Biological and Mechanistic Studies on Selected Chinese Medicines for Psoriasis

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Abstract

Psoriasis, a chronic inflammatory skin disorder affecting approximately 2–3% of the population worldwide, is characterized histologically by hyperproliferation and aberrant differentiation of epidermal keratinocytes. Many conventional therapies are offered for psoriasis treatment but there exist problems such as unsatisfactory efficacy, side effects and drug resistance. Many patients therefore turn to alternative and complementary medicines for help. Traditionally, Chinese herbal medicine has been extensively used to treat psoriasis and produced promising clinical results. The present PhD study was conducted to investigate psoriasis-treating Chinese herbal medicines with an aim to identify effective anti-psoriatic agents. Sixty Chinese medicinal materials were selected for the screening project based on their ethnomedical use in psoriasis. The ethanolic extracts of these medicinal substances were evaluated for their anti-proliferative action on cultured HaCaT human keratinocytes using microplate SRB and MTT assays. Among them, the root of *Rubia cordifolia* L. (Radix Rubiae) and realgar were found to have significant anti-proliferative effects, with IC_{50} values of 1.4 and 6.6 μg/ml, respectively as measured by MTT assay, while they exerted mild significant cytotoxicity on the human fibroblast Hs-68 cell line.

Further mechanistic studies demonstrated that both Radix Rubiae and realgar were capable of inducing cellular apoptosis on HaCaT cells in a dose- and time-dependent manner as shown by morphological inspection, DNA fragmentation, TUNEL assay, cell cycle analysis, annexin V–PI staining and Western blot analysis. HPLC fingerprintings were constructed for quality control of the Radix Rubiae extract using mollugin as the chemical marker. Further phytochemical study found that ethyl acetate fraction of this herb possessed potent growth inhibition on HaCaT cells, with

IC_{50} of 0.9 μg/ml. However, the chemical compounds obtained from commercial sources including mollugin, alizarin, purpurin, and quinizarin failed to induce growth inhibition. Meanwhile, arsenic trioxide, arsenic pentoxide and arsenic iodide, three arsenic salts presented in realgar, had significant anti-proliferative effect on HaCaT cells, with IC_{50} values of 2.4, 16 and 6.8 μM, respectively; and cellular apoptosis was found to be the underlying mechanism for the observed growth inhibitory activity. Furthermore, Radix Rubiae, realgar and arsenic compounds were also revealed to possess growth inhibition when evaluated in a PHA-activated PBMC model, and all of the substances except arsenic pentoxide significantly attenuated the release of inflammatory cytokines such as IFN-γ, TNF-α and IL-2 in PBMC, indicating an anti-inflammatory effect. The *in vivo* mouse tail model experiments demonstrated that arsenic trioxide, arsenic pentoxide and arsenic iodide were able to markedly induce mouse tail keratinocyte differentiation, while such differentiation-modulating effect observed in the fraction of Radix Rubiae was only marginal.

In summary, Radix Rubiae and realgar extracts and three arsenic compounds have been identified and characterized as potential anti-psoriatic agents. The discoveries from the present PhD project not only help put the traditional use of these medicinal substances for psoriasis treatment on a scientific footing, but also open up new opportunities for their development into novel anti-psoriatic therapies.

摘要

銀屑病是一種慢性炎症性皮膚病，其發病率約占全球總人口的 2-3%。銀屑病的病理特徵包括表皮細胞增殖過速、角化不全及炎症反應。目前針對銀屑病的治療藥物均存在療效不佳，副作用較多和抗藥性等局限性，尋求高效安全的抗銀屑病藥物仍然是當前新藥開發的熱點。傳統中醫藥治療銀屑病療效顯著，但一直以來缺乏對其系統性研究開發。本課題旨在從 60 種常用治療銀屑病的中藥中篩選和尋找具有抗銀屑病的有效藥物。

本研究首先利用 80%乙醇對這些中藥進行提取，並採用 SRB 和 MTT 方法對這些提取物進行抗 HaCaT 表皮細胞增殖測試。結果顯示茜草根和雄黃兩種中藥具有強大抗表皮細胞增生的藥理功效，其半效抑制濃度 IC_{50} 分別為 1.4 和 6.6 μg/ml，而該兩種提取物對正常成纖維細胞 Hs-68 株未具明顯細胞毒性。在隨後進行的螢光染色，DNA 凝膠電泳, TUNEL 方法，細胞週期檢測，annexin V-PI 雙染色試驗以及 Western blot 分析等一系列實驗證實了細胞凋亡乃是抑制 HaCaT 表皮細胞增殖的主要原因。

在品質檢測方面，我們成功地利用高壓液相指紋圖譜對茜草根提取物進行質量控制，同時運用大葉茜草素 Mollugin 作為質控之化學標誌物。隨後對茜草根乙醇提取物採用正己烷、乙酸乙酯、正-丁醇和水進行萃取，並測試這些萃取物對 HaCaT 細胞增殖的影響，結果顯示乙酸乙酯組份最為有效, 其 IC_{50} 為 0.9 μg/ml，顯示其抗表皮細胞增殖的活性比其乙醇提取物強。而幾種茜草單體物包括 mollugin, alizarin, purpurin 和 quinizarin 並未能有效抑制 HaCaT 細胞的增殖。

雄黃是一種含砷的礦物中藥，其所含的無機化學單體 As_2O_3, As_2O_5 和 AsI_3 也能顯

著抑制 HaCaT 細胞的增殖，其 IC_{50} 分別為 2.4, 16 和 6.8 μM。我們的實驗也證明這些砷化物是通過誘導細胞凋亡來達到抗 HaCaT 細胞生長。值得一提的是這些砷化物對 Hs-68 細胞只有輕微抑制作用，其 IC_{50} 分別為 43.4, 223 和 89 μM。

另外，我們還於被 PHA 活化的外周血單核細胞（PBMC）模型中對茜草根和雄黃乙醇提取物，茜草根的乙酸乙酯萃取物，As_2O_3, As_2O_5 和 AsI_3 進行抗炎症反應的測試，結果顯示所有葯品均能抑制活化的 PBMC 的增殖。另外，除了 As_2O_5 之外，所有葯品都能減少 PBMC 中炎症因數 IFN-γ，TNF-α 和 IL-2 的釋放，表明該些葯物具有較強的抗炎作用。最後利用小鼠尾部鱗片表皮模型對含茜草根的乙酸乙酯萃取物，As_2O_3，As_2O_5 和 AsI_3 的外用製劑進行測試，結果顯示 As_2O_3, As_2O_5 和 AsI_3 能有效地誘導表皮細胞分化，而茜草根的乙酸乙酯萃取物在這方面的作用則較弱。

總括而言，本研究課題成功發現茜草根和雄黃為兩種具很強抗銀屑病活性的中藥，茜草根的乙酸乙酯萃取物以及雄黃中的化學單體具有很好開發成治療銀屑病外用製劑的前景。

Publications Based on the Work in this Thesis

1) Tse, W.P., Che, C.T., Liu, C.K., Lin, Z.X. (2006) Evaluation of the anti-proliferative properties of selected psoriasis-treating Chinese Medicines on Cultured HaCaT cells. *Journal of Ethnopharmacology* **108**:133-141.
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3) Lin, Z.X., Tse, W.P., Cheng, C.H.K., Che, C.T. (2007). Realgar-derived Arsenic Compounds Induce Anti-proliferation and Apoptosis on Cultured HaCaT Keratinocytes. (Abstract). *Planta Medica* **73**(9): 989.
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The heavens declare the glory of God; and the firmament sheweth his handywork.

Psalms 19:1.

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List of Abbreviations and Symbols

μg	microgram (10^{-6} gram)
μl	microlitre (10^{-6} litre)
μM	micromolar (10^{-6} molar)
mg	milligram (10^{-3} gram)
ml	millilitre (10^{-3} litre)
mM	millimolar (10^{-3} molar)
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate fractional extraction of Radix Rubiae
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Agency for the Evaluation of Medicinal Products
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HLA	Human leukocyte antigens
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC_{50}	Concentration required to inhibit the growth by 50%
IFN-γ	Interferon-γ

IL	Interleukin
LFA	Lymphocyte function associated antigen
MHC	Major histocompatibility complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PI	Propidium iodide
PMN	Polymorphonuclear leukocytes
PsA	Psoriatic arthritis
PASI	Psoriasis Area Severity Index
PUVA	Psoralen plus ultraviolet A
RA	Rheumatoid arthritis
S.D.	Standard deviation
SEM	Standard error of mean
SRB	Sulphorhodamine B
TCM	Traditional Chinese Medicine
Th	T helper cell

TLC	Thin Layer Chromatography
TNF-α	Tumor necrosis factor-α
UV	Ultraviolet

Chapter One

General Introduction

1.1. Psoriasis as a Disease

The word "psoriasis" comes from ancient Greece, and means to itch. It has long been recognized that red eruptions appearing on the skin of psoriasis patients usually accompany with itchiness. In psoriatic lesions, patches of thick, red skin are covered with silvery scales. Fig. 1.1. shows the presentation of a typical plaque type of psoriasis. Psoriasis is one of the most common clinical dermatological conditions and recent evidence points to an aetiology encompassing systemic, immunological, autoimmune and genetic elements; but the exact mechanism for its initiation, development and maintenance is not known. It is important to recognize that 'psoriasis' is a term used to describe a spectrum of presentations of the disease, ranging from localized plaques to more severe generalized involvement, with or without psoriatic arthritis and the associated manifestations of other autoimmune diseases (Smith et al., 2002).

Although psoriasis is not contagious, patients with this skin condition may have discomfort, including pain and itching, restricted motion in their joints, and emotional distress. Psoriasis lesions most often occur on the elbows, knees, scalp, lower back, palms and soles of the feet, but it can affect any body locations, for example, it also affects the fingernails, toenails, and soft tissues inside the mouth and genitalia. Thus, the various clinical manifestations of psoriasis make it more than a dermatological nuisance,

as it interferes with many normal daily activities, such as use of hands, walking, sleeping, and sexual activity. At least 30% of patients with psoriasis contemplate suicide, which places psoriasis on par with other major medical diseases such as depression, heart disease, and diabetes in terms of psychological impact. It can have a significant psychological impact on the suffering individuals, which can lead to low self-esteem, social embarrassment, distorted self-perception, psychological turmoil such as depression and anxiety. Many psoriasis patients feel isolated because of their disease and become self-conscious and socially withdrawn. Once psoriasis begins, there are only remissions and relapses of varying degrees of intensity, and it often improves and worsens in a natural but unpredictable pattern. This distressing skin condition would, more often than not, accompany with the sufferer for the rest of his/her life. In short, psoriasis severely affects a patient's quality of life, both in terms of their psychological and physical well-beings (de Arruda and de Moraes, 2001). The annual cost to society due to psoriasis has been estimated to be more than a staggering sum of US$3 billion (Sander et al., 1993), making it a disease of major socioeconomic importance.

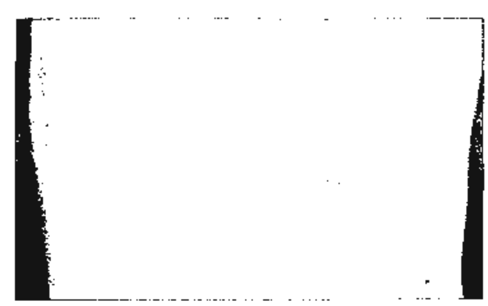


Fig. 1.1. Typical plaque of chronic psoriasis showing discoid configuration, thick adherent scales, induration and redness.

Most investigators regard psoriasis as a multi-factorial disease in which several genes interact with one another and with environmental stimuli. The disease varies in severity depending on inheritance and environmental factors. As a disease entity, psoriasis has distinct clinical presentations in the cutaneous tissue. In order to have a holistic and comprehensive understanding of this common skin disorder, it is important to understand the anatomy, physiological function of the skin as well as the epidemiology, aetiology and pathogenesis of psoriasis.

1.2. Anatomy and Physiology of the Skin

1.2.1. Structure of the Skin

The skin is the largest human organ. The total skin area of an adult covers about 1.5 - 2 m^2, and makes up of about one sixth of the total body weight. The most important function of the skin is as a barrier to protect the body from noxious external factors and to keep the internal systems intact (Gawkrodger, 2002). Structurally, skin is typically divided into three layers, viz. the epidermis, the dermis, and subcutaneous layer (Fig. 1.2.).

1.2.1.1. Epidermis

Epidermis is the outer layer of the skin and is not vascularized. Like its appendages such as hair, nails, sebaceous glands and sweat glands, epidermis has the origin of the embryonic ectoderm. The epidermis is a stratified squamous epithelium measuring about 0.1 mm in thickness. Although it is only about the thickness of a sheet of paper, the epidermis contains five distinct layers, including stratum corneum, stratum lucidium, stratum granulosum, stratum spinosum and stratum basale. The basal layer of the epidermis is composed mostly of keratinocytes, which move progressively towards the skin surface; and as ascending, they undergo a process known as "terminal differentiation" to produce the surface layer of cells (stratum corneum). A cell takes

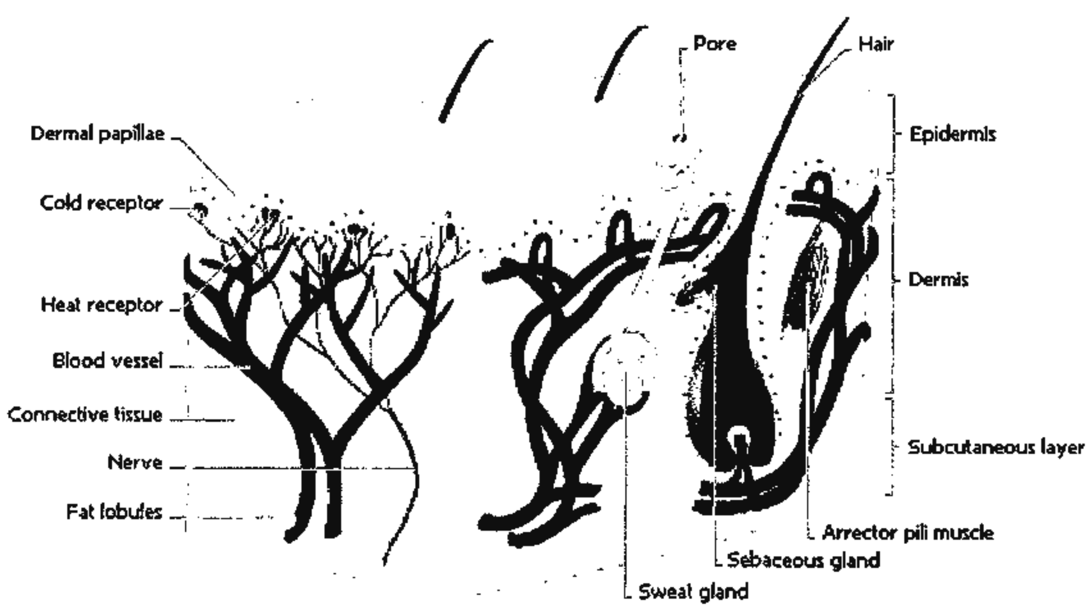


Fig. 1.2. Diagram showing the main features of the human skin. (Source: http://encarta.msn.com/media_461516297_761569048_-1_1/structure_of_the_skin.html)

approximately 8-10 weeks to pass from the basal layer to the surface of the epidermis (epidermal transit time), and loss of cells from the surface is matched by production in the basal layer so that epidermal thickness remains constant. The homeostasis of the epidermis is achieved by a delicate balance between the growth stimulators such as epidermal growth factors and the growth inhibitors including transforming growth factors alpha and beta (Seeley, 1992).

Superficial to the stratum basale is the stratum spinosum, consisting of eight to ten layers of multilateral cells. As the cells pushed to the surface, the flatten desmosomes are broken apart and new desmosomes are formed. The stratum granulosum consists of two to five layers of somewhat flattened, diamond-shaped cells that have their long axes oriented parallel to the surface of the skin. Cells become flattened and lose their nuclei in the granular cell layer. Keratohyalin granules are seen in the cytoplasm together with membrane-coating granules, which expel their lipid contents into the intercellular spaces. The stratum lucidum appears as a thin, clear zone above the stratum granulosum and consists of several layers of dead cells with indistinct boundaries. Keratin fibers are present, but the keratohyalin has dispersed around the keratin fibers, and the cells appear somewhat transparent. The last and most superficial stratum of the epidermis is the stratum corneum, which is composed of as many as 25 or more layers of dead squamous

cells joined by desmosomes. Eventually the desmosomes break apart, and the cells are desquamated from the surface of the skin (Seeley, 1992).

The differentiation of basal cells into dead but functionally important corneocytes is a unique feature of the skin. During differentiation, keratin filaments in the corneocytes aggregate under the influence of filaggrin, a process known as keratinization, and bundles of filaments form a complex intracellular network embedded in an amorphous protein matrix derived from the keratohyalin granules of the granular layer. The horny layer is important in preventing all manner of agents from entering the skin, including microorganisms and water. The epidermis also prevents the body's fluids from getting out, therefore playing a very important role in maintaining the equilibrium of the body's interior environment. Kinetic studies show that, on average, the dividing basal cells replicate every 200 to 400 hours. The resultant differentiating cells take about 14 days to reach the stratum corneum and a further 14 days to be shed (Seeley, 1992). In normal skin, skin cells grow, move to the surface and are sloughed off unnoticed at a steady rate. The cell turnover time is considerably shortened in keratinization disorders such as psoriasis. The study of keratinization is important because psoriasis is a skin disease typically resulted primarily from the malfunctions in this process (Boehm, 2006).

1.2.1.2. Dermis

The dermis consists of vascularized tissues surrounding the accessory organs (sweat glands, sebaceous glands and hair follicles) and nerves. The dermis is defined as a tough supportive connective tissue matrix, containing specialized structures, found immediately below, and intimately connected with, the epidermis. Developmentally, it is derived from the mesodermal origin. The main feature of the dermis is a network of interlacing fibres, mostly collagen with some elastin. Collagen fibres make up 70% of the dermis and impart a toughness and strength to the structure. On the other hand, elastin fibres are loosely arranged in all directions in the dermis and provide elasticity to the skin. The collagen and elastin fibres, both of which are proteins, are embedded in a ground substance of mucopolysaccharides (glycosaminoglycans). In terms of cell components, the dermis contains fibroblasts which synthesize collagen, elastin, other connective tissues and glycosaminoglycans, dermal dendrocytes which are dendritic cells with a probable immune function, mast cells, macrophages and lymphocytes (Gawkrodger, 2002).

1.2.1.3. Subcutaneous fat

The subcutaneous fat constitutes the insulation between the skin and the underlying tissue such as muscle, bone, tendon and ligament. This layer is relatively thick, typically in the

order of several millimeters. The subcutaneous fat layer is loosely connected to itself but adheres to the dermis and the underlying fascia. This allows skin to dissipate pressure, absorb shock, thereby helping cushion our skin against trauma and shearing forces. The subcutaneous fat layer also stores energy, which the body uses during periods of high activity (Seeley, 1992). It also provides a readily available supply of high-energy molecules, whilst the principal blood vessels and nerves are carried to the skin in this layer.

1.2.1.4. Accessory skin structures

(a) Hair

Hairs are found over the entire surface of the skin. The hair follicle has an input from the epidermis, which is responsible for the matrix cells and the hair shaft. The dermis contributes to the papilla, together with its blood vessels and nerves (Gawkrodger, 2002).

(b) Nails

The nail is a phylogenetic remnant of the mammalian claw and consists of a plate of hardened and densely packed keratin. The nail consists of the proximal nail root which is cover by skin and the distal nail body which is the visible portion of the nail. It protects the finger tip and facilitates grasping and tactile sensitivity in the finger pulp (Gawkrodger, 2002).

(c) Glands

The major glands of the skin are the sebaceous and sweat glands. Sebaceous glands are found all over the body, but distribute most abundantly in the scalp area and around the forehead, chin, cheeks and nose. Sebaceous glands can produce oily substance that is a natural moisturizer, prevents drying, conditions the hair and skin and provides protection against some bacteria (Seeley, 1992). Sweat glands are simple coiled tubular glands that open directly onto the surface of the skin through sweat pores, which are sweat-producing structures consisting of a single tube, a coiled body and a superficial duct. Sweat glands are involved in thermoregulation, as sweating dissipates the body heat to bring down the body temperature (Seeley, 1992).

1.2.2. Functions of the Skin

The skin not only serves as a mechanical barrier between the external environment and the underlying tissues but is also intricately involved in a defense role and several other important functions. The skin impedes passage into the body of most materials that come into contact with the body surface, including most bacteria and toxic chemicals. It also contains several types of immune cells and antibodies that can initially control the proliferation of bacteria until the immune system can mount a more specific response (Guyton and Hall, 1991).

A second function of the skin is to maintain a keratinized layer that is airtight and fairly waterproof, thus impervious to most substances. Therefore, it serves to minimize loss of water and other vital components from the body. The function of is protective layer in holding in body fluids becomes obvious when considering severe burns, when large quantities of body fluids and plasma proteins can be lost from the exposed burned surface, and the resulting circulatory disturbances can be life-threatening.

Another function of the skin is the maintenance of body temperature. This function helps maintain a constant temperature in spite of variations in the environmental temperature. Vasoconstriction reduces blood flow through the skin, thereby decreasing the amount of heat carried to the skin, which has the effect of increasing the insulating property of the skin. Vasodilatation of the skin vessels permits increased flow of blood to the skin, which promotes heat loss. To augment the dissipation of heat by the skin, sweat is secreted through specialized appendages of the skin, known as sweat glands. The heat of the skin is transferred to the sweat, which adds energy to the solution, causing it to achieve sufficient energy to leave the liquid phase and enter the gaseous phase, that is, to evaporate. This mechanism of energy transfer from skin to liquid sweat to water vapor is capable of transferring enough heat to allow a person to maintain constant body

temperature in hot environment.

A final function of the skin is its role as an endocrine-producing organ that produces the important hormone cholecalciferol, or vitamin D, upon exposure to sunlight. The active form of vitamin D (1,25-dihydroxyvitamin D3) is produced by the skin from a precursor related to cholesterol. This chemical messenger is subsequently released into the blood to act at the distant target site, the intestine (Guyton and Hall, 1991). Vitamin D functions as a hormone to stimulate uptake of calcium and phosphate from the intestines, to promote their release from bones, and to reduce their loss from the kidneys, resulting in increased blood calcium and phosphate levels (Seeley, 1992).

1.3. Epidemiology of Psoriasis

Psoriasis affects both sexes equally. The onset of the disease can occur at any age but is more frequent around puberty, which corresponds to the ages between 15 and 25. Two further peaks of increased incidence have also been observed at about 30 and 50 years of age, but there is a considerable overlap between the three groups (Baker, 2000).

Approximately 2 – 3% of the population worldwide is afflicted with psoriasis (Nickoloff and Nestle, 2004), and the total number of people afflicted with psoriasis worldwide is about 125 million according to the World Psoriasis Day Consortium (http://www.worldpsoriasisday.com/). Although all races can be affected by psoriasis, there is considerable interracial variation regarding its incidence rates. Estimations of the prevalence of psoriasis arrange from 0.5% to 4.6% among different countries and races (Lebwohl, 2003). It is generally agreed that psoriasis is more common among the American and European whites, with 4.9 million and 5.1 million respectively being affected by this disease. The prevalence of psoriasis for North America, United Kingdom, North American Black, Eastern Africa, and China are 2.5% (Gelfand et al., 2005a) , 1.5% (Nevitt and Hutchinson, 1996), 0.7% (Christophers, 2001), 0.7% (Farber and Nall, 1998) and 0.4% (Smith et al., 2002), respectively. In general, prevalence of psoriasis in the whites is more than twice as that in the blacks or Asians (Lebwohl, 2003).

Epidemiological studies have shown that 11 percent of those diagnosed with psoriasis have also developed psoriatic arthritis, a destructive and occasionally disabling joint disease. This is equivalent to a prevalence of 0.25 percent of American adults in the general population (Gelfand et al., 2005b). In different countries, between 5% and 42% of patients with psoriasis have psoriatic arthritis (Gladman and Brockbank, 2000). Psoriatic arthritis is usually seronegative for rheumatoid factor and presents in several characteristic forms: oligoarticular disease, distal interphalangeal arthritis, arthritis mutilans, and spondylitis or sacroiliitis. Thirty nine percent of patients had the polyarticular form, 16% the oligoarticular form of the disease and 8% of patients had distal interphalangeal involvement, while 2% have arthritis mutilans and 21% spondylitis. Arthritis precedes psoriasis in about 20% of patients (Scarpa et al., 1984). Even in patients without skin lesions, however, nail changes can usually be found in patients with psoriatic arthritis.

1.4. Aetiology of Psoriasis

1.4.1. Genetics

There is a strong familial association of psoriasis. Approximately one-third of the patients with psoriasis have a history of other family members with psoriasis (Ortonne, 1999). Strong evidence for a genetic basis for psoriasis was first provided by two large-scale epidemiological studies in the Faroe Islands and Sweden which revealed significantly higher incidences of psoriasis in relatives compared to the general population, or to matched controls (Baker, 2000). Thirty percent of patients have a family history of the disease; however, at present, how psoriasis is inherited is less well-known.

Further evidence for the involvement of genetic factors in psoriasis studies of monozygotic twins suggests a 70% chance of a twin developing psoriasis if the other twin has the disease. The concordance is around 20% for dizygotic twins (Krueger, 2005). These findings suggest both a genetic predisposition and an environmental influence in developing psoriasis. In both studies, the age of onset of the disease and its manifestations were very similar in the concordant monozygotic twins. Since the concordance rates are not 100% environmental factors are implicated for triggering of the disease (Baker, 2000).

The precise psoriasis gene or genes have not been identified, but several candidates have been suggested. It is frequently inherited and passed from one generation to the next, but not following a classical autosomal Mendelian profile. Psoriasis is a multifactorial disease in which several genes interact with other factors and with environmental stimuli. Recent studies have concentrated on a locus within the major histocompatibility complex (MHC) on the short arm of chromosome 6. Psoriasis susceptibility (PSORS)1 is located in the MHC (chromosome 6p21.3), which contains the human leucocyte antigens (HLA) genes as well as more than 200 other genes (Horton et al., 2004). Several HLA loci and alleles are now known associated with psoriasis, including HLA-B13, HLA-B37, HLA-B46, HLA B57, HLA-Cw1, HLA-Cw6, HLA-DR7, and HLA-DQ9 (Elder et al., 1994 ; Choonhakarn et al., 2002).

In addition to the PSORS1 locus, the PSORS2 locus is situated near the telomeric end of chromosome 17q. It was first reported by the Tomfohrde Research Group and confirmed by Samuelsson and Nair Research Groups (Tomfohrde et al., 1994; Samuelsson et al., 1999; Nair et al., 1997). The PSORS3 locus was shown on chromosome 4q34 (Mattews et al., 1996). The PSORS4, PSORS5 and PSORS6 locus are reported on chromosome 1q21 (Bhalerao and Bowcock, 1998), 3q21 (Samuelsson et al., 1999) and 19p13 (Lee et al., 2000), respectively.

Once a psoriasis gene or genes have been discovered, more light would be shed on the interaction between keratinocyte proliferation and contributions of the immune system to the development of psoriasis. It is likely that the development of psoriasis is multifactorial - patients inherit only a predisposition to the disease and require an exogenous stimulus before the disease is symptomatically expressed.

1.4.2. Immune System

Recent research has indicated that psoriasis is most likely a disorder of the immune system. It is generally believed that an abnormal T cell activity of the immune system causes the skin to become inflamed and keratinocytes to proliferate excessively. Psoriasis is therefore a T cell-mediated disease. In the early 1980's, Bos et al. (1983) showed that the majority of the dermal inflammatory infiltrate seen in psoriatic lesion consists of partially activated CD4+ and CD8+ T cells. The antigenic process is characterized by an initiation phase in which antigen-presenting cells (APC) in both the epidermis (Langerhans cells) and dermis (dermal dendritic cells) capture the antigen. Activated T cells produce in general one of two types of cytokines. Type 1 T cells produce the pro-inflammatory cytokines such as interleukin (IL)-2, IFN-γ, and TNF-α, whereas type 2 T cells release cytokines including IL5, IL-4, and IL-10. Activated T cells isolated from

psoriasis lesions produce a predominant type 1 cytokine profile (Uyemura et al., 1993; Schlannk et al., 1994). The chronic inflammation observed in psoriasis arises from an uncontrolled proliferation of T cells. The adaptive immune system and effectors of the innate immune system, including neutrophils, monocytes, macrophages, antigen-presenting cells and natural killer T cells, have all been implicated in the pathogenesis of psoriasis (Nickoloff, 1999; Bos et al., 2005; Gaspari, 2006). In conclusion, psoriasis can be described as a T cell-mediated inflammatory skin disease, with the involvement of a variety of cytokines.

1.4.3. Environmental Factors

Various environmental factors can initiate or exacerbate psoriasis, although their effects may vary among individuals presumably due to other modifying factors. For example, infection can provoke or exacerbate psoriasis. The most common factor to trigger for guttate psoriasis is the upper respiratory tract infection by β-haemolytic Streptococci. Local infection with *Staphylococcus aureus* and *Candida albicans* has also been implicated for the exacerbation of psoriasis (Noah, 1990). Recently, patients with HIV virus infection have been reported to have worsening psoriasis (Mahoney et al., 1991). Furthermore, both *Retroviruses* and *Pityrosporum* yeasts have been proposed as possible triggering factors for worsening of the disease (Baker, 2000)

Other important environmental factors for psoriasis initiation and aggravation include drugs, particularly beta-blockers, lithium, Angiotensin-converting enzyme (ACE) inhibitors and anti-malarials, and physical or psychological stress. Excessive alcohol intake is also associated with disease deterioration which makes management of this disease even more difficult. Psoriatic patients tend to drink more because of higher level of disease-induced depressive symptoms. Recently, Farkas et al., (2003) found that ethanol enhance the proliferation of keratinocytes and concluded that alcohol misuse may contribute to the exacerbation of psoriasis. Similarly, cross-sectional and case-control studies have reported a slightly increased risk of psoriasis in smokers compared with non-smokers (Davison et al., 2000).

Finally, as stress has effects on hormone secretion, the autonomic nervous and immune system function, it is not surprising that stress plays an important role as a triggering factor in the onset and exacerbation of psoriasis, but the precise mechanism of action underlying its role in the induction and aggravation of the disease is largely unknown.

1.5. Pathogenesis of Psoriasis

Both systemic and local factors are involved in the pathogenesis of psoriasis. Within the skin, several features are believed to have a primary role in the pathogenesis of the diseases, including the genetic, immunological and environmental mechanisms. A multitude of abnormalities have been described at the cellular and molecular levels, but whether any of these is of primary importance in the pathogenesis of psoriasis is uncertain. In general, psoriasis involves hyperproliferation of the epidermis in combination with the activation of inflammatory pathways and vascular changes that occur in response to a combination of genetic predisposition and environmental stimuli.

1.5.1. Hyperproliferation of Keratinocytes

Until the late 1980s, most research attention had focused on a primary fault in the keratinocyte based on the observation of increased epidermal proliferation in psoriatic plaques. The hyperplasia of the epidermis is a result of both a shortened epidermal cell cycle and an increase in the proliferative cell population. The growth rate of the psoriatic epidermis is up to ten times that of the normal epidermis (Gawkrodger, 2002). The epidermal cells have additional cohesiveness, which leads to the formation of the thick scales of psoriatic plaques (Farber et al., 1985). Psoriatic epidermis has 26.6% of the proliferative cells in the DNA synthesis (S) phase compared to only 7.8% of that in the

normal skin. The main defect in epidermal kinetics is the overall eight-fold increase in the germinative cell cycle compared to the normal skin (Camisa, 1998). The result is a cell cycle shortened from 311 to 36 hours; and accordingly, a significant reduction of epidermal turnover time, that is the time a basal cell takes to reach the stratum corneum, from 27 to 4 days (van de Kerkhof and Vissers, 2003). Given the intrinsic hyperproliferative nature of epidermal cells in psoriatic lesions, it has been postulated that acanthosis (thickening of skin lesion) of psoriasis is a direct result from diminished apoptotic cell death of keratinocytes; and indeed, resistance of epidermal keratinocytes to apoptosis has been found in psoriatic lesions (Wrone-Smith et al., 1997). Defects in epidermal apoptosis will result in hyperproliferation of keratinocytes, the underlying pathogenesis of psoriasis (Kawashima et al., 2004).

1.5.2. Differentiation of Keratinocytes

Another pathomechanism involved in psoriasis is abnormal keratinocyte differentiation. Histopathologically, the granular cell layer in the psoriatic lesion is reduced or absent, and as a result, hyperkeratosis (excessive keratinization) and parakeratosis (incomplete keratinization) develop. Cytokeratin expression is also altered in psoriatic skin compared to the normal skin. Involucrin and membrane bound transglutaminases appear prematurely in psoriatic epidermis (Parent et al., 1990). Involved psoriatic skin reveals

little or no reaction of anti-filaggrin antibody in the stratum corneum or granular layer. Involucrin staining appears paradoxically in the lower cell layer. The uninvolved epidermis of psoriatic patients shows the same staining pattern as the normal skin. In psoriatic epidermis there is apparently a downregulation of cytokeratins K1 and K10 and upregulation of K6 and K16 (Thewes et al., 1991). These quantitative differences in cytokeratins are the result of a delay in the differentiation of the basal cell layer in psoriatic epidermis.

1.5.3. Cutaneous Inflammation

The inflammatory infiltrate of the psoriatic lesion varies depending on the manifestations of the disease. The inflammatory infiltrate in psoriasis involves polymorphonuclear leukocytes (PMN), monocytes and macrophages, T lymphocytes, and various cytokines (Christophers and Mrowietz, 1995).

1.5.3.1. Polymorphonuclear leukocytes

PMN adhered to psoriatic epidermis is found to be markedly increased compared to the normal skin. PMN accumulation under the stratum corneum can be observed in the highly inflamed and therapeutically recalcitrant areas of psoriatic lesions. Stimulated

PMN are able to influence the growth and differentiation of epidermal keratinocytes as well as the activation of the T cells by aberrant expression of HLA-DR (HLA-DR is a major histocompatibility complex, MHC class II, cell surface receptor encoded by the human leukocyte antigen complex located on chromosome 6 region 6p21.31). These T cells affect the transepidermal PMN migration through the effect of their lymphokines on the keratinocyte production of pro-inflammatory mediators. As a result, a PMN-associated inflammation-boosting loop is proposed associated with the chronic psoriatic plaques and the acutely inflamed lesions of pustular psoriasis (Terui et al., 2000).

1.5.3.2. Monocytes and macrophages

Monocytes and tissue macrophages are consistently present in all manifestations of psoriasis. In two-thirds of psoriatic lesions, lining macrophages appear directly beneath the basal membrane and are in contact with basal keratinocytes (Wortmann et al., 1993; van den Oord and de Wolf-Peeters, 1994). Monocytes may directly influence keratinocyte growth by excreting mediators such as IL-6 and IL-8 (Grossman et al., 1989; Krueger et al., 1990).

1.5.3.3. T lymphocytes

The cellular infiltrate in psoriasis is composed primarily of T cells, both CD4+ and CD8+ cells (Bjerke et al., 1978; Baker et al., 1984). CD4+ cells are located mainly in the dermis, while CD8+ cells are found mostly in the epidermis (Menssen et al., 1995). The ratio of these cell types are important for both the genesis and resolution of psoriatic plaques, but it is not clear which of these T cell subsets is more important in the expression of psoriasis. The majority of the leukocytes in the dermis of psoriasis skin are CD4-positive helper T-lymphocytes of the Th1 phenotype. T cell activation requires stimulation of the T cell receptor (TCR) by the MHC I or II on the APCs.

1.5.3.4. Cytokines

Cytokines are a large family of extracellular protein mediators. They have pleiotropic effects (producing multiple effects). The cytokine network is crucial in the control of epidermal proliferation, differentiation and inflammation as an integrated process. In the Th-1 cytokine network immune response, the net effect of producing IL-2, IFN-γ, and TNF-α was to promote a T cell-mediated reaction. Psoriasis is best considered as a Th-1 type disease (Schlaak et al., 1994; Uyemura et al., 1993). IFNs are a family of glycoproteins which have several effects on epidermal cell behavior and inflammation.

IFN-γ and IFN-α are typically present in the psoriatic lesion (Livden et al., 1989). TNF-α is produced by activated keratinocytes and dermal macrophages. It is capable of activating lymphocytes, neutrophils, eosinophils and macrophages (Kristensen et al., 1992).

T cell is activated by the antigen-presenting cells which are in close contact with intracellular adhesion molecule (ICAM), lymphocyte function-associated antigen 2 & 3 (LFA 2 & 3) and cluster of differentiation 2 (CD2). Upon stimulation, Th-1 T cells release cytokines such as IL-2, IL-12 and IL-23, which will subsequently induce IFN-γ and TNF-α production resulting in informatory response. In conclusion, IL-2, IFN-γ and TNF-α play key roles in the inflammatory and proliferative process of psoriasis (Chang et al., 1997; Nickoloff, 1991; Uyemura et al., 1993; Valdimarsson et al., 1986).

1.6. Classification of Psoriasis

The symptoms of psoriasis can vary from person to person and the psoriatic lesions can occur on any part of the body. According to the appearance and the occurring sites of the lesions, psoriasis can be clinically divided into five different types, which include plaque, guttate, inverse, pustular and erythrodermic forms. Plaque-type psoriasis is the most common form of the disease, accounting for more than 80% of cases. Guttate psoriasis occurs in about 18% of patients with psoriasis, and erythrodermic and pustular psoriasis is each seen in fewer than 3% of the patients (Camisa, 2005). Patients with more severe or long-standing disease are more likely to have nail changes than those with less severe or recent-onset disease, and such changes are often accompanied with scalp and periungual involvement as well as psoriatic arthritis.

1.6.1. Plaque Psoriasis

Plaque-type psoriasis is the most common morphologic variant encountered in dermatological practice and is also called psoriasis vulgaris. Fig. 1.3.a. illustrates the clinical feature of typical plaque psoriasis. A fully developed clinical lesion of psoriasis vulgaris is a well-demarcated, red, round to oval plaque about 1 cm or larger across, surmounted by white silvery scales. If a white silvery scale is detached, it may leave bleeding points (Gawkrodger, 2002). The most common locations for plaque-type

psoriasis are the knees, elbows, scalp and trunk, although it can also appear in any other skin surface.

1.6.2. Guttate Psoriasis

Guttate means 'raindrop' and it describes the clinical appearance of guttate psoriasis that is characterized by multiple small, red, well-demarcated individual drops on the skin. Fig.1.3.b. shows the clinical image of guttate psoriasis. This type of psoriasis often appears on the abdomen, chest, back, limbs, and scalp; and it is prone to affect children and adolescents usually following an acute streptococcal throat infection (Nanda et al., 1990). The diagnosis is primarily based on clinical presentation, and investigations should include a throat swab and streptococcal serology. Guttate psoriasis may be the initial manifestation of psoriasis or it may represent an acute flare of existing chronic plaque-type psoriasis.

1.6.3. Pustular Psoriasis

Pustular psoriasis accounts for about 1.7% of cases (Camisa, 2005) and it is characterized by sterile pustules either generalized or localized on the palms and soles. The pus consists of white blood cells. Pustular psoriasis may be triggered by internal medications, irritating topical agents, overexposure to UV light, infections and emotional stress.

1.6.3.1. Palmoplantar pustular psoriasis

Palmoplantar pustular psoriasis (PPP) presents as erythematous plaques on the palms and soles. Fig.1.3.c shows the typical clinical presentation of PPP. PPP is characterized by multiple pencil eraser-sized pustules in fleshy areas of the hands and feet (Camisa, 2005). Symptoms of itching and burning are evidenced but the severity varies from patient to patient. The pustules appear in reddened plaques of skin, then turn brown, peel and become crusted. PPP is usually cyclical, with new crops of pustules followed by periods of low activity.

1.6.3.2. Generalized pustular psoriasis

Acute generalized pustular psoriasis (GPP), as is illustrated in Fig. 1.3.d, can present without any preceding history of this type of psoriasis, usually in later life. When typical psoriasis becomes unstable, often after provocation by topical drugs or other environmental factors, it can develop into this form. It is characterized by fiery-red, irregular patches with curved borders that are scattered with many 1 mm to 2 mm superficial pustules (Camisa, 2005). Provocative factors for GPP include over-zealous treatment of psoriasis with irritating topical therapies, an adverse reaction to phototherapy, or the withdrawal of systemic steroids. GPP is a serious and life-threatening medical condition, and in many cases may require hospitalization. Most patients with GPP

usually develop fever, leukocytosis, hypocalcemia and hypoablbuminemia.

1.6.4. Inverse Psoriasis

Inverse psoriasis is characterized by large, dry, smooth, vividly red plaques occurring in the folds of the skin near the genitals, under the breasts, in the armpits and other skin folds. Fig. 1.3.e. illustrates the clinical presentation of inverse psoriasis. Inverse psoriasis is usually associated with increased sensitivity to friction and sweating, and the lesional sites may be painful or itchy. The skin presentation of this type of psoriasis usually lacks the silvery scale often associated with plaque psoriasis. Inverse psoriasis is particularly sensitive to irritation from rubbing and sweating because of its involvement of skin folds and tender areas. Inverse psoriasis is more common and troublesome in overweight people who usually have deeper skin folds. Treatment application can be difficult due to the sensitivity of the skin in these areas.

1.6.5. Erythrodermic Psoriasis

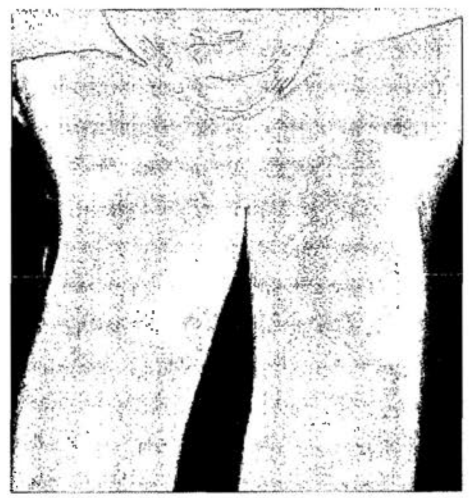
The least common form of psoriasis is exfoliative dermatitis or psoriatic erythroderma, representing 1% to 2% of all forms of psoriasis (Camisa, 1998). Erythroderma, shown in Fig. 1.3.f., is characterized by periodic, widespread, fiery redness of the skin. This rare form of psoriasis could involve the entire body surface and may be precipitated by

inappropriate use of systemic glucocorticoids, infections or even phototherapy or sun burns. Patients are febrile, and often have high white cell counts and problems with temperature control. Association with ankle edema is common in this form of the disease. Frequently, cardiac and renal decompensation is seen, particularly in the elderly (Smith et al., 2003). Precipitating factors include systemic illnesses, emotional stress, and alcoholism.

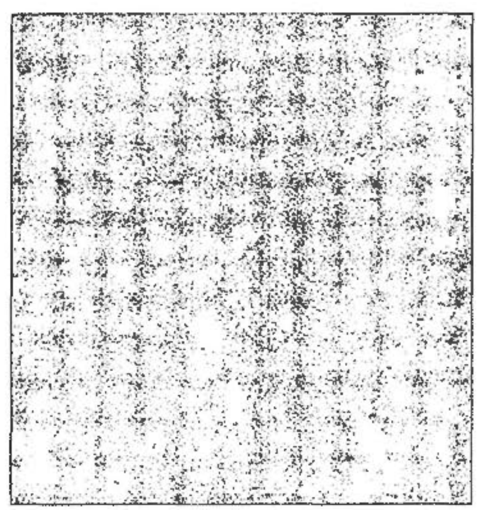
1.6.6. Psoriatic Arthritis

About 10% to 30% of people with psoriasis also develop psoriatic arthritis (PsA), which causes pain, stiffness and swelling in and around the joints. Fig. 1.3.g. shows the typical clinical presentation of PsA. PsA is a seronegative inflammatory arthritis (Camisa, 1998). PsA can result in significant symptomatology, quality of life impairment, and joint deformation. It is diagnosed when a patient with psoriasis of the skin and/or nails also has a distinctive pattern of peripheral and/or spinal arthropathy. PsA shares many characteristics with rheumatoid arthritis (RA) such as chronic course of the diseases that more likely result in damage to bone and synovial membrane, disability and increased mortality. Unlike RA, psoriatic arthritis usually affects the distal joints in fingers or toes. The lower back, wrists, knees or ankles may also be affected. Muscle or joint pain can occur without joint inflammation. The radiographic appearance of PsA differs from that

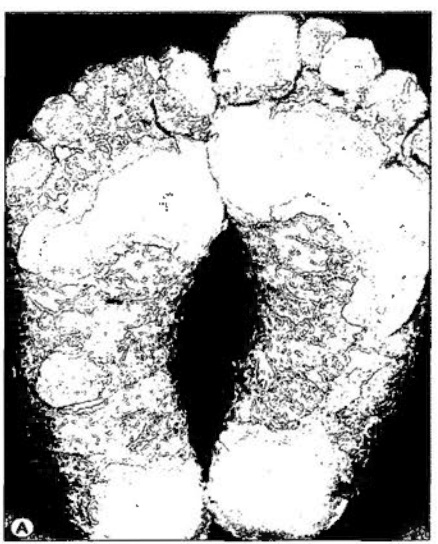
of RA, particularly with regard to sacroiliac and cervical spine involvement. Rheumatoid factor is usually absent in most patients with PsA, however, as many as 10% of this population may test positive for rheumatoid factor (Galadari et al., 2003).



(a) Plaque psoriasis

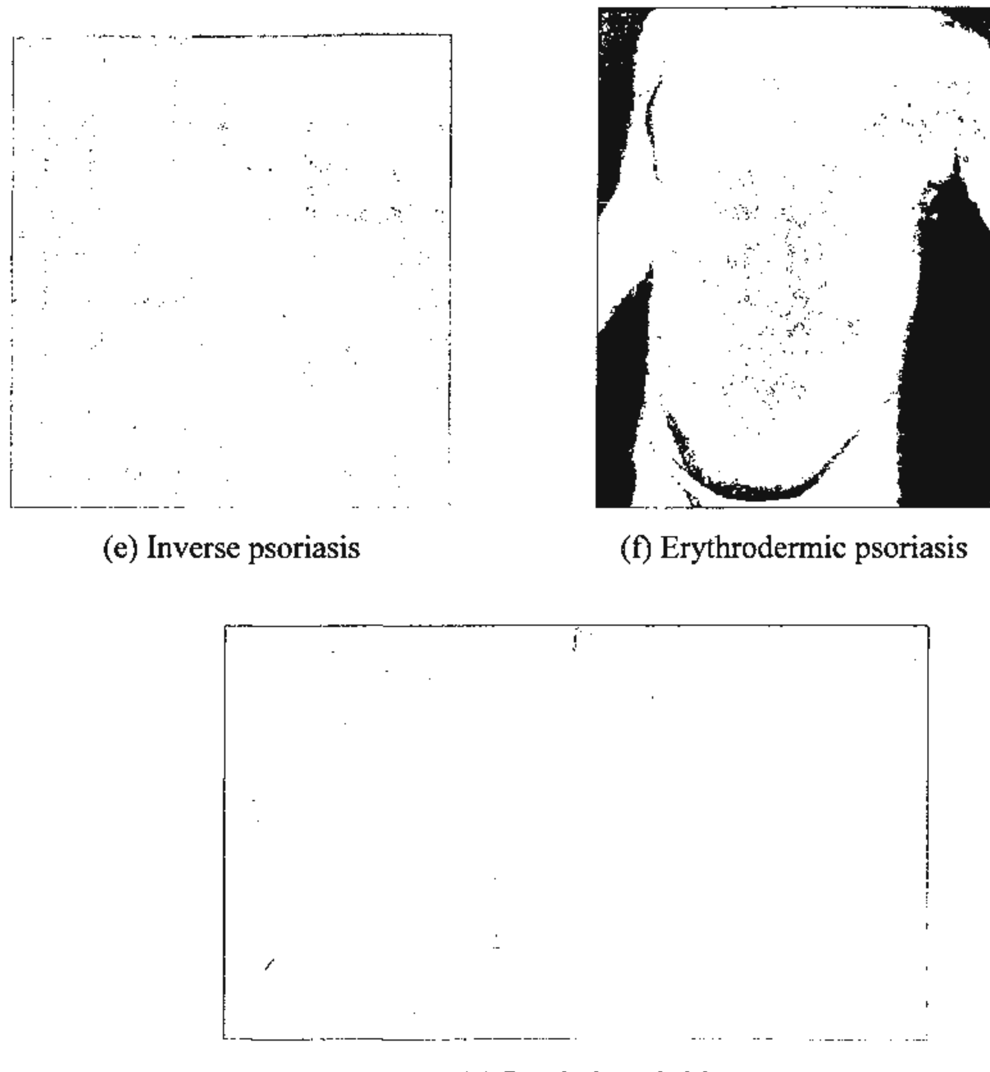


(b) Guttate psoriasis



(c) Palmoplantar pustular psoriasis

(d) Generalized pustular psoriasis



(e) Inverse psoriasis

(f) Erythrodermic psoriasis

(g) Psoriatic arthritis

Fig. 1.3. Different types of psoriasis. (a) Plaque psoriasis; (b) Guttate psoriasis; (c) Palmoplantar pustular psoriasis; (d) Generalized pustular psoriasis; (e) Inverse psoriasis; (f) Erythrodermic psoriasis; and (g) Psoriatic arthritis.

1.7. Severity of Psoriasis

Psoriasis is usually graded as mild (affecting less than 3% of the body surface), moderate (affecting 3-10% of the body surface) or severe (more than 10%) according to the definition by the National Psoriasis Foundation (NPF) of the USA. The palm of the hand roughly equals to 1% of the total body skin surface. Several scales exist for measuring the severity of psoriasis. The degree of severity is generally based on the following factors: the proportion of body surface area affected; degree of plaque redness, thickness and scaling; response to previous therapies; and the impact of the disease on the person. The Psoriasis Area Severity Index (PASI) is the most widely used measurement tool for psoriasis. PASI combines the assessment of the severity of lesions and the area affected into a single score in the range 0 (no disease) to 72 (maximal disease). Nevertheless, the PASI can be too unwieldy to use outside the context of clinical trials, which has led to attempts to simplify the index for clinical use (Louden et al., 2004).

The severity of psoriasis is also measured by how psoriasis affects a person's quality of life. Psoriasis can have a serious impact even if it involves a small area, such as the palms of the hands or soles of the feet. The majority of people with psoriasis have mild disease. Nearly one-quarter of people with psoriasis have cases that are considered moderate to severe (http://www.psoriasis.org).

1.8. Current Available Conventional Treatments for Psoriasis

Up to now, there is no proven cure for psoriasis, but a wide range of treatment modalities can give people control over the condition. It is well known that for psoriasis, no single treatment works for everyone, and different psoriasis may require different treatment method. Many patients require a regimen of different agents for different sites at different times. In general, treatment of psoriasis ranges from topical therapies for mild disease to phototherapy to systemic therapy for more widespread disease.

1.8.1. Topical Treatment

The first-line treatment for stable plaque psoriasis is topical therapy, as this type of treatment is known to be both safe and effective. There are a wide range of agents used for topical treatment including emollients, keratolytic agents, corticosteroids, vitamin D analogues, dithranol and coal tar.

1.8.1.1. Emollients

The term emollient is used interchangeably with the term moisturizer which can provide oil layer on the skin to slow water loss and thus increase the moisture content of the stratum. Emollients are an old, safe and inexpensive method for relieving psoriasis symptoms. The application hydrates and softens the thickened, scaly surface of psoriatic

plaques. Emollients used in psoriasis management include lotions, creams, ointment, glycerine and urea. The side effects of emollients inlcude irritant reactions, allergy, folliculitis and facial rashes (http://dermnetnz.org/treatments/emollients.html).

1.8.1.2. Keratolytic agents

The most widely used keratolytic agents in dermatology include urea, propylene glycol, resorcinol, and salicylic acid. 2 – 10% salicylic acid ointment is the most widely used keratolytic agent. As the name keratolytic suggests, it has the function of softening the psoriatic scales, thus can flatten and smoothen psoriatic plaques. Salicylic acid is well absorbed through the skin and can dissolve the built-up dead skin on psoriasis plaques (Camisa, 1998). However, it can be mildly irritated and so lower concentrations should be used initially; and it is better to avoid sensitive areas such as the centre of the face and around the eyes. Keratolytic agent is rarely used alone, instead it is often used in combination with other active agents such as topical corticosteroid or coal tar (Smith et al., 2002) so as to enhance effectiveness.

1.8.1.3. Coal tar

Coal tar is produced by destructive distillation of coal, and is frequently used in shampoos to reduce scalp inflammation and scaling. Coal tar is the traditional topical treatment for

psoriasis and remains as an effective anti-psoriatics today (Tham et al., 1994; Dodd, 1993; Williams et al., 1992). Although it is generally considered to be safe, theoretic concerns about its carcinogenic properties remain unabated. Coal tar elicits its anti-psoriatic action through suppressing DNA synthesis, thereby reducing the epidermal hyperproliferation that is the basic pathology of psoriasis (Walter et al., 1978). The kinetic of coal tar in psoriasis treatment is slow, although its anti-psoriatic effect can be enhanced and quickened with concomitant application of ultraviolet light (Graham-brown and Burns, 2002). In practical level, its poor physical appearance and unpleasant odour generally limit its use in the hospital setting. Moreover, coal tar ointment applications can cause folliculitis, and tar-induced sensitivity can occur in approximately 5% of patients (Clark et al., 1996).

1.8.1.4. Dithranol (Anthralin)

Dithranol is among the most effective topical anti-psoriatic treatments (Silverman et al., 1995; Lebwohl et al., 1995). Dithranol, also known as anthralin, is a tricyclic molecule derived from wood tar. Fig. 1.4. shows the chemical structure of dithranol. Pharmacologically, it has both anti-inflammatory and anti-mitotic effects on keratinocytes (Mahrle et al., 1994). In addition, dithranol inhibits DNA synthesis and cellular enzymes, in particular those involved in nucleic acid metabolism and oxidative

phosphorylation (Shroot, 1992). Dithranol can also effect a rapid action on the normalization of epidermal differentiation, with an increased expression of the differentiation-associated keratins K1, K2, and K10 and reappearance of filaggrin, which is filament-associated proteins for binding to keratin fibers in epidermal cells (Camisa, 1988). It has also been found to up-regulate interleukin-10 receptor expression on keratinocytes (Farkas et al., 2001). All these biological actions lead to the reduction of the epidermal hyperproliferation seen in psoriasis, thus ameliorate the symptoms of psoriasis. Dithranol therapy is usually initiated by application of low concentrations for a short period (de Mare et al., 1989). It has been shown that the mean PASI fell from 9.1 to 4.7 after 8 weeks of treatment with dithranol (Berth-Jones et al., 1992). Although dithranol offers a safe approach without severe adverse events, the staining and, to some extent, the obligatory irritation remains major drawbacks in its clinical use (Camisa, 1998).

OH O OH

Fig. 1.4. Chemical structure of dithranol

1.8.1.5. Topical corticosteroids

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex. Its basic structural skeleton is shown in Fig. 1.5. Corticosteroids are involved in a wide range of physiologic systems such as stress response, immune response and regulation of inflammation. Topical corticosteroids are the most commonly prescribed treatment for psoriasis. It is well-established that corticosteroids possess marked anti-inflammatory, anti-proliferative and immunomodulatory properties that are potentially relevant to their efficacy in psoriasis. However, the precise mode of action regarding corticosteroids for psoriasis treatment is not known (van de Kerkhof, 2001). It should be borne in mind that this form of treatment can produce serious side effects such as cutaneous atrophy, formation of telangiectasia and striate, and development of tachyphylaxis. Despite these drawbacks, topical corticosteroids are among the most effective treatments for psoriasis in modern dermatological practice (Mason et al., 2002). In view of its side effect, topical corticosteroids must be used under close supervision to avoid unnecessary side effects and it should only be applied for short periods of time.

Fig. 1.5. The basic chemical structure of corticosteroids.

