows significant anti-psoriatic activity, and that it reduces animal skin-relative epidermal thickness in addition to other pathological features. The study indicates that flavonoids in *C. tora* leaves can be used as natural therapeutic drugs for preventing psoriasis complications (42). Another study on *Croton sphaerogynus* showed the antiproliferative activity of seems to be related to the presence of flavonoids. The highest antiproliferative activity Among all tested samples that are composed a high number of flavonoids. Samples containing small proportions of flavonoids also showed weak antiproliferative activity (43).

HaCaT cells are human spontaneous transformed immortal keratinocytes and are often used as a functional model for anti-psoriatic activity and the data gained by this model have shown a good association with skin irritation in vivo Because of its highly conserved differential ability.

Hence, we investigated the anti-shock activity of selected medicinal plants using cultured HaCaT cells and estimated cell viability by MTT assay.

Many Indian medicinal plantsare used for healing purposes traditionally in the treatment of psoriasis like *Phyllanthus simplex Retz, Crotolaria juncea Linn,Leucas aspera Linn* and *Vitex glabrata R.Br*. This prompted the researcher to evaluate the anti-psoriatic activities of these plants (44).Antipsoriatic activity of the extracts wasassessed by MTT assay, using HaCaT cells,their findings revealed that these plants showed promising skin keratinocyte antiproliferative activity andinhibitory action against NO production in HaCaT cells proposed that the anti-psoriatic activity of the extracts was mediated by an antioxidant mechanism (44).

In many studies found there was a relation between NO levels and severity of disease.That they were found significantly high NO levels in patients with active disease like chronic plaque-type psoriasis as compared to normal individuals (45).

which is commonly employed as an in vitro test model for antipsoriatic activity.

All previous mentioned literatures documentation goes along with what have been recorded in this research that FS fruit and leaves extracts revealed in the NO production inhibition in that the fruits extract had resulted in NO inhibitory activities of28.54% and 16.57% at 250, 125 μg/ml, respectively. While, the FS leaves extract revealed the NO

inhibition of 18.64 and 16.24% at 250, 125 μg/ml, respectively. Furthermore, the qualitative phytochemicals evaluation of both FS fruit and leaf extracts indicated the presence of glycoside, phenols, flavonoids, steroids, saponins and tannins. None the less, minor variations between both extract types were recorded in those carbohydrates, proteins and amino acids as well as terpenoids are found in FS fruit extract only. While, reducing sugars are in leaf extract only. Alkaloids are found in neither fruit nor leaf extracts. These results occurred as a result of antioxidant and anti-inflammatory activities of extracts of this plant species. This obtained FS studied extracts inhibitory effect could be referred to their flavonoids phytoconstituents which was also detected in this study. Moreover, all the recorded MTT assay results monoculture and co-culture cell line viability and the morphological screening of the cells under the inverted microscope at the different extract types treatments under study displayed a concentration dependent manner. This may lead to more effective antiproliferative response at higher concentrations.

The MTT assay is widely used to measure cell viability, live cell reproduction, and cytotoxicity of new drug candidates in the 96-Wellplate format. MTT reduction is attributed to mitochondrial activity (46,47).

Lately, literature data has collected surprising results which expose that in some experimental systems and with many of the tested compounds. It appears that reducing MTT is an insufficient test of the number of viable cells, which leads to false results is a source Misinterpretation. The main

reason for such screening pitfalls and limitations are tested compounds have ability to interact directly with 3− [4,5-dimethylthiazole-2-yl] −2,5 diphenylte-trazolium bromide (MTT) (44). It has been documented, for example, that ascorbic acid reduced MTT and the reaction was significantly enhanced in the presence of retinol. Also, several plant extracts, such as polyphenols and flavonoids, severely reduced MTT in the absence of live cells. It appears necessary to exclude direct chemical reactions of the compounds tested with MTT before starting routine measurements of cell viability using this assay (48). Other reason the tested compounds could interfere with the dehydrogenase activity in the mitochondria, thus leading to overestimation (activation of MTT reduction of dehydrogenase) or underestimation (inhibition of mitochondrial dehydrogenase) results of the MTT test (48).

polyphenols reduced MTT directly in the absence of living cells. Moreover, they found that the type of cell culture medium and the filter set of the spectrophotometer had an effect on the results of the MTT assay (49).

Although the MTT test is a useful test of cell viability for cell proliferation and cytotoxicity and is widely used in many fields, particularly in drug discovery screening, many factors can interfere with results. For those reasons the results of the examination must be carefully confirmed.

