VOT 75127

BASIC IN VITRO STUDIES OF HUMAN SKIN CULTURE: *MELASTOMA MALABATHRICUM* EXTRACT ON FIBROBLASTS GROWTH

(KAJIAN DASAR KULTUR KULIT MANUSIA SECARA IN VITRO: EKTRAK MELASTOMA MALABATHRICUM KE ATAS PERTUMBUHAN FIBROBLASTS)

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RESEARCH VOTE NO:
75127

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2007

ACKNOWLEDGEMENT

The authors are grateful to Fundamental UPP grant of 75127 for financial supports. Thanks are also due to Tissue Engineering Laboratory, Hospital Universiti Kebangsaan Malaysia for their supporting in this work.

BASIC IN VITRO STUDIES OF HUMAN SKIN CULTURE: *MELASTOMA MALABATHRICUM* EXTRACT ON FIBROBLASTS GROWTH

(Keywords: Keratinocytes, fibroblasts, Melastoma malabathricum)

The objectives of this project were to isolate two types of cell, keratinocytes and fibroblasts from human skin, study the cells growth and to test the effect of *Melastoma malabathricum* extract on these two cell lines. MTT assay was used to test cell viability in the experiments which carried out in 96 wells plate. Keratinocytes and fibroblasts were successfully isolated and maintain in cultures. Keratinocytes show differentiation after 2 or 3 passages. Fibroblasts culture show better proliferation than keratinocytes culture. The study found that high seeding density (more than 20000 cells/cm2) suitable for keratinocytes and low seeding density (less than 10000 cells/cm2) suitable for fibroblasts. Extract of *Melastoma malabathricum* shown to be not effected fibroblasts attachment. Fibroblast cells are not shown any toxic effect at concentration below 10ug/ml. However at concentration ranging from 10µg/ml to 1000µg/ml, the cells relative viability decreased (93.4% to 63.9%). Keratinocytes were not used to test with the extracts since cells start differentiated only after 2 or 3 passages.

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KAJIAN DASAR KULTUR KULIT MANUSIA SECARA IN VITRO: EKTRAK MELASTOMA MALABATHRICUM KE ATAS PERTUMBUHAN FIBROBLASTS

(Keratinosit, fibroblast, Melastoma malabathricum)

Objektif projek ini ialah mengasingkan dua jenis sel, keratinosit dan fibroblas daripada kulit manusia, mengkaji pertumbuhan sel dan menguji kesan ektrak *Melastoma malabathricum* pada kedua-dua sel tersebut. Kaedah MTT digunakan untuk mengira viabiliti sel dalam eksperimen yang dijalankan pada piring 96 lubang. Keratinosit dan fibroblas berjaya diasingkan dan dikulturkan. Keratinocytes menunjukkan pembezaan selepas 2 atau 3 kali subkultur. Fibroblas menunjukkan pertumbuhan yang lebih baik daripada keratinosit. Ketumpatan pembenihan (Seeding density) tinggi (lebih daripada 20000 cells/cm2) sesuai untuk kultur keratinocytes manakala ketumpatan pembenihan rendah (kurang daripada 10000 cells/cm2) sesuai untuk kultur fibroblas. Ekstrak *Melastoma malabathricum* tidak menunjukkan kesan terhadap pelekatan fibroblas. Kepekatan ekstrak dalam lingkungan antara 10μg/ml hingga 1000μg/ml menunjukkan penurunan sel hidup relatif bagi kultur sel fibroblast (93.4% to 63.9%). Manakala ekstrak *Melastoma malabathricum* tidak dapat diuji menggunakan sel keratinosit kerana sel mula membeza selepas 2 atau 3 subkultur sahaja.

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LIST OF SYMBOLS/ABBREVIATION

c	-	Cell concentration
n	-	Number of cells counted
D	-	Dilution factor
N	-	Normality
DBSS	-	Dissection Balanced Salt Solution
DKSFM	-	Defined keratinocytes serum free media
DPBS	-	Dulbelco phosphate-buffered salines
FBS	-	Fetal Bovine Serum
HEPES	-	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
MTT	-	3- (4, 5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
OD	-	Optical density

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CHAPTER 1

INTRODUCTION

1.1 Basic Requirements

In recent year, there have been many calls for balancing society's requirement for efficacy and toxicity assessment with animal welfare. This has often taken the form of the 3Rs approach, namely *reduction* of animal numbers to the essential minimum, *refinement* of methods to minimize suffering and *replacement* of animals by alternative tests (Benford, 1992). Animal tests are not perfect. It is sometimes stated that they have not been properly validated with respects to human data. Thus human and animal data are not produced at equivalent dose levels and there are problems in extrapolating between doses. In addition there are also complications of species and strain differences, individual variation amongst the human population and interaction between different substances.

The implementation of the 7th European Amendment of the Comestic Directive (76/768/EEC) urges comestic scientists to ban animal testing and to use alternate methods in the assessment of tolerance and efficacy of products to ensure maximum consumer safety. Tissue culture cells grown in two dimensional monolayers which have been use successfully for many purposes in research and industrial production can be used as an alternative to animal testing.

In vitro skin culture have been used to study germinated seeds extract on cell metabolism in order to study the suitability of this extract as active comestic ingredient (Benaiges *et al.*, 2000); biomaterial that have potential as wound healing agents or dressing materials (Howling *et al.*, 2001); cellular process in response to the growth factors that are present in the wound (Cha *et al.*, 1996); cytotoxicity of chemical compound (Hidalgo and Dominguez, 2000); herb extract on processes involved in wound reepithelialization (Phan *et al*. 2001)

Melastoma malabathricum or more known as senduduk is a common shrub which can be found in previously cleared land and waste places. It can be found easily in Malaysia. Various parts of the plant are used for diarrhoea, anti-infection, scar prevention and post-partum recovery. The leaves can be used to stop the bleeding from a cut or wound, treat scar, pimple and black spot at skin. It is a traditional medicinal herb that are widely use at village but there are not scientific study on it. By the time of this writing, there is still not any in vitro study of Melastoma malabathricum on human dermal fibroblasts.

For these reasons, this project was establishing an in vitro human skin culture to test effect of *Melastoma malabathricum* extract on fibroblasts. It can be use for basic testing of other chemical, phytochemicals, drugs or comestic products. The establishment of standardized protocol to culture and testing the ability of *Melastoma malabathricum* extract to influence human skin fibroblasts growth were the two major research activities in this project.

1.2 Objectives

To establish human skin cell culture for basic testing of *Melastoma malabathricum* extract on fibroblasts growth

1.3 Scopes

(i) To isolate and cultivate the epidermal and dermal cells from human skin biopsy

(ii) To determine growth characteristic of human skin keratinocytes and fibroblasts

(iii) To study the effect of *Melastoma malabathricum* extract on fibroblasts growth

CHAPTER 2

LITERATURE STUDIES

2.1 Skin

2.1.1 Functions of the skin

Also called the integument which simply means “covering”, the skin is much more than an external body covering. The skin has many functions; mostly are protective. It insulates and cushions the deeper body organs and protects the entire body from mechanical damage (bumps and cuts), chemical damage (such as from acids and bases), thermal damage (heat and cold), ultraviolet radiation (in sunlight), and bacteria. The uppermost layer of the skin is full of keratin and cornified, or hardened, to prevent water loss from the body surface (Marieb, 1997)

The skin’s rich capillary network and sweat glands (both controlled by the nervous system) play an important role in regulating heat loss from the body surface. The skin acts as a mini-excretory system; urea, salts and water are lost when we sweat. The skin also manufactures several proteins important immunity and synthesizes vitamin D (modified cholesterol molecules located in the skin are converted to vitamin D). The cutaneous sensory receptors, which are actually part of the nervous system, are located in the skin. These tiny sensors, which include touch, pressure, temperature and pain receptors provides a great deal of external environment information (Marieb, 1997).

Further, skin transmits important emotional signals to the environment, such as paleness or blushing of the face and emission of scents (pheromones) (Yannas, 2000).

2.1.2 Structure of the skin

The skin is the largest organ of the body, ranging from 0.2m2 to 0.3m2 in an average newborn to 1.5m to 2.0m2 in an adult. The skin consists of two layers: the epidermis, ranging from 0.05mm thick (in areas such as the eyelids) to over 1mm thick on the soles; and the dermis, usually at least 10 times thicker than the associated epidermis. An average total skin depth is 1mm to 2mm (Lam, 1999). These two layers are separated by a basal lamina. Collagen VII is an important component of this basal lamina (Palsson and Bhatia, 2004). Deep to the dermis is the subcutaneous tissue, essentially adipose tissue. It is not considered part of the skin, but it does anchor the skin to underlying organs (Marieb, 1997).

2.1.2.1 Epidermis

The epidermis is avascular; that is, it has no blood supply of its own (Marieb, 1997). It is constituted about 95 percent of keratinocytes or dead skin cells which function as a barrier, keeping harmful substances out and preventing water and other essential substance from escaping the body. The other epidermal cells are melanocytes, which function to produce melanin for the purpose of ultraviolet protection; Langerhans cells which serve an immune function and Merkel cells which serve as mechanoreceptors. The normal epidermis also contains appendages such as hair follicles and sweat glands.

The deepest cell layer of the epidermis is called the stratum germinativum. It lies closest to the dermis and contains the only epidermal cells that receive adequate

nourishment through diffusion of nutrients from the dermis. These cells are constantly undergoing cell division (germinate = to grow), and millions of new cells are produced daily. From this layer, cells migrate outward and mature along the way to become the stratum spinosum, a layer where mitosis no longer occurs but protein synthesis is prominent. The next outward layer is the stratum granulosum where specialization into keratin production predominates. The next stage of migration is the stratum lucidum, where cells lose their nuclei and flatten, evolving into a dead layer called stratum corneum (Lam, 1999).

The stratum corneum is 20 to 30 cell layers thick. It accounts for about three-quarters of the epidermal thickness. The shingle like dead cell remnants, completely filled with keratin, are referred to as cornified or horny cell (Marieb, 1997). After serving a brief protective function, the stratum corneum is imperceptibly sloughed off. This process of a living cell's evolution is called keratinization (Marieb, 1997). Figure 2.1 shows the keratinization process of skin.

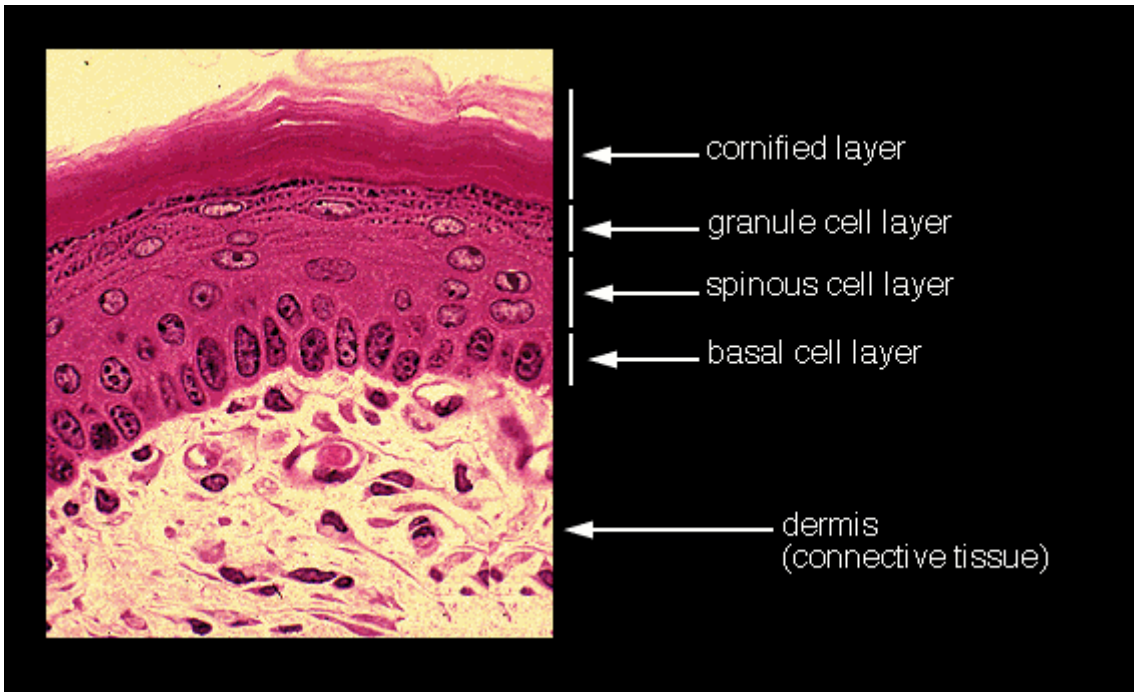

Figure 2.1: Keratinization process of skin

The net proliferative rate of skin varies with the region of the body. The turnover of skin is on the order of a few weeks, which regenerates a large amount of flakes over time. Skin is the body's third most prolific tissue (Palsson and Bhatia, 2004).

2.1.2.2 Dermis

The dermis or the "true skin" is composed of gel-like and elastic materials, water and primarily, collagen. Embedded in this layer are systems and structures common to other organs such as lymph channels, blood vessels, nerve fibers, and muscle cells, but unique to the dermis are hair follicles, sebaceous glands and sweat glands.

The fibrous connective tissue making up the dermis consists of two major regions, the papillary and the reticular areas (see Figure 2.2). The dermis varies in thickness like epidermis. The papillary layer is the upper dermal region. It is uneven and has fingerlike projections from its superior surface, called dermal papillae, which indent the epidermis above. Many dermal papillae contain capillary loops, which furnish nutrients to the epidermis (Marieb, 1997).