1.8.1.6. Topical vitamin D analogues

Vitamin D analogues such as calciportriol, calcitriol and tacalcitol can be used for the treatment of psoriasis. The chemical structures of these vitamin D analogues are shown in Fig. 1.6. Vitamin D3 analogues have a range of biological effects that, via specific binding to the vitamin D3 receptor and also non-receptor-mediated events, are relevant to their therapeutic efficacy in psoriasis. The use of vitamin D analogues lead to the reduction of proliferation, enhancement of normal keratinization of keratinocytes and inhibition of accumulation of inflammatory cells, particularly neutrophils and T lymphocytes (Kragballe and Wildfang, 1990; Binderup and Bramm, 1988). Vitamin D analogues are also able to produce a shift towards Th2 cytokine expression, with an increase in IL-10 and a decrease in IL-8, which may mediate part of the improvement in psoriasis (Kang et al., 1998). In a recent study, treatment with 3 μg/g of calcitriol showed clinical clearance in 48% of the patients in comparison with 7% of placebo, and a further 41% had a considerable improvement (Langner et al., 2001).

Fig. 1.6. Chemical structures of (a) calcipotriol, (b) calcitriol and (c) tacalcitol.

1.8.1.7. Topical retinoids

Retinoids are a class of vitamin A derivatives that constitute the most recent development of topical treatment for psoriasis. Tazarotene is a typical vitamin A derivative and is also known as a topical retinoid. Fig. 1.7. depicts the chemical structure of this compound. Tazarotene is a receptor-selective retinoid found to be effective in treating mild to

moderate plaque psoriasis (van de Kerkhof, 2001) because of its ability in modulating keratinocyte proliferation and differentiation. In a large clinical study involving 324 patients, Weinstein and co-workers demonstrated that Tazarotene could lead to a reduction in scaling and plaque thickness in the active group in comparison with placebo (Weinstein et al., 1997). In order to increase the efficiency and reduce the irritation which is often associated with the use of tazarotene, the combination of other topical agents can be considered. The combination of tazarotene with topical corticosteroids has been studied for the purpose of avoiding retinoid dermatitis (Lebwohl et al., 1998).

O
OCH_2CH_3
N
S

Fig. 1.7. Chemical structure of tazarotene.

As a chronic skin disease, psoriasis would normally develop resistance to topical agents, especially topical corticosteroids. In such case, phototherapy or/and systemic treatment may be considered as an alternative option.

1.8.2. Phototherapy

When resistance is developed for topical agents after long time exposure, phototherapy can be considered as an effective alternative option for psoriasis. Phototherapy is particularly useful when the involved body surface area is extensive and plaques are small and thin, making topical therapy time consuming (Stern et al., 1986).

1.8.2.1. Ultraviolet B (UVB)

The benefits of sunlight for psoriasis were known long before phototherapy units were introduced for the treatment of psoriasis, and the use of broadband UVB phototherapy for psoriasis can be traced back to 1920s (Muller and Perry, 1984). The use of UV light therapy is well established; the most effective wavelengths being in the medium range that comprises part of the electromagnetic spectrum of wavelengths of 290-320 nm, known as UVB. The effects of UVB on human body are multiple, but its working mechanism for psoriasis is not completely understood. The exposure to UVB can exert effects on immune system that may contribute to its keratinocyte proliferative action, hence antipsoriatic effect (Voorhees, 1996). UVB can be administered in conjunction with a variety of topical and systemic treatments to achieve faster and more effective clinical results. For instance, UVB is often administered with topical corticosteroids (Meola et al., 1991), or with short-contact dithranol therapy (Boer and Smeenk, 1986), or

with calcipotriene (Hecker and Lebwohl, 1997). However, caution must be exercised when combining phototherapy with other agents because the possibility of increased photosensitivity and burning or shortened duration of remission (Hönigsmann, 2001).

1.8.2.2. Narrowband UVB

The spectrum of UV light found to be most effective for psoriasis treatment is in a narrow range around 311 nm. In recent years high-output bulbs emitting UV light in a narrow range around 311 nm have been developed. Narrowband UVB therapy has been shown to be more effective than broadband UVB (Picot et al., 1992; Storbeck et al., 1993). Narrow-band UVB treatment offers a reduction of side effects by limiting exposure to unnecessary wavelengths. Also, it is effective with fewer treatments, safer over the long term. In addition, as the equipment for emitting narrow-band UVB becomes less expensive, this form of phototherapy is now becoming more cost-effective and convenient for application than broad-band UVB.

1.8.2.3. Psoralen plus UVA (PUVA)

A major breakthrough in the treatment of psoriasis was made with the introduction of PUVA. PUVA was developed in the 1970's and involves the ingestion of 8-methoxypsoralen (8-MOP), a classical photosensitizer, and the chemical structure of

which is shown in Fig. 1.8., followed by irradiation with UVA. UVA consists of radiation wavelengths of 320 – 400 nm. Some patients treated with PUVA are able to achieve long-term remissions even without maintenance therapies. PUVA therapy suppresses DNA synthesis by cross-linking DNA strands and conjugating proteins (Epstein and Fukuyama, 1975). Treatments are administered 2 or 3 times per week and after 20 to 30 treatments, nearly 90% of patients achieve marked improvement or clearance of their psoriasis (Melski et al., 1977). In a long-term study involving 33 patients with at least 8% affected body surface area, it was found that 90% of the patients had initial clearance and 42% of these improved patients remaining in remission (Collins et al., 1996). The most common side effect of PUVA therapy is nausea that usually develops shortly after 8-MOP ingestion. The other side effects of PUVA are related to phototoxicity. Patients must avoid prolonged sun exposure, and should wear UVA-blocking sunscreens on the days when administering 8-MOP. Exposure to excessive sunlight or overexposure to phototherapeutic UVA could easily result in significant burns (Lebwohl and Ali, 2001a). Moreover, the PUVA regimen has been linked with an enhanced possibility of developing squamous cell carcinoma and malignant melanoma (Stern et al., 1997). In practice, the concerns over the enhanced chance of developing skin cancers greatly limits it from wide application.

O=C1C=CC2=CC3=C(OC=C3)C(OC)=C2O1

Fig. 1.8. Chemical structure of 8-methoxypsoralen.

1.8.2.4. Excimer laser

Recently, an excimer laser emitting UVB at 308 nm has been introduced into psoriasis treatment (Asawanonda et al., 2000; Trehan and Taylor, 2002). The laser can provide greater energy, therefore shorter treatment time is required. Also, it can deliver through a flexible hand piece that can direct at the affected areas. Psoriatic plaques are known to be able to tolerate higher doses of UV than adjacent unaffected skin (Speight and Farr, 1994). Excimer laser treatment has been reported to produce 75% of reduction in the severity of the treated plaques (Feldman et al., 2002).

1.8.3. Systemic Treatments

Systemic medications are prescription medications that affect the entire body, and are usually reserved for patients with moderate to severe psoriasis who are not responsive to or eligible for conventional topical or UV light treatments. The three most widely used systemic agents for psoriasis are methotrexate, oral retinoid and cyclosporin.

1.8.3.1. Methotrexate

Methotrexate (MTX; (S)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid), the chemical structure of which is presented in Fig. 1.9., has a long history of use for psoriasis and is highly effective for this disease (Jeffes and Weinstein, 1995; Collins and Rogers, 1992). Methotrexate is a potent inhibitor of the enzyme dihydrofolate reductase and prevents the conversion of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is an essential cofactor for the synthesis of thymidylate and purine nucleotides required for DNA and RNA synthesis. Recently, it has been revealed that MTX can inhibit aminoimidazolecarboxamide ribotide transformylase, causing adenosine to accumulate. As adenosine is a T-lymphocyte toxin, this mechanism may be partially responsible for the immunosuppressive effect of MTX (Baggott et al., 1999). MTX is particularly beneficial for patients with psoriatic arthritis. It is also indicated for the long-term management of severe forms of psoriasis including psoriatic erythroderma and pustular psoriasis. Since MTX is immunosuppressive, it should be avoided in patients with active infections. Short-term side-effects of MTX include nausea, aphthous stomatitis, and bone marrow toxicity. In addition, long-term use of MTX has been associated with hepatic fibrosis. Moreover, MTX interacts with many other medications. Caution should be exercised when concomitantly use it with other drugs, such as penicillin, sulfonylureas, pyrimethamine etc. The list of drugs what

could have potential drug-drug interactions with MTX has been compiled (Evans and Christensen, 1985).

Fig. 1.9. Chemical structure of methotrexate.

1.8.3.2. Oral retinoids

Retinoids are a class of compounds that comprise vitamin A and its natural and synthetic analogues such as etretinate, acitretin, isotretinoin and liarozole. Retinoids elicit their effects by binding to nuclear receptors, which regulate gene transcription. *In vivo* study showed that oral retinoids induce keratinocyte differentiation and reduce epidermal hyperplasia in psoriasis (Gottlieb et al., 1996). Retinoids can be dramatically effective as monotherapy for pustular and erythrodermic psoriasis, but work more slowly and less effectively for plaque and guttate psoriasis. Nevertheless, they can effectively improve the response of these two types of psoriasis to PUVA and UVB when using concomitantly. The main side effects of the retinoids include teratogenicity, elevated liver enzymes, hyperlipidaemia, hyperostosis, and cheilitis (Mendonca and Burden, 2003).

1.8.3.3. Cyclosporin

Cyclosporin was introduced for the prevention of the rejection of kidney transplantation in the 1970s. It is an immune inhibitor that prevents the activation of T cells by blocking interleukin-2 production (Ellis, 1993). The Chemical structure of cyclosporin is illustrated in Fig. 1.10. Cyclosporin blocks the intracellular components of T-cell activation by binding to a cytosolic immunophilin. The complex then binds to a cytosolic enzyme, calcineurin phosphatase, resulting in inhibition of nuclear factor of the T cells. Because cyclosporin is an immunosuppressive agent, it is contraindicated in patients with acute infection and in patients with active malignancy. Cyclosporin has been used for treatment of psoriasis for more than a decade and is very effective. The main side effects of cyclosporin include nephrotoxicity and hypertension; therefore, careful monitoring is required when prescribing this drug for psoriasis.

Fig. 1.10. Chemical structure of cyclosporin.

1.8.3.4. Other systemic agents for psoriasis

Tacrolimus, thioguanine, mycophenolate mofetil (MMF), azathioprine, sulfasalazine and fumaric acid esters (FAE) are also systemic drugs used to treat psoriasis. Fig. 1.11. depicts the chemical structures of these anti-psoriatics. Oral tacrolimus (also FK506 or Fujimycin) is a potent immunosuppressive agent that has been approved for the prevention of organ transplant rejection. Like cyclosporin, it inhibits T cell activation. In a clinical trial, tacrolimus was effective in the treatment of psoriasis, resulting in an 83% reduction in PASI scores (Kino et al., 1987). Thioguanine, which is an analogue of the natural purines, hypoxanthine and guanine, has been postulated to deplete cutaneous T cells by inducing apoptosis (Murphy et al., 1999). MMF, which is a semisynthetic morpholinoethylester of mycophenolic acid, has been shown to have a specific lymphocyte antiproliferative effect by reversibly blocking the de novo synthesis of guanine nucleotides required for DNA and RNA synthesis (Allison and Eugui, 2000). Azathioprine, a synthetic purine analogue synthesized by attaching 6-mercaptopurine to an imidazole ring, blocks purine biosynthesis, thereby resulting in inhibiting rapidly dividing cells (Loo et al., 1968). Sulfasalazine is an anti-inflammatory agent that is commonly used in the treatment of inflammatory bowel disease and is an effective treatment for rheumatoid arthritis and psoriasis (Camisa, 1994). FAE have been shown to have inhibitory effects on keratinocytes (Thio et al., 1994) and lymphocytes (Ockenfels et

al., 1998) and were approved for the treatment of psoriasis in Europe several years ago.

(a)

(b)

(c)

(d)

(e)

(f)

Fig. 1.11. Chemical structures of (a) tacrolimus, (b) thioguanine, (c) mycophenolate mofetil, (d) azathioprine, (e), sulfasalazine, and (f) fumaric acid esters.

1.8.4. Combination Therapy

Combination of two systemic therapies, or of a systemic therapy with UVB or PUVA, are often more effective than each of the individual therapies. In addition, the toxicities of individual therapies can be reduced since the dose of the individual treatments is often reduced when used in combination. Furthermore, photochemotherapy, which is the combination of phototherapy and systemic or topical therapies, can achieve higher clearance rates, longer disease–free intervals and a lower carcinogenic risk. Topical agents for psoriasis include anthralin, vitamin D analogues, corticosteroids, emollients, salt-water baths and tar. Among these, anthralin, vitamin D analogues and topical retinoids constitute the most relevant agents for combination with phototherapy. MTX may be combined with other treatments, including PUVA, UVB, cyclosporin, retinoids, sulfasalazine or hydroxyurea to clear the remaining lesions or to reduce side effects of treatment (van de Kerkhof, 2001).

1.8.5. New Therapies for Psoriasis

Our improved understanding of the immunological processes pertinent to psoriasis has provided new therapeutic targets. Innovations in biotechnology have the potential to offer pharmaceutical agents with greater safety by building designer drugs that interfere with specific targets in the pathogenesis of psoriasis. The cutaneous autoimmune

reaction is believed to play a causative role in the development of skin lesions in psoriasis. Current therapeutic strategies involve suppression of autoimmune reaction and/or modulating immunological processes. Biologic-based agents are novel therapeutic options in the treatment of moderate-to-severe psoriasis. Unlike traditional systemic anti-psoriatic drugs, which are chemically synthesized, these agents are unique in that they are derived from living organisms and hence called "biologics". The specificity of these biologics can theoretically avoid the side effects of the chemical-based systemic agents including the effects of hepatotoxicity, nephrotoxicity, and bone marrow suppression (van de Kerkhof, 2001). However, high costs and varying efficacies remain the restricting factors for these drugs.

1.8.5.1. Alefacept

Alefacept became the first biologic agent to be approved by FDA and the European Medicines Agency (EMEA) for the treatment of moderate-to-severe psoriasis. It is administered via intramuscular injection. Alefacept is a fusion protein combining a portion of human immunoglobulin and the binding site of lymphocyte function-associated antigen-3 (LFA-3). It binds to CD2, the partner molecule of leukocyte function associated antigen (LFA)-3 located on the surface of T cells, thus inhibiting memory T-cell activation and proliferation (Strober and Menon, 2007).

Alefacept also enhances binding between natural killer cells and T cells, resulting in T cell apoptosis. Since it also acts to deplete a subset of T cells, the absolute $CD4^+$ count may decline and must be monitored during treatment (Krueger and Callis, 2003). In a multicentre, placebo-controlled, double-blind, dose-escalation study of 12 weekly intravenous injections of alefacept in 229 patients with severe psoriasis, the mean reduction in PASI in the treatment groups was 50% (Ellis and Krueger, 2001).

1.8.5.2. Efalizumab

Efalizumab is a humanized monoclonal antibody that binds to the CD11a portion of LFA-1 and interferes with the LFA-1/intercellular adhesion molecule 1 (ICAM-1) interaction, thus blocking T cell activation and migration. Efalizumab can decrease recruitment of natural killer cells in the skin; hence, enables a rapid disappearance of lesional $CD11c^+$ dendritic cells (Lowes et al., 2005). Efalizumab has been approved by FDA/EMEA for the treatment of psoriasis and psoriatic arthritis. Most of the patients who achieved greater than 75% reduction in PASI scores in the first 12 weeks of treatment sustained that response or achieved even further clearing with an additional 12 weeks of treatment.

1.8.5.3. Etanercept

Etanercept is composed of two p75 TNF- α receptors fused to human Fc portion of IgG_1. Etanercept contains two recombinant p75 extracellular receptors allowing it to bind to both TNF- α and TNF- β, and forms relatively unstable complexes with TNF- α trimmers (Gisondi et al., 2004). It reduces the amount of various cytokines and chemokines in psoriatic lesions resulting in reduction in T cell and dendritic cell infiltration and in epidermal thickness (Gottlieb et al., 2005). It is administered twice weekly by subcutaneous injection. Etanercept has now been approved by FDA for the treatment of psoriatic arthritis. In phase III trials, half of the psoriasis patients who received 50 mg Etanercept twice a week for 3 months showed a 75% reduction in PASI (Leonardi et al., 2003; Tyring et al., 2006).

1.8.5.4. Infliximab

Infliximab is a chimeric human/mouse anti-TNF- α monoclonal antibody administered by intravenous infusion. Infliximab, approved by the FDA in 2006 for the treatment of psoriasis, is a chimeric monoclonal antibody specific to TNF-α and human IgG_1. Binding of Infliximab to soluble TNF-α neutralizes its activity, thereby preventing the cytokine from binding to its cell surface receptors and thus decreasing inflammation (Gottlieb et al., 2003). Infliximab also reduces vascular endothelial growth factor (VEGF)

and thus reduce blood supply at the site of tissue damage leading to an improvement of psoriatic conditions (Goedkoop et al., 2004). Reduced keratin 16 (a marker of abnormal keratinocyte differentiation) expression, keratinocyte apoptosis, and decreased endothelial cell adhesion molecule expression have also been observed during Infliximab therapy (Goedkoop et al., 2004; Kruger-Krasagakis et al., 2006).

1.8.5.5. Adalimumab

Adalimumab is a recombinant human IgG1 monoclonal antibody specific for human TNF-α, which binds soluble and membrane-bound TNF-α with high specificity and affinity. The binding blocks its interaction with the p55 and p75 cell surface TNF receptor (Weinberg et al., 2005). After treatment with Adalimumab, normal epidermal differentiation was rapidly restored without detectable apoptosis. It also showed a significant decrease in TNF-α-positive dermal dendritic cells and macrophages (Marble et al., 2007). Adalimumab was approved by the FDA in 2002 for the treatment of rheumatoid arthritis in adults. In a phase II clinical trial, it was demonstrated that subcutaneous adalimumab was effective in moderate-to-severe psoriasis treatment (Gordon et al., 2004).

1.9. Treatment of Psoriasis with Chinese Medicine

Conventionally, treatments for psoriasis include topical therapy (Mason et al., 2002), phototherapy (Hőnigsmann, 2001) and systemic therapy (van de Kerkhof, 2001; Callen et al., 2003; Mendonca and Burden, 2003). However, the current treatments have not fully met the needs of the sufferers, largely due to the side effects so often associated with various therapies. Also, a large proportion of patients would develop drug resistance after long term drug exposure (Lebwohl and Ali, 2001a, b). Because of these limitations associated with conventional treatments, many patients turn to alternative and complementary medicine for help. As an alternative medicine, traditional Chinese medicine (TCM) has been widely used for the treatment of a variety of skin conditions including psoriasis in China (Koo and Arian, 1998; Tse, 2003). In this session, an overview would be conducted on the use of TCM in the treatment of psoriasis.

1.9.1. Psoriasis in Chinese Medicine

TCM is one of the great treasures and an indispensable part of the splendid classic Chinese culture. The Chinese medical tradition embraces more than five thousand years of medical practice, and is very much alive today with more than one billion people in the world depending on it for basic medical care. The basic theories of TCM mainly originated from the medical classic *Huang Di Nei Jing* (The Yellow Emperor's Classic of

Internal Medicine). The basic characteristics of TCM are the concepts of holism and syndrome differentiation and treatment determination. In TCM practice, treatment strategies and therapeutic methods are decided according to the result of syndrome differentiation. The basic principles of treating diseases with Chinese medicinal herbs are to eliminate causes of disease, dispel pathogenic factors and restore the harmonious functions of the body.

Psoriasis is one of the commonest skin conditions encountered in TCM dermatological practice. With regards the aetiology and pathogenesis of psoriasis in TCM, it is believed that psoriasis is caused by invasion of evil heat in the blood that is caused by various factors such as invasion of six environmental evils, internal disharmony due to excessive seven emotions and improper diet. The long course of the disease can also give rise to blood stasis as the pathogenic factor.

As TCM emphasizes that treatment of disease should base on pattern recognition and differentiation, psoriasis is commonly classified into three main syndrome types, namely blood heat, blood stasis and blood deficiency types (Liang, 1988; Koo and Arain, 1998). Each pattern type has its own clinical manifestations and, therefore, requires dissimilar treatment strategies and herbal formulae.

1.9.1.1. Oral medications for psoriasis based on pattern differentiation

(i) Blood heat type

(a) Etiopathogenesis

Blood heat is an important causative factor in human body for causing psoriasis. The seven emotions may cause internal injury leading to depression of vital energy whose prolonged stagnation may produce heat syndrome. Besides, irregular diet or over-taking of meat and fish may cause a disharmony between the spleen and the stomach, which transforms into heat which can subsequently invades blood phase. Erythema of the psoriatic skin is due to retention of heat in superficial part of the body. Heat may lead to deep red color and bleeding in the lesions of psoriasis (Zuo and Zhu, 2002).

(b) Clinical manifestations

The main symptoms and signs of blood heat type of psoriasis include serious itching, red papules on the skin, scaly erythematous lesions with red halo around them, red tongue with thin and yellow fur, and taut and rapid pulse. The lesions of this type usually have acute onset, many in numbers, punctiform or oval, and extremely red. These lesions also have numerous thin, silver squames and tiny bleeding points. During the course of the disease, new lesions continue to appear. In addition, patients may complain of thirst, dryness of the tongue, constipation, and deep-colored urine. (Koo and Arain, 1998)

(c) Internal treatment with Chinese herbs

Therapeutic principle for the blood heat type of psoriasis is to clear away pathogenic heat, and to remove toxin and cool the blood. The representative recipe achieving this treatment strategy is Lonicera and Polygoni Cuspidati Decoction (銀花虎杖湯加減) with variations. The ingredients of the formula include Flos Lonicerae Japonicae (銀花), Radix et Rhizome Polygoni Cuspidati (虎杖), Radix Salviae Miltiorrhizae (丹參), Caulis Piperis Futokadsurae (海風藤) 15 g each; Radix Rehmanniae Glutinosae (生地), Radix Angelicae Sinensis (歸尾), Radix Paeoniae Rubrae (赤芍), Flos Sophorae Japonicae Immaturus (槐花), 12 g each; Folium Daqingye (大青葉), Cortex Moutan Radicis (丹皮), Radix Arnebiae seu Lithospermi (紫草), Radix Sophorae Tonkinensis (北豆根), Radix Adenophorae seu Glehniae (沙參), 10 g each (Liang, 1988).

(ii) Blood stasis type

(a) Etiopathogenesis

Blood stasis in Chinese medicine refers to extravasations of blood that is not excreted or dispersed in time and retained in the body; or stagnation of blood in the meridians, vessels or organs and tissues due to inhibited circulation of blood. According to the blood stasis hypothesis, blood heat in psoriasis lesions can cause the injury of yin and blood leading to the increased viscosity of the blood, hence the slow circulation of the blood resulting in

blood stasis.

(b) Clinical manifestations

Blood stasis type corresponds to a chronic, relapsing form of psoriasis and the course of the disease is usually long. The main symptoms and signs of blood stasis type of psoriasis include thick, scaly, dry and dark-red lesions. The scales on erythema or the erythema with silver scales on the skin may not subside over time. Itching of lesional skin is mild or not obvious. The disease either ceases to extend or extends only slowly, and some lesions may resolve gradually. Squames, Auspitz phenomenon (appearance of punctate bleeding spots), a purple tongue, thin and astringent pulse may also be seen in psoriasis of the blood stasis type.

(c) Internal treatment with Chinese herbs

The therapeutic principle for blood stasis type of psoriasis is by invigorating the blood and removing stasis, dissipating nodules and unblocking the blood vessels. The representative formula is Radix Astragali Membranaceus, Radix Salviae Miltiorrhizae Decoction (黃芪丹參湯加減) with variations. The make-up ingredients of the formula include Radix Salviae Miltiorrhizae (丹參), Herba Lycopi Lucidi (澤蘭), Radix Rubiae Cordifoliae (茜草),Radix et Caulis Jixueteng(雞血藤)15g each; Radix Astragali Membranaceus (黃芪), Pericarpium Citri Reticulatae Viride (青皮), Rhizoma Cyperi Rotundi (香附) 10g each; Rhizoma Curcumae Ezhu (莪朮), Radix Paeoniae Rubrae (赤

芍), Rhizoma Sparganii Stoloniferi (三棱), Zaocys Dhumnades (烏蛇), Fasciculus Vascularis Luffae (絲瓜絡), Flos Campsis(凌霄花) 6 g each (Liang, 1988).

(iii) Blood deficiency type

(a) Etiopathogenesis

This type of psoriasis is actually caused by long term of illness, which leads to the deficiency of Yin-blood giving rise to wind and dryness in the muscles and skin. The long duration of blood deficiency and lack of nourishment in the skin may induce skin eruptions, or the disease may be caused by a deficiency of nutritive blood which blocks the flowing of blood to form stagnation at the superficial layer (Lin, 1990). The psoriasis of this type usually occurs at the chronic stage of the disease.

(b) Clinical manifestations

This is a typical chronic form of psoriasis. The psoriatic lesions are pale-red and thin and do not spread or resolve. These lesions are usually covered with silvery scales and itching of the lesions could be severe. Patients may have pale or sallow complexion, pale eyelids, lips and nails. Vertigo, insomnia and constipation may also be the accompanying symptoms. The tongue is usually pale in color and covered with a thin, white coating and the pulse is thin and weak (Koo and Arain, 1998).

(c) Internal treatment with Chinese herbs

Therapeutic principle is to nourish the blood and disperse the wind, to augment the qi and replenish the nutritive. The representative herbal formula is the Nourishing Blood and Dispersing Wind Decoction (養血祛風湯加減) with variations. The ingredients of the formula include Radix Astragali Membranaceus (黃芪), Radix Codonopsitis Pilosulae (黨參), Radix Angelicae Sinensis (當歸), Semen Cannabis Sativae (火麻仁) 10 g each; Radix et Caulis Jixueteng (雞血藤), Cortex Dictamni Dasycarpi Radicis (白鮮皮), Radix Paeoniae Lactiflorae (白芍), Radix Rehmanniaae Glutinosae Conquitae (熟地黃), Radix Scrophulariae (玄參), Tuber Ophiopogonis Japonici (麥冬), Fructus Tribuli Terrestris (白蒺藜), Radix Angelicae Dahuricae (白芷) 6 g each (Liang, 1988).

1.9.1.2. Topical preparations for psoriasis

In TCM dermatological practice, oral medication is the most popular form of herbal medicine used in psoriasis treatment, but the topical preparations are also commonly applied on the psoriatic lesions to elicit direct therapeutic effects on the skin. The main forms of external preparations for psoriasis treatment include tincture, ointment, lotion and soaking bath. The topical application can be single herb or many herbs combined in a formula. Over the years, many topical formulas and preparations have been developed for psoriasis treatment. The comprehensive survey of the herbal formulas is beyond the

scope of the present review. Nevertheless, a list of individual herbs or medicinal substances that are most commonly used as ingredients in various topical preparations are provided here. These include: Radix Arnebiae seu Lithospermi (紫草), Radix Sophorae Flavescentis (苦參), Cortex Moutan Radicis (牡丹皮), Radix Paeoniae Rubrae (赤芍), Radix Rehmanniae Glutinosae (生地黃), Radix Salviae Miltiorrhizae (丹參), Radix Scutellariae Baicalensis (黃芩), Rhizoma Imperatae Cylindricae (白茅根), Cortex Dictamni Dasycarpi Radicis (白鮮皮) Radix Isatidis seu Baphicacanthi (板藍根). Radix Rehmanniae Glutinosae (生地黃), Radix et Rhizome Polygoni Cuspidati (虎杖), Cortex Phellodendri (黃柏), Herba Taraxaci Mongolici (蒲公英), Fructus Cnidii (蛇床子), Flos Chrysanthemi Indici (野菊花), Flos Carthami Tinctorii (紅花), Herba Menthae Haplocalycis (薄荷), Mirabilitum (芒硝), Indigo naturalis (青黛) and Sulphur (硫磺) (Hu and Xun, 2008; Lin et al., 2006; Luo, 2000; Zhu et al., 2008)

1.9.2. Clinical Studies of Chinese Medicine in Psoriasis

TCM has a long history in treating psoriasis. Over the past several decades, many clinical studies on the use of Chinese herbal medicine for the treatment of psoriasis have been conducted in China, and many of these studies were published in Chinese medical journals. Among these studies, most of them are not randomized, controlled and blind trials, and only a small number of these studies were with randomization with control

design. Most all of these studies indicated Chinese herbal formulas were effective and beneficial in improving psoriasis symptoms. Table 1.1. summarizes the clinical response of the herbal formulas in the clinical treatment of psoriasis. It is also evidenced from these trials that the most frequently used herbal materials belonged to the categories of heat-clearing and toxin-relieving, blood invigorating and cooling, and stasis dispersing. The encouraging results shown in the clinical studies suggest that Chinese herbal medicine is a promising source from which effective anti-psoriatic agents could be found. Based on this rationale, we decided to undertake a research project to systematically investigate the commonly used psoriasis-treating Chinese medicinal materials with an explicit aim to identify effective anti-psoriatic agents.

Table 1.1. A review of clinical studies on the effectiveness of Chinese herbal medicines in the treatment of psoriasis

Authors	TCM treatment methods	Study design[a]	No. of cases	Treatment response		
				Cure	Fair response	No response
Liu et al., 2002	Qi tonifying & blood invigorating	C	40	30%	70%	
Yang and Pi, 2002	Heat clearing & toxin relieving	U	38	60%	20%	20%
Lu et al., 2002	Blood invigorating, heat clearing & stasis dispersing	U	200	75%	23.5%	1.5%
Liu et al., 2002	Heat clearing & blood invigorating	R, C	129	60%	20%	20%
Deng et al., 2002	Blood invigorating, heat clearing & stasis dispersing	C	48	16.67%	60.42%	22.91%
Zhu and Hu, 2000	Toxin relieving & blood cooling	R, C	87	59.8%	27.6%	12.6%
Wang, 2003	Heat clearing & blood cooling	U	120	55.8%	34.2%	10%
Wang et al., 2003	Qi tonifying	R, C	48	66.7%	16.7%	16.6%

[a] C, design with control group; R, design with randomization; U, Uncontrolled study.

1.10. Aims and Objectives of the Present Study

Psoriasis is a common chronic skin condition that often develops in early adulthood with typical disease duration of more than 30 years (Stern, 1997). Although clinical diagnosis of psoriasis is relative easy, the aetiopathogenesis associated with this skin condition is far from well understood. Because of this lack of clear and identified aetiology, currently there remain no aetiology-oriented therapeutic approaches available to treat this distressing skin disease, and a substantial proportion of psoriasis patients are globally dissatisfied with the most commonly used therapies today (Stern, et al., 2004).

On the other hand, TCM has been used for the treatment of many diseases for centuries; and in China today, this traditional form of treatment still remains a popular option for management of psoriasis (Koo and Arian, 1998; Tse, 2003). Data derived from evidence-based clinical investigations have indicated that Chinese herbal medicines are effective and safe in the treatment of psoriasis (see the review in Table 1.1.). It is therefore rational that systematic screening of the anti-psoriasis Chinese herbal medicines could lead to identification and characterization of new and effective therapeutic agents for psoriasis.

In view of the current understanding of psoriasis, it is believed that the pathomechanism

of psoriasis invariably involve three cardinal basic characteristics, i.e. T cell-mediated inflammation, keratinocyte hyperproliferation and aberrant differentiation. To identify agents that are capable of inhibiting keratinocyte proliferation, modulating keratinocyte differentiation and attenuating inflammatory response would be a promising pathway leading to effective treatment for this distressing skin disease. Based on the fact that Chinese herbal medicine represents a promising therapeutic approach for psoriasis, the aims and objectives of the present PhD project were established. These were:

1. To select Chinese herbs based on their ethnomedical use in the treatment of psoriasis.
2. To screen the selected Chinese herbs for anti-proliferative action on the growth of cultured human keratinocytes. In our project, an immortalized HaCaT human keratinocyte cell line would be employed as a psoriasis-relevant in vitro experimental model
3. To test and to identify the chemical components presented in the effective Chinese herbal substances for anti-proliferative action.
4. To investigate the mechanism of action underlying the observed herbs-induced anti-proliferative action. In our experiments, we would focus on the cellular apoptosis as the possible action mechanism for the herbs-mediated anti-proliferative effects.

5. To determine the anti-inflammatory properties of the identified anti-proliferative and apoptogenic agents using PHA-stimulated human peripheral blood mononuclear cell (PBMC) model.
6. To elucidate the keratinocyte differentiation-modulating properties of the identified anti-proliferative and apoptogenic agents using a mouse tail animal model.

It was anticipated that through these systematic investigations, some Chinese herbal substances and chemical compounds would be identified as having anti-proliferative, differentiation modulating effects on keratinocytes and inflammation-attenuating action. The discovery and characterization of these anti-psoriatic herbal substances and chemical constituents would open a new path and add new dimensions to our current limited options for the management of psoriasis.

Chapter Two

Selection and Screening of Chinese Medicinal Materials for Anti-proliferative Action on HaCaT Human Keratinocytes

2.1. Introduction

Psoriasis is a disease of distinct epidermal hyperplasia resulted from the hyperproliferation and abnormal differentiation of keratinocytes (Camisa, 1998). Compounds that inhibit keratinocyte proliferation are potentially useful in the treatment of psoriasis because a rebalanced homeostatic control of keratinocyte growth and differentiation is crucial for recovery from psoriatic to normal epidermis. Drugs that counteract keratinocyte hyperproliferation are still the mainstay in psoriasis management today, and identifying anti-proliferative agents against keratinocytes remains an area of active research (Takahashi and Iizuka, 2004).

Data derived from clinical investigations have indicated that Chinese herbal medicines are effective and safe in the management of psoriasis (Jia and Wu, 2002; Zhang and Qu, 2002). However, the mechanisms underlying the effects of Chinese herbal medicines have largely remained uninvestigated. Only a few articles in English such as those of Koo and Arain (1998), Zhang and Qu (2002) and Tse (2003) are currently available to provide an overview of the efficacy, mechanism of action as well as adverse effects of TCM in the treatment of psoriasis. In view of the fact that some Chinese medicinal materials have been reported to achieve positive results in the treatment of psoriasis, it is anticipated that some of these herbs might possess inhibitory effect on the growth of

keratinocytes. It is against such backdrop that in this study we aimed to search traditional Chinese herbs for anti-proliferative agents on the growth of keratinocytes. We intended to select herbs that are commonly prescribed and well documented in the literature for the treatment of psoriasis in TCM practice. The selected herbs would then be subjected to a screening programme employing HaCaT cell culture as a psoriasis-relevant *in vitro* model in a hope that agents with keratinocyte growth inhibition could be found. Before selecting appropriate Chinese herbs for screening of anti-proliferative action, it is essential to survey the use of Chinese herbal medicine in the treatment of psoriasis.

2.2. Selection and Preparation of Chinese Medicinal Materials

2.2.1. Introduction

In the herbal screening programme to find active ingredients for psoriasis, the selection of these herbs was primarily based on the empirical uses and clinical studies that have been documented in the Chinese medical literature showing promising results in the clinical treatment of psoriasis. As has been discussed in Section 1.9. in the Chapter 1, psoriasis in TCM is commonly classified into three main syndrome types according to the disease history and clinical manifestations, namely blood heat, blood stasis and blood deficiency types (Liang, 1988; Koo and Arain, 1998). Based on these pattern classifications, appropriate treatment strategies are established for its treatment, including heat clearing and blood cooling, blood invigorating and stasis removing, wind expelling and itchiness stopping, and qi tonifying and blood replenishing. Many herbal formulae, both for internal and topical uses, have over the years been put forward to achieve the treatment strategies in the management of psoriasis in TCM practice.

In our project, a broad base approach was used for the selection of Chinese medicinal materials. The Chinese medicinal substances which are commonly prescribed in the clinical studies for the treatment of psoriasis, regardless of the quality of the study design, were included in the selection list. Most of these clinical studies were

published in Chinese medical journals or dermatology books. We also focused our literature search on articles published during the period of 2000 to 2005. The Chinese medicinal materials used both externally or internally were considered. After a thorough survey of the Chinese medical literature in psoriasis treatment, 60 Chinese medicinal substances derived from plant, animal or mineral sources were selected for the current screening programme. Among these materia medica, they are broadly classified into five different groups according to their functions and therapeutic indications in Chinese materia medica. These include: (i) heat-clearing and toxin-relieving; (ii) blood invigorating and cooling, and stasis dispersing; (iii) wind and dampness dispelling; (iv) qi tonifying; and (v) substances for topical application.

2.2.2. Materials and Methods

2.2.2.1. Sources of medicinal materials

All the medicinal materials were purchased from the Hung Kei Herbal Company, Hong Kong, and their identities were authenticated by Dr. Yu-ying Zong, a seasoned pharmacognosist by macroscopically comparing with the authenticated specimens stored at the Herbarium of the School of Chinese Medicine, Faculty of Science, The Chinese University of Hong Kong. Voucher specimens have been deposited in the Herbarium of the School, with reference no. PSO. 01 – 60. Table 2.1. shows a list of

60 Chinese medicinal materials selected for the study, together with the references where they were found to have been used for the treatment of psoriasis in TCM.

Table 2.1. Chinese medicinal substances selected for the initial screening programme.

Botanical / Zoological Name	Pharmaceutical name	Pinyin	Chinese Name	Part used	References	Extract yield (%)[a]
Heat clearing and toxin relieving medicinal substances						
Aconitum bullatifolium var. homotrichum	Radix Aconitum Bullatifolium	xue shang yi zhi hao	雪上一枝蒿	Root	Xu et al., 2003	21.0
Baphicacanthus cusia (Nees) Brem.	Indigo Naturalis	qing dai	青黛	Natural dye	Zhuang, 2001; Liu et al., 2002	2.7
Camellia sinensis (L.) O. Ktze.	Folium Camellia Sinensis	lü cha	綠茶	Leave	Qiu et al., 2000	45.6
Coptis chinensis Franch.	Rhizoma Coptidis	huang lian	黃連	Rhizome	Qin, 2001; Zhuang, 2001	21.7
Cryptotympana atrata Fabr.	Periostracum Cicadae	chan tui	蟬蛻	Slough shed	Qin, 2001; Liu, 2002	2.7

Dictamnus dasycarpus Turcz.	Cortex Dictamni Dasycarpi Radicis	bai xian pi	白鲜皮	Root	Xu, 2000; Wang et al., 2002	10.8
Euphorbia fischeriana Steud.	Radix Euphorbia fischeriana	lang du	狼毒	Root	Qin, 2001; Zhuang, 2001	31.8
Forsythia suspensa (Thunb.) Vahl	Fructus Forsythiae Suspensae	lian qiao	連翹	Fruit	Xu, 2000; Liu et al., 2002	12.6
Gardenia jasminoides Ellis	Fructus Gardeniae Jasminoidis	zhi zi	梔子	Fruit	Liu, 2002; Lu et al., 2002	22.1
Heydyotis diffusa (Willd.) Roxb.	Herba Hedyotidis Diffusae	bai hua she she cao	白花蛇舌草	Whole plant	Liu, 2002; Yang and Pi, 2002	6.3
Kochia scoparia (L.) Schrad.	Fructus Kochiae Scopariae	di fu zi	地膚子	Seed	Luo, 2000; Lu et al., 2002	7.6
Lithospermum erythrorhizon Sieb. et Zucc.	Radix Arnebiae seu Lithospermi	zi cao	紫草	Root	Liu, 2002; Wang et al., 2002	4.6

Lonicera japonica Thunb.	Flos Lonicerae Japonicae	jin yin hua	金銀花	Flower bud	Deng et al., 2002; Liu, 2002	41.1
Lonicera japonica Thunb.	Caulis Lonicerae	ren dong teng	忍冬藤	Vine	Zhu, 1983; Luo, 2000	10.2
Portulaca oleracea L.	Herba Portulacae Oleraceae	ma chi xian	馬齒莧	Whole plant	Liu et al., 2005	17.5
Rheum palmatum L.	Radix et Rhizoma Rhei	da huang	大黃	Rhizome	Deng et al., 2002; Wang et al., 2002	43.3
Scutellaria baicalensis Georgi	Radix Scutellariae Baicalensis	huang qin	黃芩	Root	Liu, 2002; Wang et al., 2002	36.3
Scutellaria barbata D. Don	Herba Scutellariae Barbatae	ban zhi lian	半枝蓮	Aerial parts	Xu, 2000; Qin, 2001	8.4
Sophora flavescens Ait.	Radix Sophorae Flavescentis	ku shen	苦參	Root	Xu, 2000; Lu et al., 2002	25.9

Taraxacum mongolicum Hand.-Mazz.	Herba Taraxaci Mongolici cum Radice	pu gong ying	蒲公英	Whole plant	Xu, 2000; Qin, 2001	19.8
Tripterygium wilfordii Hook. f.	Radix Tripterygii wilfordii	lei gong teng	雷公藤	Root	Qin, 2001; Zhuang, 2001	8.8
Blood invigorating and cooling, stasis removing herbs						
Campsis grandiflora (Thunb.) Loisel	Flos Campsis	ling xiao hua	凌霄花	Flower	Xu, 2000; Lu et al., 2002	12.9
Carthamus tinctorius L.	Flos Carthami Tinctorii	hong hua	红花	Flower	Deng et al., 2002; Liu, 2002	25.2
Curcuma zedoaria (Berg.) Roscoe	Rhizoma Curcumae Ezhu	e zhu	莪术	Rhizome	Qin, 2001; Zhuang, 2001	5.8
Imperata cylindrica (L.) P. Beauv. var. Major (Nees) C.E. Hubb.	Rhizoma Imperatae Cylindricae	bai mao gen	白茅根	Rhizome	Deng et al., 2002; Lu et al., 2002	34.5

Ligusticum chuanxiong Hort.	Radix Ligustici Chuanxiong	chuan xiong	川芎	Radix	Deng et al., 2002; Wang et al., 2002	29.4
Paeonia suffruticosa Andr.	Cortex Moutan Radicis	mu dan pi	牡丹皮	Root bark	Deng et al., 2002; Liu, 2002	21.8
Paeonia veitchii Lynch	Radix Paeoniae Rubrae	chi shao	赤芍	Root	Liu, 2002; Wang et al., 2002	31.4
Prunus persica (L.) Batsch.	Semen Persicae	tao ren	桃仁	Seed	Liu, 2002; Wang et al., 2002	8.22
Rehmannia glutinosa (Gaertn.)Libosch.	Radix Rehmanniae Glutinosae	sheng di huang	生地黄	Root	Deng et al., 2002; Liu, 2002	58.9
Rubia cordifolia L.	Radix Rubiae Cordifoliae	qian cao gen	茜草根	Root	Xu, 2000; Qin, 2001	10.9

Salvia miltiorrhiza Bge	Radix Salviae Miltiorrhizae	dan shen	丹参	Root	Liu, 2002; Lu et al., 2002; Wang et al., 2002	33.8
Sinomenium acutum (Thunb.) Rehd. et Wils.	Caulis Sinomenium	xue feng teng	血楓藤	Vine	Lu et al., 2002	19.0
Sparganium stoloniferum Buch.-Ham.	Rhizoma Sparganii Stoloniferi	san leng	三棱	Rhizome	Qin, 2001; Zhuang, 2001	2.4
Spatholobus suberectus Dunn.	Radix et Caulis Jixueteng	ji xue teng	雞血藤	Vine	Deng et al., 2002; Liu, 2002	23.2
Wind and dampness dispelling medicinal substances						
Alisma plantago-aquatica L. var. *orientale* Samuels	Rhizoma Alismatis Orientalitis	ze xie	澤瀉	Rhizome	Liu, 2002; Yang and Pi, 2002	17.8
Artemisia capillaris Thunb.	Herba Artemisiae Yinchenhao	yin chen hao	茵陳蒿	Aerial parts	Yang and Pi, 2002	8.2

Bombyx mori L.	Bombyx Batryticatus	jiang can	僵蠶	Dead body	Qin, 2001; Liu, 2002	13.3
Buthus martensi Karsch	Buthus Martensi	quan xie	全蠍	Whole body	Qin 2001; Lu et al., 2002	17.3
Clematis chinensis Osbeck	Radix Clematidis	wei ling xian	威靈仙	Root/rhizome	Zhu, 1983; Luo, 2000	23.5
Coix lacryma-jobi L.	Semen Coicis Lachryma-jobi	yi yi ren	薏苡仁	Seed	Luo, 2000; Liu, 2002	5.8
Dioscorea hypoglauca Palib.	Rhizoma Dioscoreae Hypoglaucae	bi xie	萆薢	Rhizome	Luo, 2000; Liu, 2002	11.5
Elaphe taeniurus Cope, E. carinata	Exuviae Serpentis	she tui	蛇蛻	Slough shed	Lu et al., 2002	5.3
Pheretima aspergillum Perrier	Lumbricus	di long	地龍	Whole body	Qin, 2001; Liu, 2002	13.5

Piper futokadsura Sieb. et Zucc.	Caulis Piperis Futokadsurae	hai feng teng	海風藤	Vine	Jia and Wu, 2002; Wang et al., 2004	14.9
Polyporus umbellatus (Pers.) Fr.	Sclerotium Polypori Umbellati	zhu ling	猪苓	Sclerotium	Liu, 2002	1.96
Saposhnikovia divaricata (Turez.) Schischk	Radix Ledebouriella Divaricatae	fang feng	防風	Root	Zhou, 1989; Liu, 2002	27.8
Scolopendra subspinipes mutilans L. Koch.	Scolopendra Subspinipes	wu gong	蜈蚣	Whole body	Qin, 2001	29.6
Smilax glabra Roxb.	Rhizoma Smilacis Glabrae	tu fu ling	土茯苓	Rhizome	Liu, 2002; Wang et al., 2002	14.9
Tribulus terrestris L.	Fructus Tribuli Terrestris	ci ji li	刺蒺藜	Fruit	Liu, 2002; Lu et al., 2002	7.9
Zaocys dhumnades (Cantor)	Zaocys Dhumnades	wu shao she	烏梢蛇	Whole body	Deng et al., 2002; Liu, 2002	8.7

Qi tonifying herbs						
Astragalus membranaceus (Fisch.) Bge.	Radix Astragali Membranaceus	huang qi	黄芪	Root	Deng et al., 2002; Liu, 2002	32.0
Codonopsis pilosula (Franch.) Nannf.	Radix Codonopsitis Pilosulae	dang shen	黨参	Root	Xu, 2000	49.9
Glycyrrhiza uralensis Fischer	Radix Glycyrrhizae Uralensis	gan cao	甘草	Root	Liu, 2002; Lu et al., 2002	34.1
Polygonatum sibiricum Redoute,	Rhizoma Polygonati	huang jing	黄精	Rhizome	Liu, 2002	64.8
Psoralea corylifolia L.	Fructus Psoraleae Corylifoliae	bu gu zhi	補骨脂	Seed	Qin, 2001; Zhuang, 2001	21.6
Substances for topical application						
Cnidium monnieri (L.) Cusson	Fructus Cnidii Monnieri	she chuang zi	蛇床子	Fruit	Luo, 2000	89.6
Mylabris phalerata Pall.	Mylabris	ban mao	斑蝥	Whole body	Zhuang, 2001	27.9
Realgar (Oxide mineral)	Realgar	xiong huang	雄黄	Mineral	Fan and Liu, 2001	0.9

Sulphur (Mineral)	Sulphur	liu huang	硫磺	Mineral	Zhao et al., 2000; Fan and Liu, 2001	0.9

[a] Percentage extract yield (w/w) was calculated as dry extract weight / dry crude raw material weight X 100.

2.2.2.2. Preparation of extracts

For the preparation of extracts, all substances were ground to powder or pieces using an electrical blender. One hundred ml 80% aqueous ethanol was added into 30 g of the powdered medicinal substance and sonicated in an ultrasonic bath at 50 °C for 30 min. The extract was filtered and the residue was further extracted twice with 80% aqueous ethanol as before. All three filtrates were combined, and then concentrated in a rotary evaporator under negative pressure, and then finally dried in blowing air. The resultant extracts were weighed and stored at -20°C for subsequent bioassay testing.

2.2.3. Results

The last column in Table 2.1. shows the percentage of the dry extract yield (w/w) from 30 g of raw materials.

2.2.4. Discussion

A total of 60 Chinese medicinal materials were selected for the herbal screening programme. The selection was based on their ethnomedical uses for the treatment of psoriasis which have been well documented in TCM. It should be stressed that this project is not about a random screening of a library of herbal extracts. A selection of herbal ingredients based on ethnomedical knowledge would likely enhance the possibility of successfully finding the right Chinese medicinal substances with intended anti-psoriatic properties. Ethanolic extraction was employed to carry out the extraction, because ethanol is an organic solvent which has the least toxicity among the organic solvents. 80% of ethanol can provide a suitable polarity for extracting many components from the herbal materials. Moreover, ethanol can be consumed by human body, making it an ideal organic solvent for phytochemical investigation.

The medicinal substances were extracted individually in our project. Composite formulas as those often used in routine clinical practice were considered inappropriate for the present project because a formula usually contains several herbs, making it difficult, if not impossible, to study the mechanism of action for such therapy and to elucidate the active ingredients of the formula responsible for the therapeutic action. Sixty single Chinese medicinal materials belonging to different functioning categories were included in the initial screening programme; and the selection was primarily based on their common use in clinical practice for psoriasis treatment. Such selection strategy would provide a greater opportunity for finding potent anti-proliferative agents and eventually to develop promising candidates for further experiments.

2.3. Development and Validation of 96-well Plate Assays for Screening of the Selected Medicinal Materials

2.3.1. Introduction

The human HaCaT keratinocyte cell line was originally established from a Caucasian patient with psoriasis, and has been widely used as a valuable in vitro experimental model to study the pathogenesis of psoriasis and evaluate anti-psoriatic therapeutic agents (Garach-Jehoshua et al., 1999; Lehmann, 1997; Paramio et al., 1999). This cell line was thus employed as a psoriasis-relevant *in vitro* model in our screening programme. As the number of the medicinal substances to be studied for their anti-proliferative action was large, the evaluation of cell numbers by haemocytometer counting method was considered not suitable, as this method would invariably involve tedious and time consuming procedures. In the present screening project, we elected to employ a Sulforhodamine B (SRB) assay to evaluate the HaCaT cell numbers under the influence of the extracts, because this colorimetric assay has been demonstrated to be reliable and rapid in measuring cell numbers in culture (Skehan et al., 1990; Lin et al., 1999). Before the use of SRB assay to evaluate the anti-proliferative effect of the extracts on HaCaT cells in the screening programme, a validation of the linearity of the HaCaT cell densities and absorbance needs to be undertaken. In addition, growth curves of HaCaT cells in the absence of extract treatment should also be plotted in order to decide the optimal seeding densities and harvest time for the screening experiments. It is worth noting that after the initial screening programme with SRB assay, MTT assay will also be performed to confirm the findings regarding those extract with significant anti-proliferative effects.

2.3.2. Materials and Methods

2.3.2.1. General cell culture

Unless otherwise specified, chemicals and reagents used were obtained from Sigma-Aldrich (St. Louis, MO, USA.). Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS) and trypsin-EDTA (1X) were purchased from Invitrogen (Carlsbad, CA, USA). HaCaT, which is an immortalized line of human epidermal keratinocytes (Boukamp et al., 1988), was provided by the China Centre for Type Culture Collection, Wuhan, China. HaCaT cell line (unknown passage number) was routinely maintained in DMEM supplemented with 10% FCS, 10 μg/ml of streptomycin and 10 U/ml of penicillin, and incubated at 37°C in a 5% CO_2, 95% air humidified atmosphere. All cell culture experiments were carried out when the culture was approximately 60-90% confluent.

2.3.2.2. SRB assay

The SRB assay was performed using a method originally described by Skehan et al., (1990). Briefly, the cultured cells attached to the substratum of the plate were fixed by addition of 25 μl of cold 50% trichloroacetic acid (TCA, 4°C) on top of the 100 μl growth medium already present in each well. The plate was incubated at 4°C for 1 h before being gently washed five times with tap water to remove TCA, growth medium and the dead cells. The plate was then allowed to dry in air, or aided with a hair dryer; then 100 μl of 0.4% (W/V) SRB dissolved in 1% acetic acid was added into each well to stain the HaCaT cells for 30 min. At the end of the staining period, unbound SRB dye was thoroughly removed by washing four times with 1% acetic acid. The plate was air dried again, and 150 μl of 10 mM aqueous Tris base

[Tris(hydroxymethyl)aminomethane] was added into each well to solubilize the cell-bound dye. The plate was then shaken for 5 – 10 min on a gyratory shaker followed by reading the optical density (OD) at 540 nm in a microplate spectrophotometer (BMG Labtechnologies, Fluostar Optima, CA, USA).

2.3.2.3. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that can be cleaved by active mitochondria of viable cells to form a dark blue formazan product which can be measured colorimetrically. At the end of the incubation, MTT assay was performed using a method originally developed by Mosmann (1983). Briefly, MTT was added to the wells at a final concentration of 0.5 mg/ml and incubated at 37°C for 2 h. The medium was then completely removed from the wells by vacuum suction and replaced with 100 μl DMSO. The OD of the dissolved formazan dye was recorded at 540 nm using a microplate spectrophotometer (BMG Labtechnologies, Fluostar Optima, CA, USA).

2.3.2.4. Validation and optimization experiments

(a) Linearity and range (Calibration curve)

A series of density of HaCaT cells ranging from 7.5×10^5 to 5.9×10^3 cells/ml (by doubling dilutions) was plated in 96 well-plates. Each concentration had six replicates. After 4 h of incubation, the SRB and MTT assay were performed. Calibration curves were constructed using absorbance (i.e. OD) as Y-axis and cell density as X-axis.

(b) Growth curves of different seeding densities

$0.2 - 2.5 \times 10^4$ cells/ml were seeded in 96 well-plates with each concentration having six replicates. The cells were incubated with DMEM medium up to 6 days and culture

medium was replaced on day 3. At daily intervals, one of the plates was removed and processed with SRB assay. Growth curves were then constructed using absorbance as the Y-axis and time (number of days) as X-axis.

2.3.3. Results

2.3.3.1. Linearity and range

For both SRB and MTT assays, the linearity of each assay was determined by constructing a scatterplot calibration curve and calculating the regression coefficient of the relationship between absorbance and cell numbers, as shown in Fig. 2.1.. Both SRB and MTT calibration curve were linear within the working range from 7.5×10^5 to 5.9×10^3 cells/ml with the regression coefficient (R^2) being 0.992 and 0.995, respectively. The results explicitly demonstrated that both SRB and MTT assay produced a very satisfactory linearity in measuring cultured HaCaT cells. Therefore, both assay methods can be readily employed to evaluate the proliferation rate of cultured HaCaT cells with good assurance of accuracy.

2.3.3.2. Optimization of incubation conditions

Growth curves of HaCaT cells using different seeding densities were collectively shown in Fig. 2.2.. At seeding densities of up to 2.5×10^4 cells per well, exponential growth rates were observed over the 6-day incubation period. For the highest cell density (2.5×10^4 cells/well) nutrients in the medium appeared to be depleted by day 2, whereas this was not observed for lower cell densities. It is clear that a seeding density of 1×10^4 cells/well and incubation time for up to 4 days still produced near linear growth curve. Therefore, it is rational to use an initial seeding cell density of 1×10^4 cells/well for the screening programme. Moreover, because culture medium usually needed to be

replaced on day 3 for optimal cell viability, the incubation time would ideally be chosen for 3 days or less. We chose an incubation time for 2 days for the screening programme because the cells would be in an exponential growth phase, and the troublesome medium replenishing procedure would not be required.

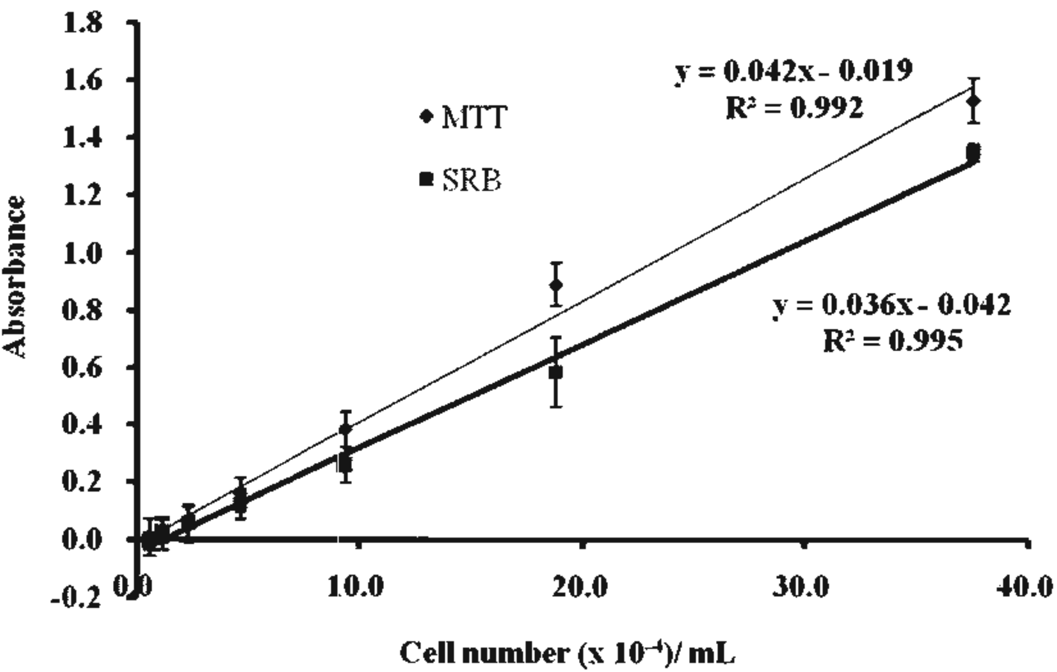


Fig. 2.1. Calibration curves of SRB and MTT assays on HaCaT cells for the validation of linearity. Data points are the means ± SD of six replicates from the same plate.