Cytotoxic drugs affect all dividing cells, including those of healthy tissue. But because psoriasis cells often divide markedly faster than normal epithelial skin cells, they are particularly sensitive to cytostatic agents

rather than cytotoxic ones. The effects on normal cells are less pronounced and healthy cells also recover faster. Cytostatic means that the compound of interest lowers the growth rate of a given cell population without direct cell killing effects. So, cell death will occur as a consequence of a too long cytostatic effect.

As it could be achieved via anti-proliferative effect (cytostatic) rather than cytotoxic effect. This explanation is confirmed by both morphological cell examinations under the inverted microscope and the MTT assay of the different FS fruits and leaves extracts studied concentrations.

Also,the phytochemical analysis of FS extract indicated the presence of terpenoids and phenols. Terpenoids are identified to possess antioxidant, anti-inflammatory and anti-microbial activities. Also, because phenolic compounds were also detected in the FS extracts, it is reasonable to assume that the relatively higher antioxidant activity of the extract was due to a joint effect of these bioactive compounds in biological systems, acting as scavengers of singlet oxygen and free radicals. Saponins, tannins and others detected in the FS extracts may also contribute to the combined effects of the extracts as useful natural inhibitors of iNOs.

Therefore, this plant could be useful in the management of inflammation exacerbated by NO over-production and hyperproliferative of psoriasis (Free radicals are the main indicators of activity of keratinocytic proliferation in psoriasis).

In conclusions, this in vitro study indicated that fruits and leaves extract from *Ficus sycomorus* was not toxic at all tested concentrations in association with higher cytostatic effects in general and of fruits in particular on the HaCaT and THP-1 monoculture system than the co-culture. While, its leave extract effects wereon HaCaT monoculture more than on co-culture. In addition, the pronounced inhibition of LPS-induced NO production on THP-1 monoculture. Hence all in all *Ficus sycomorus* fruits and leaves extracts positive recorded data would serve as a source of a novel, effective antiproliferative and NO production inhibitory bioactivitypotentialagentsagainst hyperproliferative of skin. From this point of view, this study supports to a certain degree the traditional medicinal uses of the plants in diseases therapy and reinforces the concept that ethno botanical approach to screen plants as potential sources of bioactive substances is successful.

In spite of that, further phytochemical studies for the purification and characterization of the active ingredients of the examined plant species extracts are still recommended. Moreover, further experimentation is needed in order to understand the precise mechanism of action on psoriasis by the extracts. Also, quantitative analysis of the different bioactive phytochemical constituents of the examined plant species extracts is recommended to illustrate the exact correlation between those constituents and their effect on the treatment of psoriasis.

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جامعة النجاح الوطنية

كلية الدراسات العليا

تأثير مستخلصات نبات الجميز على خلايا الانسان الجلدية كعلاج مقترح للصدفية

إعداد

ريم عدنان نمر حنايشة

إشراف

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قدمت هذه الأطروحة إستكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

2021

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الملخص

مقدمة: الصدفية مرض جلدي التهابي مزمن مفرط التكاثر يؤدي إلى نمو متزايد في الخلايا الكيراتينية. الصدفية التي تصيب 2 إلى 3٪ من السكان ناتجة عن عدة عوامل منها الوراثة وعلم التخلق والبيئات وأنماط الحياة وكذلك الإجهاد والأدوية والالتهابات والصدمات. النايتريك اكساید هو مؤشر حيوي رئيسي لمرض الصدفية. منذ الأزل، استخدم الإنسان النباتات والمنتجات الطبيعية كمصدر للغذاء والأدوية لعلاج الأمراض والوقاية منها. واحد منها الجميز الذي يستخدم في التقليد لعلاج الصدفية.

الهدف: تهدف هذه الدراسة في المختبر الى دراسة السمومية والتثبيط الخلوي وتثبيط الالتهابات لمستخلصات الإيثانول المائي لأوراق وثمار الجميز على خلايا الكيراتينية (HaCaT) و THP-1.

الطرق: تم قياس قابلية بقاء الخلايا لكل من الزراعة الأحادية والمشتركة عن طريق اختبار MTT. بالإضافة الى انه تم استخدام خلايا الدم البيضاء المتمايزة الى خلايا بلعمة المستحثة بواسطة عديد التسكر الدهني من (LPS) كزراعة أحادية ومشتركة تم استخدامها لتقييم آثار مستخلصات نبات الجميز على إنتاج النايتريك أكساید المؤيد للالتهابات بواسطة كاشف Griess reagent. علاوة على ذلك تم اجراء التحليل الكيميائي النباتي النوعي لمستخلصات ثمار وأوراق الجميز.

النتائج: أظهرت النتائج التي تم الحصول عليها أنه لا يوجد فرق ذو اهمية بين جميع التراكيز التي تم فحصها لأجزاء نبات الجميز في كل من الزراعة الأحادية والمشتركة بناءً على التحليل الإحصائي الذي تم إجراؤه ($P < 0.05$) الذي اظهر عدم وجود اختلافات بين أوراق وثمار الجميز.