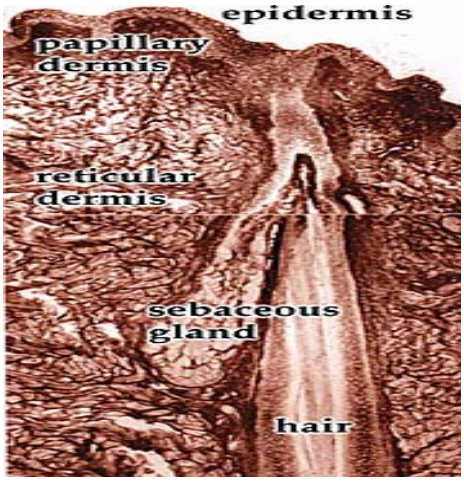

Figure 2.2: Two major regions of the dermis layer: the papillary and the reticular areas

The primary cell type in the dermis is the fibroblast, a spindle-shaped cell which does not frequently replicate but very active in producing extracellular protein, primarily collagen, specially type I collagen and elastin. Collagen is secreted into cellular matrix, where it undergoes maturation (cross-linking and coiling) into strong fibres oriented so as to allow stretch ability while providing tensile strength (Lam, 1999). Collagen fibres also attract and bind water and thus help to keep the skin hydrated. Elastin processed to form elastic fibrin, give the skin its elasticity (Marieb, 1997).

The ground substance of the skin is a non-fibrous bulk of glycosaminoglycans (GAG), proteoglycans and similar macromolecules. Hyaluronic acid is an example of a well-known and important GAG. The GAG, collagen fibrils, fibronectin join in architecture that is not amorphous and is thought to contribute to the vis-coelasitc. The ground substance provides a semi fluid matrix which lubricates the cellular and fibrillar components. It is through this ground substance that inflammatory and other cells may migrate and nutrient diffuse (Lam, 1999).

2.2 Tissue culture

2.2.1 Introduction

Tissue culture is often a generic term that refers to both organ culture and cell culture and the terms are often used interchangeably. The term organ culture will always imply a three-dimensional culture of undisaggregated tissue retaining some or all the histological features of the tissue in vivo. Cells culture refers to a culture derived from dispersed cells taken from original tissue, from a primary culture or from a cell line or cell strain by enzymatic, mechanical or chemical disaggregation (Freshney, 2000). Primary cell cultures typically will have a finite life span in culture whereas continuous cell lines are, by definition, abnormal and are often transformed cell lines

2.2.2 Advantages of tissue culture

The two major advantages of tissue culture are control of the physiochemical environment (pH, temperature, osmotic pressure, and O_2 or CO_2 tension) and the physiological conditions, giving greater reproducibility of results and facilitating investigations of mechanisms of actions (Benford, 1992).

Replicate samples in tissue culture are identical to each other and the characteristic of the line may be perpetuated over several generations. The need for statistical analysis of variance is reduced (Freshney, 2000).

In addition to the ethical reasons for using tissue cultures as alternatives to laboratory animals, tissue culture is generally less expensive to perform and produce results more quickly. The use of human cell, particularly primary cultures, can greatly assist extrapolation of animal data to man (Benford, 1992).

2.2.3 Limitations of tissue culture

Tissue culture must be carried out under strict aseptic conditions because it is easily contaminated. These imply a level of skill and understanding on the part of the operator in order to appreciate the requirement of the system and to diagnose problem as they arise (Freshney, 2000).

A major limitation of tissue culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories might be 1 to10g of cells; above 100g implies industrial pilot plant scale a level that is beyond the reach of most laboratories. But, it is possible to generate kilogram quantities if special facilities are provided.

Some dedifferentiated in cells will occur, which is commonly progressive with time in culture. If dedifferentiated properties are lost, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived (Freshney, 2000). Thus freshly derived primary cell cultures will respond in a manner most closely resembling that occurring in the same cells *in vivo* (Benford, 1992).

2.3 Skin Culture

2.3.1 Application of Human Skin Culture

Various models of human skin have been developed until now, primary for clinical objectives (burn wound, ulcer, skin lesion and etc) as a replacement for skin grafting.

Subsequently, reconstructed skin models also use as test skin for determine the potential effects and toxicity of chemicals and drugs on the skin. Test protocols have been developed for use with theses models that enable materials to be evaluated for their safety (e.g. assessment of toxicity, corrosivity, skin irritancy, and carcinogenicity (Seymour *et al.*, 1987)).

The reconstructed human skin permits studies on the skin physiology, physiopathology and pharmacology at the tissue and organ levels in vitro (Coulomb *et al.*, 1986).

A few research area of tissue engineering involves the investigation of how living cells interact and respond to synthetic biomaterial surfaces.

Now, reconstructed skin frequently used as an alternative to animal testing in assessment of safety and efficacy of comestic formulations and bioactive ingredients. Reconstructed skin supported by collagen-glycosaminoglycan-chitosan biopolymer in which human keratinocytes and dermal fibroblasts were co-cultured to form skin tissue is successfully used for demonstrate the effect of formulations containing active ingredients on the stimulation of the synthesis skin protein such as filaggrin and collagen, which play a role in the ageing process of the skin. The histological structure is preserved for at least up to 28 days (Schlotmann *et al.*, 2001).

By contrast with simple monolayer keratinocytes culture, the skin is cultivated at the air-liquid interface which allows the differentiation of the keratinocytes and the creation of a stratum corneum which exhibits a partial barrier function. In addition, the air-liquid interface condition enables the topical applications of either non-aqueous compounds or comestics as found in real life conditions. EPISKIN®, a standardized reconstructed epidermis kit used to assess in vitro the tolerance and the efficacy of comestics is reported (Roguet *et al.*, 2000).

2.3.2 Commercially Skin Substitutes

There are now several commercially available products that fall into two categories: dermal skin substitutes and combined epidermal and dermal skin substitutes. Dermal skin substitutes include AlloDerm® (LifeCell, Inc., Branchburg, NJ) a dermal matrix lacking immunogenic cells, and Integra® (Integra LifeSciences Holding Corp., Plainsboro, NJ), a combination of dermal fibroblasts and bovine collagen. Dermagraft® (Applied Tissue Sciences, La Jolla, CA) consists of non-immunogenic neonatal fibroblasts cultured on a polyglactin mesh and has been used to treat burns and diabetic foot ulcers (Rodriguez et al., 2003).

Combined epidermal and dermal skin substitutes include Apligraf® (Organogenesis, Inc, Canton, MA), the first mass-produced skin product comprised of fibroblasts and keratinocytes (derived from human neonatal foreskin) in a type I bovine collagen extracellular matrix. This product has been approved by the Food and Drug Administration (FDA) for treatment of partial thickness and full thickness skin loss due to venous stasis ulcers. Apligraf is a bilayered living skin analog with appearance and handling characteristics that are similar to normal human skin (Rodriguez *et al.*, 2003).

TestSkin II® (Organogenesis, Inc, Canton, MA) is equivalent to Apligraf and is sold for the purpose of *in vitro* research (Rodriguez *et al.*, 2003).

At present, epidermis and epithelial models are commercially available from MatTek Corporation, Ashland, MA, United States (Epiderm epidermis model), Saduc (Episkin epidermis model) and SkinEthic, Nice, France (SkinEthic epidermis model) (Schlotmann *et al.*, 2001; Freshney, 2000).

2.4 *Melastoma malabathricum*

Melastoma malabathricum which belong to the Family Melastomataceae have different local name (Kemunting (Iban), Senduduk, Seduduk, Sekeduduk, Sikadudok, Kenduduk, Keduduk (Malay); Harendong, Kluruk, Senggani (Javanese); Singapore rhododendron (English)).

2.4.1 Habitat and ecology

The plant can be found in Madagascar, India to Australia. But it is very common in Southeast Asia. It is a common shrub which can be found in previously cleared land, waste places and roadside. This showy bush rapidly colonises wastelands as their seeds are dispersed by birds. Average height of *Melastoma malabathricum* is 1m but it may grow up to 3m tall.

2.4.2 Botanical characteristics

The stems are generally bristly, covered with small rough scales and reddish. The leaves are simple, narrow with 3 prominent longitudinal veins, tapered and bristly underside. The flowers have five petals, dark purple to pinkish, on rare occasions, white. The fruits have oval shape with purple pulp and contain many tiny seeds.

2.4.3 General usage

Various parts of the plant can be used. The young leaves are eaten raw or cooked and taste sour. The pulp around the seeds can also be eaten (Indonesia). The seeds are

used to produce a black dye and the roots, a pink dye. In some places, the leaves are fed to silkworms.

2.4.4 Traditional medicinal usage

There are a lot of uses for this plant reported in folk medicine, but not supported by clinical data. Leaves can be used to stop the bleeding from a cut or wound, through the application of chewed up leaves onto the wound or by finely chopping the leaves up and squeezing the juice onto the wound or through the application of the paste produced by pounding the leaves (Ahmad *et al.*, 1993) The leaves can also be used to treat piles, dysentery, diarrhoea, prevent scarring from smallpox and as a tonic.

The liquid of the shoots can also relieve a toothache used as a mouthwash or to treat leucorrhea. In other cases, the shoots can be ingested against puerperal infections (Burkill, 1966). Eat the young shoot as ulam can treat high blood pressure and diabetes.

Mouthwash to relieve a toothache can also be compounded from roots.(Burkill, 1966; Jaganath *et al.*, 2000) In children, this liquid can be applied to lessen the soreness due to thrush.(Burkill, 1966) The root can be given to post-partum women to aid healing and womb strengthening(Anonymous, 2002; Jaganath *et al.*, 2000; Ahmad *et al.*, 1993) or to alleviate rheumatism, arthritis and tenderness in the legs (Burkill, 1966).

Powdered roots and leaves can be applied to wounds and pox scars to aid the healing process (Burkill, 1966; Anonymous, 2002) or to relieve the discomfort of haemorrhoids (Anonymous, 2002). Flowers can be used to treat stomachache.

2.4.5 Pharmacology

Tannins and flavanoids are two active constituents found at the plant. Extracts of the plant were reported to have anti-aggregation properties (Jaganath *et al.*, 2000). However, there is no clinical or scientific data to substantiate this claim and it is not known whether this activity applies to animals or humans. No other pharmacological activities were reported on *Melastoma malabathricum* in animals or humans. However, current reports have indicated antiviral (HSV-1, Poliovirus) and cytotoxic (against murine cell lines) activities for the methanolic extract (Lohézic-Le Dévéhat *et al.*, 2002).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Media and chemicals

Penicillin-streptomycin, Fungizone™ amphotericin B, gentamycin, dispase, collagenase (type 1), trypsin-EDTA, fetal bovine serum (FBS), Dulbelco phosphate-buffered salines (DPBS), HEPES, glucose, Glutamax, Defined KSFM (DKSFM) and DMEM/F12 were purchased from Gibco BRL. All others chemicals were obtained from Sigma-Aldrich unless otherwise stated. Cell culture grade chemicals and analytical grade were used in this study depending on their appropriate application. All the solutions were prepared using deionised double distilled water (Millipore).

3.1.2 Tissue source

Human eyelid was obtained from healthy donors undergoing plastic surgery where skin was to be discarded. The skin was placed into a sterile container with Dissection Balanced Salt Solution (DBSS) (DPBS, penicillin 100U/ml, streptomycin 100μg/ml, Fungizone™ amphotericin B 2.5μg/ml and gentamycin 50μg/ml) and

delivered immediately to the tissue culture laboratory or kept at 4°C for a short period of time.

3.1.3 Plant Material

The fresh leaves of *Melastoma Malabathricum* were collected from Universtiy Technology of Malaysia, Skudai, Johor.

3.2 Cell culture procedure

3.2.1 Isolation of human keratinocytes and fibroblasts

Skin samples were rinsed two to five times in DBSS until the washing solution became clear. Dispase 2.4U/ml in DKSFM was added and skin was incubated overnight at 4°C to facilitate separation of the epidermis from the dermis.

The following day, the epidermis was peeled off with two fine curved forceps, washed in DBSS and placed in the 0.25% trypsin/0.02% EDTA for 10 min at 37°C with periodic gentle agitation. Trypsin action was quenched by DBSS with 10% FBS. Epidermal cell suspension was transferred into tubes and centrifuged at 100g for 10min. Then, keratinocytes were seeded in DKSFM at 2×10^4 cells/cm2 and incubated at 37°C in a humidified 5%CO2/95% air atmosphere.

In parallel, dermal tissue from the same skin sample was washed in DBSS, minced, and placed into 0.3% collagenase (type 1) for three to four hours at 37°C until it fully digested. Cells were spun at 110g for 10min, washed with DBSS, and cultured in

DMEM/F12 supplemented by 10% FBS. Fibroblasts were seeded at 1×10^4 cells/cm2 and incubated at 37°C in 5%CO2/95% air atmosphere.

3.2.2 Trypan blue exclusion test

Trypan blue which was excluded by live cells but accumulates in dead cells was used to determined cell viability using a haemocytometer. A clear coverslip was placed on a haemocytometer slide. 50µl of cell suspension was mixed with 50µl trypan blue. The cell suspension mixture was carefully transferred to the edge of the coverslip. After 1-2 minutes, the numbers of stained (dead) cells and unstained (live) cells were counted under a light microscope. Concentration of cell was derived using the formula

$$c = n \times D \times 10^4 / 9$$

where c is the cell concentration (cells/ml), n is the number of cells counted and D is the dilution factor.

3.2.3 Routine maintenance

Cells were monitored daily for morphology, microbial contamination, and the color of the medium. Culture was feed every 2 to 3 days, and subcultured when necessary. A minimum of two 25cm2 flasks were carried for each cell line and expanded as necessary for the experiments. Several vials were frozen away and stored in liquid nitrogen when have enough cells.