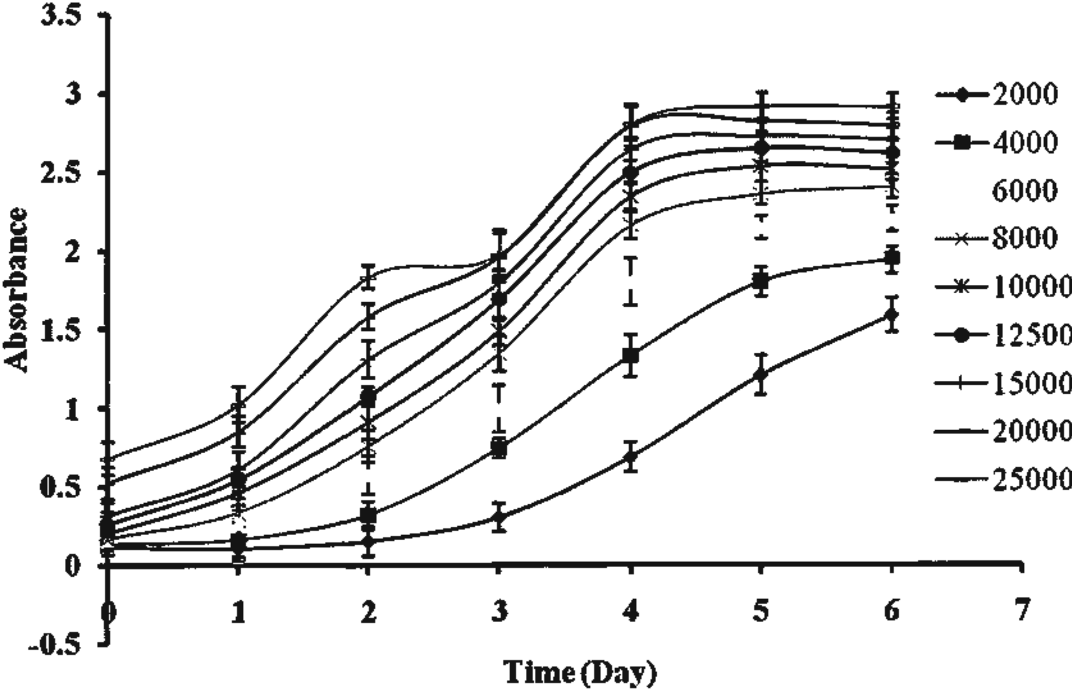


Fig. 2.2. HaCaT cell growth curves with different initial plating densities and incubation time as detected with SRB assay. The medium was replaced on day 3. Data points are the means ± SD of six replicates from the same plate.

2.3.4. Discussion

HaCaT human keratinocyte cell line is a well-established psoriasis-relevant *in vitro* model and was employed in our present screening programme. SRB assay is a rapid microplate assay that quantifies cell numbers by colorimetrically measuring cellular protein content. Due to its rapidness and simplicity in operation, SRB assay was considered to be suitable for handling a large amount of extracts in our project. As a prerequisite for the use of this assay in extract screening programme, experiments were conducted to establish the linearity of this assay on HaCaT cells, as well as the optimal seeding density and incubation time. Our experimental results demonstrated SRB that assay produced an excellent linearity between cell numbers and optical densities. Therefore, by colorimetrically measuring the absorbance, the cell number can be accurately evaluated. Our experiments also established that a seeding density at 1 x 10^4 cells/well and incubation time for 2 days would be appropriate for the screening programme.

As MTT assay would be used to confirm the significant anti-proliferative effects possessed by a number of extracts, a validation of linearity between cell numbers and absorbance was also conducted. The result showed that like SRB assay, MTT assay also produced a satisfactory linearity between the cell numbers and OD readings, indicating that this formazan-based assay was also suitable for the studies extract-induced growth inhibition on cultured HaCaT cells. In the ensuing section, experiments would be conducted to evaluate the anti-proliferative action of the extracts on the growth of HaCaT human keratinocytes using these two validated assays.

2.4. Screening 60 Medicinal Materials for Anti-proliferative Effects on HaCaT Cells Using SRB Assay

2.4.1. Introduction

Epidermal keratinocyte hyperproliferation is one of the most important features in the pathogenesis of psoriasis, and remains a valid therapeutic target for development of anti-psoriatic agents. Agents that could inhibit keratinocyte proliferation are potentially useful in the treatment of psoriasis. In this screening programme, we used anti-proliferative effect as the parameter for the screening target. Sixty commonly prescribed psoriasis-treating Chinese medicine herbs were selected for investigation into their anti-proliferative effects on HaCaT cells *in vitro*. Since SRB assay has been shown to be a more rapid and user-friendly method than MTT assay in evaluating cell numbers in culture (Lin et al., 1999), it was thus chosen in our experiments for the initial screening experiments which involved with handling a large number of extracts. The positive results obtained by SRB assay would be further confirmed by MTT assay.

2.4.2. Materials and Methods

2.4.2.1. Sources of medicinal materials and preparation of extracts

The sources and preparation of the extracts of the selected medicinal substances were described in section 2.2.2.1..

2.4.2.2. General cell culture

The details of the general cell culture procedure were described in section 2.3.2.1..

2.4.2.3. Screening Chinese medicinal extracts for anti-proliferative effects on HaCaT cells by SRB assay

For the initial anti-proliferative assay on HaCaT cells, the resultant dry extracts were redissolved in 80% ethanol to give stock solutions of 10 mg/ml, which were then sterilized by filtration (0.2-μm pore size filter, Corning, NY, USA) before testing in cell culture experiments. Dithranol, which was employed as a positive control for the screening experiments, was dissolved in 100% ethanol to give a final concentration of 0.25 mg/ml as stock solution. It was then sterilized with filtration before use. The reconstituted extracts together with HaCaT cells were cultured in 96 well-plates, with each well containing 1 x 10^4 cells in 100 μl DMEM, and the final concentrations of the extract being 250, 125 and 62.5 μg/ml. Note that the seeding density was determined based on the results of the growth curves experiment. Each concentration of an extract had six replicates in the same plate. The cells were then grown for 48 h. After 48 h, the proliferation rate of the incubated cells was determined by SRB assay using the protocol described in Section 2.3.2.2..

2.4.2.4. Confirmation of the anti-proliferative action using MTT assay

Notwithstanding that SRB assay is a robust and sensitive assay to quantify cell numbers by colorimetrically measuring the cellular protein content in culture, other assay using different mechanism should be used to confirm the results obtained by SRB assay. Thus, the 20 effective extracts that were found to have significant growth inhibition on the HaCaT cells would be further confirmed by MTT assay for their anti-proliferative effect. The cell culture procedures were similar to that described in Section 2.4.2.3., except the final extract concentrations were 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 and 0.95 μg/ml. The MTT assay protocol used was identical to that described in Section

2.3.2.3..

2.4.2.5. Confirmation of the anti-proliferative effect by trypan blue exclusion assay

For the two extracts, i.e. the root of *Rubia cordifolia* (Radix Rubiae), and realgar, that showed highly potent anti-proliferative effects on HaCaT cells, they were further investigated for the anti-proliferative effect on HaCaT cells using trypan blue exclusion assay to determine the viable cell numbers. Briefly, at the end of the incubation, culture medium was removed and cell viability was determined using trypan blue exclusion assay employing 0.6% trypan blue solution for staining. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cells unless their membrane is damaged (Guckian et al., 1978; Freshney, 1987). Therefore, the cells that exclude the dye are viable. The numbers of viable cells were counted with a haemocytometer under a phase contrast microscope (Olympus, Tokyo, Japan).

2.4.2.6. Statistical analysis

Differences between treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* Dunnett's test using medium-only treatment as control group on the SPSS for Windows (version 11.0). Differences were considered to be significant at $p < 0.05$, and denoted as *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$.

2.4.3. Results

2.4.3.1. Anti-proliferative effects of 60 Chinese medicinal extracts on HaCaT cells by SRB assay

Table 2.2. summarizes the anti-proliferative effects of the 60 Chinese medicinal substances on cultured HaCaT cells and their respective IC_{50} values. Note that IC_{50} is the concentration of the sample required to inhibit the growth of HaCaT cells by 50%.

It is evident that among the 60 medicinal substances tested, 20 extracts showed significant inhibitory effects on the growth of HaCaT cells when compared to the non-treated control, and had IC_{50} values less than 200 μg/ml. The remaining extracts either failed to significantly inhibit the cell growth at the concentrations tested, or their IC_{50} values well exceeded 200 μg/ml, suggesting that these extracts were ineffective in our experimental system and rendering them not worthy of further investigation.

Table 2.2. Anti-proliferative effects of the 60 alcoholic extracts of Chinese medicinal substances on HaCaT cells as measured by SRB assay.

Name of medicinal material	Cell number[a] (expressed as % of non-treated control) after 48 h incubation in the presence of extract at the concentration (μg/ml) of			IC_{50} (μg/ml)[b]
	250	125	62.5	
Extracts with IC_{50} < 200 μg/ml				
Bombyx Batryticatus	3.1**	68.6**	89.6	160.5
Caulis Sinomenium	8.1**	49.2**	90.0*	123.8
Cortex Moutan Radicis	22.4**	51.3**	96.3	130.6
Exuviae Serpentis	37.8**	46.9**	69.4**	116.4
Flos Campsis	2.25**	21.2**	53.4**	69.1
Folium Camellia Sinensis	7.6**	35.0**	87.0*	107.0
Fructus Cnidii Monnieri	0.4**	22.3**	85.0*	97.4

Fructus Forsythiae Suspensae	5.3**	41.9**	65.4**	103.5
Fructus Kochiae Scopariae	11.4**	86.4*	106.1	185.7
Indigo Naturalis	11.1**	56.6**	108.6**	143.1
Mylabris	5.1**	8.5**	60.1**	74.7
Radix et Caulis Jixueteng	0.0**	4.0**	31.2**	<62.5
Radix Ledebouriella Divaricatae	0.7**	10.9**	14.6**	<62.5
Radix Rubiae Cordifoliae	6.2**	8.2**	6.8**	<62.5
Radix Salviae Miltiorrhizae	30.4**	67.1**	102.1	183.2
Realgar	4.1**	8.6**	9.6**	<62.5
Rhizoma Coptidis	20.9**	33.0**	42.3**	<62.5
Rhizoma Curcumae Ezhu	9.7**	30.3**	86.0*	102.9
Scolopendra Subspinipes	3.9**	71.2*	94.3	164.4
Sulphur	0.9**	1.2**	7.3**	<62.5

Extracts with IC_{50} > 200 μg/ml				
Buthus Martensi	94.4	101.2	100.1	>250
Caulis Lonicerae	93.5	102.3	102.4	>250
Caulis Piperis Futokadsurae	45.5**	88.1	101.4	236.8
Cortex Dictamni Dasycarpi Radicis	97.1	113.3	108.8	>250
Flos Carthami Tinctorii	42.8**	88.1	101.7	230.1
Flos Lonicerae Japonicae	113.7	112.6	110.2	>250
Fructus Gardeniae Jasminoidis	104.0	104.0	108.0	>250
Fructus Psoraleae Corylifoliae	96.1	105.1	104.3	>250
Fructus Tribuli Terrestris	99.5	107.0	112.0	>250
Herba Artemisiaae Yinchenhao	81.1	100.6	103.9	>250
Herba Hedyotidis Diffusae	36.8**	74.8**	97.2	206.6
Herba Portulacae Oleraceae	85.7	95.6	98.7	>250
Herba Scutellariae Barbatae	37.8**	86.2	102.0	218.5

Herba Taraxaci Mongolici	94.7	109.3	103.3	>250
Lumbricus	88.6	97.4	95.8	>250
Periostracm Cicadae	67.2	98.2	104.5	>250
Radix Aconitum Bullatifolium	53.0**	90.7	102.9	>250
Radix Arnebiae seu Lithospermi	40.4**	84.2	100.7	222.6
Radix Astragali Membranaceus	107.2	108.2	104.6	>250
Radix Clematidis	93.3	106.6	108.0	>250
Radix Codonopsitis Pilosulae	91.9	101.4	94.9	>250
Radix et Rhizoma Rhei	53.2**	82.0**	96.4	>250
Radix Euphorbia fischeriana	104.9	106.4	106.2	>250
Radix Glycyrrhizae Uralensis	82.0	104.5	105.6	>250
Radix Ligustici Chuanxiong	48.1**	95.2	106.5	245.0
Radix Paeoniae Rubrae	56.5**	93.8	106.2	>250
Radix Rehmanniae Glutinosae	100.0	107.9	101.5	>250

Radix Scutellariae Baicalensis	53.0**	72.8**	83.1**	>250
Radix Sophorae Flavescentis	48.4**	92.9	105.9	245.5
Radix Tripterygii wilfordii	100.9	108.0	105.7	>250
Rhizoma Alismatis Orientalitis	61.2	96.9	111.1	>250
Rhizoma Dioscoreae Hypoglauca	96.5	105.1	105.0	>250
Rhizoma Imperatae Cylindricae	107.0	110.6	111.6	>250
Rhizoma Polygonati	94.0	104.3	99.6	>250
Rhizoma Smilacis Glabrae	104.2	106.5	104.7	>250
Rhizoma Sparganii Stoloniferi	96.8	107.8	111.3	>250
Sclerotium Polypori Umbellati	108.0	119.8	123.8	>250
Semen Coicis Lachryma-jobi	120.8	118.6	117.8	>250
Semen Persicae	40.2**	118.8	110.6	234.4
Zaocys Dhumnades	118.2	115.2	109.2	>250

[a] Standard deviation of all measurements were smaller than 5% of the mean OD readings.

[b] IC_{50} is the concentration of the sample required to inhibit the growth of HaCaT cells by 50%.

IC_{50} was calculated using the GraphPad PRISM software version 3.0 (GraphPad Software, San Diego, CA, USA).

*, $P < 0.01$ and **, $P < 0.001$, when compared with non-treated control (One way ANOVA, followed by *post hoc* Dunnett's test).

2.4.3.2. Anti-proliferative effects of the 20 Chinese medicinal substances on HaCaT cells measured by MTT assay

Table 2.3. shows the anti-proliferative effects of the 20 Chinese medicinal substances on the growth of HaCaT cells as determined by MTT assay. These 20 herbs have been found to have inhibitory action on the growth of HaCaT cells in the previous experiment when SRB assay was used to detect the cell numbers. Among these extracts tested, two of them, namely Radix Rubiae and realgar exhibited highly significant anti-growth effects on the HaCaT cells, with IC_{50} values of 1.4 and 6.6 μg/ml, respectively. It is noteworthy that at the final extract concentration of 20 μg/ml, the solvent (ethanol) concentration in the culture was less than 0.2% (v/v), in such low concentration, the solvent exerted either stimulatory nor inhibitory effect on the growth of HaCaT cells (data not shown).

To be a good drug candidate, the IC_{50} value of such agent should be sufficiently low to avoid any possible unspecific effects, thus we considered extracts exhibiting IC_{50} value < 20 μg/ml as promising candidates. This cut-off line is as arbitrary as it is sensible. Only two extracts, i.e. Radix Rubiae and realgar demonstrated IC_{50} values of less than 20 μg/ml. Further studies will focus on these two extracts. Fig. 2.3. depicts the growth patterns of the HaCaT cells under the influence of Radix Rubiae and realgar extracts as measured by MTT assay.

Table 2.3. Anti-proliferative effects of the 20 Chinese medicinal extracts and dithranol on HaCaT cells as measured by MTT assay.

Name of the medicinal material and the part used	Cell number[a] (expressed as % of non-treated control) after 48 h incubation when grown in the presence of extract at the concentration of (μg/ml)											IC_{50} (μg/ml)[b]
	250	125	62.5	31.3	15.6	7.8	3.9	1.9	0.98	0.49	0.24	
Extracts with IC_{50} < 20 μg/ml												
Radix Rubiae Cordifoliae	0.10**	0.36**	1.4**	1.6**	4.6**	15.4**	25.6**	40.0**	59.2**	74.0**	83.2*	1.4
Realgar	5.7**	5.8**	5.5**	7.5**	18.8	42.3**	66.4**	78.3*	82.0	101.3	105.3	6.6
Extracts with IC_{50} > 20 μg/ml												
Bombyx Batryticatus	8.3**	58.1**	64.0*	95.2	101.0	102.3	104.0	ND	ND	ND	ND	145.3
Caulis Sinomenium	33.5**	46.9**	62.2**	93.3	97.8	ND	ND	ND	ND	ND	ND	112.3
Cortex Moutan Radicis	26.7**	54.8**	101.2	113.4	108.5	ND	ND	ND	ND	ND	ND	146.4
Exuviae Serpentis	27.5**	51.4**	75.2**	92.4	101.7	ND	ND	ND	ND	ND	ND	132.3
Flos Campsis	4.9**	25.9**	60.1**	82.1**	91.2	102.3	104.0	ND	ND	ND	ND	80.9
Folium Camellia Sinensis	16.0**	30.7**	91.4	105.9	105.9	ND	ND	ND	ND	ND	ND	105.1

Fructus Cnidii Monnieri	1.2**	43.6**	82.0**	87.9	98.5	ND	ND	ND	ND	ND	ND	114.6
Fructus Forsythiae Suspensae	2.1**	23.6**	62.0**	77.6**	87.6**	101.6	104.6	ND	ND	ND	ND	82.0
Fructus Kochiae Scopariae	0.4**	50.0**	80.0**	94.3	99.3	ND	ND	ND	ND	ND	ND	125.0
Indigo Naturalis	12.3**	61.7**	76.3**	88.8	100.4	ND	ND	ND	ND	ND	ND	154.6
Mylabris	10.4**	6.3**	28.3**	84.8*	99.0	99.7	104.6	ND	ND	ND	ND	50.5
Radix et Caulis Jixueteng	23.6**	18.6**	42.9**	84.1*	103.6	110.0	105.5	ND	ND	ND	ND	57.1
Radix Ledebouriella Divaricatae	1.8**	41.0**	71.6**	86.6**	94.4	92.3	95.1	ND	ND	ND	ND	106.6
Radix Salviae Miltiorrhizae	29.4**	45.9**	51.0**	66.7**	85.2**	91.7	103.0	ND	ND	ND	ND	56.3
Rhizoma Coptidis	0.3**	11.9**	30.3**	45.8**	54.2**	61.7**	69.4**	81.7**	88.6*	96.7	98.6	23.4
Rhizoma Curcumae Ezhu	1.6**	38.3**	77.6*	90.6	95.5	ND	ND	ND	ND	ND	ND	106.4
Scolopendra Subspinipes	0.94**	59.6**	82.3*	85.3	96.0	ND	ND	ND	ND	ND	ND	145.5
Sulphur	0.0**	0.0**	13.8**	62.7**	87.8**	96.1	94.6	93.2	92.0	93.3	98.4	39.4

Dithranol (positive control)										
Concentration (μg/ml)	1.8	0.9	0.45	0.22	0.11	0.056	0.028	0.014	0.007	0.003
Cell number[a] (expressed as % of non-treated control) after 48 h incubation	0.2**	4.5**	31.2**	67.6**	74.6**	85.3	93.8	97.3	99.2	100.3
IC_{50}[b]	0.33 μg/ml or 1.47 μM									

[a] Standard deviation of all measurements were smaller than 5% of the mean OD readings.

[b] IC_{50} is the concentration of the sample required to inhibit the growth of HaCaT cells by 50%.

IC_{50} was calculated using the GraphPad PRISM software version 3.0 (GraphPad Software, San Diego, CA, USA).

*, $P < 0.01$ and **, $P < 0.001$, when compared with non-treated control (One way ANOVA, followed by *post hoc* Dunnett's test).

ND, Not done.

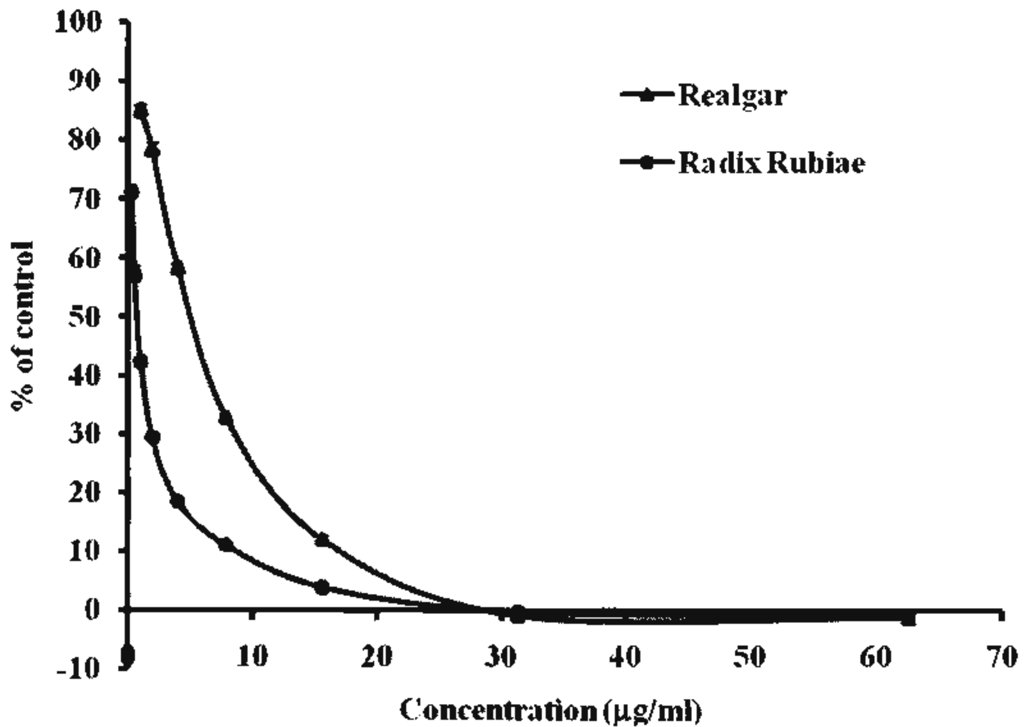


Fig. 2.3. The growth patterns of HaCaT cells in the presence of Radix Rubiae and realgar extracts as measured by MTT assay. Data represents % of non-treated control ± SEM, n=6.

2.4.3.3. Effects of Radix Rubiae and realgar on the growth of HaCaT cells as determined by trypan blue exclusion assay

The results shown in Fig. 2.4. demonstrated that Radix Rubiae and realgar extracts significantly inhibited the HaCaT cell proliferation. Comparing to the realgar extract, Radix Rubiae had more significant inhibitory effect on HaCaT cells. The results regarding the anti-proliferative effects of Radix Rubiae and realgar as counted by the trypan blue exclusion method (Fig. 2.4.) were congruent with that determined by MTT assay.

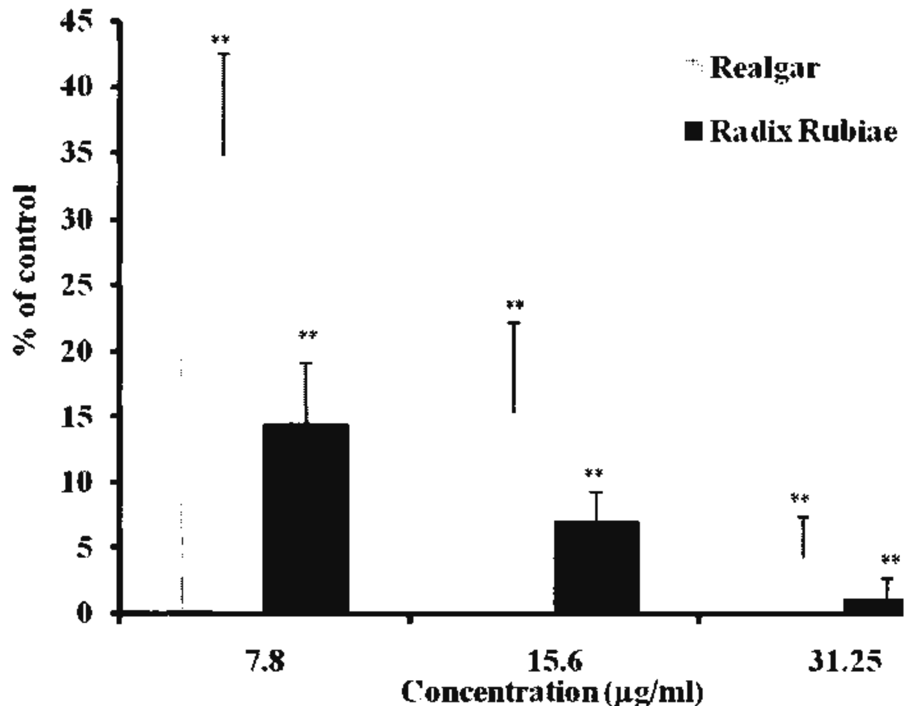


Fig. 2.4. Anti-proliferative action of the extracts of Radix Rubiae and realgar on HaCaT cells as measured by trypan blue exclusion assay. Data represents % of non-treated control ± SEM, n=6. **, $p < 0.001$ versus non-treated control.

2.4.4. Discussion

In the present screening programme, ethanolic extracts of 60 medicinal materials were investigated for their anti-proliferative effects on cultured HaCaT keratinocytes. As SRB assay is a more user-friendly colorimetric microplate assay than MTT assay, it was employed in the initial screening project in order to efficiently handle the large number of extracts. Among the 60 extracts tested, 20 of them showed significant anti-proliferative action on HaCaT cells with IC_{50} values smaller than 200 μg/ml. For these 20 effective extracts, their anti-proliferative effects were further evaluated by MTT assay, with broader and lower concentrations being tested so that precise IC_{50} values could be established. Based on the MTT results, we found that Radix Rubiae and reaglar had the most significant anti-proliferative property, with IC_{50} values of 1.4 and 6.6 μg/ml, respectively. The IC_{50} values exhibited by Radix Rubiae and reaglar are comparable with the positive control dithranol (IC_{50}, 0.33 μg/ml). Furthermore, the anti-proliferative actions of Radix Rubiae and reaglar were further confirmed by trypan blue exclusion assay, which used direct cell counting method to evaluate cell numbers. Both MTT assay and trypan blue exclusion method produced consistent results. The present screening programme successfully identified Radix Rubiae and realgar extracts as potent anti-proliferative agents on the HaCaT human keratinocytes, and the discovery opened opportunities for further studies on these two extracts in a hope to develop them into anti-psoriatic agents for clinical treatment of psoriasis. The remaining of this chapter will focus on in-depth study on Radix Rubiae and realgar.

2.5. Time Course Study and Specificity Test of the Anti-proliferative Action of Radix Rubiae and Reaglar on HaCaT Cells

2.5.1. Introduction

From the above experiment, we found that Radix Rubiae and reaglar have inhibitory effect on the growth of keratinocytes. In this section, the time-dependence of the inhibitory effects of these two extracts will be evaluated. In addition, the specificity of these two extracts will be tested on a normal fibroblast Hs-68 cell line.

2.5.2. Materials and Methods

2.5.2.1. Sources and preparation of Radix Rubiae and realgar

The source and preparation of these two extract were described in section 2.2.2.1..

2.5.2.2. General cell culture

The details of the culturing procedures for HaCaT cells were as that described in Section 2.3.2.1.. Hs-68, a human fibroblast cell line (unknown passage number) established from the foreskin of a normal Caucasian newborn male, was purchased from the American Type Culture Collection (ATCC, VA, USA). Hs-68 cells were routinely maintained in DMEM with 10% FBS (Gibco Laboratories, NY, USA), 10 μg/ml of streptomycin and 10 U/ml of penicillin, and incubated at 37°C in a 5% CO_2, 95% air humidified atmosphere. The cell culture experiments were carried out when the culture was 60-90% confluent.

2.5.2.3. Time course evaluation of the anti-proliferative effect of Radix Rubiae and realgar

Radix Rubiae extract (ranging from 62.5 μg/ml to 0.24 μg/ml by serial doubling dilutions) and realgar extract (ranging from 31.2 μg/ml to 0.24 μg/ml by serial doubling dilutions) were incubated with HaCaT cells in 96 well-plates, with each well containing 2×10^4 cells in 200 μl DMEM. The incubation time was set for 3, 6, 12, 24, 48, and 72 h. At each time point, MTT assay was carried out to evaluate the cell numbers.

2.5.2.4. Evaluation of cytotoxic effect of Radix Rubiae and realgar on Hs-68 cells

Further experiments were conducted on Hs-68 cell line to test whether these extracts have unspecific cytotoxicity on different cell type other than HaCaT keratinocytes. The experimental protocol for Hs-68 cells was similar to that of HaCaT cells, and the cell numbers were evaluated by MTT assay. In addition, the tendency of anti-proliferative effect of the two extracts on Hs-68 was determined at different incubation time points and different concentrations.

2.5.2.5. Statistical analysis

Differences between treatment groups were analyzed by one-way analysis of variance (ANOVA), followed by *post hoc* Dunnett's test using medium-only treatment as control group on the SPSS for Windows (version 11.0). Differences were considered to be significant at $p < 0.01$, and denoted as *, $p < 0.01$, and **, $p < 0.001$.

2.5.3. Results

2.5.3.1. Time course study on the effect of Radix Rubiae and realgar on HaCaT cell proliferation

The time course of the anti-proliferative action of Radix Rubiae and realgar on HaCaT keratinocytes as determined by MTT assay is shown in Fig. 2.5. and 2.6., respectively. It is evident that the Radix Rubiae and realgar extracts exerted potent anti-proliferative action on the HaCaT keratinocytes in a dose- and time-dependent manner. Table 2.4. shows the IC_{50} values of Radix Rubiae and realgar on the growth of HaCaT cells when incubated at different time points. The promising IC_{50} values exhibited by both Radix Rubiae and realgar indicate that they have good potential for further development into anti-psoriatic therapeutic agents.

Table 2.4. The IC_{50} values of Radix Rubiae and realgar on the growth of HaCaT cells when incubated at different time points as determined by MTT assay.

Incubation time (h)	3	6	12	24	48	72
IC_{50} of Radix Rubiae (μg/ml)	>62.5	18.3	11.9	5.8	1.4	2.9
IC_{50} of realgar (μg/ml)	22.0	6.9	4.4	1.9	1.6	1.6

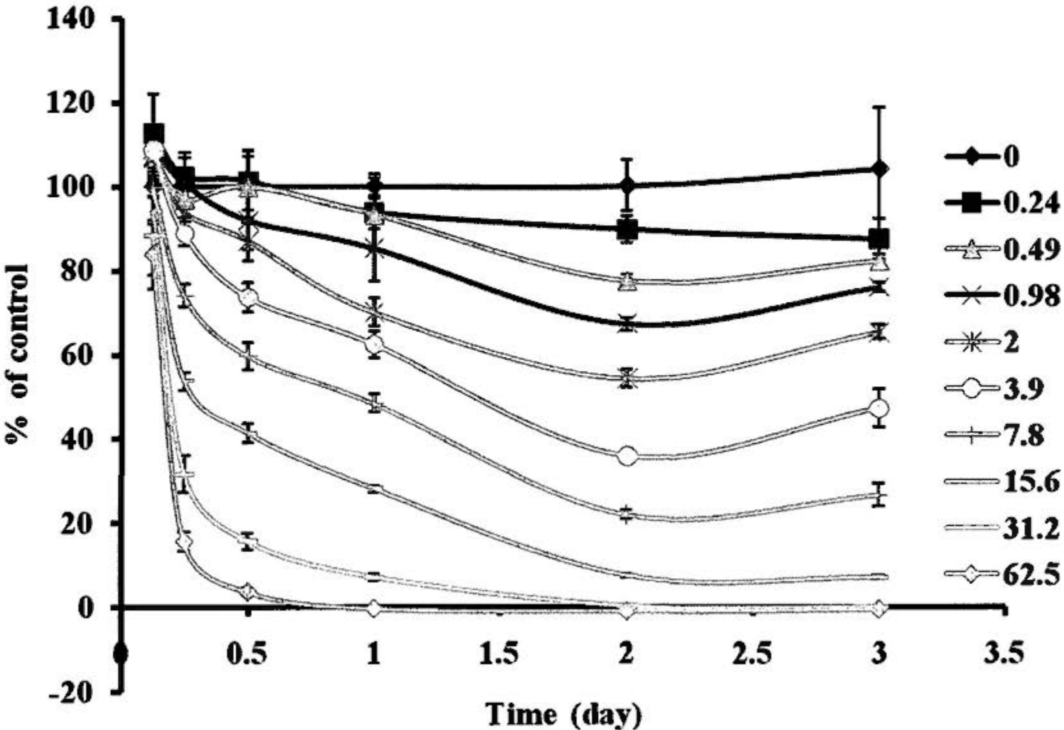


Fig. 2.5. Anti-proliferative action of Radix Rubiae extract at different concentrations on cultured HaCaT cells with different incubation times. The HaCaT cells were treated with different concentration of Radix Rubiae extract ranging from 0 to 62.5 μg/ml, and the cultures were assayed with MTT at different incubation time points. Each data point represents % of non-treated control ± SEM, n = 6.

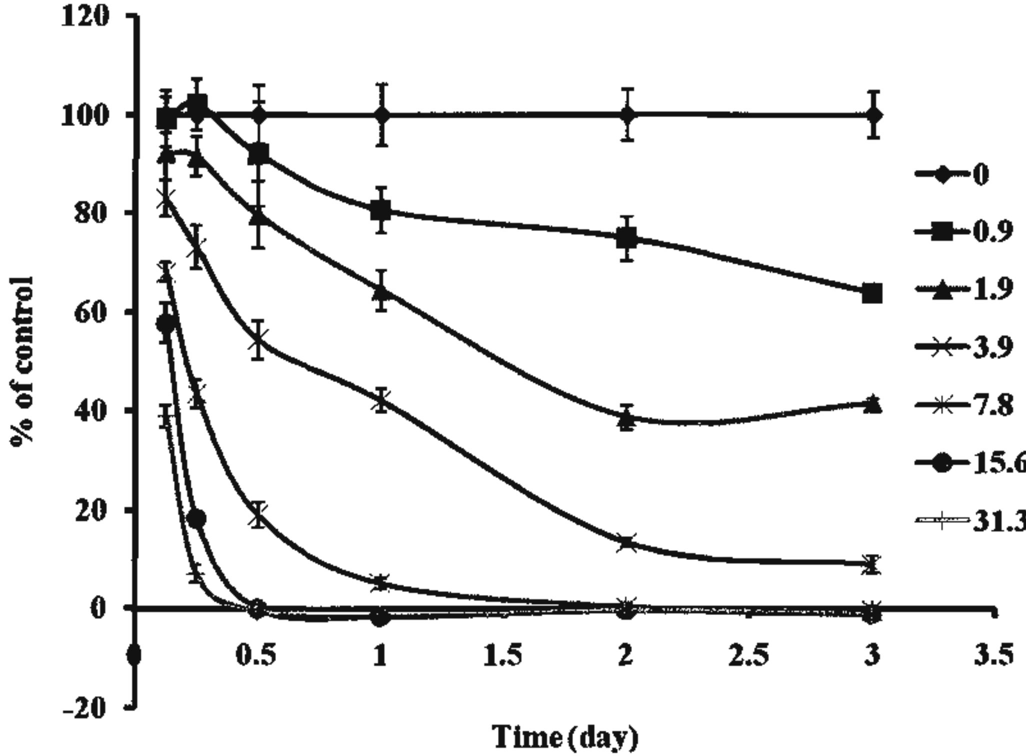


Fig. 2.6. Anti-proliferative action of realgar extract at different concentrations on cultured HaCaT cells with different incubation times. The HaCaT cells were treated with different concentrations of realgar extract ranging from 0 to 31.3 μg/ml, and the cultures were assayed with MTT at different incubation time points. Each data point represents % of non-treated control ± SEM, n = 6.

2.5.3.2. Effects of Radix Rubiae and realgar extracts on the growth of Hs-68 cells

In order to determine whether these two extracts have unspecific cytotoxicity on any cell type, we tested them on the growth of Hs-68 cells. Fig. 2.7. shows the growth patterns of Hs-68 cells under the influence of the extracts of Radix Rubiae and realgar. It is evidenced that Radix Rubiae extract did not exert significant inhibitory effect on the proliferation of Hs-68 cell line, and the result is in stark contrast to the significant anti-proliferative effects exerted by this herb on HaCaT cells which had an IC_{50} value of 1.4 μg/ml. On the other hand, realgar extract was shown to mediate modest growth inhibition on this human fibroblast line, with IC_{50} value of 48.1 μg/ml, which demonstrates a milder cytotoxic effect than that on HaCaT cell line which showed an IC_{50} value of 6.6 μg/ml.

It is interesting to know that dithranol, a positive control used in our experiments, had an IC_{50} value of 0.33 μg/ml (1.47 μM) on the growth of HaCaT cells as determined by MTT assay (Fig. 2.8.), but showed no inhibitory effect on the growth of Hs-68 cells. This provides experimental evidence to support this popular topical drug used in the effective management of psoriasis. Dithranol is usually available as a yellow powder, soluble in organic solvents such as chloroform and petroleum. Dithranol ointments, creams, and scalp solutions are commercially available in concentrations ranging from 0.1% to 1.0% (Camisa, 1998). The ready availability of this topical drug from commercial sources makes dithranol an ideal candidate for positive control used in our *in vitro* and *in vivo* experiments.

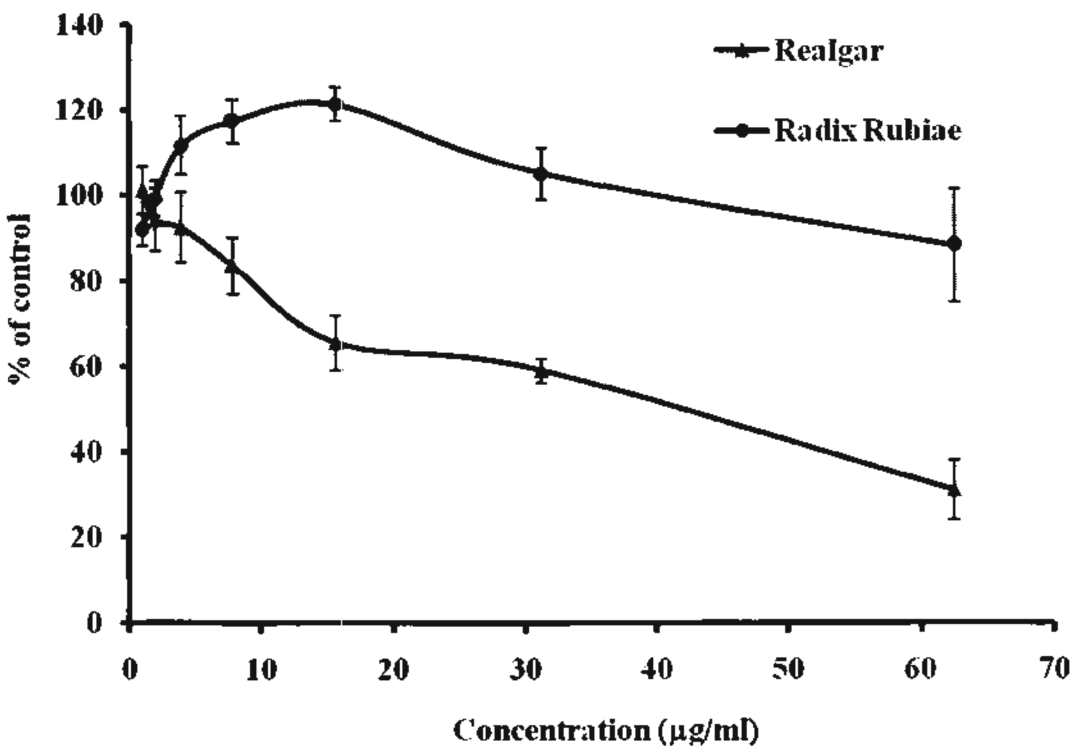


Fig. 2.7. The growth patterns of Hs-68 cells in the presence of Radix Rubiae and realgar extracts as measured by MTT assay. Data represents % of non-treated control ± SEM, n=6.

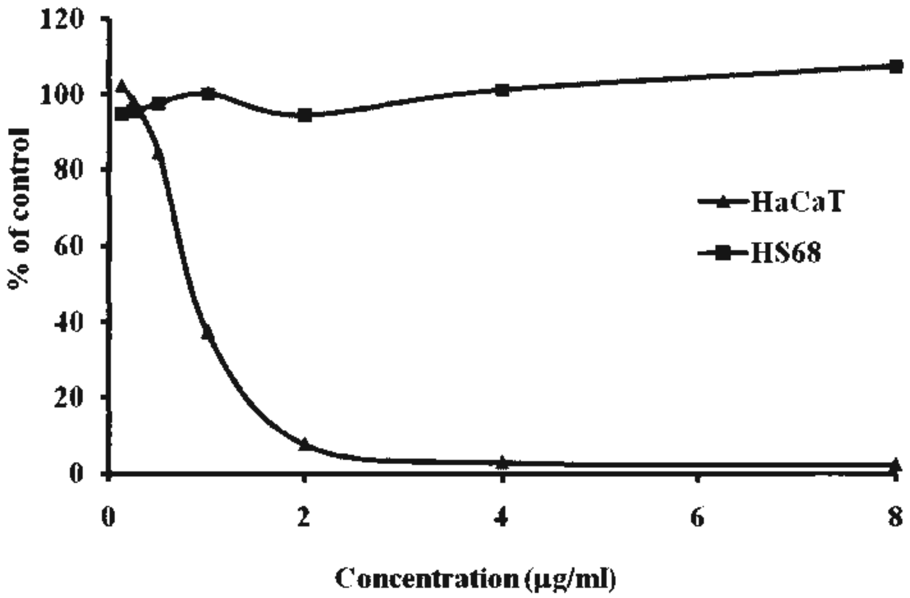


Fig. 2.8. Comparison of the anti-proliferative effects of dithranol on HaCaT and Hs-68 cells as determined by MTT assay. The IC_{50} values were 1.47 and 36 μM for HaCaT and Hs-68 cells, respectively. Each data point represents % of non-treated control ± SEM, n = 6.

2.5.4. Discussion

In the present experiments, both Radix Rubiae realgar extracts were shown to have dose- and time-dependent anti-proliferative action on the growth of HaCaT cells. In the specificity testing, Radix Rubiae showed strong selectivity towards HaCaT cells in the growth inhibition, but exhibited not inhibition on the proliferation of the normal fibroblast Hs-68 cells. The results clearly indicate that Radix Rubiae possess desirable differential effect on HaCaT cells, rendering it a promising anti-proliferative agents for further development into psoriasis treatment. Similarly, the realgar extract exhibited differential cytotoxic profile on the HaCaT and Hs-68 cells, and it only showed mild cytotoxic action towards the normal human Hs-68 fibroblasts. The differential cytotoxic profile possessed by realgar extract suggest that this mineral could be a good candidate for making topical application which can exert potent anti-psoriatic effect on hyperproliferative epidermal keratinocytes as seen in psoriasis lesions without eliciting harmful adverse effect on normal skin cells.

2.6. General Discussion

SRB assay quantifies cell numbers by colorimetrically measuring cellular protein content. The use of SRB assay for evaluating cell numbers in culture has many distinctive advantages when compared to other microplate assay such as MTT and XTT. For example, SRB assay does not involve with time-critical steps, is less sensitive to environmental fluctuation and independent of intermediary metabolism (Skehan et al., 1990; Lin et al., 1999). Moreover, it has a large linear working range for cell densities. The present study demonstrated that the SRB assay on HaCaT cell line produced excellent correlation between cell numbers and OD readings. The validation study indicated that SRB assay was a reliable and accurate method for estimating HaCaT cell numbers and was therefore employed for screening a large number of herbal extracts.

Sixty Chinese medicinal substances that are commonly prescribed in TCM practice to treat psoriasis were systematically screened for their potential anti-proliferative activities using an in vitro psoriasis-relevant HaCaT cell line. At the test concentrations of 62.5 – 250 μg/ml, 20 Chinese medicinal substances were effective in inhibiting HaCaT cell proliferation as measured by SRB assay. The anti-proliferative effects of these extracts were subsequently tested for confirmation by MTT assay which uses a mechanism different from that of SRB assay. MTT assay is cleaved by active mitochondria to form a dark blue formazan product, which is correlated with cell number, and this microplate assay has been widely used to quantitate cell proliferation and substance-induced cytotoxicity (Mosmann 1983; Maguad et al., 1988). Two extracts, i.e. Radix Rubiae, and realgar were shown to be highly potent in inhibiting the proliferation of HaCaT cells, and all had IC_{50} less than 20 μg/ml as assayed by MTT.

However, since crude extracts may affect cellular metabolism that may in turn affect formazan production without the concurrent increase or decrease in cell number. Therefore, trypan blue exclusion method was employed to further confirm the results obtained through MTT assay. The results obtained from trypan blue exclusion method indicated the similarity between trypan blue exclusion and colorimetric methods of SRB and MTT assays when assessing the anti-proliferative effects of these two extracts.

Our experiments also demonstrated that both extracts of Radix Rubiae and realgar exerted growth inhibition on HaCaT cells in a dose- and time-dependent manner. Moreover, both extracts showed specific anti-proliferation on fast proliferative HaCaT cells, but exerted much less potent growth inhibition on normal human fibroblast Hs-68 cell line. The differential anti-proliferative profiles of these two extracts make them promising candidates for further investigation and development into anti-psoriatic pharmaceuticals.

It is worth noting that dithranol, an effective topical therapeutic agent for psoriasis (Farkas et al., 2001) which has been shown to completely inhibit cell growth and inhibit DNA synthesis (Camisa, 1998), exhibited IC_{50} of 0.33 μg/ml on HaCaT cells by MTT assay in our experimental system. Given that crude extracts contain many different active and non-active ingredients, we believe that the IC_{50} values exhibited by these two medicinal substances were comparable to that of dithranol.

To conclude, the present screening programme for effective anti-proliferative agents have successfully identified Radix Rubiae and realgar as two potent HaCaT keratinocyte growth inhibitory agents. The identification of these two commonly used

anti-psoriasis medicinal substances will herald further investigations to identify and characterize the active ingredients responsible for their biological activities; and more importantly to illuminate the underlying molecular and biochemical mechanisms of action for the observed anti-proliferative action. In the ensuing two chapters, we would be studying the phytochemical and biochemical aspects of these two Chinese medicinal substances for their anti-psoriatic bioactivities.

Chapter Three

Phytochemical Studies of Radix Rubiae Extract and Apoptotic Investigation of Its Fractions and Chemical Components on HaCaT Human Keratinocytes

3.1. Introduction

In our previous studies, we have demonstrated that the ethanolic extract of Radix Rubiae possess the most significant inhibitory effect on cultured HaCaT cell line with an IC_{50} value of 1.4 μg/ml as mentioned in Section 2.4.3.2.. In addition, it did not exert cytotoxicity to human fibroblast Hs-68 cells. As a herbal medicine, Radix Rubiae has been subjected to various pharmacological and phytochemical investigations. For example, Radix Rubiae has been demonstrated to possess inhibitory action on platelet activating factor (Tripathi et al., 1993). Other activities relating to Radix Rubiae include antibacterial (Qiao et al., 1990) and anti-cancer (Adwankar et al., 1980; Tan et al., 2004). The phenolic and triterpenic constituents isolated from this herb have been shown to exhibit antioxidant and anxiogenic (anticonvulsant) properties respectively (Kasture et al., 2000; Cai et al., 2004). The results of our present project for the first time unambiguously demonstrated that Radix Rubiae has potent inhibitory action against the HaCaT human keratinocyte cell line. The positive results indicate that Radix Rubiae is a promising herb which could be developed as a therapeutic pharmaceutical for psoriasis treatment. As ethanolic extract is only a crude preparation form of this herb, it is rational to further fractionate this herbal extract to identify the most potent fraction and to investigate the active chemical constituents responsible for the observed biological activities. The fractionation of the ethanolic

extract of Radix Rubiae represents the initial step toward the final isolation and purification of active phytochemicals. We would also obtain from commercial sources the chemical constituents presented in Radix Rubiae and test their anti-proliferative action on cultured HaCaT cells in a hope to identify and characterize the active ingredients responsible for the biological activity possessed by Radix Rubiae. Finally, the mechanism of action underlying the anti-proliferative effect of Radix Rubiae would be investigated. In this regard, the apoptogenic potential of Radix Rubiae extract, its active fraction and phytochemicals would be evaluated.

3.2. Fractionation of the Ethanolic Extract of Radix Rubiae and Evaluation of the Anti-proliferative Action of the Fractions

3.2.1. Introduction

Radix Rubiae was found to have the most potent anti-proliferative action on the growth of keratinocytes among the 60 Chinese herbs in our initial screening programme. It is rational to separate and identify the most potent fraction that is responsible for the observed biological activities. Different organic solvents have different polarities which can extract different components of varying degree of polarities. The solubility of different organic solvents should be able to extract different chemical constituents presented in Radix Rubiae. Three organic solvents including hexane, ethyl acetate (EA) and n-butanol, and water are employed to separate ethanolic extract into four fractions. Each fraction will be subjected to biological investigation for the anti-proliferative action on HaCaT cells so that to determine whether the active constituents are dissolved in the fraction.

3.2.2. Materials and Methods

3.2.2.1. Fractionation of the ethanolic extract of Radix Rubiae

For the process of fractionation, 500 g of Radix Rubiae was ground to pieces in an electrical blender and refluxed with 4 L of 80% ethanol for 1 h. The ethanol was then

removed using a rotary evaporator under reduced pressure. The ethanolic extract was subsequently suspended in 300 ml of water and then sequentially partitioned with hexane, EA), n-butanol and water to afford the hexane, EA, n-butanol and the remaining water soluble fractions. Hexane, EA and n-butanol fractions were finally made to dryness using a rotary evaporator under negative pressure while the water fraction was lyophilized. The four dry fractions were weighed and then put in sealed plastic bags and stored at -20°C before use in cell culture experiment. Fig. 3.1. summarizes the working procedures of the fractionation process.

3.2.2.2. General cell culture.

The procedures of culturing the HaCaT cells have been described in detail in section 2.3.2.1..

3.2.2.3. Effects of the individual fractions of Radix Rubiae on the proliferation of HaCaT cells.

The resultant dry fractions of hexane, EA and n-butanol were redissolved in 80% ethanol to give their stock solutions of 5 and 10 mg/ml respectively. The water soluble fraction was redissolved in distilled water to give stock solution of 10 mg/ml. All these stock extracts were then sterilized by filtration (0.2-μm pore size filter, Corning,

NY, USA) before testing in cell culture experiments.

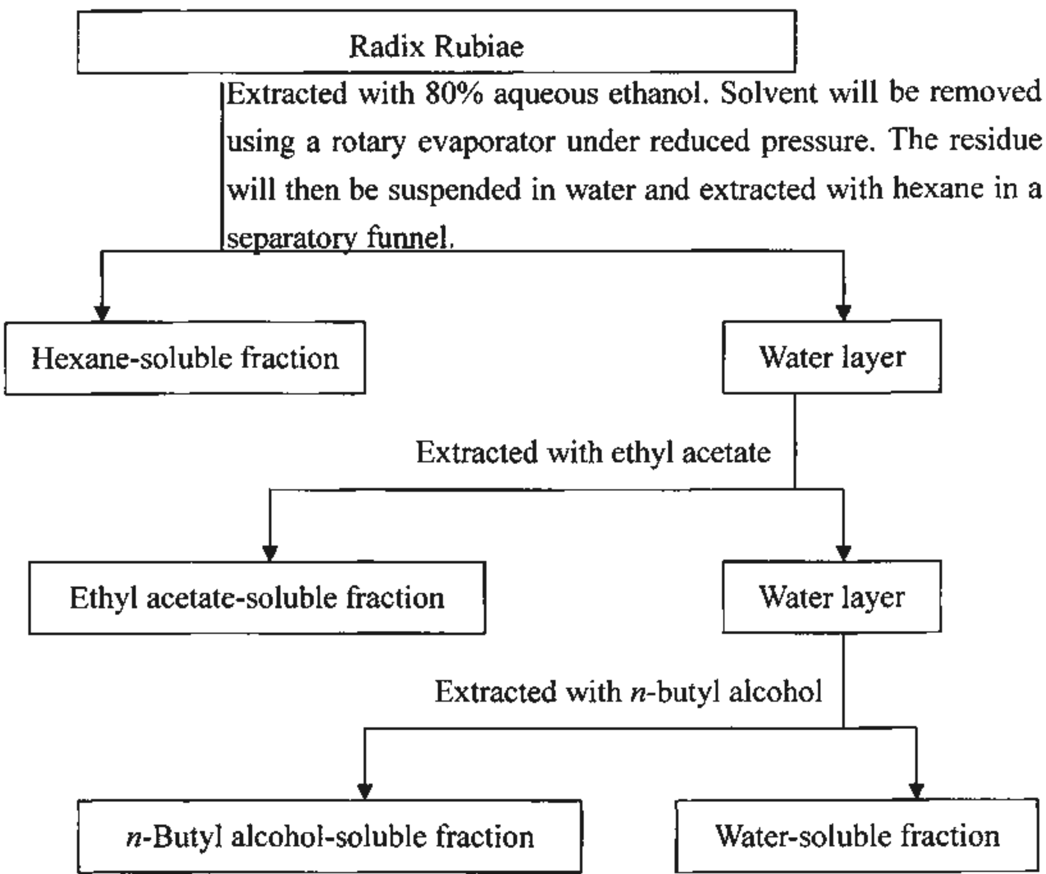


Fig. 3.1. Flow chart summarizing the extraction and fractionation of the Radix Rubiae.

The reconstituted fractions together with HaCaT cells were cultured in 96 well-plates, with each well containing 1 x 10^4 cells in 100 µl DMEM, and the final concentrations of the fractions ranging from 62.5 – 0.24 µg/ml by serial doubling dilutions. Each concentration of fractions had six replicates in the same plate. The cells were then grown for 48 h, after which the proliferation rate of the incubated cells was determined by MTT assay. The protocol of MTT assay has been described in session 2.3.2.3..

3.2.2.4. Statistical analysis.

Data were expressed as mean ± SD, with n=6. IC_{50} values were calculated using the GraphPad PRISM software version 3.0 (GraphPad Software, San Diego, CA, USA). Percentage extract yield (w/w) was calculated as dry fractionate weight/crude Radix Rubiae weight x 100.

3.2.3. Results

3.2.3.1. Yields of the fractions

The four different fractionates were collected and the results are summarized in Table 3.1. It is evidenced that the water-soluble fraction yielded the most amount of dry fractionate while the hexane-soluble fraction was the least productive.

3.2.3.2. Effect of the fractionates of Radix Rubiae on the growth of HaCaT cells

Fig. 3.2. shows the growth pattern of HaCaT cells in the presence of the four fractions of Radix Rubiae. The IC_{50} values of hexane, EA, n-butanol and water fractions were 36.4, 0.9, 7.8 and >62.5 μg/ml respectively. As shown in Table 3.1., among the four fractions tested, EA fraction showed the most potent anti-proliferative effect, with IC_{50} value being 0.9 μg/ml, which is smaller than that of the crude ethanolic extract of Radix Rubiae (IC_{50}, 1.4 μg/ml). This result indicates that EA fraction is more potent then its mother ethanolic extract in inhibiting the proliferation of HaCaT cells. On the other hand, the n-butanol and hexane fractions exhibited larger IC_{50} values than that of the crude extract. It is interesting to know that water fraction showed no inhibitory effect on cultured HaCaT keratinocytes.