ومع ذلك، ظهر تأثير بارز لمستخلص ثمار الجميز لتثبيط نمو الخلاياالكيراتينية ($P < 0.05$) في جميع التراكيز المدروسة مع تأثير أكثر وضوحًا عند 1000 و500 ميكروغرام / مل. كان هذا التأثير أعلى على الزراعة الأحادية للخلايا HaCaT منه على خلايا1-THP بطريقة تعتمد على التركيز حيث كانت قابلية النمو والحياة للخلايا **69.6%** على تركيز 31.8 ميكروغرام/ مل وصولاً إلى **57%** على تركيز 1000 ميكروغرام / مل. بينما أظهرت الزراعة المشتركة انخفاضًا بارزا في قابلية النمو والحياة للخلايا عند 1000، 500، 250 ميكروغرام/ مل بنسبة **64.2%**، **63.4%**، **64.2%** على التوالي. هذا التأثير الخلوي الواضح المسجل مشابه بين جميع مستخلصات الأوراق والثمار. لذلك، فإن التأثير الجدير بالملاحظة هو التثبيط الخلوي لمستخلصات ثمار وأوراق الجميز كان أكثر من التأثير السام على الخلايا في كل من الزراعة الأحادية للخلايا HaCaT والزراعة المشتركة. وبالمثل، ولوحظ ان التأثير لمستخلص ثمار الجميز الزراعة الأحادية لخلايا THP−1 كان معتمدا على التركيز، على العكس من ذلك، كما انه تم التعرف على تأثير مستخلص نبات الجميز على الزراعة الأحادية لخلايا THP-1 حيث تم ملاحظة ان هناك تأثير سام على خلايا بدلاً من تثبيط الخلوي بطريقة تعتمد على التركيز، بالإضافة إلى ذلك كما أنه تم توثيق قوة الكسح لمستخلصات ثمار وأوراق الجميز من خلال تقليل إنتاج النايتريك اكساید، لوحظ النشاط المثبط لأكسيد النيتروجين على خلايا البلعمة لثمار الجميز عند 250 ميكروغرام / مل حيث تسبب في إنتاج **71.46%** فقط بالمقارنة مع positive control. لذلك، نتج عن مستخلص الثمار نشاط مثبط لأكسيد النيتروجين بنسبة **16.57%**و**28.54%**عند 250، 125 ميكروغرام/ مل على التوالي. بينما أظهر مستخلص أوراق الجميز نشاط مثبط لنايتريك اكساید بنسبة 16.24% و**18.64%** عند 250، 125 ميكروغرام/ مل على التوالي، علاوة على ذلك أشار التقييم النوعي للمواد الكيميائية النباتية لكل من مستخلصات الثمار وأوراق الجميز إلى وجود الغلايكوزساید والفينول والفلافونويد والستيرويدات والصابونين والتانين، ومع ذلك تم تسجيل اختلافات طفيفة بين

نوعي المستخلصات في أن الكربوهيدرات والبروتينات والأحماض الأمينية وكذلك التربينويدات موجودة في مستخلص ثمار الجميز فقط. بينما السكريات المختزلة موجودة في مستخلص الأوراق فقط. بينما لم يتم العثور على قلويدات لا في مستخلصات الثمار ولا الأوراق.

الخلاصة: في المحصلة، أشارت هذه الدراسة في المختبر إلى أن مستخلص الثمار وأوراق الجميز لم يكن سامًا في جميع التراكيز المختبرة بالاقتران مع تأثير التثبيط الخلوي بشكل عام والثمار بشكل خاص على نظام الزراعة الأحادية لخلايا HaCaT و THP-1 مقارنة مع الزراعة المشتركة، كانت آثار مستخلص الاوراق على الزراعة الأحادية لخلايا HaCaT أعلى من الزراعة المشتركة. بالإضافة إلى ذلك، التثبيط الواضح لإنتاج النايتريك اكساید المستحث بـ LPS على الزراعة الأحادية لخلايا THP-1. ومن ثم فإن جميع البياناتالإيجابية المسجلة لجميع مستخلصات ثمار وأوراق الجميزستكون بمثابة مصدر لعوامل جديدة وفعالة مضادة للتكاثر وعوامل محتملة للنشاط الحيوي المثبط لإنتاج النايتريك اكساید ضد النمو المتزايد لخلايا الجلد، من وجهة النظر هذه تدعم هذه الدراسة إلى حد ما الاستخدامات الطبية التقليدية للنباتات في علاج الأمراض وتعزز مفهوم أن النهج العرقي النباتي لفحص النباتات كمصادر محتملة للمواد النشطة بيولوجيًا ناجح.