3.2.4 Subculture procedure

The keratinocytes were subcultured once the monolayer culture reached 60% to 75% confluency. For fibroblasts culture, 90% to 95% confluent cells were subcultured. The medium of the cells was aspirated. The cells in the flasks were rinsed with DBBS (0.2ml/cm2) to remove traces of serum which would inhibit the action of trypsin. The wash solution was removed and 0.25% trypsin/0.02%EDTA (0.1ml/cm2) was added. The flask was incubated for 5 minutes at 37°C. When the cells began to round and detach under the microscope, equal volume of DBBS/10%FBS was added to neutralize the trypsin. The cells were washed with DBBS and cell viability was checked with trypan blue exclusion test. Each keratinocytes and fibroblasts seeding concentration was prepared by diluting the cells with the culture medium to the volume required. Cells were then incubated at 37°C in 5%CO2/95% air atmosphere.

3.2.5 Cell cryopreservation

Healthy cells at log phase were used for freezing. Cells were trysinized and centrifuged. The freezing medium containing 90% FBS and 10% DMSO was slowly added to the cell suspension at approximately 10^6-10^7 cells/ml. Cells were aliquoted into 1.2ml prelabeled cryogenic vials and were brought down to freezing temperature using Nalge Nunc freezing container. After approximately 24 hours at -70°C, the vials were transferred to liquid nitrogen storage.

3.2.6 Cell Recovery

Cryogenic vial containing cells of a given passage was removed from liquid nitrogen storage. Cells were thawed by gently agitating vial in a 37ºC water bath for about 1minute or until completely thawed. Thawed cells were immediately transferred, drop by drop slowly added to medium preheated to 37ºC, centrifuged and the supernatant discarded. The cell pellet was then resuspend in medium and viable cell

count was performed. Cells were allowed to attach in T-flasks at 37ºC in a humidified incubator.

3.3 Plant extraction

Fresh leaves of *Melastoma Malabathricum* were collected, washed and blended by adding deionised water to obtain the juice. The juice was centrifuged at 1000 rpm and then filtered through Whatman filter paper. The clear solution obtained was freeze dried to yield a dried extract. The dried extract was dissolved in DMSO to form stock solution 2g/10ml which were sterilized by filtration through 0.2μm pore membrane and kept in the dark at 4°C. Various dilutions of the stock extract in DMSO were prepared on the day of the experiment.

3.4 Biochemical analysis

3.4.1 MTT assay

Supernatants of the cells were discarded. To minimize the interference of residue of the supernatant, the adherent cells were washed two times with DPBS. Then 100 μl of MTT working solution (0.5 mg/ml MTT in DPBS) was added to each well and the plates were further incubated for 4 hour at 37°C. MTT working solution was removed with care and 100 μL of MTT solvent (0.1N HCl-Isopropanol solution) was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbency was measured at wavelength of 570 nm and reference wavelength 630 nm with a microplate reader within 1 hour after adding MTT solvent.

3.5 Detailed experimented procedures

The work was conducted in three scopes such as isolation and maintenance of cells, growth characteristic of cells and effect of *Melastoma Malabathricum* extract on cells growth. The designed of the overall experimental procedures are described in Figure 3.1.

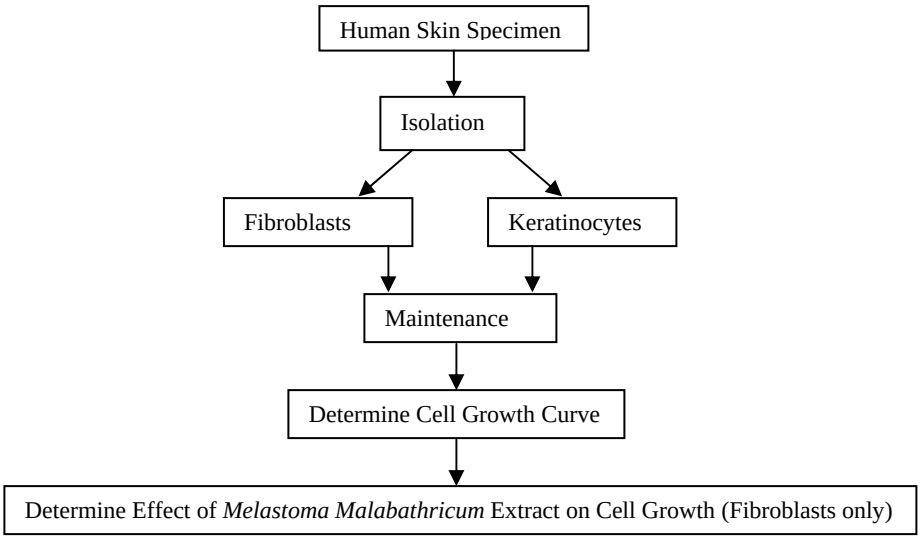

Figure 3.1 The designed of overall experimental procedures.

3.5.1 Measurement of cells growth

A series of cultures at eight different cell concentrations were set for keratinocytes and fibroblasts with 200µl of the appropriate medium per well. Ten 96 well plates were seeded for 10 days cells growth study for each cell line. Cultures were incubated at 37°C in 5%CO2/95% air atmosphere. One 96 well plate was taken for each cell line at daily intervals to determine number of viable cells using MTT assay.

3.5.2 Measurement of cells growth with *Melastoma Malabathricum* extract

Fibroblasts were seeded at 96 well plates with seeding density 10000 cells/cm2 and 200µl medium per well. After 2 days, cells were treated with various concentration of *Melastoma Malabathricum* extract. Experiment was allowed to proceed further for 24 hours, 48 hours and 72 hours at 37°C in humidified 5% CO2 atmosphere. At the end of these periods, number of viable cells was determined by MTT assay.

3.6 Statistics

Each experiment was performed at least three times. Results are expressed as mean ± standard error. Data were analyzed by using SPSS 13 for Windows. Differences at the 95% level were considered to be significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Cells

Once a primary culture is subcultured, it becomes known as cell line. This term implies the presence of several cell lineages of either similar or distinct phenotypes. If a cell line transform in vitro, it give rise to a continuous cell line (Freshney, 2000). There were two finite cell line, keratinocytes and fibroblasts being successfully isolated and maintained from human skin sample collected. Since both cell lines derived from normal skin tissue, they have limited culture lifespans. They can divide for a limited number of times and die out after a fixed number of population doublings in a genetically determined event, known as senescence.

4.1.1 Keratinocytes

The epidermis consists of multiple cell layers, with a single basal stratum of proliferating keratinocytes that differentiate as they move toward the skin surface. As differentiation progresses, cell division is reduced and eventually lost. This process gives rise to mature, differentiated cell that normally will not divide. This makes the keratinocytes hard to culture. To avoid the differentiation, serum free media was use instead of FBS to culture keratinocytes. Unfortunately, even by using serum free media, we still unable to maintain keratinocytes in undifferentiated state. After passage 2 or passage 3, the cells start to differentiate. Since the cells were being cryo before with

10% DMSO in FBS as freezing medium, both DMSO and FBS might have induces the keratinocytes differentiation.

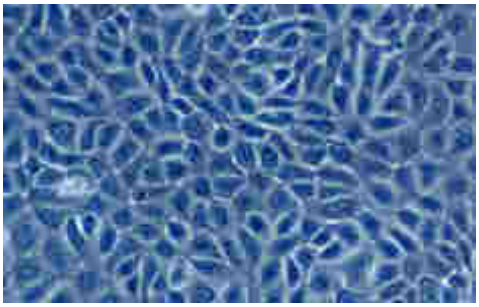

Figure 4.1: Human epidermal keratinocytes (picture taken from internet)

4.1.2 Fibroblasts

Human dermal fibroblasts are robust cells and relative ease to be cultured. Fibroblasts isolated from skin sample initially grow very rapidly, forming vigorous cultures that can be subcultured once or twice a week once they reach confluence. These cultures do not grow indefinitely. After about 60 population doublings the appearance of the cells begin to change and the growth rate of the culture diminishes (Hayflick and Moorhead, 1961)

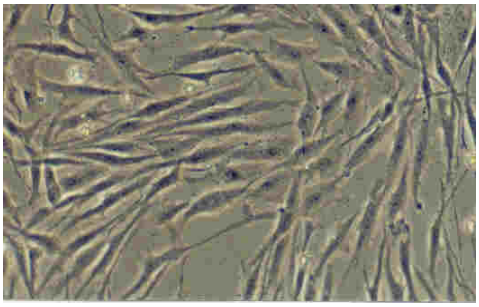

Figure 4.2: Human dermal fibroblasts (picture taken from internet)

4.2 Cells Growth

4.2.1 Effect of Human Variability

4.2.1.1 Keratinocytes

Since keratinocytes were not successfully grow, standard curve for this cells were not done. Specific growth rate and doubling were not calculated as well. From Figure 4.3, it can be observed that keratinocytes were grow very slowly and most of the. cells start to differentiate even at the beginning of the experiment when seen under microscope The growth curve was obtained by taking average OD of 6 human skin samples. From Figure A.1, we found that variation between human skin samples were very large. Our human skin samples taken mostly from eyelid, and other part of body skin and ages were varied from 20 to 60. This might be another reason for the variation to occur beside human variety.

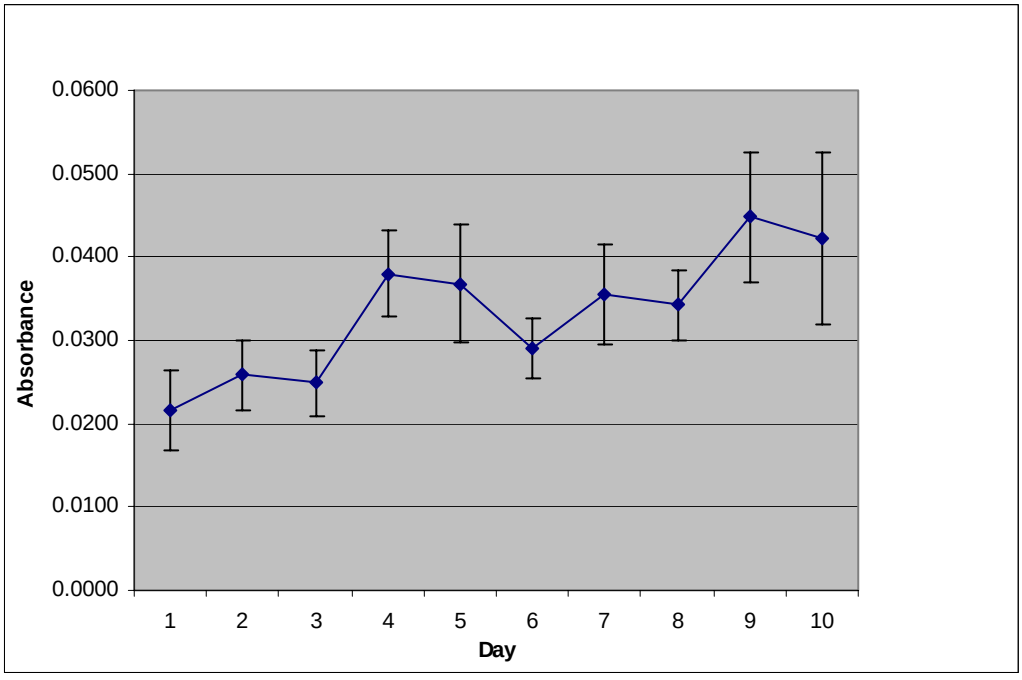

Figure 4.3: Keratinocytes growth curve using 6 human skin samples at seeding density 20000cells/cm2. n=3 replicates per sample

4.2.1.2 Fibroblasts

Cell viability as measured by MTT reduction is now widely chosen as the optimal end point. MTT is a yellow water-soluble tetrazolium dye that is reduced by live, but not dead, cells to a purple formazan product that is insoluble in aqueous solution. Figure 4.4 show good correlation between quantity of cell (tryphan blue exclusion test) and absorbance (MTT assay) of fibroblasts. The MTT response clearly reflect cell number (R^2=0.989).

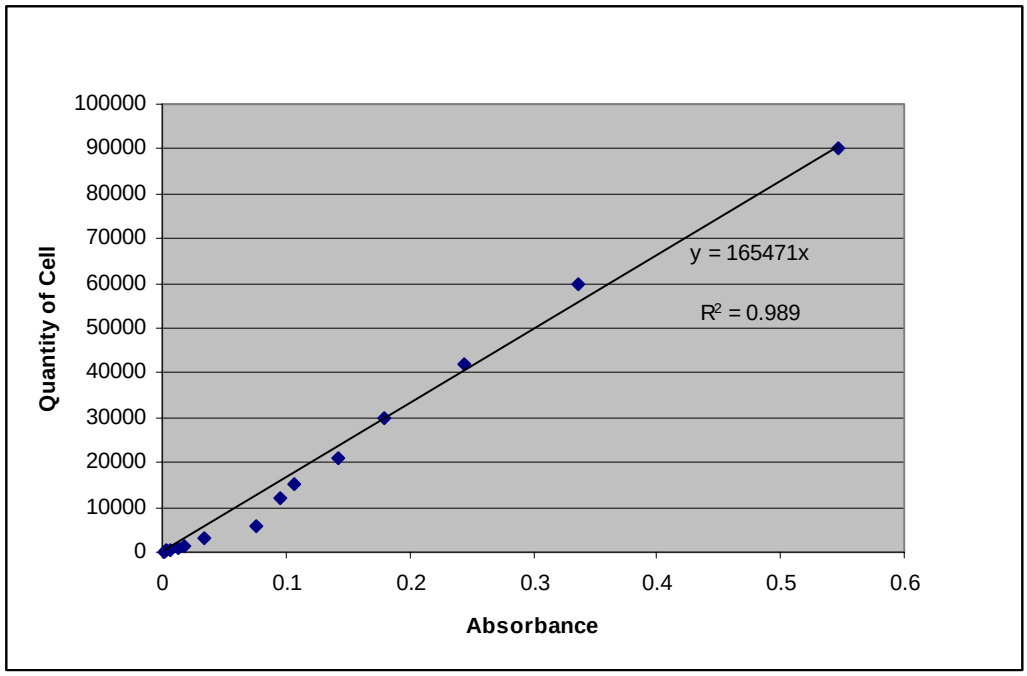

Figure 4.4: Fibroblasts standard curve using 1 human skin sample. n=2 replicates per sample

A typical growth curve showing lag phase, log phase and stationary phase can be seen from Figure 4.5 for fibroblasts growth curve. From Figure B.1, we can found that 6 samples show the almost same pattern of growth and the standard error for fibroblasts absorbance is small compare to keratinocytes. Human variation effect was not severe at

fibroblasts growth curve. Averagely, fibroblasts have 2.5 days lag period, obtained by extrapolating a line drawn through the points for exponential phase until it intersects the seeding concentration and then reading off the elapsed time since seeding equivalent to that intercept. The second parameter is the population doubling time (PDT), 2.2 days, time taken for fibroblasts to increase twofold in the middle of the log phase of growth. Specific growth rate, 0.315 h^{-1} for fibroblasts was obtained by dividing ln2 with PDT.