Table 3.1. Weight and the percent yield of the four fractionates from 500 g Radix Rubiae.

Fractionate	**Weight of the fractionate (g)**	**% yield (w/w)[a]**	**IC_{50} (μg/ml)**
Hexane-soluble	5.9	1.18	36.4
Ethyl acetate-soluble	8.7	1.74	0.9
n-Butanol-soluble	20.5	4.1	7.8
Water-soluble	27.5	5.5	>62.5

[a]Percent yield (w/w) was calculated as dry fractionate weight/crude Radix Rubiae weight x 100.

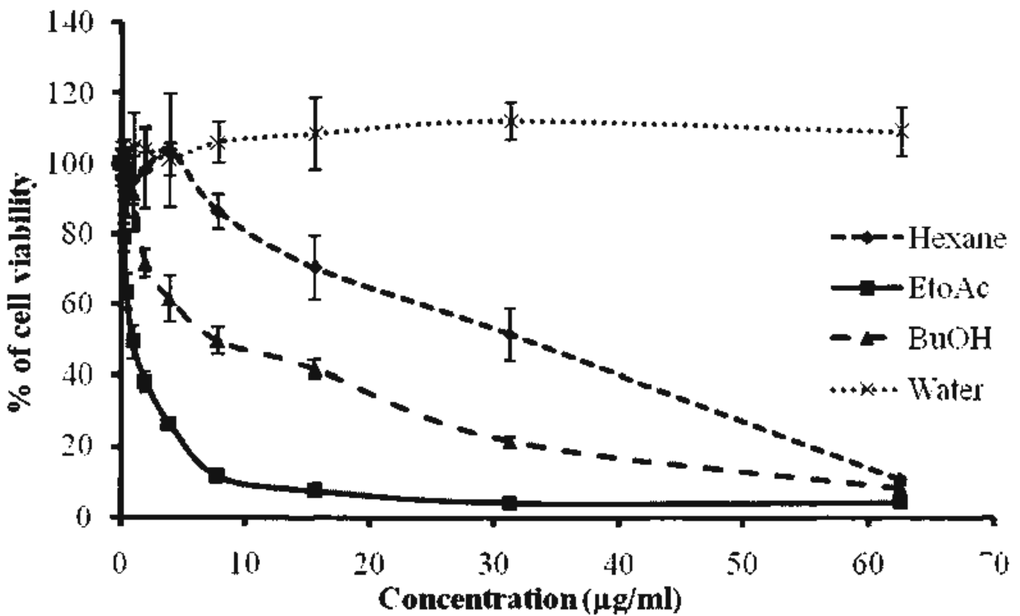


Fig. 3.2. The growth patterns of HaCaT cells in the presence of four fractions of Radix Rubiae. Each data point represents mean ± SD, n=6.

3.2.4. Discussion

We have demonstrated that ethanolic extract of Radix Rubiae possesses strong anti-proliferative activity on cultured HaCaT cells with IC_{50} of 1.4 μg/ml, which was comparable to the potency of dithranol (IC_{50}, 0.33 μg/ml), a therapeutic topical drug commonly used for psoriasis. Given that Radix Rubiae contains active and inactive components, the active components may have more powerful anti-proliferative property. Thus, it is worth isolating the pure compounds from Radix Rubiae, and the first step toward this target is to fractionate it using solvents of different polarity. In this experiment, four fractionates were obtained from Radix Rubiae. Hexane is the most non-polar solvent and water is the most polar one. The order of the polarity of solvents is water > n-butanol > EA > hexane. Our experiment results demonstrated that the EA fraction possessed the most significant anti-proliferative action with an IC_{50} value of 0.9 μg/ml, which is smaller than that of the crude Radix Rubiae extract (IC_{50}, 1.4 μg/ml). This finding indicates that the active ingredients responsible for epidermal keratinocyte growth inhibition are mainly present in the EA fraction. The EA fractionate could be formulated into topical preparation for the clinical treatment of psoriasis. For future work, this fraction would be further studied for the presence of active chemical components and elucidation of the cellular and molecular mechanisms underpinning the observed proliferation-inhibiting properties brought about by Radix

Rubiae. The identification of such ingredients is an important step towards development of new and effective pharmaceutical agents for the treatment of this common debilitating dermatological condition.

3.3. Quality Control of Radix Rubiae Extract

3.3.1. Introduction

Radix Rubiae contains a series of pigments, including purpurin, alizarin, rubiadin, munjistin and pseudopurpurin. In addition, triterpene rubifolic acid, rubicoumaric acid, rubiatriol, oleanolic acid acetate as well as sitosterol are also present in this herb (Zhu, 1998). Pharmacologically, Radix Rubiae has been shown to possess a number of biological activities, including hemostatic and hematopoietic effects in rabbits and mice models (Zhu, 1998). In our previous study, Radix Rubiae has been shown to have potent anti-proliferative properties on HaCaT cells *in vitro*. In addition, the EA fraction of Radix Rubiae showed the most potent keratinocyte growth inhibition. All these findings indicate that Radix Rubiae has good potential to be developed as a herb-based topical drug for psoriasis treatment. Before we further develop this herbal extract as a topical product, it is necessary to carry out the quality control on Radix Rubiae. As a means for quality control, high performance liquid chromatography (HPLC) fingerprinting would be established on this bioactive herbal extract.

HPLC is a powerful analytical method that is widely used for the separation, identification, and determination of the chemical components in complex mixtures. Components of a mixture are carried through the stationary phase by the flow of mobile

phase, separations are achieved based on differences in migration rates among the sample components (Skoog et al, 1988). Diode-array detector (DAD) is the most popular detector for detecting ultraviolet-visible compounds. Mass spectrometry (MS) can measure the atomic and molecular weights of charged species in a complex sample mixture. Modern mass spectrometers record both the mass-to-charge ratios of the ionized forms of atoms or molecules and also their relative abundance in the spectrum (Downard, 2004). In the quality control of Radix Rubiae, we used HPLC-DVD-MS for qualitative and quantitative analysis of Radix Rubiae extract.

3.3.2. Materials and Methods

3.3.2.1. Preparation of Radix Rubiae extract

The preparation of Radix Rubiae was described previously in Section 2.2.2.2. The resultant ethanolic extract (3.3 g) was stored at -20°C until used. Mollugin, one of the major ingredients present in Radix Rubiae, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

3.3.2.2. HPLC fingerprinting of Radix Rubiae extract

For quality control of the Radix Rubiae extract, quantitative analysis was performed and HPLC fingerprinting was constructed. Mollugin, a main ingredient present in the

Radix Rubiae (Chen and Wang, 1988), was selected as the chemical marker. The analyses were performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). Briefly, the samples were separated on an Alltech Alltima C18 column (4.6 x 250 mm, 5μm). The column temperature was set at 25°C. For fingerprinting, the mobile phase consisting of acetonitrile (A) and water (B), and a gradient programme was used as follows: a linear gradient from 30% A (V/V) to 80% A in the first 70 min, then to 100% A over 10 min and the flow rate was kept at 1.0 ml/min.

For HPLC-MS analysis, an Agilent 1100 LC/MD trap mass spectrometer was connected to the HPLC system via an APCI interface. Ultra-high purity helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. The optimized parameters in the positive ion mode were as follows: nebulizer gas pressure, 50 psi; dry gas flow, 5.0 L/min; dry temperature, 350°C; vaporizer temperature, 400°C. For full-scan MS analysis, the spectra were recorded in the range of *m/z* 100-500.

3.3.2.3. Quantitative analysis method

The quantitative analysis of Radix Rubiae was performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a secondary pump, a DAD, an autosampler, and a column compartment. The samples were separated on an Alltech

Alltima C18 column (4.6 x 250 mm, 5μm). The column temperature was set at 25°C. The DAD-recorded UV spectra in the range of 190-400 nm, and the HPLC chromatogram was monitored at 250 nm. The mobile phase for quantitative analysis was acetonitrile-water (80 : 20), and set at a rate of 0.8 ml/min. To establish the calibration curve, a stock solution of mollugin was prepared by dissolving the mollugin standard in methanol to a final concentration of 450 mg/L, then the stock was diluted to appropriate concentrations (15 - 450 mg/L). The linearity was established with two injections for each concentration of mollugin to obtain the mean peak areas; and the peak areas were plotted against the concentrations to construct a calibration curve.

3.3.3. Results

The calibration curve of mollugin was obtained ($y = 30.799\ x - 60.611$), with the correlation coefficient (R^2) value of 1, indicating that the quantification of mollugin by this HPLC method was linear in the range of 15 - 450 mg/L. Fig. 3.3. shows the overlapped HPLC chromatograms of mollugin in the standard solution and that present in the Radix Rubiae extract. The content of mollugin present in the Radix Rubiae extract was calculated to be 2.774%, which was arrived by using the following equation:

$$\text{\% of Mollugin} = \frac{\text{Amount of Mollugin extracted from Radix Rubiae}^{a}}{\text{weight of crude Radix Rubiae}} \times 100\%$$

a Calculated from the above calibration curve equation y = 30.799 x – 60.611.

The HPLC fingerprinting of Radix Rubiae extract was established and is shown in Fig. 3.4. The mollugin peak in the chromatogram of the extract sample was identified by comparing its retention time, UV absorption profile and HPLC-MS result with those of the mollugin standard solution. The established HPLC fingerprinting can be employed as a reference for the purpose of quality assurance for any future experiments on this herbal extract.

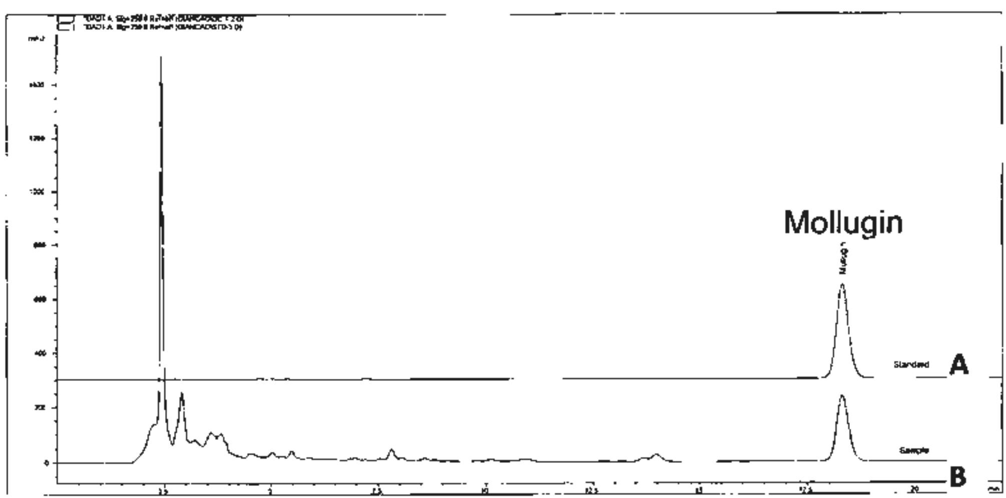


Fig. 3.3. Overlapped HPLC chromatogram for the quantitative analysis of mollugin. Conditions: Column, 4.6 x 250 mm I.D., packed with C18 (5μm); column temperature, 25°C; flow rate, 0.8 ml/min; mobile phase, acetonitrile-water (80 : 20); UV detection wavelength, 250nm. Chromatogram A and B denote the mollugin standard and the Radix Rubiae ethanolic extract, respectively.

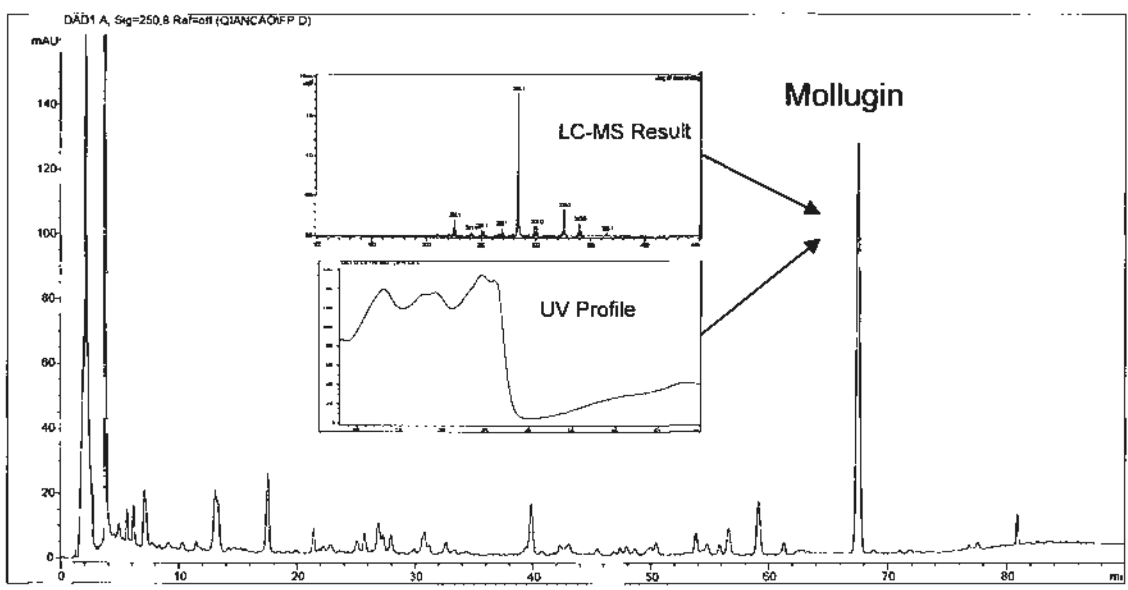


Fig. 3.4. HPLC fingerprinting for quality control of Radix Rubiae extract. Conditions: Column, 4.6 x 250 mm I. D., packed with C18 (5μm); column temperature, 25°C; UV detection wavelength, 250nm; flow rate, 1.0 ml/min; mobile phase consisted of acetonitrile and water with a linear gradient from 30% acetonitrile (v/v) to 80% acetonitrile in the first 70 min, then to 100% acetonitrile over 10 min. The peak of mollugin was identified by comparing its retention time (t_R, 67.5 min), UV absorption profile (λ_{max}, 216, 250, 272, 280 nm), and APCIMS data (M^+, *m/z* 284) with those of standard sample. For full-scan MS analysis, the mass spectra were recorded in the range of *m/z* 100-500.

3.3.4. Discussion

Quality control remains an important issue in the studies of herbal medicines. It is noteworthy that Radix Rubiae extract used in the present study was properly standardized. Considering that the bioactive constituents responsible for Radix Rubiae-mediated anti-proliferative action on human keratinocytes are still not well understood, and most possibly multiple constituents are involved in the pharmacological action, we therefore chose to quantify mollugin, a commercially available major constituent in Radix Rubiae, and to construct the HPLC fingerprinting of the extract. The concentration of mollugin and the characteristics of fingerprinting can present the quality index of the extract and make it possible to ensure a batch-to-batch consistency of the extract in future studies of this common Chinese herb.

3.4. Investigation of the Chemical Compounds in Radix Rubiae for Anti-proliferative Action on Cultured HaCaT Cells

3.4.1. Introduction

In the previous chapter, we demonstrated that Radix Rubiae was a promising anti-proliferative crude drug on cultured human keratinocytes and could be a valuable source from which to develop novel therapeutic agents for psoriasis treatment. In this section, we intended to investigate what chemical compounds are likely to be responsible for the Radix Rubiae-mediated anti-proliferative action. Bioassay-directed fractionation and isolation is a conventional way to obtain the bioactive compounds. However, this approach usually involves many time-consuming and laborious steps such as purification and the structural elucidation procedures. For this reason, we elected to by-pass this approach by using alternative method which was to purchase Radix Rubiae-derived chemical compounds from commercial sources. The pure compounds that were commercially available include mollugin, alizarin, purpurin and quinizarin. These four compounds were subjected to investigation for their anti-proliferative effect on HaCaT cells and Hs-68 cells in culture.

3.4.2. Materials and Methods

3.4.2.1. Chemical compounds from Radix Rubiae

Mollugin, Alizarin, Purpurin, and Quinizarin were purchased from Sigma Chemical Company (St. Louis, MO, USA.). Table 3.2. provides information about the names, molecular formulas and chemical structures of these four Radix Rubiae-derived compounds. All these compounds were dissolved in absolute ethanol to give stock solution of 10 mg/ml, and were sterilized by filtration (0.2- μm pore size filter, Corning, NY, USA) before being tested in cell culture experiments.

3.4.2.2. Anti-proliferative action of the chemical compounds on HaCaT cells by MTT assay

The detailed procedures of cell culture for HaCaT cells, Hs68 cells and MTT assay were described in section 2.3.2.1., 2.3.2.3. and 2.5.2.2.. In this set of experiments, the pure compounds together with HaCaT cells were cultured in 96 well-plates, with each well containing 1 x 10^4 cells in 100 μl DMEM. Each concentration of the same compound had six replicates in the same plate. Cells were then incubated for 48 h, after which the proliferation rate of the incubated cells was determined by MTT assay. Further experiments were conducted on Hs-68 cell line. The experimental protocol for Hs-68 cells was similar to that for HaCaT cells, and the cell numbers were also evaluated by

MTT assay.

Table 3.2. The names, molecular formulas and chemical structures of four Radix Rubiae-derived compounds obtainable from commercial sources.

Name of Compound	Chemical structure	Molecular formula	Molecular Weight
Mollugin	OH O O	$C_{17}H_{16}O_4$	284
Alizarin	O OH OH O	$C_{14}H_8O_4$	240
Purpurin	O OH OH O OH	$C_{14}H_8O_4$	256
Quinizarin	O OH O OH	$C_{14}H_8O_4$	240

3.4.3. Results

3.4.3.1. Effects of mollugin, alizarin, purpurin and quinizarin on HaCaT cell proliferation

Fig. 3.5a-d show the effect of mollugin, alizarin, purpurin, and quinizarin on the proliferation of HaCaT cells and Hs-68 cells. Table 3.3. summarizes their IC_{50} values on HaCaT cells and Hs-68 cells. Quinizarin (Fig. 3.5d) exhibited no inhibitory effect in both HaCaT and Hs-68 cells in all concentrations tested. On the other hand, mollugin, alizarin, and purpurin (Fig. 3.5a-c) demonstrated the inhibitory effect on HaCaT cells only at higher concentration. However, the responses are difference among these three compounds on Hs 68 cells. Purpurin exerted anti-proliferative action on Hs-68 at higher concentration (Fig. 3.5c). Interestingly, mollugin at high concentrations even caused proliferative effect on Hs-68 cells (Fig. 3.5a). No significant response on this cell line was observed for Alizarin (Fig. 3.5b).

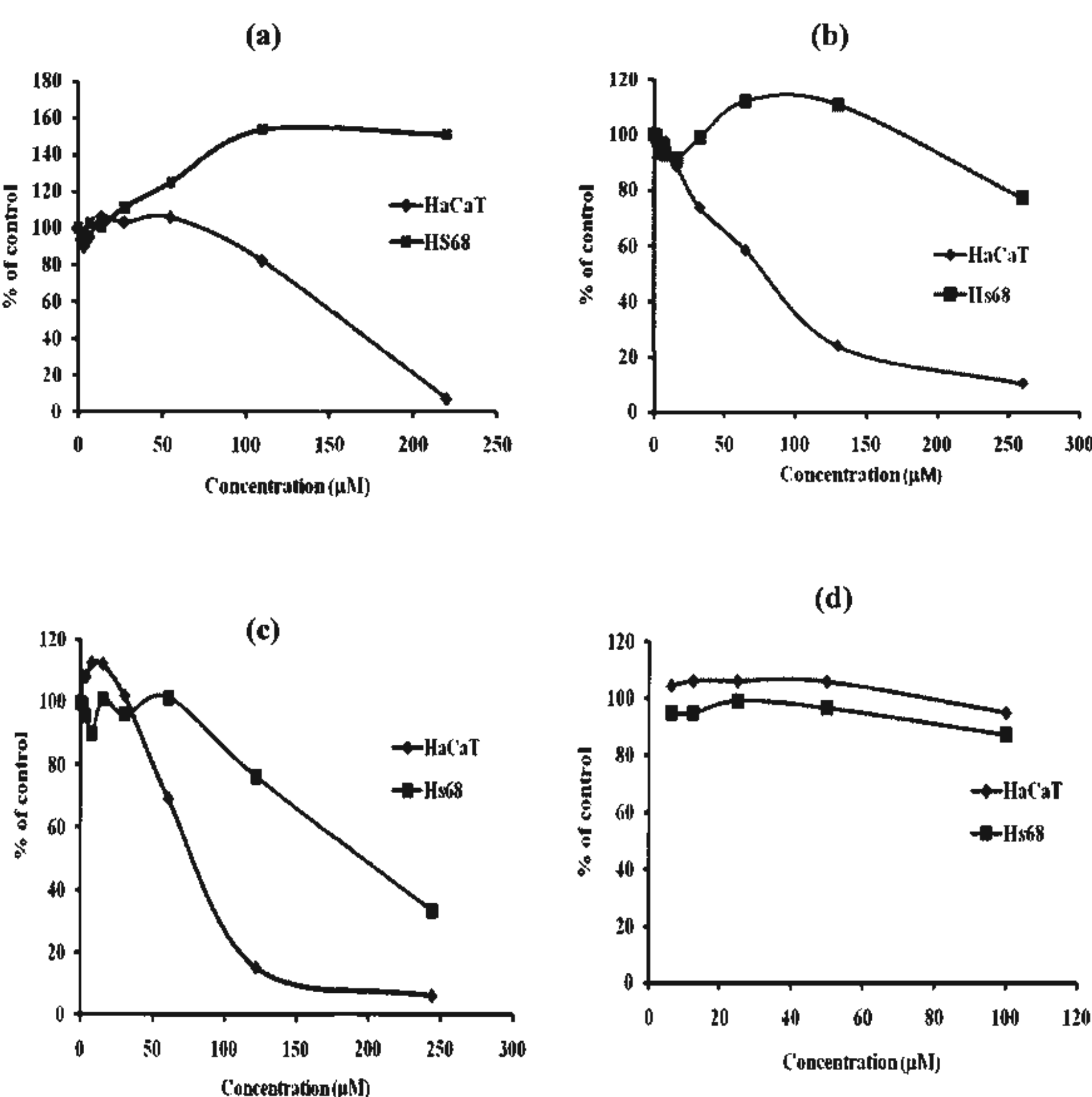


Fig. 3.5. Effects of four Radix Rubiae-derived chemicals on the growth of HaCaT and Hs-68 cells. (a). Mollugin; (b). Alizarin; (c). Purpurin; and (d). Quinizarin.

Table 3.3. The IC_{50} values of Mollugin, Alizarin, Purpurin and Quinizarin in HaCaT and Hs-68 cells.

Name of Pure compounds	Mollugin	Alizarin	Purpurin	Quinizarin	Dithranol
IC_{50} in HaCaT cells (μM)	157.3	80.73	88.1	NA	1.47
IC_{50} in Hs68 cells (μM)	NA	>260	196.7	NA	>36

NA, not applicable.

3.4.4. Discussion

Although many compounds have been reported to present in *Rubia cordifolia*, in our study, only four chemical compounds, namely mollugin, alizarin, purpurin and quinizarin, were commercially available for the experiments. In an attempt to unravel whether these compounds have anti-psoriatic activities, they were subjected to investigation for their effect on the growth of HaCaT and Hs68 cells. Interestingly, all these four compounds did not produce significant anti-proliferative action on cultured HaCaT cells with IC_{50} values greater than 80 μM. Their effects on cell growth are in stark contrast to that of dithranol, the positive control used in our experiment, which exhibited IC_{50} value of 1.47 μM only. It is therefore concluded that these compounds are not the active ingredients responsible for the anti-proliferative action observed with

Radix Rubiae; and they might not be the suitable candidates for further development into anti-psoriatic drug. The possible synergistic effect produced by all chemical constituents presented in Radix Rubiae may be responsible for the positive anti-proliferative effect of Radix Rubiae extract. Individual compound might not be able to produce significant anti-proliferative action in our experimental system. As a result of these negative findings, these compounds would not be the subjects for further in-depth investigation.

3.5. Apoptotic Studies on Radix Rubiae

3.5.1. Introduction

The maintenance of normal epidermal architecture is the result of a delicate balance between proliferation of cells in the basal cell layer and the death of keratinocytes, mainly through apoptosis, in the superficial layer of the epidermis. Epidermal hyperplasia and abnormal keratinization are two characteristic cellular events in psoriasis. Hyperproliferation of epidermal keratinocytes is the result of the aberrant expression of many regulatory molecules associated with proliferation, but defects in apoptosis are believed to play an important role in the pathogenesis of psoriasis (Boehm, 2006). Data from *in vitro* studies have shown that human keratinocytes derived from psoriatic lesions are resistant to induced apoptosis compared to keratinocytes derived from normal skin (Wrone-Smith et al., 1997). Moreover, induction of apoptosis of keratinocytes has been suggested to be responsible for the regression of psoriatic hyperplasia resulting in long-lasting clinical remissions after UVB phototherapy (Krueger et al., 1995). Other established anti-psoriatic therapies such as dithranol and vitamin D3 analogs exert their therapeutic actions through induction of keratinocyte apoptosis (Benassi et al., 1997; McGill et al., 2005). Agents that are able to inhibit keratinocyte proliferation and induce keratinocyte apoptosis therefore possess good potential for being developed into effective agents for treating psoriasis.

Apoptosis, a term coined by Kerr et al. in 1972, is a physiological process of programmed cell death responsible for the homeostasis of a variety of physiological systems in the body including skin. Morphological aspects of apoptosis comprise cellular shrinkage, chromatin condensation, blebbing, cellular fragmentation, and finally removal of apoptotic bodies via phagocytosis by neighboring cells or macrophages (Hughes and Mehmet, 2003). In the skin, apoptosis is a unique way of regulating keratinocyte proliferation, differentiation and epidermal growth (Thompson, 1995; Raskin, 1997). Balance between cell death and cell proliferation maintains homeostasis of epidermal compartment.

Given the intrinsic hyperproliferative nature of epidermal cells in psoriatic lesions, it has been postulated that acanthosis of psoriasis is a direct result of diminished apoptotic cell death of keratinocytes, and indeed, resistance of epidermal keratinocytes to apoptosis has been found in psoriatic lesions (Wrone-Smith et al., 1997). Apoptosis enables the elimination of dysfunctional cells without evoking an inflammatory response. Because of this unique function, apoptosis plays a crucial role in maintaining homeostasis in continually renewing tissues such as the skin (Bianchi et al, 1994; Reed, 1998), and counterbalances proliferation to maintain epidermal thickness and contributes to normal stratum corneum formation. On the contrary, defects in

epidermal apoptosis result in hyperproliferation of keratinocytes, a basic pathogenesis of psoriasis (Kawashima et al, 2004). Agents that induce keratinocyte apoptosis can therefore be useful in the treatment of psoriasis.

In our initial study in which 60 medicinal herbs commonly prescribed in TCM to treat psoriasis were investigated using a cultured HaCaT human keratinocyte model, we identified the ethanolic extract of the Radix Rubiae as possessing potent anti-proliferative action with an IC_{50} value of 1.4 μg/ml. The promising findings on the anti-proliferative properties of Radix Rubiae prompted us to undertake further phytochemical investigation for the most effective fraction of the extract and to investigate the underlying mechanism for the observed cellular growth inhibition. As apoptosis is a common and important mechanism contributing to a reduction of cellular proliferation, in this section we were intended to investigate whether apoptosis was responsible for the observed cellular growth inhibition. This section mainly focused on experimental findings concerning the apoptotic action of Radix Rubiae on cultured HaCaT keratinocytes.

3.5.2. Materials and Methods

3.5.2.1. Preparation of Radix Rubiae extract

Plant material of Radix Rubiae was obtained and extracted as described in section 2.2.2.1. and 2.2.2.2..

3.5.2.2. General cell culture

The source and procedures of culturing the HaCaT cells have been described in detail in section 2.3.2.1..

3.5.2.3. Fluorescent staining of HaCaT cells for morphological evaluation

Approximately 7.5×10^5 HaCaT cells were seeded in 6-well plates. The cells were treated with 16 μg/ml Radix Rubiae extract for 48 h. After the incubation, they were then washed with PBS and fixed in 4% paraformaldehyde for 30 min. Subsequently, they were stained with 20 μg/ml Hoechst 33342 (Molecular Probes, CA, USA) for 15 min at room temperature in the dark. Morphological changes in the cells treated with Radix Rubiae were evaluated using an inverted fluorescent microscope (Olympus, Tokyo, Japan) according to the method described previously (Abrams et al., 1993).

3.5.2.4. DNA fragmentation assay

A million HaCaT cells were seeded on 100-mm plates and exposed to Radix Rubiae extract at 2, 4, 8, 16 and 32 μg/ ml for 24, 48 and 72 h. After harvest, cells were lysed in 200 μl of DNA lysis buffer at 37°C for 15 min. The supernatant was sequentially incubated with 10 μl RNase (4 mg/ml) and then with 20 μl proteinase K (1.5 μg/ml) at 56°C for 1.5 h. The DNA of the cells was then precipitated with sodium acetate and centrifuged at 20,000 x g for 30 min. Finally, 30 μl of Tris-EDTA buffer was added to the sample and incubated at 37°C for 30 min. To analyze the fragmented DNA, 10 μl of the extracted cellular DNA was electrophoresed on a 1.5% agarose gel, and DNA ladders in the gels were visualized under UV light after staining with ethidium bromide.

3.5.2.5. TUNEL assay

To further analyze DNA fragmentation, the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay in which DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH termini with modified nucleotides was employed using methods described previously (Gavrieli et al, 1992; Portera-Cailliau et al, 1994; Sgonc et al, 1994). Briefly, 7.5×10^5 HaCaT cells were seeded on a 6-well plate and exposed to Radix Rubiae extract at 14 μg/ml for 48 h at 37°C. Cells were then fixed in 2% paraformaldehyde for 1 h and permeabilised with 0.1% Triton X-100

at 4°C for 2 min. The cells were then incubated at 37°C in the dark for 1 h with 50 μl TUNEL reaction mixture provided with the In Situ Cell Death Detection kit (Roche Applied Science, Philadelphia, PA, USA). Finally, the cells were re-suspended in 0.5 ml PBS and analyzed by FACSort flow cytometry (Becton Dickinson, NJ, USA).

3.5.2.6. Cell cycle analysis with PI staining

Approximately 7.5×10^5 HaCaT cells seeded on 6-well plates were exposed to Radix Rubiae at 1, 8, 16 and 32 μg/ml for 24, 48 or 72 h. After being washed in PBS. cells were fixed in 70% ethanol at 4°C overnight. The cells were then re-suspended in 10 μl PI solution (2 mg/ml) with 50 μl RNAase (10 mg/ml) and incubated in the dark at 37°C for 30 min. They were then subjected to DNA content analysis using a FACSort flow cytometer (Becton Dickinson, NJ, USA), in which the 'Cell Quest' program was used to analyze the results. Different phases of the cell cycle were assessed by collecting the signal at channel FL2-A. The percentage of the cell population at a particular phase was estimated by ModFit LT for Mac V.3.0 computer programme (Verity Software House, Topsham, ME, USA) according to the methods described previously (Nicoletti et al., 1991; Tounekti et al., 1995).

3.5.2.7. Quantitative analysis of apoptotic cells by annexin V-PI staining

In this experiment, 7.5×10^5 HaCaT cells were seeded on 6-well plates and incubated with Radix Rubiae at 1, 8, 16 and 32 μg/ml for 24, 48 or 72 h. Trypsinized cells were pooled together and stained concomitantly with annexin V and PI. The annexin V used was a chimeric recombinant protein produced by fusing green fluorescent protein (GFP) to the N-terminus of annexin V (Ernst et al., 1998). The stained cells were subsequently analyzed by flow cytometry (Becton Dickinson, NJ, USA). The signal was detected by FL1 and FL3 channels, and quadrant markers were set on dotplots of unstained and stained cells.

3.5.2.8. Western blot analysis of caspase-3

A million cells seeded on each 100-mm plate were exposed to Radix Rubiae extract at 1, 8, 16 and 32 μg/ml for 24, 48 and 72 h. The cells removed from the culture plates by scraping were lysed with lysis buffer for 3 h, and the resultant lysates were boiled for 10 min. The supernatant was collected and stored at -20°C. The protein concentrations were measured with the Bicinchoninic acid protein assay kit. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel. Separated proteins were then electro-transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA), which was then blocked with 10%

non-fat milk. Next the membrane was sequentially probed with the primary anti-caspase-3 antibody (Calbiochem, La Jolla, CA, USA) and then with the secondary peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, CA, USA). The immunoreactive bands were visualized with an ECL Western blotting detection kit (Amersham Life Sciences, Sydney, Australia) on light sensitive films (AGFA, Mortsel, Belgium). Rainbow molecular weight markers were used as size markers for the determination of protein size.

3.5.2.9. Statistical analysis

Data were expressed as mean ± SEM. Statistical comparisons between Radix Rubiae treatment and control were carried out using one-way ANOVA, followed by *post-hoc* Dunnett's test using the non-treatment as the control group on the SPSS for Windows (version 14.0). Differences were considered significant at $p < 0.05$, and were denoted as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

3.5.3. Results

3.5.3.1. Alteration of cellular morphology

After exposure to 16 μg/ml Radix Rubiae for 48 h, a greater number of HaCaT cells showed detachment from the culture plate when compared to the non-treatment control

(Fig. 3.6.a-b). They appeared to be shrunken and displayed fewer intercellular connections and showed typical apoptotic morphology characterized by chromatin condensation and DNA fragmentation.

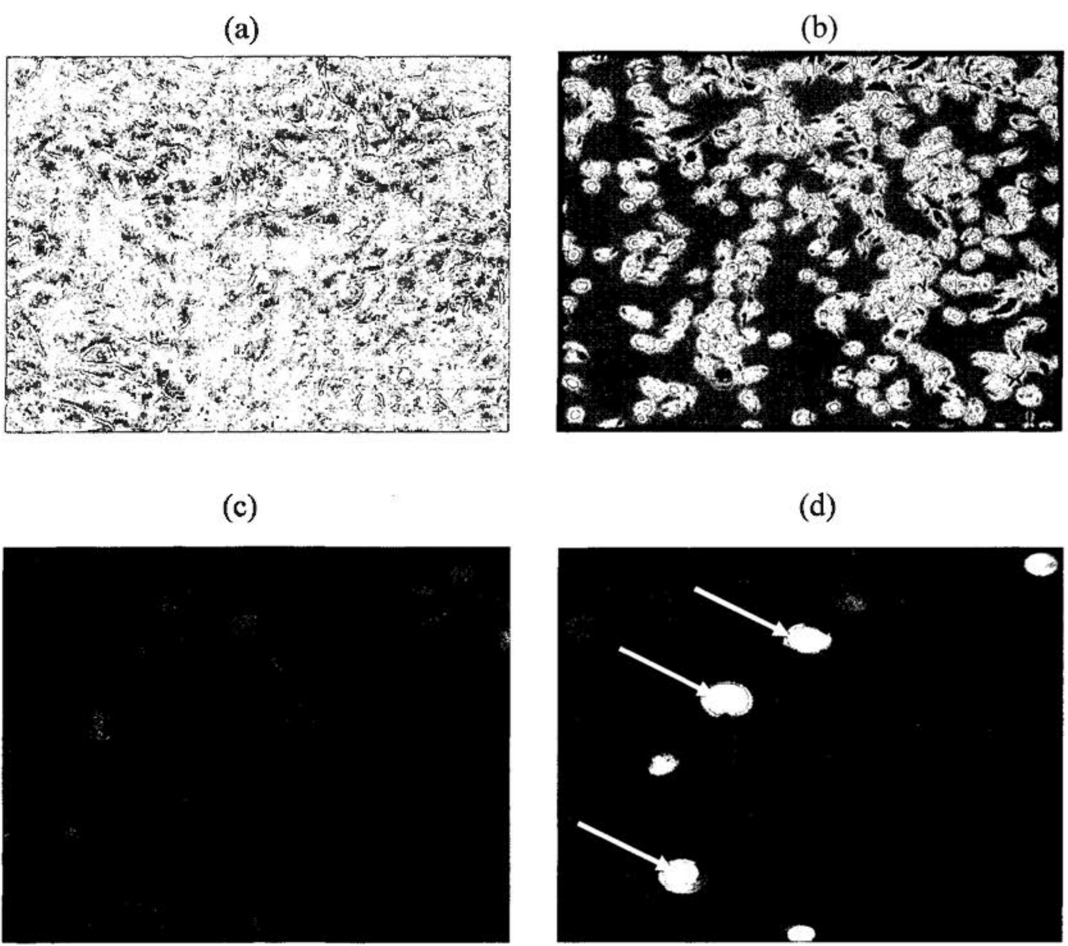


Fig. 3.6. Action of Radix Rubiae on HaCaT morphology. (a) Normal HaCaT cells. (b) HaCaT cells treated with 16 μg/ml Radix Rubiae extract for 48 h. (c) Normal HaCaT cells stained with Hoechst 33342. (d) HaCaT cells treated with 16 μg/ml Radix Rubia. Arrows point to the cells with chromatin condensation.

3.5.3.2. Detection of DNA fragmentation

Detection of DNA laddering on electrophoresis was used to confirm the morphological findings regarding the apoptotic action of Radix Rubiae. In Fig. 3.7.a, DNA laddering was evidenced when the cells were treated with a higher concentration of Radix Rubiae for 48 h. In Fig. 3.7.b, the laddering pattern of nucleosome monomer and oligomers was clearly distinguishable only after 48 and 72 h of incubation, but not at 24 h. The results indicated that the occurrence of cellular DNA fragmentation mediated by Radix Rubiae was dose- and time-related. The appearance of DNA laddering is indicative of DNA fragmentation, and a characteristic feature of cellular apoptosis.

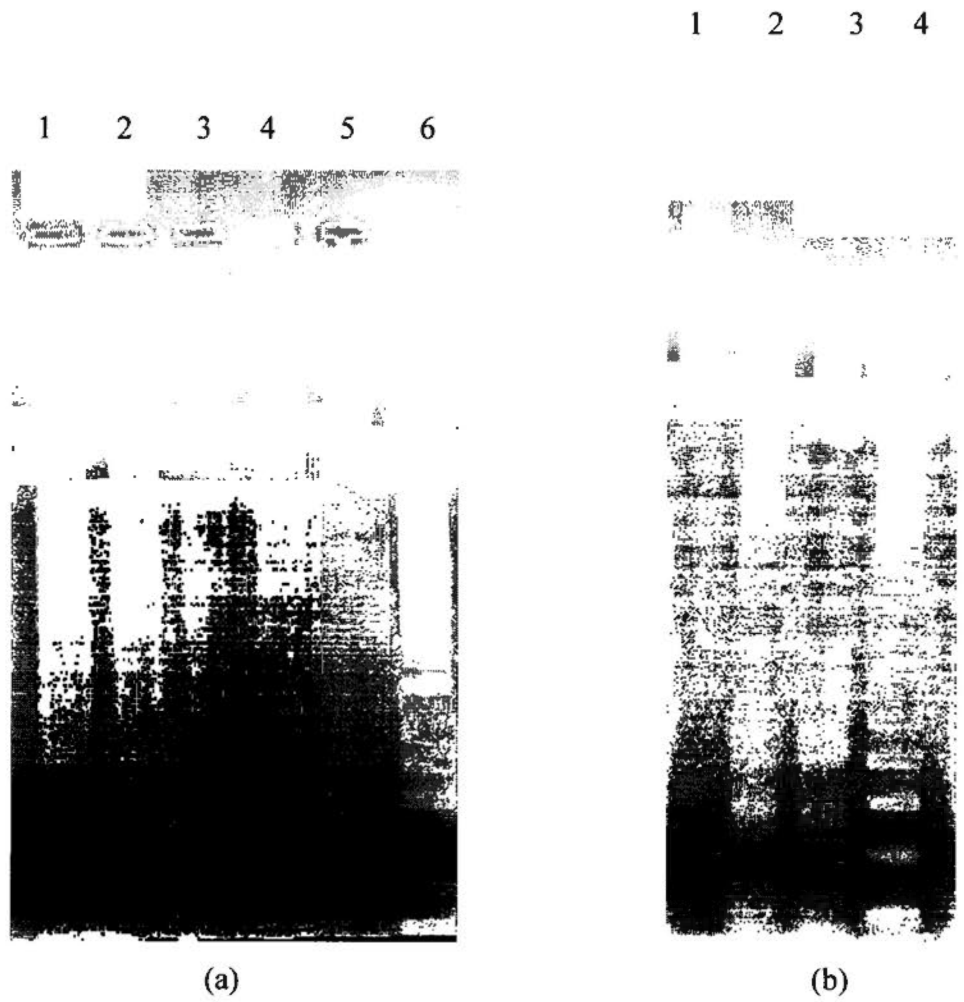


Fig. 3.7. Action of Radix Rubiae on DNA fragmentation in HaCaT cells.

(a) Lanes 1 to 6 are Radix Rubiae extract at 32, 16, 8, 4 and 2 μg/ml, 100-base-pair marker, respectively. (b) Lane 1 to 4 correspond to an incubation of 32 μg/ml Radix Rubiae extract for 24, 48, 72 and 100-base-pair marker, respectively.

These results were confirmed by the TUNEL assay which constitutes another method to detect the fragmented DNA by identifying the apoptotic cells in situ using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the strand breaks of cleaved DNA. The DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH terminal with modified nucleotides. Comparing to the control in Fig. 3.8.a, 14 μg/ml Radix Rubiae (Fig. 3.8.b) was capable of inducing the appearance of apoptotic peaks, indicative of the occurrence of apoptosis in the HaCaT cells.

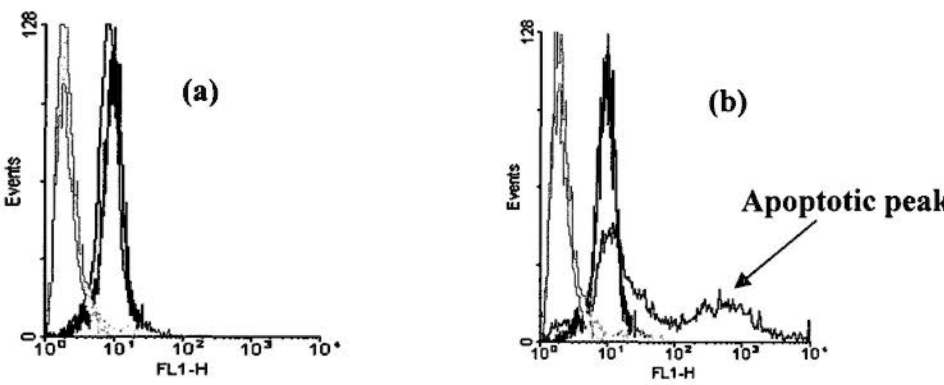
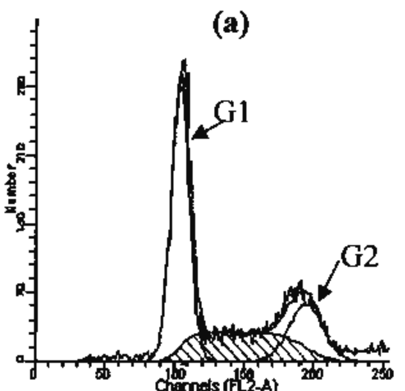
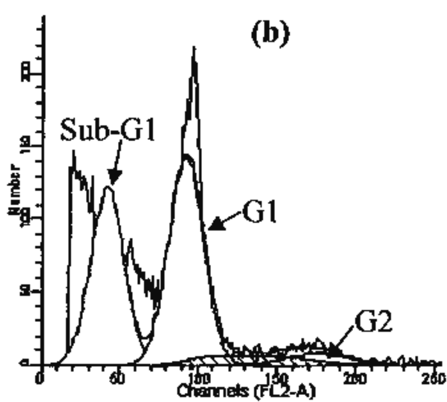


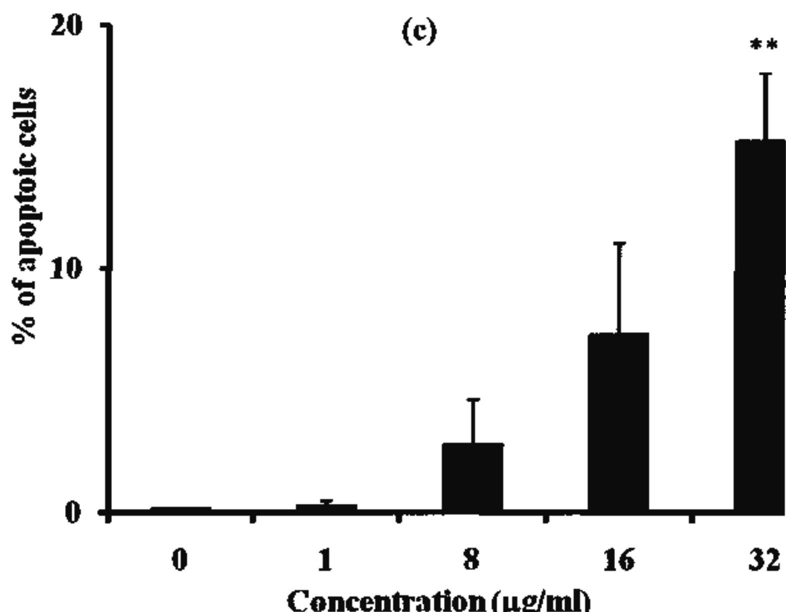
Fig. 3.8. TUNEL analysis of Radix Rubiae-induced apoptosis in HaCaT cells. (a) Cells cultured in the absence of the herbal extract. (b) Cells cultured in the presence of 14 μg/ml Radix Rubiae extract for 36 h. Green line, control for autofluorescence of cells in the absence of label or enzyme solution. Black line, non-treatment control incubated with label solution. Red area indicates cells incubated with TUNEL reaction mixture with both label and enzyme solution

3.5.3.3. Action on cell cycle progression

The flow cytometric measurement of PI-stained DNA is shown in Fig. 3.9.a and b. Radix Rubiae extract at the concentrations tested was capable of inducing the sub-G1 phase. The appearance of the sub-G1 phase is indicative of the occurrence of apoptosis (Nicoletti et al, 1991; Telford et al, 1992). After 48 h of treatment with the extract, the amount of cells in sub-G1 phase increased from 0.3 to 15.2% when the concentration was increased from 1 to 32 μg/ml (Fig. 3.9.c). Also, the sub-G1 population gradually increased from 3.3 to 34.3% when the incubation time was extended from 24 to 72 h in the presence of 32 μg/ml Radix Rubiae extract (Fig. 3.9.d). Moreover, apart from the induction of the sub-G1 peak, Radix Rubiae also altered the cell cycle distribution of the cultured HaCaT cells. After treatment for 48 h with 32 μg/ml of Radix Rubiae extract, the percentage of cells in G1, S and G2/M phases changed from 52.0, 31.1 and 16.9% to 76.3, 14.2 and 9.5%, respectively (Fig. 3.10.a). Furthermore, when exposed to longer time periods, the percentage of cells in the G1 phase increased markedly, while S and G2/M phases decreased accordingly (Fig. 3.10.b). Taken together, the results from our experiments indicated that Radix Rubiae induced HaCaT cell arrest at the G1 phase.


(a)
G1
G2
Channels (FL2-A)


(b)
Sub-G1
G1
G2
Channels (FL2-A)


(c)
% of apoptoic cells
20
10
0
**
0
1
8
16
32
Concentration (μg/ml)

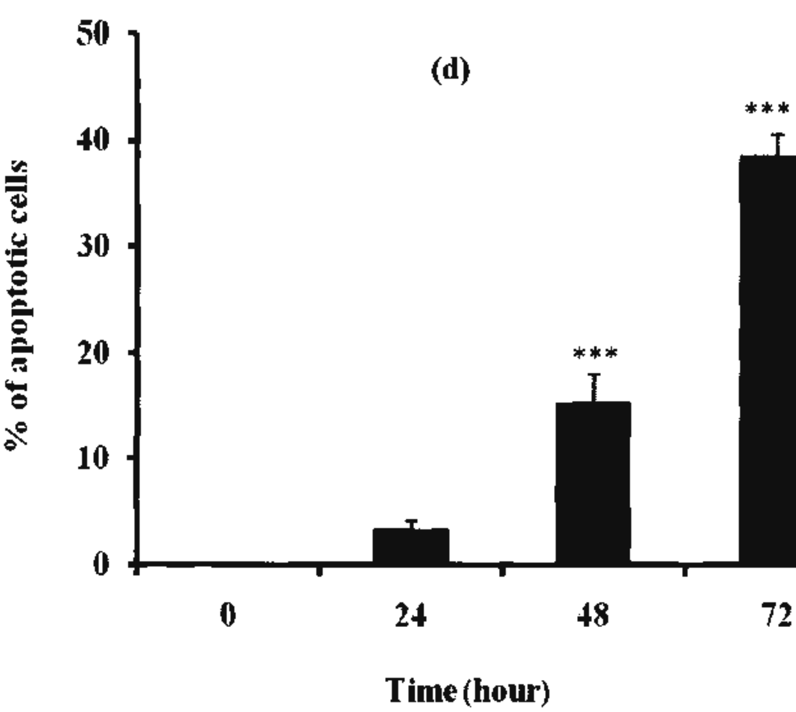


Fig. 3.9. Flow cytometric analysis of cell cycle distribution of cultured HaCaT keratinocytes with PI staining. (a) Non-treatment control only. (b) Cells treated with 32 μg/ml of Radix Rubiae extract for 72 h. Note the appearance of sub-G1 phase upon treatment with Radix Rubiae. (c) Dose-dependent action of Radix Rubiae on the induction of sub-G1 phase on HaCaT keratinocytes. HaCaT cells were cultured for 48 h. (d) Time course effect of Radix Rubiae on the induction of sub-G1 phase on HaCaT keratinocytes. HaCaT cells were cultured in the presence of 32 μg/ml Radix Rubiae extract. The values shown in c and d are the mean ± SEM with n=3. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ when compared with the non-treatment control.

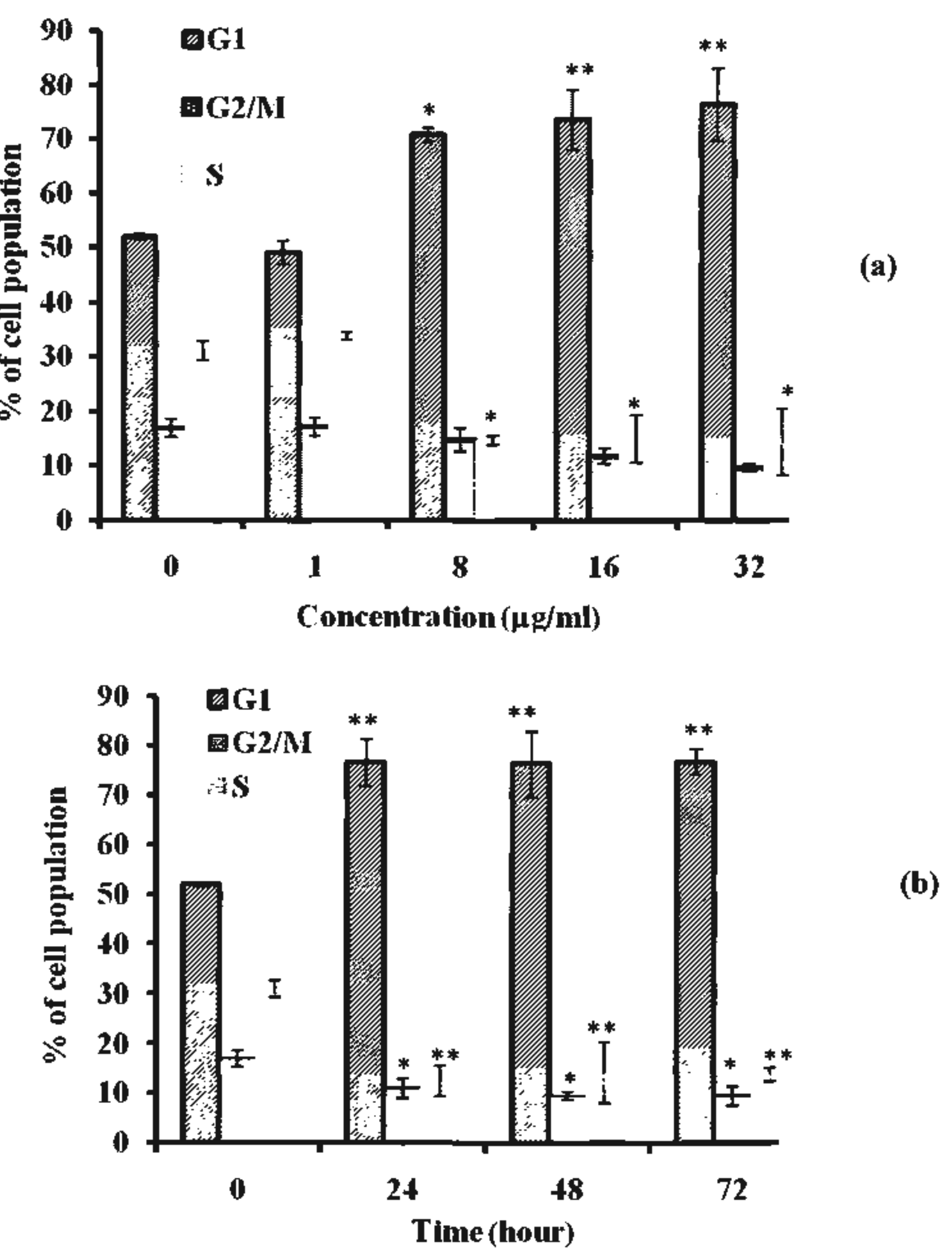


Fig. 3.10. Distribution of the cell cycle of HaCaT keratinocytes under the influence of Radix Rubiae. (a) The cells were exposed to different concentrations of Radix Rubiae extract for 48 h followed by flow cytometric analysis with PI staining. (b) The cells were treated with 32 μg/ml Radix Rubiae extract for 24, 48 and 72 h, respectively. The values shown are the mean ± SEM, with n=3. *, $p < 0.05$ and **, $p < 0.01$ when compared with the non-treatment control.

3.5.3.4. Quantitative analysis of apoptotic cells by annexin V-PI staining.

Early in apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer surface of the plasma membrane. PS exposure, therefore, represents a useful target for evaluating apoptosis (Fadok et al., 1992; Martin et al., 1995; Vermes et al., 1995). The translocation of PS from the inner to the outer surface of the plasma membrane during apoptosis can be detected by annexin V which binds preferentially to PS in the presence of Ca^{2+}. To assess plasma membrane changes, cells were stained with annexin-V and PI simultaneously. The combination of staining with annexin-V and PI which does not diffuse through intact cell membranes allows discrimination between, and quantification of, apoptotic, necrotic and viable cells. As shown in Fig. 3.11, the viable, apoptotic and necrotic cells were localized in the lower left, the lower right, and the upper right quadrant, respectively. When exposed to lower concentrations of the Radix Rubiae extract, the majority of cells were localized in the lower left quadrant, indicative of an absence of overt cell death. As the concentration of Radix Rubiae increased from 1 to 32 μg/ml, the percentage of apoptotic cells was significantly elevated from 3.5 to 49.4%; and accordingly, the percentage of viable cells decreased from 87.1 to 31.3% after 48 h of drug treatment (Fig. 3.12.a). Since apoptotic cells *in vitro* will eventually undergo 'secondary necrosis', the percentage of necrotic cells thus increased from 9.5 to 21.9%. Likewise, the percentage of apoptotic cells markedly increased from 23.3 to 60% and

the viable cells significantly decreased from 56.2 to 12.0% as the incubation time was extended from 24 to 72 h in the presence of 32 μg/ml Radix Rubiae. However, the percentage of necrotic cells did not change significantly (Fig. 3.12.b). These results unambiguously demonstrated that induction of cellular apoptosis was mainly responsible for the Radix Rubiae-mediated HaCaT keratinocyte growth inhibition, and the apoptotic action of this herb was dose- and time-dependent.

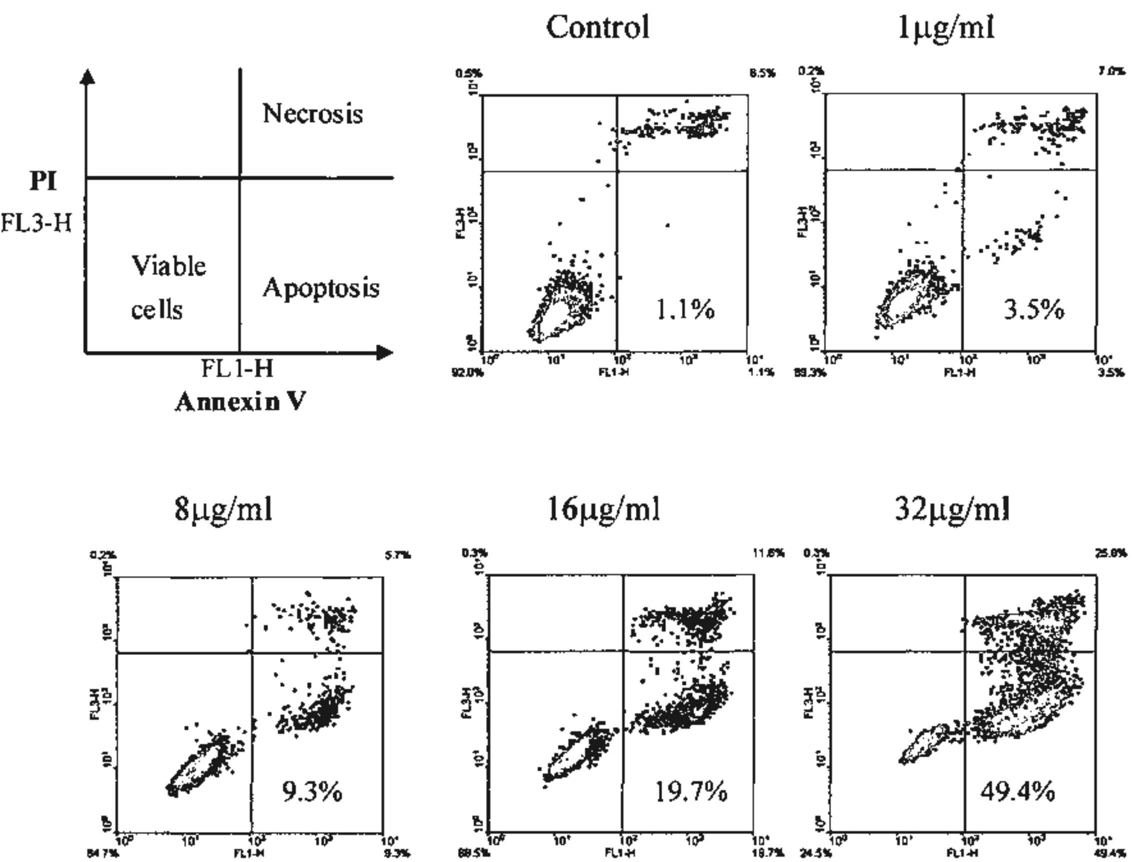


Fig. 3.11. Quantitative analysis of Radix Rubiae-induced apoptosis by annexin V-PI binding assay. Figures show the distribution of apoptotic cells in the total HaCaT cell population in the presence of Radix Rubiae extract (1,8,16 and 32 μg/ml) for 48 h followed by labeling with annexin V and PI and analysis by flow cytometry.

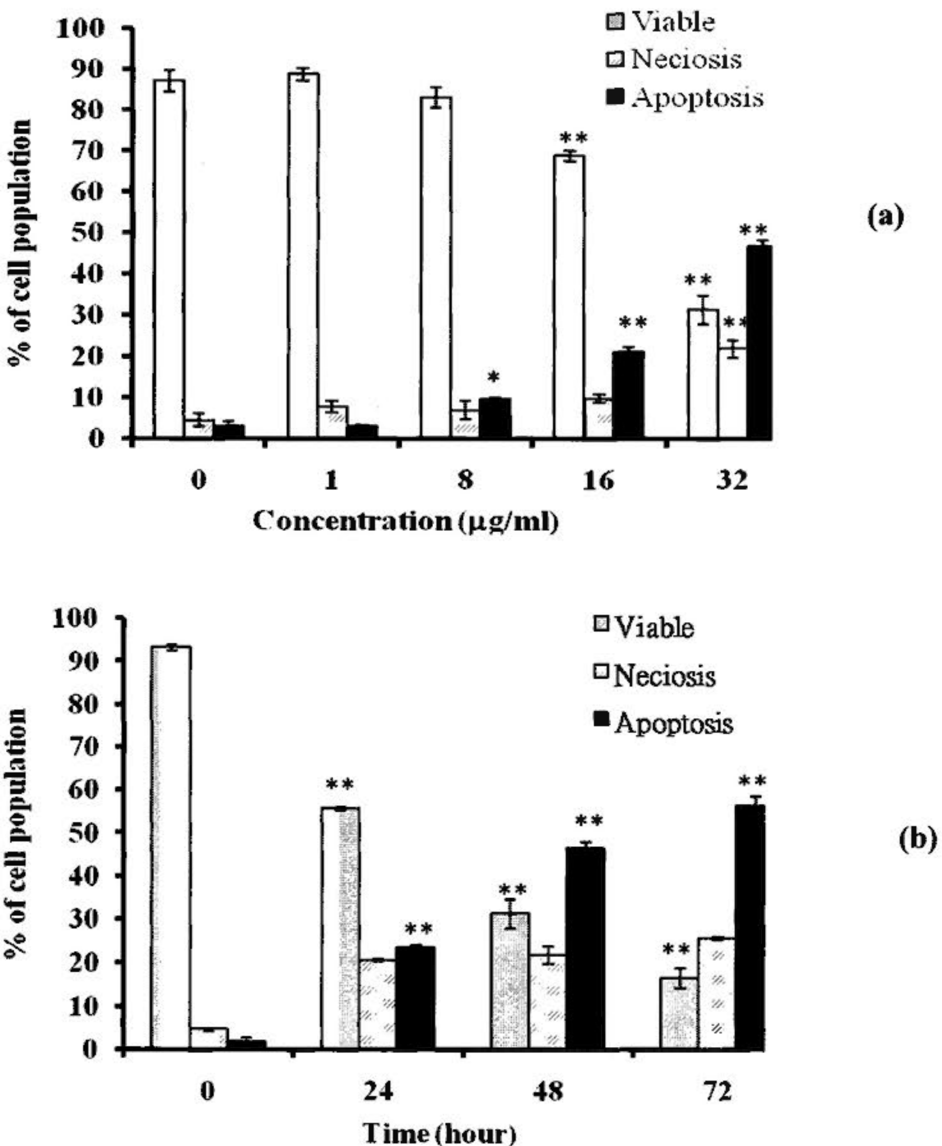


Fig. 3.12. Distribution of viable, apoptotic and necrotic HaCaT keratinocytes in the presence of Radix Rubiae extract. (a) Bar chart presentation of the distribution of viable, apoptotic and necrotic cell populations after treatment with Radix Rubiae. (b) Bar chart presentation of time course effect on the distribution of viable, apoptotic and necrotic cell in the presence of 32 μg/ml Radix Rubiae extract. The values shown represent the mean ± SEM with n=3. *, $p < 0.05$; and **, $p < 0.01$ when compared with the non-treatment control.

3.5.3.5. Western blot analysis

Caspase-3 is the apoptosis promoting enzyme responsible for cleaving cellular substrates leading to the characteristic cellular morphological alterations. The results of caspase-3 activation by different concentrations of Radix Rubiae extract are shown in Fig. 3.13.a. Radix Rubiae were able to significantly increase the activity of caspase-3 (19 and 17 kDa) and decrease procaspase-3 (32 kDa) in a dose-dependent manner. Likewise, in the presence of 32 μg/ml Radix Rubiae, the expression of procaspase-3 decreased and the activation of caspase-3 increased as the exposure time was extended (Fig. 3.13.b).

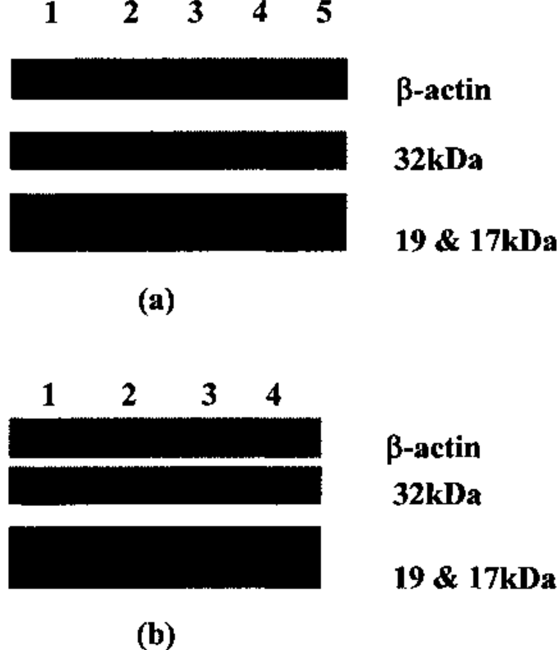


Fig. 3.13. Western blot analysis of the Radix Rubiae-induced expression of caspase-3. (a) Lanes 1 to 5 correspond to control, 1, 8, 16, and 32 μg/ml of Radix Rubiae extract, respectively. (b) Lanes 1 to 4 correspond to the incubation time of 0, 24, 48, and 72 h, respectively in the presence of 32 μg/ml Radix Rubiae extract. Note that the band sizes 19 and 17 kDa are activated caspase-3, and band size 32 kDa is procaspase-3.

3.5.4. Discussion

In our initial screening programme aimed at evaluating the anti-psoriatic properties of Chinese herbal medicines, Radix Rubiae was identified to possess potent anti-proliferative action on human keratinocytes. The elucidation of the underlying cellular and biochemical mechanisms for the observed growth inhibitory action is necessary for this bioactive herbal extract to be developed as an effective therapy for psoriasis treatment. In the present study, experiments were designed to elucidate at the morphological, molecular and biochemical levels whether induction of cellular apoptosis is responsible for the Radix Rubiae-mediated growth inhibition on human keratinocytes.

Morphologically, apoptosis is characterized by membrane blebbing and DNA fragmentation in individual cells. Radix Rubiae-treated HaCaT cells were found to have hypercondensed nuclei and DNA fragmentation when stained with the Hoechst stain followed by observation under the microscope. DNA cleavage is a biochemical hallmark of apoptosis, and assays which measure prelytic DNA fragmentation are especially useful for the determination of apoptotic cell death (Compton 1992). Electrophoresis of fragmented DNA can provide clear evidence of DNA laddering (Wyllie 1980; Barry and Eastman 1993). In our experiments, Radix Rubiae induced DNA fragmentation as illustrated by gel electrophoresis. Using the TUNEL method,

we further demonstrated that DNA strand breaks were induced in the HaCaT cells by Radix Rubiae.

Cell cycle progression analysis by flow cytometry revealed that Radix Rubiae significantly increased the population of HaCaT cells in the sub-G1 phase (apoptotic peak) while reducing the number of cells in the G2/M and S phases. This result suggests that Radix Rubiae induces cell cycle arrest at the G1 phase, thereby causing apoptosis.

The discrimination between apoptotic and necrotic cells was achieved by quantitatively estimating the relative amount of the annexin V- and PI-stained cells in the population. The results from quantitative analysis of apoptotic cells by concomitant annexin V-PI staining also revealed that Radix Rubiae induced apoptosis of the HaCaT keratinocytes in a time- and concentration-dependent manner. The physical destruction of the cell occurring during apoptosis is mediated by a class of enzymes called cysteine proteases, or caspases, which are responsible for the cleavage of specific protein substrates at an amino acid position immediately following an aspartic acid residue. Caspase-3 is the major active caspase in apoptotic cells, and its activation is a pivotal event in the execution of apoptosis (Hoshi et al., 1998; Kirsch et al., 1999). In our present study,

the activation of caspase-3 was detected when the HaCaT keratinocytes were exposed to the Radix Rubiae extract, indicating unequivocally the occurrence of cellular apoptosis. Collectively, our experimental results confirm that Radix Rubiae is capable of inducing programmed cell death in cultured HaCaT keratinocytes. The apoptotic actions observed in the present study provide an explanation for the underlying mechanism of the potent anti-proliferative property exhibited by Radix Rubiae on HaCaT cells.

3.6. General Discussion

In the previous chapter, we showed that Radix Rubiae possessed significant anti-proliferation on cultured HaCaT cells. In this chapter, we undertook the phytochemical investigation on this herbal extract and mechanistic studies for the observed anti-proliferative effect of this herb on HaCaT cell. We identified that the EA fraction possessed the most potent keratinocyte growth inhibition. It is important to control the quality of Radix Rubiae to ensure a batch-to-batch consistency of the extract in future studies. In our experiments, quality control of Radix Rubiae was established by constructing a HPLC fingerprinting with mollugin being used as a chemical marker. However, to further isolate the active ingredients from EA fraction would involved many time-consuming and laborious steps. In our project, we decided to purchase commercially available chemical compounds including mollugin, alizarin, purpurin and quinizarin; and their anti-proliferative action on HaCaT keratinocytes were evaluated. It is interesting to know that all these Radix Rubiae-derived chemical compounds did not possess anti-proliferative action.

The promising findings on the anti-proliferative properties of Radix Rubiae prompted us to undertake mechanistic investigation on the observed cellular growth inhibition. As the cellular apoptosis is one of the main mechanisms leading to the inhibition of cellular

proliferation, in this mechanistic study, various bioassay methods including morphologic observation, DNA fragmentation, TUNEL, cytometric analysis, cell cycle analysis and Western blotting were used to elucidate whether induction of apoptosis underlay the Radix Rubiae-mediated growth inhibition. Collectively, our experimental results confirmed that Radix Rubiae was capable of inducing programmed cell death in cultured HaCaT keratinocytes. The apoptotic actions observed in the present study provide a mechanistic explanation for the potent anti-proliferative property exhibited by Radix Rubiae on HaCaT cells.

Our present study was the first to report the anti-proliferative and apoptogenic effects of Radix Rubiae, and provided scientific explanation for the traditional use of this herb in the treatment of psoriasis. The successful identification of Radix Rubiae as a potent anti-proliferative and apoptogenic agent also renders it a promising candidate and lays the groundwork for its further development into a topical therapeutic agent for psoriasis. Results of further experiments to evaluate the anti-psoriatic potential of Radix Rubiae as a topical agent on psoriasis-relevant animal models *in vivo* will be presented in the Chapter 6.

Chapter Four

Anti-psoriatic Action of Realgar and Arsenic Compounds – Studies of Anti-proliferation and Apoptosis Induction on HaCaT Human Keratinocytes

4.1. Introduction

In our earlier screening programme, realgar, a mineral commonly used in TCM for topical treatment of psoriasis and the main chemical constituent of which is As_2S_2, was found to be a potent anti-proliferative agent on HaCaT cells (section 2.3.). This encouraging experimental finding stimulated us to further investigate whether other arsenic compounds also possess similar anti-proliferative properties. The identification of active anti-proliferative arsenics and the elucidation of their action mechanism would lead to the development of topical agents for effective management of psoriasis.

Arsenics are inorganic metalloids which are ubiquitously distributed throughout the Earth's crust. For centuries, some of these inorganic compounds have been used to treat a variety of ailments in many traditional medical systems, and remain an essential element in many traditional medical practices in Asia today (Lederer and Fensterheim, 1983). In TCM practice, for example, arsenic-containing minerals, such as realgar and sulfur, are primarily prescribed for the topical treatment of scabies, carbuncles, herpes zoster, enduring ulcers, psoriasis and arthritis (Jiangsu New Medical College, 1986; Hua et al., 2003). Recent decades have also seen the resurgence of use of arsenic compounds in Western medicine. For instance, arsenic trioxide, when used in

conjunction with other chemical constituents such as potassium bicarbonate, is indicated for malignant conditions such as leukemia and Hodgkin's disease, and non-malignant diseases such as eczema, psoriasis, pemphigus and asthma (Gallagher, 1998). However, the contemporary widespread use of arsenic compounds in patients with hematologic malignancies did not occur until the 1990s, when several Chinese research groups reported the dramatic clinical response in patients with acute promyelocytic leukemia (APL) when treated with arsenic trioxide (Mervis 1996; Du and Li, 2001; Suo and Jia, 2003; Berenson and Yeh, 2006). As a result of much intensive research, arsenic trioxide has now been approved by the Food and Drug Administration (FDA) of the USA for the treatment of relapsed and refractory APL. In addition to their application in hematologic malignancies, medical literature in TCM has also indicated various arsenic compounds for effective treatment of psoriasis and parasitic diseases, although the underlying mechanisms of action for these treatments have largely remained obscure.

Compounds that inhibit keratinocyte proliferation and modulate keratinocyte differentiation are potentially useful in the treatment of psoriasis because a balanced homeostatic control of keratinocyte growth and differentiation is crucial for recovery from psoriatic to normal epidermis. In this chapter, the growth inhibitory action of

three arsenic compounds, namely arsenic trioxide, arsenic pentoxide and arsenic iodide, on a cultured HaCaT human keratinocytes would be investigated. In addition, we would also elucidate whether apoptosis was the underlying mechanisms of action responsible for the anti-proliferative effect brought about by realgar extract and the arsenic compounds.

4.2. Anti-proliferative Action of Arsenic Compounds on HaCaT Keratinocytes

4.2.1. Introduction

In our initial screening programme we have found that realgar, a commonly used mineral in TCM practice for topical treatment of psoriasis, possessed potent anti-proliferative action on cultured human HaCaT keratinocytes with an IC_{50} value of 6.6 μg/ml. This promising experimental finding stimulated us to further investigate whether arsenic compounds presented in this mineral also possess similar anti-proliferative properties. The identification of active anti-proliferative arsenic compounds and the elucidation of their action mechanism would be necessary steps leading to the development of topical agents for effective treatment of psoriasis. In this section, the anti-proliferative effect of three arsenic compounds, viz. arsenic trioxide, arsenic pentoxide and arsenic iodide on the cultured HaCaT cells would be investigated. The promising keratinocyte growth inhibition seen with these arsenic compounds would herald mechanistic studies on the possible apoptosis-inducing effect of these arsenic salts.

4.2.2. Materials and Methods

4.2.2.1. Arsenic compounds

Arsenic trioxide (As_2O_3), arsenic pentoxide (As_2O_5) and arsenic iodide (AsI_3) were purchased from Sigma-Aldrich (St. Louise, MO, USA.). The compounds were dissolved in PBS in room temperature to give 10 mM stock solutions. Vigorous stirring was necessary to aid the complete dissolution of these compounds. The solutions were then sterilized by filtration (0.2-μm pore size filter, Corning, NY, USA) before use in cell culture experiments.

4.2.2.2. General cell culture

The procedures of culturing the HaCaT cells and Hs-68 cells were described in detail in section 2.3.2.1. and 2.5.2.2..

4.2.2.3. Anti-proliferative assay

The arsenic compounds together with HaCaT cells were cultured in 96 well-plates, with each well containing 2 x 10^4 cells in 200 μl DMEM. By serial dilutions, the final concentrations of arsenic trioxide, arsenic pentoxide and arsenic iodide ranged from 100 to 0.4 μM, 250 to 1 μM, and 250 to 1 μM, respectively. The treated HaCaT cells were incubated for 12, 24 and 48 h and the proliferation rates under the influence of these

inorganic compounds were determined by MTT assay, the detailed protocol of which was described in section 2.3.2.3. Similarly, Hs-68 cells were exposed to arsenic trioxide, arsenic pentoxide and arsenic iodide at concentrations from 100 to 0.4 μM by serial dilution. MTT assay was also used to determine the proliferation of Hs-68 cells after 48 h incubation.

4.2.2.4. Statistical analysis

Data were expressed as mean ± SD (n=6). IC_{50} values were determined using the GraphPad PRISM software version 3.0 (GraphPad Software, San Diego, CA, USA).

4.2.3. Results

4.2.3.1. Action of arsenic compounds on HaCaT cell proliferation

The anti-proliferative action of arsenic trioxide, arsenic pentoxide and arsenic iodide on the cultured HaCaT keratinocytes as determined by MTT assay is shown in Fig. 4.1. It is evident that all arsenic compounds exerted potent anti-proliferative action on HaCaT keratinocytes in a dose- and time-dependent manner. Table 4.1. depicts the IC_{50} values of the arsenic compounds on HaCaT and Hs-68 cells. The IC_{50} values of arsenic trioxide were 9.0, 6.9 and 5.1 μM; and those for arsenic pentoxide were 35.5, 25.0 and 18.6 μM while for arsenic iodide were 19.2, 18.0 and 7.3 μM when the cells were

incubated for 12, 24 and 48 h, respectively. These results demonstrated the significant growth inhibitory effect of the arsenic compounds on HaCaT keratinocytes.

4.2.3.2. Cytotoxicity of arsenic compounds on Hs-68 cells

To determine whether these arsenic compounds possess non-specific cytotoxicity on cells, arsenic compounds were tested on the growth of Hs-68, a line of normal human fibroblast cells. Fig. 4.2. illustrates that all three arsenic compounds only elicited modest growth inhibition on this normal human fibroblast cell line. The IC_{50} values of arsenic trioxide, arsenic pentoxide and arsenic iodide on Hs-68 cells were 43.4, 223.0 and 89.0 μM, respectively (Table 4.1.), and these values are much bigger than those for HaCaT cell line. It is clear that the arsenic compounds exhibited differential cytotoxic profiles on the HaCaT and Hs-68 cells, and they only showed mild cytotoxic action towards the normal human Hs-68 fibroblasts.

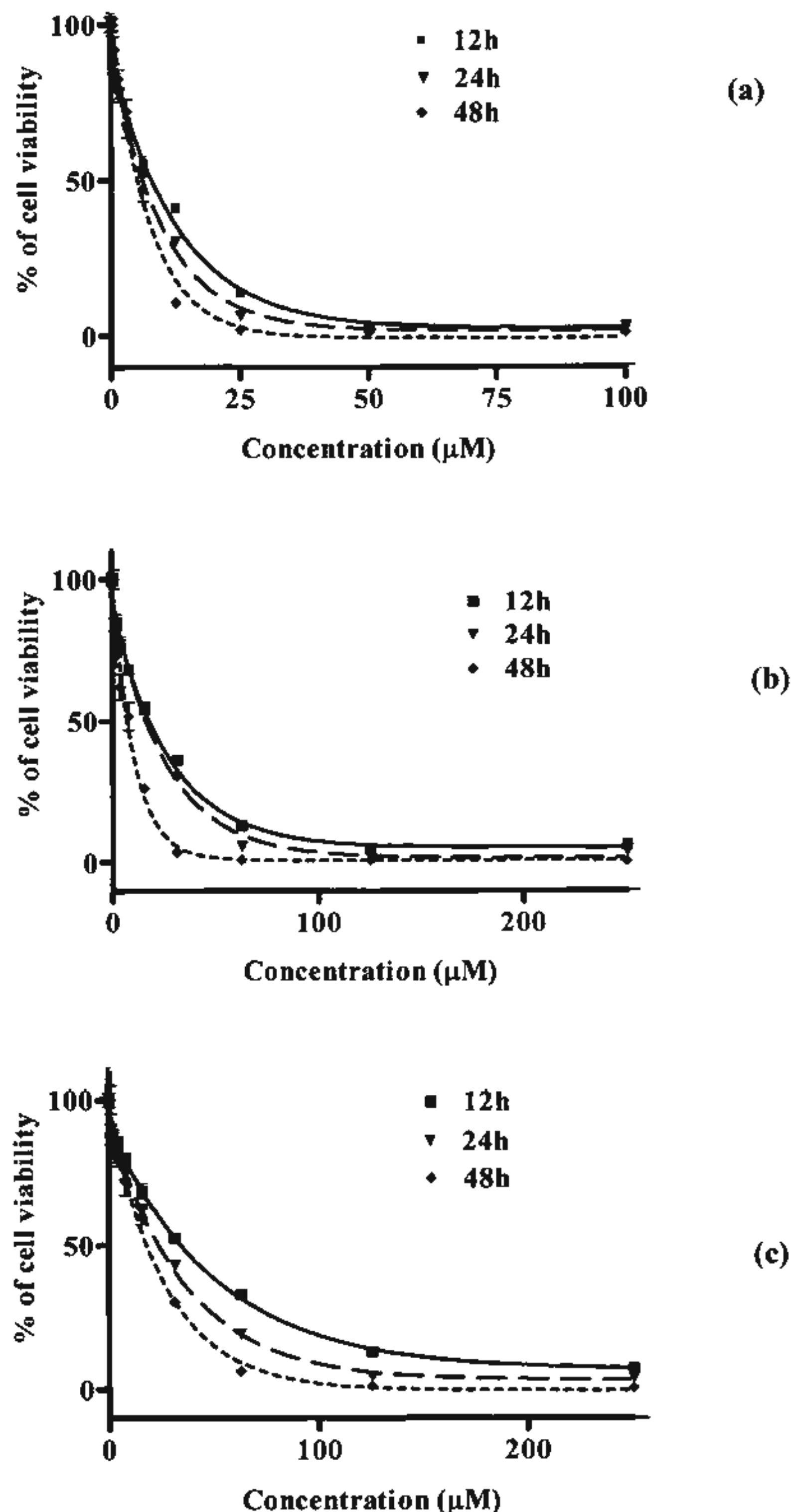


Fig. 4.1. Anti-proliferative action of arsenic compounds on HaCaT cells as determined by MTT assay. (a) Arsenic trioxide. (b) Arsenic pentoxide. (c) Arsenic iodide.

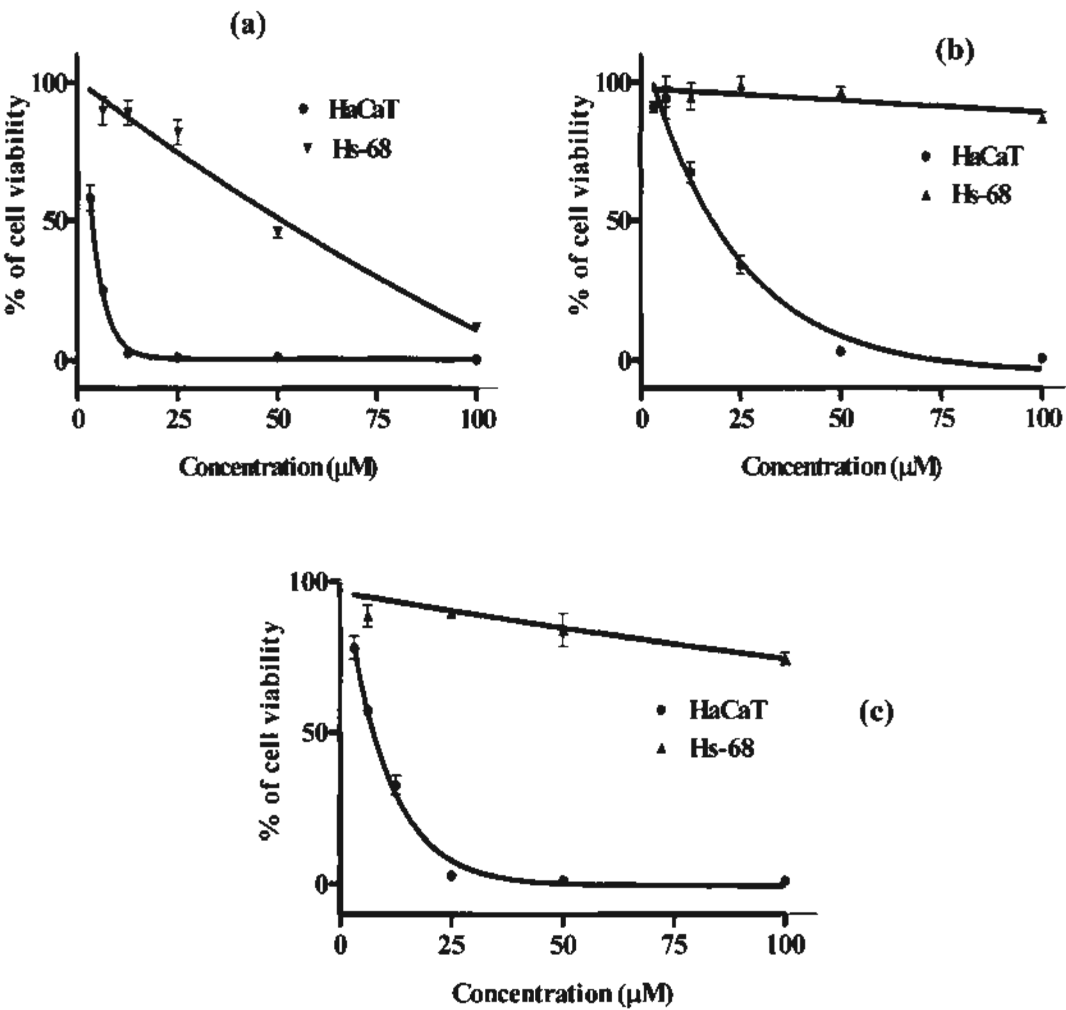


Fig. 4.2. Comparison of the anti-proliferative effects of arsenic compounds on HaCaT and Hs-68 cells as determined by MTT assay. (a) The cells were treated with arsenic trioxide; IC_{50} values were 2.4 and 43.4 μM for HaCaT and Hs-68 cells respectively. (b) The cells were treated with arsenic pentoxide; IC_{50} values were 16.0 and 223 μM for HaCaT and Hs-68 cells respectively. (c) The cells were treated with arsenic iodide; IC_{50} values were 6.8 and 89.0 μM for HaCaT and Hs-68 cells respectively.

Table 4.1. The IC_{50} values of arsenic trioxide, arsenic pentoxide and arsenic iodide on the growth of HaCaT and Hs-68 cells.

		IC_{50} (μM)		
Cell type	**Incubation time (h)**	**As_2O_3**	**As_2O_5**	**AsI_3**
	12	9.0	35.5	19.2
HaCaT	24	6.9	25.0	18.0
	48	5.1	18.6	7.3
Hs-68	48	43.4	223.0	89.0

4.2.4. Discussion

In the present study, experiments were designed to evaluate the anti-proliferative activity of three arsenic chemicals namely arsenic trioxide, arsenic pentoxide and arsenic iodide. Our experimental results demonstrated that these arsenic compounds possess potent inhibitory action on the growth of HaCaT keratinocytes, with arsenic trioxide being the most potent while arsenic pentoxide the least potent. It is also worth noting that all three arsenic compounds showed only modest inhibitory effect on the growth of normal human fibroblast Hs-68 cells, exhibiting discernible differential cytotoxic profiles between the fast growing HaCaT cells and normal human fibroblasts. This favorable toxicity profile of the arsenic compounds is important as it enables

formulating topical applications of arsenic compounds which could exert significant therapeutic effect without evoking harmful side effects on the normal skin cells. The data also showed that arsenic trioxide and arsenic iodide, as trivalent salts, possessed higher inhibitory action but also higher toxicity than the pentavalent salt arsenic pentoxide. These experimental observations are congruent with other findings that the inorganic trivalent salts of arsenic are generally more toxic than the pentavalent ones (Lederer and Fensterheim, 1983). As cellular apoptosis is one of the common mechanisms leading to growth inhibition, in the next session, the apoptosis-inducing potential of these three arsenic compounds as well as realgar extract would be investigated.

4.3. Studies of Apoptotic Effect of Realgar Extract and Arsenic Compounds

4.3.1. Introduction

Our previous studies demonstrated that ethanolic extract of realgar and three arsenic compounds found in realgar exhibited significant inhibition on the growth of human HaCaT keratinocytes. All realgar extract and arsenic compounds exerted less cytotoxicity on Hs-68 cells, indicating a desirable cytotoxic profile. As cellular necrosis, apoptosis could be responsible for the observed anti-proliferative action of realgar extract and arsenic compounds, the unraveling of the underlying action mechanism would be an important step in the drug discovery process. In the present study, we intended to investigate whether cellular apoptosis was responsible for the observed anti-proliferative action induced by realgar and arsenic compounds.

4.3.2. Materials and Methods

4.3.2.1. Fluorescent staining of HaCaT cells for morphological evaluation

Approximately $7.5x10^5$ HaCaT cells were seeded in 6-well plates. The cells were treated with 10 μg/ml realgar, 12 μM arsenic trioxide, 40 μM arsenic pentoxide and 24 μM arsenic iodide for 48 h. The details of the experimental procedures were as that described in chapter 3.5.2.3..

4.3.2.2. DNA fragmentation assay

A million HaCaT cells were seeded on 100-mm plates and exposed to realgar extract at 2, 5, 10, 15 and 20 μg/ml for 24, 48 and 72 h. Similarly, arsenic trioxide at 3, 6, 12, and 24 μM, arsenic pentoxide at 10, 20, 40, and 80 μM, and arsenic iodide at 6, 12, 24, and 48 μM respectively were exposed to 1 x 10^6 HaCaT cells on 100-mm plates and incubated for 48 h. The procedures of DNA fragmentation assay were as the same as that described in section 3.5.2.4..

4.3.2.3. TUNEL assay

7.5x10^5 HaCaT cells were seeded on a 6-well plate and exposed to realgar extract at 8 μg/ml, arsenic trioxide at 48 μM, arsenic pentoxide at 120 μM and arsenic iodide at 72 μM, respectively for 48 h. The details of TUNEL assay were as that described in section 3.5.2.5.

4.3.2.4. Cell cycle analysis with PI staining

Approximately 7.5x10^5 HaCaT cells seeded on 6-well plates were exposed to realgar at 2, 5, 10 and 20 μg/ml for 24, 48 or 72 h. In addition, arsenic trioxide at 6, 12, 24 and 36 μM, arsenic pentoxide at 40, 60, 80 and 100 μM and arsenic iodide at 24, 36, 48 and 60 μM respectively were also exposed to 7.5x10^5 HaCaT cells seeded on 6-well plates

and incubated for 48 h. The cell cycle was detected by flow cytometry using procedures described in section 3.5.2.6.

4.3.2.5. Quantitative analysis of apoptotic cells by annexin V-PI staining

In this experiment, $7.5x10^5$ HaCaT cells were seeded on 6-well plates and incubated with realgar at 2, 5, 10 and 20 μg/ml for 24, 48 or 72 h. Similar to realgar extract, arsenic trioxide at 3, 12, 24, 36 μM, arsenic pentoxide at 40, 60, 80 and 100 μM and arsenic iodide at 24, 36, 48 and 60 μM respectively were exposed to $7.5x10^5$ HaCaT cells seeded on 6-well plates and incubated for 48 h. The procedures of cell staining and analysis by flow cytometry were as that described in section 3.5.2.7..

4.3.2.6. Western blot analysis of caspase-3

A million cells seeded on each 100-mm plate were exposed to realgar extract at 2, 5, 10, 15 and 20 μg/ml, arsenic trioxide at 6, 12, 24 and 36 μM, arsenic pentoxide at 40, 60, 80 and 100 μM and arsenic iodide at 24, 36, 48 and 60 μM respectively for 48 h. The protocol of Western blot analysis has been described in section 3.5.2.8..

4.3.2.7. Statistical analysis

Data were expressed as mean ± SEM. Statistical comparisons between realgar extract, arsenic compounds treatments and control were carried out using one-way ANOVA, followed by *post-hoc* Dunnett's test using the non-treatment as the control group on the SPSS for Windows (version 14.0). Differences were considered significant at $p < 0.05$ and were denoted as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

4.3.3. Results

4.3.3.1. Alteration of cellular morphology

After exposure to 10 μg/ml realgar, 12 μM arsenic trioxide, 40 μM arsenic pentoxide and 24 μM arsenic iodide for 48 h, a greater number of HaCaT cells showed detachment from the culture plate when compared to the non-treatment control (Fig. 4.3. and 4.4.). The Hoechst-stained HaCaT keratinocytes appeared to be shrunken and displayed fewer intercellular connections and exhibited typical apoptotic morphology characterized by chromatin condensation and DNA fragmentation.

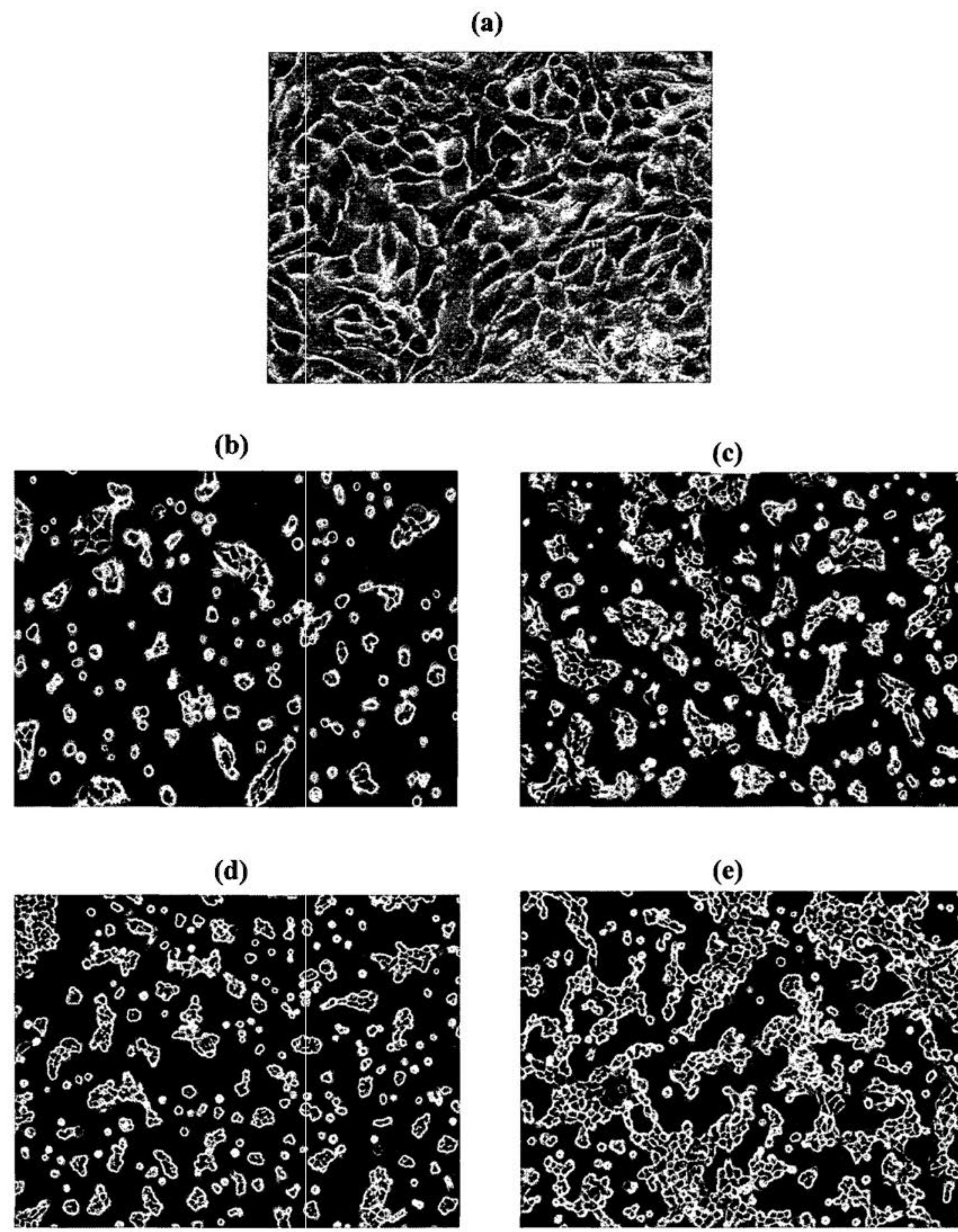


Fig. 4.3. Actions of realgar extract and arsenic compounds on HaCaT cell morphology as examined by light microscopy. (a) Normal HaCaT cells. HaCaT cells treated for 48 h with (b) 10 μg/ml realgar extract; (c)12 μM arsenic trioxide; (d) 40 μM arsenic pentoxide; and (e) 24 μM arsenic iodide.

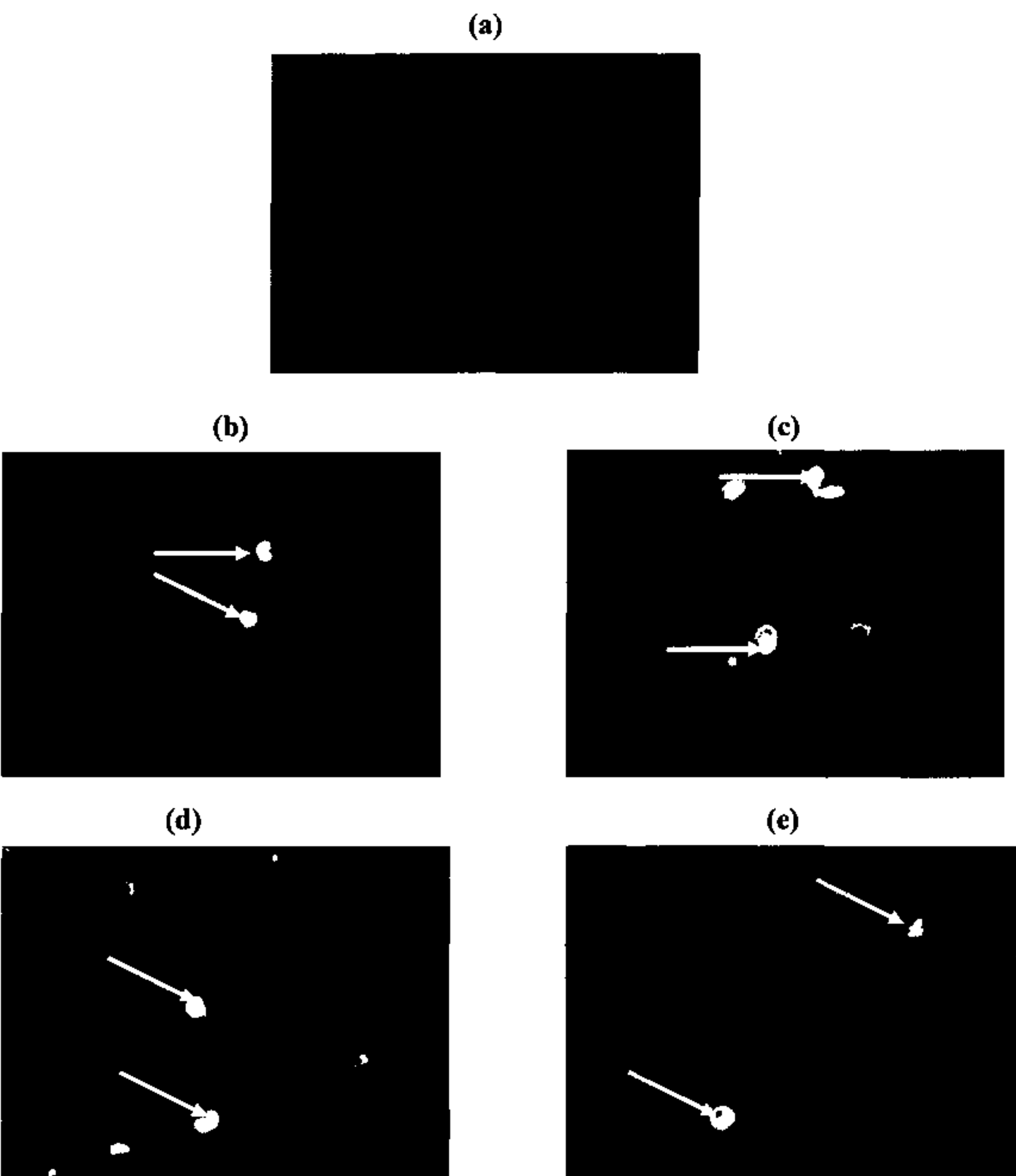


Fig. 4.4. Actions of realgar extract and arsenic compounds on HaCaT cell morphology as examined by fluorescent microscopy. (a) Control HaCaT cells stained with Hoechst. HaCaT cells treated with (b) 10 μg/ml realgar extract; (c) 12 μM arsenic trioxide; (d) 40 μM arsenic pentoxide; and (e) 24 μM arsenic iodide. Note that the realgar- and arsenics-treated HaCaT cells appeared to be shrunken and showed apoptotic morphology characterized by chromatin condensation (arrow shown).

4.3.3.2. Detection of DNA fragmentation

Detection of DNA laddering on electrophoresis was used to confirm the morphological finding regarding the apoptotic action of the realgar extract and arsenic compounds. As shown in Fig. 4.5.a, DNA laddering was clearly evident with treatment of a higher concentration of realgar for 48 h. In Fig. 4.5.b, the laddering pattern of nucleosome monomer and oligomers was clearly distinguishable only after 48 and 72 h of incubation, but not at 24 h. In Fig. 4.6., DNA laddering was evident when HaCaT cells were exposed to 48 μM arsenic trioxide, 120 μM arsenic pentoxide and 72 μM arsenic iodide for 48 h. The appearance of DNA laddering is indicative of cellular DNA fragmentation. This was confirmed by the TUNEL assay which constitutes another method to detect the fragmented DNA. Comparing to the control in Fig. 4.7.a, 8 μg/ml realgar (Fig. 4.7.b), 18 μM arsenic trioxide (Fig. 4.7.c), 65 μM arsenic pentoxide (Fig. 4.7d) and 42 μM arsenic iodide (Fig. 4.7.e) were all capable of inducing the appearance of apoptotic peaks, indicative of the occurrence of apoptosis in the HaCaT cells.

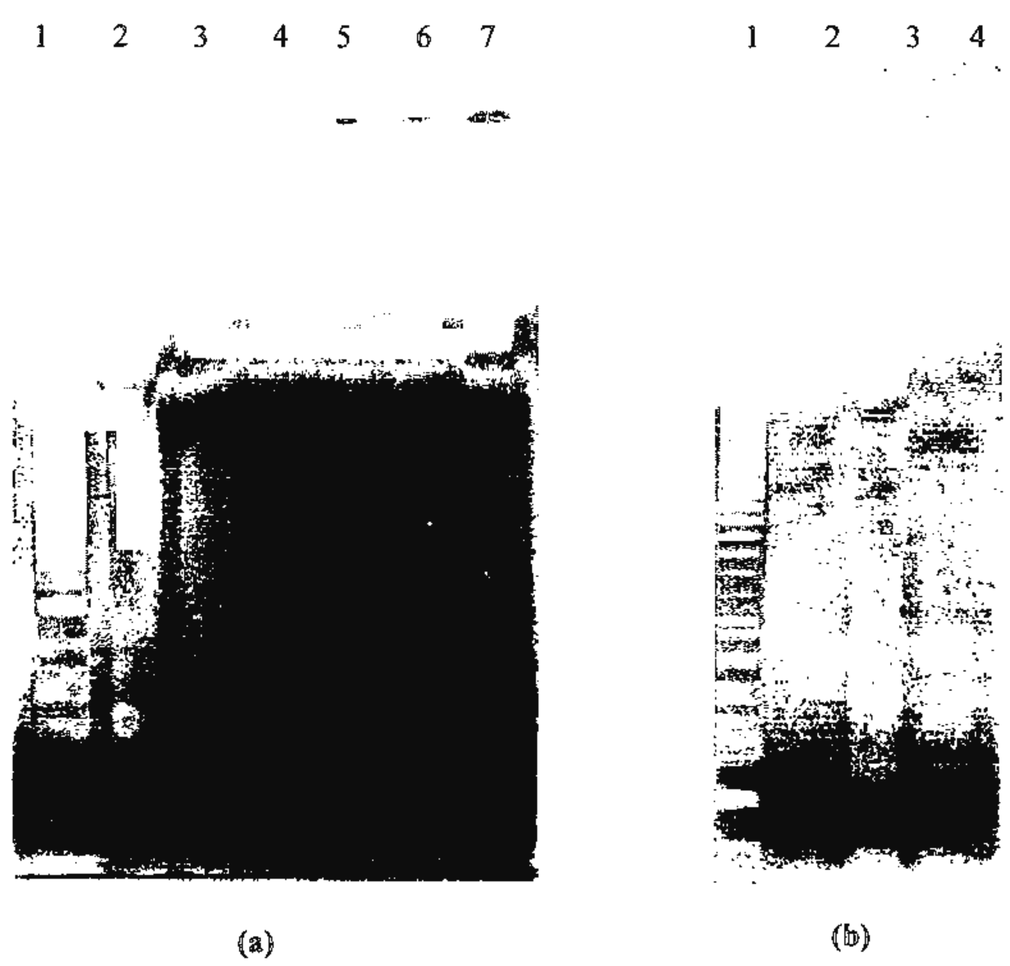


Fig. 4.5. Action of realgar on DNA fragmentation on HaCaT cells.

(a) Lanes 1 to 7 are realgar extract at 100-base-pair marker, 20, 15, 10, 5, 2 μg/ml and cell control, respectively. (b) Lane 1 to 4 corresponding to 100-base-pair marker, an incubation of 20 μg/ml realgar extract for 24, 48, and 72, respectively.

Fig. 4.6. Action of arsenic compounds on DNA fragmentation in HaCaT cells. Lane 1 to 5 were 100 base pair marker, 72 μM arsenic iodide, non-treatment control, 48 μM arsenic trioxide and 120 μM arsenic pentoxide, respectively . Note that ladder patterns were observed at 72 μM arsenic iodide, 48 μM arsenic trioxide and 120 μM arsenic pentoxide.

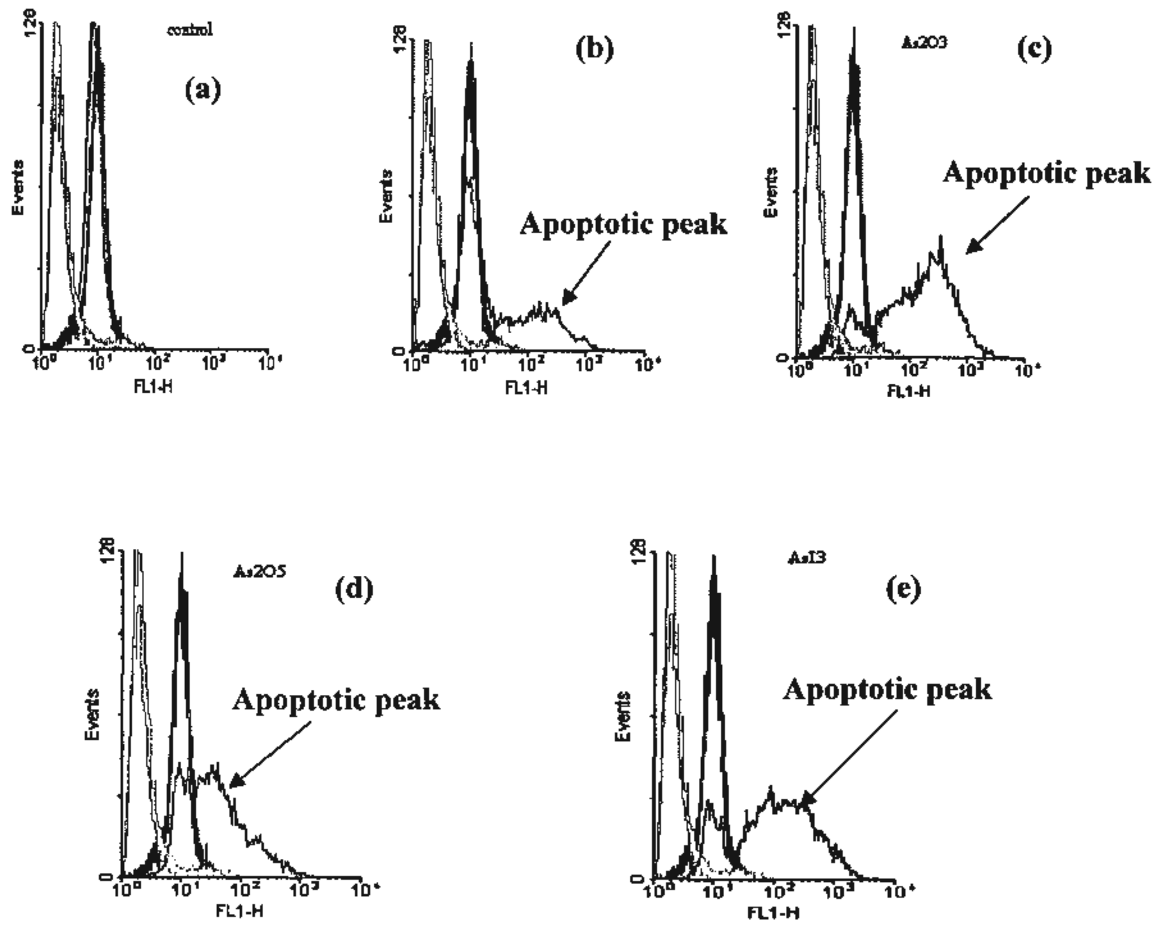


Fig. 4.7. TUNEL analysis of realgar extract- and arsenic compounds-mediated apoptosis in HaCaT cells. (a) The cells cultured in the absence of realgar extract and arsenic compounds. (b-e) The cells cultured in the presence of 8 μg/ml realgar extract, 18 μM arsenic trioxide, 65 μM arsenic pentoxide and 42 μM arsenic iodide, respectively. Green line, control for autofluorescence of cells in the presence of label or enzyme solution. Black line, non-treatment control incubated with label solution only. Red area indicates cells incubated with TUNEL reaction mixture with both label and enzyme solution.

4.3.3.3. Actions of realgar extract and arsenic compounds on cell cycle progression

The flow cytometric measurement of PI-stained DNA is shown in Fig. 4.8.b-e. Realgar extract at 10 μg/ml, arsenic trioxide at 24 μM, arsenic pentoxide at 80 μM and arsenic iodide at 60 μM, respectively were capable of inducing the sub-G1 phase when comparing with the control in Fig. 4.8 a. After 48 h of treatment with the realgar extract, the amount of cells in sub-G1 phase increased from 0.4 to 44.1% when the concentration was increased from 2 to 20 μg/ml (Fig. 4.9.a). Also, the sub-G1 population gradually increased from 32.4 to 77.1% when the incubation time was extended from 24 to 72 h in the presence of 20 μg/ml realgar extract (Fig. 4.9.b). Moreover, apart from the induction of the sub-G1 peak, realgar extract also altered the cell cycle distribution of the cultured HaCaT cells. After treatment for 48 h with 20 μg/ml of realgar extract, the percentage of cells in G1, S and G2/M phases changed from 45.8, 36.8 and 17.3% to 80.5, 12.8 and 6.7%, respectively (Fig. 4.10.a). Furthermore, when exposed to longer time periods, the percentage of cells in the G1 phase increased markedly, while S and G2/M phases decreased accordingly (Fig. 4.10.b). Also, after 48 h of treatment with arsenic trioxide and when the concentration was increased from 6 to 36 μM, the amount of cells in sub-G1 phase was elevated from 0.64% to 33.7% (Fig. 4.11a). Similarly, the sub-G1 population gradually increased from 2.9% to 28.0% when the arsenic pentoxide concentration was increased from 40 to

100 μM (Fig. 4.11b). As for arsenic iodide, an increase of concentration from 24 to 60 μM corresponded to an augmentation of sub-G1 population from 2.4% to 16.5% (Fig. 4.11c). Taken together, our experimental results clearly demonstrated that realgar extract and the arsenic compounds tested were able to induce apoptosis in HaCaT cells.