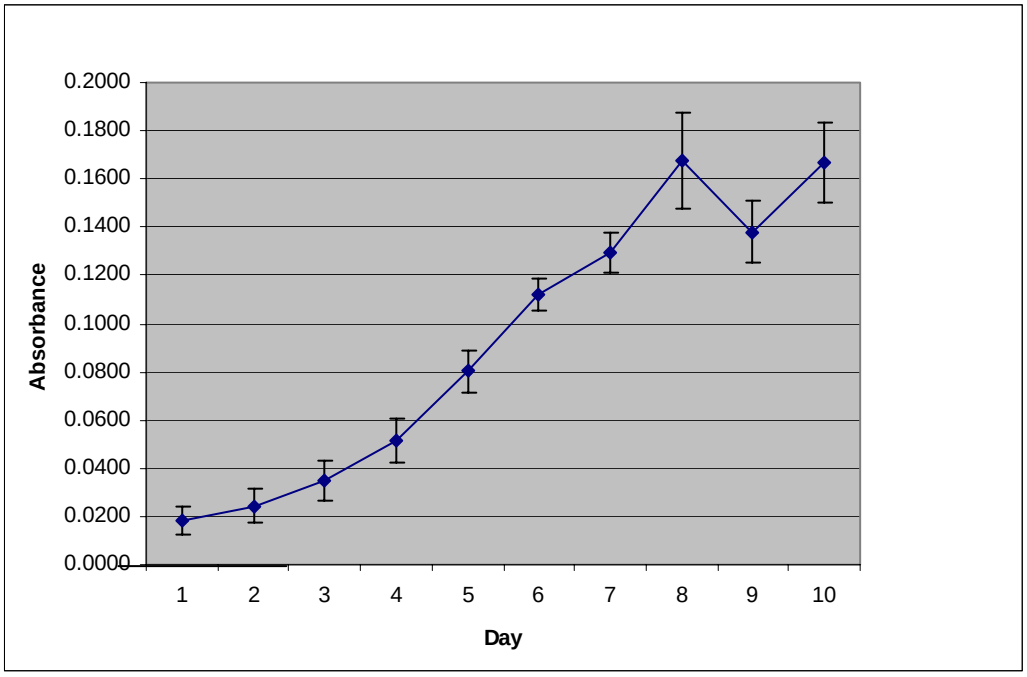

Figure 4.5: Fibroblasts growth curve using 6 human skin samples at seeding density 10000cells/cm2. n=3 replicates per sample

4.2.2 Effect of Seeding Density

4.2.2.1 Keratinocytes

Keratinoctyes with various seeding density ranging from 2500 cells/cm2 to 30000 cells/cm2 were test for their growth to obtain the optimum seeding density. From the Figure 4.6, we found that at lower seeding density, 2500 cells/cm2 and 5000

cells/cm2, the keratinocytes were not able to proliferate. The seeding density with 7500 cells/cm2 show better growth with a clear log phase starting at day 4. Log phase was started at day 4 for seeding at 25000 cells/cm2 and 30000 cells/cm2 as well. It seem like keratinocytes growth better when seed at higher seeding density than 20000 cells/cm2 since seeding at 10000 cells/cm2 and 15000 cells/cm2 did not proliferate well too.

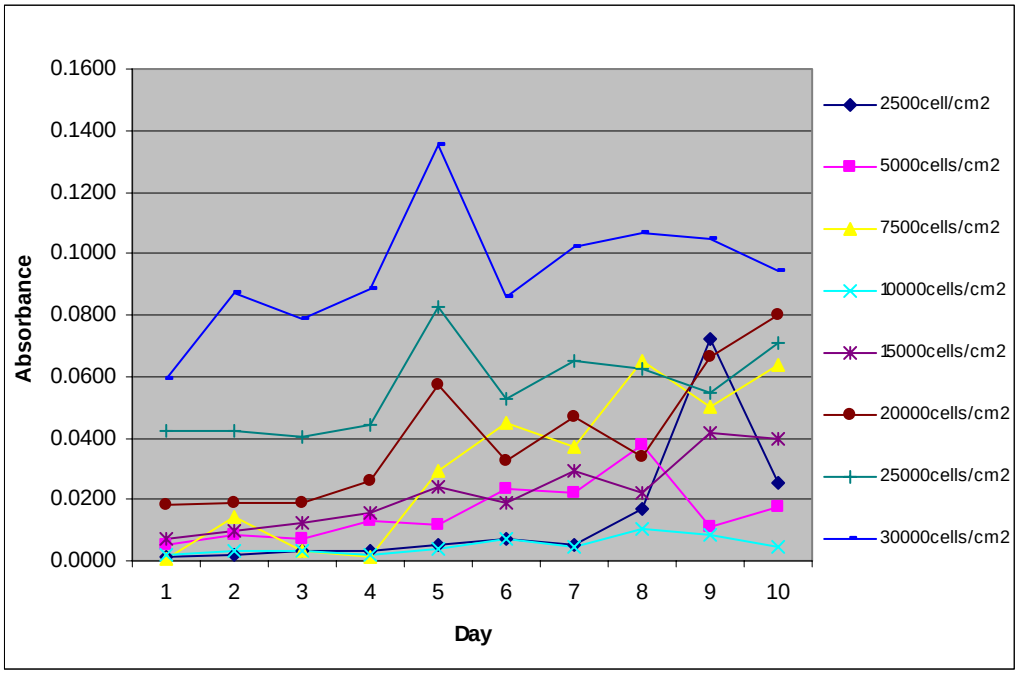

Figure 4.6: Keratinocytes growth curve with different seeding density ranging from 2500 cells/cm2 to 30000 cells/cm2. N=1 human skin samples, n=3 replicates per sample

4.2.2.2 Fibroblasts

Fibroblasts shown the almost same growth pattern, 2 days lag period and achieve saturation density at day 8 even with different seeding density. What made them different is their growth rate at log phase. From the Figure 4.7, we can seem that the growth rate for higher seeding density was slower than those seed at lower seeding density but the lag phase was shorter.

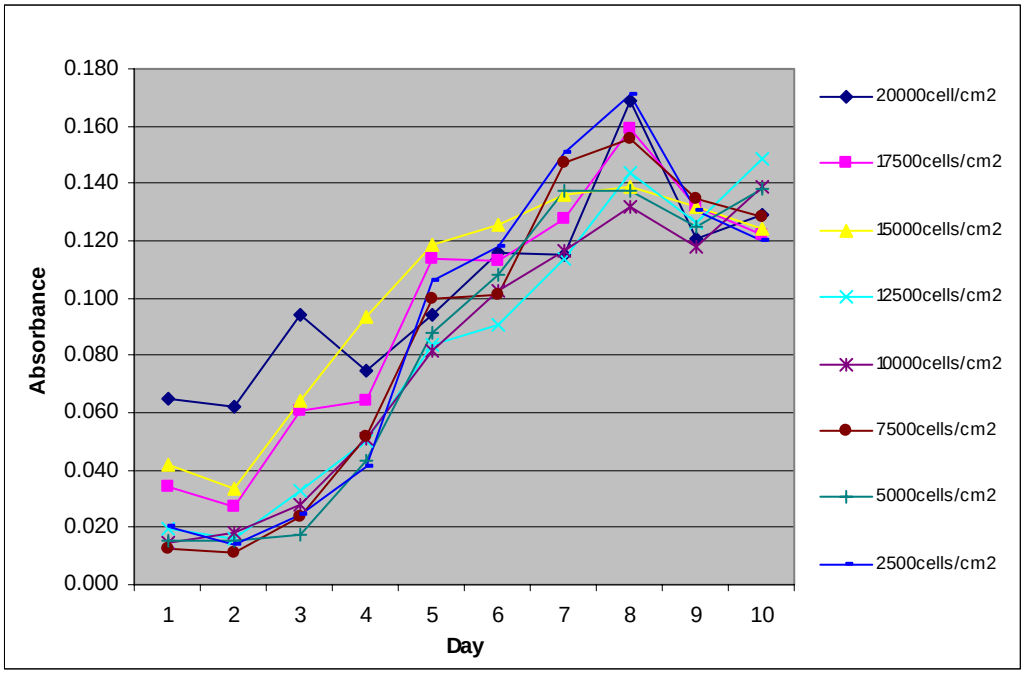

Figure 4.7: Fibroblasts growth curve with different seeding density ranging from 2500 cells/cm2 to 20000 cells/cm2. N=3 human skin samples, n=3 replicates per sample

4.3 Effect of *Melastoma malabatricum* on Fibroblasts Growth

4.3.1 Effect of Day Add In Extract

Various concentration ranging from 1000µg/ml to 0.000001µg/ml with ten time dilution were added into fibroblasts seeding at 10000cells/cm2. Extract was added in at different day. For one set of experiment, extract was added at the time of seeding cells. Another set of experiment, extract was added in two days after the cells were seeding. For the first set, the cells were not attached yet at the time of extract treatment. The cells were attached well when extract was added in after two days cell seeding. From Table D.1, data analysis shown significance effect of concentration ($p<0.05$) but effect of day added in extract was not significance. So extract *Melastoma malabathricum* did not have any effect on fibroblasts attachment. Post Hoc Tests was run and shown significance relative viability of fibroblasts effect with extract concentration 1000µg/ml compare to

control ($p<0.05$), and the rest shown not effect. Doses 1000µg/ml was toxic to fibroblasts and doses of the rest were not harmful to cell. Figure 4.8 shown various doses of extract add to fibroblasts at different day after seeding.

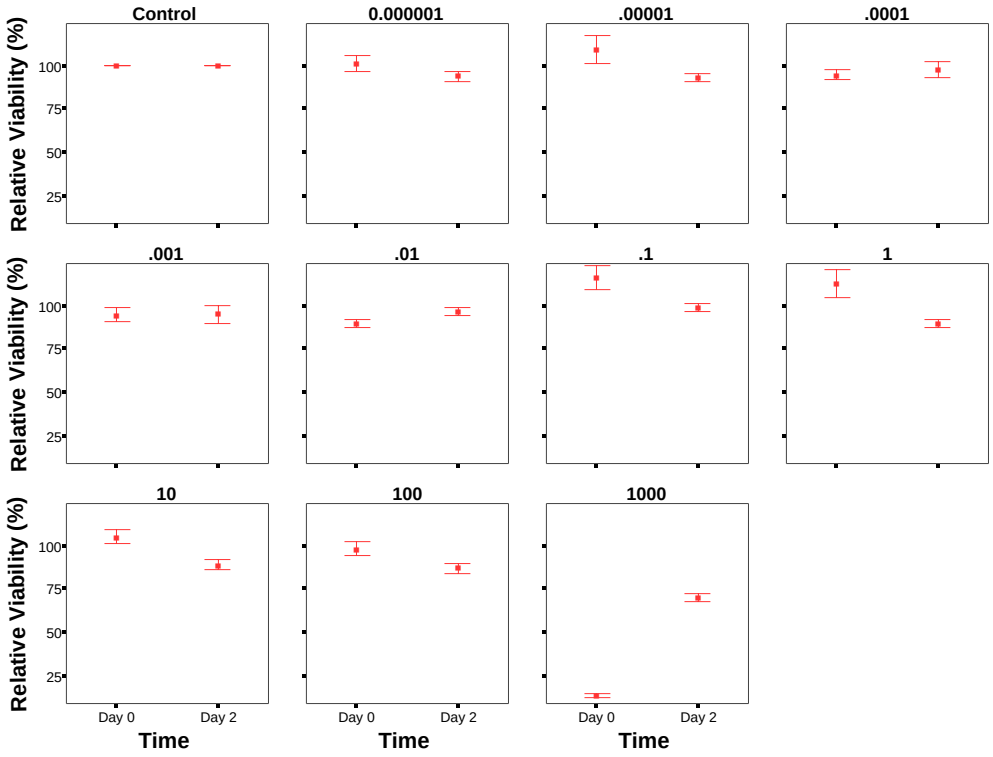

Figure 4.8: Fibroblasts relative viability when adding different concentration of *Melastoma malabatricum* extract at different day after seeding. N= 1 human skin sample, n= 8 replicates per sample, OD reading was taken 1 day after seeding cell together with *Melastoma malabatricum* extract

4.3.2 Effect of Extract Concentration

Further experiment was conducted to test the effect of extract concentration and day of treatment. After two day seeding, various doses of extract were added to

fibroblasts. MTT assay was carried out after 24 h, 48 h and 72 h each day. Data analysis from Table D.2 and Table D.3 shown significance effect for treatment day and extract concentration ($p<0.05$). Post Hoc tests shown significance effect of extract concentration 10µg/ml, 100µg/ml and 1000µg/ml compare to control. Figure 4.9 shown relative viability of fibroblasts treat with various doses of extract at different treatment day.