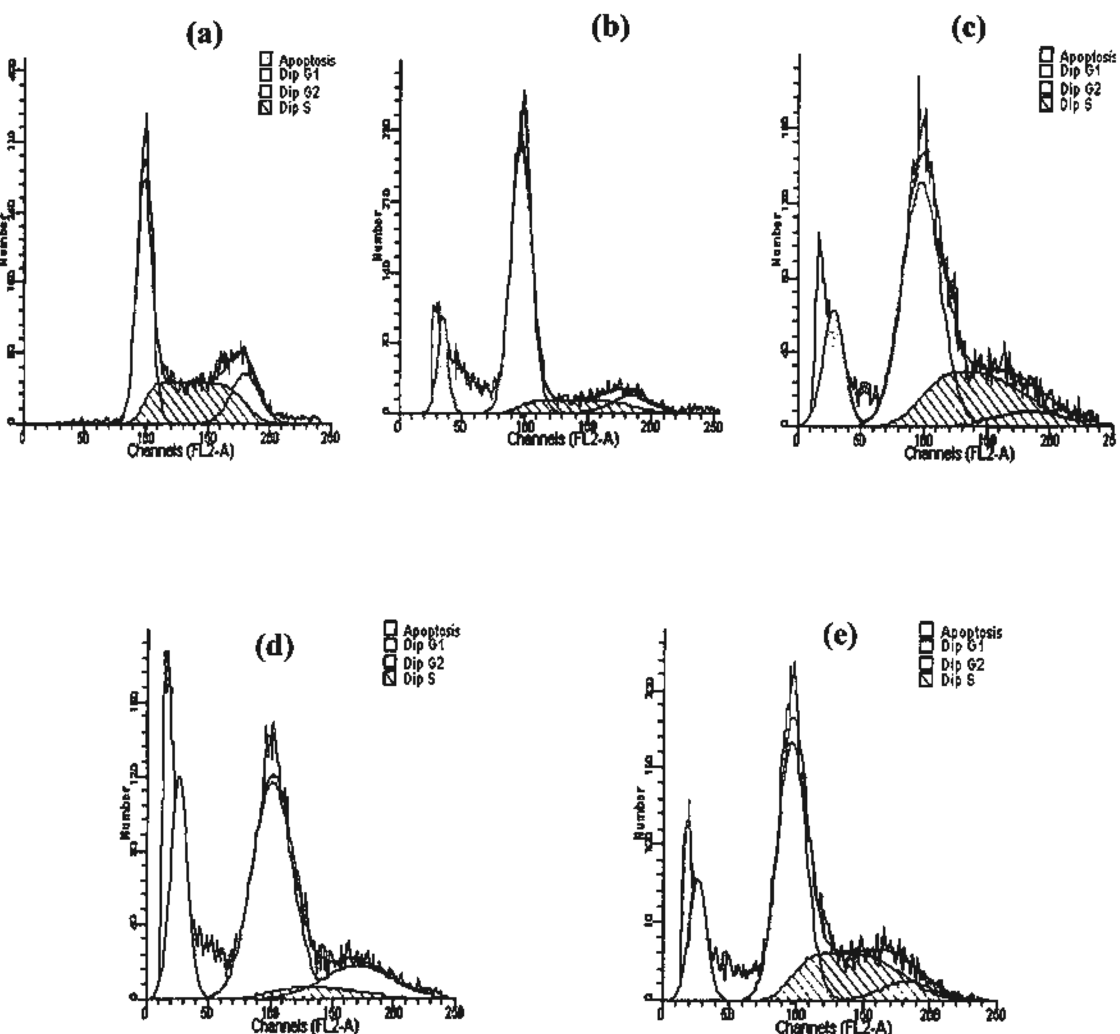


Fig. 4.8. Flow cytometric analysis of cell cycle distribution of HaCaT cells with PI staining. (a) Non-treatment control. (b-e) Cells treated with 10 μg/ml of realgar extract, 24 μM of arsenic trioxide, 80 μM of arsenic pentoxide and 60 μM of arsenic iodide for 48 h respectively. Note the appearance of sub-G1 phase upon the treatment with arsenic salts.

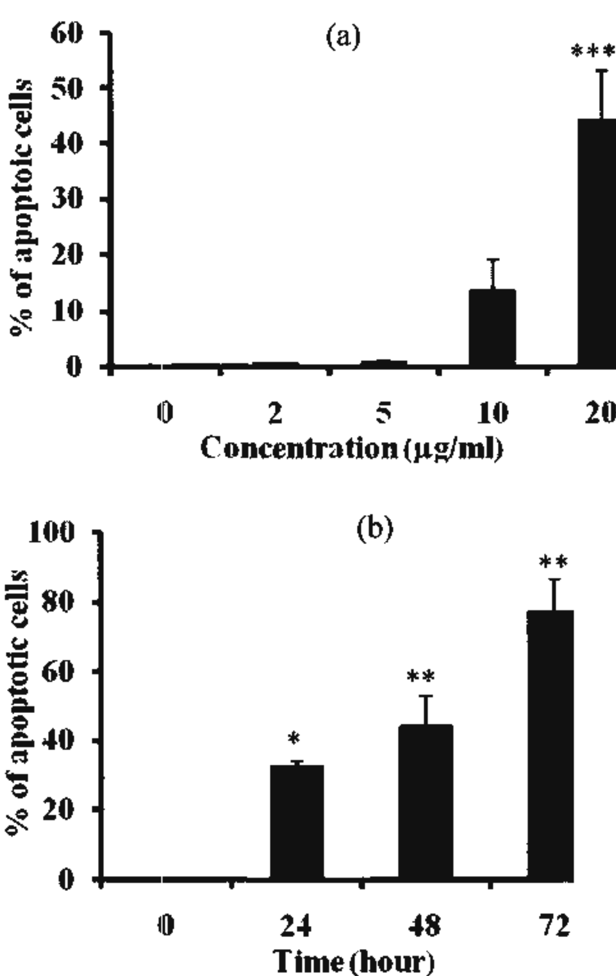


Fig. 4.9. Bar chart presentation of the occurrence of sub-G1 phase of cultured HaCaT keratinocytes with PI staining. (a) Dose-dependent effect of realgar extract on the induction of sub-G1 phase on HaCaT keratinocytes for 48 h. (b) Time course effect of 10 μg/ml realgar extract on the induction of sub-G1 phase on HaCaT keratinocytes. The values shown in Fig. 4.9.a and Fig. 4.9.b are the mean ± SEM with n=3. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ when compared with the non-treatment control.

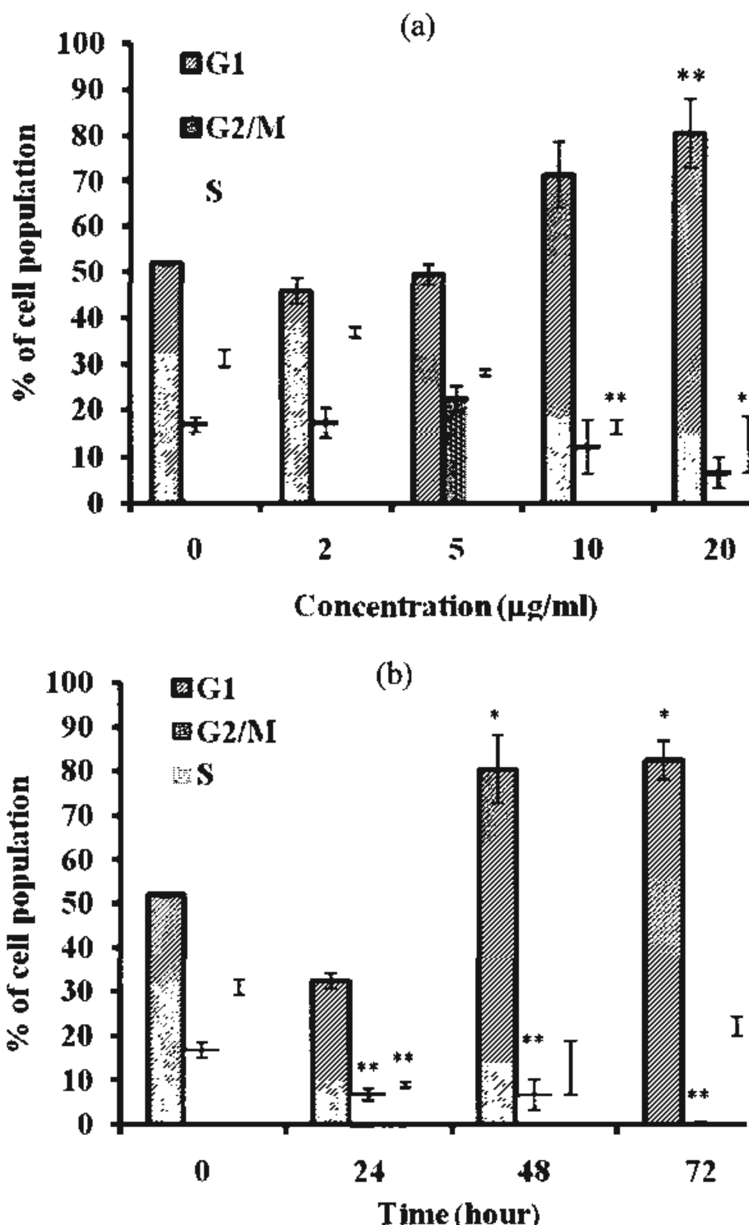


Fig. 4.10. Bar chart presentation of the distribution of the cell cycle of HaCaT keratinocytes under the influence of realgar extract. (a) Cells were exposed to different concentrations of realgar extract for 48 h followed by flow cytometric analysis with PI staining. (b) Cells were treated with 20 μg/ml realgar extract for 24, 48 and 72 h, respectively. The values shown are the mean ± SEM, with n=3. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ when compared with the drug-free control.

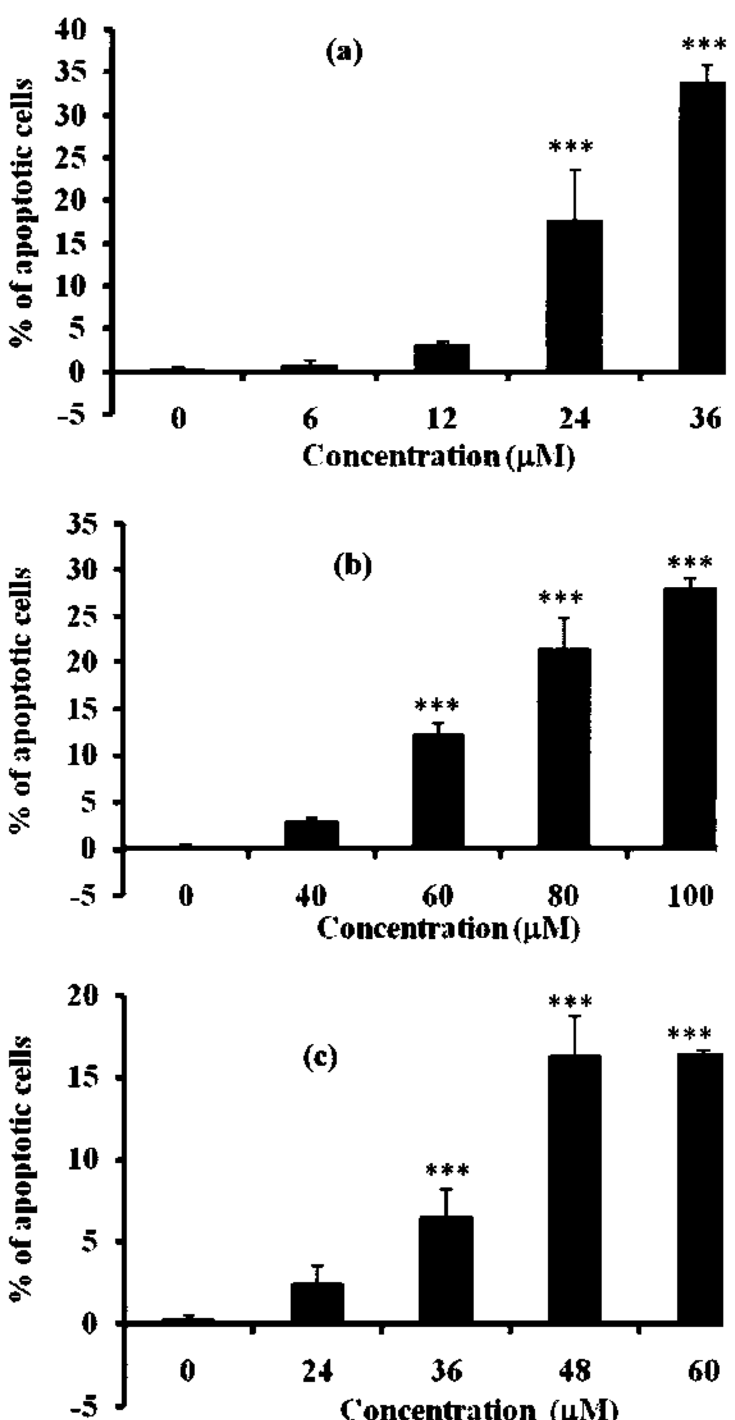


Fig. 4.11. Dose-dependent effect of arsenic compounds on the induction of sub-G1 phase on HaCaT cells. (a-c) Treatment with arsenic trioxide, arsenic pentoxide and arsenic iodide respectively for 48 h. The values shown are the mean ± SEM, with n=3. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ when compared with the drug-free control.

4.3.3.4. Quantitative analysis of apoptotic cells by annexin V-PI staining

The discrimination between apoptotic and necrotic cells could be achieved by quantitatively estimating the relative amount of the annexin V and PI-stained cells in the population. As shown in Fig. 4.12., realgar extract was able to significantly increase the percentage of apoptotic cells from 7.5 to 87.0%, but the percentage of viable cells was decreased from 80.2 to 4.7% as the concentration of realgar extract increased from 2 to 20 μg/ml after 48 h of extract treatment (Fig. 4.13.a). Likewise, at 20 μg/ml realgar, the percentage of apoptotic cells elevated from 67.3 to 88.1% as the incubation time was extended from 24 to 72 h (Fig. 4.13.b). These results illustrated that the apoptotic action of realgar is dose- and time-dependent.

As shown in Fig. 4.14., when exposed to lower concentration of the arsenic compounds, the majority of cells were localized in the lower left quadrant. As the concentration of the compound increased, the amount of cells localized in the lower right quadrant and upper right quadrant correspondingly increased. When the concentration of arsenic trioxide increased from 3 to 36 μM, the percentage of the apoptotic cells was significantly elevated from 5.5% to 63.0%; and accordingly, the percentage of viable cells was decreased from 86.1% to 16.8% after 48 h of the arsenics treatment (Fig. 4.15.a). Likewise, the percentage of apoptotic cells was markedly increased from

16.9% to 68.5% and the viable cells were significantly decreased from 64.2% to 14.0% as the concentration of arsenic pentoxide increased from 40 to 100 μM (Fig. 4.15.b). Similarly, when the arsenic iodide concentration increased from 24 to 60 μM, the apoptotic cells also were elevated from 11.7% to 61.1%, and correspondingly, the viable cells were decreased from 75.3% to 17.0% (Fig. 4.15.c). Since apoptotic cells in vitro will eventually undergo "secondary necrosis", the percentage of necrotic cells was thus increased from 7.7% to 21.9%, 17.7% to 19.2% and 12.3% to 20.6% for arsenic trioxide, arsenic pentoxide and arsenic iodide respectively as the drug concentration increased. These results unambiguously demonstrated that induction of cellular apoptosis was mainly responsible for the arsenic compounds-mediated HaCaT keratinocyte growth inhibition; and the apoptotic action of these arsenic compounds was dose-dependent.

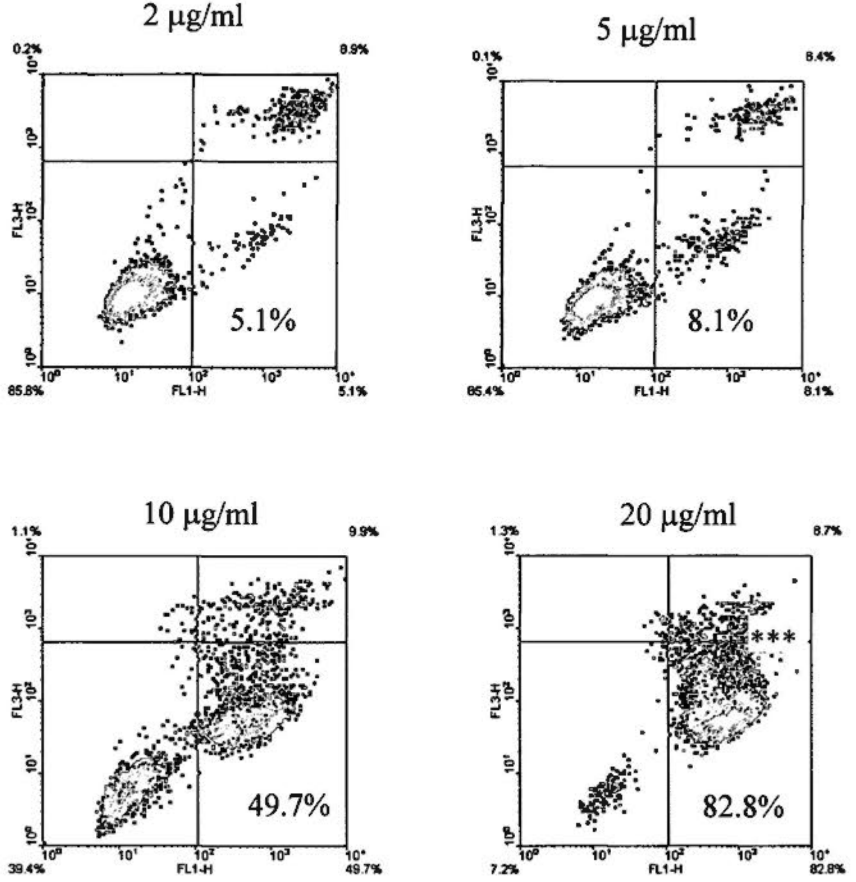


Fig. 4.12. Quantitative analysis of realgar-induced apoptosis by annexin V-PI binding assay. Figures show the distribution of apoptotic cells in the total HaCaT cell population in the presence of realgar extract (2, 5, 10 and 20 µg/ml) for 48 h followed by labeling with Annexin V and PI and analysis by flow cytometry.

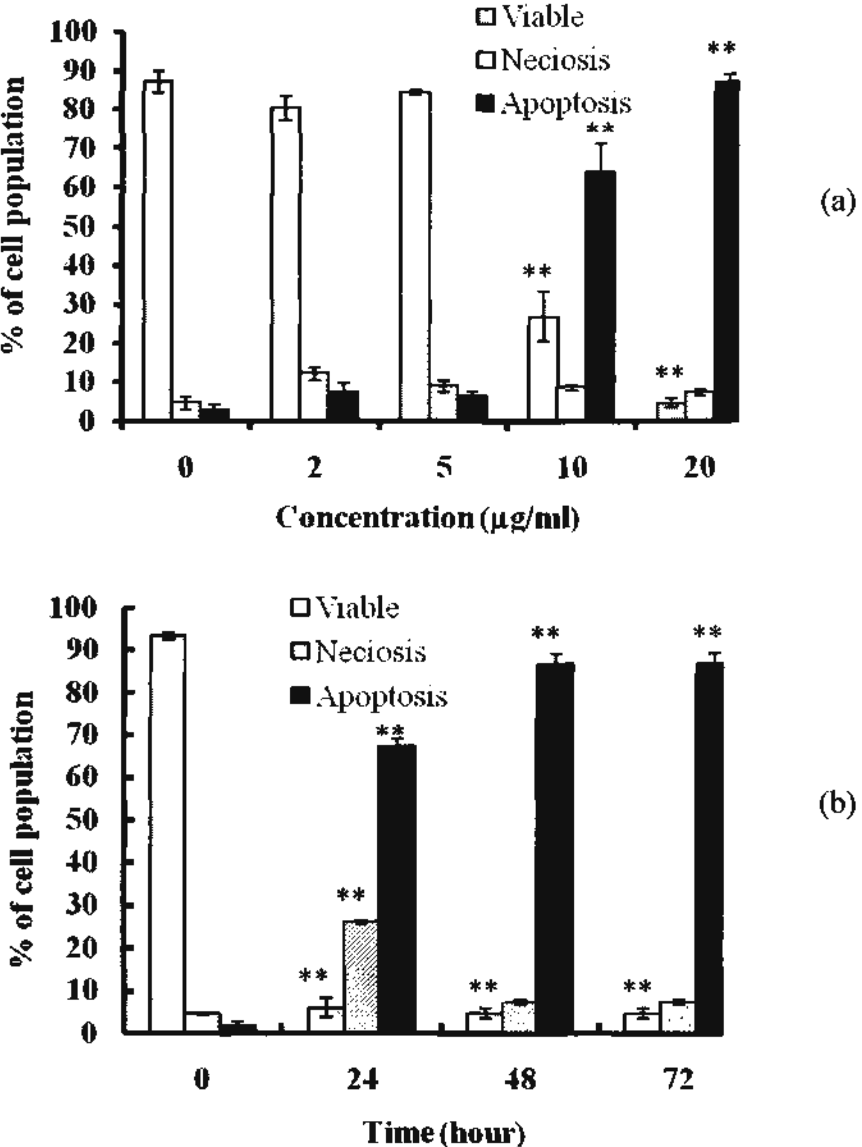


Fig. 4.13. Distribution of viable, apoptotic and necrotic HaCaT keratinocytes in the presence of realgar extract. (a) Bar chart presentation of the distribution of viable, apoptotic and necrotic cell populations after treatment with realgar extract. (b) Bar chart presentation of time course effect on the distribution of viable, apoptotic and necrotic cell in the presence of 20 μg/ml realgar extract. The values shown represent the mean ± SEM, with n=3. *, $p < 0.05$; and **, $p < 0.01$ when compared with the non-treatment control.

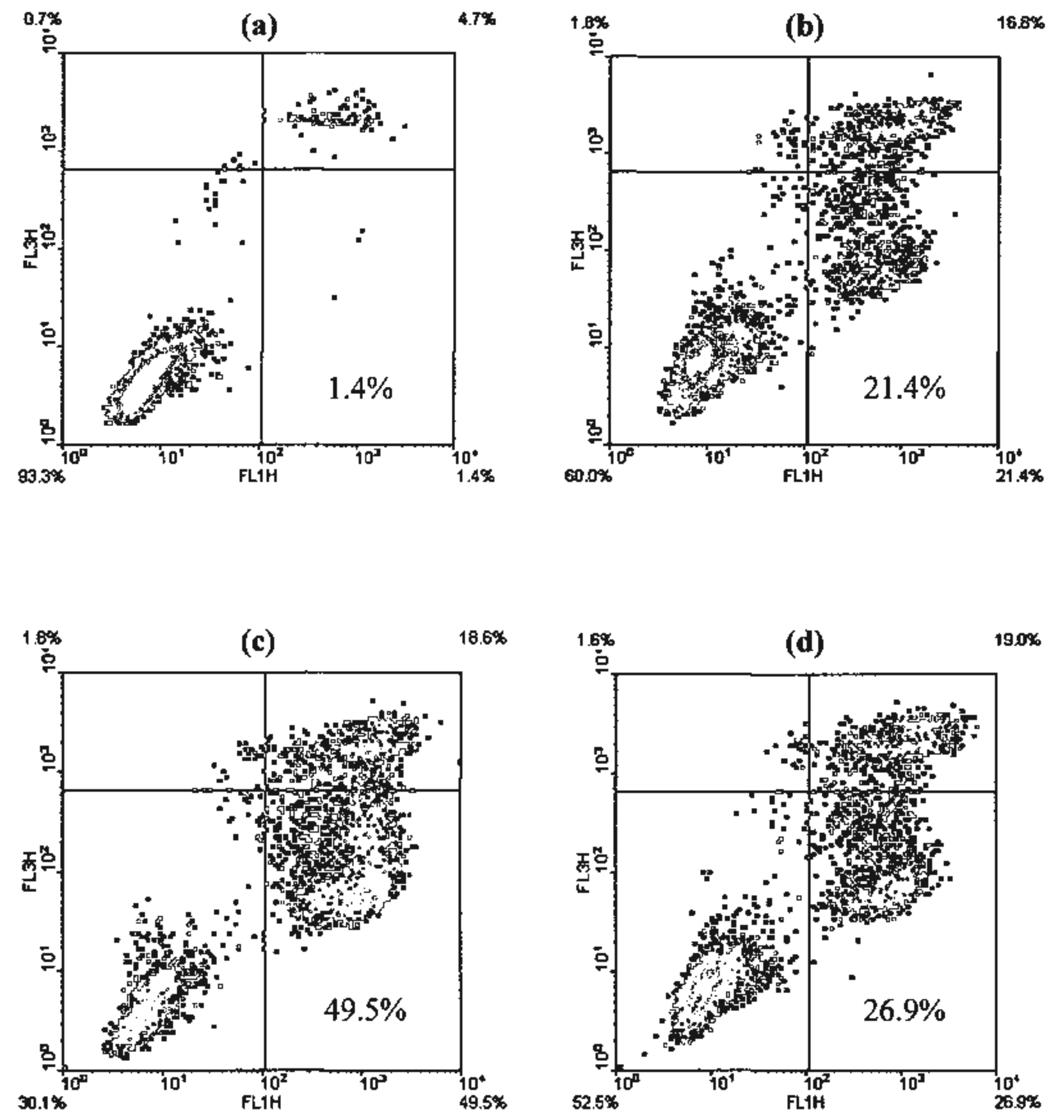
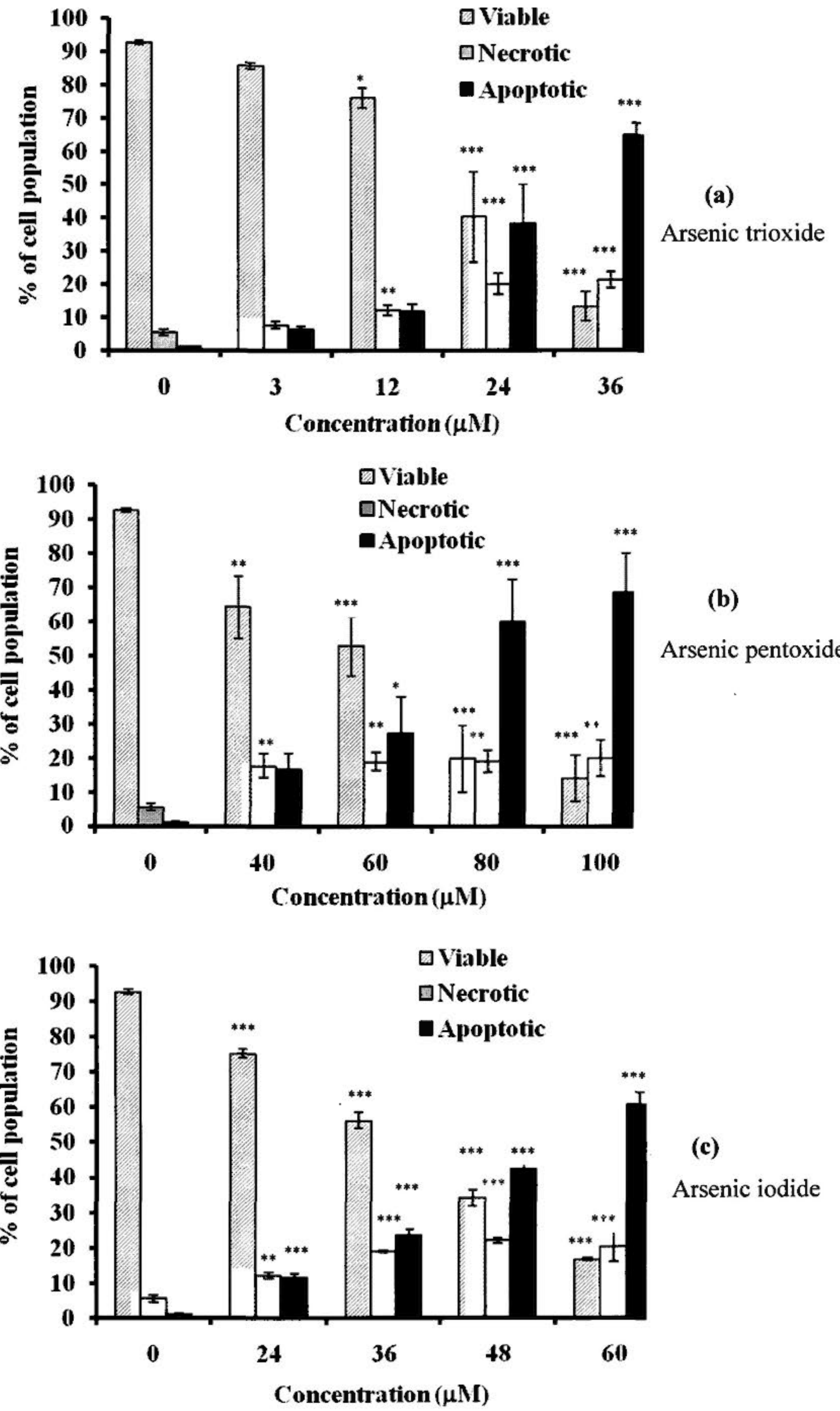


Fig. 4.14. Quantitative analysis of arsenic compounds-induced apoptosis by annexin V–PI staining assay. (a) Non-treatment control. (b-d) HaCaT cells were exposed for 48 h to 24 μM of arsenic trioxide, 60 μM of arsenic pentoxide and 36 μM of arsenic iodide, respectively.



Viable
Necrotic
Apoptotic
% of cell population
Concentration (μM)
(a)
Arsenic trioxide
(b)
Arsenic pentoxide
(c)
Arsenic iodide

Fig. 4.15. Distribution of viable, apoptotic and necrotic HaCaT keratinocytes in the presence of arsenic compounds. (a-c) Bar chart presentation of the distribution of viable, apoptotic and necrotic cell populations after treatment with arsenic trioxide, arsenic pentoxide and arsenic iodide respectively for 48 h. Differences were considered significant at $p < 0.05$ and were denoted as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

4.3.3.5. Western blot analysis

Caspase-3 is the apoptosis-promoting enzyme responsible for cleaving cellular substrates leading to the characteristic cell morphology alterations. Fig. 4.16.a illustrates the effect of realgar extract on the caspase-3 activation. It is evidenced that the activity of caspase-3 (19 and 17 kDa) was increased by realgar extract. Moreover, the results of caspase-3 activation by different concentrations of arsenic compounds are shown in Fig. 4.16.b-d. The arsenic compounds were able to significantly increase the activity of caspase-3 (19 kDa and 17 kDa) and decrease the procaspase-3 (32 kDa) in a dose-dependent manner. These results demonstrated that the underlying mechanism of the reaglar extract and arsenic compounds-induced apoptosis in HaCaT cells involves the cleavage of procaspase-3 into the activated form of caspase-3.

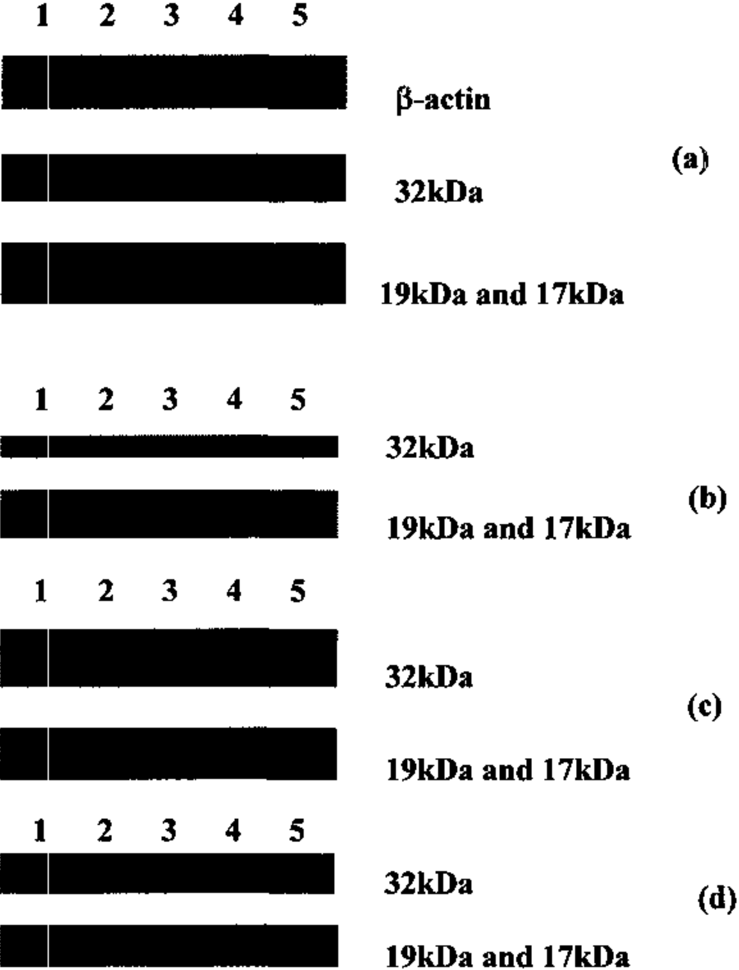


Fig. 4.16. Western blot analysis of the realgar extract- and arsenic compounds-induced expression of caspase-3. (a) Lanes 1 to 5 correspond to control, 2, 5, 10, and 20 μg/ml of reallgar extract, respectively. (b) Lane 1 to 5 correspond to control, 6, 12, 24 and 36 μM of arsenic trioxide, respectively. (c) Lane 1 to 5 correspond to control, 40, 60, 80 and 100 μM of arsenic pentoxide, respectively. (d) Lane 1 to 5 correspond to control, 24, 36, 48 and 60 μM of arsenic iodide, respectively. Note that the band sizes 19 and 17kDa are the activated caspase-3, and band size 32 kDa is the procaspase-3.

4.3.4. Discussion

Realgar extract and arsenic compounds that are able to inhibit keratinocyte proliferation and induce keratinocyte apoptosis would conceivably possess good potentials of being developed into effective agents for treating psoriasis. As cellular apoptosis and/or necrosis could be responsible for growth inhibition of cultured cells, experiments were designed in the present study to elucidate, at morphological, molecular and biochemical levels, whether induction of cellular apoptosis was responsible for the realgar extract and arsenic compounds-mediated growth inhibition on human keratinocytes.

Several assays were employed to detect realgar- and arsenics-induced apoptosis, as no single assay is capable of unambiguously confirming the occurrence of apoptosis. In our experiments, realgar extract and arsenic compounds-treated HaCaT cells were found to have hypercondensed nuclei when stained with the Hoechst stain followed by observation under the microscope. In the current investigation, realgar extract and arsenic compounds were able to induce DNA fragmentation as illustrated by gel electrophoresis and the TUNEL method. Cell cycle progression analysis by flow cytometry revealed that reaglar extract and arsenic compounds significantly increased the population of HaCaT cells in the sub-G1 phase (apoptotic peak) while reducing the number of cells in the G2/M and S phases. This finding suggests that the realgar

extract and arsenic compounds were able to induce sub-G1 phase, thereby causing apoptosis in the HaCaT cells. Quantitative analysis of apoptotic cells by concomitant annexin V-PI staining also demonstrated that the realgar extract and arsenic compounds were capable of inducing apoptosis on the HaCaT keratinocytes in a concentration-dependent manner. In our study, the activation of caspase-3 was detected when the HaCaT keratinocytes were exposed to the realgar extract and arsenic compounds, indicating unequivocally the occurrence of cellular apoptosis. All the above different experiments indicated both realgar extract and arsenic compounds were able to induce cellular apoptosis; and this mechanism of action is believed to be responsible for the inhibition on the growth of human keratinocytes

4.5. General Discussion

In our endeavor to discover anti-psoriatic therapeutic agents, we identified realgar as a potent anti-proliferative agent in our initial screening experiments. In the present chapter, three arsenic compounds found in realgar were tested for their anti-proliferative action on human keratinocyte cell line. The results showed that all three arsenic compounds had potent anti-proliferative effect on HaCaT cells. The subsequent mechanism of action studies concluded that the realgar and arsenic compounds were capable of inducing programmed cell death in cultured HaCaT keratinocytes. The apoptotic actions observed in the present study provide an explanation to the underlying mechanism of the potent anti-proliferative property exhibited by realgar extract and arsenic compounds on HaCaT cells. The successful identification of arsenic compounds as potent anti-proliferative and apoptogenic agents not only places the traditional use of arsenic-containing minerals for psoriasis on a scientific footing, but also renders them promising candidates for their further development into topical therapeutic formulae for psoriasis treatment. Further *in vivo* experiments to evaluate the anti-psoriatic potentials of several topical formulations containing arsenic compounds on psoriasis-relevant animal models will be reported in the Chapter 6.

Chapter Five

Immune Response of Radix Rubiae, Realgar, and Arsenic Compounds in Peripheral Blood Mononuclear Cells

5.1. Introduction

Psoriasis is a common inflammatory skin disease characterized by activated T cell-mediated hyperproliferation of keratinocytes and an increased expression of pro-inflammatory cytokines. A direct role of T cells in the pathogenesis of psoriasis was first suggested in 1983 (Bos et al., 1983). Evidence available so far indicates a common pathogenic pathway involving specific antigen recognized by T cells that results in stimulation of keratinocyte proliferation (Valdimarsson et al., 1986). Psoriasis is now considered to be a Th1 cell mediated inflammatory skin disease, and therapies on targeting T cells and inflammatory cytokines have been developed during the past few years to treat psoriasis, and in many cases, produced impressive clinical results (Lowes et al., 2007). Type 1 Interferons (IFNs) play an essential role in host defense against various viral infections and are associated with the pathogenesis of many autoimmune diseases (Schmidt and Ouyang, 2004). It has been reported that the activated type 1 T cells and other leukocyte populations in psoriatic lesions contribute to the production of cytokines, mainly IFN-γ, TNF-α and IL-17 (Albanesi et al., 1999). Also, pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α produced by activated T cells play key roles in the inflammatory and proliferative process of psoriasis (Chang et al., 1997; Nickoloff, 1991; Uyemura et al., 1993 and Valdimarsson et al., 1986). The IFN-γ and TNF-α are predominant in psoriatic lesions (Gearing et

al., 1990), although the exact role of TNF-α in the pathomechanism of psoriasis is still unclear. Nevertheless, anti-TNF-α therapy is highly effective in psoriasis (Schopf et al., 2002), indicating that this cytokine has, together with IFN-γ, a central role in the pathogenesis of psoriasis. Compounds that suppress the activated T cells and reduce the release of IFN-γ, TNF-α and IL-2 could be promising candidates warranting further development into a therapeutic agent for psoriasis treatment.

A peripheral blood mononuclear cell (PBMC) is a blood cell that has a round nucleus, and this cell type includes lymphocyte and monocyte. PBMC is a critical component in the immune system to fight infection. The lymphocyte population consists of T cells, B cells and natural killer (NK) cells. The mitogen phytohemagglutinin (PHA) is a mixture of glycopropteins isolated from red kidney beans and contains all five isolectins. This mitogenic agent has been widely used for activation of lymphoid cells. PHA can stimulate the T cell production, an activity found to be similar in psoriasis.

In our previous study, 60 Chinese medicinal materials commonly prescribed in TCM for psoriasis treatment were investigated using a cultured HaCaT human keratinocyte model. We identified the ethanolic extract of Radix Rubiae and realgar as possessing potent anti-proliferative action. We also confirmed that Radix Rubiae and realgar were

capable of inducing programmed cell death in cultured HaCaT keratinocytes. Further investigations have also established that arsenic trioxide, arsenic pentoxide and arsenic iodide were potent anti-proliferative and apoptogenic agents. As psoriasis is a distinct inflammatory disorder, it would be interesting and relevant to study whether these anti-proliferative and apoptogenic substances also possess anti-inflammatory action. The present study was undertaken to establish whether Radix Rubiae extract, its EA fraction, realgar extract and arsenic compounds had immunosuppressive action on PBMCs activated by PHA and whether they could attenuate the production of pro-inflammatory cytokines.

5.2. Materials and Methods

5.2.1. Medicinal Materials

The source and preparation of the extracts of Radix Rubiae and realgar were described in detail in Section 2.2.2.1. and 2.2.2.2., respectively. The details of preparation of EA fraction of Radix Rubiae was provided in Section 3.2.2.1., and the source and preparation of arsenic compounds were described in Section 4.2.2.1.. PHA was purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium and FBS were purchased from Invitrogen (Carlsbad, CA, USA). Ficoll-Pague™ Plus solution was purchased from Amersham Pharmacia Biotech Limited (Uppsala, Sweden).

5.2.2. Preparation of PBMCs

PBMCs were isolated from human buffy coat, obtainable from the Blood Transfusion Center, Hong Kong Red Cross, Hong Kong, by density gradient separation method as described previously (Zhou et al., 2002). Briefly, the fresh buffy coat was diluted with PBS at a ratio of 1 : 1. The diluted buffy coat sample was layered on equal volume of Ficoll-Paque™ Plus solution in a 50 ml centrifuge tube. The tube was then centrifuged at 800x g for 20 min at 18°C. The uppermost plasma layer was removed and the thin white middle PBMC layer was collected using a 1 ml pipette and transferred to a new centrifuge tube. The PBMCs were then washed with PBS twice

and centrifuged at 100x g for 10 min at 18°C. The supernatant was discarded and the PBMCs were resuspended in 10 ml RPMI 1640 medium supplemented with 10% FBS. The cell numbers were evaluated by trypan blue exclusion assay observed under a light microscope (IX-71, Olympus, Tokyo, Japan). Only the isolated cells with 95% or above viability tested by trypan blue exclusion assay were resuspended to the intended density. The PBMCs were resuspended at 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 10 μg/ml of streptomycin and 10 U/ml of penicillin. The isolated PBMCs were used to carry out the following experiment.

5.2.3. PBMC Proliferation Assay and Cytokine Determination

5.2.3.1. Culture of PBMCs and collection of culture supernatant

The isolated PBMCs (1 x 10^5 cells/well) were stimulated by 10 μg/ml PHA. These PBMCs were seeded in 96-well flat bottom microplates and incubated in an atmosphere of 5% CO_2 and 95% air at 37°C with Radix Rubiae extract and its EA fractionate, realgar extract, arsenic trioxide, arsenic pentoxide and arsenic iodide at various concentrations and the microplates were incubated for 72 h. At the end of the incubation period, the microplates were centrifuged at 300x g for 10 min at 18°C to obtain cell-free supernatant, which was subsequently stored in -20°C for cytokine ELISA experiments. After the collection of supernatant, proliferation rates of PBMCs

were estimated by [methyl-^{3}H]-thymidine assay and trypan blue exclusion assay.

5.2.3.2. [Methyl-^{3}H]-thymidine incorporation assay

[Methyl-^{3}H]-thymidine is a radioactively labeled compound widely used in *in vitro* experiments to measure cell proliferation. Analysis of cell proliferation is made possible by measuring the amount of DNA synthesis. Labeled DNA precursor (^{3}H-thymidine) was added to cells, and its incorporation into DNA was quantified after incubation. The amount of ^{3}H-thymidine incorporated into DNA is measured by a liquid scintillation analyzer. The addition of ^{3}H-thymidine into the cell culture allows the accurate study of the replication of DNA in the cells (Cleaver, 1977). In our experiment, PBMCs were seeded at 1 x 10^{5} cells/well in 96-well plate. After treatment with different concentrations of the substances for specified time period, 0.5 μl [methyl-^{3}H]-thymidine solution (1 μCi/μl, Amersham Bioscience, Judy Moore, UK) was added to each well and the culture was maintained at 37°C for 6 h. The cell suspension was then transferred to glass microfiber filters (Whatman, Kent, UK) by using a cell harvester, and the nucleic acids incorporated with [methyl-^{3}H]-thymidine were trapped on the filter paper. Radioactivity in the filters was measured by Packard TopCount NXT^{TM} Microplate Scintillation and Luminescence Counter (PerkinElmer Inc., MA, USA).

5.2.3.3. Determination of IFN-γ, TNF-α and IL-2 production in PBMCs

The PBMC supernatants were subjected to test for the presence of cytokines such as IFN-γ, TNF-α and IL-2 by Enzyme-linked immunosorbent assay (ELISA). ELISA is a commonly used laboratory test to detect antibodies in the serum. Double-antibody sandwich ELISA was conducted in this study. The basic principle is that a monoclonal anti-human cytokine antibody is bound onto polystyrene microplate wells. Human cytokine present in a measured volume of sample is captured by the antibody on the microplate plate, and non-bound material is removed by washing. Subsequently, a biotinylated polyclonal antibody to human cytokine is added. This antibody binds to the cytokine-antibody complex present in the microplate. Excess biotinylated antibody is remoaved by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the cytokine sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A colored product is formed in proportion to the amount of cytokine present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a plate reader. From the absorbance of samples and those of a standard curve, the concentration of cytokine can be determined by interpolation with the standard curve (Casadevall et al., 1992; Todaro-Luck et al., 1989). Our diagnostic kits for human IFN-γ, TNF-α and IL-2 contain the components

necessary to develop ELISA for natural or recombinant human IFN-γ, TNF-α and IL-2 in cell culture supernatants, respectively. Briefly, a 96-well ELISA microplate was coated with 100 μl/well capture antibody. Then the microplate was sealed and incubated overnight at 4 °C. After the incubation, the wells were washed with wash buffer. The microplate was blocked by assay diluents and incubated at room temperature for 1 h, followed by washed with wash buffer again. Then, 100 μl of the standards (purified Baculovirus-expressed recombinant human IFN-γ, TNF-α and IL-2) and cell culture supernatants were added into appropriate wells. The microplate was sealed and incubated for 2 h at room temperature. After the incubation, the wells were washed with wash buffer and 100 μl of working detector (containing detection antibody + avidin-HRP reagent) was added to each well. The microplate was sealed and incubated for 1 h at room temperature followed by washing with wash buffer and addition of 100 μl of substrate solution to each well. Subsequently, the microplate was incubated in the dark for 30 min at room temperature. Finally, 50 μl stop solution was added to each well and the absorbance at 450 nm was read with a BMG FLUO star Optima microplate reader (BMG Labtech, Offenburg, Germany) within 30 min of stopping reaction.

5.2.4. Statistical Analysis

All data were expressed as mean ± SEM (n = 12, except for trypan blue exclusion assay where n=3). Statistical comparisons between compound treatments and control were carried out using one-way ANOVA, followed by *post-hoc* Dunnett's test using the non-treatment as the control group on the GraphPad PRISM software version 3.0 (GraphPad Software, San Diego, CA, USA).

5.3. Results

5.3.1. PBMC Proliferation Assay

In our experiment, [methyl-^{3}H]-thymidine uptake was used to assess the proliferation rate of PBMCs in the presence of various test compounds. As shown in Fig. 5.1, ethanolic extract and the EA fraction of Radix Rubiae significantly inhibit the DNA synthesis of PBMCs thereby suppressing the proliferation of the cells.

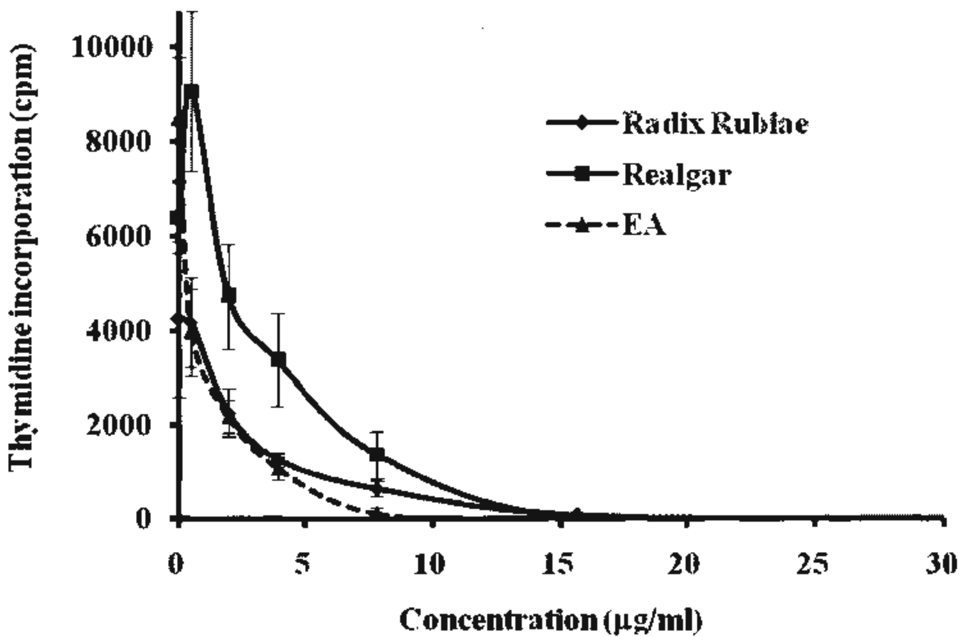


Fig. 5.1. Effect of Radix Rubiae, EA fraction of Radix Rubiae and realgar on [^{3}H]-thymidine uptake by PBMCs. The cells (1 x 10^5 cells/well) stimulated with PHA (10 µg/ml) were incubated in 96 well-plates in the presence of Radix Rubiae, realgar and EA fraction of Radix Rubiae and incubated for 72 h. Data were expressed as the means ± SEM (n=12).

However, it is interesting to note that when assessing the percentage of viable cells by trypan blue exclusion method for detection of the viable cells, the Radix Rubiae extract and its EA fraction showed no significant inhibitory effect on PBMC proliferation (Fig. 5.2). These results indicate that Radix Rubiae did not exert cytotoxic to the cells while arresting or inhibiting the cellular DNA synthesis thus preventing the cells from proliferating. In contrast to Radix Rubiae, realgar extract, arsenic trioxide, arsenic pentoxide and arsenic iodide demonstrated biphasic pattern of both proliferation-stimulating and inhibiting effects on PBMCs. At lower concentrations, these substances enhanced the proliferation of PBMCs, but exerted inhibitory effect at higher concentrations. Fig. 5.1, 5.3.a, 5.4.a and 5.5.a, show the biphasic pattern of these compounds on the growth of PBMCs as determined by [methyl-^{3}H]-thymidine incorporation assay for realgar, arsenic trioxide, arsenic pentoxide and arsenic iodide, respectively. In addition, similar to Radix Rubiae, these compounds did not exhibit significant cytotoxic effect on the viability of the PBMCs when detected by trypan blue exclusion assay. Fig. 5.2, 5.3.b, 5.4.b, and 5.5.b illustrate the proliferation pattern of PBMCs in the presence realgar, arsenic trioxide, arsenic pentoxide and arsenic iodide, respectively as detected by trypan blue exclusion assay. Table 5.1. summarizes the IC_{50} of all the extracts and arsenic compounds on the growth inhibition of PBMCs as determined by [methyl-^{3}H]-thymidine assay.

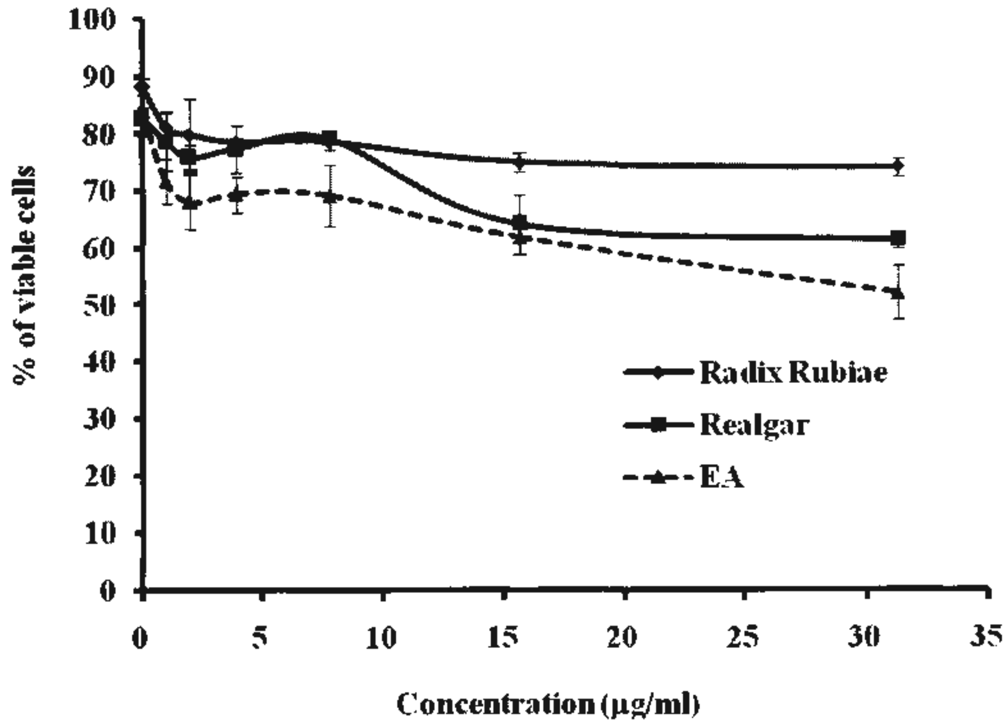


Fig. 5.2. Determination of the viability of PBMCs in the presence of Radix Rubiae, EA fraction of Radix Rubiae and realgar as estimated by trypan blue exclusion assay. The cells (1 x 10^5 cells/well) stimulated with PHA (10 μg/ml) were incubated in 96 well-plate in the presence of Radix Rubiae, its EA fraction of Radix Rubiae and realgar. After 72 h incubation, the numbers of viable cells were assessed by counting the cells with trypan blue dye. Data were expressed as the means ± SEM (n=3).

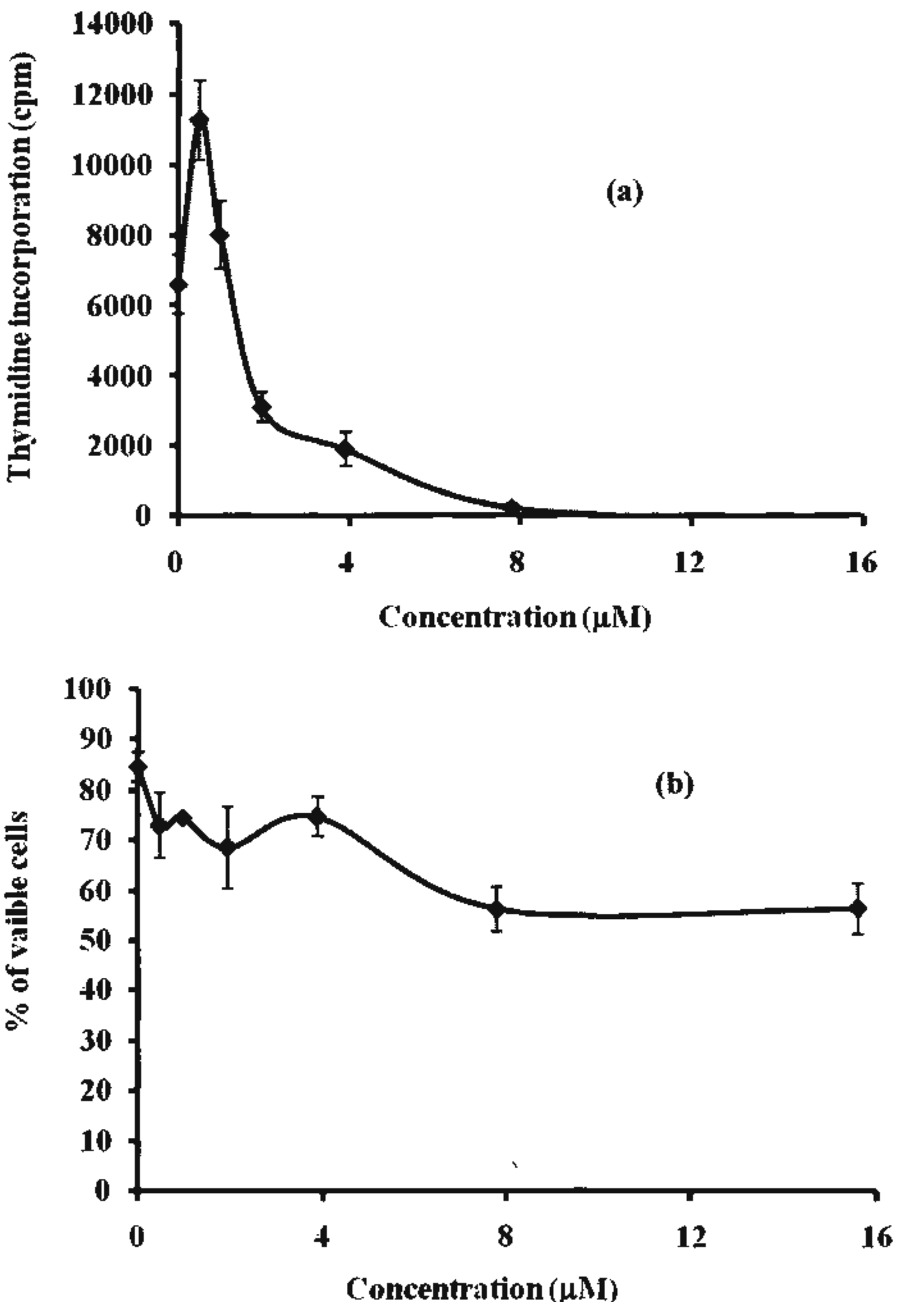


Fig. 5.3. Effect of arsenic trioxide on PBMC proliferation. The cells (1 x 10^5 cells / well) stimulated with PHA (10μg/ml) were incubated in 96 well-plates in the presence of arsenic trioxide for 72 h. Proliferation was determined by (a) [3H]-thymidine incorporation assay (n=12); and (b) trypan blue exclusion assay (n=3). Data were expressed as the means ± SEM.