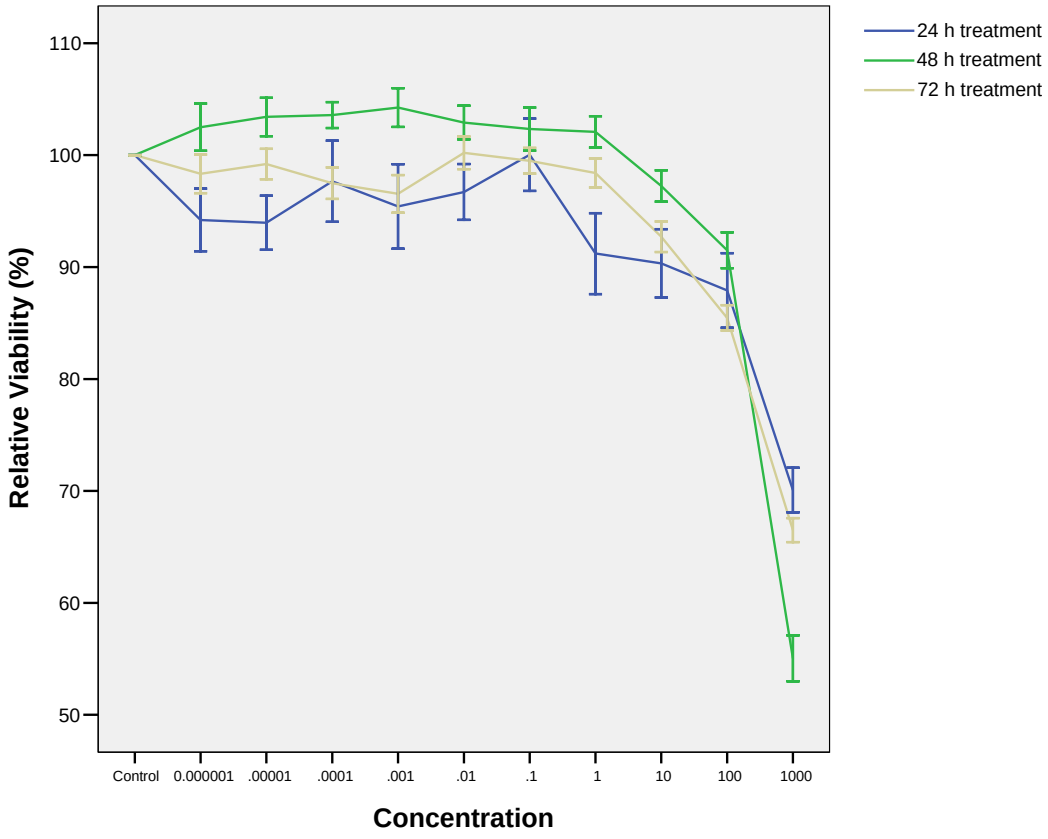

Figure 4.9: Fibroblasts relative viability when adding different concentration of *Melastoma malabatricum* extract ranging from 0.000001µg/ml to 1000µg/ml at 24 h, 48 h and 72 h treatment

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Keratinocytes and fibroblasts were able to isolate and maintain in culture. Keratinocytes show differentiation after passage 2 or passage 3, and fibroblasts at passage 7 still show good proliferation. Human variation was noticed at keratinocytes but not fibroblasts culture. Seeding density 20000 cells/cm2 is suitable for keratinocytes and 10000 cells/cm2 for fibroblasts.

Melastoma malabathricum extract show no effect on fibroblasts attachment at concentration range tested. Extract with concentration 10μg/ml, 100μg/ml and 1000μg/ml show slightly toxicity to fibroblasts.

5.2 Recommendations

Since all the experiment were conducted in 96 well plate, the cells growth might be different when grow in different culture environment. So further experiment can be conduct at different culture environment to determine the cell growth behaviour. The better freezing way to freeze keratinocytes should be find so that the cell can be preserved for further use.

Melastoma malabathricum healing effect on skin scar and wound claimed by traditional remedies need further experiment to proof it. Different extract preparation can be try to test keratinocytes proliferation; keratinocytes and fibroblasts migration; and protein synthesis.

APPENDIX A
Keratinocytes growth curve

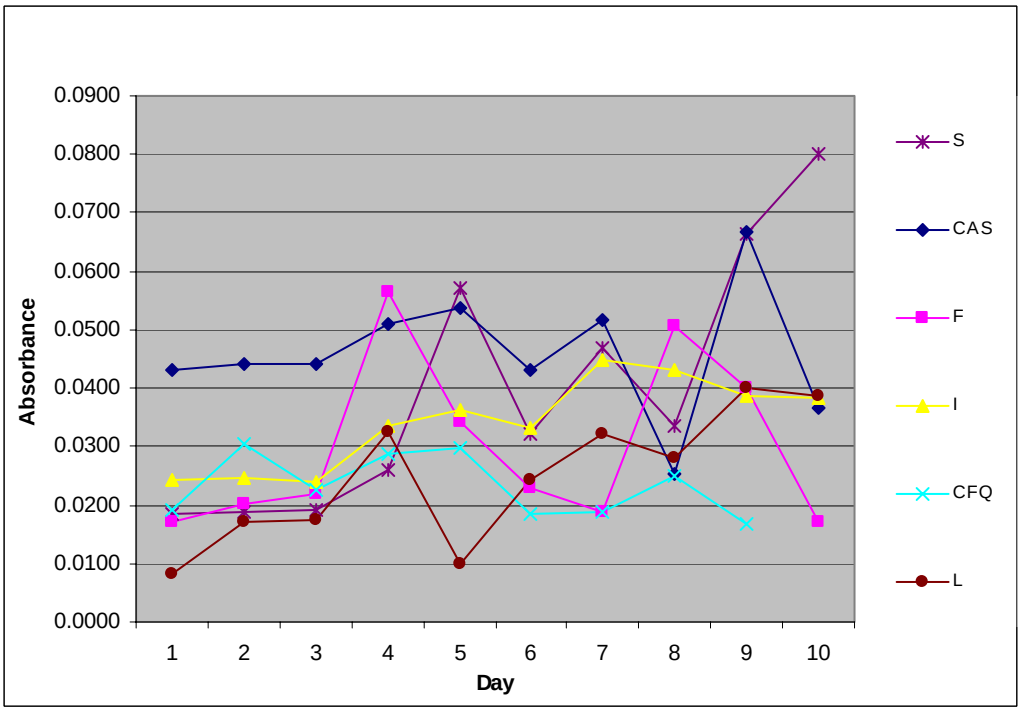

Figure A.1: Keratinocytes growth curve using 6 human skin samples at seeding density 20000cells/cm2. n=3 wells per sample

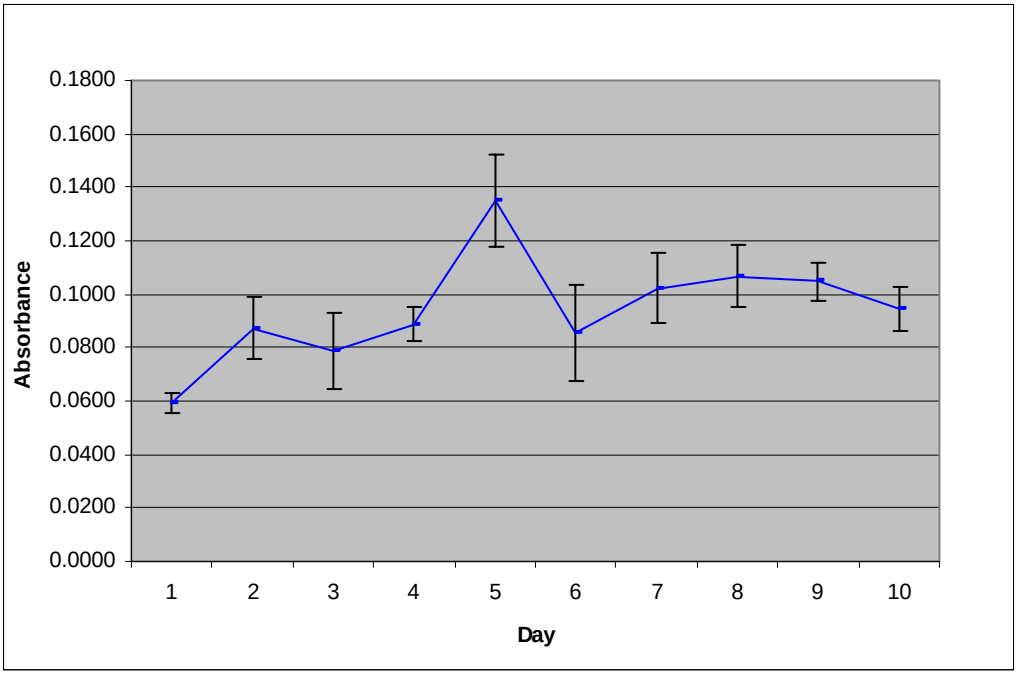

Figure A.2: Keratinocytes growth curve with seeding density 30000 cells/cm2

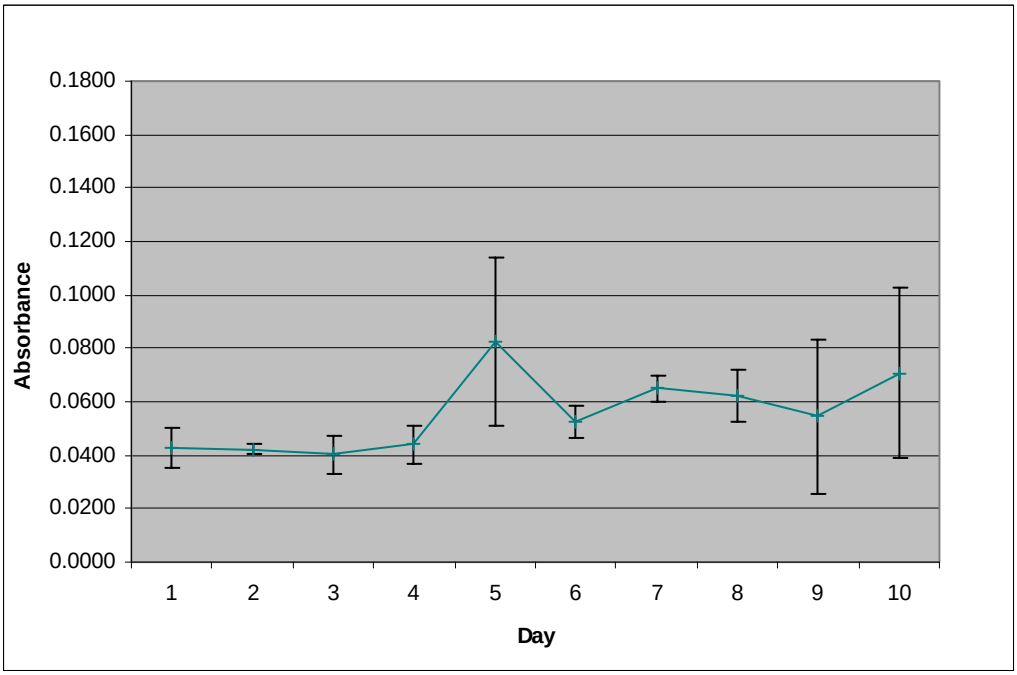

Figure A.3: Keratinocytes growth curve with seeding density 25000 cells/cm2

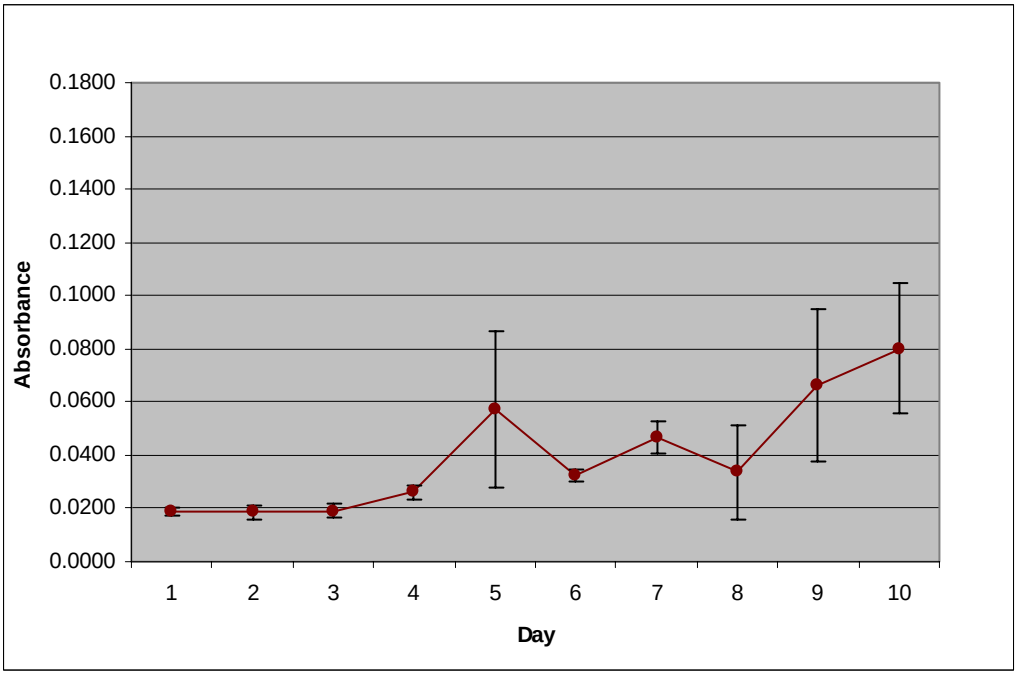

Figure A.4: Keratinocytes growth curve with seeding density 20000 cells/cm2

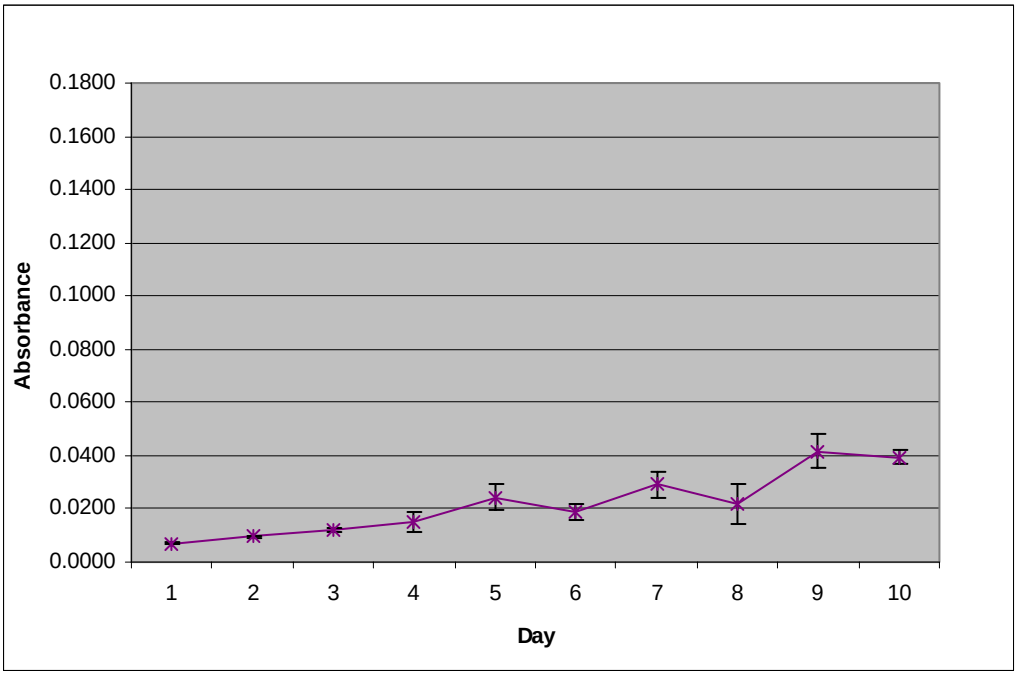

Figure A.5: Keratinocytes growth curve with seeding density 15000 cells/cm2

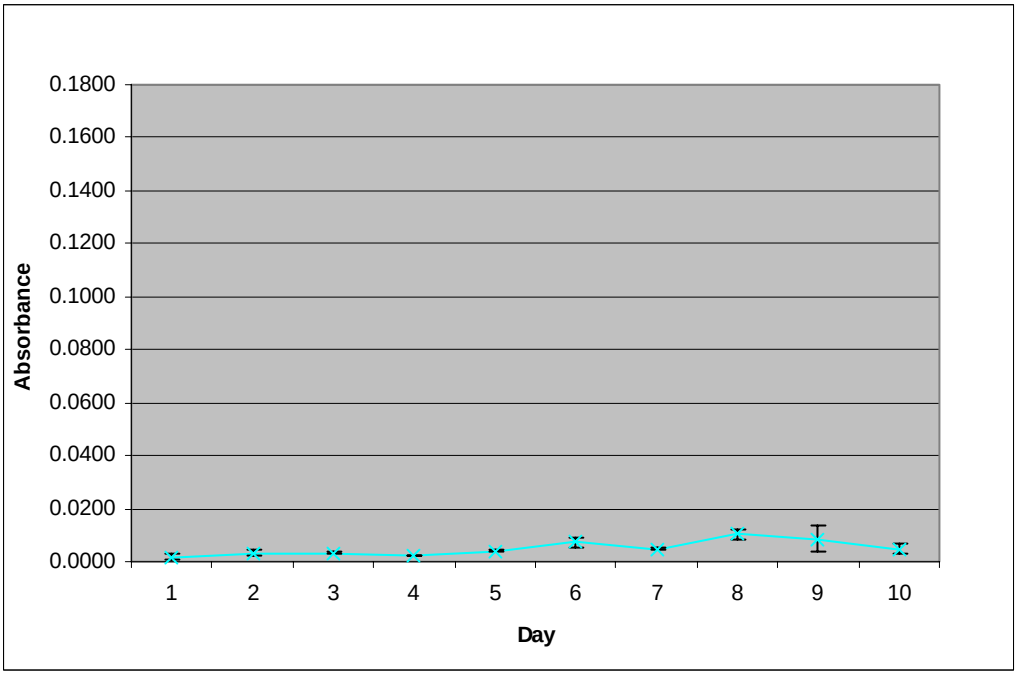

Figure A.6: Keratinocytes growth curve with seeding density 10000 cells/cm2

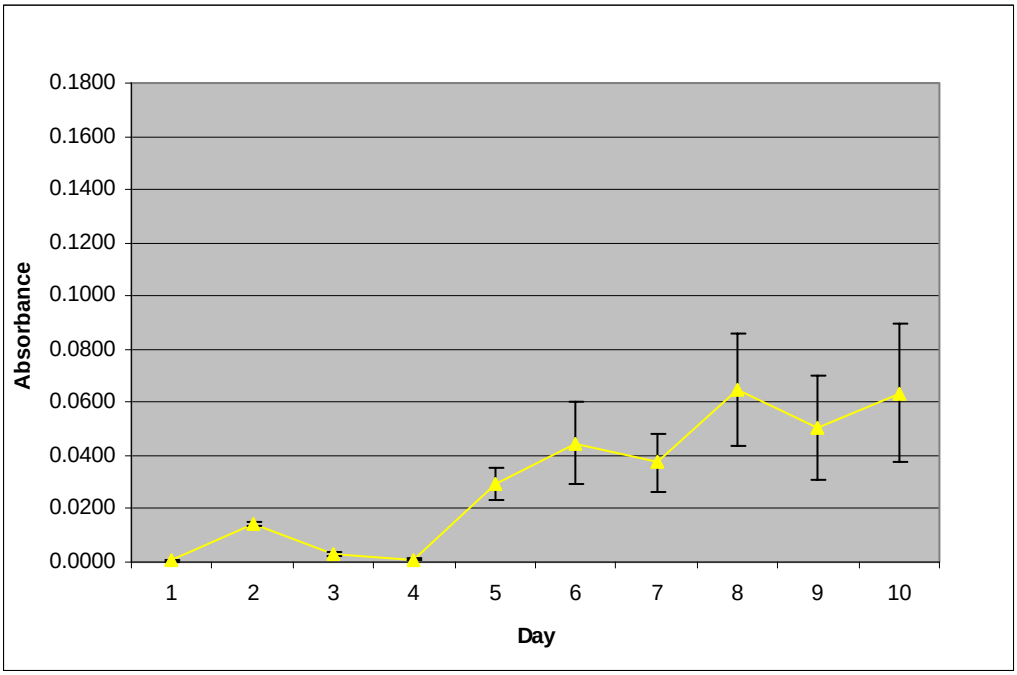

Figure A.7: Keratinocytes growth curve with seeding density 7500 cells/cm2

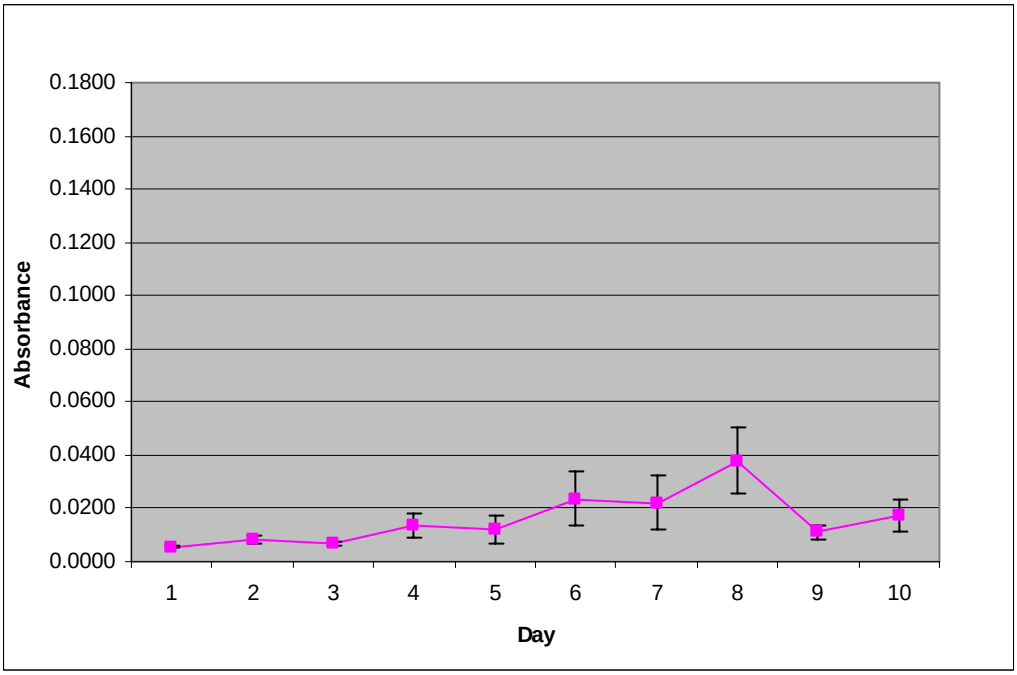

Figure A.8: Keratinocytes growth curve with seeding density 5000 cells/cm2

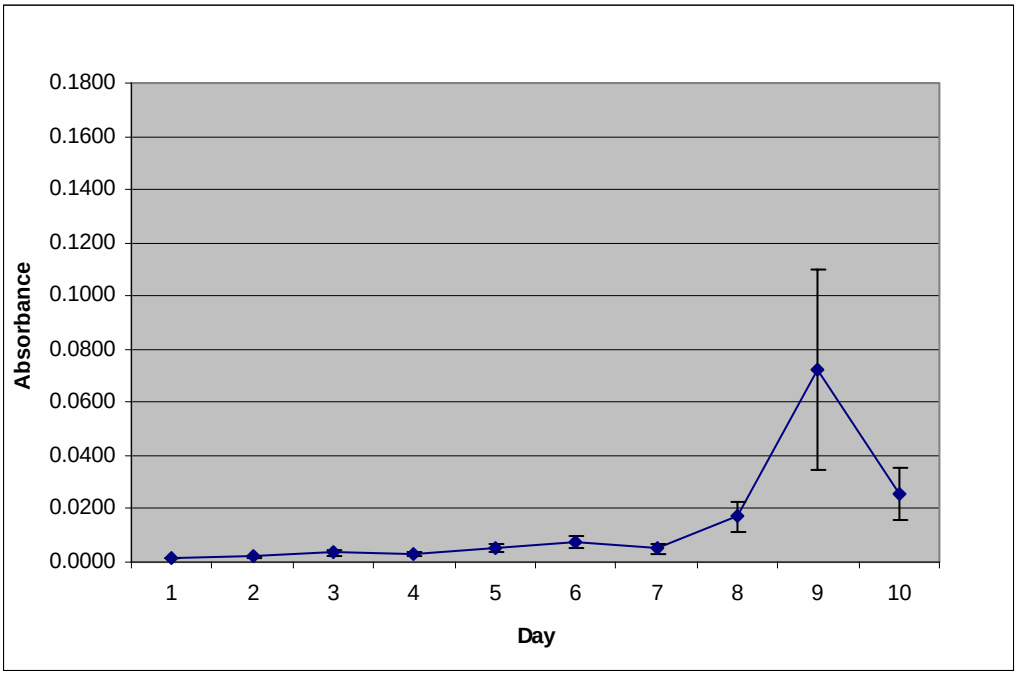

Figure A.9: Keratinocytes growth curve with seeding density 2500 cells/cm2

APPENDIX B
Fibroblasts growth curve

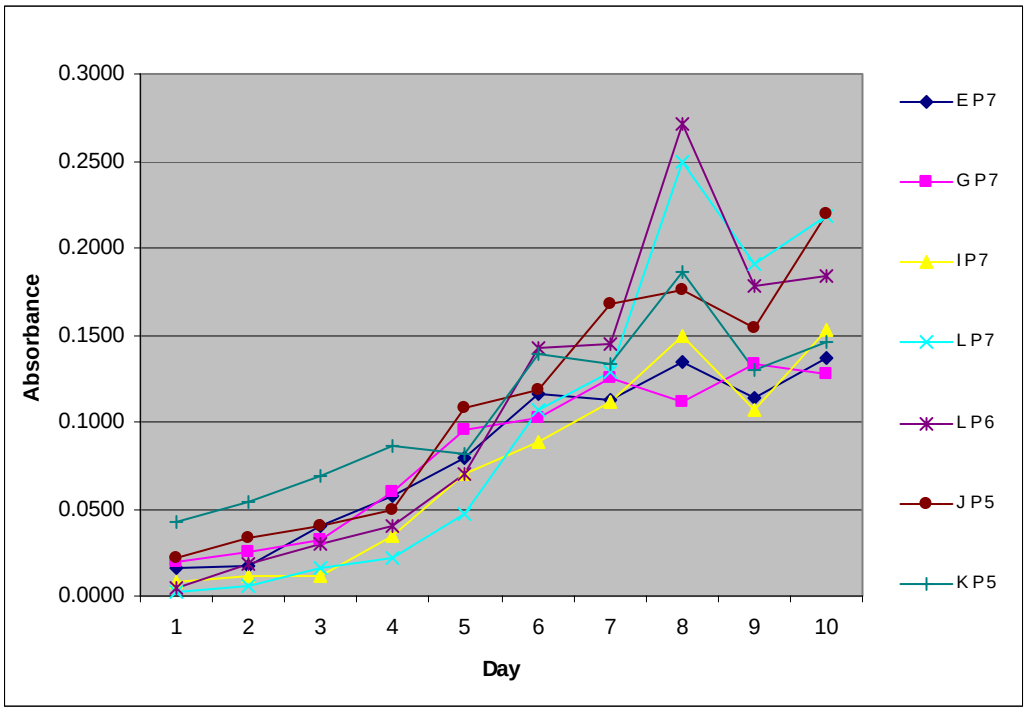

Figure B.1: Fibroblasts growth curve using 6 human skin samples at seeding density 10000cells/cm2. n=3 wells per sample