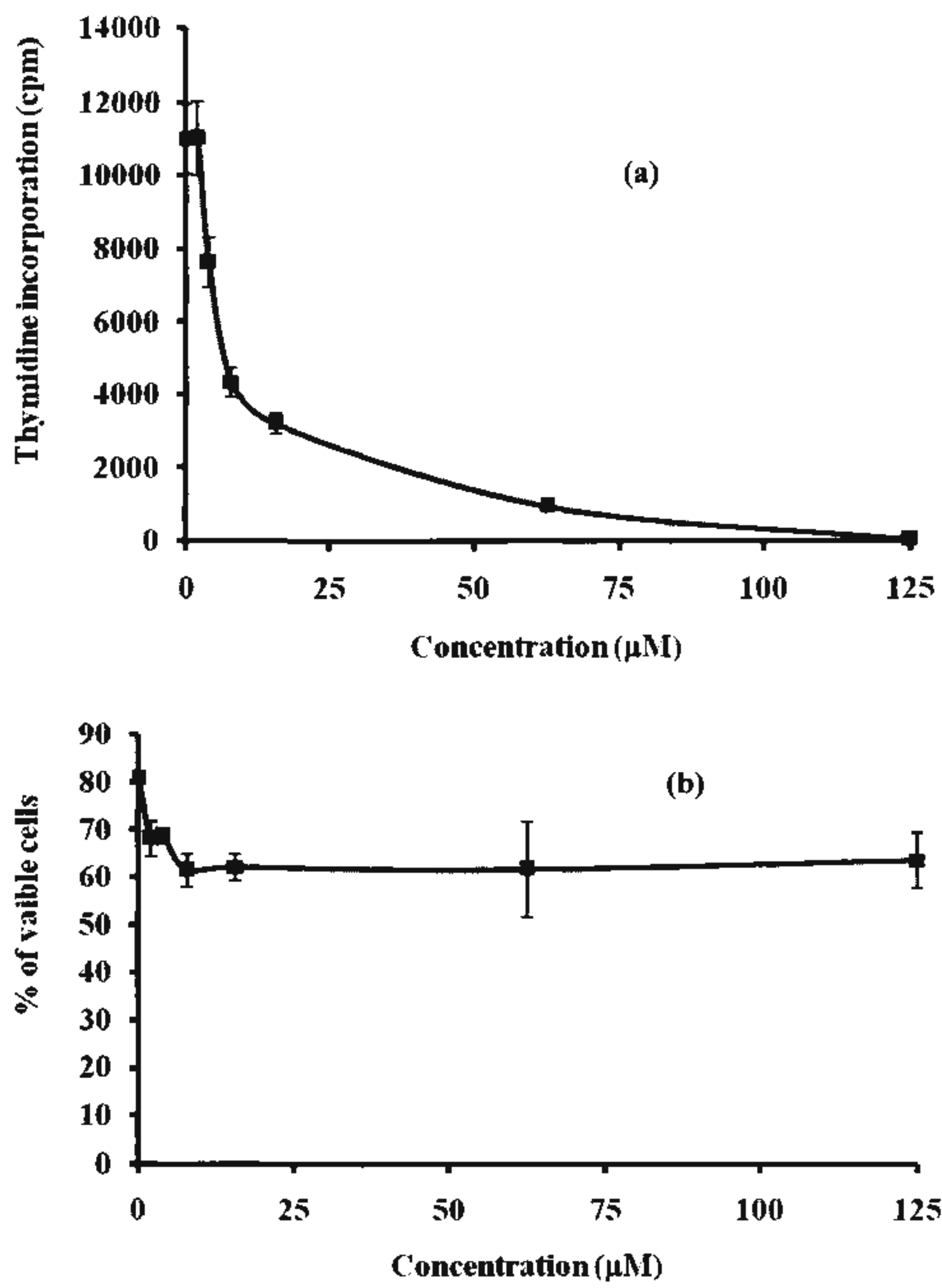


Fig. 5.4. Effect of arsenic pentoxide on PBMC proliferation. The cells (1 x 10^5 cells / well) stimulated with PHA (10 μg/ml) were incubated in 96 well-plates in the presence of arsenic pentaoxide for 72 h. Proliferation was determined by (a) [3H]-thymidine incorporation assay (n=12); and (b) trypan blue exclusion assay (n=3). Data were expressed as the means ± SEM.

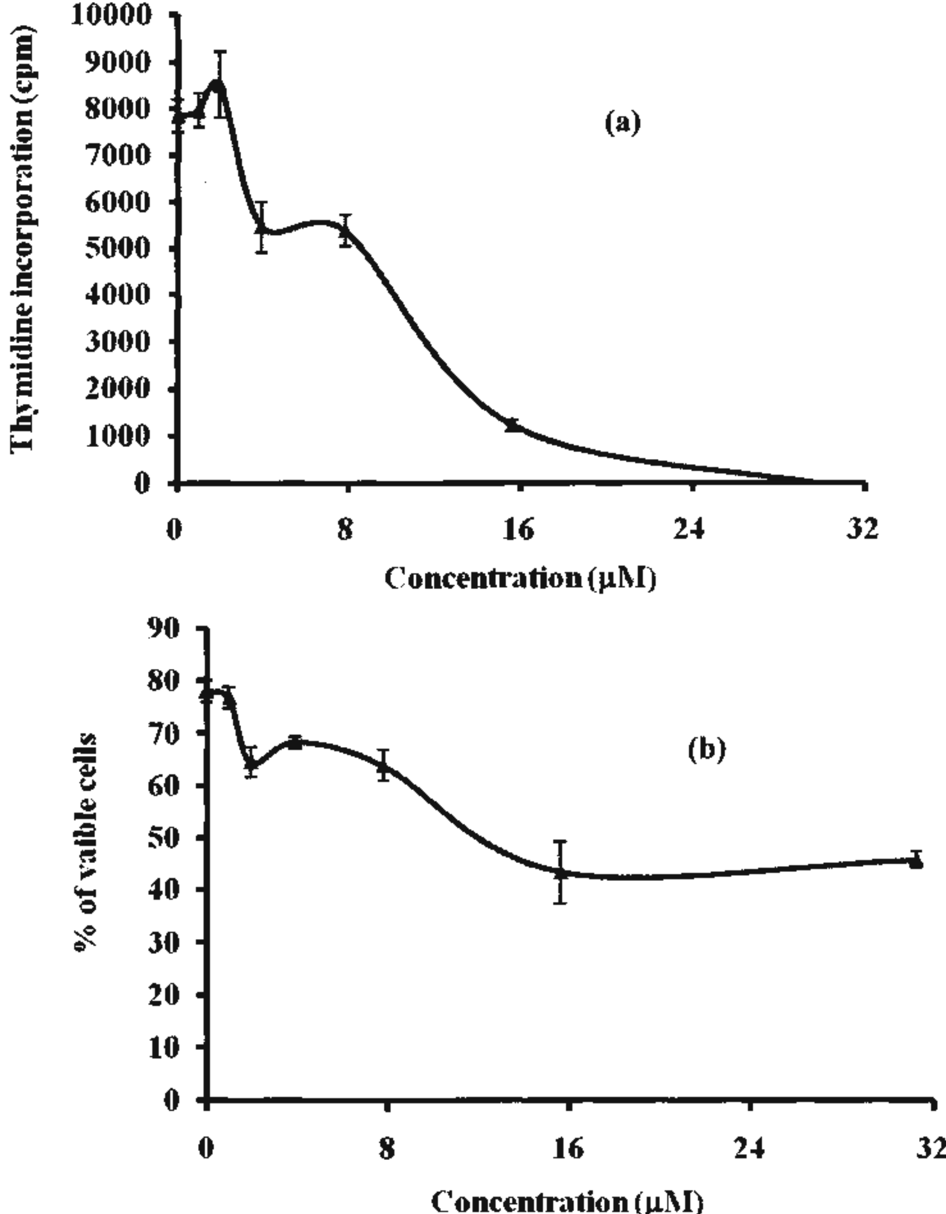


Fig. 5.5. Effect of arsenic iodide on PBMC proliferation. The cells (1 x 10^5 cells / well) stimulated with PHA (10 μg/ml) were incubated in 96 well-plates in the presence of arsenic iodide for 72 h. Proliferation was determined by (a) [3H]-thymidine incoporation assay (n=12); and (b) trypan blue exclusion assay (n=3). Data were expressed as the means ± SEM.

Table 5.1. IC_{50} values of Radix Rubiae extract, EA fraction of Radix Rubiae, realgar extract, arsenic trioxide, arsenic pentoxide and arsenic iodide on the growth of PBMCs as determined by [methyl-^{3}H]-thymidine incorporation assay

Compounds	Radix Rubiae extract (μg/ml)	EA fraction of Radix Rubiae (μg/ml)	Realgar extract (μg/ml)	As_2O_3 (μM)	As_2O_5 (μM)	AsI_3 (μM)
IC_{50} on T cell-activated PBMCs	1.4	0.18	1.2	0.67	3.1	15.1

Since the IC_{50} value of EA fraction was smaller than Radix Rubiae extract, it is suggestive that the active components are more concentrated in EA fraction. When considering the results of proliferation and apoptotic effects on HaCaT cells together with the present finding, it is clear that this fraction is a more suitable candidate for further *in vivo* investigation. Among the arsenic compounds, arsenic trioxide was most potent in inhibiting the PHA-activated PBMC proliferation. In view of this finding, arsenic trioxide is therefore a promising compound warranting further development into pharmaceutical treatment for psoriasis.

5.3.2. Effects of Extract and EA Fraction of Radix Rubiae, Realgar Extract and Arsenic Compounds on IFN-γ, TNF-α and IL-2 Release in PBMCs

The release of IFN-γ, TNF-α and IL-2 in PBMC in the presence of various test substances was determined by ELISA. As shown in Fig. 5.6, 5.7 and 5.9, Radix Rubiae, realgar extracts and arsenic trioxide showed a biphasic pattern on the production of the cytokines. These test substances up-regulated the release of IFN-γ, TNF-α and IL-2 at lower concentrations while were able to significantly down-regulate their release at higher concentrations. On the other hand, EA fraction of Radix Rubiae dose-dependently reduced the release of IFN-γ and TNF-α at our working concentration range (Fig. 5.8), but it enhanced the release of IL-2 at lower concentrations while

exerted inhibitory effect at higher concentrations.

Arsenic iodide was able to attenuate the release of IFN-γ at our working concentration range (Fig. 5.11.). However, the release of TNF-α and IL-2 were accentuated at lower concentrations and decreased at higher concentrations. Unlike arsenic iodide, arsenic pentoxide had no significant effect on the release of TNF-α and IL-2 (Fig. 5.10). However, it was able to increase the release of IFN-γ at lower concentrations and reduce its production at higher concentrations.

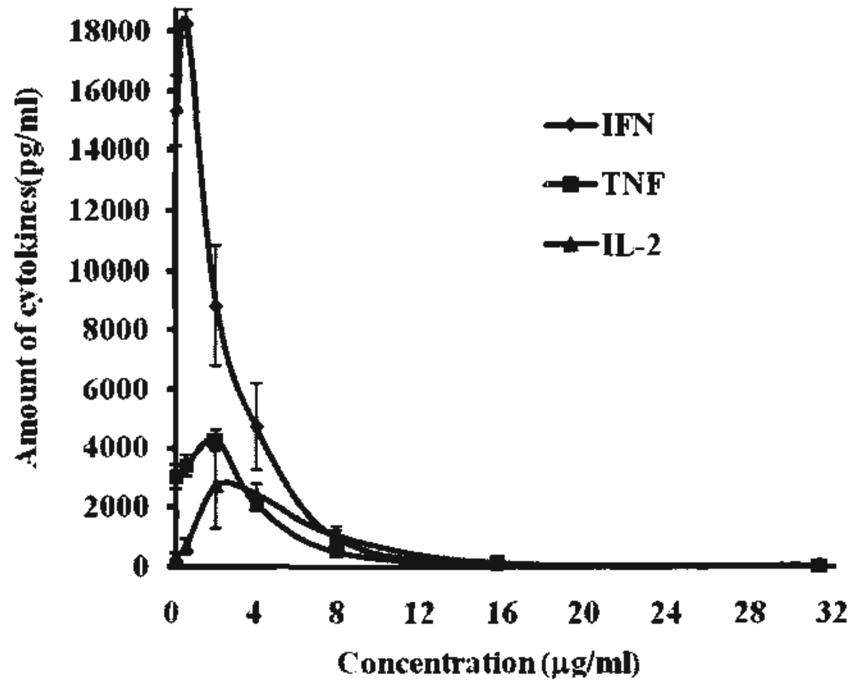


Fig. 5.6. Effect of Radix Rubiae extract on the release of cytokines. The release of IFN-γ, TNF-α and IL-2 from the PHA-stimulated PBMCs in the presence of Radix Rubiae after 72 h incubation was determined by ELISA. Data were expressed as the means ± SEM (n=6).

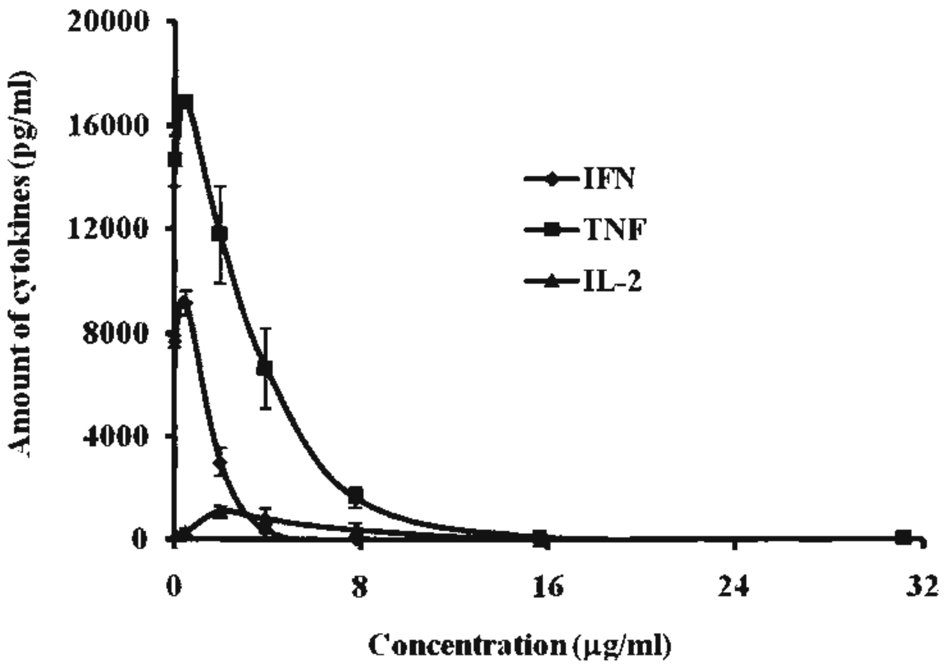


Fig. 5.7. Effect of realgar extract on the release of cytokines. The release of IFN-γ, TNF-α and IL-2 from the PHA-stimulated PBMCs in the presence of realgar extract after 72 h incubation was determined by ELISA. Data were expressed as the means ± SEM (n=6).

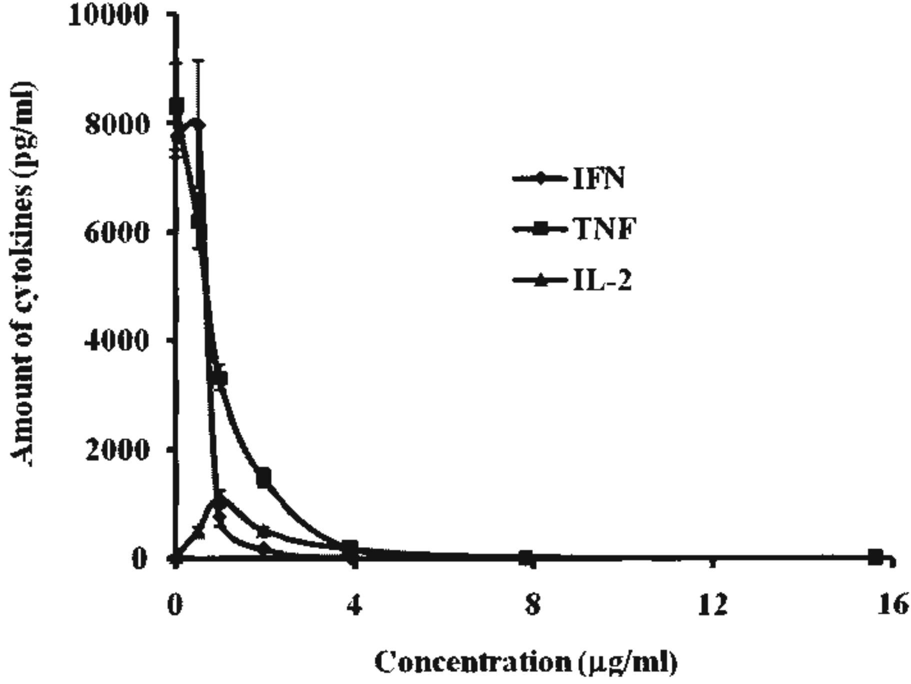


Fig. 5.8. Effect of EA fraction of Radix Rubiae on the release of cytokines. The release of IFN-γ, TNF-α and IL-2 from the PHA-stimulated PBMCs in the presence of EA fraction of Radix Rubiae after 72 h incubation was determined by ELISA. Data were expressed as the means ± SEM (n=6).

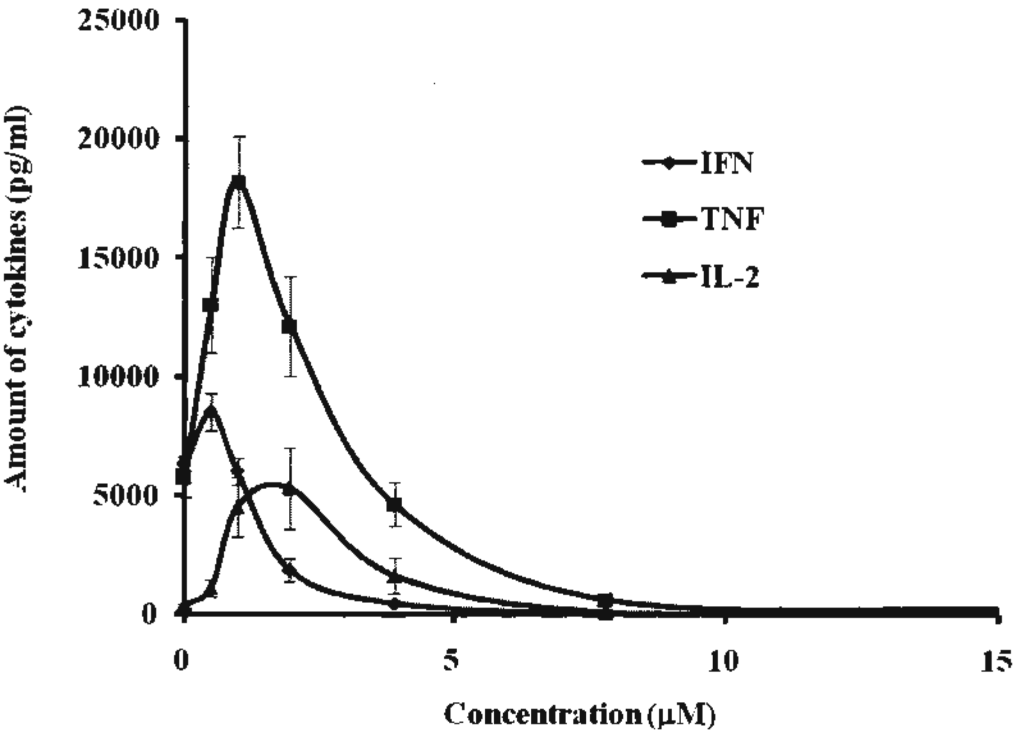


Fig. 5.9. Effect of arsenic trioxide on the release of cytokines. The release of IFN-γ, TNF-α and IL-2 from the PHA-stimulated PBMCs in the presence of arsenic trioxide after 72 h incubation was determined by ELISA. Data were expressed as the means ± SEM (n=6).

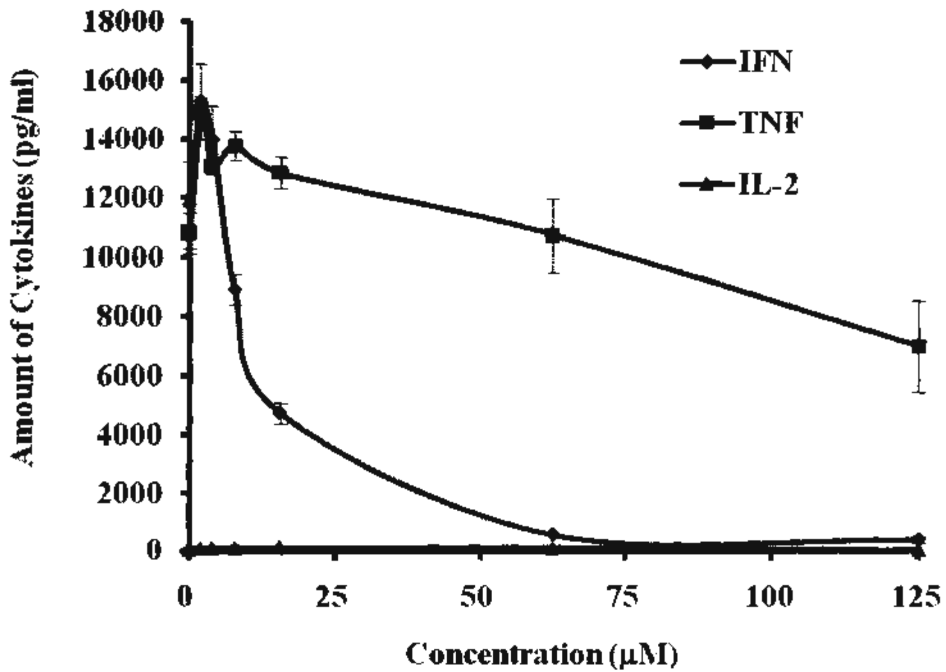


Fig. 5.10. Effect of arsenic pentoxide on the release of cytokines. The release of IFN-γ, TNF-α and IL-2 from the PHA-stimulated PBMCs in the presence of arsenic pentoxide after 72 h incubation was determined by ELISA. Data were expressed as the means ± SEM (n=6).

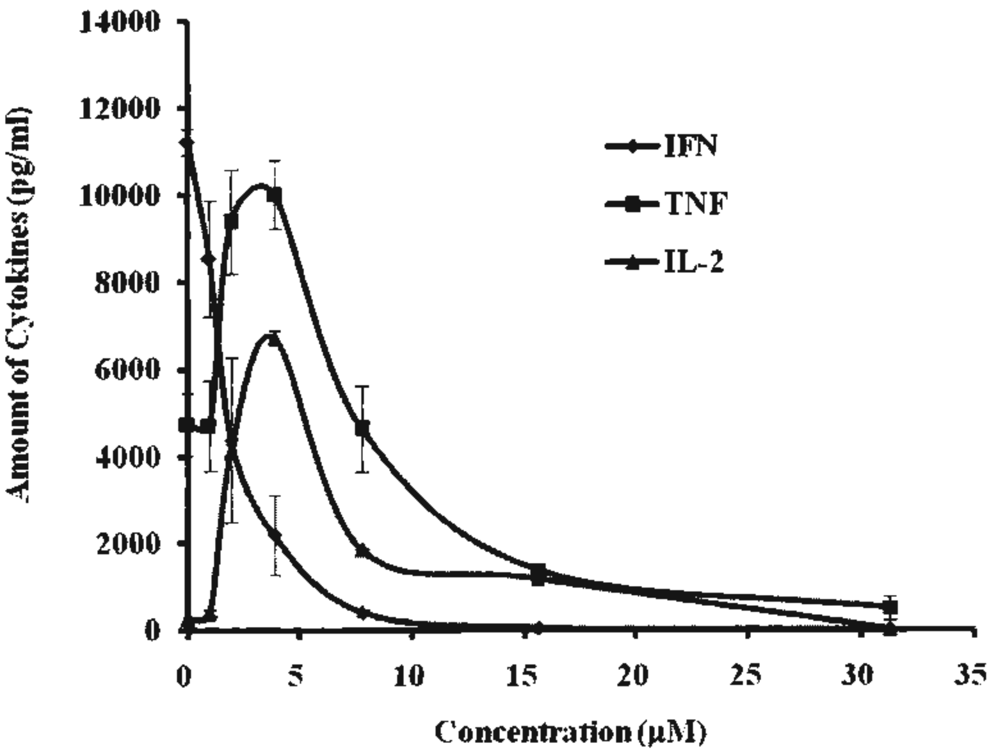


Fig. 5.11. Effect of arsenic iodide on the release of cytokines. The release of IFN-γ, TNF-α and IL-2 from the PHA-stimulated PBMCs in the presence of arsenic iodide after 72 h incubation was determined by ELISA. Data were expressed as the means ± SEM (n=6).

5.4. Discussion

Psoriasis is characterized by epidermal hyperplasia and accumulation of inflammatory cells, particularly neutrophils, monocytes and T lymphocytes. Downregulation of T-cell activation by cyclosporine is known to be a very effective treatment in patients with psoriasis (Stoof et al, 2001). Activated T cells in general produce one of two types of cytokines, known as type 1 (T1) and type 2 (T2) cytokines. T1 cytokines including IL-2 and IFN-γ mediate cellular immunity. Cells cloned from psoriatic lesions have been shown to produce IL-2 and IFN-γ (Uyemura et al. 1993; Schlaak et al. 1994). For this reason, psoriasis is considered to be a T1 dominant disease. The critical importance of IL-2 and IFN-γ in disease expression is clear because these cytokines are capable of inducing the appearance of psoriasis when it is administered to patients with a propensity for psoriasis (Lee et al. 1988; Fierlbeck et al. 1990). Thus, agents that can reduce the release of IL2 and IFN-γ are useful for the treatment of psoriasis. Another cytokine TNF-α is implicated in the pathogenesis of chronic inflammatory diseases including psoriasis (Valesini et al., 2007). The new pharmacological agents that are able to block the TNF-α have offered new hope in the treatment of psoriasis.

In the present study, the human PBMCs, which have been used as an investigative tool

for studying antipsoriatic drugs (Stoof et al, 2001), were used as an *in vitro* model to study the anti-inflammatory action of several potential anti-psoriatic agents. Psoriatic plaque typically over-expresses Th1 cytokines including IFN-γ, TNF-α and IL-2 (Austin et al., 1999). The suppression of these cytokines could lead to the improvement of psoriasis symptom. In our study, the Radix Rubiae extract and its EA fraction, realgar extract, arsenic trioxide, arsenic pentoxide and arsenic iodide compounds were evaluated for their effects on the proliferation and cytokine production in the PHA-stimulated PBMCs.

The results of our experiments showed that all the extracts and arsenic compounds were able to suppress the proliferation of the PBMCs pre-activated by PHA when determined by [3H]-thymidine incoporation assay, indicating that these substances could effectively arrest DNA synthesis. However, when trypan blue assay was employed to evaluate their effects on PBMC growth, no significant inhibition was found, indicative of absence of cytotoxicity. Among these tested substances, the EA fraction of Radix Rubiae exhibited significant immunosuppressant activities because it strongly inhibited the growth of PBMCs while exerted no cytotoxicity even at the higher concentration. The EA fraction of Radix Rubiae possesses most potency as it has the lowest IC_{50} value (0.18 μg/ml). In addition, it also demonstrated anti-inflammatory properties as it was

able to reduce the release of inflammatory cytokines IFN-γ and TNF-α. In this regard, the EA fraction of Radix Rubiae has good potential for further development into an novel anti-psoriatic agent.

Unlike EA fraction of Radix Rubiae, ethanolic extract of Radix Rubiae showed a mixed trend toward cytokine responses. At lower concentrations, this herbal extract showed stimulating effect on the release of IFN-γ, TNF-α and IL-2 but produced opposite effect at higher concentrations. The discrepancy between Radix Rubiae extract and its EA fraction of Radix Rubiae may be explained by the fact that Radix Rubiae extract contains many more chemical components, which may make it likely produce a mixed response in PBMC growth. On the other hand, the EA fraction of Radix Rubiae contains more uniformed chemical components, hence more likely to produce concerted response in PBMCs.

With regard to realgar extract, arsenic trioxide and arsenic iodide, all of them caused a biphasic response in PBMC. At lower concentrations, they stimulated the proliferation of PBMCs and enhanced the release of IFN-γ, TNF-α and IL-2; while at higher concentrations, they produced opposite effects. Their biological effects in PBMCs are clearly concentration dependent and they could be regarded as immunomodulators. As

a result of this special feature, dose range will become an important factor for these agents to be developed as optimal anti-psoriatic drugs. Finally, arsenic pentoxide elicited an anti-proliferative effect on the growth of PBMCs and suppressed INF-γ elease, but it also caused a significant stimulating effect on TNF-α and IL-2 production, a fact renders it less desirable for further development as an anti-psoriatic drug.

Taking the above experimental results together, we conclude that all the extracts and arsenic compounds were capable of inhibiting the growth of PBMCs without showing cytotoxicity to the PBMCs stimulated by PHA. In addition, the EA fraction of Radix Rubiae, realgar, arsenic trioxide and arsenic iodide could reduce the release of IFN-γ, TNF-α and IL-2 at higher concentrations. The findings from the present experiments provide evidence that these herbal extracts and arsenic salts may work in psoriasis through the anti-inflammatory mechanism. The successful identification of these anti-inflammatory agents, particularly the EA fraction of Radix Rubiae and arsenic trioxide, renders them promising candidates for development into psoriasis treatment. Further work to test these components for their *in vivo* anti-psoriatic action in a psoriasis-relevant animal model will be discussed in Chapter 6.

Chapter Six

In vivo Anti-psoriatic Effects of Topical Preparations

- Mouse Tail Tests on Arsenic Trioxide, Arsenic Pentoxide, Arsenic Iodide and EA Fraction of Radix Rubiae

6.1. Introduction

Modeling psoriasis in experimental animals would be of great benefit for psoriasis research and anti-psoriatic drug development. However, an animal model that completely resembles the complex human disease psoriasis is not yet available to date. Investigation into the pathogenesis of psoriasis has been severely hampered by this lack of suitable laboratory animal models that mimic the complex phenotype and pathogenesis of psoriasis (Boehncke et al., 2007). It appears that no single feature within the complex pathologic mosaic in many psoriatic animal models is truly psoriasis specific. In some animal models, metabolic and biochemical abnormalities are found similarly in psoriasis but inflammatory mediators or adhesion molecules may be found in diseases unrelated to psoriasis. Also, in other animal models, although some genetic markers show only statistical associations with psoriasis, psoriasis phenotype cannot be established. In short, there is no animal model that meets all the pathologic alterations seen in psoriasis.

Although no perfect animal is available for psoriasis, during the past several decades, several experimental animal models have been recommended for the studies of anti-psoriatic drugs (Marks et al., 1973; Krueger et al., 1975; du Vivier and Stoughton, 1975; Lowe and Stoughton, 1977; McCullough et al., 1978; Boehncke et al., 1994; and Sundberg et al., 1994). Recent decades have witnessed an increasing effort in the research on animal models of psoriasis. A large variety of spontaneous mutations, genetically engineered rodents, immunological reconstitution approaches, and xenotransplantation models have all been used for studying specific pathophysiology of psoriasis and anti-psoriatic drug development (Boehncke and Schon, 2007). Several commonly used psoriasis animal models will be discussed in the ensuing sections.

(a) Spontaneous mutation models

Several spontaneous mutations in mice are associated with psoriasis-like phenotype. Asebia (Gates and Karasek 1965) is the first spontaneous mutation psoriasis model. The asebia mouse mutation features include moderate epidermal acanthosis, increased dermal vascularity, and a dermal infiltrate composed of macrophages and mast cells. However, one significant drawback of this model is that the infiltrate seen in this model distinctly lacks T cells and neutrophils. Another spontaneous mutation method is chronic proliferative dermatitis (*cpd*) (HogenEsch et al, 1994). The *cpd* mouse mutation features epidermal hyperproliferation, enlarged and dilated blood vessels as well as mixed inflammatory infiltrate with neutrophils forming epidermal microabscesses. However, the drawback of this model is that the hemopoietic cells fail to induce skin lesions in syngeneic recipients. Similarly to the phenotype of *cpd*, flaky sin (*fsn*) (Beamer et al, 1995) mice also exhibit epidermal hyperproliferation, a mixed inflammatory infiltrate with neutrophils forming epidermal microabscesses, and dilated dermal blood vessels, but it appears to be independent of T cells, because cyclosporine A has no therapeutic efficacy for this model.

(b) Animal models with epidermal overexpression of cytokines

More specific genotype-phenotype studies have been performed in transgenic animals. Cytokine transgenic animals have great potential particularly for the studies aimed at developing specific cytokine or cytokine receptor antagonists to treat psoriasis (Schon, 1999). A frequently used approach was epidermal overexpression of cytokines of interest under the control of the keratin 14 promoter, because K14 promoter-driven epidermal overexpression of keratinocyte growth factor or TGF-α resulted in marginal

alterations of keratinocyte proliferation and differentiation. These cytokine models include single cytokine transgenics involving transforming growth factor-α (TGF-α) (Vassar and Fuchs, 1991), interferon-γ (IFN-γ) (Carroll et al, 1997), keratinocyte growth factor (Guo et al., 1993), and IL-1α (Groves et al., 1995). Therefore, transgenic mice with targeted cytokine expression within the skin provided promising tools for studying possible roles of these cytokines in the pathogenic cascade of psoriasis.

(c) Mice overexpression of adhesion molecules in epidermis

Besides cytokines, adhesion molecules have extensively been studied in transgenic mouse models. Tissue-specific localization of immune cells including T cells, granulocytes, and macrophages, is considered to be crucial in the pathogenesis of psoriasis. Thus, animals overexpressing or lacking certain adhesion molecules are interesting tools for studying pathogenic events. When human β_1 integrins were expressed in the suprabasal epidermis under the control of the involucrin promoter, the resulting phenotype resembled psoriasis in hyperkeratosis, acanthosis, parakeratosis, and inflammatory infiltrates (Carroll et al, 1995). In β_1 integrins transgenic mice, epidermal hyperplasia and inflammatory infiltrates were 64%-89% and 36%-68%, respectively. The result indicate that keratinocyte hyperproliferation have occurred in the absence of infiltrating leukocytes. However, such hypothesis needs to carry out more experiments to clarify.

(d) Xenotransplantation models

To date, the most appropriate animal model for psoriasis can be obtained by grafting human psoriatic skin on to immunodeficient mice (Krueger et al., 1975; Briggaman and Wheeler, 1980; Lowe, 1988). Xenotransplantation of human skin would be recognized

and rejected through T cells and other immune cells, so immunodeficient recipient animals are needed for humanization. The immunodeficient mouse strain of mutation is the most widely used one for xenotransplantation experiment. With the use of T cell deficient nude mice as recipients, the psoriatic phenotype can last for more than 2 months after transplantation of lesional psoriatic skin (Krueger et al, 1975). Another xenotransplantation model, the psoriasis-SCID mouse model, becomes more widely used in translational research today (Boehncke, 1999). When lesional skin is grafted, the efficacy of potential therapeutics for psoriasis can be assessed. The dermis taken from clinically affected sites is overgrown by murine keratinocytes to form a multilayer psoriaislike epidermis that is usually not seen in murine skin. The results drive to proliferation and differentiation of the epidermis (Boehncke et al., 1994). When nonlesional skin is grafted, triggering factors for the onset of psoriasis can be studied, such as adaptive immune system (Wrone-Smith and Nickoloff, 1996). Another study, purified lymphocyte subpopulations were transferred, and activated T cells were identified as the key effector cells to induce psoriasis. With the similar approach, xenotransplantation models are clearly appropriate tools for mechanistically studying local events underlying the pathogenesis of psoriasis, including interactions of certain cell types, cytokines, and exogenous factors in an *in vivo* setting (Schon, 1999). It is now known that psoriasis-SCID mouse model is a powerful tool for studying pathogenesis of psoriasis and evaluating anti-psoriatic drugs (Boehncke et al., 1999). However, this method is not without shortcomings. The SCID mice are usually expensive and require special sterile environment for maintenance; and the techniques involving with transplantation are often complicated and require experienced hands to operate. Furthermore, it is difficult to get access to psoriatic lesional skin samples for transplantation purpose. Perhaps this is the most significant limiting factor for

adoptation of this model in psoriasis research, especially in small or medium size research units without the access to clinical patients.

(e) Mouse tail test for psoriasis

The mouse tail model for psoriasis was first described by Jarrett and Spearman in 1960s (Jarrett and Spearman, 1964), and modifications of this model have been reported by other authors (Bosman et al, 1992; Bosman, 1994). In this model, the normal adult mouse tail is used because mouse tail scale epidermis, unlike the rest of mouse body skin, is histologically and biochemically similar to psoriatic skin. There is a granular layer in the orthokeratotic interscale regions around the hair follicles, and the stratum corneum is histologically similar to that of normal human epidermis. However, in the scale regions, the granular layer is thin or absent, and nucleated cells are visible in the stratum corneum. These regions of parakeratotic stratum corneum are microscopically similar to those found in the involved stratum corneum of individuals with psoriasis. The mouse tail test measures the induction of granular layer in the epidermis of adult mouse tail which change from parakeratotic to orthokeratotic state when treated with anti-psoriatic agents. Longitudinal histological sections are prepared from the tail skin, and the degree of orthokeratosis can be determined by measuring the length of the granular layer, number of granular layers and the entire epidermal thickness. Mouse tail test is well-known for its ability to evaluate the antipsoriatic efficacy of topical drugs with regard to the induction of orthokeratosis. It is a physiological model of cell differentiation requiring no artificial induction, and it is a relatively sensitive method (Hofbauer, 1988) which allows the quantitative evaluation of the effects of anti-psoriatic agents on keratinocyte differentiation. Unlike xenotransplantation method, this model is easy to handle and the mice are ready to obtain and do not require special housing

conditions such as sterile environment. The other advantage of this model is that it is relatively inexpensive and technical requirement for this model is minimal. The mouse tail test thus presents a very useful experimental model for screening potential anti-psoriatic agents. This psoriasis-relevant animal model is readily available in our laboratory. Thus, mouse tail model would be employed in our project to determine the anti-psoriatic action of several topical agents including Radix Rubiae and arsenic compounds.

6.2. Objectives of the *in vivo* Studies

In our previous studies we found that EA fraction of Radix Rubiae possesses very effective inhibitory effect via induction of apoptosis on cultured HaCaT cells. EA fraction of Radix Rubiae was also found to have immunosuppressive effect on activated T cells and reduction of cytokine production. In addition, we successfully identified arsenic compounds as potent anti-proliferative and apoptogenic agents on HaCaT cells. These arsenic salts also exerted immunomodulatory effect on activated T cells. All these *in vitro* experimental results demonstrated that Radix Rubiae and arsenic compounds are potential candidates for further development into pharmaceutical treatment for psoriasis. In this drug development process, *in vivo* animal testing of the anti-psoriatic properties of these agents would provide valuable information regarding their potential in the treatment of psoriasis. In this study, mouse tail test would be used as the psoriasis-relevant animal model. The objectives of this study were established as below:

1. To evaluate the effect of the topical preparation containing EA fraction of Radix Rubiae on the induction of epidermal differentiation using mouse tail test;
2. To evaluate the effect of the topical preparations containing arsenic trioxide, arsenic pentaoxide, arsenic iodide on the induction of epidermal differentiation using mouse tail test.

6.3. Materials and Methods

6.3.1. Source and Preparation of Chinese Medicinal Materials

The purchasing of arsenic trioxide (As_2O_3), arsenic pentoxide (As_2O_5) and arsenic iodide (AsI_3) was described in Section 4.2.2.1. The preparation of EA fraction of Radix Rubiae was described in Section 3.2.2.1. The aqueous cream BP was purchased from Adams Healthcare Limited (Leeds, United Kingdom). 1% (w/w) Dithranol ointment (trade name DithraSal® Ointment 1%) was obtainable from DermaTech Laboratories (Bella Vista, Australia).

6.3.2. Preparation of Topical Drugs

For preparation of topical arsenic compounds, 1, 2 and 5 g of Arsenic compounds were weighted and put into 100 g of aqueous cream base, and the arsenic compounds were then evenly mixed with the cream base using a blender to make 1% (w/w), 2% (w/w) and 5% (w/w) of topical arsenic compound creams, which were then stored at 4°C- 8°C until further use. For making EA fraction of Radix Rubiae topical preparation, 0.5 g of EA fraction of Radix Rubiae were weighted and dissolved in 1 ml DMSO, followed by mixing with 50 and 5 g of aqueous cream base to make up 1% (w/w) and 10% of EA fraction of Radix Rubiae topical preparations. The EA topical preparations were then stored at 4°C- 8°C before their use in *in vivo* experiments.

6.3.3. Mice for *in vivo* Experiments

Male BALB/c mice of 22-25 grams in weight were used for the mouse tail test. They were housed in the Animal Room at the Institute of Chinese Medicine, The Chinese University of Hong Kong. The ethics approval for the mouse tail experiments was granted by the Animal Research Ethics Committee, The Chinese University of Hong

Kong, and license [(07-187) in (DH/ORHI/8/2/1 pt.9] for performing the animal experiment was granted by the Department of Health, Hong Kong SAR. The male mice were kept in animal cages at controlled temperature (23 ± 2°C) under an artificial 12 h light-dark cycle and were allowed to normal mouse feedings and water freely.

6.3.4. Treatment with Topical Preparations

For the mouse tail test, the mice were put into immobilizing cages, and only the tails were left out of the cage. Creams were applied with cotton wool onto the tail about 2 cm in length starting 1 cm proximal to its body. The topical agents were left on the tails for 2 h each day. At the end of each treatment, the remaining of the ointment were washed off with wet cotton and the immobilizing plastic tubes were removed, and the mice belonging to the same treatment group were placed in the same animal cage. The treatment was carried out once a day, 7 times a week for a consecutive 2 or 3 weeks. Three animals were used for each topical agent. At the end of the entire treatment period, the mice were sacrificed by cervical vertebrate dislocation. Then, the tails were cut off and the skin tissue layer was removed from the cartilage and fixed in 10% formaldehyde. The tail skin tissues were then embedded in the paraffin and longitudinal histological sections were made at the treated parts of the tail skin. Paraffin sections of 5 μm were cut and hematoxylin-eosin staining was performed. The stained sections were inspected under a light microscope (IX-71, Olympus, Tokyo, Japan). The specimens were histometrically analyzed for: (1) the number of granular layer which is defined as the layer of orthokeratotic cells. To measure the number of granular layer, a total of 5 counts were made for each scale, 50 scales were counted in each animal in total and altogether 3 animals were employed for each topical preparation; (2) the vertical thickness of the granular layer defined as the vertical length

of the orthokeratotic layers. To gauge the vertical thickness of the granular layer, 5 counts were made for each scale, and 50 scales were measured for each animal and a total of 3 animals were used; and (3) the vertical epidermal thickness defined as the distance between the dermo-epidermal junction and the lowest part of the stratum corneum. Similar to the above measurement, 5 counts were made for each scale, and 50 scales were measured for each animal and a total of 3 animals were employed.

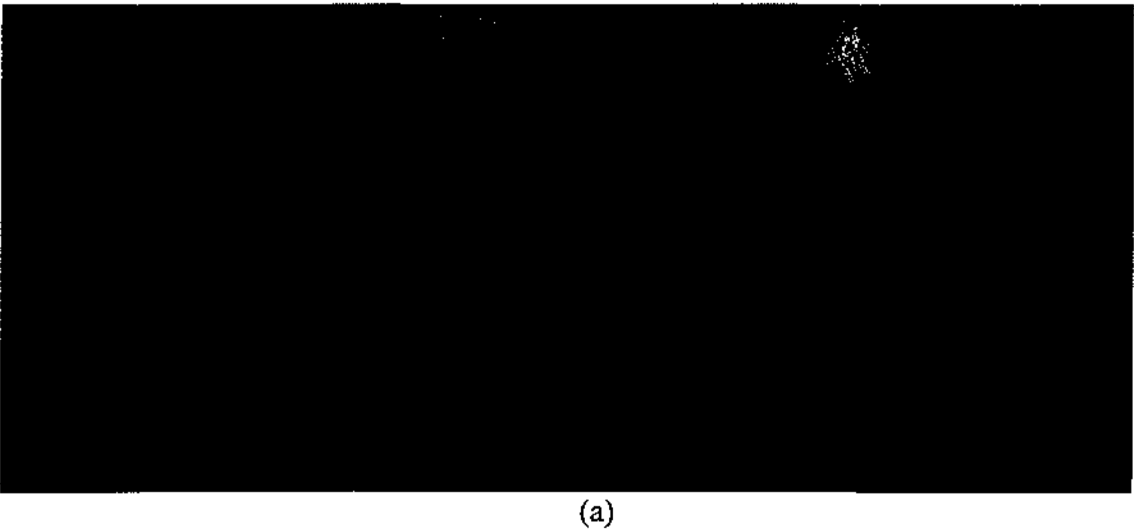
6.3.5. Statistical Analysis.

Data were expressed as mean ± SD. Statistical comparisons between the topical preparation treatments and cream base control were carried out using one-way ANOVA, followed by *post hoc* Dunnett's test using the cream control as the control group on the SPSS for Windows (version 14.0). Differences were considered significant at $p < 0.01$ and were denoted as *, $p < 0.01$.

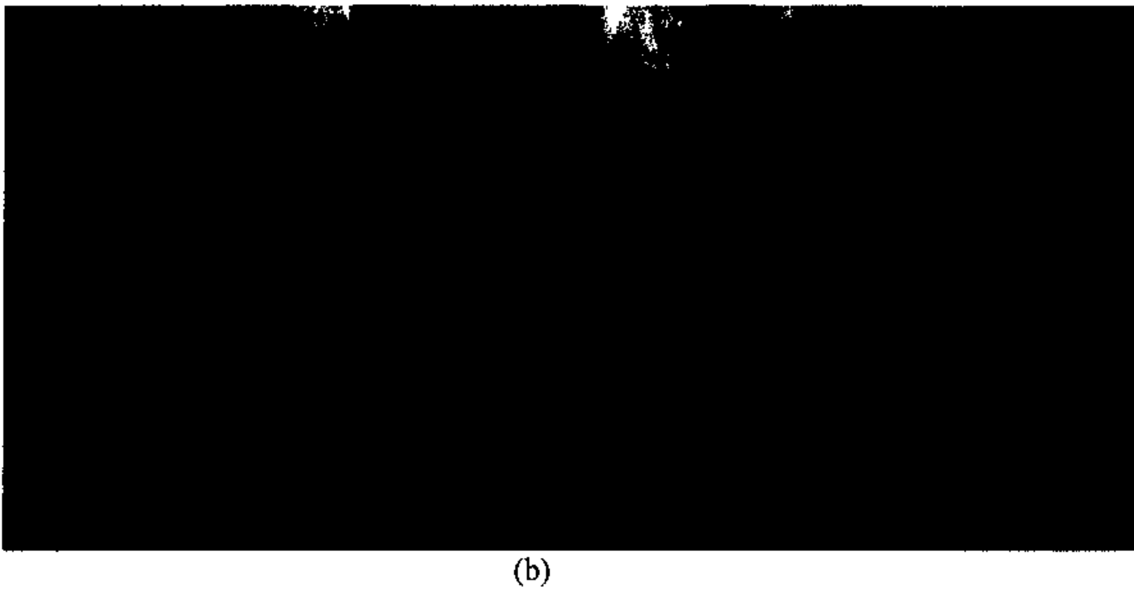
6.4. Results

6.4.1. Macroscopic Appearance of the Tail Skin after Topical Treatment

Fig. 6.1. presents the digital photographic image of the mouse tails after topical treatment. In the positive control dithranol group, the mouse tails were stained by the drug to dark yellow color, and varying degrees of swelling and skin damage were observed. The appearance of tail skin treated with different concentration of As_2O_3 is similar to the cream control and showed no apparent color or texture change when compared with the control. However, in contrast to As_2O_3, the tail skin treated with 5% AsI_3 and As_2O_5 were stained to yellow color. Moreover, treatment with 5% AsI_3 and 5% As_2O_5 also caused skin damages manifesting as redness, swelling and tissue ulcer in the tail.

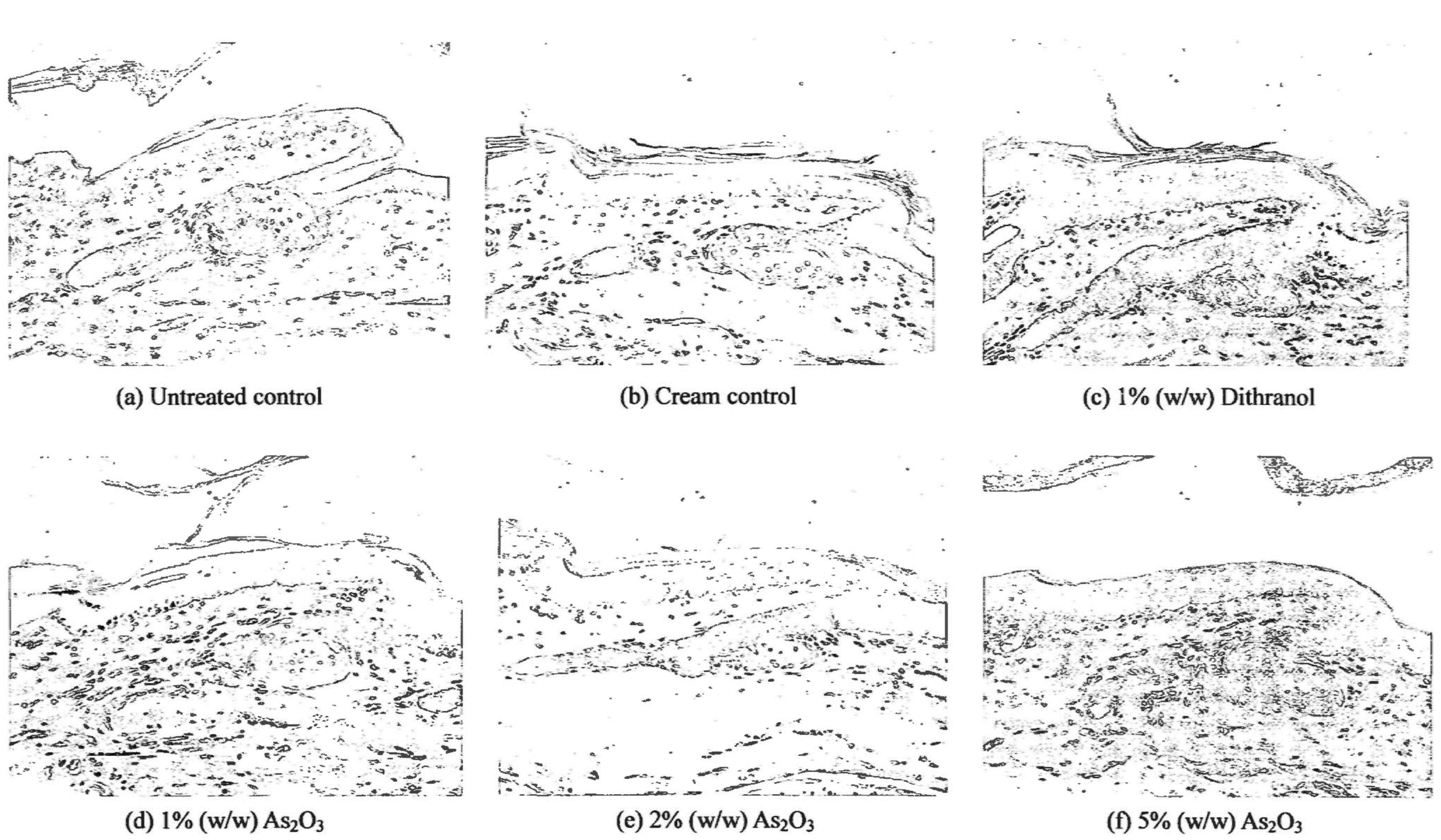


(a)



(b)

Fig. 6.1. Macroscopic appearance of BALB/c mice tails after two-week treatment with various topical preparations. (a) Mice from left to right: untreated mouse (Control), treated with cream only (Cream Control), 1% (w/w) Dithranol, 1% (w/w) As_2O_3, 2% (w/w) As_2O_3, and 5% (w/w) As_2O_3. (b) Mice from left to right: 1% (w/w) AsI_3, 2% (w/w) AsI_3, 5% (w/w) AsI_3, 1% (w/w) As_2O_5, 2% (w/w) As_2O_5, 5% (w/w) As_2O_5. Note that the skin damages were observed for mouse tails treated with 1% Dithranol and 5% AsI_3 and As_2O_5.