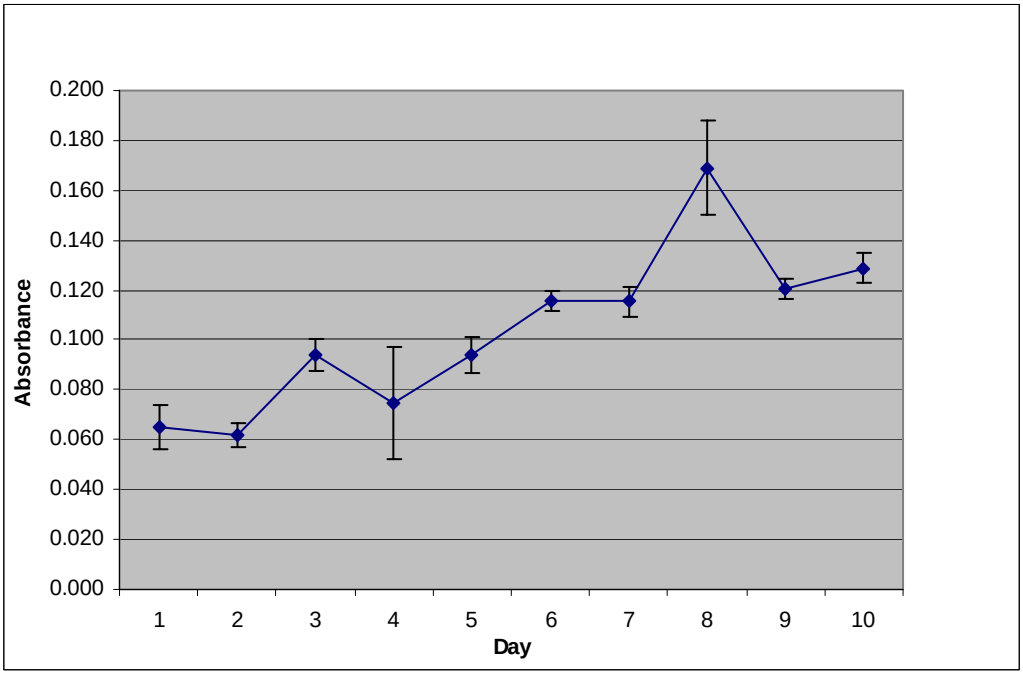

Figure B.2: Fibroblasts growth curve with seeding density 20000 cells/cm2

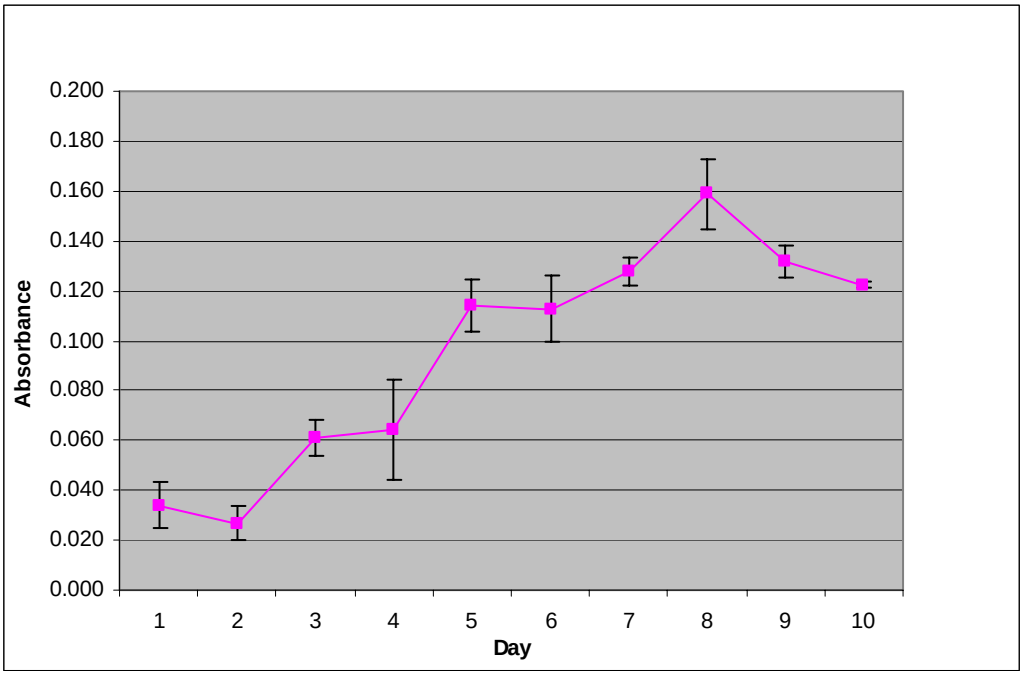

Figure B.3: Fibroblasts growth curve with seeding density 17500 cells/cm2

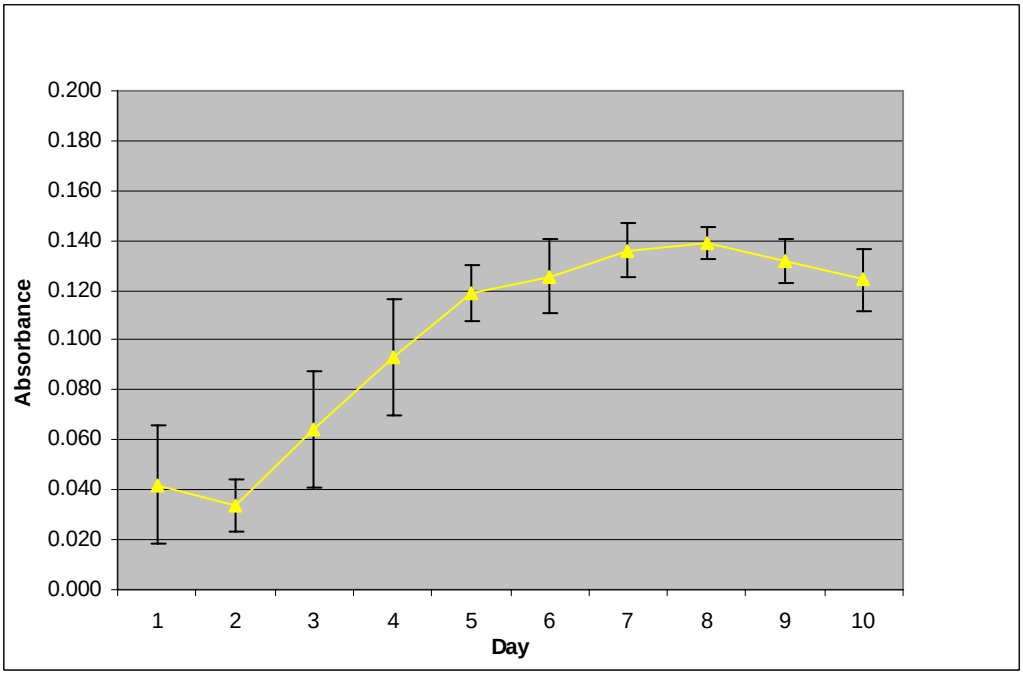

Figure B.4: Fibroblasts growth curve with seeding density 15000 cells/cm2

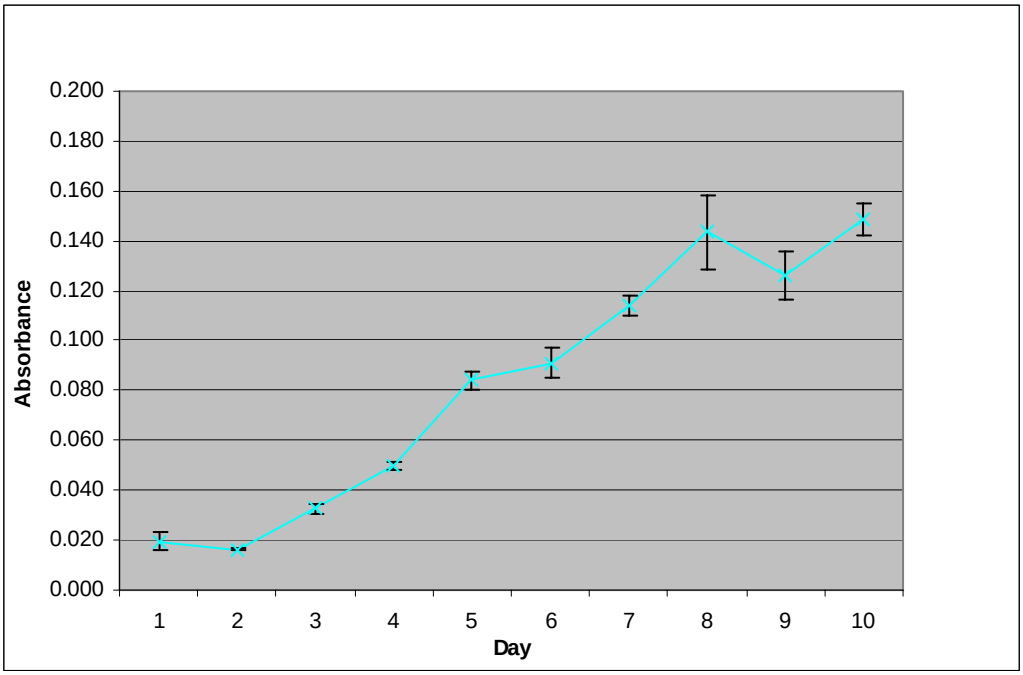

Figure B.5: Fibroblasts growth curve with seeding density 12500 cells/cm2

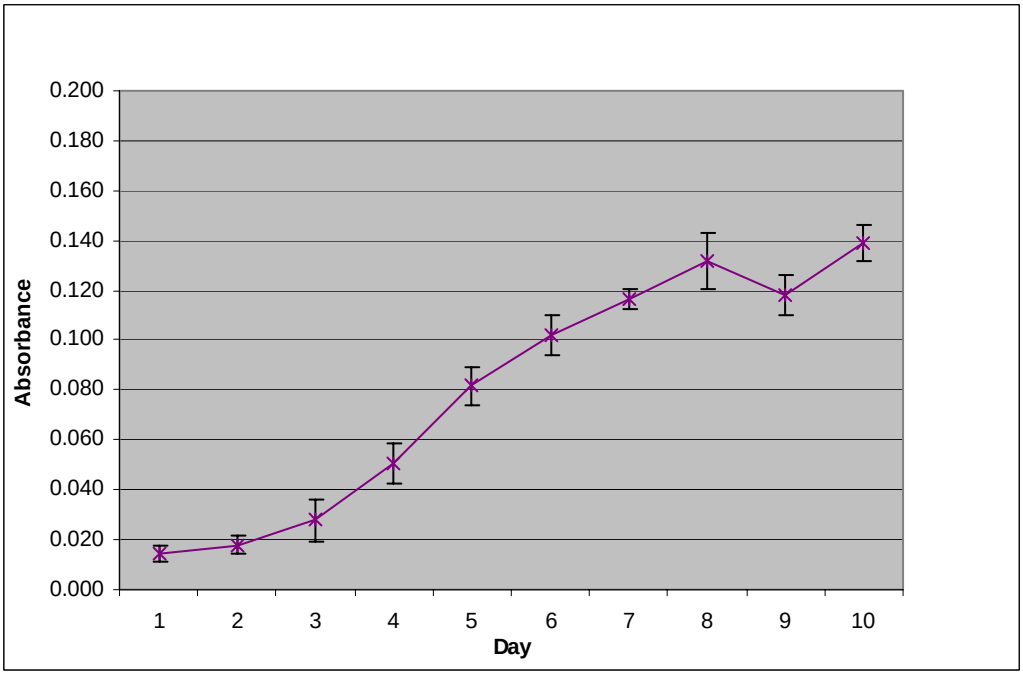

Figure B.6: Fibroblasts growth curve with seeding density 10000 cells/cm2

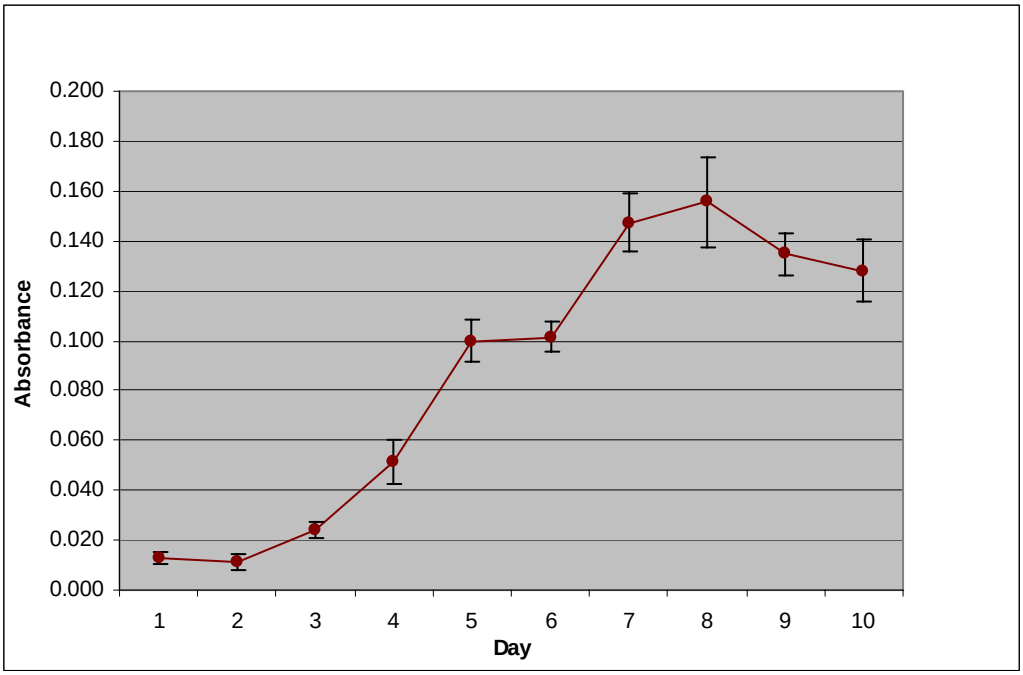

Figure B.7: Fibroblasts growth curve with seeding density 7500 cells/cm2

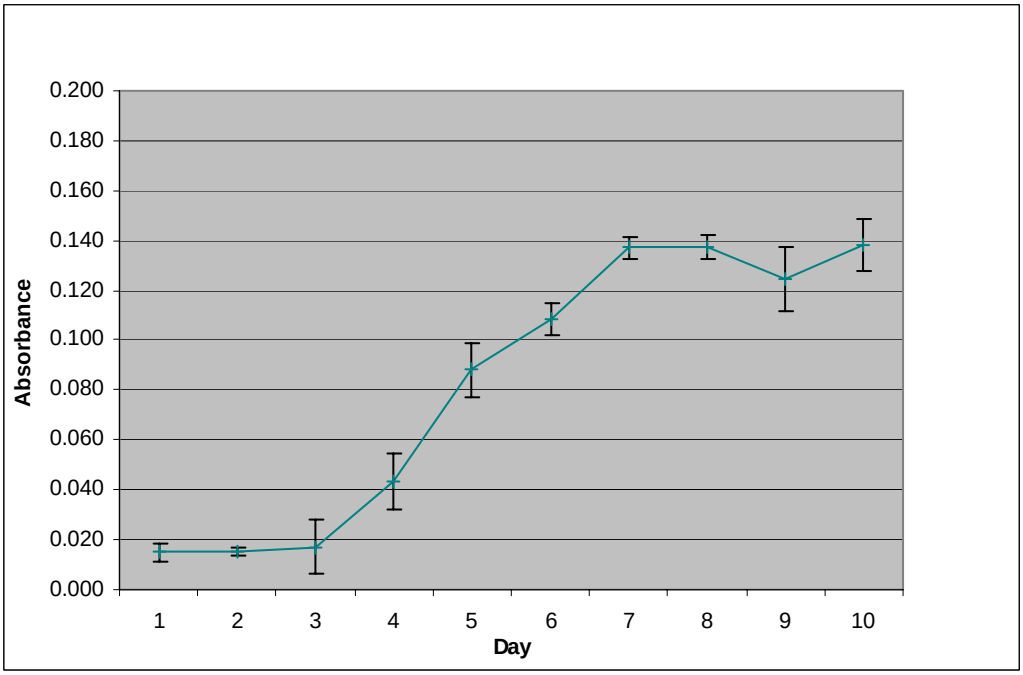

Figure B.8: Fibroblasts growth curve with seeding density 5000 cells/cm2

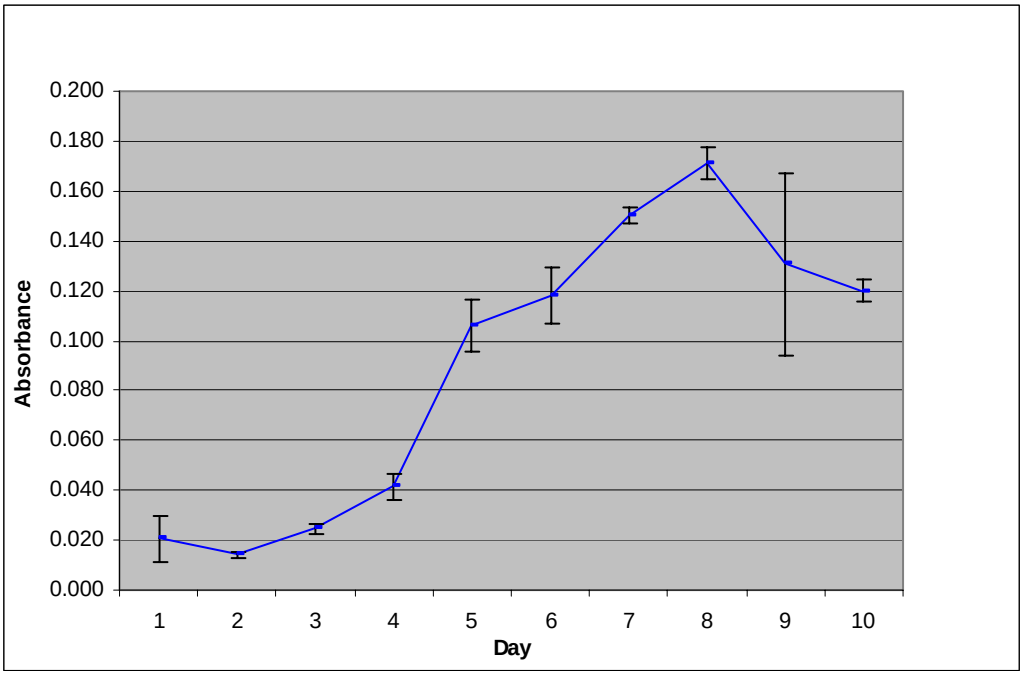

Figure B.9: Fibroblasts growth curve with seeding density 2500 cells/cm2

APPENDIX C

Melastoma malabathricum

C.1: *Melastoma malabathricum* flower

C.2: *Melastoma malabathricum* fruit

APPENDIX D
Data analysis

Table D.1: Data analysis for effect of day adding extract Melastoma malabathricum and its concentration on fibroblasts

Tests of Between-Subjects Effects

Dependent Variable: Relative Viability (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	68131.283[a]	21	3244.347	24.456	.000
Intercept	1508532.749	1	1508532.749	11371.314	.000
Concentration	48914.646	10	4891.465	36.872	.000
Day	209.945	1	209.945	1.583	.210
Concentration * Day	19006.691	10	1900.669	14.327	.000
Error	20429.833	154	132.661		
Total	1597093.865	176			
Corrected Total	88561.116	175			

a. R Squared = .769 (Adjusted R Squared = .738)

Table D.2: Data analysis for effect of different concentration of *Melastoma malabathricum* extract and treatment day on fibroblasts

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
day	Sphericity Assumed	2512.040	2	1256.020	12.143	.000
	Greenhouse-Geisse	2512.040	1.533	1638.606	12.143	.000
	Huynh-Feldt	2512.040	1.601	1568.567	12.143	.000
	Lower-bound	2512.040	1.000	2512.040	12.143	.001
day * Concentratio	Sphericity Assumed	7118.876	20	355.944	3.441	.000
	Greenhouse-Geisse	7118.876	15.330	464.365	3.441	.000
	Huynh-Feldt	7118.876	16.015	444.517	3.441	.000
	Lower-bound	7118.876	10.000	711.888	3.441	.000
Error(day)	Sphericity Assumed	52339.083	506	103.437		
	Greenhouse-Geisse	52339.083	387.858	134.944		
	Huynh-Feldt	52339.083	405.176	129.176		
	Lower-bound	52339.083	253.000	206.874		

Table D.3: Data analysis for effect of different concentration of *Melastoma*

malabathricum extract and treatment day on fibroblasts

Tests of Between-Subjects Effects

Measure: MEASURE_1
Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	7065011.122	1	7065011.122	67597.641	.000
Concentration	83523.419	10	8352.342	79.915	.000
Error	26442.458	253	104.516		

REFERENCES

Ahmad, F., Hamid, R. A. and Raji, H. Medicinal Plants Used by Bajau Community in Sabah, in Trends in Traditional Medicine Research. In: Chan, K. L. et al. eds. *Proceedings of the International Conference on the Use of Traditional Medicine and Other Natural Products in Health Care*. Penang: The School of Pharmaceutical Sciences. Universiti Sains Malaysia. 1993. 494.

Anonymous. *Melastoma malabathricum L., in Compendium of Medicinal Plants Used in Malaysia*. Herbal Medicine Research Centre. Institute for Medical Research. Kuala Lumpur. 2002. 2: 131-132.

Benaiges, A., Armengol, R., Mateu, B., Sagrista, M. L. and Mora, M. (2001). Effect of Germinated Seeds Extract on the Respiratory Activity of Human Skin Fibroblasts and Sheep Liver Mitochondria. Influence on Cell Viability and Proliferation and Their Usefulness as Active Comestic Ingredient. *International Journal of Comestic Science.* 23: 245-255.

Benford, D. J. (1992). The Use of Animal Cells as Replacements for Whole Animals in the Toxiciy Testing of Chemicals and Pharmaceuticals. In: Spier, R. E. and Griffiths, J. B. eds. *Animal Cell Biotechnology.* Volume 5. United States: Academic Press Inc. 98-121.

Burkill, I. H. *M. malabathricum in A Dictionary of Economic Products of Malay Peninsular*. Ministry of Agriculture and Cooperatives Malaysia. 1966: 1463-1465.

Cha, D., O'Brien, D., O'Toole, E. A., Woodley, D. T. and Hudson, L. G. (1996). Enhanced Modulation of Keratinocytes Motility by TGF-a Relative to Epidermal Growth Factor. *Journal Invest Dermatology.* 106: 590-597.

Doyle, A. and Griffiths, J. B. eds. (2000). Cell and Tissue Culture for Medical Research. Canada: Wiley-Liss Inc. 352-363.

Garcia, N., doucet, O., Bayer, M., Fouchard, D., Zastrow, L. and Marty, J. P. (2002). Characterization of the Barrier Function in a Reconstituted Human Epidermis Cultivated in Chemically Defined Medium. *International Journal of Comestic Science.* 24: 25-34.

Hidalgo, E. and Dominguez, C. (2000). Growth-altering Effects of Sodium Hypochlorite in Cultured Human Dermal Fibroblasts. *Life Science.* 67: 1331-1344.

Howling, G. I., Dettmar, P. W., Goddard, P. A., Hampson, F. C., Dornish, M. and Wood, E. J. (2001). The Effect of Chitin and Chitosan on the Proliferation of Human Skin Fibroblasts and Keratinocytes in vitro. *Biomaterial.* 22: 2959-2966.

Jaganath, I. B. and Ng, L. T. eds. *Melastoma malabathricum, in Herbs: The Green Pharmacy of Malaysia*. Kuala Lumpur: Vinpress. 58-59; 2000

Lam, P. K. (1999). *Evaluation of Human Skin Substitute for Burn wound Coverage based on Cultured Epidermal Autograft.* The Chinese University of Hong Kong: Ph.D. Thesis.

Lohézic-Le Dévéhat F., Bakhtiar, A., Bézivin, C., Amoros, M. and Boustie, J. Antiviral and Cytotoxic Activities of Some Indonesian Plants. *Fitoterapia*. 2002. 73(5): 400-405.

Naughton, G. K. and Naughton, B. A. (1993). *Three Dimensional Skin Culture System.* (U. S. Patent 5,266,480).

Palsson, B. Ø. and Bhatia, S. N. (2004). *Tissue Engineering.* New Jersey: Pearson Prentice Hall.

Perkin, M. A., Osborne, R., Rona, F. R., Ghassemi, A. and Robinson, M. K. (1999). Comparison of in Vitro Human Skin Responses to Consumer Products and Ingredients with a Range of Irritancy Potential. *Toxicological Sciences.* 48: 218-229.

Phan, T. T., Hughes, M. A. and Herry, G. W. (2001). Effects of an Aqueous Extract from the leaves of Chromolaena odorata (Eupolin) on the proliferation of Human Keratinocytes and on Their Migration in an in vitro Model of Reepithelialization. *Wound Repair and Regenration.* 9: 305-313.

Rodriguez, H., Jaruga, P., Birincioglu M.,. Barker, P. E., Connell, C. O' and Dizdaroglu, M. (2003). Oxidative DNA Damage Biomarkers used in Tissue Engineered Skin. *Advances in Experimental Medicine and Biology.* 534:132-133.

Roguet, R., Cohen, C. and Leclaire, J. (2000). Use of a Standardized Reconstructed Epidermis Kit to Assess *In Vitro* the Tolerance and the Efficacy of Comestics. *International Journal of Comestic Science.* 22: 409-419

Schlotmann, K., Kaeten, M., Black, A. F., Damour, O., Waldmann-Laue, M. and Forster, T. (2001). Comestic Efficacy Claims In Vitro Using a Three Dimensional Human Skin Model. *International Journal of Comestic Science.* 23: 309-318.

Seymour, C. B., Mothersill, C., Cusack, A. and McDonnell, M. (1987). Development of a Model for the Study of Toxicity and Carcinogenicity In Vitro. In: Spier, R. E. and Griffiths, J. B. eds. Modern Approaches to Animal Cell Technology. United Kingdom: Butterworth and Company Limited. 756-771.

Yannas, I. V. (2000). Artificial Skin and Dermal Equivalents. In: Bronzino, J. D. ed. *The Biomedical Engineering Handbook.* 2nd ed. Boca Raton: CRC Press LLC.