(a) Untreated control

(b) Cream control

(c) 1% (w/w) Dithranol

(d) 1% (w/w) As_2O_3

(e) 2% (w/w) As_2O_3

(f) 5% (w/w) As_2O_3

(g) 1% (w/w) AsI_3

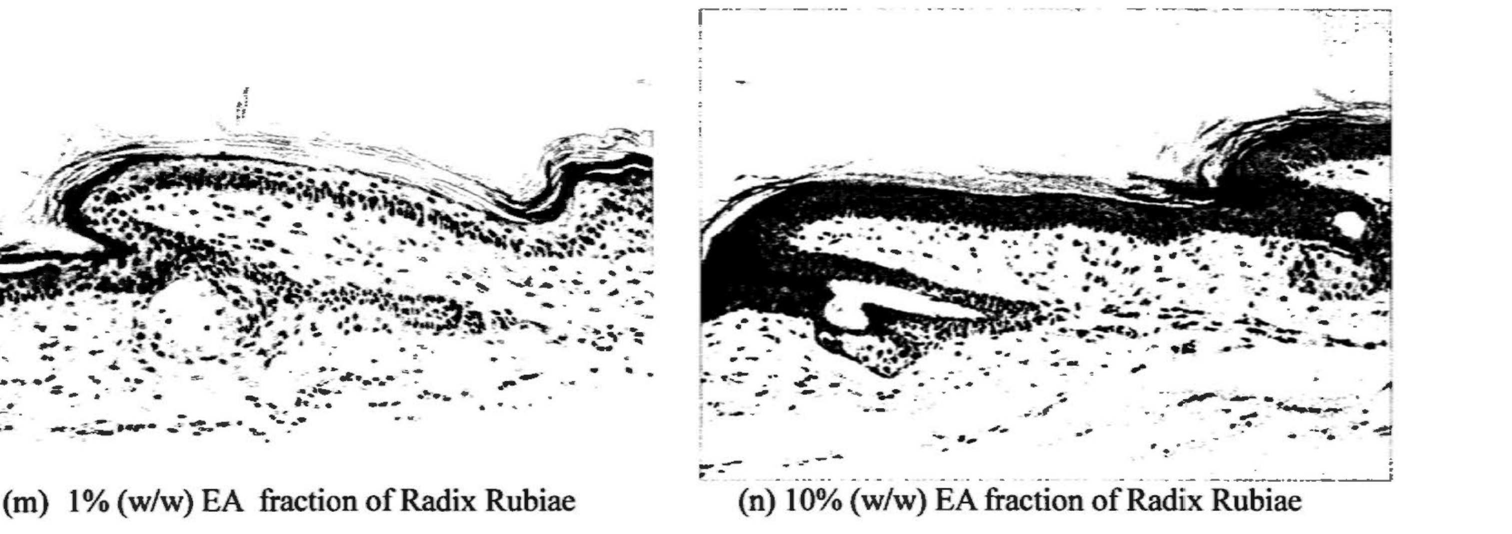
(h) 2% (w/w) AsI_3

(i) 5% (w/w) AsI_3

(j) 1% (w/w) As_2O_5

(k) 2% (w/w) As_2O_5

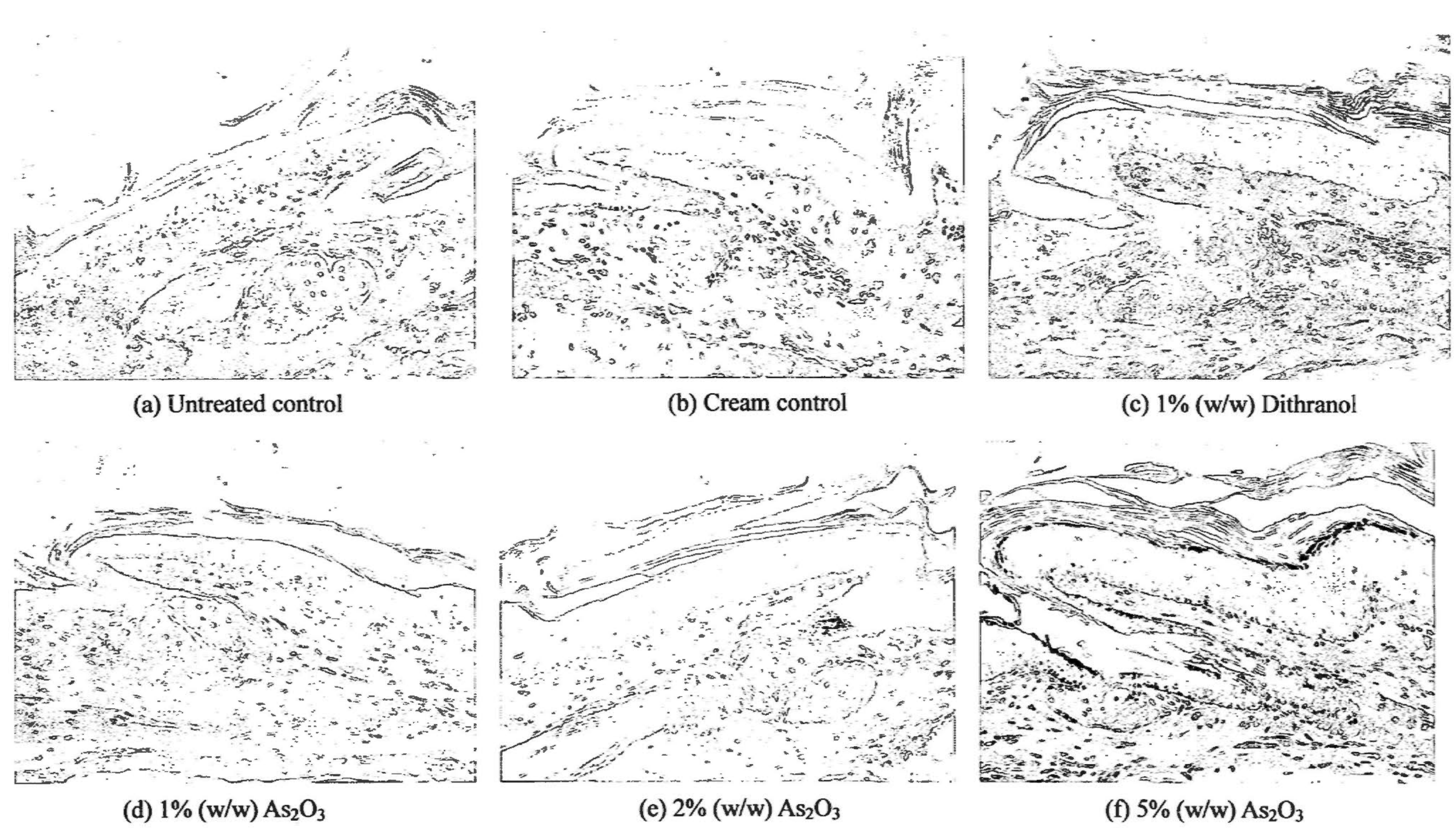
(l) 5% (w/w) As_2O_5



(m) 1% (w/w) EA fraction of Radix Rubiae

(n) 10% (w/w) EA fraction of Radix Rubiae

Fig. 6.2. Microscopic images of the longitudinal histological sections of the mouse-tail skin treated for two weeks and stained with haematoxylin and eosin. Magnification: 200X. The animals were treated topically with 1% (w/w) Dithranol, 1% (w/w) As_2O_3, 2% (w/w) As_2O_3, 5% (w/w) As_2O_3, 1% (w/w) AsI_3, 2% (w/w) AsI_3, 5% (w/w) AsI_3, 1% (w/w) As_2O_5, 2% (w/w) As_2O_5, 5% (w/w) As_2O_5, 1% EA fraction of Radix Rubiae, and 10% EA fraction of Radix Rubiae for 2 weeks. (a) untreated control; (b) cream control; (c) 1% (w/w) Dithranol; (d) 1% (w/w); As_2O_3; (e) 2% (w/w) As_2O_3; (f) 5% (w/w) As_2O_3; (g) 1% (w/w) AsI_3; (h) 2% (w/w) AsI_3; (i) 5% (w/w) AsI_3; (j) 1% (w/w) As_2O_5; (k) 2% (w/w) As_2O_5; (l) 5% (w/w) As_2O_5; (m) 1% (w/w) EA fraction of Radix Rubiae; and (n), 10% (w/w) EA fraction of Radix Rubiae, respectively.



(a) Untreated control

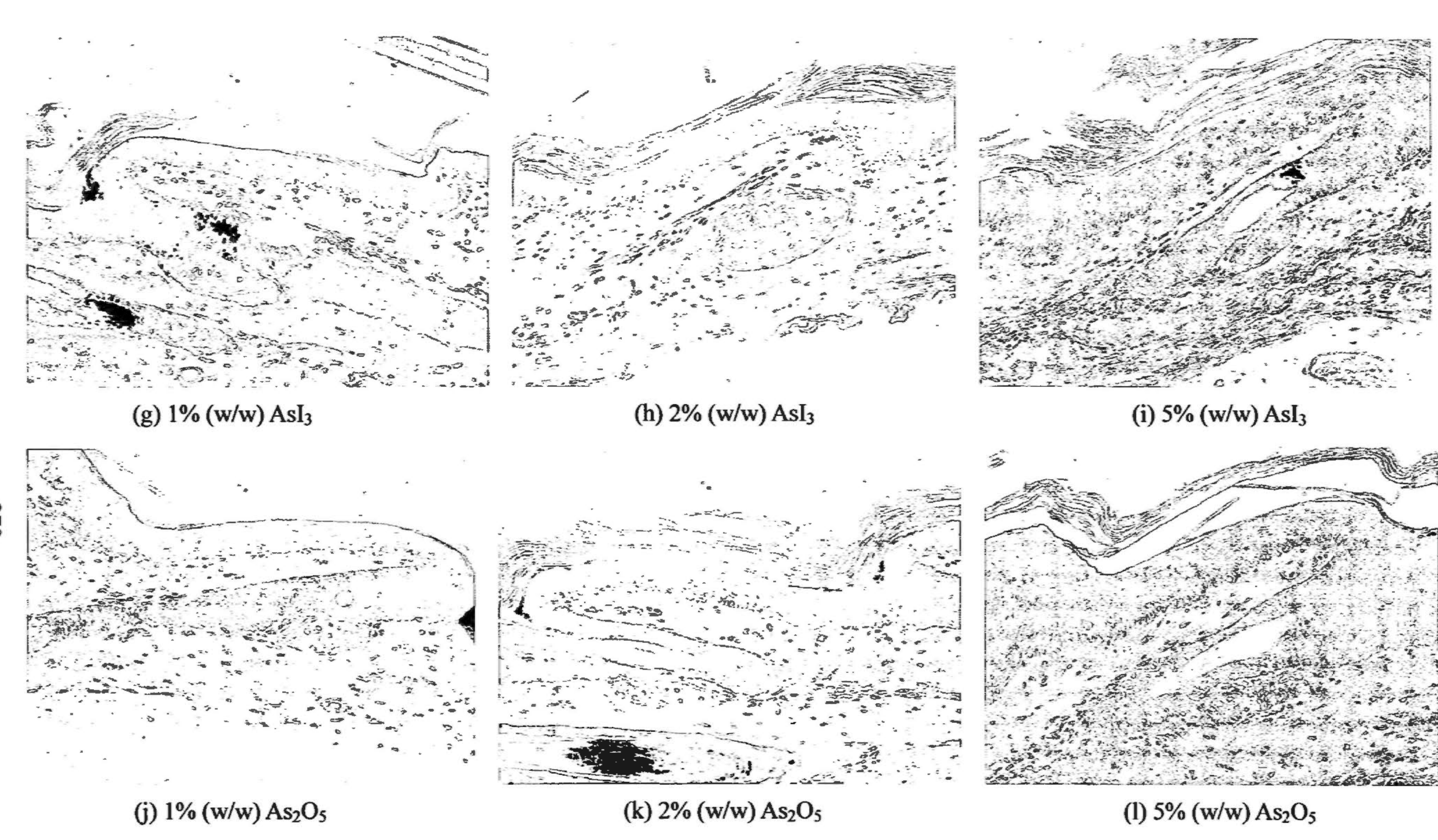
(b) Cream control

(c) 1% (w/w) Dithranol

(d) 1% (w/w) As_2O_3

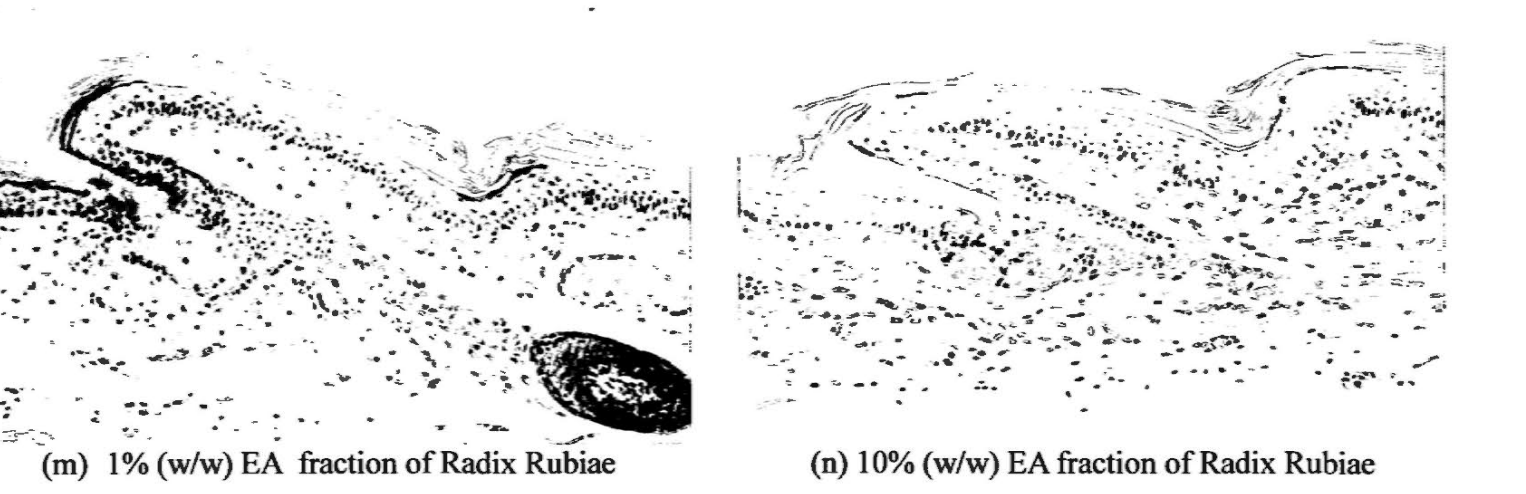
(e) 2% (w/w) As_2O_3

(f) 5% (w/w) As_2O_3



(g) 1% (w/w) AsI_3 (h) 2% (w/w) AsI_3 (i) 5% (w/w) AsI_3

(j) 1% (w/w) As_2O_5 (k) 2% (w/w) As_2O_5 (l) 5% (w/w) As_2O_5



(m) 1% (w/w) EA fraction of Radix Rubiae

(n) 10% (w/w) EA fraction of Radix Rubiae

Fig. 6.3. Microscopic images of the longitudinal histological sections of the mouse-tail skin treated for three weeks and stained with haematoxylin and eosin. Magnification: 200X. The animals were treated topically with 1% (w/w) Dithranol, 1% (w/w) As_2O_3, 2% (w/w) As_2O_3, 5% (w/w) As_2O_3, 1% (w/w) AsI_3, 2% (w/w) AsI_3, 5% (w/w) AsI_3, 1% (w/w) As_2O_5, 2% (w/w) As_2O_5, 5% (w/w) As_2O_5, 1% EA fraction of Radix Rubiae, and 10% EA fraction of Radix Rubiae for 3 weeks. (a) untreated control; (b) cream control; (c) 1% (w/w) Dithranol; (d) 1% (w/w) As_2O_3; (e) 2% (w/w) As_2O_3; (f) 5% (w/w) As_2O_3; (g) 1% (w/w) AsI_3; (h) 2% (w/w) AsI_3; (i) 5% (w/w) AsI_3; (j) 1% (w/w) As_2O_5; (k) 2% (w/w) As_2O_5; (l) 5% (w/w) As_2O_5; (m) 1% (w/w) EA fraction of Radix Rubiae; and (n) 10% (w/w) EA fraction of Radix Rubiae, respectively.

Table 6.1. Quantitative evaluation of the effect of the topical preparations containing arsenic compounds and EA fraction of Radix Rubiae on the number of granular layers, the granular layer thickness and the epidermal thickness after two and three weeks treatment.

Week		Control (a)	Cream control (b)	Dithranol (c)	1% As_2O_3 (d)	2% As_2O_3 (e)	5% As_2O_3 (f)	1% AsI_3 (g)	2% AsI_3 (h)	5% AsI_3 (i)	1% As_2O_5 (j)	2% As_2O_5 (k)	5% As_2O_5 (l)	1%EA Radix Rubiae (m)	10%EA Radix Rubiae (n)
2	Number of Granular Layer	3.4	4.1	6.2*	3.8	4.4	4.9*	4.1	4.3	7.5*	4.7	6.7*	7.3*	4.0	4.3
	Granular Thickness (µm)	23.7	27.5	48.8*	25.7	27.9	34.3	27.9	31.8	61.3*	33.9	53.8	60.0*	29.5	32.2
	Epidermal Thickness (µm)	32.8	37.5	63.5*	34.8	36.9	43.8	36.7	41.2	74.6*	43.7	65.0	71.8*	38.3	41.3
3	Number of Granular Layer	3.4	4.3	6.3*	4.3	6.1	7.2*	4.1	4.2	7.3*	4.5	4.8	6.5*	4.3	4.6
	Granular thickness (µm)	21.2	27.4	47.5*	27.8	41.2	54.2*	28.3	29.0	59.1*	32.5	35.5	48.4*	32.8	33.4
	Epidermal thickness (µm)	29.1	35.2	57.7*	36.2	50.5	65.0*	36.6	37.1	70.5*	41.1	44.5	58.6*	41.4	42.5

Differences were considered significant at $p < 0.01$ and were denoted as *, $p < 0.01$ (One-way ANOVA followed by *post hoc* Dunnett's test).

Data were expressed as the means ± SD (n=3) and all SDs were < 19% of the mean.

6.4.2. Histological Findings

Histological alterations of the mice tails after treatment by various topical preparations for two and three weeks are shown in the Fig. 6.2 and 6.3, respectively. The quantitative measurements of the number of granular layers, the granular layer thickness and the epidermal thickness are summarized in Table 6.1. The experimental results were also presented as bar chart as illustrated in Fig. 6.4.

(a) Cream control on mouse tail test.

The data showed that there were no significant differences in the number of granular layers, the granular layer thickness and the epidermal thickness between the untreated and the cream treated tails. In addition, no significant difference was observed between the untreated control and cream treated group even when the treatment was extended to 3 weeks.

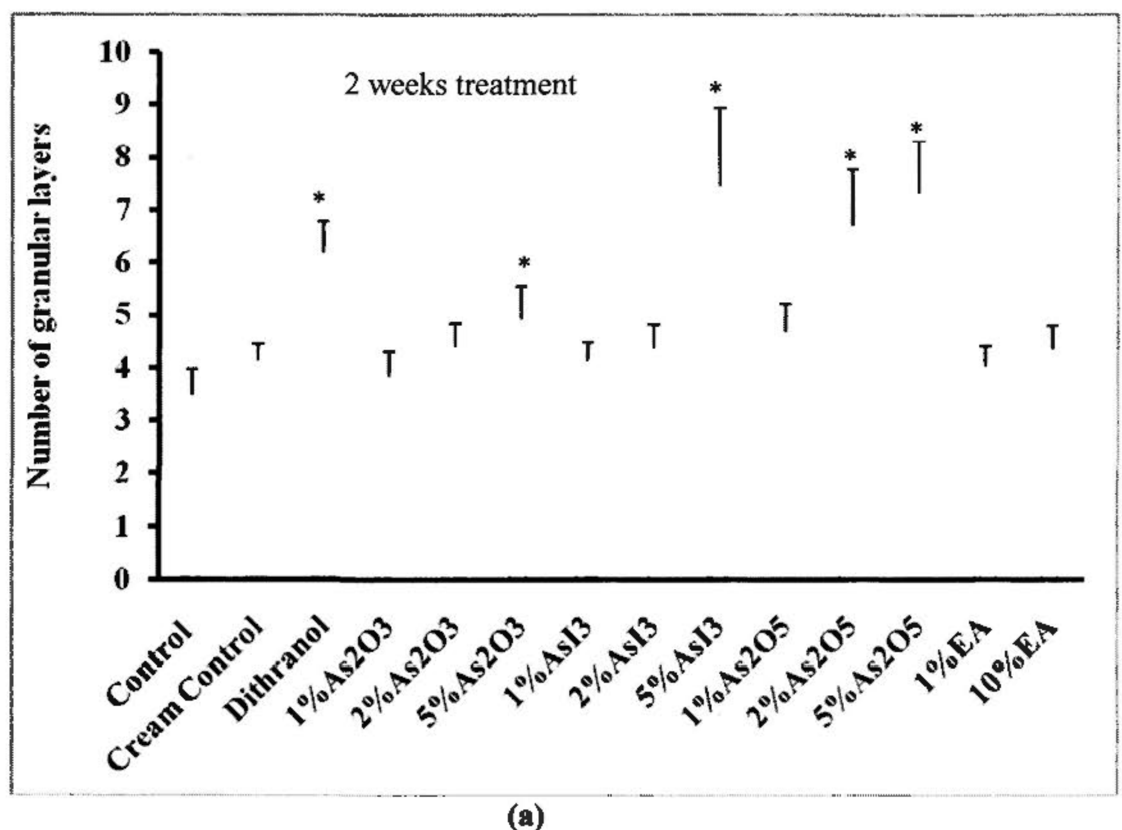
(b) Dithranol on mouse tail test.

On the other hand, the positive control, 1% (w/w) dithranol ointment markedly increased the number of granular layers, granular layer thickness and the epidermal thickness. Dithranol is well-known as a potent compound with ability to converse the parakeratosis to orthokeratosis of the epidermis (Hofbauer et al., 1988). We have also shown in our previous experiments that dithranol significantly reduced the proliferation rate of the cultured HaCaT keratinocytes (Tse et al., 2006). Moreover, dithranol has also been shown to possess ability to induce keratinocyte differentiation on various experimental models including mouse tail test (Bosman et al, 1992; Bosman 1994; Sebok et al, 2000 and Sebok et al, 1996). To date, dithranol is being used as an effective clinical drug for the treatment of psoriasis. In view of its multiple biological effects on keratinocytes that are relevant to psoriasis treatment, in our present study, dithranol was used as a positive control. Statistical comparisons were made between

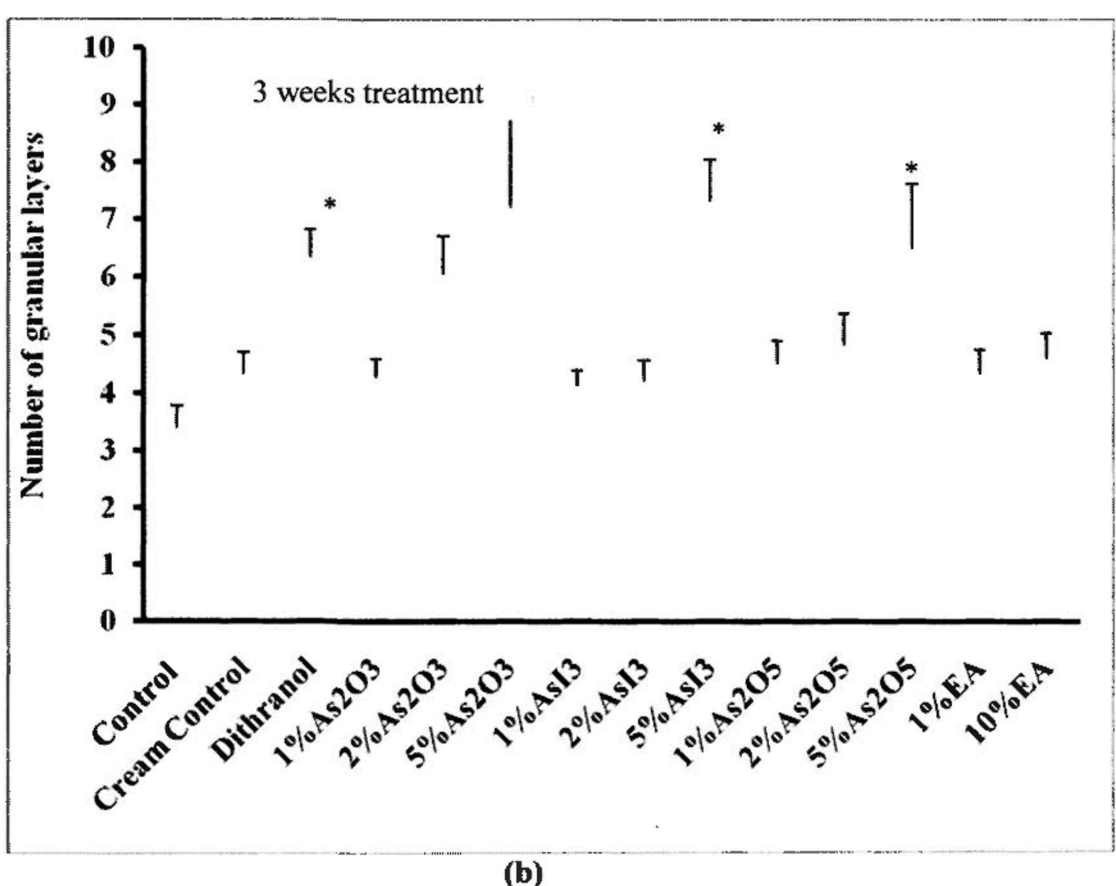
dithranol treatment with cream control only. The results from dithranol treatment also demonstrated that the number of granular layers, the granular layer thickness and the epidermal thickness are closely correlated.

(c) Arsenic trioxide on mouse tail test.

Arsenic trioxide dose-dependently increased the number of granular layers from 3.8 to 4.9 as concentration increased from 1% (w/w) to 5% (w/w) after two weeks treatment. It exhibited a significant effect in the number of granular layers but not in the thickness granular layer and the epidermal thickness when compared with the cream control for two weeks treatment. However, when the treatment was extended to three weeks, the number of granular layers, granular layer thickness and the epidermal thickness significantly differed from the cream control. With 5% (w/w) arsenic trioxide treatment, the granular layer, granular layers and epidermal thickness markedly increased from 4.9 to 7.2, 34.5 to 54.2 μm and 43.8 to 65.0 μm, respectively, when treatment extended from two to three weeks. The results indicated that arsenic trioxide may require a longer treatment period in order to obtain satisfactory anti-psoriatic effect in the mouse tail test, and the effect of As_2O_3 on granular thickness of the mouse tail was clearly dose and time dependent.



(a)



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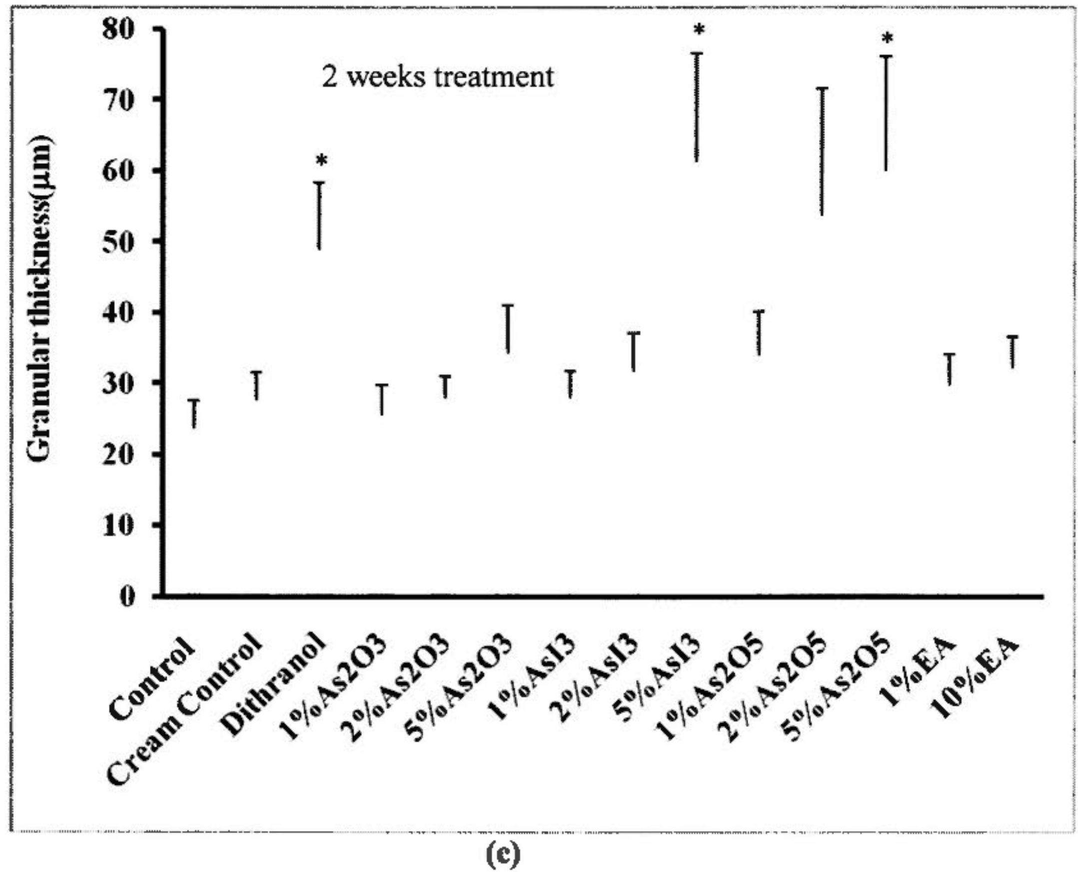

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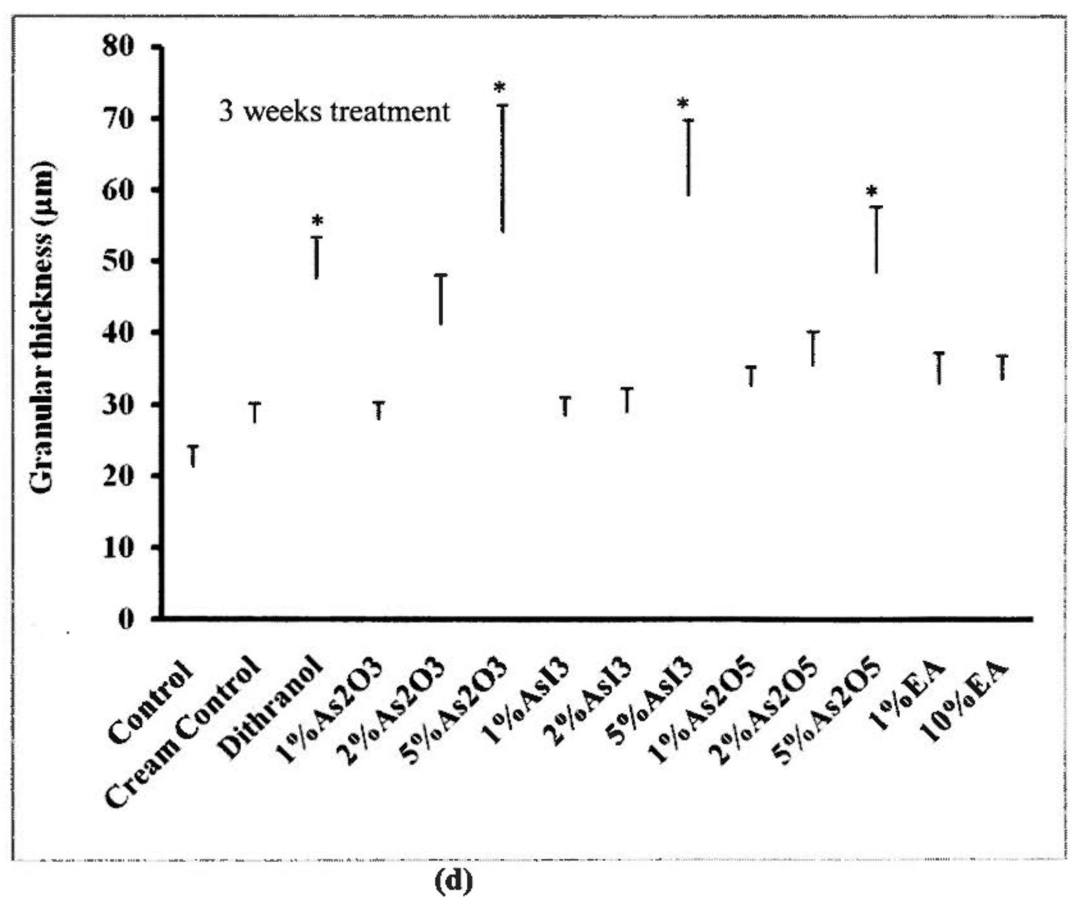

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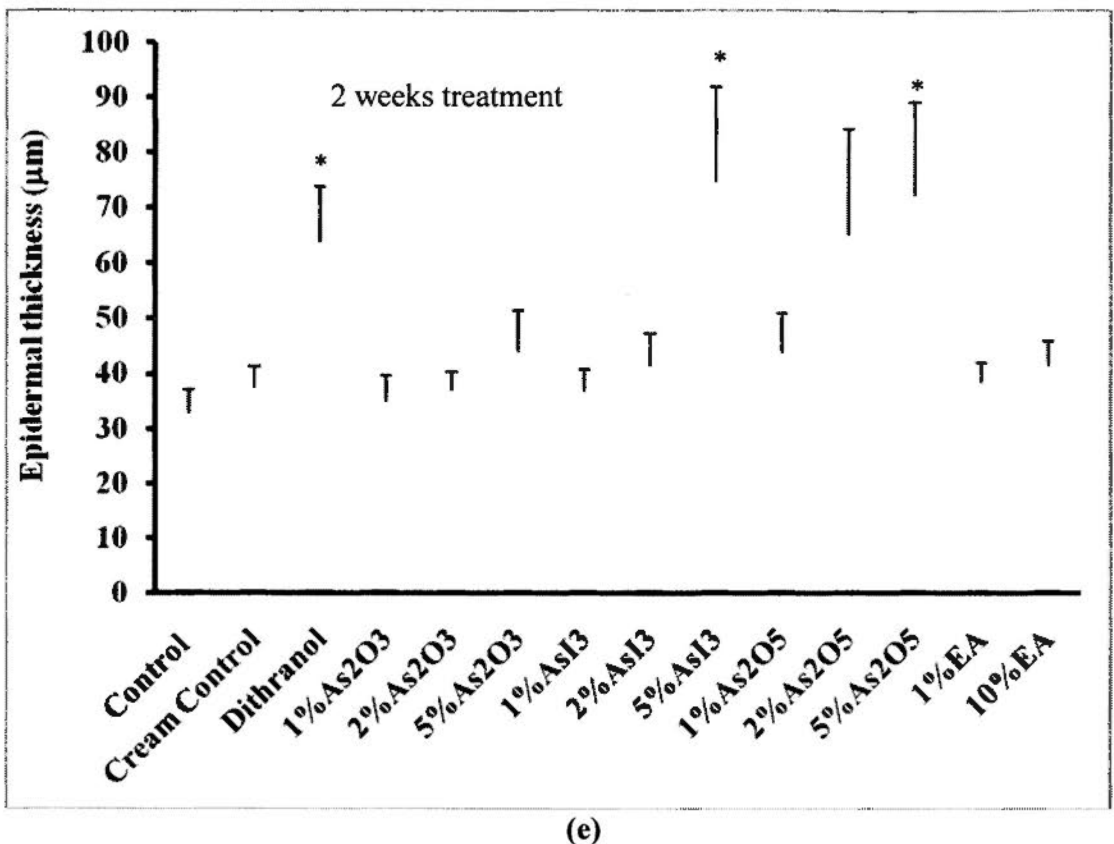
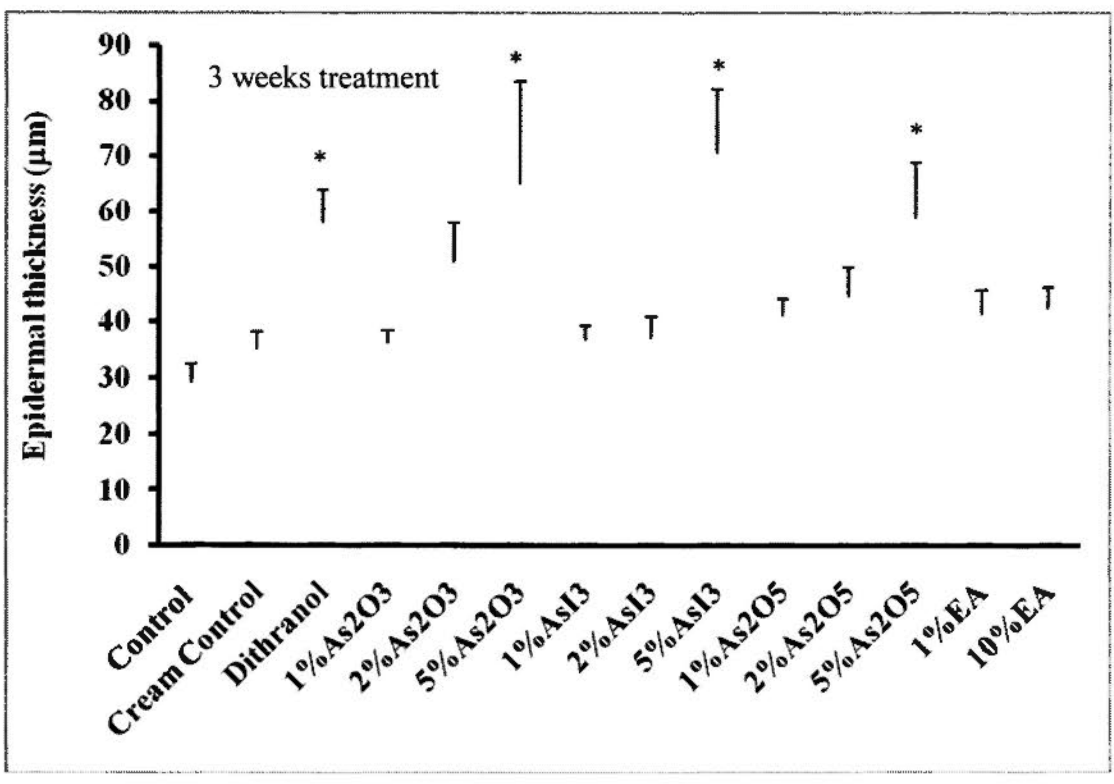


(e)



(f)

Fig. 6.4. Bar chart presentation of the effects of various topical preparations on mouse tail test. (a) the number of granular layers after two weeks treatment; (b) the number of granular layers after three weeks treatment; (c) the granular layer thickness after two weeks treatment; (d) the granular layer thickness after three weeks treatment; (e) the epidermal thickness after two weeks treatment; and (f) the epidermal thickness after three weeks treatment, respectively. *, $p < 0.01$ (One-way ANOVA followed by *post hoc* Dunnett's test.)

(d) Arsenic iodide on mouse tail test.

Arsenic iodide at 5% (w/w) concentration was shown to have significant effect on granular layers, granular layer thickness and epidermal thickness for both two- and three-week treatments. The extension of treatment time to 3 weeks did not augment the granular inducing effect. . However, 1% (w/w) and 2% (w/w) arsenic iodide for two and three weeks produced no significant change to the number of granular layers, granular layer thickness and epidermal thickness when compared to cream control. Macroscopically, arsenic iodide at 5% (w/w) substantially stained the mouse tail skin to yellow colour, as shown in Fig. 1, and the damage to the surface of mouse tail skin was evident in that the skin was red and swollen. In addition, the ulceration was also found to occur on the surface of the skin.

(e) Arsenic pentoxide on mouse tail test

Another arsenic compound arsenic pentoxide exhibited capability to increase the granular thickness from 33.9 to 60.0 μm and from 32.5 to 48.4 μm for two weeks and three weeks treatment, respectively. As_2O_5 produced change in granular thickness in a dose but not time related manner. Like arsenic iodide, arsenic pentoxide also caused certain degree of skin damage to the mouse tail epidermis, in which bleeding was observed to the treated skin.

(f) EA fraction of Radix Rubiae on mouse tail test

In our experiments, the topical preparation containing EA fraction also marginally augmented the epidermal thickness of the mouse tail, although such increases did not produce statistical significance. Also, the EA fraction was able to increase the thickness of the granular layer but the response is not statistically significant from that of the cream control. Because the magnitude of the effect of EA preparation is smaller than that of arsenic compounds, a larger number of animals may be required in the

treatment group in order to detect the treatment effect. Another reason to explain this observation is that EA was not completely dissolved in the cream, this may somehow reduced the actual concentration of the EA fractionate. In future experiments, better pharmaceutical techniques should be used to increase the solubility of the Radix Rubiae and improve the quality of the topical application. Also, a larger number of animals should be used in the treatment arm so that the therapeutic efficacy possessed by Radix Rubiae can be revealed.

6.5. Discussion

Psoriasis is a chronic immune-mediated inflammatory skin disease characterized by hyperproliferation and abnormal keratinocyte differentiation of keratinocytes. The parakeratosis, the characteristic feature of psoriatic lesions, is the results of the combination of over proliferation and aberrant differentiation of keratinocytes. The modulation of keratinocyte differentiation may reduce the proliferation potential of the keratinocytes, and lead to improvement of the skin lesions in psoriasis patients. Corticosteroids, a common form of therapeutic modality for psoriasis, can reduce the proliferation of keratinocytes, thereby effect dramatic improvements in the psoriatic skin lesions. However, this form of topical treatment can produce many unwanted adverse effects such as telangiectasia and collagen atrophy (Shuster, 1969). Also, corticosteroids can become ineffective as a treatment goes on for a longer period of time. Topical coal tar preparations have helped treat the scaling, inflammation and itchiness associated with psoriasis for centuries (Pegum, 1972), but it can irritate the skin and stain clothing. Calcipotriol, a synthetic form of vitamin D3, is currently used to treat mild to moderate psoriasis and can flatten psoriasis lesions and remove scales (Segaert and Duvold, 2006). Tazarotene is a retinoic acid receptor-specific retinoid, which can down-regulates markers of keratinocyte differentiation, keratinocyte proliferation, and inflammation (Duvic et al., 1997). However, tazarotene can also cause skin irritation. In view of the above drawbacks of current therapeutic modalities for psoriasis, there clearly exists a need for safer and more efficacious topical applications for the desirable management of psoriasis.

Our previous studies have shown that Radix Rubiae and arsenic compounds are potently effective in inhibiting cultured keratinocyte proliferation. The study of this chapter

aimed at evaluating whether these anti-proliferative agents also possess keratinocyte differentiation modulating effect. For this purpose, we employed the mouse tail test to examine how cell differentiation is affected by the *in vitro* anti-proliferative compounds including arsenic trioxide, arsenic pentoxide, arsenic iodide and EA fraction of Radix Rubiae in comparison with the classical topical anti-psoriatic drug dithranol. A substantial drawback to the development of new antipsoriatic drugs stems from the fact that psoriasis does not occur nor can it be induced in laboratory animals. Mouse tail model cannot fully exhibit the pathologic alterations seen in human psoriasis; nevertheless this model is a morphometry-based, relatively sensitive and well reproducible method which allows the quantitative evaluation of the effects of antipsoriatic drugs on epidermal differentiation (Hofbauer et al, 1988; Sebok et al, 1996). The other advantage of the mouse tail test lies in its simplicity in operation and cost effectiveness. To date, this model is widely used in screening anti-psoriatic drugs. On the practical side, the mice used for mouse tail mouse are readily available in the Laboratory Animal Services Centre, the Chinese University of Hong Kong, and they do not require special housing condition and handling procedures.

In our previous studies, arsenic compounds and EA fraction of Radix Rubiae extract were found to have potent anti-proliferative action on HaCaT keratinocytes in culture (Tse et al., 2007; Tse et al., 2008). *In vivo* experiment is essential for further evaluation of the anti-psoriatic activities of the topical preparations containing these substances. All arsenic compounds were able to enhance the number of granular layers and the granular thickness of the mouse tail epidermis, a change suggestive of their ability to induce keratinocyte differentiation. Among the three arsenic compounds, arsenic trioxide showed the most potent keratinocyte

differentiation-modulating effect in a dose and time dependent manner. Given that arsenic trioxide possesses highly potent anti-proliferative effect on keratinocytes *in vitro*, the current results lend further experimental support to suggest that arsenic trioxide is a promising candidate for further development into anti-psoriatic drug. In this study, EA fraction of Radix Rubiae was shown to marginally increase the granular thickness, although such increase was not statistically significant.

In view of the encouraging results obtained in this *in vivo* experiment, further investigation using alternative animal model and ultimately clinical trial is warranted to confirm the anti-psoriatic properties of arsenic compounds and EA fraction of Radix Rubiae. Further studies employing immunochemical staining techniques to study proliferation marker such as Ki67 and differentiation marker such as K10 should shed light into the underlying biochemical mechanism of action for the observed anti-psoriatic properties brought about by these agents.

Chapter Seven

General Discussion and Conclusions

Psoriasis is a common inflammatory and hyperproliferative skin disease characterized by abnormal keratinocyte proliferation and differentiation, accumulation of polymorphonuclear leukocytes in the skin, and T-cell activation (Camisa, 1994). Psoriasis is a non-contagious skin disorder and does not normally impinge significant threat on the life of the patient. However, not only does it have a physical impact such as itching in the skin and pain and restricted motion in their joints, psoriasis can also adversely affects the psychological feelings of the sufferers leading to anxiety, anger, embarrassment, low self-esteem and even depression. Worldwide, psoriasis affects about 2-3% of the population (Nickoloff and Nestle, 2004) and it has a tendency of becoming chronic thereby requires ongoing treatments. During the past several decades, its pathogenesis has been intensively investigated, and we now have a better understanding about its genetic causes, precipitating factors and general pathogenesis, current pharmaceutical treatments for this skin disease, however, remain largely unsatisfactory.

On the other hand, base on the concept of holism and syndrome differentiation and treatment determination, TCM is a popular choice of therapy for psoriasis for a long time and in general produces good clinical results. Since TCM usually uses tailor-made formulas for individual psoriasis patients, and there are often too many

herbs in a formula, it is therefore difficult to study the mechanism of action for such therapy and to elucidate the active ingredients of the formulas responsible for the therapeutic action. For the above reasons, in this study, we only focused on the investigation of individual Chinese medicinal materials for anti-psoriatic action. Sixty Chinese medicinal materials were selected for the project based on ethnopharmacological use of these herbs in Chinese medicine practice for the treatment of psoriasis. Among these 60 herbs selected, most of them are heat clearing and toxin relieving herbs; the other classes of the herbs include blood invigorating and cooling, and stasis removing; wind and dampness dispelling as well as Qi tonifying.

In our experiments, ethanol was elected as the solvent for the extraction of these herbs, owing to the fact that ethanol is the least toxic among the organic solvents and safe for use in human body. Also, the medium range of polarity of ethanol makes it possible to extract from the herbs many chemical components with a wide range of polarity.

As the hyperproliferation of keratinocytes is the main feature of psoriasis pathogenesis (Weinstein and Frost, 1968), in the initial stage of the project, we attempted to screen the selected Chinese medicinal materials in a hope to identify the active ingredients for anti-proliferative activity using a psoriasis-relevant *in vitro* model. A HaCaT cell line,

which is an immortalized line of human epidermal keratinocytes (Boukamp et al., 1988), and has been extensively used for the studies of pathogenesis of psoriasis and screening anti-psoriatic drugs (Garach-Jehoshua et al. 1999; Farkas et al., 2003; Thielitz et al., 2004), was used as the cell culture model in our initial screening programme. The advantages of HaCaT cells over primary human keratinocytes for the herbal screening programme were that the culture of HaCaT cells was relatively easier to maintain, and a large number of cells could be obtained to fulfill the needs of the screening programme. In additional, the genetic heterogeneity of primary human keratinocytes inherited from different donors could lead to non-reproducible results. Further experiments were also conducted on Hs-68 cell line, which was a normal human fibroblast cell line, in order to test whether the active herbal extracts had unspecific cytotoxicity on different cell types.

For measuring the HaCaT cell numbers in the culture, a colorimetric protein-staining assay using sulphorhodamine B (SRB) dye was adopted in our experiments. By having carefully validated the SRB assay on HaCaT cells, it was found that this colorimetric protein assay produced a good linearity between the cell numbers and OD readings (correlation coefficient $R^2 = 0.995$), thus providing an extremely useful tool in accurate quantitation of cell numbers in the screening programme.

Out of the 60 different Chinese medicinal materials tested, most of them were not able to inhibit the growth of the cultured HaCaT cells. However, two of the medicinal substances, viz. Radix Rubiae and realgar showed potent anti-proliferative effect on HaCaT cells with IC_{50} values of 1.4 and 6.6 μg/ml respectively as measured by SRB assay. In contrast, Radix Rubiae extract did not exert significant inhibitory effect on the proliferation of Hs-68 cell line. Meanwhile, realgar extract was shown to mediate modest growth inhibition on this human fibroblast cell line, demonstrating a milder cytotoxic effect than that on the HaCaT cell line. The findings regarding the anti-proliferative action of Radix Rubiae and realgar were further corroborated by MTT assay and trypan blue exclusion method. The advantages of using in-vitro anti-proliferation screening method include low cost, minimal technical requirement, relative sensitiveness and efficiency when compared with other therapeutic targets such as modulation of differentiation and immune functions.

As cellular apoptosis and/or necrosis could be responsible for growth inhibition of cultured cells, experiments were subsequently designed to elucidate, at morphological, molecular and biochemical levels, whether induction of cellular apoptosis was responsible for the Radix Rubiae and realgar extracts-mediated growth inhibition on the cultured HaCaT keratinocytes. It was well recognized that hyperproliferation of

epidermal keratinocytes seen in psoriasis was the result of the aberrant expression of many regulatory molecules associated with proliferation, and defects in apoptosis were believed to play an important role in the pathogenesis of psoriasis (Boehm, 2006). Several assays were employed to detect whether Radix Rubiae and realgar extracts were capable of inducing apoptosis, as no single assay was capable of unambiguously confirming the occurrence of apoptosis. Cell cycle progression analysis by flow cytometry revealed that these two extracts significantly increased the population of HaCaT cells in the sub-G1 phase (apoptotic peak), thereby causing apoptosis. Morphologically, Radix Rubiae and realgar-treated HaCaT cells were found to have hypercondensed nuclei and DNA fragmentation when stained with the Hoechst stain, two of the characteristic morphological features of cellular apoptosis. In addition, both of these extracts induced DNA fragmentation in HaCaT cells as illustrated by gel electrophoresis and TUNEL method. Moreover, the results from quantitative analysis of apoptotic cells by concomitant annexin V-PI staining also revealed that both of them induced apoptosis of the HaCaT keratinocytes in a time- and concentration-dependent manner. Finally, the activation of caspase-3 was detected when the HaCaT keratinocytes were exposed to the Radix Rubiae and realgar extracts, indicating unequivocally the occurrence of cellular apoptosis. The apoptotic actions observed in the present study provide a mechanistic explanation for the potent anti-proliferative

property exhibited by Radix Rubiae and realgar on HaCaT cells. The successful identification of Radix Rubiae and realgar as the potent anti-proliferative and apoptogenic agent renders them promising anti-psoriatic candidates and lays the groundwork for their further pharmaceutical development.

For quality control of the Radix Rubiae extract, A HPLC fingerprinting was established, and quantitative analysis was performed using mollugin, a major chemical constituent presented in Radix Rubiae, as the chemical marker. The quality control parameters resulted from this work provides valuable reference to ensure the batch-to-batch consistence of this extract when used in future laboratory experiments and clinical studies.

Since Radix Rubiae exhibited pronounced apoptogenic action on HaCaT cells, naturally we wanted to investigate whether the Radix Rubiae-derived chemical compounds which were available from commercial sources were responsible for the observed anti-proliferative effect. Altogether, mollugin, alizarin, purpurin and quinizarin were tested for their anti-proliferative action on cultured HaCaT cells and none of them showed significant growth inhibition on this cell line. As only very limited number of compounds were available for the test, and majority of the chemical compounds such as

many of the anthraquinones presented in the Radix Rubiae were not available for testing in our assay system, further bioassay-guided isolation work may help to obtain more chemicals from this herbal extract for activity testing. At the time of writing this thesis, funding from Hong Kong Research Grants Council has been secured for undertaking the bioassay-directed purification and characterization of the anti-psoriatic chemical components from this promising psoriasis-treating herb.

As a first step towards the final purification of the anti-psoriatic chemical compounds from Radix Rubiae, some phytochemical work was conducted on Radix Rubiae to identify which fraction was the most potent in eliciting anti-proliferative activity on cultured HaCaT cells. Four fractions of various solvents viz. hexane, ethyl acetate, n-butyl alcohol and water were obtained and they were subjected to test for anti-proliferative effect on cultured HaCaT system. Among these fractions tested, EA fraction showed the most potent growth inhibition with IC_{50} value of 0.9 μg/ml, which is more potent than the crude ethanolic extract ($IC_{50,}$ 1.4 μg/ml). The identification of the EA fraction of the Radix Rubiae as the most potent fraction in inhibiting proliferation is significant as topical preparations containing such potent fraction could have better potential in treating psoriasis.

As realgar is an effective agent with potent anti-proliferative effect on HaCaT keratinocytes, we therefore investigated whether several inorganic compounds presented in this mineral, i.e. arsenic trioxide, arsenic pentoxide and arsenic iodide also possess the anti-psoriatic activities. All these arsenic compounds could be dissolved in PBS. Our results showed that all these inorganic salts were able to induce significant growth inhibition on HaCaT cells with IC_{50} values of 5.1, 18.6 and 7.3 μM, respectively after 48 h incubation. It was noteworthy that all three arsenic compounds showed only modest inhibitory effect on the growth of normal human fibroblast Hs-68 cells, exhibiting discernible differential cytotoxic profiles between fast growing HaCaT cells and normal human fibroblasts. Similar to realgar, all arsenic compounds were also found to induce apoptosis on HaCaT cells. We believe that arsenic compounds that were able to inhibit keratinocyte proliferation and induce keratinocyte apoptosis would possess good potentials for development into effective pharmaceutical agents for the treatment of psoriasis.

As psoriasis is a distinct T cell-mediated inflammatory skin condition, it is therefore logical to evaluate whether the above Radix Rubiae extract and the EA fraction, realgar and arsenic compounds also possess anti-inflammatory properties. Our experiments using *in vitro* PBMC model showed that all of these compounds inhibited the activated

PBMC proliferation and reduce the production of IFN-γ, TNF-α and IL-2 by PBMCs. Since IFN-γ, TNF-α and IL-2 are predominant inflammatory cytokines in psoriatic lesions, reduction of these cytokines could offer a new opportunity for the treatment of psoriasis. Among these substances, the EA fraction has more pronounced growth inhibitory action on activated PBMCs than that of Radix Rubiae ethanolic extract. In view of this finding, it is believed that EA has high hope to be developed into effective treatment for psoriasis. On the other hand, arsenic trioxide and arsenic iodide have biphasic effects on PBMCs, as they stimulated the proliferation of PBMCs and enhanced the release of IFN-γ, TNF-α and IL-2 at lower concentrations, but produced opposite effect at higher concentration. They were able to attenuate the release of inflammatory cytokines including IFN-γ, TNF-α and IL-2 at certain concentration range. The immunomodulatory properties of the arsenic trioxide and arsenic iodide are important as they enable formulating topical applications of arsenic compounds which can exert significant therapeutic effect without evoking harmful side effect on the skin. It is interesting to know that arsenic pentoxide did not possess anti-inflammatory action, thus is less ideal for further development into anti-psoriatic agent.

The EA fraction of Radix Rubiae and arsenic compounds were further investigated for their differentiation-modulating properties because altered differentiation was

considered to be an important pathogenesis of psoriatic skin lesions (Voorhees, 1978). Mouse tail model was considered to be the most suitable in our study because of its availability, reproducibility and low cost. Also, the results based on mouse tail test have been shown to be relevant in evaluating efficacy of anti-psoriatic drugs. Our experimental results demonstrated that all arsenic compounds were able to enhance the number of granular layers and the granular thickness of the mouse tail epidermis. Among the three arsenic compounds, arsenic trioxide showed the most potent keratinocyte differentiation-modulating effect in a dose and time dependent manner. Taken the anti-proliferative, apoptogenic, anti-inflammatory and keratinocyte-modulating effects of arsenic trioxide into consideration, it is concluded that arsenic trioxide is a promising candidate for further development into antipsoriatic drug. In our experiment, topical preparations containing EA fraction of Radix Rubiae showed only marginal effectiveness in inducing keratinocyte differentiation in the mouse tail test. Further experiments usually better formulation and larger number of animals would help to evaluate the anti-psoriatic action of this herbal extract.

To conclude, the present PhD project has successfully identified two Chinese medicinal substances, namely Radix Rubiae and realgar, and realgar-derived arsenic compounds particularly arsenic trioxide as potent anti-psoriatic agents. These novel discoveries

have opened up a new avenue for research into developing safe and effective pharmaceutical agents for psoriasis. The following areas of further investigations are suggested as an extension to the current project. These include:

1. Purification and characterization of natural compounds derived from EA fraction of Radix Rubiae with anti-psoriatic activity.

As EA fraction of Radix Rubiae has been found to have potent anti-proliferative, apoptosis-inducing, immunosuppressive properties and mild differentiation-modulating effects, it would be rational to identify by bioassay-guided purification technique chemical components with marked anti-psoriatic action. The isolation of single components possessing the therapeutic activity is clearly desirable for future drug development. Various chromatographic techniques such as column chromatography, thin layer chromatography, HPLC will be used to implement the separation and isolation process. The bioassays to be carried out will include anti-proliferative, apoptosis-inducing, immunosuppressive and differentiation-modulating assays. The successful identification and characterization of the anti-psoriatic pure chemical compounds are necessary if the final products are to be protected by intellectual property right.

2. Determining the cell signaling pathway of apoptosis induced by Radix Rubiae and arsenic compounds

Evidence has been provided thus far that induction of apoptosis is the underlying mechanism for the observed anti-proliferative action of Radix Rubiae and arsenic compounds. However, it remains to be elucidated whether intrinsic (mitochondria-mediated) and/or extrinsic (death receptor-mediated) pathway is involved in the Radix Rubiae and arsenic compounds-mediated apoptosis. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors, located on the cell membrane and with subsequent activation of caspase 8. In contrast, the intrinsic pathway is initiated through the release of signalling factors by mitochondria within the cell, and caspase 9 is induced in this process. Further investigation of the Fas ligand and Bax family and determination of caspase 8 and caspase 9 by Western blot analysis can facilitate our understanding about the mechanisms of action associated with the apoptosis induced by Radix Rubiae and arsenic compounds in human keratinocytes.

3. Clinical trials evaluating the efficacy of topical preparations containing EA fraction of Radix Rubiae or arsenic trioxide.

Obviously, the development of anti-psoriatic agents for clinical treatment of psoriasis

would invariably involve clinical trials to evaluate their effectiveness for psoriasis. Particularly, randomized, placebo control clinical studies would be most desirable in evaluating an anti-psoriatic preparation. At the time of writing this thesis, a pilot clinical study evaluating the anti-psoriatic efficacy of a topical preparation containing the EA fraction of Radix Rubiae in gel form has been initiated at the Institute of Chinese Medicine, London, UK. This collaborative clinical project presents an important step in our endeavour in developing Radix Rubiae as a pharmaceutical agent for psoriasis treatment. Further clinical study to evaluate the anti-psoriatic potential of arsenic trioxide will be conducted in the near future once funding is available.

Because the actual pathogenesis of psoriasis is far from illuminated and the current conventional treatments have many limitations, development of effective and safer therapeutic topical applications still remains a worthy scientific pursuit. It is anticipated that investigation into the above mentioned future work will further advance our understanding of the underlying mechanisms concerning the anti-psoriatic effects of the EA fraction of Radix Rubiae and arsenic trioxide for psoriasis. More importantly, the future studies would contribute to the successful development of these promising substances into effective therapeutics for the treatment of psoriasis.

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